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PRODUCTION DE POLYHYDROXYALKANOATE (PHA) EN UTILISANT LES EAUX USÉES COMME SOURCE DE CARBONE ET LES BOUES ACTIVÉES COMME SOURCE DE MICRO-ORGANISMES

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RÉSUMÉ

Depuis plusieurs décennies, le développement de plastiques biodégradables présente un intérêt considérable compte tenu des problèmes et des effets nocifs que présentent les plastiques conventionnels vis-à-vis l'environnement.

Dans cette optique, le poly(3-hydroxyalkanoate) (PHA), un thermoplastique biodégradable et biocompatible, a attiré une attention particulière du point de vue industriel. Les méthodes courantes pour la production de ce polymère au niveau commercial utilisent le plus souvent des cultures pures de microorganismes exigeant des sources pures de carbone et des procédures complexes et coûteuses de bio-réaction. Par conséquent, des recherches ont été entreprises pour développer des processus de production plus rentable des PHAs. Ces procédés basés sur des cultures microbiennes mélangées (c.-à-d. boues activées) sont étudiés comme une technologie pouvant réduire les coûts de production, puisqu'aucune stérilisation n'est exigée et que les microbes en question peuvent s'adapter aux substrats complexes comme par exemple des déchets. Ceci permettrait de produire des plastiques biodégradables en utilisant des rejets renouvelables comme substrat de base et donc de réduire les coûts d'obtention du PHA. Dans la plupart des études de production de ce polymère en employant des boues activées, des eaux usées synthétiques ont été utilisées pour obtenir les boues destinées à la synthèse du PHA. Dans notre étude, la production de PHA se base sur une nouvelle approche, soit l'utilisation d'une part de microorganismes présents dans des boues activées provenant d'usines de traitement des eaux résiduaires et d'autre part des eaux usées comme substrat de fermentation.

Dans cette optique, quatre types de boues activées d'usines de traitement des eaux résiduaires (municipales, industries de pâtes et papier, usine d'amidon et fromageries) ont montré beaucoup de potentiel, comme sources de boues activées (micro-organismes), pour produire des plastiques biodégradables en erlenmeyers ; Les boues d'eaux usées des pâtes et papier permettant d'accumuler le plus de PHAs.

Les boues activées d'usines de traitement des eaux usées d'industries de pâtes et papier ont donc été employées comme sources de micro-organismes pour produire des PHAs à 25oC, à pH 7 et à différentes concentrations initiales de solides en suspension (SS) (5, 10,

15 et 20 g/l) dans des fermenteurs de 15 L gérés par informatique. L'acétate a été ajouté à des concentrations variables (5 à 20 g/l) comme unique source de carbone. L'impact du modèle d'alimentation sur la production des PHAs a été étudié. Différentes stratégies de fermentation (Batch, Fed-batch etalimentation continue) ont été évaluées. La concentration maximale obtenue a été de 60.9% avec une concentration initiale de solides solubles dans les eaux de 15 g/l ; Une addition continue d'acide acétique permettant de maintenir le rapport de C/N à 144. Par ailleurs, avec des eaux usées de pâtes et papier utilisées au début de l'expérience et avec un ajout constant d'acide acétique pour un rapport de C/N de 144, le taux maximum de PHA atteint était de 54.9%.

Six souches bactériennes produisant PHA ont été isolées des boues de pâtes et papier et leurs capacités de production de ce bioplastique ont été évaluées. Les teneurs en bioplastiques produits étaient sensiblement différentes avec ces six isolats. Un contenu maximal de 35.45% de PHA a été obtenu par l'isolat PHA-P5 même dans sans contrôle du pH et de la concentration d'oxygène dissous. Ces micro-organismes ont été identifiés en se basant sur les séquences des rDNA 16S et PHA-P5 produisant le maximum de PHA a été identifié comme *Comamonas* sps.

Douze souches bactériennes produisant PHA ont aussi été isolées dans des boues municipales et une teneur maximum de 27.5% de biopolymères a été obtenue avec l'isolat PHA-M3. Ces micro-organismes ont été également identifiés par séquençage des rDNA 16S et PHA-M3 produisant le plus de PHA a été identifié comme *Citrobacter* sp.

Les résultats de cette étude montrent que les eaux usées et les boues activées présentent beaucoup de potentiel pour la production de PHAs et ce, comme une nouvelle approche à l'obtention d'un produit à valeur ajoutée par le recyclage et donc la gestion des déchets.

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ABSTRACT

There has been a considerable interest in the development of biodegradable plastics since last few decades due to the problem and harmful effects of conventional plastics on the environment.

Poly(3-hydroxyalkanoate) (PHA), one of the biodegradable and biocompatible thermoplastics, has attracted considerable industrial attention. Current methods for PHA production at the industrial scale are mostly pure cultures requiring pure carbon sources and complex and costly processing procedures. Therefore, attempts have been made to develop more cost-effective processes. PHA production processes based on mixed microbial cultures (i.e. activated sludge) are being investigated as a possible technology to reduce production costs, since no sterilization is required and bacteria can adapt well to the complex substrates that may be present in waste material. This would allow the production of biodegradable plastics from renewable waste streams, with reduced costs on materials and processing. In most of the studies of PHA production by activated sludge, synthetic wastewaters were used to cultivate PHA producing sludge. In this study, the PHA production on the new approach involves the use of activated sludge from full-scale wastewater treatment plant as the microorganisms and wastewater as the substrate for the PHA production is investigated.

Four types of activated sludge from different full-scale wastewater treatment plants (municipal, pulp and paper industry, starch manufacturing and cheese manufacturing wastewaters) showed the good potential to be used as the source of microorganisms to produce biodegradable plastics in shake flask experiments. Pulp and paper wastewater sludge was found to accumulate maximum concentration.

Activated sludge from full-scale pulp and paper industry wastewater treatment plants was used as a source of microorganisms to produce PHA at 25oC, pH 7 and at different initial sludge suspended solids (SS) concentration (5, 10, 15 and 20 g/l) in computer controlled fermentors (15 L). Acetate was used as sole carbon source at varying concentration (5 to

20 g/l). Impact of feeding pattern on polyhydroxyalkanoates (PHA) production was investigated. Batch, fed-batch and continuous feed strategies were conducted. The maximum PHA content obtained was 60.9% (of sludge suspended solid-SS) with the sludge SS concentration of 15 g/l, acetic acid continuous feed under the C/N ratio of 144. While the pulp and paper wastewater used at the beginning of the experiment, with acetic acid continuous feed under the C/N ratio of 144, the maximum PHA content reached 54.9%.

Six PHA accumulating bacterial strains were isolated from the pulp and paper sludge and their capabilities of the PHA production had been evaluated. The PHA amounts produced significantly varied among the six strains. The maximum PHA content of 35.45% was obtained by strain PHA-P5 even under uncontrolled conditions of pH and dissolved oxygen concentration. These sludge microorganisms were identified based on their 16S rDNA sequences and the PHA-P5 with the maximum PHA production was identified as *Comamonas* sp.

Twelve PHA accumulating bacterial strains were isolated from the municipal sludge and the maximum PHA content of 27.5% was obtained by strain PHA-M3. These sludge microorganisms were also identified based on their 16S rDNA sequences and the PHA-M3 with the maximum PHA production was identified as *Citrobacter* sp..
The results from this study showed the promising potential of wastewater and activated sludge for the PHA production, a new approach to a new value added product for the waste management.

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ABBREVIATIONS

AS	Activated sludge
ATP	Adenosine triphosphate (metabolic energy equivalent)
COD	Chemical oxygen demand
DO	Dissolved oxygen
DAS	Dairy activated sludge
DM	Dry mass
DWW	Dairy wastewater
EBPR	Enhanced Biological Phosphorous Removal
ED	Entner-Doudoro
EMP	Embden-Meyerhof-Parnas
GAO	Glycogen accumulating organisms
HAc	Acetic acid
MAS	Municipal activated sludge
MLSS	Mixed liquor suspended solid
MWW	Municipal wastewater
PAO	Polyphosphate accumulating organisms
PAS	Pulp-paper activated sludge
PHA	Poly-hydroxyalkanoate
PHB	Poly-hydroxybutyric acid
P(3HB-co-3HV)	poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
PHV	poly-hydroxyvalerate
P(3H2MB)	poly(3-hydroxy-2-methyl butyrate)
P(3H2MV)	(3-hydroxy-2-methyl valerate)
PolyP	Polyphosphate
WW	Wastewater
PWW	Pulp-paper wastewater
SAS	Starch activated sludge
SRT	Sludge retention time
SWW	Starch wastewater
TAC	Tricarboxylic acid cycle
VFAs	Volatile fatty acids
WW	Wastewater
ABR	Anaerobic baffled reactor
AMW	Apparent molecular weight
A/O	Anaerobic/oxic
AODC	Acridine orange direct count
API	Analytical profile index
AODC	Acridine orange direct count
ATP	Adenosine triphosphate
BAP	Biomass associated products
BMP	Biochemical methane potential
BOD	Biochemical oxygen demand
CAS	Conventional activated sludge
COD	Chemical oxygen demand
CSTR	Continuously stirred tank reactor
DAPI	4’,6-diamidino-2-phenylindole
DGGE	Denaturing gradient gel electrophoresis

DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
EBPR	Enhanced biological phosphate removal
ECP	Extracellular polymers
FISH	Fluorescence *in situ* hybridization
GAC	Granular activated carbon
GAO	Glycogen accumulating organisms
GC	Gas chromatography
GNS	Green nonsulfur
GPC	Gel Permeation chromatography
HPC	Heterotrophic bacteria
HRT	Hydraulic retention time
LUASB	Lighted upflow anaerobic sludge blanket
MAR	Microautoradiography
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids
MPN	Conventional most probable number
MSAS	Membrane separation activated sludge process
MW	Molecular weight
OLR	Organic loading rate
OTUs	Operational taxonomic units
OMW	Olive mill wastewater
OUR	Oxygen uptake rate
PAB	Polyphosphate-accumulating bacteria
PAC	Powdered activated carbon
PAOs	Phosphorus accumulating organisms
PACT	Powdered activated carbon treatment
PHA	Polyhydroxyalkanoates
PHB	Poly(3-hydroxybutyrate)
PHBV	Poly(3-hydroxybutyrate-co-hydroxyvalerate)
Pi	Phosphates
PP, polyP	Polyphosphates
RPAO	*Rhodocyclus*-related PAO
rRNA	Ribosomal ribonucleic acid
SBR	Sequencing batch reactor
SMP	Soluble microbial products
SOC	Soluble organic carbon
SON	Soluble organic nitrogen
SRP	Soluble residual products
SRT	Sludge retention time
THM	Trihalomethane
TOC	Total organic carbon
UAP	Utilisation associated products
UASB	Up flow anaerobic sludge blanket
UF	Ultrafiltration
VSS	Volatile suspended solids
VFA	Volatile fatty acids
WWTP	Wastewater treatment plant

CHAPITRE 1.

SYNTHÈSE

1.1 Introduction

Le développement de plastiques biodégradables présente un intérêt considérable, surtout depuis ces dernières décennies, compte tenu des problèmes et des effets nocifs des matières plastiques conventionnelles sur l'environnement.

En 1926, le polyhydroxyalkanoate (PHA) a été identifié comme un biopolymère constitutif de Bacillus megaterium (Lemoigne, 1926). Plus tard, le poly- b-hydroxybutyrate (PHB), un des PHA les plus communs et d'autres PHAs ont été découverts; 40 PHAs ayant été caractérisés (Steinbuchel, 1991). Parmi eux, le poly-hydroxy-butyrate (PHB) est accumulé par un grand nombre de bactéries, généralement, dans des conditions de limitation des éléments nutritifs et en présence d'excès de source de carbone.

1.2 Conditions de productions de biopolymères

Généralement, les polymères sont synthétisés par des bactéries sous certaines conditions de croissance. Par exemple, les PHAs et PHBs sont accumulés lorsqu'une source de carbone est abondamment disponible , alors que l'azote, un autre élément essentiel de croissance, est à une concentration limite ou même nulle dans le milieu (Hrabak, 1992).

Certaines bactéries (*Alcaligenes eutrophus, Protomonaas extorquens, Pseudomonas olevorans*, etc.) ont besoin d'une limitation de certains éléments nutritifs essentiels, tels que N, P, Mg, K, O ou S pour synthétiser des PHA. Un excès de source de carbone permet une synthèse efficace de ces polymères. Toutefois, d'autres bactéries (*A. latus*, une souche mutante de Az. Vinelandii, des *E. coli*. recombinants) n'ont pas besoin de limites en nutriments (Lee, 1996a). Par ailleurs, des bactéries produisent du PHB en présence de minéraux contenant des métaux à l'état de traces (comme Co, Cr, Cu, Zn) (Repaske et Repaske, 1976). Selon Findlay et White (1983), les PHBs sont produits en grandes quantités dans les nappes phréatiques (oligotrophes environnementaux). En revanche, la production peut être inhibée par Mg et SO_4^{2-}. A. eutrophus, peut accumuler 80% de son poids sec en PHAs alors que dans le cadre d'un excédent de carbone (par exemple le glucose) et d'une limitation de P ou de N, le polymère accumulé agit comme

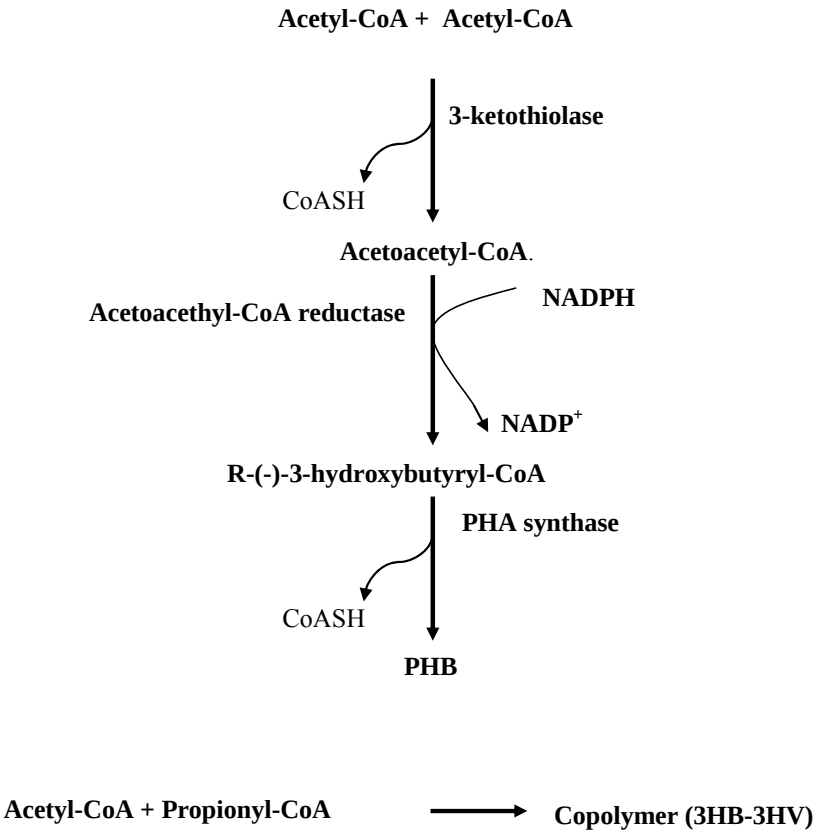
une réserve de carbone et de puits d'électrons (Anderson et Dawes, 1990). Quand les conditions limites sont réduites (ajout de P ou N), le PHB est catabolisé en acétyl-CoA (Yves et al., 1995). Généralement, les PHAs sont accumulés sous forme de granules (Page et al., 1992) visibles en microscopie électronique (Lageveen et al., 1988; Hrabak, 1992; Vincent et al., 1962).

1.3 Biosynthèse, structure et propriétés de biopolymères

1.3.1 Voies biosynthétiques

La majorité des données sur le mécanisme de production des PHAs ont été obtenues avec A. eutrophus. Cette bactérie synthétise PHB à partir de l'acétyl-CoA au cours de trois réactions catalysées par trois enzymes (thiolase, réductase et synthétase). Au début, la condensation réversible de deux molécules d'acétyl-CoA, sous l'action de la 3 - ketothiolase, produit l'acétoacétyl CoA. Par la suite l'acétoacyl CoA réductase réduit l'acétyl-CoA en R - (-) - 3 - hydroxybutyryl CoA; La NADPH réductase, étant liée. Enfin, R - (-) - 3 - hydroxybutyryl CoA est polymérisé en PHB sous l'action de la PHA synthétase. PHA est formé par polymérisation de 103 à 104 monomères et s'accumule dans les cellules en inclusions de 0,2 µm de largeur et de 0,5 µ m de diamètre (Yves et al., 1995; McCool et Cannon, 2001; Fukui et Doi, 1997). En fait, les séquences des nucléotides sont connues pour plus de 30 PHA synthases et elles sont identifiées en trois classes (Classe I: catalysation de la polymérisation de chaîne courte durée HACoAs; classe Π: catalysation de la polymérisation de longueur de moyenne chaîne de HACoAs et de la classe IIΠ: catalyse de la polymérisation de chaîne à courtes durées, HACoAs) (Rehm et Steinbuchel., 1999). Par conséquent, la nature du PHA produit par A. eutrophus, est liée à la structure de la source de carbone présente dans le milieu de culture. Les PHA produits sont composés de différents monomères ayant 3 à 5 atomes de carbone (C3 à C5) (Steinbuchel, 1991). L'adjonction d'acide propionique ou d'acide valérique aux milieux contenant du glucose permet l'accumulation de copolymère (P (3HB - 3HV): copoly (3 - hydroxybutyrate - 3 - hydroxyvalérate)) (Yves et al., 1995). Toutefois, le système de synthèse des PHAs permet la saturation en acides 3, 4 et 5 - hydroxyalcanoique avec un maximum de cinq atomes de carbone. C'est pourquoi, dans ce cas, les acides hydroxyalkanoique à six atomes de carbone ou plus n'ont pas été détectés

dans les PHAs produits par cette bactérie (Yves et al., 1995; Liebergesell et al., 1991; McCool et Cannon, 2001; Fukui et Doi, 1997). Cependant, un grand nombre de bactéries notamment Pseudomonas olevorans accumulent des polymères composés de monomères ayant 6 à 14 atomes de carbone (C6 à C14) (Steinbuchel, 1991). Par conséquent, la flexibilité de la voie des PHAs permet la conception de nouveaux biopolymères ayant potentiellement des propriétés physiques spécifiques (Yves et al., 1995).



Le schéma 1. Voie de biosynthèse de PHA

1.3.2 Structure de PHBs

Les polymères PHAs ont un certain nombre de caractéristiques intéressantes et peuvent être utilisés de manières similaires à de nombreux plastiques classiques synthétiques . Généralement, les PHAs sont formées par polymérisation de monomères d'acide R - (-) - hydroxyalkanoique ayant 3 à 14 carbones (C3 à C14); PHB étant le plus intéressant et le plus connu (Budwill et al., 1992). Beaucoup d'unités monomériques ont été identifiés

dans diverses espèces de bactéries comme constituants des PHAs (plus de 90 monomères) (Steinbuchel, 1995), par exemple des unités monomères sont des: 3 - hydroxyalcanoates (de 3 à 12 atomes de carbone), 4 - hydroxypentanoate (ayant 4 à 8 atomes de carbone), 5 - hydroxypentanoate, 5 - Hydroxyhexanoate et 6 Hydroxydodécanoate. Toutefois, seul un petit nombre de ces PHA ont été produits et caractérisés.

1.3.3 Propriétés

Les propriétés des PHBs sont comparables à celles des plastiques conventionnels tel que le polypropylène. Ce sont des biopolymères naturels 100% résistants à l'eau et à l'humidité, thermoplastiques et 100% biodégradables (Hrabak, 1992). Cependant, il ont des propriétés spécifiques telles qu'une faible perméabilité aux gaz.

L'homo PHB est un polymère rigide et un thermoplastique relativement cassant (Holmes, 1988). Son point de fusion est de 175 ° C et il a une faible résistance relative aux réactifs (acides et bases). Cependant, il a une bonne résistance au rayonnement UV. Par rapport à d'autres plastiques biodégradables, le PHB est insoluble dans l'eau et relativement résistant à la dégradation hydrolytique. Il se caractérise également par une bonne perméabilité à l'oxygène (Holmes, 1988).

L'utilisation des PHBs est limitée par des points de fusion élevés et un faible taux de dégradation. Ces propriétés peuvent être modifiées en changeant la configuration de la polymérisation des PHBs pouvant conduire à une composition différente de PHAs comme copolymères (Cox, 1994). Par exemple, l'intégration de monomères 3HV (3 - hydroxyvalérate) en polymères PHB entraîne une diminution de la cristallinité et du point de fusion par rapport à l'homopolymère PHB (Holmes, 1988). Ainsi, le copolymère permet de diminuer la raideur et d'augmenter la ténacité. Par conséquent, le copolymère P (3HB - 3HV) a été adapté à de nombreuses applications . Le polyhydroxyoctanoate a les propriétés d'un élastomère comme un faible point de fusion, une faible cristallinité, mais une mauvaise résistance à la traction et une sensibilité accrue à la rupture (Gagnon et autres, 1992).

1.3.4 Biodégradabilité

Beaucoup de micro-organismes tels que des bactéries et des champignons dégradent les PHAs et les utilisent comme des sources de carbone (Matavulj et Molitoris, 1991; Jendrossek et al., 1993). PHA peut être dégradé rapidement en anaérobiose, mais lentement dans les eaux usées ou l'eau de mer (Luzier, 1992). Les produits finaux de la biodégradation sont du CO_2 et de l'eau sous conditions aérobies. Cependant, en conditions anaérobies, PHBs peut être transformé en CH_4 et CO_2. (Budwill et al., 1992; Lee, 1996b; Cox, 1994).

La biodégradation se produit par l'attaque des biopolymères par les bactéries qui secrètent une enzyme extracellulaire appelé dépolymérases. Certains polymérases extracellulaires (hydrolysant PHB, P (3HB - 3HV) et PHO: Poly 3 - hydroxyoctanoate) ont été identifiées et leurs gènes clonés (Schirmer et al., 1993; Saito et al., 1989). En outre, une PHV dépolymérase a été isolée et caractérisée à partir de Pseudomonas lemoignei (Muller et al., 1993). PHB est aussi connu comme un matériau biocompatible. La dégradation in vivo est lente (Fiechter, 1990) par rapport à la dégradation in vitro, (dans le sol et dans les boues) et son produit dérivé est l'acide R - (-) - 3 - hydroxybutyrique (Yves et al., 1995).

La biodégradation de plastiques à base de PHB a été étudiée dans différents types de compost et par différentes techniques de compostage (Hrabak, 1992). Une perte de poids de 18 à 40% a été signalée après 12 semaines d'exposition dans un engrais vert à base de compost. Cependant, la forme de compactage de produits PHB s'est révélée défavorable pour la dégradation (Hrabak, 1992). Le compostage sous température plus élevée et l' agitation mécanique permet une dégradation rapide des bouteilles de copolymère (P (3HB - 3HV))' Après 15 semaines, une perte de 80% du poids a été observée (Yves et al., 1995). Toutefois, une perte de poids d'environ 50% pour le même matériel a été notée sous conditions simulées d'enfouissement géré à 35^0C et après une période de plus de 40 semaines (Yves et al., 1995). Généralement, le taux de biodégradation des bioplastiques est contrôlée par certains facteurs environnementaux (pH, température, éléments nutritifs, et l'humidité) et des paramètres des copolymères (co-monomère HV: 3 - hydroxyvalérate) tel que le poids moléculaire, le degré de cristallinité, la superficie et la

formulation). Par conséquent, les paramètres peuvent être contrôlés, au cours de la synthèse, afin d'optimiser le taux de biodégradation. Par exemple, l'augmentation de la la teneur en HV P (HB - HV) copolyesters peut améliorer la biodégradabilité (Cox, 1994).

1.4 Diversité des micro-organismes et des substrats pour produire des biopolymères

L'accumulation de PHAs à plus forte concentration a été observée avec une variété de micro-organismes tels que Clostrodusim, Sytrophomonas, Pseudomonas et Alcaligene. Certaines cyanobactéries produisent PHB à un niveau inférieur (Fiechter, 1990).

Alcaligenes eutrophus produit des PHA à chaînes courtes (polymérisation de C3 à C5 monomères) (Anderson et dawes, 1990; Steinbuchel, 1991). A. eutrophus est le plus utilisé sous de rudes conditions car il produit PHB et P (HB - HV) au taux le plus élevé (Ramsay et al., 1992). A. eutrophus peut accumuler environ 80% de son poids sec (Byrom, 1987). C'est un chimiolithotrophe qui croît avec un taux moyen de minéraux. Le milieu d'enrichissement moyen contient (en g / l) : Extrait de levure: 10; de peptone: 10; extrait de viande: 5 et (NH4) 2SO4: 5 (Yoshiharu et al, 1988). La croissance de A. eutrophus dans ce milieu ne peut pas synthétiser de polyester. Afin d'en produire, ces bactéries doivent être transférées dans un autre milieu limité en azote (Repaske et Repaske, 1976) et en présence d'un excès de carbone à un pH de 7, à 300C et sous oxygénation.

La nature et la proportion des polymères produits sont influencées par la source de carbone utilisée (Steinbuchel, 1991). Les acides, valérique et propionique, conduisent à la production de copolymère P (3HB - 3HV) (mélange de 3HB et 3HV). Avec la même souche sur un milieu à base d'acide valérique et à un taux moyen, la proportion de 3HV atteint 90% des PHA produits (Doi et al., 1988; Steinbuchel et al., 1991).

A. eutrophus est inhibé par l'acide propionique à 0,1% (Byrom, 1987), toutefois, il accumule des P (3HB - 3HV). L'utilisation de glucose à 2% ou d'amidon à 2% permet

d'augmenter la production de PHBs. Généralement ils sont produits dans le cadre d'une concentration minimale d'ammonium (Heinzle et Laffery, 1980).
Pseudomonas olevorans est un chimiolithotrophe qui produit des chaînes de moyenne longueur de PHAs par incorporation de C6 à C14 monomères (Anderson et dawes, 1990; Steinbuchel, 1991) et qui se développe dans un milieu minéral sous oxygénation à 30 ° C et à pH 7.

Un certain nombre de bactéries peuvent accumuler des copolymères PHA contenant à la fois des chaînes courtes ou moyennes . Parmi elles, P. VIM produit un copolymère composé de C5 à C10 monomères tout en croissant sur valérate (Timm et Steinbushel, 1990). De la même manière, P. resinovarans cultivée sur hexanoate accumule des PHA de C4, C6, C8 et C10 monomères (Ramsay et al., 1992).

Azotobacter vinelandii produit une grande quantité de PHB sur une variété de sources de sucre (Page et al., 1992), sans nécessiter des conditions de croissance limitées. Cette bactérie est utilisée comme une alternative pour produire des PHBs (Page et Knops, 1989). Elle accumule un maximum de PHB sans besoin d'azote et d'excès de carbone (le glucose). L'utilisation d'autres substrats (méthanol, acétate, butyrate) a été évaluée par Yamane (1992). La souche UWD d'azotobacter vinelandii synthétise des PHAs efficacement dans des milieux à base de mélasse de betterave, de mélasse de canne et de sirop (Page, 1989; 1990). En utilisant comme substrat de la mélasse, le PHA accumulé représente plus de 80% de son poids sec. L'obtention d'un copolymère par cette bactérie a également été étudiée par Pagé et al. (1992). La croissance sur glucose comme source principale de carbone et l'addition d'autres sources secondaires (valérianate, pentanoate, etc) et l'effet de l'ajout de poissons ont été étudiés pour Azotobacter vinelandii croissant en peptones dans deux types de cultures (lot par lot - et nourris). L'utilisation accrue de poissons de peptone augmente la synthèse de PHB. La peptone utilisée comme une source d'azote permet donc une rapide accumulation de PHB (Page et Cornish, 1993).

D'autres bactéries sont aussi connues pour leur potentiel à produire des PHA, toutefois, leur application est limitée. Certaines études ont montré que des souches de Rhizobium

sont capables d'accumuler du PHB à un niveau significatif (Hayward et al., 1959; Vincent et al., 1962). Le Methylobacterium organophilum montre un optimum de croissance à 0,5% (p / v) de méthanol. La concentration de méthanol à 4% (p / v) et le manque de cations divalents réduit la croissance bactérienne. Toutefois, en vertu d'un taux limite de potassium et avec du méthanol comme source de carbone, le PHA (P (3HB)) contenu atteint 52% du poids sec des cellules. (Kim et al., 1996). La croissance de Chromobacterium violaceum sur acide valérique à une concentration moyenne donne un polymère composé de 100% du monomère 3HV (Doi et al., 1988; Steinbuchel et al., 1991). Par ailleurs, Rhodococcus cultivée sur glucose produit un copolymère avec 75 mole% 3HV et 25 mole% 3HB (Haywood et al., 1991).

1.5 Production industrielle des biopolymères

1.5.1 Technologies de production

Généralement, les batchs et les techniques de culture en continu sont utilisées pour l'accumulation de la plus forte concentration de PHB. Le Fed - batch est surtout employé pour les bactéries (*Alcaligenes eutrophus, Protomonas extorquens, Protomonas. Oleovorans, ...*) nécessitant des facteurs limitants (N, P, Mg, K, O ou S) et d'un excédent de source de carbone pour l'efficacité de la production de PHA (Lee , 1996a). Au début, les cellules sont cultivées jusqu'à une concentration de nutriments sans limite. Après cela, un élément nutritif essentiel est éliminé pour permettre plus d'accumulation de PHB dans la cellule. L'élimination complète de P ou N dans le cas de *A. eutrophus* augmente significativement l'accumulation de polymères (jusqu'à 80% de la cellule de poids pour P (3HB) et P (3HB - 3HV)) (Byrom, 1987; Kim et al. , 1994). En outre, d'autres bactéries (*P. extorquens* et *P. oleovorans*) avec une élimination totale de N ou P ne présentent pas une bonne accumulation de polymères (Preusting et al., 1993; Suzuki et al., 1986). Deux étapes chémostat doivent être employées pour ce type de micro-organismes – La 1ère étape est de générer des cellules en masses et la 2e permet d'accumuler PHB. Cependant, pour certains souches tels que *P. oleovorans*, une étape du processus pourrait être utilisée (Preusting et al., 1993).

L'application de la stratégie Fed – batch, pour les bactéries qui ne nécessite pas une limitation des conditions de nutriments (*A. latus*, un mutant de *Az. Vinelandii*, etc.) est importante pour le succès de fermentation (Lee, 1996a). Des sources d'azote (liqueur de maïs, extrait de levure, de peptone obtenues de poissons, etc.) ont été ajoutées pour augmenter la croissance des cellules et par conséquent l'accumulation de PHA (Lee et Chang, 1994, Page et Cornish, 1993). Cependant, la culture donne la plus grande accumulation de PHA. Un l*atus* et *P. oleovarans* ont été cultivées avec succès dans un processus continu pour la production de PHA (Preusting et al., 1993). Pour ces types de bactéries qui n'exigent pas des conditions de limitation de nutriments, l'étape chémostat pourrait également être utilisée. Toutefois, il est très important d'optimiser les stratégies en continu ou nourriture par lots dans le but de comparer la productivité des différentes souches en fonction de la nature du support utilisé. En outre, l'analyse économique devrait être effectuée afin de choisir la méthode la plus appropriée.

En 1975, l'ICI (Imperical industrie chimique, Royaume Uni) a commencé la production de PHBs en réponse à la hausse du prix du pétrole (Yves et al., 1995). Une souche mutante de *A. eutrophus* (Byrom, 1987, 1990), cultivée avec du glucose et de l'acide propionique (teneur moyenne), a été utilisée pour produire ce copolymère (P (3HB - 3HV)). Afin d'éviter la toxicité et de contrôler la composition du copolymère (proportion d'HV unité), l'ajout d'acide propionique est contrôlé. La dernière teneur en PHA atteint 70 à 80% du poids sec cellulaire. Selon le substrat utilisé, HV représente de 0 à 30% du copolymère. À la fin de la fermentation, le PHA est purifié par extraction par solvant. Toutefois, les solvants ont été remplacés par des enzymes et des détergents pour solubiliser les composés cellulaires et extraire les polymères (Byrom, 1987, 1990). Les copolymères P (3HB - 3HV) sont commercialisés sous le nom de BIOPOL par Zeneca Bio. Le polymère est produit par culture en batch et la production est d'environ 1000 tonnes par ans (Byrom, 1994).

Par ailleurs la société autrichienne Chemie Linz GmbH produit l'homopolymère PHB par fermentation avec *A. latus* avec du saccharose comme base (Hrabak, 1992). La souche utilisée (*A. latus* btF - 96) permet la production de plus de 100 kg de PHB en une semaine

dans un fermenteur de 15 m 3. Après bioréaction, les cellules doivent être récoltées, lavées à l'eau du robinet, ceci suivi par l'extraction du polymère. Pendant ce processus, les cellules en suspension sont traitées par des solvants (chlorure de méthylène) et le PHB séparé des débris cellulaires, par décantation. Il est précipité (par addition de l'eau) et séché. L'utilisation de ce processus conduit à obtenir du PHB d'une pureté de 99% (Hrabak, 1992; Lafferty et Braunegg, 1984a, 1984b).

1.5.2 Applications

Les propriétés de PHB sont comparables aux plastiques conventionnels (polypropylène). Par conséquent, il peut être traité de la même manière que le polypropylène. Ainsi, l'homo et les copolymères conviennent à certaines applications spécialisées, telles que la libération lente ou l'encapsulation d'appareils biomédicaux (Seebatch et al., 1987). L'unité monomère (3 - hydroxybutyric acide) peut être utilisée pour produire des dérivés pharmaceutiques chiraux complexes ou des agents agrochimiques (Seebatch et al., 1987). En outre, l'application d'une matrice PHB pour des produits pharmaceutiques à libération lente a été étudiée (Lafferty et al., 1988; Korsatko et Wabnegg, 1983).

Les propriétés du copolymère P (3HB - 3HV) (connu sous le nom commercial de BIOPOL), telles que la résistance à l'eau et l'imperméabilité à l'oxygène, le rendent approprié pour beaucoup d'applications, y compris le matériel d'emballage (films et bouteilles), le revêtement de matériaux fibreux tels que papiers et cartons (Cox, 1994; Holmes, 1988; Marchessault et al., 1990).

Toutefois, l'application du PHB est limitée en raison de sa dégradation thermique pendant le traitement, d'une cristallisation relativement lente et de sa rigidité. Ces propriétés peuvent être contrôlées en modifiant la configuration de la chaîne de polymères PHB, produisant différentes compositions ou en utilisant des formulations contenant des coproduits (Cox, 1994). Afin de surmonter ces problèmes (dégradation thermique et crsitasllisation relativement lente), l'utilisation de P (HB - HV) copolyesters avec nucleants a été considérée comme le plus grand succès. En outre, elle permet la réduction de la rigidité, ce qui élargit la gamme d'applications.

1.6 Coût de production de bioplastiques

Les facteurs les plus importants à considérer pour une production économique de PHBs sont le rendement en produit (Yp / s, - PHB g de produit par g de substrat consommé), la productivité (Pr -g PHB produit / L / h) du contenu des cellules de PHB (Yp / X g de PHB / g de cellule sèche) et le rendement des cellules (Yx / s, - g de cellule formée par g de substrat consommé). Yp / s et Yx / l détermine le substrat ou l'exigence des matières premières ou des valeurs plus élevées de ces facteurs de rendement d'un bas niveau de requirement pour substrat. La productivité a un impact direct sur les coûts fixes (équipement) en capital. Le facteur Yp / X a un impact sur le recouvrement des coûts par le biais de L'extraction des PHB par solvant, l'étape suivant la fermentation. Des efforts ont été consacrés à l'optimisation du processus de production du PHB afin de réduire les coûts de production dans les directions suivantes: développement de souches, optimisation / amélioration des processus de fermentation, des méthodes de séparation et utilisation de sources de carbone bon marché.

Comme indiqué précédemment, un grand nombre de supports tels que les hydrates de carbone, des huiles, des alcools, des acides organiques et des hydrocarbures peuvent être utilisés par les microbes producteurs de PHA. Sur la base de la source de carbone, le rendement théorique de P (3HB) a été estimé (Yamane, 1993) et a été utilisé pour déterminer le coût de la production. Basé sur la stoechiométrie biochimique, les valeurs théoriques de rendement varient entre 0.32 PHB / kg (pour le glucose) et de 1.16 kg PHB / kg (pour butanol) (Yamane, 1992). Les coûts et les rendements de substrat affectent les coûts de production. Généralement, le coût des plastiques de synthèse (polypropylène et polyéthylène) est de 1 $ / kg de moins que celui des plastiques biodégradables (reportage sur la commercialisation des produits chimiques, 1994). En outre, le coût de production dépend de celui des matières premières, les coûts d'opération et des processus d'extraction et de purification des polymères utilisées dans la production à grande échelle.

Le rendement des PHA a été amélioré grâce au développement de microorganismes recombinants à rendement élevé (Lee et al., 1994) ou par l'intermédiaire de l'amélioration

des processus dans les stratégies de culture (Shirai et al., 1994). Une autre méthode permettant d'obtenir un rendement élevé des PHA est d'optimiser la phase de croissance (biomasse pour améliorer le rendement, Yx / s) et la phase de production (pour améliorer le rendement PHA, Yp / x) ou de procéder à deux phases (phase de croissance et la phase de production) Séparément (Shimizu et al., 1992). Malgré ces efforts, le coût de production des PHA reste élevé par rapport au coût de production des plastiques synthétiques (Choi et Lee, 1997).

Une plus large utilisation des plastiques biodégradables dans les emballages et les produits jetables comme une solution aux problèmes de l'environnement dépend fortement de la réduction des coûts de production et de la découverte de nouveaux plastiques biodégradables aux propriétés améliorées. PHB représente, environ 25-29% du coût total de production en capital fixe et des coûts directs et la matière première représente 40-50% du coût total de production (Choi et Lee, 1999). Ainsi, le recours à des sources moins chères de carbone (mélasse de betteraves, lactosérum, huiles végétales, hydrolyats d'amidon, etc.) est nécessaire pour réduire sensiblement le coût de production élevé du PHB (Brandl et al., 1990). Le développement de nouvelles souches pour la production à grande échelle sur un substrat moins onéreux et complexes peut aussi contribuer à réduire le coût de production.

1.7 Nouvelles stratégies pour la production de PHA

Un des problèmes majeurs empêchant l'application commerciale des PHA est leur coût élevé de production. Beaucoup d'efforts ont été faits pour réduire ces coûts. Cette nouvelle approche implique l'utilisation de ressources renouvelables de carbone provenant de l'agriculture ou de déchets industriels comme substrat et / ou de boues activées d'une usine de traitement des eaux usées comme source d'accumulation de PHA (Chua et al., 2003; Khardenavis et al., 2007; Kumar et al., 2004). Ces approches ont l'avantage d'économiser sur les coûts de production des bioplastqiues tout en réduisant le volume des déchets en extrayant des boues activées produisant ces polymères. Les économies réalisées sur l'élimination des déchets pourrait réduire le coût de production de PHA attribuant ainsi un avantage économique pour ce processus (Khardenavis et al.,

2007). Cet examen porte sur les récents travaux de recherche sur l'utilisation des déchets et / ou de boues activées de station d'épuration pour la production de PHA.

1.7.1 Déchets de carbone comme substrats pour la production de PHA en utilisant des cultures pures.

D'un point de vue économique, le coût du substrat (principalement la source de carbone) contribue de façon très significative à l'ensemble du coût de production de PHA. Il a été estimé à environ 40% du total des coûts de production de PHA (Choi et Lee, 1999; Dias et al., 2006). Durant la dernière décennie, une grande variété de substrats de carbone à faible coût (par exemple, les sources renouvelables de carbone), telles que les eaux usées, les déchets municipaux, agricoles et industriels, des résidus ou produits (par exemple, de l'amidon, de tapioca hydrolysat, lactosérum, xylose, de mélasse , de malt, de soja et de déchets), le brut carboné (déchets alimentaires ou produits) ont été testés pour la production de PHA par des cultures pures de microbes en raison de leur faible prix et de la disponibilité potentielle (Lee et Gilmore, 2005). Deux sources bon marché de carbone et d'azote sont disponibles à partir de sources industrielles ou agricoles, les déchets et l'excédent des matériaux permettant d'apporter une contribution substantielle à la réduction des coûts de production de PHA (Koller et al., 2005). De cette façon, les déchets municipaux, industriels ou agricoles, tout en abaissant le coût de leur élimination, sont convertis en bioplastiques à valeur élevée et durable (Koller et al., 2005). Par conséquent, les déchets sont prometteurs en tant que sources de carbone pour la croissance bactérienne et leur utilisation peut résoudre le problème environnemental de leur élimination tout en réduisant simultanément le coût de production de PHAs.

Il a été rapporté que *Methylobacterium ps*. ZP24 et *Pseudomonas cepacia*, peuvent produire PHB à partir de lactosérum ou de ses principaux composants tels que le lactose (Yellore et Desai, 1998; Young et al., 1994). ***P. cepacia***, a également été évalué pour la production de PHB xylose, un important sucre hemicellulosique de feuillus (Ramsay et al., 1995; Young et al., 1994). Outre *A. chroococcum*, *Haloferax mediterranei* a été utilisée pour produire des PHB en employant de l'amidon comme source de carbone (Lillo Garcia Rodriguez et Valera, 1990). Une productivité élevée de PHB a été obtenue

par culture de *Ralstonia eutropha* à partir d'hydrolysats de tapioca (Kim Chang Beom et Ho, 1995). *Azotobacter vinelandii* UWD est utilisé pour produire une concentration relativement élevée de PHB (22 g de longueur - 1) avec de la mélasse comme source de carbone (Page et Cornish, 1993).

Le lactosérum est le principal sous-produit de l'industrie de fromagerie qui représente 80 - 90% du volume de lait transformé. Le lactosérum est disponible en grandes quantités mais il est partiellement utilisé comme aliments pour animaux. Sa forte production exige donc d'autre moyen d'élimination et pour accroître la valeur ajoutée de ce matériau. Le lactosérum a été le plus largement étudié pour l'accumulation de PHA à partir de déchets par différents micro-organismes (Khardenavis et al., 2007; Park et al., 2002; Povolo et Casella, 2003). Le lactose est le principal composant du lactosérum et de nombreuses souches de *E. coli* peuvent l'utiliser pour leur croissance (Khardenavis et al., 2007; Park et al., 2002; Povolo et Casella, 2003).

Povolo et Casella (2003) ont constaté que le lactosérum doux contient environ 5% de lactose, 0,2% d'acide lactique et 1% des protéines ainsi que des graisses, des vitamines et des minéraux, et *Hydrogenophaga pseudoflava* DSM 1034 a été un bon candidat pour la production de PHA avec du lactosérum comme source de nutriments. Toutefois, le P (3HB), la concentration et P (3HB) contenus, obtenus à partir des déchets ont été nettement inférieurs à ceux obtenus en utilisant des substrats de carbone purifiés. Par conséquent, les souches recombinantes utilisant une source de carbone bon marché et des stratégies plus efficaces de fermentation ont été élaborées pour accroître l'efficacité de la production de PHA. Lee et al. (1997) ont examiné différentes souches recombinantes d'*Escherichia coli* pour leur capacité à accumuler de grandes quantités de PHB en employant du lactosérum. La plus forte concentration en PHB obtenue était de 5,2 g / L et de 81% en poids sec de la cellule, respectivement.

Wong et Lee (1998) ont étudié la souche recombinée d'*Escherichia coli* CSGC 6576, qui héberge un grand nombre de copies de plasmides contenant les gènes de *Ralstonia eutropha* produisant polyhydroxyalkanoate (PHA) et la synthèse de *E. coli* ftsZ gène.

Cette souche a été employée pour produire du PHB) à partir de lactosérum. Ils ont obtenu des cellules ayant de hautes concentrations de 87 g de l - 1 80% PHB à 49 h (Wong et Lee, 1998).

Des déchets à base d'huiles comestibles provenant de l'industrie et des services alimentaires sont en grande partie détruits par incinération ou perdus dans l'environnement - les deux itinéraires étant inacceptables dans une perspective environnementale verte. La production de PHA a été réalisée par *Ralstonia eutropha* dans des déchets d'huiles et de graisses comestibles comme sources de carbone (Taniguchi et al., 2003). L'efficacité de ce processus est intéressante et élevée et le polymère peut être facilement extrait. Ainsi, un déchet est converti par une méthode sans danger pour l'environnement en un produit valable (Taniguchi et al., 2003).

Les huiles végétales et les graisses sont des coproduits agricoles renouvelables et peu coûteux et donc, ce sont les candidats les plus qualifiés pour la production microbienne de PHA. Plusieurs *Pseudomonas* produisent ces polymères à partir de suif et d'huiles végétales. *Aeromonas caviae* également accumule PHBx en employant de l'huile d'olive, mais les rendements sont assez faibles, dans tous les cas (Doi et al., 1995; Fuchtenbusch et al., 2000; Taniguchi et al. ., 2003). Des graisses / huiles agricoles renouvelables et peu coûteuses et leurs déchets sont potentiellement utiles pour la production de PHA par fermentation par *R. eutropha* (Taniguchi et al., 2003).

Des déchets huileux agro-industriels ont également été utilisés pour obtenir des PHAs par *Pseudomonas* VIM 42A2 (Fernandez et al., 2005). Un taux de 54,6% accumulé a été obtenu lorsque l'acide oléique technique (TOA) est utilisé comme source de carbone. L'accumulation du PHA atteint 66,1% quand des déchets d'acides gras libres à partir d'huile de soja (WFFA) sont employés comme substrat de carbone, 29,4% avec des déchets d'huile de friture (WFO) et 16,8% quand le glucose a été utilisé. Selon le substrat fourni, un large éventail de composants a été observé (Fernandez et al., 2005). Il a été constaté que les déchets résiduels de friture et d'autres déchets huileux donnent des

résultats satisfaisants comme substrats pour la production de PHAs (Fernandez et al., 2005).

De grandes quantités d'homopolymères contenant β - hydroxybutyrate (PHB) et des copolymères β - hydroxyvalérate (P [HB - HV al.aboration]) sont produites par croissance d'*Azotobacter chroococcum* , souche H23 dans un milieu de culture modifié avec alpechìn (eaux usées provenant d'usines d'huile d'olive) comme seule source de carbone. Copolymère est formé quand du valérate (pentanoate) est ajouté en tant que précurseur de l'alpechìn moyen, mais il n'a pas été formé par l'addition d'un précurseur du propionate. *A. chroococcum* synthétise des homo et des copolymères de PHA et ce, jusqu'à 80% du poids sec des cellules, lorsqu'elle est cultivée sur NH4 + - complété avec 60% (v / v), alpechı'n, après 48 h d'incubation à 100 rév Mn - 1 et 30 ° C. La production de PHA par la souche H23 utilisant alpechı'n semble prometteuse, comme un substrat bon marché pour l'obtention de ces bioplastique; ceci étant essentiel pour les rendre compétitifs (Pozo et al., 2002). Le contenu en polymère (88,6% de la masse sèche des cellules) obtenu au cours de la présente étude est le plus élevé à partir de déchets organiques, et il est comparable au contenu de PHA au cours de la fermentation avec du saccharose pur (Wang et Lee, 1997).

Les déchets solides organiques, toutefois, sont généralement sous forme complexe qui ne peut pas être directement digéré et utilisé par les microbes produisant des PHAs telles que *Ralstonia eutropha*, un représentant pour de la synthèse bactérienne de ces bioplastiques (Du et al., 2004; Yu, 2001) Pour améliorer la production en PHA en utilisant des déchets organiques, une phase de prétraitement peut être favorable. L'hydrolyse et l'acidogénèse sont la première étape pour convertir les solides biodégradables en acides gras volatils à chaînes courtes tels que, acétique, propionique, butyrique et autres acides pouvant être utilisés par les bactéries productrices de PHA.

Yu (2001) a étudié la production de PHA en employant des eaux usées de féculents selon un processus en deux étapes microbiennes, l'acidogénèse et la polymérisation de l'acide. Les déchets organiques en question ont été digérés dans un réacteur anaérobie pour les

boues (UASB) pour synthétiser des acides, acétique (60-80%), propionique (10-30%) et butyrique (5-40%) acides. Les effluents acides, après microfiltration, ont été introduits dans un deuxième réacteur dans lequel *A. eutrophus* a produit des acides PHA. Celui- ci a été formé à partir de l'acide butyrique et de poly (hydroxybutyrate - hydroxyvalérate) obtenu de l'acide propionique avec 38% d'hydroxyvalérate (Yu, 2001); Le PHA étant un copolymère de poly (3 - hydroxybutyrate - co- 3 - hydroxyvalérate) (PHA) avec 2,8% d'hydroxyvalérate (Du et al., 2004).

Si des déchets produits / flux peuvent être utilisés comme substrat pour la production de P (3HB), les avantages de la réduction des coûts d'élimination et de la production de produits à valeur ajoutée seront très valables.

1.7.2 Cultures mixtes de boues activées pour la production de PHA

Une nouvelle stratégie de production de PHA utilisant des cultures bactériennes mélangées à des boues activées a été proposée. De nombreux travailleurs ont montré que les boues activées ont la capacité de produire des bioplastiques (Dionisi et al., 2004; Lemos et al., 2004; Lemos et al., 2003; Lemos et al., 1998; Reis et al., 2003; Satoh et al., 1998a; Serafim et al., 2004a; Serafim et al., 2006; Yan et al., 2006).

Il est bien connu que les PHAs jouent un rôle important en matière de stockage du carbone dans les micro-organismes présents dans des boues activées, en particulier dans les processus anaérobie - aérobie de ces boues ou dans le cycle du phosphore (Enhanced Biological Removal (EBPR) processus) (Mino et al., 1998). Dans ce processus EBPR, les micro-organismes consomment du polyphosphate comme source d'énergie pour l'absorption en anaérobie du carbone contenu dans les substrats : les substrats carbonés repris étant temporairement stockés en PHA. En conditions aérobie, les PHAs sont utilisés pour la croissance et le polyphosphate de régénération. Les micro-organismes présents dans le processus EBPR possèdent donc posséder les caractéristiques de déphosphatation et d'accumulation de PHA. L'idée de la production de PHA à l'aide de boues activées est fort intéressante. En comparaison avec les procédés de fermentation par des cultures pures, la production de PHA par des boues activées présente les

avantages suivants: réduction des coûts de production de PHA; plus simple facilité de construction; réutilisation des boues excédentaires de traitement des eaux usées (WWTPs), contribution à la réduction de l'excédent de la production de boues de station d'épuration et création d'une nouvelle méthode pour recycler par conversion des polluants organiques en plastiques biodégradables (Chua et al., 2003; Satoh et al., 1998a).

1.7.2.1 Biosynthèse de PHAs par la culture dans des boues activées

- Production par anaérobie - aérobie de PHA par des boues sous conditions anaérobies

Deux populations microbiennes connues sont capables en anaérobie d'utiliser des substrats organiques selon le processus EBPR: (i) accumulation de Polyphosphate (PAO) par les organismes et (ii) accumulation de glycogène (GAO) (Cech et Hartman, 1993). Plusieurs modèles métaboliques pour la stoechiométrie de l'absorption de l'acétate dans des conditions anaérobies mixtes par culture de PAO et de GAO ont été élaborés (Filipe et al., 2001a, b; Hesselmann et al., 2000; Pereira et al., 1996). La suppression biologique du phosphore est fondé sur l'enrichissement de boues activées par des organismes accumulant du Polyphosphate (PAO). En anaérobiose, le PAO est pélevé des substrats organiques (de préférence, des acides gras volatils) et il est entreposé en PHA. En 'aérobiose ou en phases anoxiques, les PHAs internes sont oxydés et utilisés pour la croissance, la synthèse de glycogène et l'entretien (Smolders et al., 1994b). Il a été signalé que des microbes accumulant glycogène non polytoxicomanie P (GAO, aussi appelé G bactéries) peuvent contribuer lors de l'enlèvement biologique du P dégradé (Cech et Hartman, 1993; Liu et al., 1994; Matsuo, 1994; Satoh et al. ., 1994). Ces organismes sont également en mesure d'utiliser en anaérobie, des substrats organiques qui sont métabolisés et stockés en PHA, tandis que l'énergie et la réduction des équivalents sont fournis uniquement par la dégradation du glycogène sans implication de la polyconsommation du P. En absence de glycogène, l'absorption du susbtrat en anaérobie par PAO ou GAO ne peut se produire (Brdjanovic et al., 1998; Mino et al., 1996).

L'acétate est un des substrats les plus étudiés pour la production de PHA par des cultures mixtes. Lorsque c'est la seule source de carbone disponible dans la phase d'anaérobie, l'unité de 3 - hydroxybutyrate est la principale unité constitutive dans les PHAs (Satoh et

al., 1992). Le 3 - Hydroxyvalérate est formé par conversion du glycogène en propionyl CoA via le succinate de - propionate et réaction avec l'acétyl-CoA (Pereira et al., 1996).

Satoh et al. (1992) et Satoh et al. Inoue et al. (1996) ont montré que le PHA produit par des boues activées en anaérobie - aérobie est un mélange de P (3HB - al.aboration 3HV - co3H2MB - al.aboration 3H2MV) de différentes compositions. La présence et la proportion relatives des différents PHAs sont fonctions du type de substrat de carbone disponible. Lorsque l'acétate est la seule source disponible dans la phase d'anaérobie, le 3HB unité est la principale unité des PHAs (Satoh et al., 1992; Smolders et al., 1994a). Par ailleurs, la composition de PHA formés à partir d'autres sources de carbone peuvent être théoriquement fondée sur les modèles stœchiométriques développés par Mino et al. (1994) et Satoh et al. (1996).

Au cours du traitement des eaux, des matériaux de stockage de PHA sont généralement accumulés par les microorganismes dans le cadre d'un régime d'abondance et en cas de famine, le stockage des PHAs dans l'EBPR est largement accepté (Van Loosdrecht et al., 1997). Les PHAs sont non seulement accumulés sous des conditions anaérobies, mais aussi, ils peuvent être produits en phases aérobies ou anoxiques. Au cours de la dernière décennie, de nombreux chercheurs ont signalé la production de PHAs par des cultures mixtes dans des conditions dynamiques de boues activées. La croissance de la biomasse et le stockage des polymères se produisent simultanément quand il y a un excès de substrat externe (période de fête) ; Lorsque tous les substrats externes sont consommés, le stockage sous forme de polymères a lieu comme une source de carbone et d'énergie (période de famine).

Il est important de choisir le substrat approprié pour optimiser la production de PHA. La nature du substrat non seulement détermine le contenu de PHA synthétisé par les cellules, mais aussi sa composition, ce qui, par la suite affecte les propriétés finales des polymères obtenus. En outre, le coût de la source de carbone contribue de manière significative au prix global de revient de la production de PHA (Yamane, 1992, 1993) : le prix des sources de carbone pouvant représenter jusqu'à 70 à 80% des matières

premières, celles-ci représentant 40 - 50% du total des coûts d'exploitation (Choi et Lee, 1997). Le prix des PHA peut être sensiblement réduit avec des substrats organiques bon marché, tels que les déchets de l'agriculture et des industries alimentaires (par exemple, le lactosérum et de la mélasse) ou autres.

L'acétate est le plus utilisé comme unique source de carbone pour la production de PHA en cultures mixtes ou avec des boues activées. Seules quelques études ont estimé le potentiel d'acides gras volatils (VFAs) comme le propionate, le butyrate ou leurs mélanges (Beccari et al., 1998; Lemos et al., 1998) Les effets d'autres composés carbonés, comme le lactate, le succinate, le pyruvate, le malate (Satoh et al., 1992), l'éthanol (Beccari et al., 1998; Majone et al., 2001), l'aspartate, le glutamate (Satoh et al., 1998b) et le glucose (Dircks et al., 2001) sont encore moins étudiés.

Hollender et al. (2002) a montré (voir plus haut) que les PHAs accumulés par des boues activées pendant la phase d'anaérobie avec de l'acétate comme source de carbone est de 20 en mgC comme PHA / g de matière sèche. Avec le glucose, ils n'atteignent que 6 en mgC comme PHA / g de matière sèche, DM . Ceci est conforme à la libération observée du phosphate en anaérobie / aérobie parce que la production de l'énergie pour la formation de PHA est réalisée par la polyconsommation de l'hydrolyse du P.

Au cours de l'accumulation de PHA, le plus haut taux de consommation de substrat a été observé avec l'acétate comme source de carbone, suivie du propionate et du butyrate finalement (Lemos et al., 1998) en utilisant des boues activées. Les rendements des polymères produits par la consommation de carbone (Yp / l: mg de polymère / mgCOD) lors de l'utilisation de l'acétate, du propionate et du butyrate sont de 0,97, 0,61 et 0,21, respectivement. Ces résultats montrent que, dans ce système, l'acétate est le meilleur substrat par l'accumulation de phosphore par les bactéries pour la production de PHA.

Lemos et al. (1998) a remarqué que lors de l'utilisation de supports mixtes, la valeur Yp / l est la somme des contributions individuelles de chaque substrat. Avec une concentration de carbone de 320 mgCOD / l, des montants identiques d'unités de HB et HV ont été

produites. L'utilisation du substrat le plus concentré en carbone conduit à la consommation de propionate d'abord et au P (HB - al.aboration HV) enrichis en HV unités.

1.7.2.2 Effet des paramètres d'exploitation.

DO.

Bien que les boues activées acclimatées sous conditions aérobies, anaérobies accumulent des PHAs, il n'ya aucune garantie que le fonctionnement du processus anaérobie - aérobie de boues activées soit meilleur pour accroître de l'accumulation de PHA par les micro-organismes. Satoh et al. (1998) ont constaté que les boues accumulent plus de PHB en aérobiose que dans des conditions anaérobies lors de l'utilisation de l'acétate comme substrat. L'accumulation de PHA augmentant de façon linéaire avec l'accroissemengt des taux d'alimentation en oxygène. Toutefois le maximum d'accumulation de PHA se maintient à 33% des boues de poids sec (pour une alimentation en taux d'oxygène de 8 mg O_2/min/gMLVSS). Afin d'accroître encore davantage le contenu en PHAs produits par des boues activées, Satoh et al. (1998) ont introduit un nouveau processus de boues activées, provisoirement baptisée "microaerophilic - aérobie" boues activées. Le processus anaérobie - aérobie des boues activées augment l'accumulation en PHA par des boues capables d'utiliser le glycogène et / ou le polyphosphate, car ces métabolites sont nécessaires en aérobie pour l'absorption du substrat. Satoh et al. (1998) s'attendent à ce que l'introduction de petites quantités d'oxygène dans la zone du processus anaérobie anaérobie - aérobie favorise l'accumulation de PHA si les boues activées ne sont pas capables d'accumuler du glycogène et / ou du polyphosphate. Une teneur de 62% en PHA aété atteinte en utilisant des boues activées acclimatées lors d'une incubation de 30 heures en microaerophilic - aérobie avec l'acétate. Les résultats des troisièmes et al. (2003) montrent que le rendement en PHA avec acétate atteignait 0.49 g de PHA. G de - 1 substrat lorsque l'oxygène est limitant et 0,34 g de PHA g de - 1 substrat en excès d'oxygène.

Température

L'influence de la température (15, 20, 25, 30 et 35^{o}C) sur l'accumulation de PHB par des boues activées contenant les microbes identifiés par séquençage croissant en bio-réacteur avec de l'acétate a été étudiée par Krishna et Van Loosdrecht (1999). Le taux de formation de PHB a diminué avec l'augmentation de la température en raison d'une élévation des taux d'anabolisants à des températures plus élevées; Le niveau de PHB obtenu étant le plus haut (0,21 Cmmol PHB / Cmmol biomasse) à 15oC et le plus bas (0.06 Cmmol PHB / Cmmol biomasse) à 30^{o}C.

Chinwetkitvanich et al. (2004) ont également déterminé les effets de la température sur la production de PHB et son stockage par des boues activées par la biomasse. L'opération en deux temps, soit une phase de croissance suivie d'une phase de limitation des éléments nutritifs, a été appliquée pour induire l'accumulation de PHA. Les effets de températures pré-sélectionnées de 10, 20 et 30°C ont été étudiés en utilisant les systèmes SBR aérobie. Il a été constaté que la production de PHA est plus importante à 10 ° C qu'à 20°C et 30°C. Toutefois, il y avait peu de différence entre les deux plus hautes températures : Le maximum de fractions de PHA atteignant 52, 45 et 47% des SAT pour les trois températures respectivement de la plus basse température à la plus haute.. Ces résultats sont partiellement en accord avec ceux de Krishna et Van Loosdrecht (1999). La production de PHA augmente avec une baisse de la température, à des températures plus élevées, les tendances sont inversées.

pH

Chua et al. (2003) ont évalué le comportement des boues activées pour la production de PHA en utilisant l'acétate comme source de carbone sous différentes conditions de pH. De 6 à 9, le taux de PHA produit par les boues augmente. À pH 6 et 7, il ya très peu d'accumulation de PHA, soit un contenu inférieur à 5% en poids sec des boues . À pH 8 et 9, l'accumulation de PHA est stimulée, et le taux de PHA atteint 25-32% du poids sec des boues. Ces résultats indiquent que le contrôle du pH est essentiel à l'optimisation du processus de production PHA et le pH $\geq$ 8 est recommandé.

La conservation de la boue (SRT)

Chua et al. (2003) ont étudié la conservation des boues (3 et 10 jours) sur la production de PHAs avec l'acétate comme source de carbone. Ils ont observé que plus la conservation est longue, plus la teneur en biomasse est élevée dans le bioréacteur; Cela pouvant entraîner à la hausse, la capacité des boues activées à produire des PHAs en minuscules MLSS (ou moins SRT, c'est-à-dire 3 jours). En outre, le processus de boues activées avec un accroissement de SRT conduit normalement à un taux élevé de la biomasse inerte; ceci pouvant par contre contribuer à abaisser la teneur en PHA.

Rapport C / N

Chua et al. (1997) ont examiné l'effet du rapport C / N sur la production des PHBs . Le contenu des cellules en polymère augmente jusqu'à un maximum de 37,4% en poids sec lorsque le ratio C: N passe de 24 à 144, alors que le rendement des cellules (g de cellules formées par g de TOC utilisés) diminue suite à l'augmentation du rapport C: N. Un taux optimal du C / N soit 96 fournit le plus haut rendement, soit 0,093 g de polymère par g du substrat consommé Le rapport C / N dans les boues activées processus doit être maintenu autour de 24 pour permettre une synthèse normale des cellules microbiennes.

L'azote et / ou de la limitation de phosphore

L'accumulation de PHA par les bactéries peut être stimulée dans des conditions de croissances défavorables telles que privation d'oxygène, d'azote, de phosphate, de soufre, de magnésium ou de potassium et ce, en présence d'un excès de carbone (Chinwetkitvanich et al., 2004). Ces Auteurs ont constaté que toutes les expériences avec le P ont conduit à limiter de grandes accumulations de PHA, exprimé en% du SAT dans la biomasse; Les rendements en mg de PHA par mg de CODu et en productivités (en mg / u) sont sensiblement inférieurs à ceux obtenus au cours d'une limitation d'azote (Chinwetkitvanich et al., 2003).

Procédés utilisant des boues activées pour la production de PHA

Ces dernières années, l'intérêt pour la production de PHA par des cultures mixtes a fait l'objet de nombreuses études. Les stratégies de fermentation visant à améliorer le taux de

PHA produit, le rendement et la productivité dans les cultures pures sont très bien définis. Cependant, ces données concernant les cultures mixtes ou les boues activées sont encore très limitées.

Parmi les différents procédés décrits pour la production des PHAs par des cultures mixtes, la stratégie de l'abondance suivie d'une limitation des éléments nutritifs est la plus prometteuse en raison de la forte teneur de PHA produite par les boues et de la productivité observée. Le substrat est ajouté pendant une courte période de temps, suivi d'une longue période de restriction de nutriments. Cette période de famine est nécessaire pour stimuler la capacité de production et de stockage de PHA par les cellules. Ce processus est effectué le plus souvent dans un réacteur avec un ajout séquencé de nutrition (SBR). Les SBRs sont très valables pour sélectionner en réacteurs, des populations microbiennes robustes ayant de fortes capacités de stockage de PHA , car la biomasse se développe selon en régime instable. En outre, ce type de réacteur est très souple et facile à contrôler, permettant une rapide modification des conditions de bio-réaction (durée de l'alimentation et la longueur du cycle).

Serafim et al. (2004) ont constaté que les boues soumises à une alimentation en aérobie dynamique peuvent accumuler des quantités élevées de PHA et cela, en manipulant les concentrations des nutriments et les paramètres de fermentation dans le réacteur. Une teneur maximale de PHB (67,5%), a été obtenue pour 180 Cmmol / l d'acétate fourni en une seule impulsion. Toutefois, cette forte concentration de substrat s'est révélée inhiber les mécanismes de stockage, ce qui entraîne un ralentissement du taux d'accumulations spécifiques de PHB. Afin d'éviter l'inhibition par le substrat, 180 Cmmol / l d'acétate ont été ajoutés par différentes manières: addition en continu ou trois impulsions de 60 Cmmol / l chacune. Dans les deux cas, le taux de stockage spécifique de PHB a augmenté atteignant respectivement 56,2% et 78,5%. Cette dernière valeur du contenu en PHB est analogue à ce qui est obtenu avec des cultures pures et n'avait jamais été signalé, jusqu'à présent pour des cultures mixtes.

Dionisi et al. (2005) ont étudié le fonctionnement d'un nouveau procédé pour la production de biopolymères (PHA), appliquant différents valeurs de la charge organique (OLRs) dans un SBR. Le processus est fondé sur l'enrichissement en aérobie des boues activées pour obtenir des cultures mixtes capables de stocker les PHA à des niveaux et des rendements élevés. La meilleure performance du processus a été obtenue à un niveau intermédiaire OLR (20 gCOD / L / jour), où la productivité de la biomasse et le stockage de PHA étaient suffisamment élevés.

Pour un système de production de PHA désiré, une configuration possible pourrait comprendre deux réacteurs en série dont un plug-in flux réacteur (PFR) suivi d'un réservoir réacteur en agitation continue (CSTR), couplé à un colon ou une membrane filtrante. Beccari et al. (1998) a utilisé un CSTR avec interruptions d'alimentation. Ce processus sélectionne et produit des boues à haute capacité de stockage. Celui-ci et l'accumulation de rendement de PHA varient de 0,06 à 0,5 g COD / g de COD , ce qui représentent jusqu'à 40-50% du poids sec total des boues activées.

La plupart des études sur la production de ces polymères par des cultures mixtes ou des boues activées ont été menées avec des boues activées acclimatées avec des eaux usées obtenues synthétiquement afin d'évaluer la faisabilité de la production de PHA par ces boues, mais leur capacité dans la phase de traitement maximal des eaux usées n'a pas été signalée (Beun et al., 2000a; Beun et al., 2000b; Chua et al., 2003; Lemos et al., 1998; Satoh et al., 1998a; Satoh et al., 1998b; Satoh et al., 1996).

Le peu de données montre que l'homo et les copolymères et leur poids moléculaire moyen obtenus par des cultures mixtes sont semblables à ce qui est produit par l'utilisation de cultures pures (Lemos et al., 1998).

1.7.3 Production de PHA en utilisant des boues activées comme micro-organismes et les déchets comme sources de carbone

Le processus de production de matières plastiques biodégradables par des cultures mixtes microbiennes,en particulier bactériennes, en utilisant des déchets disponibles (ressources

renouvelables) fait l'objet de la plupart des études actuelles. (Chua et al., 2003; Khardenavis et al., 2007; Md Din et al., 2006; Rhu et al., 2003).

Chua et al. (2003) ont examiné la production de PHAs par des boues activées provenant d'usines de traitement d'eaux usées municipales. Il ont constaté que les boues acclimatées dans des eaux usées municipales avec addition d'acétate peuvent accumuler des PHAs jusqu'à 30% de leur poids sec, tandis que pour des boues acclimatées avec seulement des eaux usées municipales, ce taux atteint seulement 20% du poids sec.

Dans une autre étude, Rhu et al. (2003) évaluent la production de PHAs avec SBRs employant des déchets alimentaires. Les souches de microbes ont été collectées à partir d'une usine d'épuration biologique fonctionnant selon un procédé d'élimination des nutriments. Ces microorganismes ont été acclimatés avec substrat synthétique provenant de la fermentation de déchets alimentaires. Une teneur maximale de PHA de 51% a été atteinte avec un cycle anaérobie / aérobie comprenant une limitation de P.

La production de PHA par des boues activées a également été étudiée avec des eaux usées synthétiques (65,8% du poids sec des cellules) et en anaérobie des eaux usées (58%) (Khardenavis et al., 2005; Khardenavis et al., 2007). La production de biomasse bactérienne et du PHB a été accrue par l'aération dans un milieu synthétique à l'échelle du laboratoire.

Khardenavis et al. (2007) a évalué le potentiel de production de PHA de boues activées isolées à partir d'une station d'épuration traitant des rejets laitiers et d'industries alimentaires. Les résultats ont montré que des déchets de distillerie de grains déprotéinisés permettent d'obtenir un taux de PHB de 42,3% (p/p), alors qu'un filtrat à base de rejets de distilleries de riz , donne un taux de PHB de 40% lorsqu'ils sont utilisés comme substrats. L'adjonction d'ammonium di- hydrogène phosphate (DAHP) a augmenté la production de PHB à 67% lorsque le grain de riz brut a été utilisé. Pour les mêmes eaux usées, mais après élimination des matières solides en suspension par filtration et avec addition de DAHP, il y a eu une baisse de production (57.9%).

Toutefois, l'enrichissement d'autres déchets avec du DAHP conduit à une diminution substantielle de la synthèse de PHB par rapport à ce qui est observé en l'absence de DAHP. Bien que le contenu en PHB obtenu dans la présente étude (67%) soit inférieur à celui constaté avec des cultures pures (jusqu'à 80%), les résultats sont parmi les plus élevés rapportés jusqu'à ce jour en ce qui a trait à des boues activées et en utilisant les eaux usées en tant que substrat (Khardenavis et al., 2007).

Md Din et al. (2006) ont étudié la production de PHA unique à l'aide de lots de cultures mixtes et de ressources renouvelables. Un réacteur SBR a été utilisé pour ces travaux. La culture mixte initiale a été collectée en utilisant 10% des boues activées d'une usine de traitement d'eaux usées et 90% des effluents d'usines d'huile de palme (POME). La culture a été maintenue en un Fed-Batch réacteur et opéré en deux étapes soit croissance et stade d'accumulation. En premier lieu, ce système permet une croissance extensive (en utilisant le milieu nutritif) puis une limitation des éléments nutritifs (pas de milieu nutritif l'adaptation) survient à l'étape suivante. La production moyenne de PHA a atteint seulement 44% du poids sec des cellules. Cependant, des facteurs favorables (par exemple, la température et le temps de récolte) sont appliqués lors de la prochaine étape pour induire la production de PHA (Md Din et al., 2006).

1.8 Isolement à partir de boues d'eaux usées municipales ou industrielles, de souches bactériennes accumulant des PHAs

Le processus à base de boues activées est le plus largement utilisé pour la technologie de traitement des eaux usées municipales et industrielles. La communauté microbienne appelée "boues activées" est une population mixte contenant de nombreuses espèces de virus, de bactéries, de protozoaires, de champignons, de métazoaires et d'algues. Les mouvements démographiques au sein de cette communauté peuvent résulter des changements dans les conditions d'exploitation des usines de traitement et qui causent des problèmes de qualité pour la clarification des boues, leur compactage et leur déshydratation (Chipasa et Medrzycka 2004; Nielsen et Nielsen 2002a; Nielsen et al. 2004; Wagner Et Loy 2002). La plupart des procédés modernes de traitement des eaux usées reposent maintenant sur la composition et l'activité microbienne des boues

activées. Par conséquent, l'identification des microorganismes présents dans les boues activées est d'un intérêt considérable. La détermination du rôle dominant de chacun des microbes dans l'écosystème des boues est directement liée au contrôle du procédé de traitement pour accroître la performance des WWTPs (Nielsen et al. 2004).

De nombreuses espèces bactériennes sont présentes dans les boues activées. Elles le sont soit en tant que cellule unique formant des microcolonies ou en tant que bactéries filamenteuses. Les quantités de bactéries dans les boues activées sont de l'ordre de 1-10 × 10^{12}/g de VSS. Parmi elles, généralement, 80% sont actives ou vivantes (Nielsen 2002, Nielsen et al. 2004). Dans une usine de traitement pour éliminer des nutriments, différentes souches bactériennes ayant des fonctions diverses peuvent être présentes, y compris des microorganismes accumulant du phosphore (PAOs) (Ansa - Asare et al. 2000; Carucci et al. 2001; Crocetti et al. 2000; Das et al. 2004; Kawaharasaki et al. 1999; Lina et al. 2003; Nakamura et al. 1995b; Nielsen et al. 1999; Oehmen et al. 2005; Pijuan et al. 2005; Reddy et Bux 2002; Sidat et al. 1999a; Wong et al. . 2005; Zhang et al. 2003).

Des bactéries oxydant l'ammoniac (Purkhold et al. 2000); des bactéries oxydant les nitrites (Nogueira et al. 2002), d'autres réduisant les nitrates (Dionisi et al. 2002; Drysdale et al. 1999 ; Etchebehere et al. 2001; Jetten et al. 2001; Juretschko et al. 2002b; Khan et Hiraishi 2001; Nogueira et al. 2002); certains organismes accumulant du glycogène (GAO) (Liu et al. 1996; Seviour et al. 2000) ; Des micro-organismes filamenteux (Blackall et al. 1996b; Francis et al. 2002; Kämpfer 1997; Kanagawa et al. 2000; Kitatsuji et al. 1996; Rossett et al. 1997), des bactéries réductrices du Fe (III) ou des sulfates (Kjeldsen et al. 2004), des bactéries productrices de méthane (Nielsen et Nielsen 2002a; Nielsen et al. 2004). Les groupes réducteurs de fer ou des sulfates et les méthanogènes sont généralement considérées comme moins importants dans le processus d'action normale des boues activées. Sous certaines conditions (par exemple, lorsque des problèmes de papier bouffant apparaissent), une domination des bactéries oxydant les sulfures ou autres peut être observée. Il est important de noter que jusqu'à présent, la majorité des bactéries présentes dans les boues activées sont très peu connues comme par exemple des hétérotrophes aérobies ou dénitrifiantes. De plus, très peu de choses sont diffusées au sujet de leurs principales fonctions dans l'activité des boues (Nielsen et al.

2004; Strous et al. . 2002; Wagner et Loy 2002). Ces groupes de bactéries peuvent être observées dans la plupart des types d'usines de traitement. Toutefois, l'identité taxonomique directe au niveau de l'espèce est pratiquement inconnue, sauf pour quelques groupes. La plupart de ces groupes et d'autres espèces de bactéries présentes dans les boues ne sont pas encore cultivées , ce qui ne permet pas leur identification par les méthodes moléculaires indépendantes de la boue (Coþkuner 2002; Forster et al. 2002; Kim et al. 2004; Nielsen et Nielsen 2002a; Nielsen 2002 ; Radajewski et al. 2000; Wagner et Loy 2002). En fait, un grand nombre de micro-organismes ont été isolés et les genres les plus fréquemment trouvés cités sont les suivants: *Pseudomonas, Bacillus, Achromobacter, Enterococcus, Acinetobacter, Aeromonas, Alcaligenes, Arthrobacter, Escherichia, Salmonella, Proteus, Streptococcus, Staphylococcus, Micrococcus, Corynebacterium, Clostridium , Penicillium* (Mehandjiyska 1995), etc. De nombreux chercheurs rapportant que la majorité des bactéries dans les boues activées appartiennent aux Gram négatifs (Pick 1995; Sharifi -Yazdi et al., 2001).

1.8.1 Méthodes d'isolement et d'identification

Les approches actuelles pour l'identification des microbes présents dans les boues activées comprennent: une analyse systématique traditionnelle, une observation sous microscope, une analyse moléculaire systématique utilisant le séquençage des acides ribonucléiques ribosomiques 16S (ARNr), des sondes oligonucléotides16S ARNr, l'analyse de la communauté de16S ARNr, le concept de sondes de diagnostic, et / ou des sondes génomiques (Blackall et al. 1998; Lajoie 1997).

Les communautés microbiennes présentes dans les WWTPs ont été analysées depuis des décennies, soit par la lumière ou par l'observation microscopique dépendant de techniques de culture ; L'identificaton étant basée sur la forme (par exemple, les bâtonnets, coccus), des parois cellulaires de type (Gram négatif ou Gram positif) et sur une série de tests biochimiques (par exemple, oxydase positive ou négative), et le nombre total de bactéries est généralement évalué par coloration au 4 ',6 - diamidino - 2 - phenylindole (DAPI) et par la fraction positive soit par hybridation in situ en fluorescence (FISH) avec des sondes oligonucléotidiques de toutes les bactéries avec un

contenu significatif de ribosomes, ou la fraction permettant de compter les substrats radioactifs tels que mesurés par microautoradiographie (MAR) (Nielsen et Nielsen 2002a).

L'hybridation in situ par fluorescence (FISH) est une technique de détection de séquences d'ADN ou d'ARN dans les cellules. Ce procédé de biologie moléculaire permet de localiser des séquences d'ADN spécifiques dans le génome des micro-organismes et donc l'identification de microbes dans des conditions environnementales différentes. La quantification directe des souches bactériennes est possible par comptage manuel des cellules hybridées par fluorescence incidente et laser en confocal, soit par l'analyse des images de photos numériques (ainsi que les microscopes) ou comptage automatique avec un cytomètre de flux. FISH est devenu rapidement l'une des techniques de coloration fluorescentes les plus largement utilisées en raison de sa sensibilité, de sa polyvalence et de l'amélioration des technologies actuelles ainsi que de la rentabilité : son utilisation va sûrement continuer à s'accroître (Blackall et al. 1998; Lajoie 1997).

Le DAPI est connu pour former des complexes fluorescents avec l'ADN, montrant une spécificité de fluorescence à l'observation sous microscope. En raison de cette propriété, le DAPI est un outil utile dans diverses analyses cytochimiques (Nielsen et Nielsen 2002a).

La technique la plus sensible et la plus utilisée pour l'énumération des bactéries métaboliquement actives est la méthode en microautoradiographie (MAR) (Hoppe 1976). Les bactéries qui sont activement engagées dans l'absorption du substrat sont détectées par cette méthode et qui sont mesurées à l'aide d'un traceur marqué radioactivement tels que généralement la thymidine (un nucléoside) ou d'un mélange d'acides aminés (Nielsen et Nielsen 2002a).

La systématique moderne est fondée sur la détermination des séquences nucléotidiques de l'ARN dans les sous-unités 16S des ribosomes (16sRNA). Tous les RNA sont composés des quatre nucléotides, adenine (A), cytosine (C), guanine (G) et uracile (U). La séquence de ces quatre bases des RNA 16S est utilisée pour classifier et identifier des

micro-organismes; L'approche dite à cycle complet des ARNr impliquant la création d'une bibliothèque des gènes ARNr 16S ainsi que la conception et l'utilisation de clones de sondes spécifiques pour l'analyse FISH (Juretschko et al. 2002b).
La méthode du cycle complet ARNr offre une alternative pour l'identification de nouveaux micro-organismes. Il convient toutefois de souligner que même une étude complète des ARNr ne peut se substituer à l'isolement et à la caractérisation des micro-organismes. Actuellement, seule une partie des applications de l'approche ARNr englobe le séquençage et le sondage. Il n'est pas surprenant que les demandes initiales du procédé ARNr sont axées sur l'identification des micro-organismes présents dans les échantillons les moins complexes. Une fois que son adaptation est démontrée, il sera possible de généralisera son application (Juretschko et al. 2002a).

1.8.2 Souches bactériennes isolées de boues activées pour la production de PHA

À l'heure actuelle, seules quelques souches bactériennes capables d'accumuler des PHAs ont été isolés du système anaérobie / oxic (A / S) (Liu et al., 2000; Sawayama et al., 2000; Vincenzini et al., 1997). L'espèce la plus couramment isolée est *Acinetobacter* ps dont la souche *Acinetobacter* SP. PHB pourrait accumuler jusqu'à 11,5% en poids sec dans la cellule après croissance dans un milieu contenant de l'acétate comme source de carbone (Vincenzini et al., 1997). Un autre isolat du système A / O appartient au nouveau genre *Amaricoccus* de la subdivision *alpha Proteobacteria* (Liu et al., 2000).

Récemment (Dionisi et al., 2006), après avoir confirmé la spéciation de la population pour l'accumulation de PHA par DGGE, ont construit une bibliothèque de clones en se basant sur l'ADN total extrait des boues SBR. Le dépistage des clones a été réalisé à l'aide d'ADN ribosomique amplifié et par analyse de restriction (ARDRA). Toutefois, les isolats n'ont pas encore été associés par rapport à l'accumulation de PHAs. Un examen plus poussé concernant ces groupes microbiens est nécessaire pour déterminer leur statut de producteurs de ces polymères.

Une approche différente a été employée par Serafim et al. (2006) par application de l'hybridation par fluorescence in situ (FISH); Le principal organisme présent dans un

SBR avec abondance et limitation des nutriments a donné un signal positif à la sonde *Azoarcus ps.*. L'emploi de la coloration au Nil bleu, a permis d'établir une corrélation entre la présence d'inclusions de PHA et les *Azoarcus* ps., confirmant que c'est un producteur de ces biopolymères.

1.9 Conclusions

En se basant sur la littérature, les problèmes majeurs étudiés au xours de ces travaux de R&D peuvent être résumés comme suit:

1.9.1 Problèmes posés par les plastiques conventionnels

Le problème des matières plastiques conventionnels concerne l'environnement et cei relié à l'accroissement de la production et des difficultés d'élimination des déchets de ces produits. Des effets néfastes apparaissent au cours de leur production, de leur transformation et leur utilisation. De plus, leur rejet dans l'environnement engendre un problème écologique majeur parce qu'ils ne sont pratiquement pas dégradables. Ce type de matières plastiques pouvant être remplacé avantageusement par des plastiques biodégradables à base de PHAs.

1.9.2 Coût élevé de la production de plastiques biodégradables

La généralisation de l'application des PHAs est limitée par les coûts trop élevés de production en culture avec une source unique de carbone et qui sont de dix fois plus élevés que ceux des plastiques pétrochimiques.

1.9.3 Eaux usées , boues activées, gestion des déchets et réutilisation

La gestion des déchets municipaux et industriels (boues et eaux usées) représente un problème environnemental croissant et urgent. Les eaux usées requièrent un traitement efficace, afin de satisfaire aux réglementations environnementales. En outre, les usines de traitement de ces rejets génèrent des boues dont le coût de manipulation et d'élimination représente environ 50% des coûts d'exploitation d'une installation typique. Le fait que les boues activées contiennent des cultures mixtes pouvant produire des PHAs, et que les

eaux usées contiennent des composés organiques biodégradables, donc du carbone, de l'azote, du phosphate et autres éléments nutritifs, permet d'envisager l,emploi et donc la valorisation de ces boues pour l'obtention de PHAs.

1.10 Objectifs et hypothèses

1.10.1 Hypothèse

La production de PHA dépend des micro-organismes et des sources de carbone. Les cultures pures et les boues activées (c'est-à-dire les cultures mixtes), peuvent être utilisées comme microoganismes producteurs en employant des substrats purs ou des déchets comme sources de carbone. Toutes les études et la production industrielle de PHA actuellement en service combinent trois systèmes: Des cultures pures, des sources pures de carbone, des déchets purs et des boues activées (ou cultures mixtes) et des sources de carbone pur. Les boues activées ont la capacité d'accumuler des PHAs à des degrés divers dépendant du type de matériaux utilisés. Les eaux usées biodégradables contiennent du carbone, de l'azote, du phosphore et de nombreux autres éléments nutritifs nécessaires à la croissance des micro-organismes et au processus de production de PHAs, de sorte qu'elles peuvent être utilisées comme matières premières pour l'accumulation des ces bioplastiques. Par conséquent, la production de PHA par des boues activées employées comme sources de micro-organismes et des eaux usées comme sources de carbone peut être établie; Les conditions d'obtention devant être déterminées et optimisées. Par cette approche, il est possible de convertir les polluants organiques contenus dans les eaux usées en plastiques biodégradables (par exemple APS) et donc de réduire la charge de COD devant être traitée en station d'épuration.Cette approche permettrait de réduire sensiblement le coût du traitement des boues et de leur élimination.

1.10.2 Principaux objectifs

L'objectif principal de ces recherches est de produire des PHAs par des boues activées en utilisant les déchets comme sources de micro-organismes et des eaux usées comme substrats de carbone.

Les objectifs spécifiques de recherche pour la production de PHAs sont les suivants:

- Étudier les possibilités d'employer des boues activées provenant de différentes eaux usées municipales et industrielles et / ou des eaux usées ;

- Déterminer les boues activées possédant les meilleures capacités de production de PHAs et poursuite de l'étude des processus de développement;

- Évaluer l'effet de différentes concentrations de boues activées;

- Éstimer la validité de diverses stratégies d'alimentation (batch ou en continue), respectivement;

- Préciser les effets de différents ratios C / N ;

- Concevoir des stratégies pour inciter des bactéries présentes dans des boues activées prélevées dans une usine de traitement des eaux usées municipales à accumuler et à stocker des polymères (PHA). L'isolement des PHAs accumulés par ces bactéries ainsi que la comparaison de leurs possibilités d'accumuler des PHA devant être estimés.

- Identifier les bactéries isolées et accumulant des PHAs par séquençage des ADN r 16S.

1.11 Originalité

En tenant compte de la littérature, il a été déduit que l'emploi de boues activées prélevées comme microorganismes d'eaux usées industrielles pour produire des PHAs et les eaux usées comme substrat de fermentation est un domaine qui reste encore à explorer. L'utilisation de boues activées à une concentration supérieure à 10 g / l. n'a jamais été réalisée pour produire des PHAs. Par conséquent, l'idée d'effectuer des travaux de R&D pour conceptualiser et développer un processus d'obtention par cette méthode est originale et présente beaucoup de potentiel. De plus, l'isolement et

l'identification des souches bactériennes contenues dans ces boues activées accumulant des PHAs devraient permettre d'obtenir des résultats très valables et inédits.

1.12 Résumé du contenu de la thèse

1.12.1 Chapitre 2: Production de PHA par des cultures pures et des culture mixtes (boues activées)

Le chapitre se compose de deux parties. Le premier fournit les informations complètes les plus récentes relativement aux exigences pour l'obtention des PHAs. Des réserves d'énergies en polymères sont couramment accumulées par une grande variété de bactéries. Certaines peuvent en stocker jusqu'à 70% en poids sec des cellules, sous certaines conditions de culture. Le PHB est dérivé de l'acétyl-CoA et représente le PHA le plus abondant dans la nature. Ces biopolymères partagent de nombreuses propriétés avec les plastiques conventionnels tel que le polypropylène. Afin de protéger l'environnement, certaines industries tentent de développer la production de polymères biodégradables. Dans ce but, de nombreuses stratégies sont utilisées en particulier l'isolement de nouvelles souches pouvant accumuler de plus fortes concentrations de polymère. Aussi, afin de réduire le coût de production de PHA, de nombreux déchets ont été testés comme supports pour la croissance de ces bactéries. Par conséquent, la deuxième partie du chapitre 1 présente une nouvelle approche de production des PHAs en employant des déchets et / ou des cultures mixtes (boues activées). La synthèse des PHAs par les cultures mixtes peuvent permettre de recycler des matériaux moins onéreux et moins chers avec des équipements non stériles pour réduire significativement les coûts du processus. Par conséquent, si le contenu de PHA stocké par des cultures mixtes peut être accru, cela augmentera considérablement le potentiel de production de ces polymères. À ce propos, les résultats de nombreuses études démontrent que les cultures mixtes ont une grande capacité de stockage de PHA, des rendements élevés, et une haute productivité spécifiques. La teneur maximale intracellulaire signalée jusqu'à présent en PHAs est d'environ 70% du poids sec des cellules (Serafim et al., 2004b), ce qui est comparable au taux obtenu avec certaines cultures pures. Toutefois , ceci reste inférieur à celui obtenu avec des *E.coli* recombinants (environ 90% du poids sec des cellules).

1.12.2 Chapitre 3: Production de PHA en erlenmeyers et fermenteurs. en utilisant différentes boues activées et / ou eaux usées municipales ou industrielles.

Dans nos travaux de R&D, des boues activées prélevées de différentes usines de traitement d'eaux usées (municipales, industrie des pâtes et papiers, industrie d'amidon et fromageries) ont été utilisées comme source de microorganismes pour produire en erlenmeyers des plastiques biodégradables. L'acétate, le glucose et différentes eaux usées agissaient comme sources de carbone. Des boues activées d'eaux uséées d'industries de pâtes et papier ont été testée comme source de microorganismes pour produire des PHAs en bioréacteurs; L'acétate étant la source de carbone. Les effet de différentes concentrations de boues activées ont été examinés pour la production. Diverses stratégies d'alimentation (batch, Fed-Batch et continue) ont été évaluées pour produire des PHAs. Des boues activées d'eaux usées de pâtes et papier comme sources de microorganismes ont fait l'objet d'ecpérimentations pour produire des PHAs en bioréacteurs de15 L. Quatre types d'eaux usées municipales et industrielles et de l'acétate ont été utilisés séparément comme sources de carbone. Les diverses stratégies d'alimentation citées ont été appliquées.

1.12.3 Chapitre 4: Isolement et identification des souches bactériennes accumulant des PHAs et présentes dans les boues activées utilisées dans ces expériences.

Ce chapitre comprend cinq parties. La première partie porte sur les récentes avancées en microbiologie des boues activées. La population bactérienne dans ce système a été examinée. La procédure standard, les méthodes d'analyse et de techniques de caractérisation biochimique nécessaire à l'isolement ainsi que l'identification de bactéries responsables des processus clés des systèmes de traitement des eaux usées (élimination des nutriments, aérobies, anaérobies, etc ...) sont abordées dans cette étude. Les effets saisonniers (hiver et été), les variations de température et de salinité sur les espèces de bactéries pour le traitement des eaux usées sont examinés. La deuxième et la troisième parties concernent l'isolement des bactéries des boues activées municipales et industrielles de boues activées produisant des PHAs, respectivement; La quatrième et la cinquième parties traitant de l'identification des isolats bactériens en fonction de la séquence des ADNr 16S.

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CHAPITRE 2

PRODUCTION BACTÉRIENNE DE BIOPLASTIQUES (POLYHYDROXYALKANOATE-PHA)

Part I

PHA production by waste materials and/or activated sludge - review

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Résumé

L'utilisation généralisée des polyhydroxyalcanoates (PHA) est actuellement limitée par les coûts de production élevés de ces bioplastiques qui, pourtant ont beaucoup d'avantages par rapport aux matières plastiques conventionnelles. Des efforts importants ont été faits pour réduire les coûts de production. Ce document passe en revue une nouvelle approche impliquant l'emploi de boues activées comme sources de micro-organismes et / ou des déchets comme substrats pour la production des PHAs. D'un point de vue économique, le coût du substrat (principalement la source de carbone) contribue de façon très significative à l'ensemble du prix d'obtention des PHAs. Il a été estimé à environ 40% du total des coûts de production. Durant la dernière décennie, une grande variété de substrats de carbone peu onéreux (par exemple, des sources renouvelables de carbone), telles que les eaux usées, les déchets municipaux, agricoles et industriels, des résidus ou des sous-produits, des substrats carbonés (déchets alimentaires ou produits) ont été testés pour la production de PHAs par des cultures pures en raison de leur faible prix et de leur potentiel. De cette façon, les déchets municipaux, industriels ou agricoles sont convertis en bioplastiques ayant une valeur élevée et durable. Dans ce sens, ce type de recyclage des déchets peut contribuer à résoudre le problème environnemental de leur gestion et simultanément aider à réduire le coût d'obtention des PHAs. Le processus de production de ces bioplastiques basé sur des cultures microbiennes mixtes est à l'étude comme une technologie réaliste pour diminuer les coûts , puisque la stérilisation n'est pas exigée et que les bactéries peuvent très bien s'adapter à la complexité des substrats présents dans les déchets; Le contenu en PHAs pouvant atteindre environ 70% du poids sec des cellules. Ce processus pourrait donc être très compétitif avec des cultures pures synthétisant des PHAs en plein développement. Ces approches ont l'avantage d'économiser les coûts de production des bioplastiques et de réduire le volume des déchets par extraction des boues activées pouvant stocker des PHAs.

***Mots-clés*:** polyhydroxyalcanoates, boues activées, eaux usées, ordures ménagères, déchets industriels, déchets agricoles.

Abstract

Widespread use of polyhydroxyalkanoates (PHAs) is presently limited by their high production costs although PHAs have advantages over the conventional plastics. Much effort has been made to reduce the production costs. This paper reviews the new approach involving the use of activated sludge as the microorganisms and/or waste products as the substrate for the PHA production. From an economical point of view, the cost of substrate (mainly carbon source) contributes most significantly to the overall production cost of PHA. It has been estimated to be about 40% of the total PHA production costs. In the past decade, a wide variety of low-cost carbon substrates (e.g., renewable carbon sources), such as wastewater, municipal wastes, agricultural and industrial residues or by-products, crude carbon substrates (food wastes or by-products) have been tested for PHA production by pure cultures due to their low price and potential. This way, municipal, industrial or agricultural waste are converted into high-value and sustainable bioplastics. Therefore, the use of waste materials can solve the environmental problem of waste disposal and concomitantly help to reduce the cost of PHAs production. PHA production processes based on mixed microbial cultures are being investigated as a possible technology to decrease production costs, since no sterilization is required and bacteria can adapt quite well to the complex substrates that may be present in waste material. The PHA content can reach around 70% of the cell dry weight, showing that this process could be competitive with pure culture PHA production when fully developed. These approaches have the advantages of saving cost on PHA production and volume reduction of waste activated sludge by extracting PHA.

Key words: polyhydroxyalkanoates, activated sludge, wastewater, municipal wastes, industrial waste, agricultural waste

1. Introduction

Recently, there is a growing demand for biodegradable polymers as a solution to the problem of conventional plastic pollution of the global environment (Lee and Yu 1997; Dionisi et al. 2001a; Takabatake et al. 2002; Chua et al. 2003). Poly(3-hydroxyalkanoate) (PHA), one of the biodegradable and biocompatible thermoplastics, has attracted considerable industrial attention (Anderson and Dawes 1990). Current methods for PHA production at the industrial scale are mostly pure cultures requiring pure carbon sources and complex and costly processing procedures (Steinbüchel and Füchtenbusch 1998; Choi and Lee 1999a). Therefore, attempts have been made to develop more cost-effective processes.

The new approach involves the use of renewable carbon resources derived from agriculture or industrial wastes as substrate and/or excess activated sludge from a wastewater treatment plant as a source for polyhydroxybutyrate (PHB) accumulation (Chua et al. 2003; Kumar et al. 2004; Khardenavis et al. 2007). These approaches have the advantages of saving cost on PHA production and volume reduction of waste activated sludge by extracting PHA. The saving on waste activated sludge disposal cost following volume reduction could reduce PHA production cost thereby attributing economic advantage to the process (Khardenavis et al. 2007). This review focuses on the recent researches on use of waste materials and/or activated sludge from wastewater treatment plant for PHA production.

2. Waste materials as carbon substrates for PHA production using pure culture

From an economical point of view, the cost of substrate (mainly carbon source) contributes most significantly to the overall production cost of PHA. It has been estimated to be about 40% of the total PHA production costs (Choi and Lee 1999a; Dias et al. 2006). In the past decade, a wide variety of low-cost carbon substrates (e.g., renewable carbon sources), such as wastewater, municipal wastes, agricultural and industrial residues or by-products (e.g., starch, tapioca hydrolysate, whey, xylose, molasses, malt, and soy wastes), crude carbon substrates (food wastes or by-products) have been tested for PHA production by pure cultures due to their low price and potential availability (Lee and Gilmore 2005). Both cheap carbon sources and cheap nitrogen sources are available from industrial or agricultural waste and surplus materials and make a substantial contribution for minimizing

PHA production costs (Koller et al. 2005). This way, municipal, industrial or agricultural waste and problematic surplus materials that until today have to be disposed are converted into high-value and sustainable bioplastics (Koller et al. 2005). Therefore, Waste materials offer the greatest promise as a source of carbon for bacterial growth, their use can solve the environmental problem of waste disposal and concomitantly help to reduce the cost of PHAs production.

Table 1 summarizes PHA production from different inexpensive substrates as carbon source by various microorganisms. It has been reported that *Methylobacterium* sp. ZP24 and *Pseudomonas cepacia* could produce PHB from whey or its major component, lactose (Young et al. 1994; Yellore and Desai 1998). *P. cepacia* was also evaluated for PHB production from xylose, a major hemicellulosic sugar of hardwoods (Young et al. 1994; Ramsay et al. 1995). Besides *A. chroococcum*, *Haloferax mediterranei* was used to produce PHB from starch as a carbon source (Garcia Lillo and Rodriguez-Valera 1990). The high PHB productivity was obtained by fed-batch culture of *Ralstonia eutropha* from tapioca hydrolysate (Kim Beom and Chang Ho 1995). *Azotobacter vinelandii* UWD was used to produce relatively high concentration of PHB (22 g l^{-1}) from molasses as a carbon source (Page and Cornish 1993).

Whey is the major byproduct from the cheese manufacturing industry, representing 80 - 90% of the volume of milk transformed. Since whey is available in large amounts and only partially used as animal feed, its high production claims for an alternative way of disposal or for enhancing the added value of this material. Whey has been the most extensively studied waste for PHA accumulation by a variety of microorganisms (Park et al. 2002; Povolo and Casella 2003; Khardenavis et al. 2007). Lactose is the major component of whey and many *E. coli* strains can utilize lactose for their growth (Park et al. 2002; Povolo and Casella 2003; Khardenavis et al. 2007).

Povolo and Casella (2003) found that sweet whey contains approximately 5% lactose, 0.2% lactic acid and 1% proteins as well as fats, minerals and vitamins, and *Hydrogenophaga pseudoflava* DSM 1034 was a good candidate for production of PHAs. However, the

P(3HB) concentration and P(3HB) content obtained from waste products were considerably lower than those obtained using purified carbon substrates. Therefore, Recombinant strains utilizing a cheap carbon source and corresponding more efficient fermentation strategies have been developed for efficient production of PHA. Lee et al. (1997) examined various recombinant *Escherichia coli* strains for their ability to accumulate a large amount of PHB in whey-based medium. The highest PHB concentration and PHB content obtained were 5.2 g/L and 81% of dry cell weight, respectively.

Wong and Lee (1998) studied the recombinant *Escherichia coli* strain GCSC 6576, harboring a high-copy-number plasmid containing the *Ralstonia eutropha* genes for polyhydroxyalkanoate (PHA) synthesis and the *E. coli* ftsZ gene, was employed to produce poly-(3-hydroxybutyrate) (PHB) from whey. They obtained high cell concentration of 87 g l^{-1} with 80% PHB in 49 h by fed-batch culture of strain selected (Wong and Lee 1998).

The medium formulation and robust process modeling for PHA production by fermentation from industrial waste (ice cream residue) was studied by employing novel crossed experimental design (Lee and Gilmore 2005). A crossed design, mixture design combined with process factor (fermentation time), was performed to find the optimal medium formulation and process time. The optimal settings for three major components (50.00 ml of ice cream, 8.64 ml of buffer, and 41.36 ml of distilled water) characterized by lipid (16.2 mg/ml) and % lipid (89.5%) values were found and further investigated to find robust process conditions (51.52 ml of ice cream, 7.94 ml of buffer, 40.54 ml of distilled water, and 215.37 h of fermentation time) for PHA production (16.2 mg/ml for lipid, 87.7% for % lipid) by applying propagation of error (POE). This is the highest PHB content by *Ralstonia eutropha* H16 reported in the production by wastes, the composition of the media and the pretreated detail were shown in Lee and Gilmore (2005).

Waste edible oils exhausted from the food industry and the food service industry are largely destroyed by incineration or lost into the environment – both routes being unacceptable from a green chemistry perspective. PHA production was conducted by *Ralstonia eutropha* with waste edible oils and fats as carbon sources (Taniguchi et al., 2003). The efficiency of

this interesting process is high and the polymer can be easily extracted. Thus a waste is converted by an environmentally benign method to a valuable product (Taniguchi et al. 2003).

Plant oils and fats are renewable and inexpensive agricultural co-products, and thus, waste oils would be one of the most suitable candidates for microbial production of PHAs. Several pseudomonades produce PHA from plant oils and tallow, and *Aeromonas caviae* also accumulates PHBHHx from olive oil, although the productivities of them are quite low in all the cases (Doi et al. 1995; Fuchtenbusch et al. 2000; Taniguchi et al. 2003). By using *R. eutropha*, renewable and inexpensive agricultural fats/oils and their waste products are potentially useful feedstocks for the PHA fermentation processes (Taniguchi et al. 2003).

Agro-industrial oily wastes was also used for production of poly(3-hydroxyalkaonates) (PHA) by *Pseudomonas aeruginosa* 42A2 (Fernandez et al. 2005). A 54.6% PHA accumulation was obtained when technical oleic acid (TOA) was used as carbon source. PHA accumulation ranged between 66.1% when waste-free fatty acids from soybean oil (WFFA) were used as carbon substrate, 29.4% when waste frying oil (WFO) was used and 16.8% when glucose was used. Depending on the substrate supplied a wide range of components was observed (Fernandez et al. 2005). It was found that the residual waste frying and other oily wastes are suitable substrates for PHA production (Fernandez et al. 2005).

Large amounts of homopolymers containing β-hydroxybutyrate (PHB) and copolymers containing β –hydroxyvalerate (P[HB-co-HV]) are produced by *Azotobacter chroococcum* strain H23 when growing in culture media amended with alpechìn (wastewater from olive oil mills) as the sole carbon source. Copolymer was formed when valerate (pentanoate) was added as a precursor to the alpechìn medium, but it was not formed with the addition of propionate as a precursor. *A. chroococcum* formed homo- and copolymers of polyhydroxyalkanoates (PHAs) up to 80% of the cell dry weight, when grown on NH_4^+-medium supplemented with 60% (v/v) alpechı´n, after 48 h of incubation at 100 rev min^{-1} and 30 °C. Production of PHAs by strain H23 using alpechı´n looks promising, as the use of

a cheap substrate for the production of these materials is essential if bioplastics are to become competitive products (Pozo et al. 2002). The polymer content (88.6% of dry cell mass) reported in this study is the highest one obtained from organic wastes and is comparable with the PHA content from pure sucrose fermentation (Wang and Lee 1997).

Khardenavis et al. (2007) evaluated the waste activated sludge generated from a combined dairy and food processing industry wastewater treatment plant for its potential to produce biodegradable plastic, poly b-hydroxybutyric acid (PHB). Deproteinized jowar grain-based distillery spentwash yielded 42.3% PHB production (w/w), followed by filtered rice grain-based distillery spentwash (40% PHB) when used as substrates. Addition of di-ammonium hydrogen phosphate (DAHP) resulted in an increase in PHB production to 67% when raw rice grain-based spentwash was used. Same wastewater, after removal of suspended solids by filtration and with DAHP supplementation resulted in lower PHB production (57.9%). However, supplementing other wastes with DAHP led to a substantial decrease in PHB content in comparison to what was observed in the absence of DAHP.

Organic solid wastes, however, are usually in complex form that cannot be directly digested and utilized by PHA-producing microbes such as *Ralstonia eutropha*, a representative bacterium for PHA synthesis (Yu 2001; Du et al. 2004)To enhance the PHA production from the organic wastes, a pretreatment stage may be favorable. Hydrolysis and acidogenesis are the first step to convert biodegradable solids into short-chain volatile fatty acids such as acetic, propionic, and butyric acids that can be further utilized by PHA-producing bacteria.

Yu (2001) studied the production of PHA from a starchy wastewater in a two-step process of microbial acidogenesis and acid polymerization. The starchy organic waste was first digested in a thermophilic upflow anaerobic sludge blanket (UASB) reactor to form acetic (60–80%), propionic (10–30%) and butyric (5–40%) acids. The acid effluent, after microfiltration, was introduced into a second reactor where bacterium A. eutrophus took up the acids to form PHA. Polyhydroxybutyrate was formed from butyric acid and poly(hydroxybutyrate-hydroxyvalerate) formed from propionic acid with 38%

hydroxyvalerate (Yu 2001). In further studies, Du et al. (2004) investigated a novel technology at bench-top scale to produce PHAs from food scraps. The harvested cell mass had a high PHA content (72.6% of dry cell mass), the same as obtained from pure glucose and organic acids. The organic solid was first digested in an acidogenic reactor in which about 60% solid was converted to fermentative products, including short-chain fatty acids. The four major acids were acetic, propionic, butyric, and lactic acids at concentrations of 6, 2, 27, and 33 g/L, respectively. The acids were transported through a membrane barrier via molecular diffusion to an airlift bioreactor, where the acids were utilized by an enriched culture of Ralstonia eutropha for PHA synthesis. By using a dialysis membrane as the barrier, the dry cell mass concentration and PHA content reached 22.7 g/L and 72.6%, respectively. The PHA was a copolymer of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) with 2.8 mole % of hydroxyvalerate (Du et al. 2004).

If waste product/stream can be used as a substrate for the production of P(3HB), combined advantages of reducing disposal cost and production of value-added products can be realized.

3. Mixed cultures in activated sludge used as the microorganisms for PHA production

A novel PHA production strategy, which is to utilize the mixed bacterial culture in activated sludge for PHA production has been proposed. Many workers have shown that the activated sludge could produce bioplastics (Lemos et al. 1998; Satoh et al. 1998a; Lemos et al. 2003; Reis et al. 2003; Dionisi et al. 2004; Lemos et al. 2004; Serafim et al. 2004b; Serafim et al. 2006; Yan et al. 2006).

It is well-known that PHA serves as an important carbon storage material in microorganisms in activated sludge, especially in the anaerobic –aerobic activated sludge process or the Enhanced Biological Phosphorous Removal (EBPR) process (Mino et al. 1998). In EBPR process, microorganisms in activated sludge consume polyphosphates as an energy source for anaerobic uptake of carbon substrates, the carbon substrates taken up are temporarily stored as PHA. When the condition turns aerobic, PHA is utilized for growth and polyphosphate regeneration, the microorganisms in EBPR process should therefore

possess the characteristic of phosphate removal and PHA accumulation. For these reasons, the idea of PHA production by using activated sludge was ignited. When compared with pure culture fermentation processes, the merits of PHA production system by activated sludge will have following advantages: cost reduction in cultivating PHA producing bacterial cultures, lowering the PHA production cost, simpler facility construction, reuse of excess sludge from wastewater treatment plants (WWTPs), contribution to the reduction of excess sludge production from WWTP and to supply of a new way of carbon recycle by the conversion of organic pollutants into valuable biodegradable plastics (Satoh et al. 1998a; Chua et al. 2003).

3.1 Biosynthesis PHA by mixed culture in activated sludge

PHA production by anaerobic-aerobic sludge under anaerobic condition

There are two known microbial populations capable of anaerobic utilization of organic substrate in EBPR processes: (i) the polyphosphate accumulating organisms (PAO) and (ii) the glycogen accumulating organisms (GAO) (Cech and Hartman 1993). Several metabolic models for the stoichiometry of acetate uptake under anaerobic conditions by mixed culture of PAO and GAO were developed (Pereira et al. 1996; Hesselmann et al. 2000; Filipe et al. 2001a; Filipe et al. 2001b). Biological phosphorus removal is based on the enrichment of activated sludge with polyphosphate-accumulating organisms (PAO). Under anaerobic conditions PAO take up organic substrates (preferably volatile fatty acids) and store them as PHA. In the subsequent aerobic or anoxic phase, the internal pool of PHA is oxidized and used for growth, phosphate uptake, glycogen synthesis and maintenance (Smolders et al. 1994b). It has been reported that glycogen-accumulating non-poly-P organisms (GAO, also called G bacteria) may appear when biological P removal deteriorates (Cech and Hartman 1993; Liu et al. 1994; Matsuo 1994; Satoh et al. 1994). These organisms are also capable of anaerobic utilization of organic substrates that are converted and stored as PHA, while the energy and reduction equivalents are provided only by glycogen degradation without involvement of poly-P. In the absence of glycogen, anaerobic substrate uptake by PAO or GAO can not occur (Mino et al. 1996; Brdjanovic et al. 1998).

The energy required for anaerobic substrate uptake comes from the hydrolysis of stored polyphosphate for PAO (Maurer et al. 1997). Since PHA is a reduced polymer, its synthesis requires reducing power, the reducing power is originated from two possible sources: in the Mino model (Mino et al. 1987), the reducing power is from the glycolysis of glycogen, whereas in the Comeau-Wentzel model, substrate degradation in the tricarboxylic acid cycle (TAC) is considered to be the required reducing power (Comeau et al. 1986; Wentzel et al. 1986). According to Mino et al. (1996), the anaerobic metabolism of GAO resembles that of PAO (glycolysis of stored glycogen and substrate conversion to PHA through either acetyl-CoA or propionyl-CoA, and propionyl-CoA production by the succinate-propionate pathway), except that there is no involvement of poly-P. Glycogen in activated sludge is usually consumed simultaneously with anaerobic acetate uptake. For GAOs, ATP comes from the hydrolysis of glycogen. Glycolysis of glycogen may occur via the Embden-Meyerhof-Parnas (EMP) pathway (yielding 3ATP) (Filipe et al. 2001a) or via the Entner-Doudoro (ED) pathway (yielding 2 ATP) (Maurer et al. 1997; Hesselmann et al. 2000). The simplified metabolic pathway was shown as Figure 1.

Acetate is one of well-studied substrates for PHA production by mixed cultures. When acetate is the only carbon source available in the anaerobic phase, the 3-hydroxybutyrate unit is the major unit in the PHA formed (Satoh et al. 1992). 3-Hydroxyvalerate is formed by conversion of glycogen to propionyl-CoA via the succinate-propionate pathway and subsequent reaction with acetyl-CoA (Pereira et al. 1996).

Satoh et al. (1992) and Satoh et al. (1996) demonstrated the formation of very unique PHA containing monomeric units 3HV, 3H2MB (3-hydroxy-2-methyl butyrate), and 3H2MV (3-hydroxy-2-methyl valerate). Inoue et al. (1996) showed that the PHA produced by anaerobic-aerobic activated sludge is a mixture of P(3HB-co-3HV-co3H2MB-co-3H2MV) of different compostions. The presence and relative proportion of different PHAs is dependent on the type of carbon substrate available. When acetate is the only carbon source available in the anaerobic phase, the 3HB unit is the major unit in the PHA formed (Satoh et al. 1992; Smolders et al. 1994a), the composition of PHA formed from some other carbon

sources can be predicted theoretically based on the stoichiometries developed by Mino et al. (1994) and Satoh et al. (1996).

In wastewater treatment processes microorganisms can accumulate storage materials as PHA are generally exist under a feast and famine regime though the PHA storage in EBPR process is only widely accepted (van Loosdrecht et al., 1997). Not only accumulated under anaerobic condition, the PHA can also be produced under aerobic or anoxic conditions. In the last decade, many researchers reported the PHA production by mixed culture under dynamic conditions in activated sludge processes. The growth of biomass and storage of polymers occur simultaneously when there is an excess of external substrate (feast period), when all the external substrate is consumed, stored polymer can be used as a C- and energy source (famine period).

Table 2 gives a summary of PHA composition (molar %), the experiments yields, the PHA content (w% of dry cell) and the specific PHA production rate of various substrates and operation conditions of mixed culture or activated sludge systems.

It is important to select an appropriate substrate in the optimization of PHA production. The nature of the substrate not only determines the PHA contents of cells but also its composition, which subsequently affects the final polymer properties. Furthermore, the cost of the carbon source contributes significantly to the overall production cost of PHA (Yamane 1992; Yamane 1993); the cost of the carbon sources may account for 70–80% of the raw materials, raw materials may account for 40–50% of the total operating costs (Choi and Lee 1997), the price of PHAs can be substantially reduced if cheap organic substrates, such as waste materials from agriculture and food industries (e.g. whey and molasses) and other industries are used.

Acetate is the most used single carbon source for PHA production in mixed cultures or activated sludge. Only a few studies considered other volatile fatty acids (VFAs) like propionate, butyrate or their mixtures (Beccari et al. 1998; Lemos et al. 1998) and even less investigated the effects of other carbon compounds like lactate, succinate, pyruvate and

malate (Satoh et al. 1992), ethanol (Beccari et al. 1998; Majone et al. 2001), aspartate and glutamate (Satoh et al. 1998b) and glucose (Dircks et al. 2001).

Hollender et al. (2002) presented that the highest PHA accumulation using activated sludge during the anaerobic phase was observed with acetate as carbon source (20 mgC as PHAs/g dry matter, DM) and smallest with glucose (6 mgC as PHAs /g dry matter, DM). This is in accordance with the phosphate release in anaerobic/aerobic process because the energy for PHA formation is produced by poly-P hydrolysis.

Hollender et al. (2002) also found that PHV concentration as well as its percentage in the PHA increased with time in all experiments. As expected, the highest percentage was observed with glucose as carbon source. This indicated that a major part of the glucose was converted by the succinate-propionate pathway to propionyl-CoA that together with acetyl-CoA produce 3-hydroxyvaleryl-CoA, the monomer of PHV. This corresponded to the report from Satoh et al. (1994, 1996) that after an anaerobic phase longer than 2 h with acetate as sole carbon source the 3-HV portion of the PHA could increase up to 40%. According to the metabolic model, glycogen supplies the carbon for 3-HV formation (Mino et al. 1998).

Lemos et al. (1998) stated that the composition of the polymers formed was found to vary with the substrate used. Acetate leads to the production of a copolymer of HB and HV with the HB units being dominant (75.25%), with propionate, HV units are mainly produced (71.95%), and only a small amount of HB is synthesized. When butyrate is used, the amount of polymer formed is much lower with the HB units being produced to a higher extent. Matsuo et al. (1992) observed that when acetate was used as carbon source, 87% of the PHA produced were HB, 11% were HV, and the remaining 2% was 3H2MB and 1% 3H2MV. While with propionate as carbon sources obtained 3% HB with the rest as HV (43%), 3H2MB (6%) and 3H2MV (50%). The yield of polymer produced per carbon (g) consumed ($Y_{P/S}$) was found to diminish from acetate (0.97) to propionate (0.61) to butyrate (0.21). Using a mixture of acetate, propionate, and butyrate and increasing the carbon concentration, although maintaining the relative concentration of each substrate, propionate is primarily consumed and consequently, PHA synthesized was enriched in HV units.

During the PHA accumulation, the highest substrate consumption rate was observed for acetate followed by propionate and finally butyrate (Lemos et al. 1998) using activated sludge. The yield of polymer produced per carbon consumed ($Y_{p/s}$: mg polymer/mgCOD) obtained when using acetate, propionate, and butyrate were 0.97, 0.61, and 0.21, respectively. These results showed that in this system, acetate is the best substrate for PHA production by the phosphorus-accumulating bacteria.

Lemos et al. (1998) noticed that when using mixed substrates, the $Y_{p/s}$ value is the sum of the individual contributions of each substrate. With a carbon concentration of 320 mgCOD/l, identical amounts of HB and HV units were produced. The utilization of more concentrated carbon substrate leads to the consumption of propionate primarily, the P(HB-co-HV) being enriched in HV units.

3.2 Effect of operation parameters

DO

Although activated sludge acclimatized under anaerobic–aerobic conditions accumulates PHA, there is no guarantee that anaerobic–aerobic operation of the activated sludge process is best for enrichment of PHA accumulating microorganisms. Satoh et al. (1998) found that sludge accumulated more PHB under aerobic conditions than under anaerobic conditions when using acetate as the substrate. The accumulation of PHA increased linearly with the increase in oxygen supply rate, but the maximum PHA accumulation remained at 33% of sludge dry weight (at oxygen supply rate of 8 mgO_2/min/gMLVSS). In order to further increase the PHA content of activated sludge, Satoh et al. (1998) introduced a new activated sludge process tentatively named ‘microaerophilic–aerobic’ activated sludge process. The anaerobic–aerobic activated sludge process enriches PHA accumulators that are capable of glycogen and/or polyphosphate accumulation, because glycogen and/or polyphosphate are required for anaerobic substrate uptake. Satoh et al. (1998) expected that introduction of small amounts of oxygen into the anaerobic zone of the anaerobic–aerobic process may promote the accumulation of PHA accumulators that are not capable of accumulating glycogen and/or polyphosphate. They achieved 62% of PHA accumulation by using activated sludge acclimatized in the microaerophilic–aerobic process by incubation with

acetate for 30 h. The results from Third et al. (2003) presented that the yield of PHA on acetate was 0.49 g PHA. g^{-1} substrate when oxygen was limiting and 0.34 g PHA g^{-1} substrate under excess oxygen.

Temperature

The influence of temperature (15, 20, 25, 30 and 35^{o}C) on the accumulation of PHB by activated sludge in sequencing batch reactor cultures fed with acetate has been studied by Krishna and Van Loosdrecht (1999). The PHB formation rate was shown to decrease with increasing temperature because of an increase of the anabolic rate at higher temperatures, the PHB level reached the highest (0.21 Cmmol PHB/Cmmol biomass) at 15^{o}C and the lowest (0.06 Cmmol PHB/Cmmol biomass) obtained at 30^{o}C.

Chinwetkitvanich et al. (2004) also investigated the effects of temperature on PHA production and storage by activated sludge biomass. The two-stage operation approach, i.e. a growth phase followed by a nutrient limitation phase, was applied to induce PHA accumulation. The pre-selected temperatures of 10, 20 and 30°C were investigated using fully aerobic SBR systems. It was found that PHA production was greater in the 10°C system than in the 20°C and 30°C systems but there was little difference between the two higher temperatures. The maximum PHA fractions of the sludge were 52, 45 and 47%TSS for the three temperatures from low to high. This result partly agreed with that from Krishna and Van Loosdrecht (1999), PHA production increased with the decrease of the temperature, anyway, in the higher temperature, the trends showed differently.

pH

Chua et al., (2003) investigated the PHA production behaviour of activated sludge using acetate as carbon source under different pH conditions. As pH increased from 6 to 9, PHA content of sludge increased as well. At pH 6 and 7, there was very little PHA accumulation, and PHA content was less than 5% of sludge dry weight. At pH 8 and 9, PHA accumulation was stimulated, and PHA content reached 25-32% of sludge dry weight. The results indicated that pH control is essential in optimizing the PHA production process and pH≥8 is recommended.

Sludge retention time (SRT)

Chua et al. (2003) studied sludge retention time (3 and 10 days) on PHA production with acetate as the carbon source, it was noticed that the longer the SRT, the higher the biomass concentration in the reactor. Microorganisms in reactor with lower MLSS had a chance to take up about more organic substrate than those in higher MLSS. This might have led to the higher PHA production capability of activated sludge in lower MLSS (or lower SRT, i.e. 3 days). In addition, activated sludge process with longer SRT normally contains higher amount of inert biomass and this might also contribute to the lower PHA content.

C:N ratio

Chua et al. (1997) studied the effect of C:N ratio on PHB production.. The polymer content of cells increased to a maximum of 37.4% (cell dry weight) when C:N ratio was increased from 24 to 144, whereas the cell yield (g of cells formed per g of TOC used) decreased with increased C:N ratio. An optimum C:N ratio of 96 provided the highest polymer yield 0.093 g of polymer per g of carbon substrate consumed The C:N ratio in activated sludge process must be kept around 24 in order to enable normal microbial cell synthesis.

Nitrogen and/or phosphorus limitation

PHA accumulation in bacteria can be stimulated under conditions of unfavorable growth such as the deprivation of oxygen, nitrogen, phosphate, sulfur, magnesium or potassium in the presence of excess carbon (Chinwetkitvanich et al. 2004). Chinwetkitvanich et al. (2004) found that all experiments with P limitation resulted in large accumulations of PHA, expressed as % of TSS in the biomass, the PHA yields (mg PHA/mg CODu) and productivities (mg/l-d) were significantly lower than those obtained during the nitrogen limitation experiments (Chinwetkitvanich et al., 2003).

Processes using activated sludge for PHA production

In recent years the interest for PHA production by mixed cultures has increased and many studies addressed this subject. Cultivation strategies to improve the PHA content, yield and productivity in pure cultures are very well defined. However, for mixed cultures or activated sludge this knowledge is still very limited.

Among the different processes described for PHA production by mixed cultures, the feast and famine process is the most promising because of the high sludge PHA content and productivity (Table 4.4). The substrate is fed during a short period of time, followed by a longer period of substrate lack. This period of famine is needed to stimulate the PHA storage capacity of cells. The feast and famine process is commonly carried out in a sequencing batch reactor (SBR). MRs are ideal reactors for a selection of robust populations with high ability of PHA storage, because biomass grows under transient (unsteady) conditions. Furthermore, this kind of reactor is easy to control and is highly flexible, allowing for a quick modification of the defined process conditions (length of feed and cycle length).

Serafim et al. (2004) found that sludge submitted to aerobic dynamic feeding could accumulate high amounts of PHA by manipulating feeding concentrations and the reactor operating parameters. The maximum PHB content, 67.5%, was obtained for 180 Cmmol/l of acetate supplied in one pulse. However, such high substrate concentration proved to be inhibitory for the storage mechanism, causing a slowdown of the specific PHB storage rate. In order to avoid substrate inhibition, 180 Cmmol/l of acetate was supplied in different ways: continuously fed and in three pulses of 60 Cmmol/l each. In both cases the specific PHB storage rate increased and the PHB content obtained were 56.2% and 78.5%, respectively. The latter value of PHB content is similar to that obtained by pure cultures and was never reported for mixed cultures.

Dionisi et al. (2005) studies the operation of a new process for the production of biopolymers (polyhydroxyalkanoates, PHAs) at different applied organic load rates (OLRs) applied to an SBR. The process is based on the aerobic enrichment of activated sludge to obtain mixed cultures able to store PHAs at high rates and yields. As a consequence the best performance of the process was obtained at an intermediate OLR (20 gCOD/L/day) where both biomass productivity and PHA storage were high enough.

If a continuous system for PHA production is desired, then a possible configuration could include two reactors in series in which a plug flow reactor (PFR) is followed by a

continuous stirred tank reactor (CSTR), coupled to a settler or membrane filter. Beccari et al. (1998) used a CSTR operated with intermittent feeding. This process selects and produces sludge with high storage capacity. The PHB storage or accumulation yield ranged from 0.06 to 0.5 g COD/g COD and the sludge stored up to 40–50% of the total dry weight.

Most of the studies on PHA production by mixed culture or activated sludge were conducted with activated sludge acclimatized with synthetic wastewater (Satoh et al. 1996; Lemos et al. 1998; Satoh et al. 1998a; Satoh et al. 1998b; Beun et al. 2000a; Beun et al. 2000b; Chua et al. 2003) in order to assess the feasibility of PHA production by activated sludge, the capability of activated sludge from full scale wastewater treatment plants had been hardly reported.

Takabatake et al. (2002) conducted studies with activated sludge from the municipal WWTPs in Tokyo Japan in batch experiments using acetate as carbon source to evaluate their potential for PHA production at pH of 7.0 to 7.2. The activated sludge samples tested had the capability to accumulate PHA up to 18.8% of dry cell weight on average, with the range of 6.0% to 29.5%. The results showed that the maximum PHA content was dependent on the influent of wastewater more than on the operational conditions of the activated sludge process, and that conventional activated sludge produced PHA as much as anaerobic-aerobic activated sludge did.

The limited data shows that the homo and copolymers obtained by mixed cultures bear average molecular weights, in the same range of variation as those obtained by the use of pure cultures (Lemos et al., 1998).

3.3 PHA production by using activated sludge as microorganisms and wastes as carbon sources

The process for the production of biodegradable plastic material (polyhydroxyalkanoates, PHAs) from microbial cells by mixed-bacterial cultivation using readily available waste (renewable resources) is the main consideration nowadays (Chua et al. 2003; Rhu et al. 2003; Md Din et al. 2006; Khardenavis et al. 2007).

Chua et al. (2003) investigated the production of biodegradable plastics polyhydroxyalkanoates (PHA) by activated sludge treating municipal wastewater. It was found that sludge acclimatized with municipal wastewater supplemented with acetate could accumulate PHA up to 30% of sludge dry weight, while sludge acclimatized with only municipal wastewater achieved 20% of sludge dry weight.

In the further study, Rhu et al. (2003) attempted the PHA (polyhydroxyalkanoate) production with SBRs from food waste. Seed microbes were collected from a sewage treatment plant with a biological nutrient removal process, and acclimated with synthetic substrate prior to the application of the fermented food waste. The maximum content of 51% PHA was obtained with an anaerobic/aerobic cycle with P limitation.

PHA production from activated sludge has also been investigated with synthetic wastewater (65.8% of dry cell weight) and anaerobic wastewater (58%) (Khardenavis et al., 2005; Khardenavis et al., 2007). The PHB producing bacterial biomass was enriched by aeration in synthetic medium in lab-scale.

Khardenavis et al. (2007) evaluated waste activated sludge generated from a combined dairy and food processing industry wastewater treatment plant for its potential to produce biodegradable plastic, PHA. The results showed that, deproteinized jowar grain-based distillery spentwash yielded 42.3% PHB production (w/w), followed by filtered rice grain-based distillery spentwash (40% PHB) when used as substrates. Addition of di-ammonium hydrogen phosphate (DAHP) resulted in an increase in PHB production to 67% when raw rice grain-based spentwash was used. Same wastewater, after removal of suspended solids by filtration and with DAHP supplementation resulted in lower PHB production (57.9%). However, supplementing other wastes with DAHP led to a substantial decrease in PHB content in comparison to what was observed in the absence of DAHP. Though the PHB content achieved in this study (67%) was lower than that obtained with pure cultures (up to 80%), the results were amongst the highest obtained so far with activated sludge using wastewater as substrate (Khardenavis et al. 2007).

Md Din et al. (2006) studied the production of PHA by using single fed-batch mixed cultures and renewable resources. A sequencing batch reactor (SBR) was used in the study. The initial mixed culture was developed using 10% activated sludge from the sewage treatment plant and 90% from palm oil mill effluent (POME). The cultivation was kept maintained in a single fed-batch reactor and operated in two steps: growth and accumulation stage. Firstly, the system will allow an extensive growth (using nutrient medium) and then the limiting nutrient (no nutrient medium adaptation) will be introduced in the next step. The average production of PHA could only reach up to 44% of cell dry weight. However, the favourable factors (e.g. temperature and harvesting time) have been made in the next stage to induce the PHA production (Md Din et al. 2006).

4. Conclusions and research needs

By the review on production of PHA by activated sludge as mixed culture and/or wastes as carbon sources, the following conclusions could be drawn:

- PHA production by mixed cultures may allow use of cheaper substrates and cheaper nonsterile equipment for an important reduction on the process operating costs. Therefore, if the PHA content of mixed cultures can be improved, the mixed-culture process has high potential for PHA production.
- The results of many studies demonstrate that mixed cultures for the PHA production have high PHA storage capacity, high PHA yields, and high specific PHA productivity. The maximum intracellular PHA content reported so far (65% on cell dry weight) (Serafim et al. 2004a) is comparable with that of some pure cultures, howevere, it is lower than that obtained in recombinant *E. coli* (about 90% on cell dry weight).
- Using the mixed culture, the polymer yield (0.56 g PHB. g^{-1} substrate)(Serafim et al. 2004a) and the specific productivity (0.77 g PHB. g^{-1} cell dry weight . h^{-1}) obtained so far are very promising. The specific productivity of mixed cultures is approximately tenfold that of recombinant *E. coli* cultures. Anyway, the volumetric PHA productivity reported for mixed cultures are still lower than that by most pure cultures. The reason for this is due to the difficulty in reaching high biomass concentrations in the mixed-culture process. Therefore, the main challenge regarding

the mixed culture process is the development of culture selection strategies of fast-growing organisms that have, at the same time, a high PHA storage capacity.

- The physical properties of the PHAs produced by mixed culture of activated sludge were comparable to PHAs produced by pure cultures.
- Compared to the pure culture, the knowledge of cultivation strategies and the process control to improve the PHA content, yield and productivity in mixed cultures are limited. There are many research challenges that need to be further studied.

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Table 1. Waste used for the production of PHAs by some bacterial strains

Waste	Bacteria	Time of growth (h)	Cell conc. (g/l)	PHA conc. (g/l)	PHA content (%)	Type of PHAs (HB:HV)	References
Alpechı´n (wastewater from olive oil mills)	*Azotobacter chroococcum* strain H23	48	5.36	4.75	88.6	76:24	(Pozo et al. 2002)
Waste edible oils	*Ralstonia eutropha*	72	6.3	4.9	78	100:0	(Taniguchi et al. 2003)
Soybean and rapeseed	*Ralstonia eutropha*	72	6.1	3.5	57	100:0	(Taniguchi et al. 2003)
Soybean, rapeseed, corn, and lard	*Ralstonia eutropha*	72	6.5	5.1	79	98:2	(Taniguchi et al. 2003)
Palm and lard	*Ralstonia eutropha*	72	6.8	5.7	83	99:1	(Taniguchi et al. 2003)
Tallow	*Ralstonia eutropha*	72	7.3	5.8	80	99:1	(Taniguchi et al. 2003)
Wey	osmophilic wild-type strain	120	11.1	5.5	49.6		(Koller et al. 2005)
Gycerol liquid phase (GLP)	osmophilic wild-type strain	120	21.3	16.2	76.0		(Koller et al. 2005)
Gycerol liquid phase + meat and bone meal (GLP + MBM)	osmophilic wild-type strain	120	7.9	5.91	75.0		(Koller et al. 2005)

Agro-industrial oily wastes Technical oleic acid (TOA)	*Pseudomonas aeruginosa* 42A2	72	4.2	2.3	54.6	NA	(Fernandez et al. 2005)
Agro-industrial oily wastes waste frying oil (WFO)	*Pseudomonas aeruginosa* 42A2	72	NA	NA	29.4	NA	(Fernandez et al. 2005)
Agro-industrial oily wastes waste-free fatty acids from soybean oil (WFFA)	*Pseudomonas aeruginosa* 42A2	72	NA	NA	66.1	NA	(Fernandez et al. 2005)
Ice cream (pretreated) + Buffer media	*Ralstonia eutropha* H16	240	18.1	16.2	89.5	100:0	(Lee and Gilmore 2005)
Cheese whey permeate	*Hydrogenophaga pseudoflava* DSM 1034	96	0.375	0.017	4.4	NA	(Povolo and Casella 2003)
Food scraps with pretreatment of anaerobic digestion	*Ralstonia eutropha.*	73	22.7	16.5	72.6	97.2:2.8	(Du et al. 2004)

Table 2. Summary of different processes used for PHA production by mixed culture or activated sludge systems.

Substrate	PHA molar ratio (%)	$Y_{P/S}$	% PHA	Ref.
Anaerobic-aerobic PAO				
Acetate	90:10:0:0	1.33	16.7	Smolders et al.,1994
VFAs	62:38:0:0	1.71	23.0	Levantesi et al., 2002
Acetate	100:0:0:0	0.80	–	Bond et al., 1999
Acetate	69:31:0:0	1.43	2.6	Satoh et al., 1992
Propionate	7:59:0:34	1.85	2.5	Satoh et al., 1992
Malate	21:75:0:4	1.50	0.9	Satoh et al., 1992
Lactate	21:76:0:3	1.25	1.8	Satoh et al., 1992
Pyruvate	46:54:0:0	0.67	1.1	Satoh et al., 1992
Succinate	8:74:0:18	0.93	1.6	Satoh et al., 1992
Acetate	75:25:0:0	1.21	20.1	Lemos et al., 1998
Propionate	28:72:0:0	0.81	12.8	Lemos et al., 1998
Butyrate	60:40:0:0	0.27	13.1	Lemos et al., 1998
VFAs	55:45:0:0	0.77	12.1	Lemos et al., 1998
Acetate	88:10:2:0	1.34	16.4	Satoh et al., 1996
Propionate	2:45:3:50	1.82	26.7	Satoh et al., 1996
VFAs	14:51:5:30	1.68	28.3	Satoh et al., 1996
Lactate	32:53:5:10	1.63	10.2	Satoh et al., 1996
Aspartate	14:48:13:25	0.93	–	Satoh et al., 1998
Feast and famine (aerobic)				
Acetate	100:0:0:0	0.60	31.0	Beccari et al., 1998
VFAs	50:50:0:0	0.45	62.0	Beccari et al., 1998
Acetate	100:0:0:0	0.41–0.62	12.0	Beun et al., 2000a
Acetate	100:0:0:0	0.40	–	Beccari et al., 2002
Ethanol	100:0:0:0	0.33	–	Beccari et al., 2002
Acetate	100:0:0:0	0.41–0.61	66.8	Dionisi et al., 2001b
Feast and famine (anoxic/anaerobic)				
Acetate	100:0:0:0	0.40	16.1	Beun et al., 2000a,b
Acetate	100:0:0:0	0.24–0.49	–	Dionisi et al., 2001a
MA/AE (microaerophilic–aerobic)				
Acetate	100:0:0:0	–	62.0	Satoh et al., 1998b

Units: PHA molar ratio (%) HB: HV: HMB: HMV units, $Y_{P/S}$ in C-mol HA/C-mol substrate, % PHA in g of polymer/g biomass.

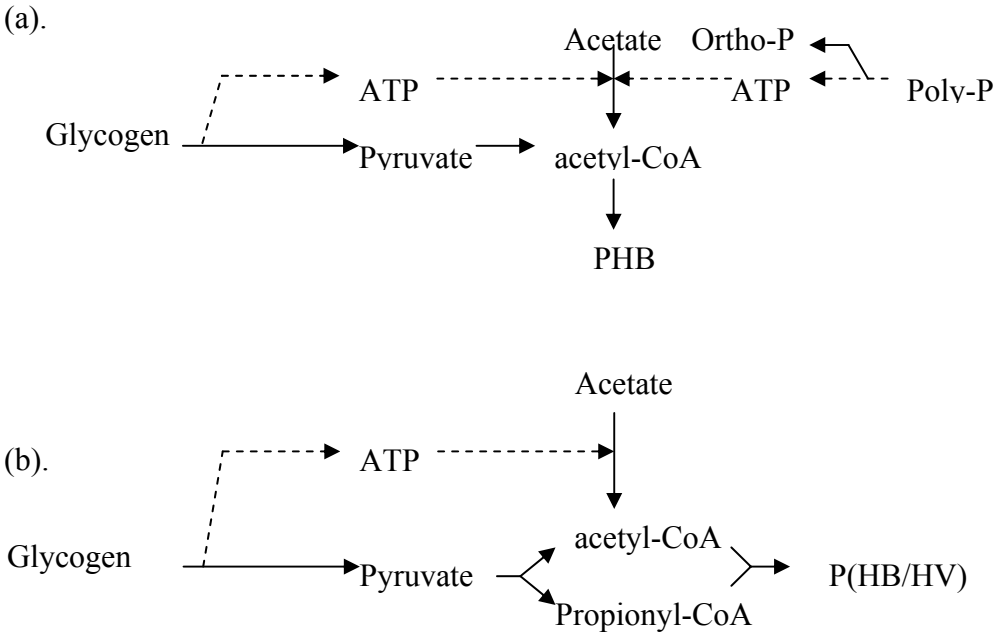

Figure 1a, b. Simplified metabolic pathways of acetate and glycogen conversion to poly-hydroxybutyrate (PHB)/poly-hydroxyvalerate (PHV) by (a) polyphospate-accumulating organisms (PAO) and (b) glycogen-accumulating organisms under anaerobic conditions (GAO)

Part II

Bacterial Production of Bioplastics

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Résumé

Des bioplastiques sont couramment accumulées comme réserves d'énergie par une grande variété de microorganismes; Certaines bactéries pouvant en accumuler jusqu'à 70% en poids sec des cellules, sous certaines conditions de culture. Le polyhydroxybutyrate (PHB) dérivé de l'acétyl-CoA représente le pHA le plus abondant dans la nature. Ces biopolymères partagent de nombreuses propriétés avec les plastiques conventionnels tel que le polypropylène. Afin de protéger l'environnement, certaines industries développent des procédés de production de polymères biodégradables. De nombreuses stratégies sont appliquées pour développer ce secteur, en particulier l'isolement de nouvelles souches bactériennes accumulant des concentrations plus élevées de polymères. Aussi, afin de réduire le coût de production de PHA, de nombreux déchets ont été testés comme supports pour la croissance des bactéries.

Mots-clés: Bioplastiques, biopolymères, PHB, PHA, bactéries, déchets industriels

Abstract

Bioplastics are energy reserve polyester commonly accumulated by a large variety of bacteria. Some bacteria can accumulate as much as 70% of cell dry weight under certain cultivation conditions. Polyhydroxybutyrate (PHB) is derived from acetyl-CoA and represents the most abundant PHAs in nature. These biopolymers share many properties with the conventional plastics (polypropylene). In order to protect the environment, industries are encouraged to develop the production of biodegradable polymers. Many strategies are used to develop this sector through development of new strains which could accumulate higher polymer concentration. Also, in order to reduce the cost of PHAs production, many wastes have been used as growth media for bacteria.

Key words: Bioplastics, biopolymers, PHB, PHA, bacteria, industrial waste

1. Introduction

Degradable plastics can be classified in two categories: the photodegradable plastic and biodegradable plastic (Yves et al., 1995). The photodegradable plastic is sensitive to the light. The photodegradation subdivides the polymer in non-biodegradable fragments and consequently the polymer loses its structure. However, biodegradable polymers can be broken up partially or completely into biodegradable fragments by microbial enzymatic action or by other hydrolysis reagents. The use of non-biodegradable plastics is an increasing environmental problem because of the increase of the production and the disposal of waste plastics. United States produces alone fifty million tons of plastics per year (Atlas, 1993). The elimination of plastics in the environment is the most difficult problem. Generally, plastics are eliminated by the discharge into marine environment, land filling and incineration.

The incineration generates harmful products such as hydrochloric acid and hydrogen cyanide. However, land filling is the most safe and easiest method of disposal of the non-biodegradable plastics (Fiechter, 1990). Approximately 40% of the produced plastics per year are discarded into landfills. This way of disposal is limited by the surface of land reserved for this practice (Fiechter, 1990). The recycling has also used as another option, but is limited because of the fact that recycling changes the plastic material proprieties (Fiechter, 1990).

The non-biodegradable plastics can cause harmful effects during the production, the transformation and the utilisation. Moreover, its discharge in the environment causes an ecological problem (Fiechter, 1990). In order to confirm the new environmental legislations, industries are encouraged to reduce the utilisation of the non-biodegradable plastics and develop the production of biodegradable plastics. Because, bioplastics present desirable properties and can be used for human, a lot of biodegradable plastics are incorporated in many materials including packaging material, coating of fibrous materials and also in pharmaceutical or agrochemical agents. Hence, the world consumption of biodegradable plastics is estimated at 1.3 billion Kg per year (Lindsay, 1992).

In 1926, polyhydroxyalkanoate (PHA) was observed as a constituent of *Bacillus megaterium* and identified as biopolymer (Lemoigne, 1926). After that the poly-β-hydroxybutyrate (PHB) which is the most frequently found members of PHAs and other PHAs were discovered and more than 40 PHAs were characterized (Steinbuchel, 1991). PHB is accumulated by a large number of bacteria, generally, under conditions of nutrient limitation and in the presence of excess carbon source.

2. Production conditions of biopolymers

Generally, polymers are produced by bacteria under specific growth conditions. For example, PHAs and PHBs are accumulated if a carbon source is abundantly available but another essential growth component such as nitrogen is limiting or missing in the medium (Hrabak, 1992). Generally, Bacteria produce PHAs under some specific conditions. Some bacteria (*Alcaligenes eutrophus*, *Protomonaas extorquens*, *Pseudomonas olevorans*, etc) need limitation of some essential nutrient such as N, P, Mg, K, O or S. The presence of an excess carbon source permits an efficient PHA synthesis. However, other bacterial strains (*A. latus*, a mutant strain of *Az. Vinelandii*, and recombinant *E. coli*. etc) do not require nutrient limitations (Lee, 1996a). Moreover, bacteria produce PHB while growing in mineral medium containing metals in trace (such as Co, Cr, Cu, and Zn) (Repaske and Repaske, 1976). According to Findlay and White (1983), the PHBs are produced in large quantity in ground water (oligotrophic environment). However, the production can be inhibited by Mg^{2+} and SO_4^{2-}. *A. eutrophus*, can accumulate 80% of its dry weight while growing under an excess supply of carbon (glucose) and limitation of P or N. Accumulated polymer acts as a carbon reserve and electron sink for the bacteria (Anderson and Dawes, 1990). When limiting conditions are alleviated (addition of P or N), PHB will be catabolized to acetyl-CoA (Yves et al., 1995). Generally, PHAs are accumulated in the form of granule form (Page et al., 1992). These granules are visible under an electron microscope (Lageveen et al., 1988; Hrabak, 1992; Vincent et al., 1962).

3. Biosynthesis, structure and proprieties of biopolymers

3.1. Biosynthetic pathways

The majority of data on the production mechanism of PHA are obtained on the basis of *A. eutrophus*. In this bacterium, PHB is synthesized from acetyl-CoA in series of three reactions catalyzed by three enzymes (thiolase, reductase and synthase). The PHB pathway is shown in Figure 1. At the beginning, the reversible condensation of two molecules of acetyl-CoA, by the action of the 3-ketothiolase, produces the Acetoacetyl-CoA. After that, the acetoacetyl-CoA reductase enzyme reduces the acetyl-CoA into R-(-)-3-hydroxybutyryl-CoA. The reductase enzyme is NADPH-linked (Figure 1). Finally, R-(-)-3-hydroxybutyryl-CoA is polymerized to form PHB under the action of PHA synthase. PHA is formed by the polymerisation of 10^3 to 10^4 monomers and accumulate in cells as inclusions of 0.2 width 0.5μm in diameter (Yves et al., 1995; McCool and Cannon, 2001; Fukui and Doi, 1997). Actually, nucleotide sequences are known for more than 30 PHA synthases and they are arranged into three classes (Class I : catalyse polymerisation of short-chain length HACoAs; Class Π : catalyse polymerisation of medium-chain length HACoAs and class IIΠ : catalyse polymerisation of short-chain length HACoAs) (Rehm and Steinbuchel., 1999). Therefore, the nature of PHAs produced, by *A. eutrophus*, is related to the structure of the source of carbon used in the growth medium. PHAs produced are composed with different monomers having 3 to 5 carbon atoms (C3 to C5) (Steinbuchel, 1991). The addition of propionic acid or valeric acid to the growth media containing glucose permits the accumulation of copolymer (P(3HB-3HV): copoly(3-hydroxybutyrate-3-hydroxyvalerate)) (Yves et al., 1995). However, the PHA biosynthetic system allows the incorporation of saturated 3-, 4-, and 5-hydroxyalcanoic acids with up to five carbon atoms. Hence, in this case, hydroxyalkanoic acids with six or more carbon atoms were not detected in PHA produced by this bacterium (Yves et al., 1995; Liebergesell et al, 1991; McCool and Cannon, 2001; Fukui and Doi, 1997). However, large number of bacteria including *Pseudomonas olevorans* accumulate polymer composed of monomers having 6 to 14 carbon atoms (C6 to C14) (Steinbuchel, 1991). Therefore, the flexibility of the PHA

pathway will help the design of a new biopolymer having potentially specific physical proprieties (Yves et al, 1995).

3.2 Structure of PHB

PHAs polymer have a number of interesting characteristics and can be used in various ways similar to many conventional synthetic plastics. Generally, PHAs are formed by polymerisation of R-(-)-hydroxyalkanoic acid monomers having 3 to 14 carbons (C3 to C14). PHB is the most interesting and most known polymer (Budwill et al., 1992). The β-hydroxybutyric acid monomer is a chiral molecule and the natural polymer consist only of D (-) monomers (Yves et al., 1995, Anderson and Dawes., 1990; Steinbuchel, 1991). A large number of monomer units have been identified in various bacterial species as constituents of PHAs (more than 90 monomers) (Steinbuchel, 1995). Example of these monomer units are: 3-hydrxyalkanoates (having 3 to 12 carbon atoms), 4-hydroxypentanoate (having 4 to 8 carbon atoms), 5-hydroxypentanoate, 5-Hydroxyhexanoate and 6-Hydroxydodecanoate (Figure 2). However, only a few of these PHAs have been produced and characterized.

3.3 Proprieties

The proprieties of PHBs are comparable to conventional plastics (polypropylene). They are natural biopolymer which is 100% resistant to water and moisture, thermoplastic and 100% biodegradable (Hrabak, 1992). However, it has some specific proprieties such as low permeability to gases (Table 1).

The homo-polymer PHB is a stiff and relatively brittle thermoplastic (Holmes, 1988). It has a melting point of 175 °C and a relatively poor resistance to reagents (acids and bases). However, it has a good UV resistance. Compared to some other biodegradable plastics, PHB is insoluble in water and relatively resistant to hydrolytic degradation. It is characterized also by a good oxygen permeability (Holmes, 1988).

The use of PHBs is limited because higher melting point and a slow rate of degradation. These proprieties can be modified by changing the configuration of the PHB polymer

which can give different PHAs composition such as the copolymers (Cox, 1994). For example, the incorporation of 3HV (3-hydroxyvalerate) monomers into PHB polymer resulted in a decrease in the crystallinity and in the melting point compared to the homopolymer PHB (Holmes, 1988). Hence, the copolymer represent a decrease in stiffness and an increase in toughness. Therefore, the copolymer P(3HB-3HV) has suitable proprieties for many commercial application. The polyhydroxyoctanoate has the proprieties of an elastomer such as low melting point, low crystallinity, poor tensile and higher extension to break (Table 1) (Gagnon et al, 1992).

3.4 Biodegradability

Many micro-organisms such as bacteria and fungi degrade PHA polymers and use the by-products as carbon sources (Matavulj and Molitoris, 1991; Jendrossek et al., 1993). PHAs can be degraded rapidly in anaerobic sewage and slowly in sea water (Luzier, 1992). The final products of biodegradation are CO_2 and water under aerobic conditions. However, in anaerobic conditions, PHBs can be transformed to CH_4 and CO_2. (Budwill *et al*, 1992; Lee, 1996b; Cox, 1994).

The biodegradation occurs by the attack of biopolymer by bacteria which secrete an extracellular enzyme called depolymerases. Some extracellular depolymerases (which hydrolyse PHB, P(3HB-3HV) and PHO : Poly 3-hydroxyoctanoate) have been recognized and their specific genes are cloned (Schirmer et al., 1993; Saito et al., 1989). Also, a PHV depolymerase has been isolated and characterized from *Pseudomonas Lemoignei* (Muller et al., 1993). Moreover, PHB is known as a biocompatible material. The degradation in vivo is slow (Fiechter, 1990) compared to in vitro degradation (in soil and in sludge) and its byproduct is R-(-)-3-hydroxybutyric acid (Yves et al., 1995).

The biodegrdation of PHB based plastics were studied in different types of compost and by different composting technologies (Hrabak, 1992). A PHB weight loss of 18 to 40% was reported after 12 weeks of exposure in green manure compost. However, compacting the shape of PHB products was shown to be unfavourable for degradation (Hrabak, 1992). Composting under higher temperature and mechanical disruption permit a rapid degradation of bottles made of copolymer (P(3HB-3HV)) and, after 15 weeks, 80%

weight lost was observed (Yves et al., 1995). However, a weight loss of about 50% for the same material was observed under simulated managed landfill condition at 35°C and after a period more than 40 weeks (Yves et al., 1995). Generally, the rate of bioplastic biodegradation is controlled by some environmental factors (pH, temperature, nutrients, and moisture) and by material parameters (co-monomer HV: 3-hydroxyvalerate) content, molecular weight, crystallinity degree, surface area and the formulation). Therefore, the material parameters can be controlled, during synthesis, in order to optimise the biodegradation rate. For example, increasing the HV content of P(HB-HV) copolyesters can enhance the biodegradability rate (Cox, 1994).

4. Diversity of micro-organisms and substrates for producing biopolymers

The accumulation of PHA at higher concentration has been observed in a variety of micro-organisms such as *Clostrodusim*, *sytrophomonas*, *Pseudomonas* and *Alcanegene*. However, some cyanobacteria produce PHB at lower level (Fiechter, 1990). Table 2 summarizes the PHA production by various bacteria and substrates. Generally, bacteria produce short-chain PHAs, medium-chain PHAs and both short and medium chain PHAs. Some strains are studied and the polymers are characterized.

Alcaligenes eutrophus produces short-chain PHAs (polymerisation of C3 to C5 monomers) (Anderson and dawes, 1990; Steinbuchel, 1991). *A. eutrophus* is the most used strain because it produces PHB and P(HB-HV) at higher rate (Ramsay et al, 1992). *A. eutrophus* can accumulate about 80% of its dry weight (Byrom, 1987). *A. eutrophus* is a chimiolithotrophic bacterium grows in mineral medium. The enrichment medium contains (in g/l) yeast extract :10; peptone :10; meat extract : 5 and $(NH_4)\ 2SO_4$: 5 (Yoshiharu et al, 1988). Growth of *A. eutoophus* in this medium can not produce polyester. In order to produce polymers, this bacteria should be transferred into another medium under nitrogen limitation (Repaske and Repaske, 1976) and in the presence of an excess of carbon at pH 7, 30°C and under oxygenation.

The nature and the proportion of polymers produced are influenced by the carbon source used (Steinbuchel, 1991). Propionic and valeric acid permit the production of copolymer P(3HB-3HV) (mixture of 3HB and 3HV). However, while growing the same strain only

on valeric acid based-medium, the poroprtion of 3HV reached 90% of PHA produced (Doi et al., 1988; Steinbuchel et al., 1991).

A. eutrophus is inhibited while growing in propionic acid (0.1%) (Byrom, 1987), however, it sustains the accumulation of P(3HB-3HV). The use of glucose (at 2%) and starch (2%) are the ideal condition of higher production of PHBs. Generally PHBs are produced under limited concentration of ammonium (Heinzle and Laffery, 1980).

Minrou et al. (1992) showed that the production of poly-3-hydroxalkanoates by a new *Alcaligene* strain growing on various carbon sources (n-alcanoic acid, oil of plants and animal fatty acids) with carbon chain ranged between C2 and C22. While cultivated in mineral medium containing salts and n-alcanoic acid, homopolymer (polymerization of 3-hydroxybutyrate : P(3HB)) was produced from n-alkanoates having a regular carbon number. However, the copolymers were produced by the remained n-alkanoates. Higher yields (cell and P(3HP)) were obtained by using n-alkanoates (C12 to C16) as a carbon source. Experiments (Yoshiharu et al, 1988) done with two strains of *A. eutrophus* (ATCC 17699 and NCIB11589) cultivated in three types of carbon sources (glucose, butyric and propionic acid) showed the production of homopolymers P(3HB) with identical carbon number. Polymers were also produced from organic acids with irregular carbon number (propionic and pantanoic acids). On the basis of NMR (Nuclear Magnetic resonance) these polymers were identified as mixture of two monomer units (3HB and 3HV). The molar fraction of these units were also determined by NMR method and indicated that a higher molar fraction of polymer 3HV (90%mol) was produced by the strain NCIB11599. However, biopolymer represents about 31 to 54% of the cell weight depending on the organic acid used. Moreover, the addition of pentanoic acid to the growth medium decreased the 3HB unit fraction in the copolymer. Under this condition the polyester cell content increased with the addition of the two organic acid types (increased from 30 to 55%). The molar portion of 3HV unit varied between 0 and 85%. According to the authors, the higher fraction of 3HV in the produced polyester explained by the biosynthetic pathway and it seemed that the pentanoic acid was incorporated in polyester as 3HV unit without the decomposition of its skeletic carbon in the cell (Yoshiharu, 1988).

Pseudomonas olevorans is a chimiolithotrophic bacterium which produces medium-chain of PHAs by incorporation of C6 to C14 monomers (Anderson and dawes, 1990; Steinbuchel, 1991) and grows in a mineral medium under oxygenation at 30°C and at pH 7. The Culture medium as described by Tsuchiya et al. (1980). In order to produce the polyester by the bacterium, carbon source should also be added to the medium. This strain accumulates PHA copolymers containing monomers (C6 to C14) while growing in alkanoic acid based medium (Steinbuchel, 1991). The same observation was noted for *Pseudomonas putida. P. oleovorans* can not produce PHA while cultivated in sugar medium. In this case the major constituent of the polymer were 3-hydroxydecanoate (Timm and Steinbuchel, 1990; Haywood et al, 1990). Polymer analyses showed the presence of unsaturated C12 and C14 units (3-hydroxy-5-cis-dodecenoate and 3-hydroxy-7-cis-tetradecenoate) (Huijbert et al., 1992). According to authors, PHA produced from sugars seems to be a derivative from the fatty acid biosynthesis pathway. This pathway has been validated by using acetate marked by ^{13}C labelled acetate as a substrate (Saito and Doi, 1993; Huijbert et , 1994). Polymer composition is also related to the nature of the substrate used and the polymer produced contained 2C less (less 2n carbon) than the used substrate. For example, with octanoate (C8) as a substrate, *P. olevorans* produced a PHA containing: 89 mol % of C8 monomer and 11 mol % of C6 monomer. However, with dodecanoate (C12) the PHA contained 31 mol % of C12 monomer, 36 mol% of C10 monomer, 31 mol % of C8 monomer and 2 mol % of C6 monomer (Lageveen *et al*, 1988).

A number of bacterial strains are able to accumulate the copolymer PHA containing both the short and the medium-chains. Among these strains *P. aeruginosa* produces a copolymer composed of C5 to C10 monomers while growing on valerate (Timm and Steinbushel, 1990). In the same way, *P resinovarans* cultivated on hexanoate accumulates the PHA from C4, C6, C8 and C10 monomers (Ramsay et al, 1992).

Azotobacter vinelandii produced a large quantity of PHB while growing on a variety of sugar sources (Page et al, 1992) without requiring limited growth conditions. This bacterium is used as an alternative for PHB production (Page and Knops, 1989). It accumulates a maximum of PHB without nitrogen and under an excess of carbon

(glucose). The use of other substrates (methanol, acetate, butyrate) have been evaluated by Yamane (1992). The *UWD Azotobacter vinelandii* strain was able to produce PHA efficiently while cultivated in beet molasses, cane molasses and core syrup (Page, 1989; 1990); while using molasses as a substrate it accumulated PHA more than 80% of its weight. The production of a copolymer by this strain was also studied by Page et al. (1992). Growth on glucose as a principal source of carbon added by other secondary sources (valerianate, pentanoate, etc) and the effect of the addition of fish peptone was studied by growing *Azotobacter vinelandii* in two type of cultures (batch and fed- batch). The use of fish peptone enhanced the PHB synthesis. It seemed to be used as a nitrogen source wich allowed a rapid accumulation of PHB (Page and Cornish, 1993).

Other bacterial strains are also known for their PHA producing potential, however, their application is limited. Some studies showed that the rhizobia strains are able to accumulate PHB at a significant level (Hayward et al, 1959; Vincent et al, 1962). The *Methylobacterium organophilum* strain showed an optimum growth at 0.5% (w/v) of methanol. Methanol concentration of 4% (w/v) and lack of divalent cations reduced the bacterial growth. However, under potassium limited condition and with methanol as carbon source, the PHA (P(3HB)) content reached 52% of the cell dry weight. (Kim *et al.*, 1996). The growth of *chromobacterium violaceum* on valeric acid based-medium gave a polymer composed of 100% of the monomer 3HV (Doi et al., 1988; Steinbuchel et al., 1991). However, *Rhodococcus rubber* grown on glucose produces copolymer with 75 mol % 3HV and 25 mol % 3HB (Haywood *et al*., 1991).

5. Industrial production of biopolymers

5.1. Technologies for the production

Generally, fed-batch and continuous cultivation techniques have been used for accumulation of higher concentration of PHB. Fed-batch culture is mostly employed for bacteria (*Alcaligenes eutrophus*, *Protomonas extorquens*, *Protomonas. oleovorans*, etc) requiring limiting factors (N, P, Mg, K, O or S) and an excess carbon source for an efficient PHA production (Lee, 1996a). In the beginning, cells are grown to reach a desired concentration without nutrients limitation. After that, an essential nutrient is

eliminated to allow higher PHB accumulation in the cell. A complete elimination of P or N in the case of *A. eutrophus* significantly increases the polymer accumulation (up to 80% of the cell weight for P(3HB) and P(3HB-3HV)) (Byrom, 1987; Kim et al., 1994). In addition to that, for other bacteria (*P. extorquens* and *P. oleovorans*) a total elimination of N or P did not permit an efficient accumulation of polymers (Preusting *et al*., 1993; Suzuki *et al*., 1986). A two-stage chemostat should be employed for this type of microorganisms – 1st stage to generate cell mass and the 2nd stage to accumulate PHB. However, for some strain such as *P. oleovorans*, one stage process could be used (Preusting *et al*., 1993).

The application of Fed-batch culture strategy, for bacteria that does not require nutrient limitating conditions (*A. latus*, a mutant of *Az. Vinelandii*, etc), is important for fermentation success (Lee, 1996a). Nitrogen sources (corn steep liquor, yeast extract, fish peptone, etc) were added to increase the cell growth and consequently PHA accumulation (Lee and Chang, 1994, Page and Cornish, 1993). However, continuous cultivation gave the highest PHA accumulation. *A latus* and *P. oleovarans* were successfully cultivated in continuous process for producing PHA (Preusting *et al*., 1993; 199). For these type of bacterial strains (that did not require conditions of nutrients limitation) single stage chemostat could also be used. However, it is very important to optimize continuous and fed-batch processes in order to compare the productivity of different strains depending on the nature of the media used. Moreover, economic analysis should be conducted in order to choose an appropriate process.

In 1975, the ICI (Imperical Chemical Industry, United Kindom) started the production of PHBs in response to the increased oil price (Yves et al., 1995). A mutant strain of *A. eutrophus* (Byrom, 1987; 1990), grown in glucose and propionic acid based medium, was used to produce copolymer (P(3HB-3HV)). In order to avoid toxicity and to control the composition of the copolymer (proportion of HV unit), the addition of propionic acid was controlled. The final PHA contents reached 70 to 80% of the cellular dry weight. Depending on the substrate used, HV represents 0 to 30% of the copolymer. At the end of fermentation, the PHA was purified by solvent extraction. However, the use of solvents is replaced by enzymes and detergents to solubilize the cellular compounds and to extract

the polymers (Byrom, 1987; 1990). The copolymer P(3HB-3HV) is marketed under a commercial name BIOPOL by Zeneca Bio. The polymer is produced by fed batch culture and the production is about 1000 tonnes per years (Byrom, 1994).

Another company (Austrian Company Chemie Linz GmbH) produced homopolymer PHB by a process of continuous fermentation of *A. latus* strain in sucrose-based medium (Hrabak, 1992). The strain used (*A. latus* btF-96) permit the production of more than 100 kg of PHB in a week in a 15-m^3 fermenter. After fermentation, cells should be harvested, washed with tap water followed by the extraction of the polymer. During this process, cell suspension is treated by solvent (methylene chloride) and PHB is separated from cell debris by decantation. Then PHB is precipitated (by addition of water) and dried. The use of this process permit to get a PHB purity of 99% (Hrabak, 1992; Lafferty and Braunegg, 1984a; 1984b).

5.2. Applications

The proprieties of PHB are comparable to conventional used plastics (polypropylene). Therefore, it can be processed in the same way as polypropylene. Hence, bacterial homo and copolymers are suitable for some specialized application, such as slow release encapsulation and biomedical devices (Seebatch, et al., 1987). The monomer unit (3-hydroxybutyric acid) can be used for the production of complex chiral pharmaceutical or agrochemical agents (Seebatch, et al., 1987). Moreover, the application of PHB as a matrix material for slow release of pharmaceuticals has been investigated (Lafferty et al., 1988; Korsatko and Wabnegg, 1983).

The useful proprieties of the copolymer P(3HB-3HV) (known under the commercial name of BIOPOL) such as the water resistance and oxygen impermeablity make it suitable for large applications including packaging material (films and bottles), coating of fibrous materials such as paper and board (Cox, 1994; Holmes, 1988; Marchessault *et al*., 1990).

However, PHB application is limited for some use because of the thermal degradation during melt processing, relatively slow crystallization and stiffness. These proprieties can

be controlled by changing the PHB polymer chain configuration, producing different PHA composition or using formulations containing coproducts (Cox, 1994). In order to overcome these problems (thermal degradation and relatively slow crystallization), the use of P(HB-HV) copolyesters with nucleants has been considered as the most successful approach. Moreover, it permit the reduction of stiffness which enlarge the range of applications.

6. Cost of bioplastics production

The most important factors to consider for economical production of PHBs are product yield (Yp/s - g of PHB produced per g of substrate consumed), productivity (Pr – g PHB produced/L/h), PHB content of cell (Yp/x - g of PHB/g dry cell) and cell yield (Yx/s – g cell formed per g of substrate consumed). Yp/s and Yx/s determines the substrate or raw material requirement; higher values of these yield factors means low requirement for substrate. Productivity has a direct impact on the fixed (equipment) capital cost. The factor Yp/x has an impact on the recovery cost of PHB through solvent extraction, a step after fermentation. Efforts have been spent in optimizing the PHB production process and reducing the cost of production in the following directions: strain development, optimization/improvement of fermentation and separation processes, utilisation of cheap carbon sources.

As indicated in the preceeding text, a large number of substrates such as carbohydrates, oils, alcohols, organic acids and hydrocarbons can be used by PHA-producing microorganisms. On the bases of carbon source, the theoretical yield of P(3HB) has been estimated (Yamane, 1993). The theoretical yield was used to determine the cost of the production. Based on the biochemical stoichiometry, values of theoretical yield varied between 0.32 PHB/Kg (for glucose) and 1.16 Kg PHB/Kg (for butanol) (Yamane, 1992). The Substrate costs and yields affect the production cost. Generally, the cost of synthetic plastic (polypropylene and polyethylene) is 1 $/kg lower than biodegradable plastics (Chemical marketing reporter, 1994). Moreover, the cost of production depends on the raw material cost, operation cost, polymer extraction process and purification methods used in large-scale production.

Yield of PHAs has been improved through the development of high yielding recombinant microorgnisms (Lee *et al*., 1994) or through process improvement in fed batch culture (Shirai et al., 1994). Another method to obtain high yield of PHAs is to optimize the growth phase (to enhance biomass yield, Yx/s) and the production phase (to enhance the PHAs yield, Yp/x) or to conduct two phases (growth phase and production phase) separately (Shimizu *et al*., 1992). Despite these efforts, the cost of production of PHAs remains high in comparison to the production cost of synthetic plastics (Choi and Lee, 1997).

Broader use of biodegradable plastics in packaging and disposable products as a solution to environmental problems would heavily depend on further reduction in costs and discovery of novel biodegradable plastics with improved properties. In PHB production, about 25-29% of the total production cost is for direct fixed capital cost and 40-50% of the total production cost is for raw material (Choi and Lee, 1999). Thus, the use of cheaper carbon source (beet molasses, cheese whey, plant oils, hydrolystates of starch, etc) is required in order to substantially reduce the high production cost of PHB (Brandl *et al.*, 1990). Development of new bacterial strain for large scale production on cheaper and complex substrate can also help in reducing the cost of production.

7. New strategies to enhance the the bioplymer poduction

7.1 Waste management strategy

Efforts have been concentrated to use various waste materials as carbon source and activated sludge as a source of microorganisms to accumulate PHAs and PHBs to reduce the cost of production (Table 3). Waste materials offer the greatest promise as a source of carbon for bacterial growth. Therefore, their use can solve the environmental problem of waste disposal and concomitantly help to reduce the cost of PHAs production.

Glucose obtained by enzymatic (enzyme from *A. oryzae*, *B. licheniformis* and Barley malt) hydrolysis of potato starch waste was used for the PHB production by *A. eutrophus* (DSM545). The use of barley malts as a source of α-amylase enzyme to produce fermentable substrate is the lowest in cost compared to the other sources. A ratio of malt

(used as a liquefying agent) to potato waste of 10:90 allow the highest conversion of starch to glucose (96%). The growth of the used strain in one litre cyclone bioreactor and under some conditions (addition of mineral salts and di-ammonium sulphate as a nitrogen source) permitted the production of 5 g/l of PHB (76,9% of the biomass dry weight) (Rusendi and Sheppard, 1995).

Starch and whey were used to produce poly (3-hydroxybutyrate) (PHB) in fed batch cultures of *Azotobacter chroococcum* and recombinant *E. coli*, respectively (Kim, 2000). Oxygen limitation increased PHB contents in both fermentations. In fed-batch culture of *A. chroococcum*, cell concentration of 54 g/L with 46% PHB was obtained with oxygen limitation, whereas 71 g/L of cell with 20% PHB was obtained without oxygen limitation. Under oxygen limiting conditions, comparatively a low cell concentration was obsrved, however, PHB content and PHB productivity was high.

Soya, and malt wastes from a beer brewery plant were used as nutrients for *Alcaligenes latus* to study the synthesis of bioplastics (Yu et al., 1999a). The production of biopolymers by *A. latus* was best using malt wastes. The biomass and polymer yield were 32.4 g/L and 22.7 g/L from soya wastes, 18.4 g/L and 6.0 g/L from soya waste, and 28 g/l and 10.2 g/L from sucrose as the carbon source. The polymer content in cells of *A. latus* using sucrose, malt and soya wastes as carbon sources was 36.3%, 70.1% and 32.6%, respectively. The maximum PHB content of cell (about 70% w/w) was obtained in a two stage system (stage 1 for growth and stage 2 for PHA production) using a mixture of malt waste and sucrose.

Lee *et al*. (1997) examined various recombinant *E. coli* strains for their ability to accumulate a large amount of PHB in whey-based medium. A high cell concentration of 87 g/L with 80% PHB in 49h of fed-batch culture using recombinant *E.coli* (Wong and Lee, 1998). During the fermentation, however, portions of the culture broth had to be removed due to volumetric limitation of the fermentor. In order to overcome this problem, a new operational method was developed to control the timing of PHB biosynthesis in recombinant *E. coli* (Kim, 2000). By limiting the maximum agitation

speed during the cultivation, cells containing 70-80% PHB could be produced without removig culture broth (Kim, 2000). It was claimed that recombinant *E. coli* did not require nutrient limitation for PHB biosynthesis and could accumulate PHB during growth (Lee and Chang, 1995).

The wastewater from olive oil mills (alpechin) was, also, used as a growth media for *Az. Chroococcum* H23. This strain was able to produce PHB up to 50% of the cell dry weight after 24 hours of growth in NH_4 medium supplemented with alpechin. The yield was about 70% in chemically-defined media containing 1% glucose, fructose mannitol, sucrose or starch (Martinez-Teledo et al., 1995).

Use of activated sludge to accumulate PHB

Many workers have shown that the activated sludge could produce bioplastics. Using activated sludge as the source of bacteria to convert nutrients into PHAs would not only produce biodegradable plastics (Yu *et al.*, 1996, 1999a, b; Chua *et al*., 1997; Hu *et al.*, 1997; Chuang *et al*., 1998), but would also alleviate the problem of disposing of municipal and industrial activated sludge. Chua *et al*. (1997) has shown that about 37% of excess sludge could be reduced through PHB production therefore reducing the sludge treatment. In addition, the use of the less value food wastes as carbon sources to produce bioplastics would tremendeously reduce the cost of the production of environment friendly bioplastics.

Activated sludge obtained from wastewater treatment plant treating different wastewater contains different amount of PHB. Activated sludge from a petrochemical complex wastewater treatment plant contained about 28% (w/w) extracellular polysaccharides (EPS) and 1.8% PHB (Dave et al., 1996). Deinema (1972) reported 0.2% PHB in domestic sewage sludge while Wallen and Rohwedder (1974) have shown 1.3% of polyhydroxyalkanoate (PHA) in activated sludge from municipal and grain processing industry wastewater treatment plants.

Several investigators reported that synthesis of PHB is favoured by excess carbon under nitrogen limiting conditions (2-6; Bryom, 1987; Anderson and Dawes, 1990; Lenz et al., 1991; Evans and Sikdar, 1990; Byrom, 1991). To examine the production of PHB, carbon was supplemented in the form of glucose, sucrose, acetate, butyrate and propionate to activated sludge obtained from the treatment of petrochemicals industry wastewaters (Dave *et al*., 1996). About 13 –fold increase in PHB content was claimed with glucose, giving 24% (w/w) yield of PHB. The optimum concentration of glucose for maximum PHB production was found to be 20 g/L. Acetate and propionate also resulted in higher PHB accumulation without increasing the biomass. Addition of nitrogen and/or phosphorus with carbon source resulted in good growth but with less PHB accumulation. The control of pH was essential to inhibit the production of extracellular polysaccharides (other than bioplastics) without affecting the yield of PHB (Dave *et al*., 1996).

Dave *et al*. (1996) also tested the sludge from acrylonitrile wastewater treatment plant and a fertilizer complex wastewater treatment plant (FCTP) for their potential to synthesize PHB. A marginal increase in PHB content (from an initial level of about 0.6-1.0% to 2.5-3.0% w/w) on incubation with glucose or acetate under nitrogen and phosphorus limited and carbon excess conditions was observed. Dave *et al*. (1996) found a number of bacterial species (*Acinetobacter, Aeromonas, Bacillus, Flavobacterium, Pseudomonas* etc.) in sludge, however, the excess accumulation of PHB was due to the domination of *Bacillus sp*. after incubation the sludge with high concentration of carbon. Many researchers have also indicated that *Bacillus spp*. synthesise and accumulate PHB (Byrom, 1987; Anderson and Dawes, 1990; Chen *et al*. 1991). Dave *et al*. (1996) also isolated a strain of *Bacillus* from activated sludge that accumulated PHB up to 52% (w/w). Thus, the sludge containg *Bacillus sp* has the ability to accumulate PHB.

The activated sludge produced in an SBR fed with synthetic wastewater was inoculated in a fermentor to produce PHAs (Hu *et al*., 1997). The medium in fermentor contained different proportions of butyric acid and valeric acids. There was no polymers, PHB (poly-3-hydroxybutyrate) or PHBV (poly 3-hydroxybutyrate-co-3-hydroxyvalerate), in activated sludge in SBR. When butyric acid was used as a sole carbon source, PHB was

produced. When valeric acid was added to the medium, PHBV was produced. The 3-hydroxyvalerate (3HV) mole fraction in the PHBV reached a maximum of 54% when valeric acid was used as a sole carbon source. In this way the composition and the mechanical properties of the co-polymer produced by activated sludge could be controlled by adjusting the medium composition (Hu *et al*., 1997).

Biopolymer production of microorganisms isolated from municipal activated sludge using malt waste was also investigated by Yu et al. (1999a). The isolated microorganisms were grown on sucrose and malt wastes. The final biomass and polymer concentration were 15.13, and 6.6 g/L dry weight, respectively. The biopolymers content of the cell was 43.3% w/w.

Activated sludge from household wastewater treatment was cultivated on hydrolysed soya waste in a Sequencing Batch Reactor for the production of bioplastics (Wong *et al*., 2000). Melting point, thermostability and NMR spectrum were determined for the biopolymers produced by activated sludge utilizing different carbon sources (malt waste, soy waste, glucose, fructose). It was suggested that different copolymers were formed by activated sludge for different types of carbon because the activated sludge composed of a wide variety of bacteria, many different species of which could produce PHAs. Different type of PHA synthatase with different substrate specificity could be present in the mixed bacterial culture in activated sludge. A PHB-producing bacterium (*Klebsiella pneumoniae* – a known pathogen) was also isolated from activated sludge and employed to produce bioplastics using synthetic media and waste materials (Wong et al, 2000). Different co-polymers were produced with a ratio of HB:HV as 93:7 and 79:21 utilizing malt and soy waste, respectively.

Activated sludge from laboratory scale anaerobic-aerobic reactors, when fed on acetate, accumulated up to 20% PHA under anaerobic conditions whereas up to 33% under aerobic conditions (Satoh et al., 1998). The cellular content of PHA was found to increase up to 62% by using “microaerophilic-aerobic” proces, where limited amount of oxygen was supplied into the anaerobic zone of the anaerobic-aerobic process.

Biodegradable thermoplastics (PHAs) were produced from municipal sludge in a two-stage bioprocess: thermophilic anaerobic digestion of sludge in the first stage and production of PHAs from soluble organic compounds in the supernatant of the digested sludge by *Alcaligenes eutrophus* in the second phase (Lee and Yu, 1997). The amount of PHAs accumulated in *A. eutrophus* was 34% of dry cell mass and was comparable to the amount of PHAs produced from pure volatile fatty acids (33% of cell mass). The conversion of four major acids in the supernatant by *A. eutrophus* were found to be 87.6% (acetic acid), 62.6% (propionic acid), 56.8% (butyric acids) and 32% (valeric acids). The sludge PHA was a co-polymer containg mainly c4 monomers (74 wt %) with 9^{o}C lower melting point than PHB and hence better impact and tensile strength. The sludge thermoplastic could be degraded by more than 70% mass in 5 weeks in sludge suspensions, and has a similar biodegradability as PHB does in soil (Lee and Yu, 1997).

Activated sludge bacteria in a conventional wastewater treatment (SBR system) were induced, by controlling carbon to nitrogen ratio in the reactor liquor, to accumulate polymers (Chua *et al*., 1997). Unlike previous researchers, the wastewater treatment and the polymer accumulation was accomplised in a single stage process. The polymer content of cells increased to a maximum of 37.4% (cell dry weight) when C:N ratio was increased from 24 to 144, whereas the cell yield (g of cells formed per g of TOC used) decreased with increased C:N ratio. Hence, the extraction of polymer could reduce 37.4% of sludge treatment and disposal. The optimum C:N ratio of 96 provided the highest polymer yield 0.093 g of polymer per g of carbonaceous substrate consumed. Sporadic change in the C:N ratio did not significantly affect the wastewater treatment efficiency in the SBR. The C:N ratio in activated sludge process must be kept around 24 in order to enable normal microbial cell synthesis. Threfore, it was suggested that if nitrogen deficiency was prolonged to have continual polymer production (C:N ratio of 96-144), cell growth and TOC removal efficiency could be adversely affected. As a result, an intermittent nitrogen feeding program was suggested in order to optimize the polymer production without affecting the treatment efficiency. The analysis of polymers accumulated in activated sludge contained mainly poly-β-hydroxybutyric acid, and co-polymers of β-hydroxybutyric (3-HB) and β-hydroxyvaleric (3-HV) acids.

7.2. Genetic modification strategy

The biosynthetic path way of PHA producing bacteria can be transferred to bacteria that do not produce PHAs. Genes involved in biosynthesis have been identified and cloned and recombinant bacteria producing PHAs more efficiently were obtained (Steinbuchel *et al.*, 1995; Steinbuchel *et al*., 1992). Moreover, two genes types such as PHA producing genes and cheaper substrate using gene should be taken into consideration while developing new recombinant strains. This permits the reduction of PHA production cost (Lee and Chang, 1995). PHA biosynthesis genes include the 3-ketothiolase (*phaA*), acetoacetyl-CoA reductase (*phaB*), PHA depolymerase (*phaZ*) and the gene of the granule-association protein (GA14) (Steinbuchel *et al*., 1992, Pieper-Furst *et al*., 1994). However, substrate utilisation genes can be introduced in PHA producing bacteria.

Many recombinant bacteria (*E. coli*, *K. aerogenes*, *A. eutrophus*, *P. oleovarans* and *Mycoplana rubra*) have been produced by the incorporation of the PHA biosynthesis genes (Lee and Chang, 1995; Lee *et al*., 1994; Fidler and Dennis, 1992; Lee and Chang, 1994; Follner et al., 1995; Preusting *et al*., 1993; Kolibachk *et al*., 1999; Hahn *et al*., 1995; Liu and Steinbuchel, 2000). For example, *E. coli* recombinant strain was used to produce P(3HV) at higher concentration (Lee *et al*., 1994; Fidler and Dennis, 1992; Lee and Chang, 1994). Because of the fact that *E. coli* can not utilize propionic acid, the production of copolymer P(3HB-3HV) is limited by the recombinant strain. However, by using a mutant of *E. coli* produced by inducing the bacterial growth with acetic or oleic acid (Slater et al., 1992; Yim et al., 1995), this problem could be eliminated.

A recombinant strain *K. aerogenes* has been developed to produce P(3HB) by using molasses as a substrate. Moreover, a recombinant of *A eutrophus* obtained by amplification of enzyme activities gave PHA production rate 1.24 times higher than that of the original strain (Park *et al*., 1995).

A. eutrophus PHA biosynthesis genes could be expressed in *P. oleovorans* in order to produce P(3HV) and medium chain length PHAs (Preusting *et al*, 1993). Moreover, it is possible to introduce specific genes into *A. eutrophus* for enhancing the growth of this

bacteria in some substrates. This strategy can be exploited to reduce the cost of PHA production by using cheap substrates (Lee, 1996).

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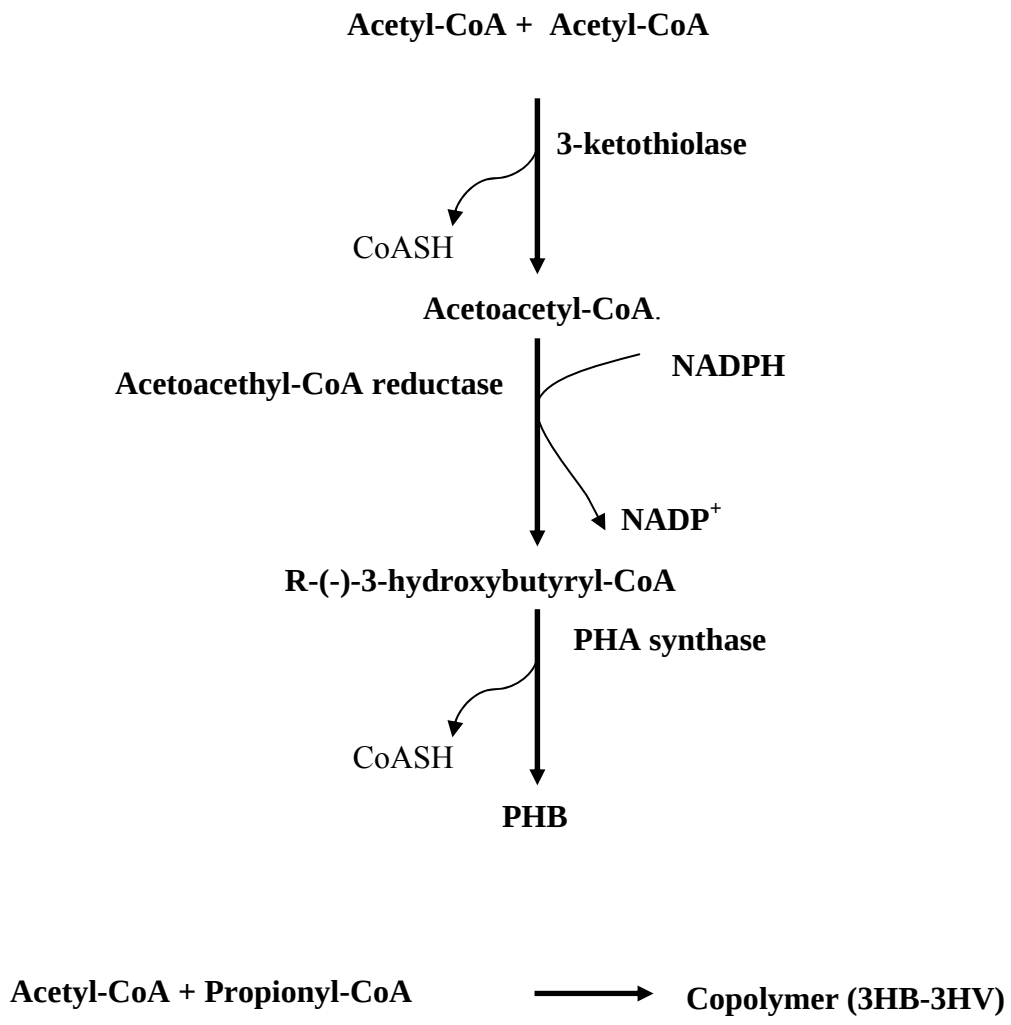
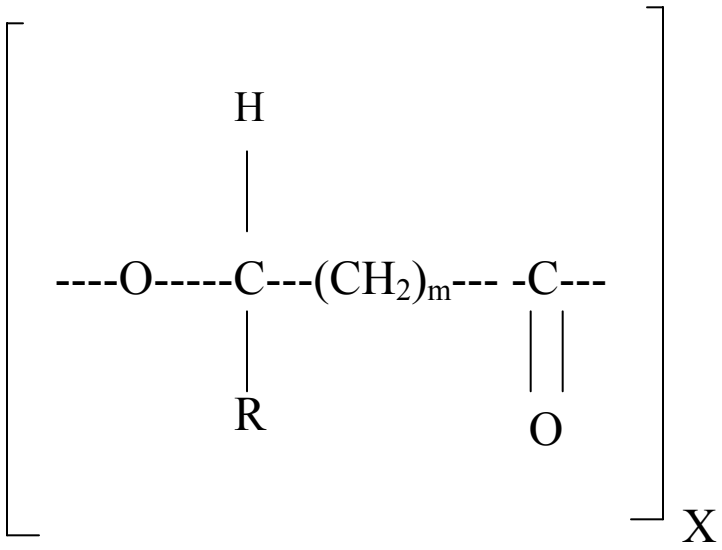


Figure 1. Pathway of PHA biosynthesis



m=1 R= Hydrogen	Poly 3-hydroxypropionate (3HP)
R= Methyl	Poly 3-hydroxybutyrate (3HB)
R= Ethyl	Poly 3-hydroxyvalerate (3HV)
R= Propyl	Poly 3-hydroxycaproate (3HC)
R= butyl	Poly 3-hydroxyheptanoate (3HH)
R= Pentyl	Poly 3-hydroxyoctanoate (3HO)
R= Hexyl	Poly 3-hydroxynonanoate (3HD)
R= Heptyl	Poly 3-hydroxydecanoate (3HUD)
R= Nonyl	Poly 3-hydroxydodecanoate (3HDD)
m=2 R=Hydrogen	Poly 4-hydroxybutyrate (4HB)
R= Methyl	Poly 4-hydroxyvalerate (4HV)
m=3 R=Hydrogen	Poly 5-hydroxyvalerate (5HV)
R= Methyl	Poly 5-hydroxyhexanoate (5HH)
m=4R=Hexyl	Poly 6-hydroxydodecanoate (6HDD)

Figure 2. Chemical structure of the major polyhydroxyalkanoates produced in bacteria

Table 1. Some proprieties of some various PHAs compared to polypropylene.

Proprieties	**PHB**	**P(HB-HV)**	**PHO**	**Polypropylene**
Molecular Weight (1000 Da)	500	-	130	200
Glass-transition temperature (°C)	15	-1	-35	-10
Melting temperature (°C)	175	145	61	176
Crystallinity (%)	80	40	30	70
Extension to break (%)	6	-	300	400
Tensile strength (MPa)	40	32	10	38
Oxygen permeability (cm3/m2/atm/day)	45	-	-	1700
Resistance to regent	Weak	-	-	Good
Resistance to UV	Good	-	-	weak

PHB : polyhydroxybutyrate

P(3HB-3HV) : copoly(3-hydroxybutyrate-3-hydroxyvalerate)

PHO : poly 3-hydroxyoctanoate)

Table 2. Some bacterial strain producing PHAs

Bacteria	**Carbon sources**	**PHA content (% dry cell weight)**	**PHAs**	**References**
Alcaligenes eutrophus	Butyric +pentanoic acid	43 to 55	P(3HB-3HV)	Yoshiharu et al., 1988
A. eutrophus	Pentanoic acid	46	P(3HB-3HV)	Yoshiharu et al., 1988
A. eutrophus	Butyric acid	48	P(3HB)	Yoshiharu et al., 1988
A. eutrophus	Glucose	54	P(3HB)	Yoshiharu et al., 1988
A. eutrophus	Olive oil	47	P(3HB)	Minoru et al., 1992
A. eutrophus	Corn oil	39	P(3HB)	Minoru et al., 1992
A. eutrophus	Palm oil	40	P(3HB)	Minoru et al., 1992
Rhizobium trifolii	Mannitol	40 t0 50	-	Vincent et al., 1962
A. latus	Sucrose	50 to 80	P(3HB)	Yamane et al., 1996 ; Wang and Lee, 1997
Azotobacter vinelandii	Glucose + fish peptone	79.8	P(3HB)	Page and comish, 1993
Azotobacter vinelandii	Valeric acid	78	P(3HB-3HV)	Page et al., 1992
Azotobacter vinelandii	Propionic acid	75	P(3HB)	Page et al., 1992
Azotobacter vinelandii	Butyric acid	78	P(HB)	Page et al., 1992
Chromobacterium violaceum	Valeric acid	62	P(3HV)	Steinbuchel and Schmack, 1995
Methylobacterium organophilum	Methanol	52	P(3HB)	Kim et al., 1996
Protomonas extorquens	Methanol	64	P(3HB)	Suzuki et al., 1986
Pseudomonas oleovorans	n-Octane	33	P(3HHx-3HO)	Preusting et al., 1993
Erwinia sp.	Palm oil	46	P(3HB)	Majid et al., 1999
Erwinia sp.	Palm oil + propionic acid	40	P(3HB-3HV)	Majid et al., 1999
Erwinia sp.	Palm oil + valeric acid	34	P(3HB-3HV)	Majid et al., 1999

P(3HB): poly(3-hydroxybutyrate).

P(3HV) : poly(3-hydroxyvalerate) ;

P(3HB-3HV) : copoly(3-hydroxybutyrate-3-hydroxyvalerate) ;

P(3HHx-3HO) :copoly(3hydroxyhexanoate-3-hydroxyoctanoate)

Table 3. Waste used for the production of PHAs by some bacterial strain

Waste	Bacteria	Time of growth (h)	Cell conc. (g/l)	PHB conc. (g/l)	PHB content (%)	References
Sucrose	*A. latus*	50	28	10.2	36.3	Yu et al., 1999
Malt	*A. latus*	50	32.4	22.7	70.1	Yu et al., 1999
Soya	*A. latus*	50	18.4	6	32.6	Yu et al., 1999
Soya	Activated sludge micro-organisms	168	-	0.172	-	Wong et al., 2000
Anaerobic digestion sludge	*A. eutrophus*	48	1.79	0.61	34	Lee and Yu,1997
starch	*A. eutophus*	45h	6.5	5	76.9	Rusendi and Sheppard, 1995
Starch	*Az. chroococcum*	70	54	25	46	Kim, 2000
Starch	*Az. chroococcum*	58	1.17	0.864	73.9	Kim, 2000
Starch	*Az. chroococcum*	72	5.19	3.85	74.2	Martinez-Teledo et al., 1995
Starch	*Haloferax mediterranei*		10	6	60	Lillo and Rodriguez-Valera, 1990
Whey	*Metylobacterium sp.*	48	9.9	5.9	59.6	Yellore and Desai, 1998
Whey	Recombinant *E. coli*	52	31	25	80	Kim, 2000
Whey	Recombinant *E. coli*	35	55	32	57	Kim, 2000
Whey	Recombinant *E. coli*	47	109	50	-	Wong and Lee, 1998
Alpechin	*P. putida*	72	1.32	21.25	1.60	Garcia-Ribera et al., 2001
Alpechin	*P. putida* (Recombinant)	72	4.24	126.95	3.59	Garcia-Ribera et al., 2001
Alpechin	*Az. Chroococcum*	24	-	-	50	Martinez-Teledo et al., 1995
Cane sugar	*Ralstonia eutropha*	45-50	125-150	-	65-70	Vincente 1998ab
Cane sugar	*Bhurkolderia sp*	45-50	125-150	-	65-70	Da Silva and Gomes, 1998
Molasses	*Az. vinlandii*	36	33	22	66	Page and Cornish, 1993
Xylose	*P. cepacia*	-	-	-	48.8	Young et al., 1994
Xylose	*P. cepacia*	60	2.59	1.55	60	Ramsay et al., 1995
Xylose	*P. pseudoflava*				22	Bertrand et al., 1990
Tapioca hydrolysate	*Ralstonia eutropha*	59	106	61	58	Kim et Chang, 1995

CHAPITRE 3

ÉTUDE DE LA PRODUCTION DE POLYHYDROXYALKANOATE (PHA) EN UTILISANT LES EAUX USÉES COMME SOURCE DE CARBONE ET LES BOUES ACTIVÉES COMME SOURCE DE MICRO-ORGANISMES

Part I

Polyhydroxyalkanoates (PHA) production using wastewater as carbon source and activated sludge as microorganisms

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Résumé

Des boues activées prélevées de rejets de différentes usines de traitement des eaux usées (municipales, industrie de pâtes et papiers, fabrication d'amidon et fromageries) ont été utilisées comme sources de microorganismes pour produire des plastiques biodégradables en erlenmeyers. De l'acétate, du glucose et différentes eaux usées ont été employés comme sources de carbone. Il a été constaté que des boues d'épuration de pâtes et papier ont accumulé la plus forte concentration de PHAs; Ceci atteignant au maximum 43% du poids sec des matières solides en suspension, avec de l'acétate comme source de carbone. Parmi les différentes eaux usées testées comme source de carbone, les eaux usées de l'industrie des pâtes et papiers et d'industrie d'amidon se sont révélées être les meilleures sources de carbone tout en employant des boues activées des pâtes et papiers pour un maximum d'accumulation de PHAs. Une forte concentration d'acides gras volatils dans ces eaux usées en serait probablement responsable.

Mots-clés: boues activées, eaux usées municipales, eaux usées industrielles, bioplastiques, polyhydroxyalcanoates, PHA.

Abstract

Activated sludge from different full-scale wastewater treatment plants (municipal, pulp and paper industry, starch manufacturing and cheese manufacturing wastewaters) was used as a source of microorganisms to produce biodegradable plastics in shake flask experiments. Acetate, glucose and different wastewaters were used as carbon source. Pulp and paper wastewater sludge was found to accumulate maximum concentration (43% of dry weight of suspended solids) of polyhydroxy alkanoates (PHA) with acetate as carbon source. Among the different wastewaters tested as a source of carbon, pulp and paper industry and starch industry wastewaters were found to be the best source of carbon while employing pulp and paper activated sludge for maximum accumulation of PHA. High concentration of volatile fatty acids in these wastewaters was the probable reason.

Key words: Activated sludge, municipal wastewater, industrial wastewater, bioplastics, polyhydroxyalkanoates, PHA.

1. Introduction

There has been a considerable interest in the development of biodegradable plastics due to the problem and harmful effects of conventional plastics on the environment. Polyhydroxyalkanoates (PHA) are attractive substitute among the biodegradable plastics, because they can be used in various ways similar to many conventional petrochemical derived plastics currently in use and due to complete biodegradability upon disposal under various environments (Kim et al., 1994; Lee and Yu, 1997; Chua et al., 2003). Poly-beta-hydroxybutyric acid (PHB) and its copolymer poly(3-hydroxybutyrate-co-hydroxyvalerate ((P(3HB-co-HV)) are the most widespread PHAs, although other forms are possible. Many microorganisms can accumulate PHA. However, its widespread applications have been limited due to high production costs. Much effort has been made to reduce the production costs.

Many researches have been conducted to investigate the possibilities to produce PHA by using activated sludge as a mixed culture. By this approach, the quantity of excess sludge generated from activated sludge process will reduce and be reused, therefore reducing the costs for sludge treatment and disposal, and a new way for carbon recycle by the conversion of organic pollutants in waste into valuable products. Satoh et al. (1998) reported that activated sludge from laboratory scale anaerobic-aerobic reactors accumulated PHA up to 20% (wt.% of sludge dry weight) under anaerobic conditions whereas up to 33% (wt.% of sludge dry weight) under aerobic condition.

The activated sludge used in most of the researches of PHA production was acclimatised with synthetic wastewater in laboratory scale, very little was known about the activated sludge from full scale wastewater treatment plants, especially from certain industrial ones. Furthermore, the wastewater used as carbon sources for PHA production is also hardly studied. Therefore, the objective of this study was to evaluate the potential of waste activated sludge (AS) from full-scale municipal and industrial wastewater treatment plants using different carbon sources for PHA production.

2. Materials and methods:

2.1. Activated sludge:

Four types of fresh activated sludges for PHA production were used from municipal (MAS), pulp-paper (PAS), starch (SAS) and dairy (DAS) wastewater treatment plants (WWTP) in Quebec Province, Canada. The characteristics of four types of sludges are listed in Table 1.

2.1. Carbon sources

Three different sole carbon sources were used for PHA production. I) Sodium acetate (NaAc) with concentration of 2.5, 1.5, 1.0, 0.5 and 0.25 g-Ac^-/l. II) Glucose with following concentration 2.5, 1.5, 1.0, 0.5 and 0.25 g/l. III) Wastewaters were sampled from different wastewater treatment plants municipal (MWW), pulp-paper (PWW), starch (SWW) and dairy (DWW) (cheese manufacturing) in Quebec Province, Canada. The characteristics of different wastwaters are shown in Table 2.

Table 1. Characteristics of 4 types of sludges.

Items	Activated sludge*			
	Municipal MAS	Pulp-paper PAS	Starch SAS	Dairy DAS
Operation process	Biofilter	Aerobic	Aerobic	Aerobic(SBR)
pH	6.5	6.9	3.5	6.8
SS	19.6	19.3	19.2	19.6
COD_{tot}(mg/l)	236.5	1675.8	8611.2	231.2
COD_{sol}(mg/l)	78.3	120.3	7203.1	96.1
TKN(mg/l)	128.3	25.3	2351.2	165.2
NH_4^+(mg/l)	71.2	12.9	1758.9	78.2
P_{tot}(mg/l)	40.1	13.3	34.5	16.2
Orth-P(mg/l)	11.2	5.2	25.6	9.1

*Activated sludge data were on the basis total solids concentration of 20 g/l for sludge.

Table 2. The characteristics of different wastewaters used as carbon source.

Items	Wastewaters			
	Municipal	Pulp-paper	Starch	Dairy
pH	6.5	6.9	3.5	11.4
COD_{tot}(mg/l)	57.2	989.6	8231.2	201.2
COD_{sol}(mg/l)	37.2	417.2	5167.6	93.1
TKN(mg/l)	77.34	22.6	2357.2	103.2
NH_4^+(mg/l)	19.8	11.3	17.5	56.8
P_{tot}(mg/l)	10.2	13.3	25.7	23.3
Orth-P(mg/l)	1.95	0.2	1.3	5.8
C:N	0.73	43.8	3.49	1.95

2.3 Experiment

Shake flask experiments:

The sludge was concentrated by gravity settling, the suspended solids (SS) of the activated sludge were measured. The experiments were conducted within a couple of hours after the sampling. Activated sludge (200 mL, 0.5 g/l SS) was transferred to 500 mL Erlenmeyer flask and appropriate amount of acetate or glucose was added to obtain desired concentration. In case of wastewater as carbon source, a pre-determined volume of sludge was added to wastewater so as to obtain a 0.5 g/L of suspended solids concentration. For all experiments, pH was adjusted to 7.0 by 2N H_2SO_4 or 2N NaOH. The flasks were incubated at 25^oC for 24 hours in a rotary shaker at 250 rpm. Samples were drawn at predetermined time.

2.4 Analytical techniques

Four-ml culture broth was sampled in a pre-weighed centrifuge tube and centrifuged at 5000 rpm for 10 minutes. The settled biomass was washed 3 times with distilled water and centrifuged, then put in a desiccator for at least 48 hours. The final weight of the tube was measured to calculate the sludge dry weight. The extraction of PHA was made by the methanol and chloroform extraction method described by Comeau et al. (1988)

using benzoic acid as an internal standard. The co-polymeric composition was measured with a gas chromatograph (GC) (Varian Model 3800) equipped with a Capillary column Zebron ZB-5 and a Shimadzu C-R5A Chromatopac flame-ionization detector. Poly-(3-Hydroxybutyrate-co-3-hydroxyvalerate)(3HB-co-3HV) (PHV content 12wt.%, Aldrich Chemical Company, Inc.) was used as a standard.

The supernatant of the centrifuged samples was used to determine dissolved chemical oxygen demand (DCOD), acetate (Ac^-) and glucose concentration. The concentration of acetate was measured using ICS-2000, Ion chromatography systems (Dionex Co.) with the column of Ion Pac AS14 anion-exchange columns. The glucose concentration was determined utilizing the glucose oxidase˜peroxidase˜o-dianisidine enzymatic assay kit, Sigma-Aldrich, Inc) (Bergmeyer and Bernt, 1974). The ammonium nitrogen (NH_4^+-N), total kjeldahl nitrogen (TKN), orthophosphate (ort-P) and total phosphate, COD, and suspended solids (SS) were analysed according to standard methods (APHA, 1992). Volatile fatty acids in wastewater were determined by gas chromatograph.

3. Results and discussions

3.1. Acetate as the carbon source for PHA production

*Pulp paper activated sludge**:***

The summary of the results at different acetate concentration using PAS is presented in Table 3. At the concentration of 1.0, 0.5 and 0.25 gAc^-/l (under substrate limiting conditions), PHA concentration increased, reached maximum and then decreased due to limitation of carbon source. At non-limiting acetate concentration (2.5 and 1.5 gAc^-/l), the biomass growth and PHA accumulation occurred simultaneously until 24 hrs (results not shown). The maximum PHA accumulation was obtained at acetate concentration of 1.0 g Ac^-/l at 16 hrs. The maximum PHA concentration obtained at acetate concentration of 1.0 gAc^-/l was 2 times higher than that obtained at 2.5 gAc^-/l, and 3.8 times that obtained at 0.25 gAc^-/l. At 0.5 and 0.25 g Ac^- /l, the maximum PHA content was low due to the shortage of the carbon source, and the maximum PHA content of cells at 1.5 and 2.5 g Ac^- /l was also less than that at 1 g Ac^- /l. Therefore, it is important to select

optimum concentration of carbon sources. At acetate concentration greater than 1.0 g Ac^-/l, the rate of PHA accumulation as well as PHA concentration decreased, this could be due to inhibition at high acetate concentration. This inhibition could be alleviated by: (a) increasing solids concentration and (b) operating the system in a fed-batch culture by maintaining acetate concentration lower than 1.0 g Ac^-/l.

Municipal activated sludge (MAS), dairy activated sludge (DAS) and starch activated sludge (SAS)

A similar phenomenon was noticed as that using PAS and the maximum PHA content (wt% of biomass) was obtained at acetate concentration of 1.0 g-Ac^-/l irrespective of sludge type. However, the maximum accumulation of PHA reached 30.64% using MAS, 4.7% using SAS and 21.73% using DAS at 24 hrs, instead of 43.06% at 16 hrs using PAS (Table 4). Thus, the PHA production potential of the PAS was significantly higher than that of MAS, DAS and SAS.

The study on PHAs production was also conducted by Takabatake et al. (2002) using 18 activated sludge samples from 4 municipal wastewater treatment plants (WWTPs) in aerobic batch experiments with excess supply of acetate as the sole carbon source. The highest PHA content obtained was 29.5% of MLSS, with the lowest 6.0% and average 18.8%(w/w). The results obtained in this study showed almost similar PHA content (30.64%w/w) for MAS as that obtained by Takabatake et al. (2002), however, significantly higher PAH content was observed for PAS.

Table 3.Summary of results at different acetate concentration using PAS.

Acetate conc.(g/l)	Time for Max.PHA (h)	Max.PHA conc.(g/l)	HB:HV at max. PHA	Biomass conc. at max.PHA (g/l)	Max. PHA content (%w/w)	Productivity (g/l/h)
0.25	4	0.0964	100:0	0.6700	14.39	0.024
0.50	8	0.1538	100:0	0.6725	22.87	0.019
1.0	16	0.3628	100:0	0.8425	43.06	0.023
1.5	24	0.2388	100:0	0.7800	30.62	0.010
2.5	24	0.1802	100:0	0.7500	24.03	0.008

Table 4. Comparison of results for four types of activated sludge (acetate concentration – 1g/L).

Activated Sludge	Time for Max.PHA (h)	Max.PHA conc.(g/l)	HB:HV	Biomass conc. at max.PHA (g/l)	Max PHA content (%)	Productivity (g/l/h)
PAS	16	0.3628	100:0	0.8425	43.06	0.023
MAS	24	0.1953	100:0	0.6375	30.64	0.008
SAS	24	0.02552	87:13	0.5429	4.7	0.001
DAS	24	0.1136	92:8	0.5228	21.73	0.005

3.1. Glucose as the carbon source

Experiments were conducted for PHA production (4 types of activated sludge) using glucose as carbon source (Table 5). The biomass growth and PHA accumulation occurred simultaneously at glucose concentration of 2.5, 1.5 1.0, and 0.5 g/l during the period of 24 hrs. At glucose concentration of 0.25 g/l, PHA concentration decreased when all glucose was consumed (after 12h). The maximum PHA concentration increased with the increase of the glucose concentration used. The maximum PHA production was

obtained at glucose concentration of 2.5 g/l at 24 hrs. It seemed that there was no inhibitory effect of glucose as observed in case of acetate. Also, there was no significant difference between the maximum PHA concentration at different glucose concentration (0.5 to 2.5 g/l).

Table 5. Summary of results using PAS at different glucose concentration (0.5g/l SS).

Glucose Conc. (g/l)	Time for Max.PHA (h)	Max.PHA conc.(g/l)	HB:HV at max. PHA	Biomass conc. at max.PHA (g/l)	Max PHA content (%)	Productivity (g/l/h)
0.25	12	0.0153	81:19	0.6338	2.41	0.001
0.50	24	0.0232	85:15	0.7150	3.25	0.001
1.0	24	0.0232	87:13	0.7100	3.27	0.001
1.5	24	0.0246	92:8	0.7163	3.43	0.001
2.5	24	0.0274	87:13	0.7475	3.66	0.001

Municipal activated sludge (MAS), dairy activated sludge (DAS) and starch activated sludge (SAS)

The maximum PHA concentration was observed at 2.5 g/l glucose concentration irrespective of sludge type. The results of different sludges obtained at 2.5 glucose concentration are compared in Table 6. The maximum PHA accumulation was almost similar irrespective of sludge type. However, the maximum PHA content with glucose as carbon source was significantly lower than that using acetate as carbon source.

Table 6. Comparison of PHA production by four types of activated sludge at 2.5 g/l glucose concentration.

Activated sludge	Time for Max.PHA (h)	Max.PHA conc.(g/l)	HB:HV	Biomass conc. at max.PHA (g/l)	Max PHA content (%)	Productivity (g/l/h)
PAS	24	0.0274	87:13	0.7475	3.66	0.0057
MAS	24	0.0240	90:10	0.7180	3.40	0.0050
SAS	24	0.0160	79:21	0.5430	2.90	0.0033
DAS	24	0.0169	81:19	0.5620	3.01	0.0035

3.3 wastewater (WW) as the carbon sources for PHA production

The results obtained using four different types of wastewater as carbon source and four types of activated sludge used as source of microorganisms (biomass concentration of 0.5 g/l) for PHA accumulation are presented in Fig 1. Among four types of watewaters, the PWW showed the highest potential for PHA production. Since volatile fatty acids (VFA) are substrates, which can readily be converted to PHA; higher volatile fatty acids concentration in the influent wastewater was measured (Table 7). It was clear that, the highest concentration of VFA and high C:N (Table 2) ratio in PWW might be an explanation for the highest PHA production using PWW. This suggested that industrial wastewater rich in volatile fatty acids could be a good carbon source for PHA production. Thus, this approach is a possibility of production of bioplastics coupled with wastewater treatment. About 40% (420.3 to 248.6 mg-COD/l) of COD was converted to PHA and this reduction in COD would decrease the pollution load to be treated at wastewater treatment plant. This will also decrease the cost of wastewater treatment and result in less sludge production.

Table 7. Volatile fatty acid concentration in wastewaters used for PHA production.

WW	Volatile fatty acid (mg/l)					
	acetic	propionic	iso-butyric	n-butyric	iso-valeric	n-valeric
PWW	256.09	3.27	1.86	3.75	5.03	2.38
MWW	13.50	3.20	0.52	0	2.10	1.50
DWW	9.2541	2.01	0	0	0	2.01
SWW	247.79	7.98	0	2.85	1.62	2.05

4. Conclusions

Different types of activated sludge were tested employing different carbon sources for the production of PHA. The pulp and paper activated sludge showed the highest potential for PHA production. Acetate and pulp and paper industry wastewater was found to be the best carbon source for highest PHA accumulation. The amount of carbon source fed, and in particular, the type of carbon source used was the most important. The use of activated sludge to convert carbon sources into PHAs can produce bioplastics and also reduce a part of the problem of the disposal of municipal and industrial activated sludge production/disposal. The selection of industrial and municipal wastewater as a carbon source can further reduce the cost of production of PHAs and minimise the treatment cost.

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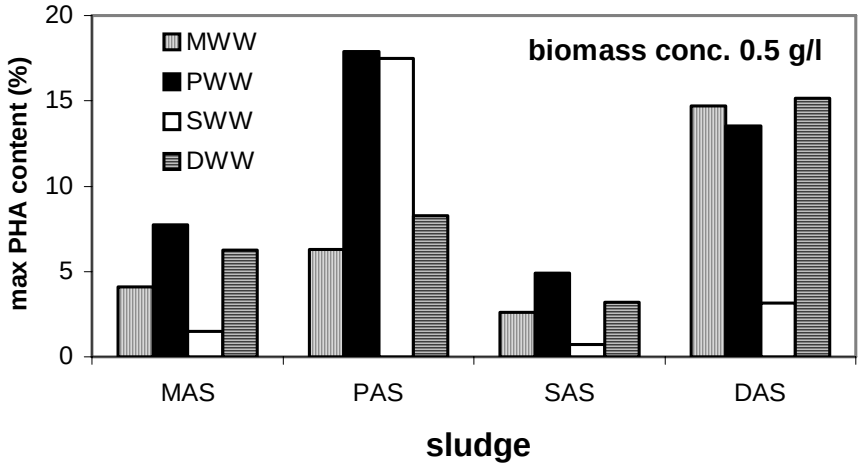

Figure 1. Comparison of maximum PHA content (w/w % of cell) of various types of WW as the carbon sources using different activated sludge.

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Part II

Bioplastics from activated sludge: Batch process.

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Résumé

Des boues activées provenant d'usines de traitement des eaux usées d'industries de pâtes et papier ont été évaluées comme source de microorganismes pour produire des plastiques biodégradables (polyhydroxyalcanoates - PHA) à 25^{o}C, à pH 7 et à différentes concentrations initiales de solides en suspension (SS); Ces concentrations (5, 10, 15 et 20 g / l.) étant contrôlées par ordinateur en fermenteurs (15 L). De l'acétate a été employé comme seule source de carbone à différentes concentrations (5 à 20 g / l.). Les échantillons de boues fraîches contentenait déjà des PHAs dont la teneur variait avec la date de l'échantillonnage. La productivité en bioplastiques varie avec la concentration initiale de boues et celle de l'acétate. Un maximum d'accumulation de PHAs (39,6% m / m de boues sèches SS) a été observé pour 15 g / l de SS et 10 g / l d'acétate. La biomasse a augmenté au cours de ce processus, toutefois, le taux net en PHA des boues résiduelles après extraction de matières solides a baissé à des degrés variables selon les boues et la concentration de l'acétate (5,5% à 36,9% m / m). Une concentration plus élevée d'acétate parait inhiber l'accumulation de PHA et la croissance. Des copolymères tels que polyhydroxybutyrates (PHB) et polyhydroxyvalérates (PHV) ont été produits par les boues et dont la composition molaire varie avec le temps de croissance en erlenmeyers ou en fermenteurs.

Mots-clés: boues activées, pâte et papier, usine de traitement des eaux usées industrielles, bioplastiques, polyhydroxyalcanoates (PHA), acétate, fermentation.

Abstract

Activated sludge from full-scale pulp-paper industry wastewater treatment plants was used as a source of microorganisms to produce biodegradable plastics (polyhydroxyalkanotes - PHA) at 25^{o}C, pH 7 and at different initial sludge suspended solids (SS) concentration (5, 10, 15 and 20 g/l) in computer controlled fermentors (15 L). Acetate was used as sole carbon source at varying concentration (5 to 20 g/l). The fresh sampled sludge was found to contain PHA and the amount varied with the sampling date. The PHA productivity and concentration varied with intial sludge SS and acetate concentrations. Maximum accumulation of PHA (39.6% w/w of dry sludge SS) was observed at 15 g/l SS and 10 g/l acetate concentration. The sludge biomass increased during the process, however, net amount of the sludge residual solids after extraction of PHA was decreased to varying degrees depending on sludge SS and acetate concentration (5.5% to 36.9% w/w). Higher concentration of acetate was found to be inhibitory for PHA accumulation and biomass growth. Copolymers (polyhydroxybutyrates - PHB and polyhydroxyvalerates - PHV) were produced by the sludge whose molar composition varied with the batch process time.

Key words: Activated sludge, pulp-paper industrial wastewater treatment plant, bioplastics, polyhydroxyalkanoates (PHA), acetate, fermentation.

1. INTRODUCTION

Polyhydroxyalkanoate (PHA) is one of the biodegradable plastics produced mainly by bacteria; it is also material which has similar properties to conventional plastics. Due to the problem and harmful effects of conventional plastics on the environment, there has been a considerable interest in the development of biodegradable plastics since last few decades(Lee and Yu 1997; Dionisi et al. 2001b; Takabatake et al. 2002; Chua et al. 2003; Kumar et al. 2004). Poly-beta-hydroxybutyric acid (PHB) and its copolymer poly(3-hydroxybutyrate-co-hydroxyvalerate [(P(3HB-co-HV)] are the most widespread PHAs, although other forms are possible. Many microorganisms can accumulate PHA. However, its widespread applications have been limited due to high production costs. Much effort has been made to reduce the production costs.

Using activated sludge as a mixed culture to produce PHA has been studied by many researches (Lemos et al. 2004; Dionisi 2005; Dionisi et al. 2005a). By this approach, it could significantly reduce the cost of PHA production and at the same time, reduce the quantity of excess sludge from the wastewater treatment process that required further treatment, and a new way for carbon recycle by the conversion of organic pollutants in waste into valuable products. Satoh et al. (1998) reported that activated sludge from laboratory scale anaerobic-aerobic reactors accumulated PHA up to 20% under anaerobic conditions whereas up to 33% under aerobic condition.

Chua et al. (2003) investigated a two stages process for PHA production by activated sludge, in the first step, an anaerobic-aerobic SBR fed with real municipal wastewater was enriched in PHA accumulating organisms through manipulation of the operational conditions (such as sludge retention time - SRT, pH, and substrate concentration). In the second stage, the enriched sludge was fed with acetate in a batch aerobic reactor. A maximum PHA content of 30% and a specific productivity of 50 mg PHA. g^{-1} cell dry weight . h^{-1} were obtained.

However, the activated sludge used in most of the researches of PHA production was acclimatised with synthetic wastewater in laboratory scale, very little was known about PHA production capability of activated sludge from full scale industrial wastewater treatment plants. In a previous study (Yan et al. 2006), it was found that activated sludge from pulp paper industry wastewater treatment plant (PAS) showed the best potential for PHA production among four types of municipal and industrial activated sludge (municipal, starch industrial, and dairy industrial wastewater treatment plants) in shake flask experiments. However, the concentration of the sludge suspended solids (biomass) in shake flask was almost always lower than 3000 mg/l, and the experiments were under un-controlled conditions of pH and oxygen concentration. Therefore, the objectives of this study was to study the PHA production using activated sludge from a full scale pulp paper industry wastewater treatment plant in a 15 L fermentor under controlled conditions and at varying concentration of sludge solids and substrate concentration.

MATERIALS AND METHODS:

Activated sludge

Fresh pulp-paper activated sludge (PAS) for PHA production was obtained from a pulp-paper industrial wastewater treatment plants (WWTP) in Quebec Province, Canada. The activated sludge was concentrated by gravity settling (at 4^{o}C), the suspended solids (SS) of the activated sludge were measured. Different concentrations (suspended solids concentration of 5, 10, 15 and 20 g/l) of activated sludge were prepared by dilution or concentrating the sludge used in various experiments. The experiments were conducted within a couple of hours after receiving the sludge samples. The characteristic of the sludge is listed in Table 1.

Carbon source

Sodium acetate (NaAc) was used as the sole carbon source with the concentration corresponding to different sludge concentration.

Experiments (controlled reactor conditions)

Experiments were conducted in two bench scale bioreactors (total capacity of 15 liter, and a working volume of 10 liter) equipped with accessories and automatic control systems for dissolved oxygen, pH, antifoam, impeller speed, aeration rate and temperature. The pH was controlled with an automatic controller by using either 4N NaOH or 4N H_2SO_4. The air was sparged in the medium through a filter (Gelman, PTFE, 0.2μm). The dissolved oxygen in the medium was measured by a DO probe (InPro 6000 of Mettler Toledo, U.S.A). The data on pH, and dissolved oxygen (DO) were collected with a Rosemount Analytical Model 1054A from Rosemount Analytical Inc (U.S.A) with a computer on a constant frequency (6 sec). The computer program (Fix 3.5, Intellution, USA) used was allowed automatic set-point control and registration of all stated parameters. Polypropylene glycol (PPG, Sigma-Canada) (0.1% v/v) solution was added to control the foam when necessary.

Batch operations

Activated sludge (10L) was transferred to bioreactors and appropriate amount of acetate was added to obtain desired concentration. The fermentation was carried out for 60 hrs. Samples were drawn at predetermined time for all analysis. For all experiments, temperature was automatic controlled at set-point of 25^{o}C. The agitation speed (300-500 rpm) and aeration rate (0.3-0.5 vvm) were varied in order to keep the dissolved oxygen (DO) values above 50% of saturation, which ensured the oxygen concentration above the critical level as already investigated in a preliminary study for optimal batch production of PHA (data not shown). The pH was controlled at 7.0± 0.1 using either 4N NaOH or 4N H_2SO4 through computer-controlled peristaltic pumps.

Analytical techniques

Four-ml culture broth was sampled in a pre-weighed centrifuge tube and centrifuged at 5000 rpm for 10 minutes. The settled biomass was washed 3 times with distilled water and centrifuged, after centrifugation, the biomass was freeze-dried for at least 48 hours. The final weight of the tube was measured to calculate the sludge dry weight (SDW). The extraction of PHA was made by the methanol and chloroform extraction method

described by Comeau et al. (1988) using benzoic acid as an internal standard. The co-polymeric composition was measured with a gas chromatograph (GC) (Varian Model 3800) equipped with a Capillary column Zebron ZB-5 and a Shimadzu C-R5A Chromatopac flame-ionization detector. Poly-(3-Hydroxybutyrate-co-3-hydroxyvalerate)(3HB-co-3HV) (PHV content 12 wt.%, Aldrich Chemical Company, Inc.) was used as a standard. The PHA content was defined as the ratio of PHA concentration to dry sludge SS concentration given as a percentage. The yield of PHA (Yp/s – PHA produced per unit weight of acetate consumed) on substrate consumed were calculated by dividing the amount of PHA formed by the amount of acetate consumed.

The supernatant of the centrifuged samples was used to measure acetate (Ac^-) concentration. The concentration of acetate was measured using ICS-2000, Ion chromatography systems (Dionex Co.) with the Ion Pac AS14 anion-exchange columns.

The ammonium nitrogen (NH_4^+-N), total kjeldahl nitrogen (TKN), total phosphorus, and orthophosphate as PO_4 were determined by Technicon Analyser (Technicon Instruments Corporation, New York). Suspended solids (SS) was analysed according to standard methods (APHA, 1998). Measurements were carried out in duplicate and average of two samples was presented.

RESULTS AND DISCUSSIONS

Batch experiments

A proper substrate (acetate) concentration was required during fermentation to achieve maximum PHA productivity. In the previous study (Yan et al., 2006), experiments were performed in shake flask in order to identify the optimal substrate (acetate) concentration for certain activated sludge concentration. The results showed that high acetate concentration had inhibitory effect for PHA production. Thus the acetate concentrations of 5, 10, 15 and 20 g/l for PHA production were selected (Table 2) to examine the impact on acetate consumption, increase in sludge SS, PHA production and change of molar ratio of HB/HV during batch process.

Acetate consumption

At sludge concentration of 5 g/l (Fig. 1), the rate of acetate consumption increased with acetate concentration. The rate of consumption was found to be of zero order with overall rate constant of 0.096, 0.135 and 0.169 at 5, 7.5 and 10 g/l acetic acid concentration, respectively.

Initial PHA content

Four series of experiments were conducted and each series was carried out at one solids concentration but different acetate concentration. For each series of experiments, the sludge was sampled at four different dates. Each time, the sludge sample (before its use) was analyzed for PHA content. The PHA content of four samples sampled at different dates was found to be 4.15, 5.08, 5.64, and 3.12% w/w and these samples were used to conduct four series of experiments using solids concentration 5, 10, 15 and 20 g/L, respectively. The PHA content in present samples was higher than reported earlier in municipal activated sludge (0 to 1.3% w/w of dry sludge) (Wallen and Rohwedder 1974). This observation is in concordance with our earlier findings that activated sludge from pulp and paper wastewater treatment possesses higher capacity to accumulate PHA.

The initial PHA content (PHA_0) of sludge after mixing with sludge solids with acetate are presented in Table 2. It is apparent that during mixing of acetate in the fermentor, pH adjustment and sampling and their manipulation (approximately 15 – 20 minutes), there was a substantial increase in sludge PHA content. It increased from a minimum of 3.1% to a maximum of 12.5% (SS-10 g/l, acetate – 10 g/l). There was almost no increase of PHA content at sludge SS- 20 g/l and acetate – 30 g/l. At high initial acetate concentration microorganisms contained in activated sludge require a certain adaptation time before they could start accumulating PHA.

PHA Accumulation

The profiles of PHA concentration and PHA content of sludge SS on dry weight basis at different sludge SS concentration and initial acetate concentration are presented in Figs. 2 and 3, respectively. All profiles could be divided in two parts. During the first part (0-

12h) there was a rapid increase of PHA concentration followed by a slow increase and then again a rapid increase until PHA concentration as well as PHA content of cell reached a maximum value. The time to attain maximum PHA concentration varied with sludge SS and initial acetate concentration (Table 2). A lag in PHA production was also observed at SS concentration of 20 g/l irrespective of acetate concentration. This lag could be due to acclimation of biomass to high concentration of acetate.

At sludge SS concentration 5 g/l, the maximum PHA content and maximum PHA concentration increased as the acetate concentration increased from 5 to 7.5 g/l. However, at all other sludge SS concentration (10, 15, and 20 g/l), the increase of acetate concentration did not result in the increase of the maximum PHA concentration or PHA content. This might be due to the inhibitory effect of a too high concentration of acetate.

PHA concentration also increased concomitantly with acetate consumption, however, reached its maximum value at different time during the batch process which was 36, 36 and 24 hr with maximum PHA content of 29%, 32% and 32.5% of cell dry weight at 5, 7.5 and 10 g/l acetic acid concentration, respectively. The acetate concentration reached zero in case of 5 g/L initial concentration at about 36 h and coincided with the maximum concentration of PHA. At 7.5 and 10 g/l initial concentration of acetate, a substantial concentration of residual acetate (1.03 and 5.01 g/l, respectively) was observed in the medium (Figs 1, 2 and 3) at the time when maximum PHA accumulation occurred. The PHA concentration started decreasing when substantial concentration of residual acetic acid was still present in the medium at 7.5 and 10 g/l initial concentration of acetate. This phenomenon was also observed at higher sludge SS concentration. Similar results were also reported by Ciggin et al. (2007).

On the other hand, in some case it was observed that after depletion of acetate, PHA did not start depleting immediately. For example, at 15 g/l sludge SS concentration, the acetate concentration became zero between 36 and 45 h batch time, however, PHA concentration kept increasing until 48 h. A similar trend has also been reported by Goel et al. (1998). It could be explained assuming the accumulation of some intracellular

intermediates which are utilized by biomass before switching to stored PHA (Goel et al. 1998). In the proposed model described the kinetic of substrate metabolism and related variables under a range of operating conditions conducted It was also proposed by Dionisi et al. (2001) that the accumulated compound(s) acts (act) as an internal pool of substrate that increases as external substrate depletes. This possible presence of a low-capacity accumulation (or buffer) can conceptually justify the experimental evidence of this study as well as other studies. Anyway, such hypothesis requires further confirmation by identification and measurement of accumulated compound(s) including a complete carbon balance.

PHA content at all SS and acetate concentrations increased with time and the maximum content varied in the range of 25.9% to 39.6% of cell dry weight (Table 2). High PHA content of cell dry weight is desirable due to the fact that it is one of the most important factors which affect the cost of PHA extraction (i.e. downstream processing cost). The other factor that is also very important is the production rate of PHA and has a great influence on the establishment (or equipment cost). These values of PHA content are in general higher than those reported (16-36% w/w) by Chua et al. (2003) who used activated sludge from a laboratory SBR to accumulate PHA at pH 8.0.

It was observed that the rate of PHA accumulation was almost constant in the first 12 hr of the process (Fig. 2). Therefore, initial rate of PHA production was calculated for all cases and the values thus obtained are presented in Table 2. The PHA production rate varied from 0.102 to 0.34 gPHA/l/h. At low sludge SS concentration (5 g/l), the rate of PHA production increased with acetate concentration whereas it decreased for all other SS concentration with acetate concentration. Increase in PHA production rate with acetate concentration at low SS was due to absence of acetate inhibition (acetate concentration was low in these experiments). At high acetate concentration, even in spite of high solids concentration (or similar food to microorganisms ratio), acetate inhibition could be responsible for decrease of PHA production rate. Further, initial specific PHA production rates were also calculated and the values varied between 15-20 mgPHA/g SS dry weight/h. These values are comparable with those reported by Chua et al. (2003) in

the range of 12-22 mgPHA/g SS dry weight/h. Very low values of specific PHA production rates were observed at high acetate concentration and 20 g/L SS concentration and was attributed to inhibition by acetate concentration. Overall specific PHA production rates were also calculated and presented in Table 2.

The maximum PHA content of 34.1, 39.6 and 32.1% w/w was observed at sludge SS concentration of 10, 15 and 20 g/l, respectively and acetate concentration of 10 g/l. An increase in initial acetate concentration more than 10 g/l resulted in decrease of PHA concentration and PHA content (Table 2). These results thus showed that during batch cultivation acetate concentration of 10 g/l could be assumed as optimum.

Further, the highest sludge PHA content (39.6% w/w) was obtained at sludge SS of 15 g/l and acetate concentration of 10 g/l. It was also noticed that at sludge SS concentration of 15 g/l, the PHA content produced at all acetate concentrations were highest than those from other sludge concentrations used. It should be pointed out here that fresh settled sludge from pulp and paper industry wastewater treatment contains SS concentration of approximately 15 g/l and therefore no manipulation is required to adjust the initial SS concentration. It is possible that during sludge solids manipulation (centrifugation, dilution etc) may bring injury to active cells leading to their low PHA accumulation capacity. This could be the reason that fresh sludge without manipulation (centrifugation, solids amendment etc) showed the best PHA production. Low accumulation of PHA at high solids concentration may also be due to low availability of oxygen or oxygen transfer due to high viscosity and thus affecting the cellular metabolism.

The value of PHA produced with respect to acetate consumed (yield coefficient – $Y_{P/S}$) at the instant of maximum accumulation of PHA at 5 g/l SS was almost same irrespective of initial acetate concentrate (0.27, 0.25 and 0.24 at 5, 7.5 and 10 g/l initial acetate concentration, respectively). The yield coefficient, in general, increased with SS concentration (Table 2) and the highest value (0.49) was observed at 15 and 20 g/l SS and acetate concentration 10 g/l.

Biomass growth and sludge reduction

The increase in sludge suspended dry weight (or biomass concentration) during PHA accumulation experiments at different concentrations of sludge SS and acetate are presented in Fig. 4. The results are quite scattered but there is a clear indication of growth of biomass irrespective of initial sludge SS and acetate concentration. The biomass concentration also increased concomitantly with acetate consumption and PHA accumulation. In certain cases biomass kept increasing even after PHA concentration reached its peak value because a substantial amount of acetate was still present in the medium. Growth of biomass decreased with increase of initial sludge SS concentration; the change in biomass concentration (from zero to the time at which PHA attained maximum concentration) was 17 to 34.9%, 14.5 to 25%, 9.4 to 13.8% and -10 to 7.2% w/w at 5, 10, 15 and 20 g/l sludge SS concentration, respectively. In general, at a given sludge SS concentration, the percent increase in biomass value was lower at higher initial acetate concentration. There was a net reduction of biomass at sludge SS of 20 g/l and acetate concentration of 20 and 30 g/l, as is also evident from Fig.4 This decrease in biomass growth could be attributed to inhibition by high acetate concentration as well as low oxygen transfer from gas phase to liquid phase due to high sludge viscosity at high sludge SS concentration (Brar et al. 2005). It should be underlined here that sludge was supplemented only with acetate and no other nutrient was added. Thus, the sludge contained enough nutrients required for biomass growth.

PHAs that accumulate in sludge solids shall be extracted through solvent extraction method (Lemos et al. 1998) in order to be used as bioplastics. Thus the amount of solids will be decreased after PHA extraction. The sludge solids reduction thus was calculated with respect to the solids at the start of each experiment. The amount of biomass increased during PHA accumulation was also taken into account while calculating the sludge reduction. The amount of sludge reduced varied with different initial sludge solids SS and acetate concentration used in different experiments. The values of sludge reduction were 5.5 to 21.5%, 11.9 to 22.9%, 26 to 32.3% and 24.8 to 36.8% w/w at 5, 10, 15 and 20 g/l initial sludge SS concentration, respectively, as presented in Table 2. Thus, after extraction of PHAs, a considerable less amount of sludge shall be required to

dispose and hence les expenses shall be incurred in overall sludge safe disposal and management.

Copolymer composition

The presence of co-polymers or their ratio affects the physical property of the bioplastics, namely melting point, and mechanical properties such as tensile strength, compressibility and shear strengths. Therefore, composition of co-polymers (polyhydroxybutyrate and polyhydroxyvalerate) was determined in all batch experiments and the results are presented in Fig. 5.

The polymers detected in this study were copolymers of polyhydroxybutyrate/ polyhydroxyvalerate (HB/HV) with a variable molar ratio. Even though the same organic substrate was used (acetate) in this study, the HB/HV ratio of the polymers was different under different operation conditions (i.e. different initial sludge SS and acetate concentrations) and varied with the process time (Fig. 5).

At initial sludge SS of 5 g/l, the polyhydroxybutyrate/polyhydroxyalkanoates (HB/HA) ratio increased with batch time until the maximum value of 73, 100, and 100%, at 48, 57 and 48 h with acetate of 5, 7.5 and 10 g/l, respectively. After that the HB/HA ratio decreased, due to degradation of stored polymers. The HB/HA ratio, at initial sludge SS of 10 g/l, increased and attained the maximum value of 77% at 10 g/l of acetate concentration followed by a decrease. At acetate concentration of 15 and 20 g/l, the HB/HA ratios increased monotonically until the end of the batch process. At sludge SS of 15 g/l, the HB/HA ratio increased until the maximum value of 72% and 75% for acetate concentration of 10 and 15 g/l, respectively, was attained. At acetate concentration of 30 g/l, the HB/HA ratio increased until the end of the experiments. At sludge SS concentration of 20 g/l, as in other cases, the HB/HA ratio increased until the maximum value of 95, 95 and 76% for acetate concentration of 10 and 20, and 30 g/l, respectively followed by a decrease in all cases.

The polymer compositional variation with different acetate concentration could be due to the fact that PHA carbon could be recycled multiple times during the batch process. In this way, or due to the existence of other VFA or glycogen carbon in the activated sludge used, PHV could be produced in varying degree. Another reason for change in PHB/PHV ratio in these experiments might be due to the fact that PHB or PHV production and degradation could occur simultaneously under certain conditions (Doi 1990). Moreover, different bacterial strains present in activated sludge could produce PHAs with different composition when growing on same substrate (Renner et al. 1996). Tsuge (2002) stated that various bacteria present in activated sludge are capable of synthesizing random copolymer of (*R*)-3HB with other HA units of C3 to C12, depending on both their intrinsic PHA biosynthesis pathways and the carbon sources used. Therefore, it is possible that the composition of polymer varied significantly under low to high acetate and sludge SS concentrations in the present study. Punrattanasin et al. (2001) showed that the compositions (i.e., the PHV/PHA ratios) of the copolymers produced were different under different operating conditions. They also demonstrated that copolymers produced were different under different cultivation approaches, i.e., PHB/PHV ratios obtained from the batch and fed-batch bioprocess were different from those of continuously feeding process.

Normally, it is well known that copolymer composition is primarily influenced by the type of substrate used (Doi 1990). Acetate is one of the well-studied substrate for PHA production. Generally, acetate is transformed into a homopolymer of PHB. However, production of copolymer of HB and HV was reported using acetate as sole carbon source in some studies using activated sludge (Lemos et al. 1998; Punrattanasin 2001). When propionate was fed as the sole carbon source to activated sludge, a homopolymer of HV was produced (Lemos et al. 2006), while a copolymer of poly(3HB-co-3HV) or a terpolymer (HB/HV/HMV) was reported by Dionisi et al. (2004). Butyrate, was converted into a homopolymer P(3HB) (Lemos et al. 2006). Valerate was converted and stored as a terpolymer of P(HB/HV/HMV). In addition to VFA, other substrates such as lactate, ethanol, and glutamate can be converted into PHB in activated sludge system. Chua et al. (1999) found that when butyric acid was used as the sole carbon source, only

the homopolymer P(3HB) was produced. The molar fraction of 3HV in the polymer tended to increase with the valeric acid concentration in the medium. Valeric acid alone resulted in a copolymer poly(3HB-co-HV) with a HV molar fraction of 54%. The incorporation of valeric acid in the substrate affected the polymer yield per substrate.

Thus, based on all previous studies, the production of different co-polymers could occur due to: 1) multiple recycle of carbon during a batch process associated with simultaneous production and degradation of PHB and PHV, 2) the existence of various carbon sources in the medium, 3) the presence of different microbial communities and 4) type of process operation (batch versus fed-batch). In this study the difference in polymer composition seems to be more likely due to differences in the microbial population structure and it is obvious from the fact that activated sludge from different wastewater treatment plant operating under different conditions and utilising different carbon sources will give rise to different microbial communities. Simultaneous production and degradation of polymers could be another reason for varying molar ratios. Furthermore, in the present study, the activated sludge was obtained from a pulp and paper wastewater treatment plant; in this plant the influent wastewater contained VFA. The characteristics of the influent wastewater were expected to affect the composition of activated sludge and its potential to accumulate PHA as well as the co-polymers. The activated sludge initially contained copolymer of HB and HV with the ratio of 20:80. Therefore, though acetate was used as the sole carbon source, the polymer produced in the study was a copolymer of HB and HV.

Potential of PHA production using waste activated sludge as a source of microorganisms

Wastewater sludge disposal has been a problem since a long time and the producers have always been looking for alternate and economical ways to dispose the sludge. One of the most important advantages of using activated sludge to accumulate PHA is that after PHA extraction there will be less amount of sludge to dispose in the environment and thus reducing the cost of final disposal. More importantly sludge pathogens are eliminated or reduced drastically during PHA extraction process (as shown by the measurement of total colifom, fecal coliform and streptococci in fresh sludge and in sludge solids left after extraction of PHA, results not presented) and this adds a new dimension for safe sludge disposal practice.

The PHA content achieved by *Ralstonia eutropha* (Kim et al. 1994), *Alkaligenes latus* (Yamane et al. 1996) and recombinant *E. Coli* (Kim 2000) has been reported to be 74%, 50%, and 76% of cell dry weight with specific productivity 31, 31, and 42 mg PHA/gSS/h, respectively. These PHA contents and production rates are higher than those reported in the present study (39.6% w/w PHA content and 20.8 mgPHA/gSS/h as maximum production rate and 10 mgPHA/l/h overall productivity). PHA content of more than 80% has been reported to make the process economically feasible (Chua et al., 2003). In case of sludge it seems difficult to reach such a high PHA content and specific production rate due to the fact that sludge also contain a large quantity of inert solids or microorganisms that do not play any role in accumulating PHA, in other words, the total sludge mass is not active in terms of PHA accumulation, or only a fraction of the sludge mass is active in the form of active bacterial strains. Therefore, PHA accumulating bacteria in sludge might have accumulated much higher concentration of polymers than that experimentally observed, however, the inert part of sludge solids may have artificially lowered the PHA content. Thus, in case of sludge utilisation as a source of microorganisms to accumulate PHA, only high PHA content as a criterion of economical process may be misleading.

It is important to mention here that most of the earlier studies (Chua et al. 2003; Lemos et al. 2006) carried out using activated sludge to produce PHA used low concentration of activated sludge (less than 5 g/l). Chua et al. (2003) used the sludge acclimatized with municipal wastewater supplemented with acetate could accumulate PHA up to 30% of sludge dry weight, while sludge acclimatized with only municipal wastewater achieved 20% of sludge dry weight, the sludge dry weight/mixed liquor suspended solid (MLSS) used was in the range of 0.5 to 2.5 g/l. In the present study, the sludge concentration higher than 5 g/l was used to augment productivity of the PHA production process. These experiments demonstrated that it is possible and possesses high to develop a PHA production system for biodegradable plastics using activated sludge biomass at high concentration.

In case of sludge, cost savings on sludge solids reduction (or less sludge solids to be disposed), sludge pathogens reduction (otherwise a treatment process application is required to reduce pathogens) and savings on the carbon that must be used to produce biomass (that accumulates PHA) in case of pure culture should also be considered in cost estimation and process comparison. Moreover, use of activated sludge to accumulate PHA offers many other advantages that may contribute to lower the overall cost of production. In general, high PHA accumulating strains are grown as pure culture and aseptic conditions are required to eliminate contamination. However, in case of sludge no aseptic conditions are required. Culture maintenance is also not necessary and this should reduce the routine microbiological work. Thus activated sludge should be viewed as having a high potential to produce PHA.

Further, the PHA content and production rate in case of activated sludge can be improved in many ways. The experiments in this study were carried out at pH 7.0, whereas pH for maximum accumulation has been worked out to be above 7 (8 to 9) (Chua et al. 2003). Further, added acetate showed some what inhibitory effects, especially when high acetate concentration was used. Therefore, proper acetate feeding strategy needs to be developed (fed batch operation, continuous or intermittent feeding etc.) that could alleviate the inhibition. Further, oxygen concentration should also be optimised as high or low

oxygenation rate may be deleterious to PHA accumulation. These strategies shall be explored in the near future.

CONCLUSIONS

The production of polyhydroxynoates (bioplastics) was studied in batch process in laboratory fermentor employing different initial concentration of sludge suspended solids and acetate as sole carbon source. The following conclusions were drawn from the foregoing study.

- Acetate consumptions followed zero order kinetics irrespective of initial sludge SS and acetate concentration.
- The sludge samples at different dates found to contain different amount of PHA.
- There was a substantial increase in PHA content during manipulation of sludge which was low at high solids and acetate concentration than at low solids and low acetate concentration.
- High sludge solids concentration (20 g/l) exhibited a lag in PHA production.
- PHA increase with time and exhibited two phases. Initial phase that last during first 12 h showed the highest PHA production (accumulate) rate.
- The activated sludge from a full-scale pulp-paper industrial wastewater treatment plant could be used as the mixed culture for PHA production.
- The activated sludge SS concentration of 15 g/l and acetate concentration of 10 g/l showed the optimum concentration for higher PHA production providing highest content of PHA (39.6% w/w).
- Higher acetate concentration exhibited an inhibition of PHA production.
- Sludge SS increased during PHA accumulation irrespective of SS and acetate concentration, however, after extraction of PHA there was a net reduction in sludge solids left for disposal.
- Polyhydroxybutyrate and polyhydroxyvalerate copolymers were synthesized by the activated sludge used and their composition changed with batch process time.

- Indicator microorganisms were reduced below the detection limits after PHA extraction.
- For a pragmatic economic evaluation and comparison of PHA production process with conventional process, sludge reduction, indicator microorganisms elimination and other factors must be considered.

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Table 1. Characteristics of sludge.

Activated sludge	Operation process	pH	SS (g/l)	COD_{tot} (mg/l)	COD_{sol} (mg/l)	TKN (mg/l)	NH_4^+ (mg/l)	P_{tot} (mg/l)	Orth-P (mg/l)
PAS	Aerobic	6.9	19.3	1675.8	120.3	25.3	12.9	13.3	5.2

* Activated sludge data were on the basis suspended solids concentration of 20 g/l for sludge.

Table 2. Summary of the results from batch experiments at different activated sludge SS concentration.

No	PHA_0 (g/l)	SS_0 T=0 (g/l)	A_0 (g/l)	Tm (h)	PHAm (g/l)	IPR (mg/l/h)	PHA_m content (w/w%)	$Y_{p/s}$ (g/g)	PD_{m*} (mg/l/h)	ACR_{m*} (g/l/h)	SR_{m*} (%)	SPR_{m*} (mg/g/h)
1	0.2(4.0)	5	5	36	1.72	92.3	29.7	0.27	48	0.140 (0.096)	5.5	17.5
2	0.33(6.6)	5	7.5	36	2.03	102	32.1	0.25	56	0.190 (0.131)	14.9	19.0
3	0.45(9.0)	5	10	24	1.71	114	32.5	0.24	70	0.210(0.169)	21.5	20.8
4	1.25(12.5)	10	10	48	4.16	199	34.1	0.31	60	0.197 (0.172)	22.9	17.6
5	0.99(9.5)	10	15	45	4.08	166	33.2	0.27	68	0.250 (0.206)	18.7	15.6
6	0.88(8.8)	10	20	30	3.65	148	29.5	0.25	58	0.245 (0.197)	11.9	14.5
7	1.60(10.6)	15	10	48	6.46	284	39.6	0.49	101	0.218 (0.163)	32.2	18.3
8	1.34(8.9)	15	15	45	5.98	224	38.2	0.37	102	0.280 (0.214)	32.3	13.6
9	1.52(10.1)	15	30	48	5.83	256	35.2	0.367	90	0.245 (0.197)	26.1	15.1
10	2.01(10.0)	20	10	36	7.07	340	32.1	0.498	140	0.282 (0.150)	24.8	15.8
11	1.00(5.0)	20	20	30	4.97	189	29.3	0.301	110	0.366 (0.211)	36.9	10.6
12	0.62(3.1)	20	30	36	4.93	161	25.9	0.327	120	0.366 (0.204)	26.6	8.9

PHA_0: initial PHA concentration after acetate addition (values in parenthesis are weight %); **SS_0** : initial sludge suspended solids concentration; **A_0**: initial acetate concentration; **T_m** : time at which maximum PHA concentration occurred; **PHA_m** : maximum PHA; **IPR**: initial PHA production rate (mg/l/h); **Yp/s**: (g polymer/g Ac-) polymer formed by substrate consumed; **PD**: PHA production rate; **ACR**: acetate consumption rate; values in parentheses are that of zero order rate constant; **SR**: sludge reduction; **SPR**: (mg PHA/g dry SS/h), specific PHA production rate; **m*** values when the maximum PHA content occurred.

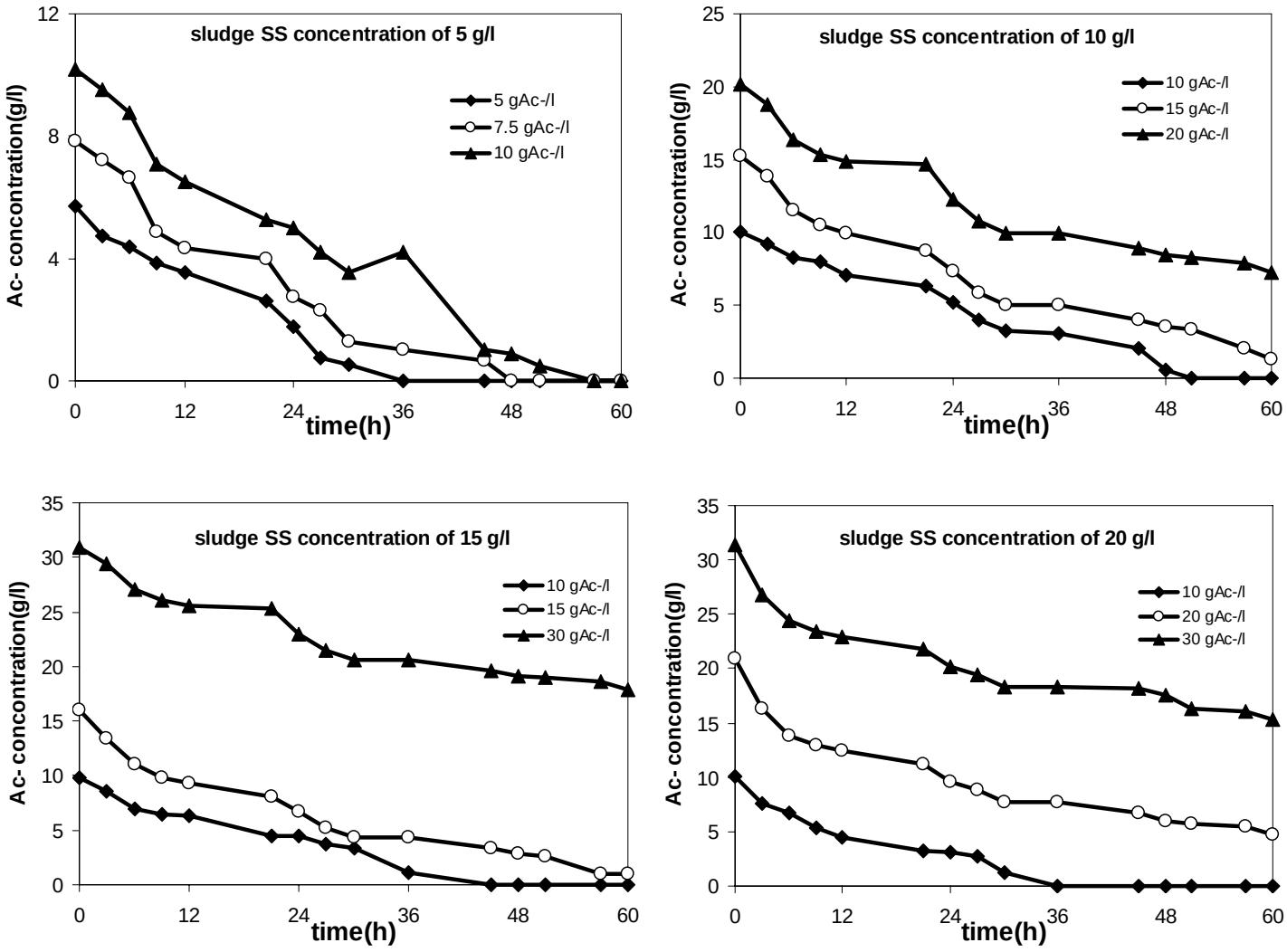

Figure 1. Profiles of acetate concentrations during batch process of PHA production at different sludge solids and acetate concentrations.

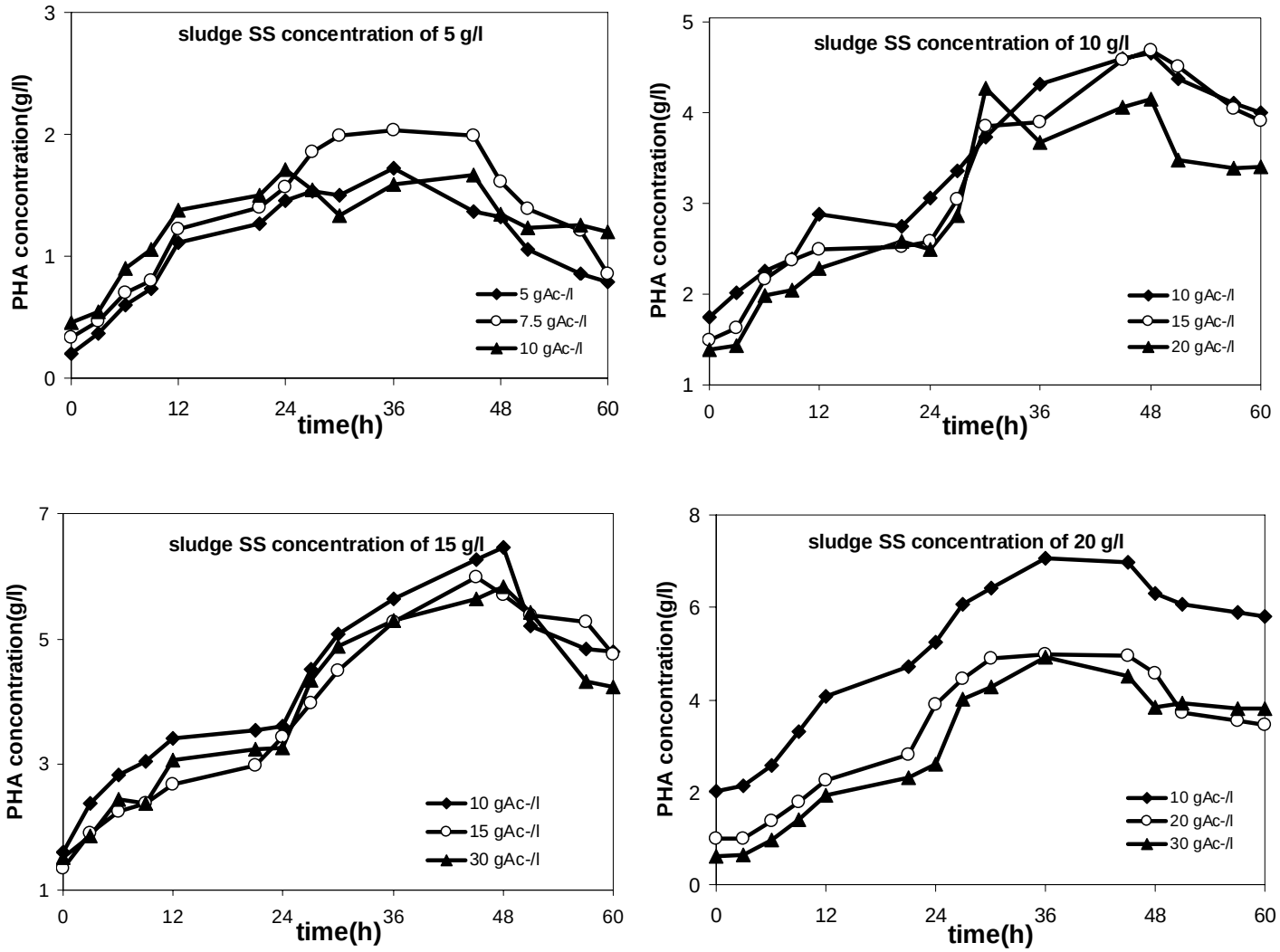

Figure 2. Profiles of PHA concentrations during batch process at different sludge solids and acetate concentrations.

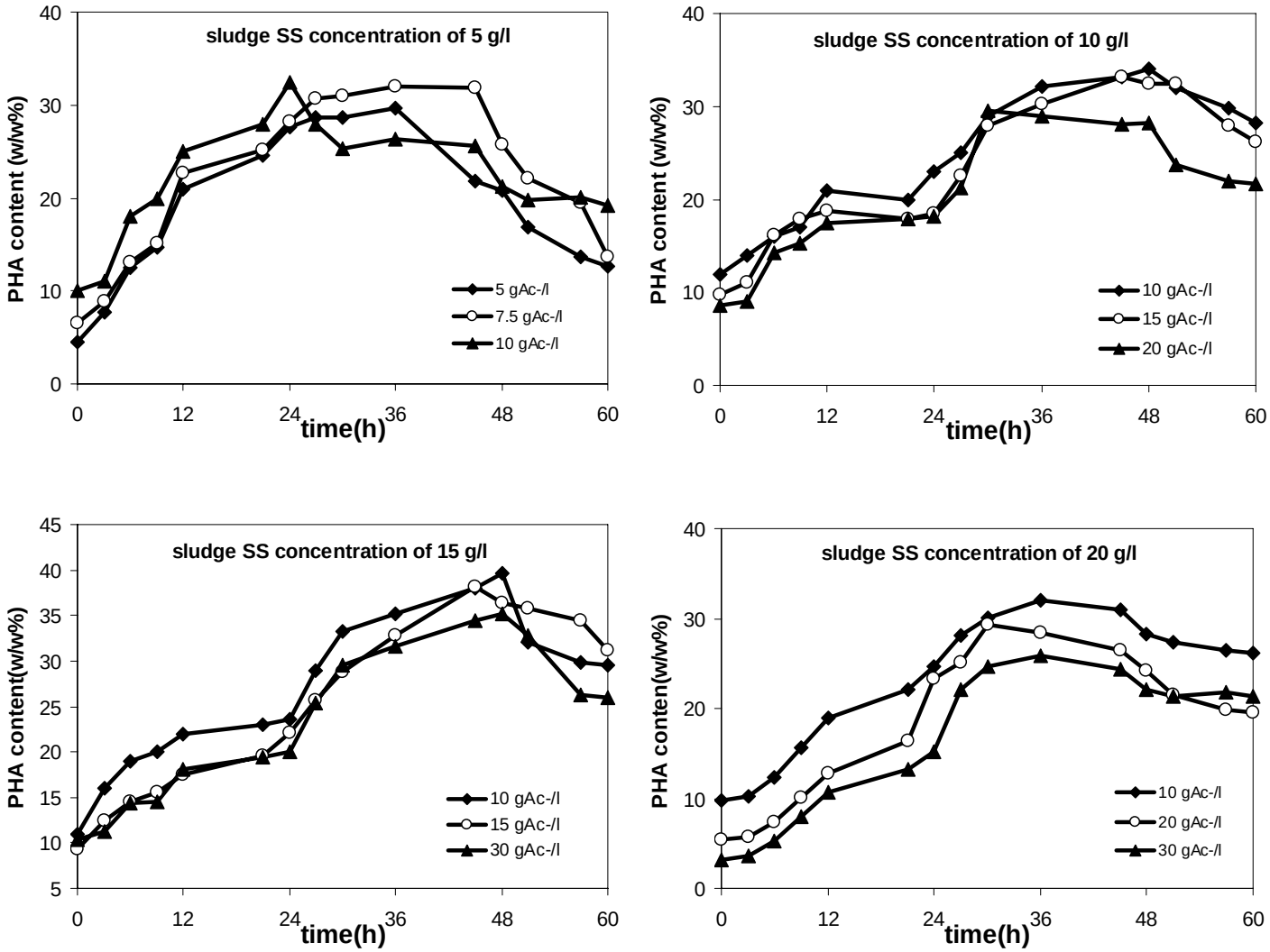

Figure 3. Profiles of PHA content (w/w%) of dry sludge SS during batch experiments at different sludge solids and acetate concentrations.

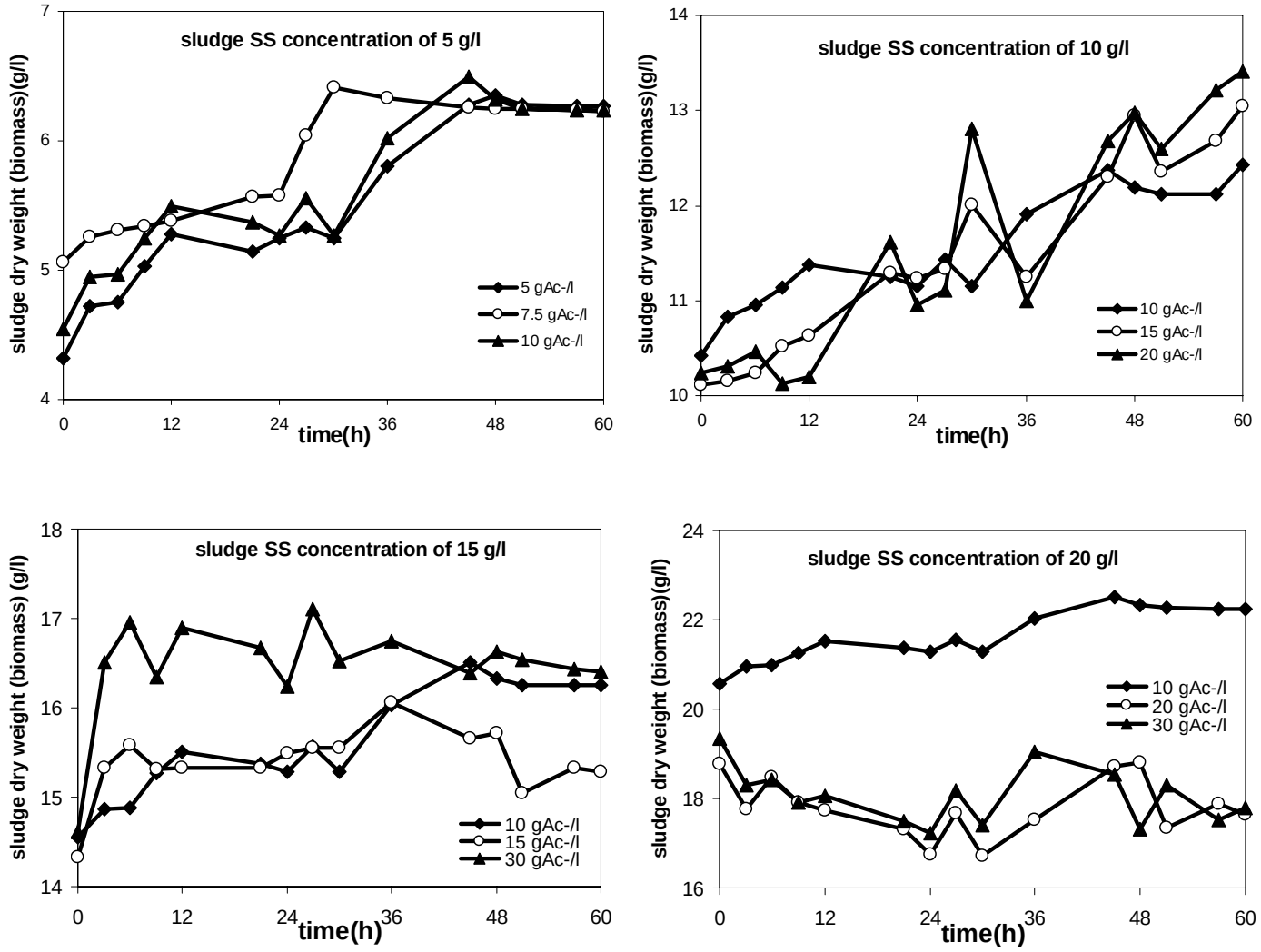

Figure 4. Profiles of sludge dry weight (biomass) (g/l) change during batch process of PHA production at different sludge SS and acetate concentrations.

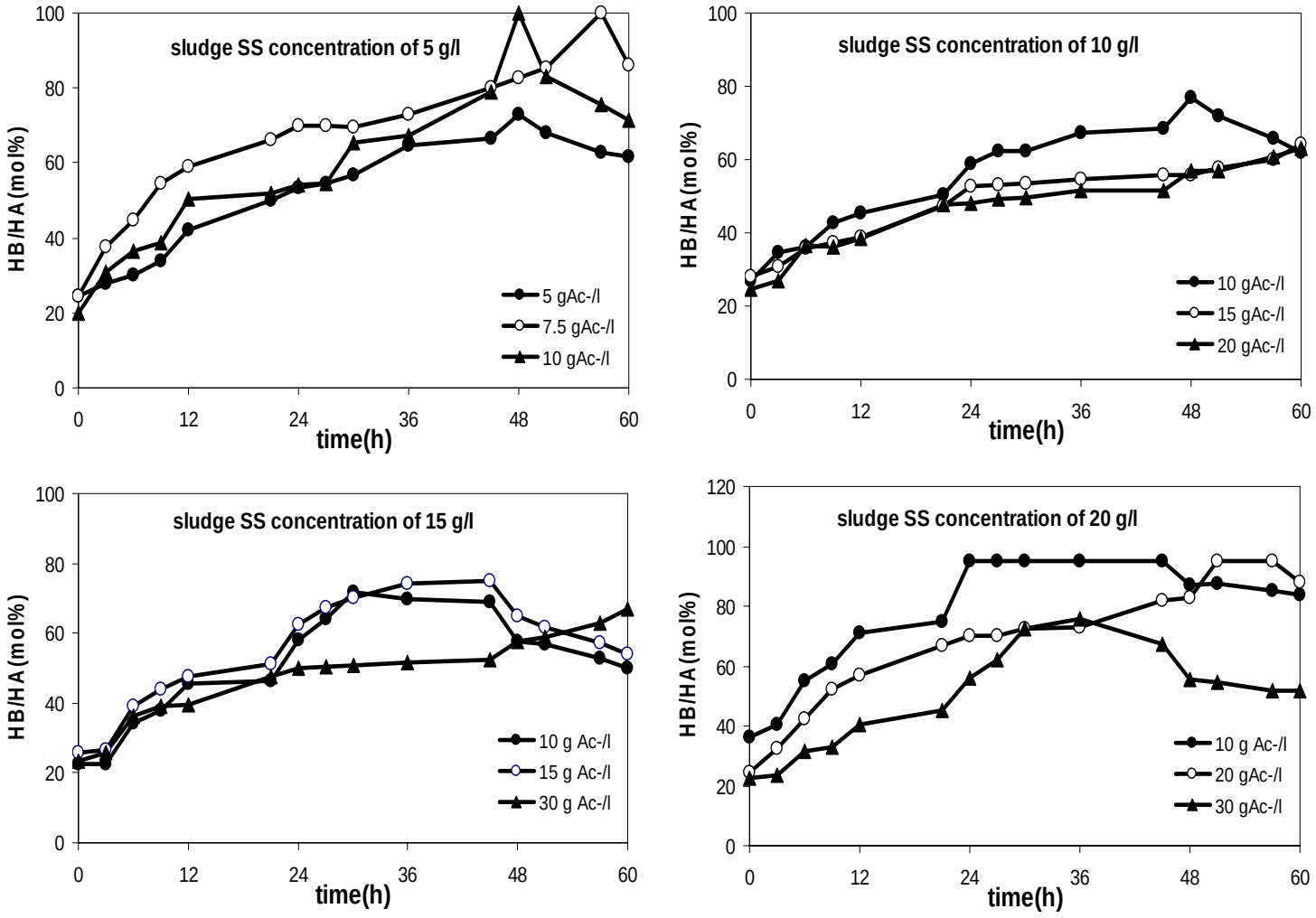

Figure 5. Profiles of mol HB/HA ratio during batch process of PHA production at different sludge SS and acetate concentration.

Part III

Impact of feeding pattern on Polyhydroxyalkanoates (PHA) production using activated sludge from a pulp-paper industry wastewater treatment plant

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Résumé

Des boues activées prélevées dans des usines de traitement des eaux usées d'industries de pâtes et papier ont été utilisées comme sources de microorganismes pour produire des plastiques biodégradables (PHA) en bioréacteurs. L'acétate a été utilisé comme seule source de carbone. Les effets des boues activées en différentes concentrations de matières solides en suspensions été évalués pour la production initiale de PHAs à différentes concentrations d'acétate et divers systèmes d'alimentation (batch, Fed-batch et alimentation en continu). Les résultats ont montré qu'une concentration de 15 g / l en boues activées permet d'obtenir la concentration la plus élevée en PHAs . Une concentration supérieure de matières solides en suspension, réduit ce taux ainsi que la productivité. L'alimentation continue en acide acétique a augmenté la teneur en PHA et la productivité. Un ratio de C: N de 144 s'est révélé permettre le plus haut taux de production et de teneur en ces biopolymères. Considérant le poids équivalent en PHA, éventuellement extrait (ou accumulés PHA) pouvant être retiré , ces résultats permettent de penser qu'une quantité importante de boues pourrait être réduite, et donc moins de déchets à éliminer définitivement.

Mots-clés: boues activées, pâtes et papier , usine de traitement des eaux usées industrielles, bioplastiques, polyhydroxyalcanoates (PHA), alimentation par batch-, continue, rapport C / N, acétate, fermentation.

Abstract

Activated sludge from full-scale pulp-paper industrial wastewater treatment plants was used as a source of microorganisms to produce biodegradable plastics (PHA) in bioreactors. Acetate was used as sole carbon source. Effect of different activated sludge suspended solids concentrations was studied on PHA production with different initial acetate concentration and employing different operating system (fed-batch and continuous feeding strategy) The results showed that activated sludge of 15 g/l was the optimum concentration for PHA production. At higher suspended solids concentration, the PHA productivity and PHA content was decreased. The continuous feeding of acetic acid further enhanced the PHA content and productivity. C:N ratio ($C_{acetate}/NH_4^+$-N) of 144 was found to furnish the highest PHA production rate as well as PHA content**.** Considering the sludge weight loss equivalent to PHA extracted (or PHA accumulated), a substantial amount of sludge could be reduced and therefore less sludge was left for final disposal.

Key words: Activated sludge, pulp-paper industrial wastewater treatment plant, bioplastics, polyhydroxyalkanoates (PHA), fed-batch, continues feed, C:N ratio, acetate, fermentation.

INTRODUCTION

Recently, there is a growing demand for biodegradable polymers as a solution to the problem of conventional plastic pollution of the global environment (Lee and Yu 1997; Dionisi et al. 2001a; Takabatake et al. 2002; Chua et al. 2003). Poly(3-hydroxyalkanoate) (PHA), one of the biodegradable and biocompatible thermoplastics, has attracted considerable industrial attention ((Anderson and Dawes 1990). Current methods for PHA production at the industrial scale are mostly pure cultures requiring pure carbon sources and complex and costly processing procedures (Steinbüchel and Füchtenbusch 1998; Choi and Lee 1999a). Therefore, the attempts have been made to develop more cost-effective processes. PHA production processes based on mixed microbial cultures (i.e. activated sludge) are being investigated as a possible technology to reduce production costs, since no sterilization is required and bacteria can adapt well to the complex substrates that may be present in waste material (Dias et al. 2006). This would allow the production of biodegradable plastics from renewable waste streams, with reduced costs on materials and processing (Chua et al. 2003; Dionisi 2005). In most of the studies of PHA production by activated sludge, synthetic wastewaters were used to cultivate PHA producing sludge, The intracellular PHA content can reach around 70% of the cell dry weight, suggesting that this process could be competitive with pure culture PHA production when fully developed (Serafim et al. 2004b).

In order to assess the impact of varying sludge suspended solids (SS) and acetate concentration, the PHA production was also studied in computer controlled fermentors (Yan et al, 2007). The PHA concentration as well as PHA content of suspended sludge solids increased to 36.9% w/w. However, it was found that high acetate concentration inhibited the biomass growth as well as PHA production. Therefore, fed-batch and continuous mode of acetate feed were explored in this study to keep low concentration of acetate and thus minimising high initial acetate concentration and thus augment PHA production. Impact of C: N ratio on PHA production/accumulation was also studied while employing continuous feed of acetate.

MATERIALS AND METHODS

Activated sludge:

Fresh pulp and paper activated sludge (PAS) for PHA production was obtained from a pulp and paper industry wastewater treatment plant (WWTP) in Quebec City (Canada). The activated sludge (AS) was concentrated by gravity settling (at 4^oC), the suspended solids (SS) of the AS was measured. The experiments for PHA production were conducted within a couple of hours after receiving the sludge samples. The characteristic of the sludge is listed in Table 1. Sodium acetate (NaAc) was used as the sole carbon source with the concentration corresponding to different sludge concentration. The experiments were conducted in two different modes (Fed-batch and continuous).

Fermentor operation:

In each experiments, ten liter volume of sludge with appropriate SS concentration was filled in each fermentor. The pH was controlled (7.0) with an automatic controller by using either 4N NaOH or 4N H_2SO_4 (if not identified). The air passes through a filter (Gelman, PTFE, 0.2μm) beforehand pressure-sealed, then conveyed by a tube perforated in the culture medium. The percentage of DO in the medium was measured by a DO probe (InPro 6000 of Mettler Toledo, U.S.A). The data on pH, and DO concentration were collected with a Rosemount Analytical Model 1054A from Rosemount Analytical Inc (U.S.A) with a computer on a constant frequency (6 sec). The computer program (Fix 3.5, Intellution, USA) used allowed automatic set-point control and registration of all stated parameters. Polypropylene glycol (PPG, Sigma-Canada) (0.1% v/v) solution was added to control the foam when necessary. The samples were drawn from the fermentors at regular intervals to measure PHA, residual acetate and sludge SS concentration. PHA content of dry sludge SS was also determined.

Fed batch operation:

Two different initial SS concentration (15 and 20 g/l) were used in these experiments. The sludge solids were concentrated by gravity settling. The experiments were conducted simultaneously in two bench scale bioreactors (total capacity of 15 liter, and a working

volume of 10 liter) equipped with accessories and automatic control systems for dissolved oxygen (DO), pH, antifoam, impeller speed, aeration rate and temperature. Three different types of acetate feed systems (two, three and four feeds) were used. The time and amount acetate fed each time is presented in Table 2. Each time the experiment was started in batch mode with 10 g/l acetate concentration. Additional dose of acetate (additional carbon source) was added to the sludge in the fermentor at 12, 24, or 36 h of the batch process. Afterwards, the fermentation process was continued in batch mode until 60 h.

Continuous feed operation:

Fermentation was started in batch mode with initial sodium acetate concentration of 10 g/l and was run in batch mode until 24h. During this time pH was controlled at 7.0 using H_2SO_4 or NaOH. At 24 h of batch process, the control of pH was switched from utilization of H_2SO_4 to acetic acid solution (6N) i.e. H_2SO_4 was replaced with acetic acid solution (6N) and was fed until the end of experiments. During this time pH tend to increase due to consumption of acetic acid by PHA accumulating bacteria and therefore acetic acid was added automatically by the pH control system the computer controlled fermentors.

C: N ratio

Effect of C/N ratio on production of PHA was also studied in this work. The experiments were conducted in continuous feed mode. The carbon, nitrogen ratios (defined as $C_{acetate}/NH_4^+$-N) used were 48, 96, 120, 144, 168, and 192, respectively. In order to adjust desired C:N ratio, appropriate amount of acetate or acetic acid was mixed with ammonium chloride. Fermentation was started in batch mode, sodium acetate (1 g/l or 3 g/l of acetate and appropriate amount of ammonium chloride to achieve desired C:N ratio). After one hour, acetic acid solution (6N) containing appropriate amount of ammonium chloride to obtain desired C:N ratio was continuously fed to the fermentor to adjust the medium pH (i.e. after 1 h H_2SO4 was replaced with acetic acid and ammonium chloride solution as a pH control agent). The samples were drawn regularly to measure concentration of acetate, PHA and sludge SS.

Analytical techniques:

Four-ml culture broth was sampled in a pre-weighed centrifuge tube and centrifuged at 5000 rpm for 10 minutes. The settled biomass was washed 3 times with distilled water and centrifuged again. After centrifugation, the biomass was freeze-dried for at least 48 hours. The final weight of the tube was measured to calculate the sludge dry weight (SDW). The extraction of PHA was made by the methanol and chloroform extraction method described by Comeau et al. (1988) using benzoic acid as an internal standard. The co-polymeric composition was measured with a gas chromatograph (GC) (Varian Model 3800) equipped with a Capillary column Zebron ZB-5 and a Shimadzu C-R5A Chromatopac flame-ionization detector. Poly-(3-Hydroxybutyrate-co-3-hydroxyvalerate)(3HB-co-3HV) (PHV content 12 wt.%, Aldrich Chemical Company, Inc.) was used as a standard.

The supernatant of the centrifuged samples was used to measure acetate (Ac^-) concentration. The concentration of acetate was measured using ICS-2000, Ion chromatography systems (Dionex Co.) with the Ion Pac AS14 anion-exchange columns.

The ammonium nitrogen (NH_4^+-N), total kjeldahl nitrogen (TKN), total phosphorus, and orthophosphate as PO_4 were determined by Technicon Analyser (Technicon Instruments Corporation, New York). Suspended solids (SS) were analysed according to standard methods (APHA, 1998). Measurements were carried out in duplicate.

RESULTS AND DISCUSSIONS

Fed-batch experiments:

Industrial fermentations are conducted predominantly as batch and fed-batch operations because long-term, continuous cultures are susceptible to contamination and strain degeneration. Commercial production of PHB also relies on batch and fed-batch cultures. Hence, the optimization studies reported here were conducted in those modes, rather than in chemostat continuous culture.

Sludge solids concentration of 15 g/l

From previous batch experiment results (Yan et al., 2007), it was observed that at low sludge SS concentration (5 g/) the maximum PHA content (% w/w) enhanced slightly with increase in the concentration of carbon source (acetate). At high sludge SS concentration (10, 15 and 20 g/l) the PHA content increased with acetate concentration, however, an inhibitory effect was observed at high concentration of acetate (>10 g/l acetate concentration). Therefore, the fed-batch strategies were conducted by starting the batch experiment at 10 g/l acetate concentration and then feeding the acetate intermittently after 12h so that the acetate concentration was kept low (Table 2).

Moreover, the maximum PHA content is the most important factor from the industrial point of view because the maximum PHA content significantly affects the effectiveness of PHA recovery process from microbial cells and thus the total cost of PHA production (Choi and Lee, 1999). Therefore, the sludge SS concentration of 15 g/l, determined in our previous experiments (Yan et al. (2007), was used in this set of experiments. Furthermore, a high sludge SS concentration (20 g/l) was also tested in fed-batch experiment to achieve high concentration of PHA; as well as to find if by maintaining low concentration of acetate in a fed-batch process a high PHA concentration (content) could be achieved. Therefore, two series of fed-batch experiments were conducted with the sludge sampled at two different dates and each series was carried out at one solids concentration (15g/l or 20 g/l) but utilizing different acetate concentration with different feed numbers (Table 2).

PHA Accumulation:

The profiles of acetate, PHA, sludge SS concentrations and PHA content with initial sludge SS at 15 g/L and different feed patterns are presented in Figure 1. In case of two feeds system, the PHA profile could be divided in two parts. During the first part (0-12h) there was a rapid increase of PHA concentration followed by a slow increase (even in spite of slug addition of acetate dose at 12h) and then again a rapid increase until PHA concentration as well as PHA content of cell reached a maximum value (45h).

Similar to two feeds system, in case of three feeds, there was an initial rapid increase of PHA production until 12h followed by a slow down in their production. The second slug dose did not boost the PHA production. In fact, the third slug dose (at 24 hrs process time) delayed the production of PHA during the next 6 hrs (24-30h) This delay in PHA production between 24-30 could be attributed to the acclimation of sludge biomass to sudden increase in acetate concentration at 24 hrs (Fig 1a). Further, after acclimation, there was a sudden increase PHA production between 30-54h of the batch process (in comparison to two feeds profile).

The four feed case, followed some what different profile for PHA accumulation. The PHA production increased until 9 h and there was a somewhat slow down until 21h. There seemed to be no impact on PHA production of the second slug dose (at 12h). There was a sudden increase between 19 -30h and 45 – 54 of the batch process. This also showed that the addition of third (at 24h) and fourth (at 36 h) slug doses of acetate did not instantly affect the course of PHA production. The maximum PHA concentration and PHA content of the dry sludge SS reached at 54 h in case of three and four feeds system. The PHA accumulation with respect to % dry sludge SS followed the same trend as that of PHA concentration (Fig.1).

This is to be noticed that in case of two feeds, the rate of accumulation of PHA was lower than that of three or four feeds systems (Table 3). Similarly, the acetate consumption was also low in case two feeds than compared to others (Fig 1a). A substantial amount of acetate was present at the end of the batch process. In fact, acetate consumption was negligible after 36 hrs of batch process for two feed system. However, maximum % accumulation of PHA in dry sludge SS reached at about 45 hrs and was some what lower than the other two cases (Table 3). The low accumulation rate as well as low maximum content of PHA in two feed system could be explained taking into account the initial PHA content of the sludge SS. The initial PHA content (13.4% w/w) was higher than three (10.3%) or four feed (6.7%) systems. It appears that as the initial content of PHA in sludge solids increased, the acetate consumption rate, PHA accumulation rate (or productivity of PHA), specific productivity of PHA (mg PHA produced per g of dry

SS/h), initial PHA production rate (first 12h of the process), time required to reach maximum concentration of PHA and maximum PHA content decreased (Table 3). Thus, initial PHA content of sludge seems to play a substantial role in PHA production and accumulation. In fact, high initial PHA content reduced the gradient or capacity of the sludge solids to accumulate PHA.

On the other hand, the yield coefficient (g PHA produced per g of acetate consumed) increased with increase in initial sludge PHA content.

Change in sludge suspended solids concentration

The sludge SS concentration for two feed system increased from 14.9 to 15.1 g/l in the first three hours of the process and then practically remained constant, then increased again between 21-36 h followed by a decrease (Fig. 1c). In case of three feed system, the biomass (sludge SS) concentration showed an abrupt increase during the first three hours and then remained practically constant until PHA content and concentration reached maximum (54 h). Four feed system showed an entirely different profile as compared to the other two systems; the biomass concentrate increased monotonically from beginning of the process until the PHA concentration attainted maximum (54h). It seemed that profile of biomass (sludge SS) concentration was independent of slug acetate feeding irrespective of the system (two, three or four feeds) and could be attributed to the fact that acetate was always present in sufficient quantity and the biomass growth was not limited by the availability of carbon source. Therefore, it may be worth to study the application of sludge dose in such a manner so that carbon remains in limited supply.

The net increase in biomass concentration when PHA concentration reached maximum in two, three and four feed systems was 0.243, 1.93 and 4.5 g/l, respectively. The initial concentration of biomass in two, three and four feed system was 14.98, 14.75 and 15.4 g/l, respectively. Thus, a maximum increase in biomass occurred in four feed system and was in agreement with the highest acetate consumption in this case. The overall amount of acetate consumed and biomass formed per unit weight of acetate consumed was 8.13, 13.5, 18.4 g/l and 0.03, 0.142, 0.245 g/g, respectively.

After extraction of PHA, the weight of sludge SS shall decrease and this decrease will depend on the biomass increased during the process of PHA production. Therefore, taking into account the biomass increase, decrease in overall sludge weight was calculated and is presented in Table 3. The sludge SS reduction of 43.1% was highest in case of two feed system compared lowest (35.6%) in case of four feed system. Thus at the end of the process a substantially less amount of sludge will be required to dispose safely in the environment as compared to the amount of sludge required to be disposed without PHA production/accumulation. This may lead to a substantial increase of the economic benefit in overall sludge disposal as well as the PHA production process.

Sludge solids concentration 20 g/l

In earlier set of fed-batch experiment three and four feed pattern were found to be better and therefore these patternof acetate feeding were studied at approximately 20 g/l initial sludge SS concentration. The exact initial biomass concentration (sludge SS) in three and four feed system was 18.37 and 18.55 g/l and the total amount of acetic acid fed was 17.5 g/l and 20 g/l, respectively. The amount of acetate added in each slug dose is presented in Table 2. The profiles of acetate and PHA concentration, PHA content and biomass (sludge SS) concentration during batch experiments with three and four slug feeds of acetate are presented in Fig. 2. The acetate concentration increased after each slug dose (Fig 1a) indicating that the consumption rate of acetate was lower than the acetate fed in both the systems used.

PHA concentration increased monotonically and reached maximum in 36 hrs and 45 hrs in three (6.07 g/l) and four (8.08 g/l) feed system, respectively (Fig 1b). PHA content of dry sludge SS followed a similar pattern. The residual acetate concentration in three and four feed systems at maximum PHA concentration was 12.65 and 14.41 g/l and the residual concentration of acetate at the end of experiment (60h) was 1.2 and 1.27, respectively. The biomass concentration also reached maximum at 36 and 45 hrs in three and four feed systems and after that started decreasing. Thus a large quantity of acetate (11.45 and 13.14 g/l) was consumed during the period where PHA concentration as well

as biomass concentration decreased. This phenomenon of acetate consumption is somewhat strange and inexplicable. The only explanation that could be plausible is that after attaining maxima in PHA, all residual acetate was consumed for biomass maintenance purpose. This observation is different than earlier fed batch system operated at 15 g/l where most of the acetate was consumed during PHA production and biomass growth. This also reflects the fact that excess amount of carbon source alone does not augment the PHA concentration or PHA accumulation.

However, the yield coefficient (g PHA produced/g acetate consumed) of PHA at the time of maximum concentration was highest 0.57 for three and 0.83 for four feed system (Table 3). The PHA productivity, initial PHA production rate, acetate consumption rate and specific PHA production rate at 20 g/l SS concentration were also computed at the time of maximum PHA concentration and were found to be lower than at 15 g/l Sludge SS concentration (Table 3).

The results obtained in fed batch system (or slug feeding of acetate) are better than those obtained in batch culture (Yan et al., 2007). The highest PHA concentration (10.1g/l), highest PHA content (50.3% of dry SS) and PHA productivity (167 mg/l/h) were obtained at initial sludge SS 15 g/l employing three feed system (Table 3). The highest PHA concentration, content and productivity obtained at sludge SS 20 g/l with four feed system were 8.08 g/l, 42% (of dry SS), 130 mg/l/h. In batch process highest PHA concentration and productivity obtained were 7.07 g/l and 140 mg/l/h, respectively, at 20 g/l initial sludge SS and initial acetate concentration of 10 g/l (Yan et al., 2007). However, highest PHA content of 39.6% w/w was observed at 15 g/l and 10 g/l initial sludge SS and acetate concentration, respectively with PHA concentration and productivity of 6.46 and 101 mg/l/h, respectively. Thus, there was a net improvement in PHA productivity, PHA content and PHA concentration in fed-batch system (or slug feeding of acetate).

Continuous feed system:

In fed batch process there was a net gain in PHA concentration, content, productivity and PHA yield coefficient. However, a substantial quantity of excess (or unused or used for maintenance purpose of cells) acetic acid was still present in the system which might cause an inhibition to PHA production. In order to minimize the excess concentration of acetate in the system, a continuous system was designed and operated. Approximately 15 g/l sludge SS was used in this experiment and 10 g/l of acetic was fed in the beginning of the experiment. Acetic acid was fed to the fermentor after 24 h to adjust the pH and the amount fed between 24 and 60h (end of the experiment) was 13.6 g/l. Thus, a total of 23.6 g/l acetate was fed to the system.

The profiles of acetate consumption, PHA concentration and content and biomass change (sludge SS) are depicted in Figure 3. The acetate concentration reached zero within 24 h and remained zero through rest of the process in spite of intermittent addition of acetic acid to adjust pH of the system.

The initial PHA content of the sludge was 9.36% (w/w) of dry sludge SS. During initial manipulation (addition of acetate, adjustment of pH, sample collection and centrifugation) the PHA content increased to 14.7%. The PHA concentration increased from 2.3 g/l to 5.38 g/l in the first 24 h before the commencement of acetic acid feed as pH adjusting agent. The PHA concentration increased abruptly soon after acetic feed was started (Fig. 3b) and attained a maximum (10.11 g/l) at 36 h. An abrupt increase in PHA production rate may be due to the fact that biomass started starving due to deficiency of carbon source and as soon as the additional carbon was available, an immediate boost occurred in PHA concentration as well as content. After 36 h, PHA concentration as well as PHA content started decreasing and continued decreasing until the end of the experiment. This decrease may be due to again deficiency of carbon in the system. This deficiency of carbon source might have developed due to increased concentration of biomass (Fig. 3c) and probably the system could not keep up with the required supply of the same and started degrading the stored carbon and energy source (PHA).

The biomass concentration increased from 15.63 g/l at the beginning to 17.65 g/l at 36 h, the time at which maximum PHA concentration and content was achieved. This increase in biomass concentration was taken into account to calculate the sludge SS reduction after extraction of PHA. The solids reduction of 52.2% (w/w) was one of the highest as compared to batch and fed batch systems. This fact underlines the net advantage of employing the continuous system for PHA production.

The continuous feeding or maintaining low concentration of acetic acid in the medium could relieve the inhibition on cell growth and PHA production by acetic acid. This resulted in a higher PHA production compared to batch and fed-batch strategies. The maximum PHA content of 57.6% w/w and PHA productivity of 219 mg/l/h obtained in continuous system was higher than batch or fed batch systems (Table 3). The acetate consumption rate (0.39 g/l/h) observed in continuous feeding was also the highest.

Copolymer composition (fed-batch and continuous systems):

According to Punrattanasin *et al*. (2001), polymer compositions, i.e., the PHV/PHA ratios of the copolymers produced were different under different operating conditions. In this study, copolymers produced were also different under different cultivation conditions, i.e., PHB/PHV ratios obtained from the batch and fed-batch bioprocess were different from those of continuously feeding bioprocess, even though the type of the substrate (carbon source) in the feed was the same. In the previous study of batch experiments, even though the acetate was used as the sole carbon source, the copolymer produced with the HB/HA ratio was quite different at initial sludge SS of 5 g/l, 10, 15, and 20 g/l.

The initial HB/HA ratio (20%) was same in all fed batch and continuous processes and it increased with the process time (Figs 4 and 5). In fed-batch experiments, the results showed that at initial sludge SS concentration of 15 g/l, with two feeds of 10 and 5 g/l of acetate at T=0 and 12 hour, the HB/HA ratios increased from 25.9% (T=0) to 76.8% (T=36h), then decreased to 53.6% at the end of experiments (60 h). In case of three and four feeds the maximum HB/HA obtained was 80.5% at 48h and 80.9% at 57h, respectively and the HB/HA trends were similar with that of two feeds. At the initial

sludge SS concentration of 15 g/l, with three feeds, the HB/HA ratios increased from 24.2% (T=0) to 85.5% (T=51h), then decreased to 80.1% at the end of experiments of 60 h. With four feeds, the HB/HA ratios increased from 24.4% (T=0) to 95.0% (T=57h), then decreased to 88.1% at the end of experiments of 60 h (Figure 4).

In continuous feed experiment, the HB/HA ratio increased (almost linearly) from the beginning until the end of the process (60 h). This was a different trend compared to the fed-batch experiments (Figure 5) as well as the batch results reported earlier (Yan et al., 2007), where the HB/HA ratio reached maximum and started decreasing after that. This different trend could be due to limiting carbon source strategy adopted in continuous feed system compared to other systems where, in general, carbon source was present in excess.

Influence of carbon to nitrogen (C:N) ratio of the substrate on PHA production:

Bacteria synthesize their cellular material (protein etc.) and grow but during nutrient limiting conditions bacteria may shift its metabolism from one product to another. In order to exploit this bacterial characteristics, the experiments were carried out in nutrient limiting conditions (by varying C:N ratio from 48 to 192).

The profiles of acetate, PHA and sludge SS concentration and PHA content are presented in Fig.6. The C:N ratio was varied from 48 to 192 with 1g/l acetate feed at the beginning followed by continuous feeding of acetic acid after 1h process time. One experiment at C:N ratio 144 with initial acetate concentration of 3 g/l followed by continuous feed after 1 h was also conducted to make sure that carbon source was not limiting and to assess its impact on PHA production. The acetate added in the beginning was consumed rapidly and reached zero within an hour. The acetate concentration stayed almost zero until 12 h, started increasing thereafter and stabilized after 30 h, irrespective of C:N ratio, indicating that the added acetic acid was consumed instantly. Further, the acetate consumption pattern was same irrespective of C:N ratio (Fig 5b, d).

The initial PHA content of the sludge used during the experiments with C:N ratio 48, 96 and 144 was 7.96% w/w dry SS, whereas for the sludge sample that was used in the experiments with C:N ratio 144, 168 and 192 was 9.55% w/w dry SS. The increase of PHA during sludge manipulation and before starting the experiments in all experiments is presented in Table 4 and shows that there was a substantial increase in PHA content.

The PHA concentration and content increased with process time and reached a maximum value and decreased thereafter. Similar trend of PHA increase was observed irrespective of C:N ratio. The maximum PHA concentration, content and productivity increased as the C:N ratio was increased from 48 to 144. Further increase in C:N ratio resulted in decrease of values of these parameters. At C:N ratio 144 with initial acetate concentration of 3 g/l PHA concentration, content and productivity substantially decreased (Table 4). The maximum values of PHA concentration (16.23 g/l), productivity (301 mg/l/h) and content (60.95 of dry sludge SS) at C:N ratio 144 was the highest compared to all systems (batch, fed batch, continuous and initial biomass as well as acetate concentration. These results in general are similar to those of Kumar et al. (2004).

The profiles of biomass at different C:N ratio showed a similar pattern irrespective of C:N ratio. Maximum biomass concentration was observed at C:N ratio of 144. Taking into account the increase in biomass concentration, a reduction in sludge solids was calculated assuming the sludge weight loss equivalent to PHA extraction. The solids reduction thus calculated was presented in Table 4 and was found to be maximum (28.4% reduction) at C:N ration of 144.

Copolymer composition (C:N Ratio):

During the experiments of different C/N ratios under continuous feed mode, the HB/HA ratios increased from the beginning until 12 h of the process and then remained fairly constant with slight change towards the end of the process (Figure 7), irrespective of C:N ratio. However, maximum HB/HA ratio varied with C:N ratio. This trend was entirely different than those observed in batch, fed batch and continuous systems.

The variations of the HB/HA ratios in this study could be due to the different operation conditions. Another reason for this might be due to the fact that PHA production and degradation could occur simultaneously under certain conditions (Doi 1990), therefore, the composition of polymer varied significantly under different acetate feed strategies. Furthermore, it was found that various bacteria present in activated sludge are capable of synthesizing random copolymer of (*R*)-3HB with other HA units of C3 to C12, depending on both their intrinsic PHA biosynthesis pathways and the carbon sources used (Tsuge 2002). Therefore, the difference in polymer composition observed in these studies is probably due to differences in the microbial population structure in the activated sludge from the pulp and paper treatment plants.

It was found that the polymer becomes tougher (increase in impact strength) and more flexible (decrease in Young's modulus) as the fraction of 3-hydroxyvalerate increases. The elongation to break also increases as the comonomer fraction increases. For example, the copolymer of P(3HB-co-3HV) with the 9 mol% of 3HV, the melting temperature is 162^{o}C, the Young's modulus is 1.9 GPA, the Notched izod impact strength is 95 J/m, the tensile strength is 37 MPa, while with 25 mol% of 3HV, the melting temperature is 137^{o}C, the Young's modulus is 0.7 GPA the tensile strength is 30 MPa, the Notched izod impact strength is 400 J/m (Lee 1996).

Furthermore, the decrease of melting temperature with increasing 3-hydroxyvalerate fraction without affecting degradation temperature allows thermal processing of the copolymer as a melt without thermal degradation. The material properties can be controlled by adjusting the fraction of 3-hydroxyvalerate during the fermentation (Lee 1996).

The family of PHAs exhibits a wide variety of mechanical properties, from hard crystalline to elastic, depending on the composition of monomer units, which broadens its application area (Lee 1996). The results from this study showed that more effort should be made to control the HB:HV ratios during the process using activated sludge mixed culture as the microorganisms for PHA production.

CONCLUSIONS

The following conclusions can be drawn from this work:

The activated sludge from a full-scale pulp-paper industry wastewater treatment plant could be used as the mixed culture for PHA production. The activated sludge concentration of 15 g/l showed the optimum concentration for higher PHA production. Continuous feeding of the substrate (acetic acid in the study) was the better strategy for higher PHA production. Maximum PHA content (%) reached 60.9% of the dry sludge weight and PHA concentration of 16.23 g/l at C:N ratio of 144. This was comparable with some of the results by using pure culture. The co-polymer concentration changed with process time and their final concentration varied with initial sludge suspended solids concentration, initial acetate concentration and type of system (batch, fed-batch or continuous) used for PHA production.

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Table 1. Characteristics of sludge.

Activated sludge	Operation process	pH	SS (g/l)	COD_{tot} (mg/l)	COD_{sol} (mg/l)	TKN (mg/l)	NH_4^+ (mg/l)	P_{tot} (mg/l)	Orth-P (mg/l)
PAS	Aerobic	6.9	19.3	1675.8	120.3	25.3	12.9	13.3	5.2

* Activated sludge data were on the basis suspended solids concentration of 20 g/l for sludge.

Table 2. Summary of the feed patterns in fed-batch experiments at different adding mode of acetate.

Activated sludge concentration T=0	Number of feed	Feed pattern (acetate added at different time) (g/l)				
		T=0 h	T=12 h	T=24 h	T=36 h	Total acetate(g/l)
15	2	10	5			15
15	3	10	5	2.5		17.5
15	4	10	5	2.5	2.5	20
20	3	10	5	2.5		17.5
20	4	10	5	2.5	2.5	20

Table 3. Summary of the results from fed-batch and continuous feed experiments at different activated sludge SS concentration.

SS_0 T=0 (g/l)	PHA_0 (g/l)	Number of feed	AA_0 (g/l)	Tm (h)	PHAm (g/l)	IPR (mg/l/h)	PHAm content (w/w%)	$Y_{p/s}$ (g/g)	PD_{m*} (mg/l/h)	ACR_{m*} (g/l/h)	SR_{m*} (%)	SPR_{m*} (mg/g/h)
Fed-batch mode:												
15	2.0(13.4)	2	10	45	6.73	209(9h)	44.2	0.58	105	0.18	43.1	6.9
15	1.5(10.3)	3	10	54	8.26	272(9h)	49.5	0.47	125	0.26	42.8	7.5
15	1.0(6.7)	4	10	54	10.1	406(9h)	50.3	0.47	167	0.35	35.6	8.4
20	2.1(11.3)	3	10	36	6.07	131(12h)	31.9	0.57	111	0.19	29.3	5.8
20	2.2(12.0)	4	10	45	8.08	235(6h)	42.0	0.83	130	0.16	39.9	6.8
Continuous feed												
15	2.3(14.7)		10	36	10.18	183(9h)	57.6	0.55	219	0.39	52.2	12.4

PHA_0: initial PHA concentration after acetate addition (values in parenthesis are weight %); **SS_0** : initial sludge suspended solids concentration; **A_0**: initial acetate concentration; **T_m** : time at which maximum PHA concentration occurred; **PHA_m** : maximum PHA; **IPR**: initial PHA production rate (mg/l/h); **Yp/s**: (g polymer/g Ac-) polymer formed by substrate consumed; **PD**: PHA production rate; **ACR**: acetate consumption rate; values in parentheses are that of zero order rate constant; **SR**: sludge reduction; **SPR**: (mg PHA/g dry SS/h), specific PHA production rate; **m*** values when the maximum PHA content occurred.

Table 4. Summary of the results from continuous feed experiments with different C/N ratios at activated sludge SS concentration of 15 g/l.

No	C/N ratio	PHA_0 (g/l)	SS_0 T=0 (g/l)	AA_0 (g/l)	Tm (h)	PHAm (g/l)	IPR (mg/l/h)	PHAm content (w/w%)	$Y_{p/s}$ (g/g)	PD_{m*} (mg/l/h)	ACR_{m*} (g/l/h)	SR_{m*} (%)	SPR_{m*} (mg/g/h)
1	48	1.51(9.9)	15	1	48	10.76	261(12h)	41.5	0.50	193	0.38	1.3	7.4
2	96	1.24(8.5)	15	1	48	11.09	293(12h)	44.2	0.52	205	0.40	4.4	8.2
3	120	1.72(11.9)	15	1	51	11.78	392(9h)	48.0	0.49	197	0.40	11.8	8.0
4	144	1.73(12.0)	15	3	57	6.57	164(6h)	35.5	0.27	95	0.35	17.4	5.1
5	144	2.68(18.4)	15	1	45	16.23	218(9h)	60.9	0.73	301	0.41	28.4	11.3
6	168	2.28(15.0)	15	1	36	9.10	298(6h)	37.0	0.47	189	0.40	3.3	7.7
7	192	1.87(11.8)	15	1	45	8.49	268(6h)	35.1	0.35	140	0.40	4.8	6.0

PHA_0 : initial PHA concentration after acetate addition (values in parenthesis are weight %); **SS_0** : initial sludge suspended solids concentration; **A_0**: initial acetate concentration; **T_m** : time at which maximum PHA concentration occurred; **PHA_m** : maximum PHA; **IPR**: initial PHA production rate (mg/l/h); **Yp/s**: (g polymer/g Ac-) polymer formed by substrate consumed; **PD**: PHA production rate; **ACR**: acetate consumption rate; values in parentheses are that of zero order rate constant; **SR**: sludge reduction; **SPR**: (mg PHA/g dry SS/h), specific PHA production rate; **m*** values when the maximum PHA content occurred.

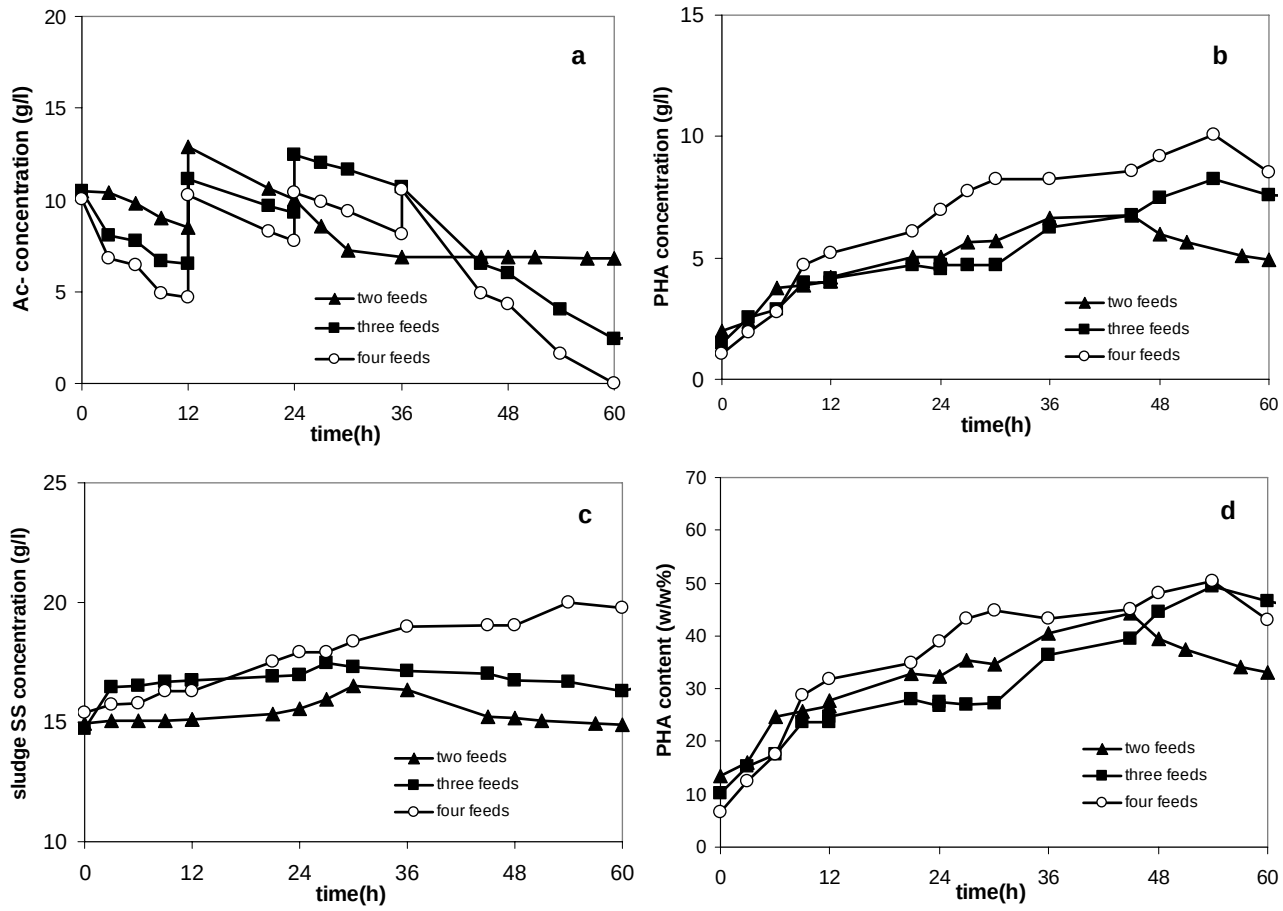

Figure 1. Profiles of acetate, PHA, sludge SS concentrations and PHA content (w/w% of SS) during fed-batch experiments at sludge SS concentration of 15 g/l with different acetate feeds.

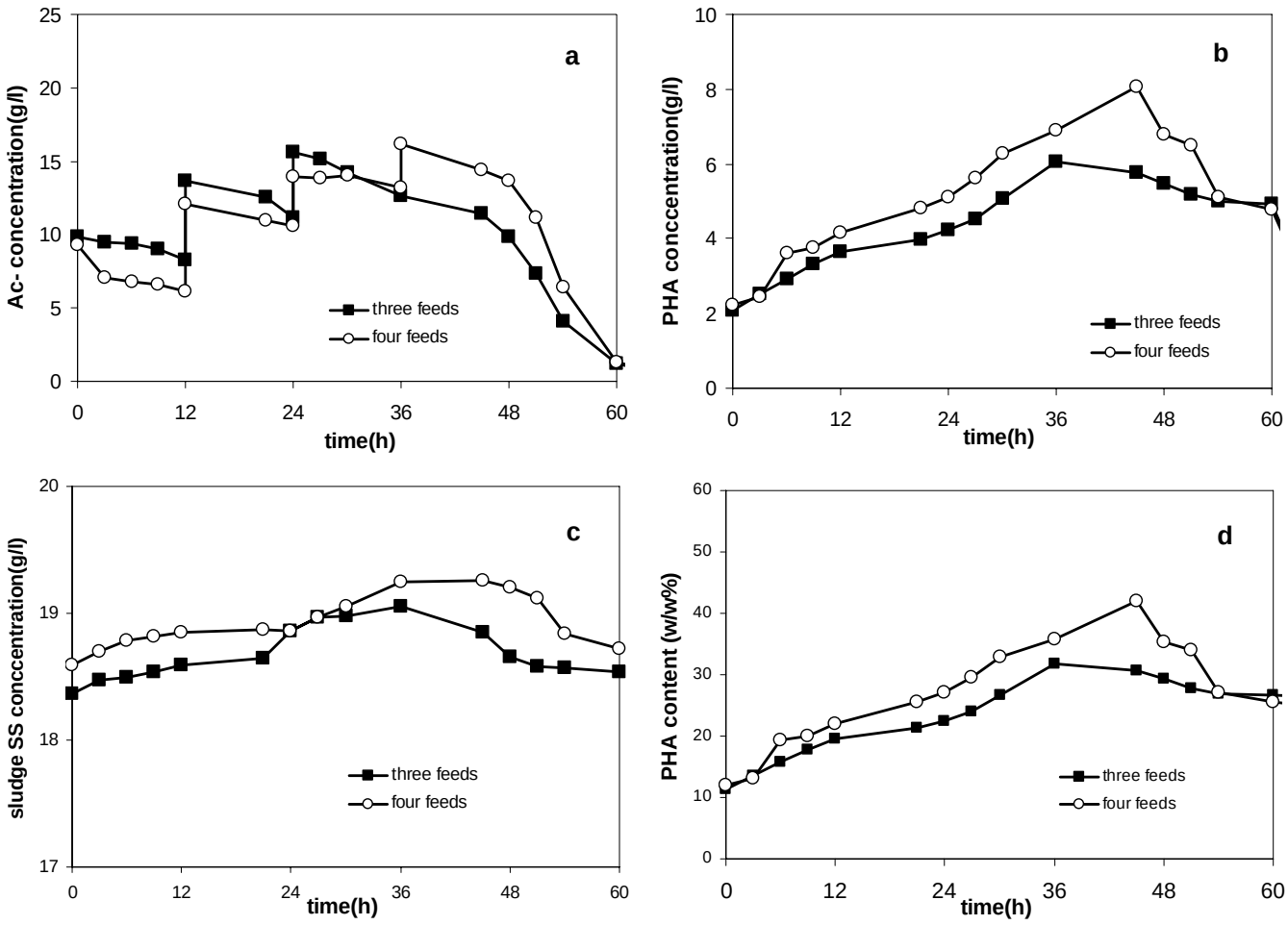

Figure 2. Profiles of acetate, PHA, sludge SS concentrations and PHA content (w/w% of SS) during fed-batch experiments at sludge SS concentration of 20 g/l with different acetate feeds.

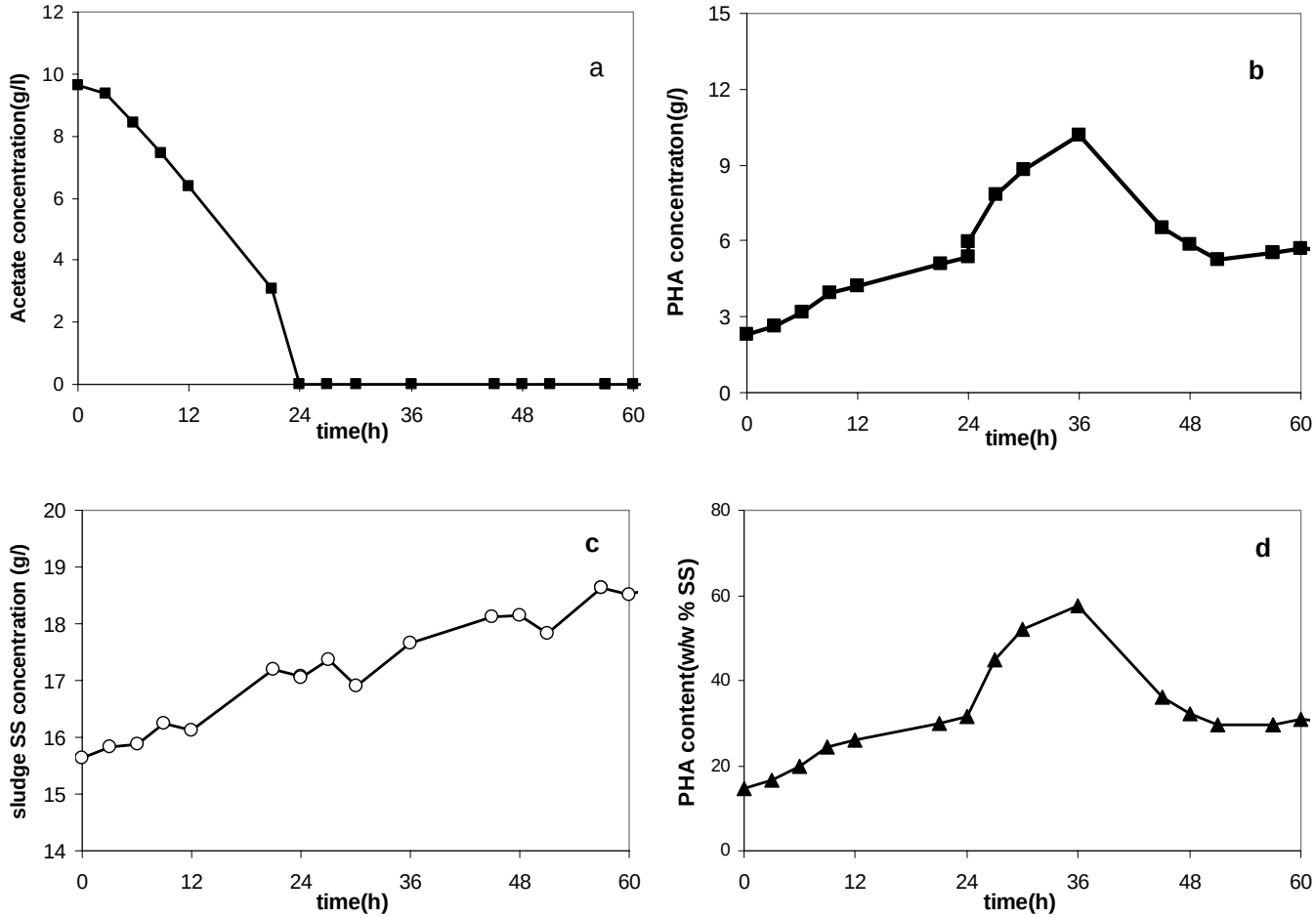

Figure 3. Profiles of acetate, PHA, sludge SS concentrations and PHA content (w/w% of SS) during continuous feed experiment at sludge SS concentration of 15 g/l.

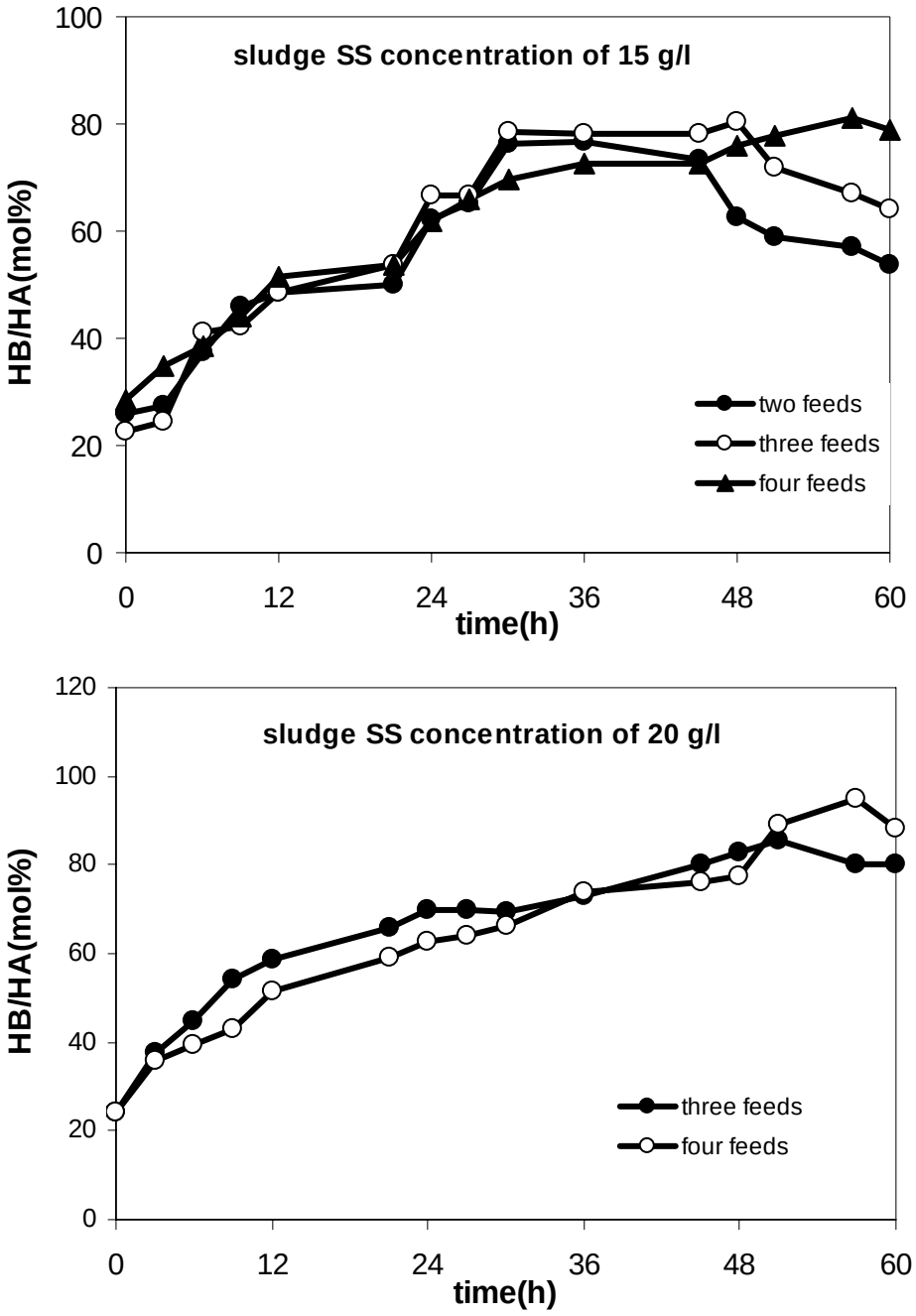

Figure 4. Profiles of HB/HA(mol%) during fed-batch experiment at sludge SS concentration of 15 and 20 g/l.

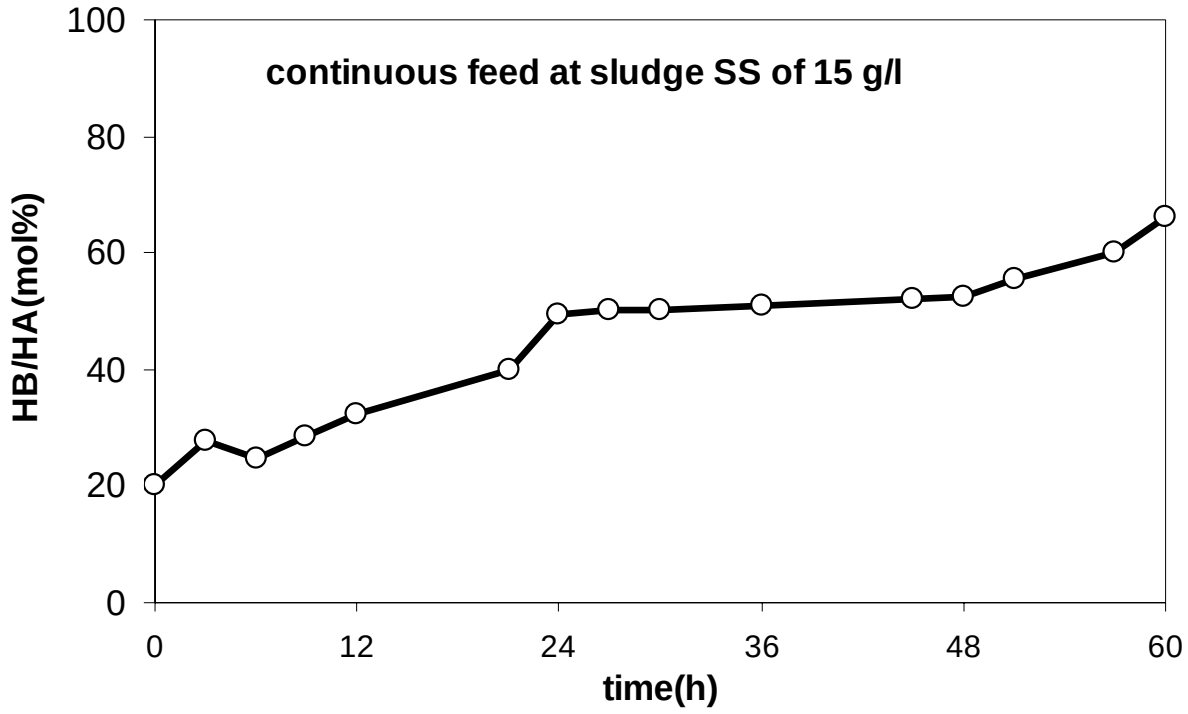

Figure 5. Profiles of HB/HA(mol%) during continuous feed experiment at sludge SS concentration of 15 g/l.

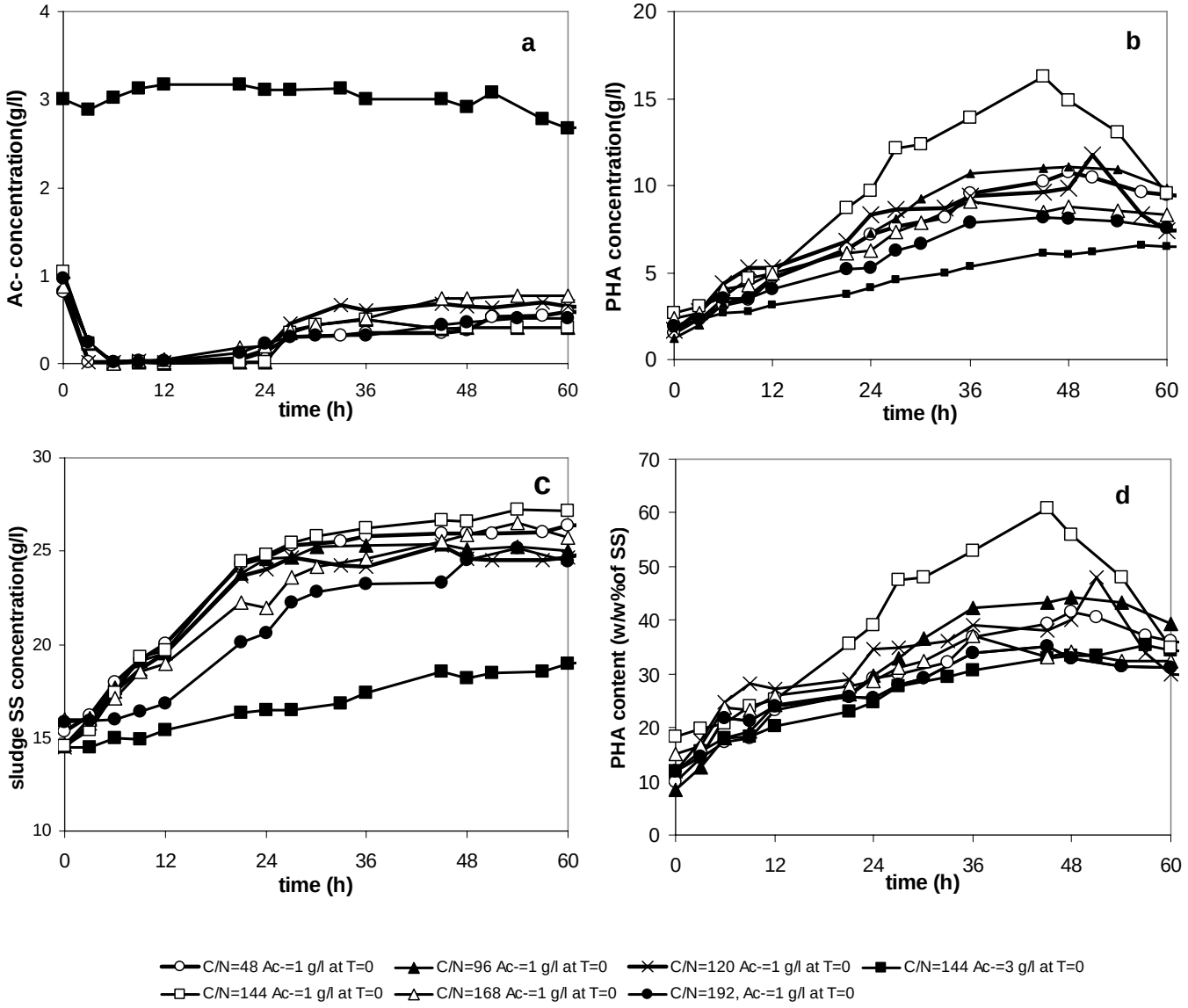

Figure 6. Profiles of acetate, PHA, sludge SS concentrations and PHA content (w/w% of SS) during continuous feed experiments with different C/N ratios at sludge SS concentration of 15 g/l.

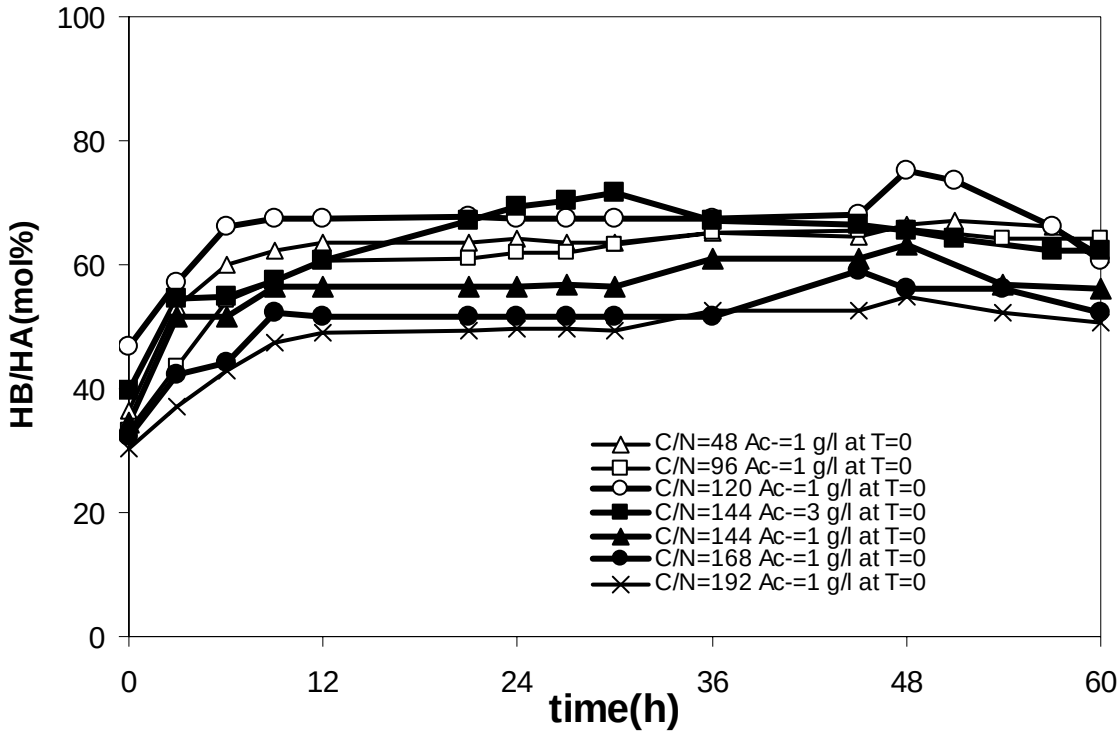

Figure 7. Profiles of HB/HA(mol%) during continuous feed experiment at different C/N ratios.

Part IV

Bioplastics production using activated sludge from pulp-paper wastewater treatment plant with continuous feed of carbon source

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Résumé

Cette étude a porté sur la production de PHA en utilisant différentes eaux usées comme substrats. Des boues activées d'usines de traitement des eaux usées d'industries de pâtes et papiers ont été employées comme source de micro-organismes. Quatre types d'eaux usées municipales et industrielles et de l'acide acétique ont été utilisés éparément comme sources de carbone. Des expériences de stratégie d'alimentation continue ont été conduites en bioréacteurs de 15 l. et donc en conditions contrôlées. Les résultats ont montré que les quatre types d'eaux usées peuvent servir de substrats pour produire des PHAs à des taux différents. La teneur de PHA maximale obtenue fut de 60,2% à l'aide d'acide acétique comme source de carbone au début de croissance et suivie d'une alimentation continue. Les eaux usées de pâtes et papier ont permis d'atteindre la plus forte concentration en PHA (54,9%) parmi les quatre types testées. Ce processus est donc un onc moyen prometteur pour réduire le coût de production de PHAs ainsi que pour recycler des polluants organiques de déchets en produits à valeur ajoutée.

Mots-clés: boues activées, polyhydroxyalcanoates (PHA), pâtes et papier, industrie des eaux usées, bioplastiques.

Abstract

This study investigated the PHA production using different wastewaters as substrate. Activated sludge from full-scale pulp and paper industry wastewater treatment plants was used as a source of microorganisms. Four types of wastewaters from municipal and industrial wastewater treatment plants, respectively and acetic acid were used as carbon source. The continuous feed strategy experiments were conducted in the controlled condition fermentors (15 L). The results showed that the four types of wastewater can used as substrate to produce PHA at different value. Maximum PHA content obtained was 60.2% of SS using acetic acid as carbon source at the beginning followed by the continuous feed. Pulp and paper wastewater showed the highest PHA content (54.9%) among the four types of wastewater. The present process was a promising way to reduce the cost of PHA production as well as the conversion of organic pollutants in waste into valuable products.

Key words: Activated sludge, polyhydroxyalkanoates (PHA), pulp-paper industry wastewater, bioplastics

INTRODUCTION

Due to the problem and harmful effects of non-biodegradable plastics on the environment, Polyhydroxyalkanoates (PHAs) have been drawing much attention as biodegradable polymer that can be used in place of conventional petrochemical-based plastics (Chua et al. 2003; Dionisi et al. 2005b). Poly-β-hydroxybutyric acid (PHB) and its copolymer poly-3-hydroxybutyrate-co-hydroxyvalerate [P(3HB-co-HV)] are the common types of PHAs, and other forms also exist. Many microorganisms have the capability to accumulate PHA. To realize sustainable demand of PHA biopolyester, the high manufacturing cost needs to be reduced by establishing an efficient production process of PHA with the desired properties (Lee and Yu, 1997; Chua et al., 2003). Much efforts have been made to reduce the production costs during last decades (Yu et al., 1998; Dionisi et al., 2001; Chua et al., 2003).

PHA production using activated sludge as a mixed culture was studied by many researchers (Lee and Yu 1997; Dionisi et al. 2001a; Chua et al. 2003; Yan et al. 2006). This approach could significantly reduce the cost of PHA production and at the same time, decrease the quantity of excess sludge from the wastewater treatment process that required further treatment and disposal, and a new way of carbon recycle through the conversion of organic pollutants in waste into valuable products.

In our previous studies (Yan et al., 2006), activated sludge from pulp and paper wastewater treatment process was used as the microorganism for PHA production. It was found that in continuous feed of acetic acid mode under C/N ratio of 144, the PHA content reach up to 60.8% of sludge suspended solids. The purpose of this study was to investigate the PHA production using different types of wastewater from full scale WWTP as the substrates in the fermentors under controlled conditions. These wastewaters utilized in the study were composed of volatile fatty acid which was reported to be one of the good substrates for PHA production.

MATERIALS AND METHODS:

Activated sludge:

Fresh pulp-paper activated sludge (PAS) for PHA production was obtained from a pulp-paper industrial wastewater treatment plants (WWTP) in Quebec Province, Canada. The activated sludge was concentrated by centrifugation at 5000 rpm for 20 minutes (at 4^{o}C); the suspended solids (SS) of the activated sludge were measured. Suspended solids concentration of 15 g/l of activated sludge were used for various experiments as in previous study (Yan et al., 2007), sludge concentration of 15 g/l was found to be the optimal). The experiments were conducted within a couple of hours after receiving the sludge samples. The characteristic of the sludge is listed in Table 1.

Carbon sources:

Four types of wastewaters indicated as municipal (MWW), pulp-paper (PWW), starch (SWW) and dairy (DWW) (cheese manufacturing) sampled from different wastewater treatment plants in Quebec Province, Canada were utilized as the carbon source, respectively of fermentation experiments. The characteristics of different wastwaters are shown in Table 2. Experiment was also conducted using acetic acid (1 g/l) as the carbon source. Acetic acid (6 N) was used as the pH control agent during the fermentation course. Ammonium chloride (NH_4Cl) was used as nitrogen source, if required, to adjust C/N ratio 144 which was found to be the optimal in the previous study.

Experiments (controlled reactor conditions):

Experiments were conducted in two-bench scale bioreactors (total capacity of 15 litre, diameter of 0.2 m and liquid height of 0.3m and a working volume of 10 litre) equipped with accessories and automatic control systems for dissolved oxygen, pH, antifoam, impeller speed, aeration rate and temperature. The air was supplied through a filter (Gelman, PTFE, 0.2μm) beforehand pressure-sealed, then conveyed by a tube perforated in the culture medium. The dissolved oxygen concentration (%) in the medium was measured by an oxygen probe (InPro 6000 of Mettler Toledo U.S.A). The computer

program (Fix 3.5, Intellution, USA) used for automatic set-point control and all stated parameters was saved. Polypropylene glycol (PPG, Sigma-Canada) (0.1% v/v) solution was added to control the foam.

Experimental operation:

10 L acetic acid solution (concentration of 1 g/l) or wastewater (10 L) was transferred to bioreactors and appropriate amount of concentrated (centrifuged) pulp-paper activated sludge was re-suspended to obtain concentration of 15 g/l as total solids. At the beginning of all experiments, pH was adjusted to 7.0 by 6 N acetic acid or 2 N NaOH. During the experimental process, the pH was controlled at 7.0 by using 6 N acetic acid fed continuously to the fermentor by the computer-controlled peristaltic pumps. In this mode, the acetic acid served as pH control agent as well as the carbon source for PHA production. The fermentation was carried out for 60 h. Samples were drawn at predetermined time for analysis.

For all experiments, temperature was automatically controlled at set-point of 25^{o}C. The agitation speed (300-500 rpm) and aeration rate (0.3-0.5 vvm) were varied in order to keep the dissolved oxygen (DO) values above 50% of saturation, which ensured the oxygen concentration above the critical level.

Analytical techniques:

Four-ml of culture broth was sampled in a pre-weighed centrifuge tube and centrifuged at 5000 rpm for 10 minutes. The settled biomass was washed 3 times with distilled water and centrifuged. After centrifugation, the biomass was freeze-dried for at least 48 hours to obtain the dry weight. The final weight of the tube was measured to calculate the sludge dry weight. The extraction of PHA was carried out according to the methanol and chloroform extraction method described by Comeau et al. (1988) using benzoic acid as an internal standard. The co-polymeric composition was measured with a gas chromatograph (GC) (Varian Model 3800) equipped with a Capillary column Zebron ZB-5 and a Shimadzu C-R5A Chromatopac flame-ionization detector. Poly-(3-

Hydroxybutyrate-co-3-hydroxyvalerate) (3HB-co-3HV) (PHV content 12wt.%, Aldrich Chemical Company, Inc.) was used as a standard.

The supernatant of the centrifuged samples was used to determine dissolved chemical oxygen demand (DCOD). Total solids (TS) and suspended solid (SS), COD were analysed according to standard methods (APHA, 1998). The ammonium nitrogen (NH_4^+-N), total kjeldahl nitrogen (TKN), orthophosphate (ort-P) and total phosphate were determined by Technicon Analyser (Technicon Instruments Corporation, New York). Measurements were carried out in duplicate and the average value was reported.

RESULTS AND DISCUSSIONS

In the previous study (Yan et al., 2007; Yan et al., 2007), it was found that under the C:N ratio of 144, with 1g/l acetate feed at the beginning followed by continuous feeding of acetic acid after 1 h process time, the highest PHA content (w/w% of SS) obtained was 60.9% among the batch, fed-batch and continuous feeding strategies.

The maximum PHA concentration, content and productivity was obtained using the C/N ratio of 144. In order to enhance the production of PHA by using different types of wastewater in this study, a continuous system was designed and operated. Approximately 15 g/l sludge SS was used in this experiment and 1 g/l of acetic acid was used at T=0 as the reference. While the other experiments fed in the beginning of four types of wastewater, respectively, then the process was operated under the continuous feed with acetic acid (6N), from 1h till the end of the experiment (60). As well as to adjust the pH.

The profiles of acetate consumption, PHA concentration and content and biomass change (sludge SS) are depicted in Figure 1. The COD concentration reached zero within 1 h and remained zero through rest of the process in spite of intermittent addition of acetic acid to adjust pH of the system.

During initial manipulation (addition of substrate, adjustment of pH, sample collection and centrifugation) the PHA contents in the filve experiments were 10.2, 7.8, 5.2, 5.3 and 5.1%, respectively. The PHA concentrations were 1.55, 1.21, 0.77, 1.47 and 0.76 g/l, respectively. before the commencement of acetic acid feed as pH adjusting agent. The PHA concentration increased abruptly soon after acetic feed was started (Fig. 1b).

Effect of carbon source

Acetic acid as substrate at T=0

The concentration profiles of PHA and sludge dry weight as well as the PHA content (wt% of dry sludge weight) by the five substrates (acetic acid and four types of wastewaters) are shown in Figure 1, respectively. It clearly indicates that the concentration of acetic (carbon source) remained very low throughout the experiments. The sludge dry weight increased with the fermentation time then kept a relative constant values, anyway, PHA concentration and PHA content (%) increased and reached the maximum value of PHA concentration 14.30 g/l, and maximum PHA content of 60.2% respectively, at about 45 h then followed by a decrease.

Pulp-paper wastewater (PWW) as the carbon source at T=0

The concentration profile of dry sludge weight, PHA and PWW (carbon source) as well as the PHA content (wt% of dry sludge weight) is shown in Figure 1. Maximum PHA content (wt.% of dry sludge weight) obtained was 54.9%, with PHA concentration was 12.70 g/l at 36 h.

Municipal wastewater (MWW) as the carbon source

The concentration profile of sludge dry weight, PHA and MWW (carbon source) as well as the PHA content (wt% of dry sludge weight) is shown in Figure 1. Maximum PHA content (wt.% of dry sludge weight) obtained was 52.3%. at 45 h while sludge dry weight and PHA concentration were 22.40 and 11.73 g/l, respectively.

Starch wastewater (SWW) as the carbon source

The concentration profile of sludge dry weight, PHA and SWW (carbon source) as well as the PHA content (wt% of dry sludge weight) is shown in Figure 1. Maximum PHA content (wt.% of dry sludge weight) obtained was 51.4% at 45 h with the sludge dry weight and PHA concentration of 30.22 and 15.53 g/l, respectively.

A higher solid concentration was observed in starch wastewater (SWW), this could be due to higher suspended solid concentration of SWW and the available carbohydrate (soluble starch) and nitrogen contents of SWW, which might have prolonged the exponential growth phase.

Dairy (DWW) (cheese manufacturing) as the carbon source

The concentration profile of sludge dry weight, PHA and DWW (carbon source) as well as the PHA content (wt% of dry sludge weight) is shown in Figure 1. Maximum PHA content (wt.% of dry sludge weight) obtained was 50.6% with the sludge dry weight and PHA concentration of 22.82 and 11.55 g/l, respectively.

The highest PHA content was observed by using acetic acid as the substrates (60.2%), while among four types of wastewaters, PWW showed the highest PHA content (%) of 54.9%. Comparing the results, there was no significant differences of the PHA content (maximum value of 54.9, 52.3, 51.4 and 50.6%) among the 4 types of wastewaters. The reason could be due to that in the present study, the wastewater served only as part of the substrates, while acetic acid also served as the substrate throughout the experiment process.

Four types of wastewater could be used as the carbon source for PHA production, by this simple strategy of the acetic acid continuous feeding as the pH control agent as well as part of the substrate for PHA production. On the other hand, they could be treated by this way to reduce the COD, by this approach, the organic pollutants in waste was converted into valuable products. Pulp-paper showed a little higher PHA production among four

types of wastewater, this agreed with the trends obtained from the shake flask experiments shown in Table 3 (Yan et al. 2006).

Solid reduction:

The summary of the solid reduction rates by acetic acid and four types of WWs as the substrates was shown in Table 5. The best reduction (47.2%) was observed with the starch industry wastewater as the substrate at T=0, while with dairy industry wastewater as the substrate at Y=0, the solid reduction rate was the lowest (24.5%). It was very clear that by the way of pulp and paper activated sludge as the microorganism for the PHA production, the excess sludge to be treated and disposal would be reduced around 20-50%.

COD level in Wastewaters

COD profile during the experiments course was shown in Figure 1a, the original COD of acetic acid, PWW, MWW, and DWW was around of 1000 mg/l, decreased along the fermentation course till below 160 mg/l at the end of the fermentation. While for SWW, the original COD was 5500 mg/l, decreased during the fermentation course, till reached 4000 mg/l at the end of experiment. This is shown that by the way of WWs as the substrates for PHA production, the COD in WWs would be reduced and converted to the value added product.

Copolymer composition:

In this study, it was found that the HB/HV ratios of the copolymers produced by the pulp-paper activated sludge were different when utilizing different substrates (wastewater or acetic acid), This might be due to the acetic and other volatile fatty acid (VFA) composition in different wastewaters (Table 2). Many researches reported that acetic acid resulted in 3HB and propionic resulted of 3HV. As these wastewater contained VFA, the PHA produced were copolymers at varying HB/HV ratios. The copolymer composition at the maximum PHA content (%) of the experiments was shown in Table 4. the results showed that that the copolymer composition, i.e., HB/HV ratio, is primarily influenced by the substrate used (Satoh et al. 1998a).

Further more, the HB/HV ratios of the copolymers produced by the activated sludge as the mixed culture presented a large variation during the time course of each experiment. This may be due to that the HB/HV ratios of the copolymers is also influenced by the fermentation conditions and time. According to Punrattanasin et al. (2001), polymer compositions, i.e., the PHV/PHA ratios of the copolymers produced were different under different operating conditions.

In the study, the PHA contents of 50.6 to 60.2% were comparable to those by using pure culture as the microorganisms (Akar et al. 2006). Hence, it is very promising that PHA production by using full scale activated sludge should a great potential as a competitive alternative to pure culture, due to its low production cost and operation flexibility.

Economic analysis

It has been well known that PHAs are attractive biopolymers for biodegradable plastics production because they have similar physical and mechanical properties to conventional plastics. Anyway, one of the major problems preventing the commercial application of PHAs is their high production cost, especially raw material costs. Lee (1996) reported that the price of BIOPOL is $16/Kg, while the price of synthetic plastics is a lot less expensive, e.g., less than $1/Kg for polypropylene. The synthesis of PHA in bacteria and subsequent extraction of the biopolymer is estimated to cost approximately 3–4 US$/kg (Lee et al., 1997). The lowest production cost of $2.6/ kgPHB was obtained from A. latus. The low production cost resulted from the high PHA content (88% PHB) and high productivity (4.94 gPHB/L/hr) of A. latus (Choi and Lee, 1999).

In this study, the PHA productivity achieved with activated sludge and wastewater was comparable to those obtained using pure cultures. Although it was lower than the maximum one till to date (around 90% of dry cell). Choi and Lee (1999) stated that there are other factors affecting the economics of PHA production include oxygen requirement and scale-up of the system. The PHA production cost decreases with the production scale

increases. However, the cost fraction of the substrate increases as the production scale increases.

Hence, to reduce the production cost of PHA production using activated sludge as microorgansims, it might be very promising that activated sludge PHA production systems utilizing wastewater as the substrate is coupled as a side-stream process with a main-stream wastewater treatment system. In this context, the production of PHA in wastewater treatment plants can be regarded as a promising alternative for the large-scale and low cost production of biopolymer. However, the process of this study needs fully developed, the intense analysis of the economic still requires considerable research.

CONCLUSIONS:

The following conclusions can be drawn from this work:

The activated sludge from a full-scale pulp-paper industrial wastewater treatment plant could be used as the mixed culture for PHA production. Maximum PHA content was 60.2% using acetic acid as carbon source at the beginning and the continuous feeding. Pulp-paper wastewater showed a little higher PHA production among the four types of wastewater. The present process was a promising way to reduce the cost of PHA production as well as the conversion of organic pollutants in waste into valuable products.

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Table 1. Characteristics of sludge.

Activated sludge*	Operation process	pH	SS (g/l)	COD_{tot} (mg/l)	COD_{sol} (mg/l)	TKN (mg/l)	NH_4^+ (mg/l)	P_{tot} (mg/l)	Orth-P (mg/l)
PAS	Aerobic	6.9	19.3	1675.8	120.3	25.3	12.9	13.3	5.2

* Activated sludge data were on the basis suspendid solids concentration of 20 g/l for sludge.

Table 2. The characteristics of different wastewaters used as carbon source.

Items	Wastewaters			
	Municipal	Pulp-paper	Starch	Dairy
pH	6.5	6.9	3.5	11.4
COD_{tot}(mg/l)	57.2	989.6	8231.2	201.2
COD_{sol}(mg/l)	37.2	417.2	5167.6	93.1
TKN(mg/l)	77.3	22.6	2357.2	103.2
NH_4^+(mg/l)	19.8	11.3	17.5	56.8
P_{tot}(mg/l)	10.2	13.3	25.7	23.3
Orth-P(mg/l)	1.95	0.2	1.3	5.8
C:N	0.73	43.78	3.49	1.95
Volatile fatty acid (mg/l)				
Acetic	13.50	256.09	247.79	9.25
Propionic	3.20	3.27	7.98	2.01
Iso-butyric	0.52	1.86	0	0
n-butyric	0	3.75	2.85	0
Iso-valeric	2.10	5.03	1.62	0
n-valeric	1.50	2.38	2.05	2.01

Table 3. Summary of the results from continuous feed experiments with different wastewaters as substrates at activated sludge SS concentration of 15 g/l under C/N ratio of 144**.**

Substrate at T=0	PHA_0 (g/l)	SS_0 T=0 (g/l)	AA_0 (gCOD/l)	Tm (h)	PHAm (g/l)	IPR (mg/l/h)	PHAm content (w/w%)	$Y_{p/s}$ (g/g)	PD_{m*} (mg/l/h)	ACR_{m*} (g/l/h)	SR_{m*} (%)	SPR_{m*} (mg/g/h)
Acetic acid	1.55(10.2)	15	1.28	45	14.30	736(3h)	60.2	0.61	283	0.46	38.0	11.9
PWW	1.21(7.8)	15	0.73	36	12.70	333(12h)	54.9	0.72	319	0.45	32.9	13.8
MWW	0.77(5.2)	15	0.28	45	11.73	276(12h)	51.4	0.71	312	0.44	27.9	10.9
SWW	1.47(5.3)	15	5.5	45	15.53	333(12h)	35.5	0.27	95	0.35	47.2	10.3
DWW	0.76(5.1)	15	0.354	48	11.55	313(12h)	50.6	0.54	225	0.42	24.5	9.8

PHA_0: initial PHA concentration after substrate addition (values in parenthesis are weight %); **SS_0** : initial sludge suspended solids concentration; **A_0**: initial substrate concentration; **T_m** : time at which maximum PHA concentration occurred; **PHA_m** : maximum PHA; **IPR**: initial PHA production rate (mg/l/h); **Yp/s**: (g polymer/g COD polymer formed by substrate consumed; **PD**: PHA production rate; **ACR**: substrate consumption rate; values in parentheses are that of zero order rate constant; **SR**: sludge reduction; **SPR**: (mg PHA/g dry SS/h), specific PHA production rate; **m*** values when the maximum PHA content occurred.

Table 4. Maximum PHA content (wt.% of dry sludge weight) results of four types of wastewater by pulp-paper activated with shake flask experiments:

Activated sludge	wastewater			
	MWW	PWW	SWW	DWW
PAS	6.3%	17.9%	17.5%	8.26%

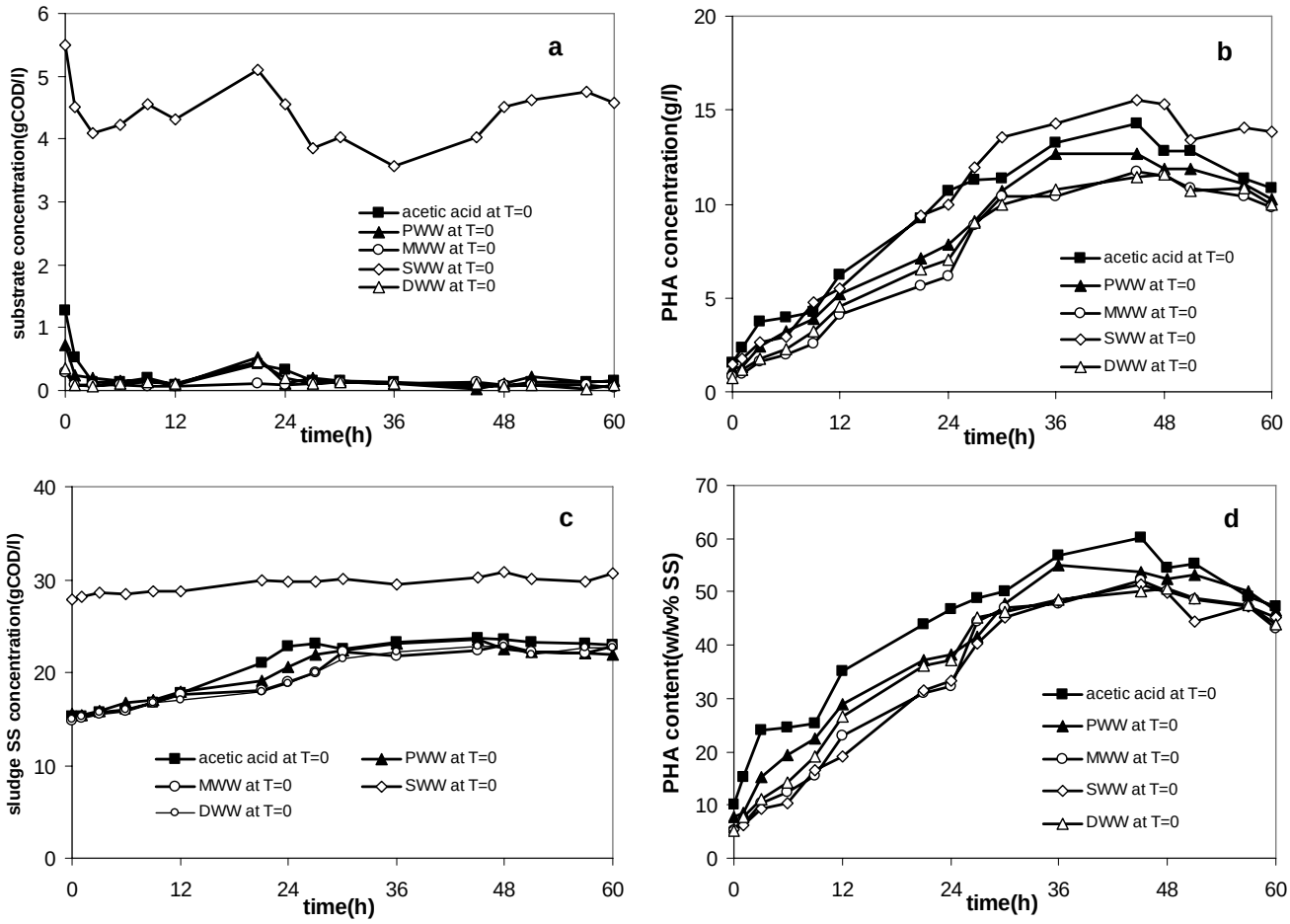

Figure 1. Profiles of acetate, PHA, sludge SS concentrations and PHA content (w/w% of SS) during continuous feeding experiments at sludge SS concentration of 15 g/l with different wastewater and acetic acid as the substrate at T=0 (PWW, pulp and paper wastewater; MWW, municipal wastewater; SWW, starch industry wastewater; and DWW, dairy industry wastewater).

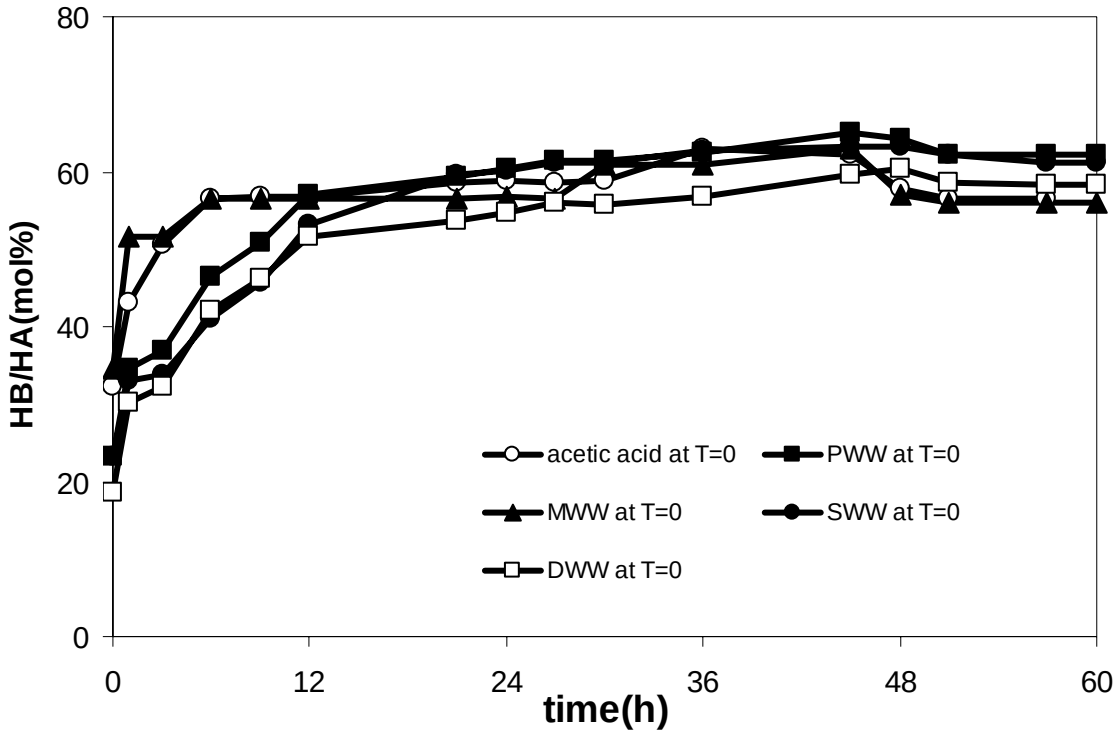

Figure 2. Profiles of HB/HA(mol%) during continuous feeding experiment at sludge SS concentration of 15 g/l with different wastewater and acetic acid as substrate at T=0.

CHAPITRE 4

ISOLEMENT ET IDENTIFICATION DES SOUCHES BACTÉRIENNES PRÉSENTES DANS DES BOUES ACTIVÉES ET ACCUMULANT DES PHAs

Part I

Isolation, characterization and identification of bacteria from activated sludge and soluble microbial products (SMP) in wastewater treatment systems

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Résumé

La méthode faisant appel aux boues activées est le processus le plus largement utilisé pour la technologie de traitement des eaux usées municipales et industrielles. L'écosystème microbien des boues activées est une population mixte contenant de nombreuses espèces de virus, de bactéries, de protozoaires, de champignons, de métazoaires et d' algues. Ces travaux examinent les récentes avancées en microbiologie des boues activées, principalement en ce qui a trait à la population bactérienne . La procédure standard, les méthodes d'analyse et les techniques de caractérisation biochimiques nécessaires à l'isolement ainsi qu'à l'identification de bactéries responsables des processus clés des systèmes de traitement des eaux usées (élimination des nutriments, aérobies, anaérobies, etc ...) sont abordées dans cette étude. Les effets saisonniers (hiver et été), les variations de température et de salinité sur la répartition des espèces de bactéries pour le traitement des eaux usées sont examinés. En outre, les produits microbiens solubles (SMP) qui sont des facteurs importants qui affectent non seulement les activités microbiennes, et par conséquent la qualité des effluents des systèmes de traitement biologique des eaux usées, sont identifiés et caractérisés ; La signification et les implications des SMP dans le contexte d'activation des boues étant également abordées. La plupart des procédés modernes de traitement des eaux usées sont fondés sur la composition et l'activité des communautés microbiennes présentes dans les boues activées. L'évolution récente des méthodes d'analyse moléculaire des communautés microbiennes ont décuplé l'intérêt pour la microbiologie des boues activées. Les approches traditionnelles d'analyse semblent avoir atteint leur maximum de rendement. C'est pourquoi, l'analyse moléculaire a le potentiel d'accroître notre compréhension du processus de boues activées, et donc d'améliorer ce procédé de contrôle.

Mots-clés: Boues activées, produits microbiens solubles (SMP), isolement, identification.

Abstract

Activated sludge process is the most widely used technology for municipal and industrial wastewater treatment. The microbial community of activated sludge is a mixed population of microorganisms containing many species of viruses, bacteria, protozoa, fungi, metazoa and algae. This review focuses on the recent advances in microbiology of the activated sludge process. The bacterial population in activated sludge system is examined. The standard procedure, medium used, analytical methods and biochemical characterization techniques required for isolation, and identification of bacteria responsible for the key process of wastewater treatment systems (nutrient removal, aerobic, anaerobic, etc.) are discussed in the review. Effect of seasonal (winter and summer) temperature variations and salinity variation on the bacterial species for wastewater treatment is examined. In addition, soluble microbial products (SMP) is one of the important factors that affects not only microbial activities, and consequently the quality of the effluents from biological wastewater treatment systems; the identification, characterisation, significance and implications of SMP in the context of activated sludge processes are also covered in this review. Today, most modern wastewater treatment processes rely on the composition and activity of their microbial communities in activated sludges. Recent developments in molecular methods for analysis of the microbial communities have re-triggered public interest in the microbiology of activated sludge. Whereas traditional approaches may have reached the point of diminishing returns, the molecular analysis has the potential to increase our understanding of the activated sludge process, and thereby improve the process control.

Key words

Activated sludge, soluble microbial products (SMP), isolation, identification

Abbreviations

ABR	Anaerobic baffled reactor
AMW	Apparent molecular weight
A/O	Anaerobic/oxic
AODC	Acridine orange direct count
API	Analytical profile index
AODC	Acridine orange direct count
ATP	Adenosine triphosphate
BAP	Biomass associated products
BMP	Biochemical methane potential
BOD	Biochemical oxygen demand
CAS	Conventional activated sludge
COD	Chemical oxygen demand
CSTR	Continuously stirred tank reactor
DAPI	4',6-diamidino-2-phenylindole
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
EBPR	Enhanced biological phosphate removal
ECP	Extracellular polymers
FISH	Fluorescence *in situ* hybridization
GAC	Granular activated carbon
GAO	Glycogen accumulating organisms
GC	Gas chromatography
GNS	Green nonsulfur
GPC	Gel Permeation chromatography
HPC	Heterotrophic bacteria
HRT	Hydraulic retention time
LUASB	Lighted upflow anaerobic sludge blanket
MAR	Microautoradiography
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids
MPN	Conventional most probable number
MSAS	Membrane separation activated sludge process
MW	Molecular weight
OLR	Organic loading rate
OTUs	Operational taxonomic units
OMW	Olive mill wastewater
OUR	Oxygen uptake rate
PAB	Polyphosphate-accumulating bacteria
PAC	Powdered activated carbon
PAOs	Phosphorus accumulating organisms
PACT	Powdered activated carbon treatment
PHA	Polyhydroxyalkanoates
PHB	Poly(3-hydroxybutyrate)

PHBV	Poly(3-hydroxybutyrate-co-hydroxyvalerate)
Pi	Phosphates
PP, polyP	Polyphosphates
RPAO	*Rhodocyclus*-related PAO
rRNA	Ribosomal ribonucleic acid
SBR	Sequencing batch reactor
SMP	Soluble microbial products
SOC	Soluble organic carbon
SON	Soluble organic nitrogen
SRP	Soluble residual products
SRT	Sludge retention time
THM	Trihalomethane
TOC	Total organic carbon
UAP	Utilisation associated products
UASB	Up flow anaerobic sludge blanket
UF	Ultrafiltration
VSS	Volatile suspended solids
VFA	Volatile fatty acids
WWTP	Wastewater treatment plant

1. Introduction

Activated sludge process is the most widely used technology for municipal and industrial wastewater treatment. The microbial community named "activated sludge" is a mixed population of microorganisms containing many species of viruses, bacteria, protozoa, fungi, metazoa and algae. Population shifts within the microbial community may result from changes in the plant operating conditions and cause sludge quality problems such as poor sludge settling, compaction, and dewatering (Wagner and Loy 2002; Nielsen and Nielsen 2002a; Chipasa and Me˛drzycka 2004; Nielsen et al. 2004). Today, most modern wastewater treatment processes rely on the composition and activity of their microbial community in activated sludge. Therefore, the analysis of the microorgansms in activated sludge is of considerable interest. Determination of the dominant role of the microorganisms in the ecosystem of the activated sludge allows the treatment process to be controlled to improve the performance of WWTPs (Nielsen et al. 2004).

Effluents from biological treatment processes contain a variety of soluble organic matter, including residual influent substrate, soluble microbial products (SMP), and non- or slowly biodegradable organic materials. The SMP are defined as the organic compounds that are released into solution from substrate metabolism (usually with biomass growth) and biomass decay (Barker and Stuckey 1999; Chipasa and Me˛drzycka 2004; Chipasa and Medrzycka 2004).

Many researchers (Kuo and Parkin 1996a; Fang and Jia 1998; Schiener et al. 1998; Barker and Stuckey 1999; Etchebehere et al. 2001; Lu et al. 2001; Laspidou and Rittmann 2002b; Liu and Rols 2002; Shin and Kang 2003) have shown that only a small fraction of the effluent was original influent substrate in terms of biodegradable organic matter, while the majority of the soluble organic matter in effluents are SMP. Therefore, an important factor affecting the quality of effluent and overall organic matter removal in biological treatment processes is the presence of SMP that are produced during biological treatment (Ritmann et al., 1987; Gaudy and Blachy, 1985; Parkin and McCarty, 1981b). Because of their many characteristics (Boero et al. 1996; Chipasa and Me˛drzycka 2004;

Chipasa and Medrzycka 2004), including flocculating and toxic properties, SMP can affect the microbial activities, and consequently the performance of treatment processes. The production and characteristics of the SMPs are dependent on the microbial species, growth environment, rate of substrate utilization, temperature, pH and oxidation/reduction potential etc (Barker and Stuckey 1999; Huang et al. 2000; Aquino and Stuckey 2002; Aquino and Stuckey 2003; Shin and Kang 2003; Aquino 2004; Aquino and Stuckey 2004; Chipasa and Medrzycka 2004).

There is an increasing concern that effluent toxicity may actually be created in the biological treatment process itself. In other words, SMP may be more toxic than the original organic compounds present in the wastewater (Eckenfelder, 1988). The effluents from biological treatment processes contain complex organic compounds that must frequently be removed in tertiary treatment systems. The physical and chemical characteristics of these organic compounds affect both the selection and performance of the subsequent treatment processes and the ultimate environmental impact of the treated wastewater.

Hence, the recent literature on the microbiological study and the SMP in activated sludge process is reviewed in this paper. This review focuses on the isolation, characterization and identification of bacteria which may be present in activated sludge and is responsible for the major processes (nutrient removal, aerobic, anaerobic etc.) of wastewater treatment as well as activated sludges from different types of industries (i.e. pulp-paper, starch, dairy industries, etc). Effect of seasonal (winter and summer) temperature variations on the activity of bacterial species for wastewater treatment is also examined. The identification and significance of SMP in the context of activated sludge processes, and their influence on microbial activities are very important in the treatment efficiency and are also reviewed in this paper.

2. Bacteria in activated sludge system

2.1. Bacterial composition and function in activated sludge

Many different bacterial groups are present in activated sludge systems. The bacteria are present either as single cell microcolonies or as filamentous bacteria. The bacterial count in activated sludge is in the range of 1–10 × 10^{12}/g VSS. Of these, typically 80% are active or alive (Nielsen 2002; Nielsen et al. 2004). In a nutrient removal treatment plant, many bacterial strains having different functions may be present including phosphorus accumulating organisms (PAOs) (Nakamura et al. 1995b; Kawaharasaki et al. 1999; Nielsen et al. 1999; Sidat et al. 1999a; Ansa-Asare et al. 2000; Crocetti et al. 2000; Carucci et al. 2001; Reddy and Bux 2002; Lina et al. 2003; Zhang et al. 2003; Das et al. 2004; Oehmen et al. 2005; Pijuan et al. 2005; Wong et al. 2005); ammonia-oxidizing bacteria (Purkhold et al. 2000); nitrite-oxidizing bacteria (Nogueira et al. 2002); nitrate-reducing bacteria (Drysdale et al. 1999; Etchebehere et al. 2001; Jetten et al. 2001; Khan and Hiraishi 2001; Dionisi et al. 2002; Juretschko et al. 2002a; Nogueira et al. 2002); glycogen accumulating organisms (GAO) (Liu et al. 1996; Seviour et al. 2000); filamentous micro-organisms (Blackall et al. 1996b; Kitatsuji et al. 1996; Kämpfer 1997; Rossett et al. 1997; Kanagawa et al. 2000; Francis et al. 2002); Fe(III)-reducing bacteria; sulfate-reducing bacteria (Kjeldsen et al. 2004); and methane-producing bacteria (Nielsen and Nielsen 2002a; Nielsen et al. 2004). Iron reducers, sulfate reducers and methanogens groups are usually considered less important in the normal activated sludge processes. Under some conditions (e.g. when bulking problems appear), domination of sulfide and useful oxidizing bacteria can be observed. It is important to note that up until now, the majority of the bacteria in activaed sludge are just roughly known as aerobic heterotrophs or denitrifiers, and very little is known about their main functions in the sludge (Strous et al. 2002; Wagner and Loy 2002; Nielsen et al. 2004). These groups of bacteria can be observed in most types of treatment plants. However, the direct taxonomical identity on a species level is presently almost unknown except a few bacterial groups. Most of these groups and other bacterial species in the sludge are still uncultured so that they can only be detected by molecular methods independent of cultivation (Radajewski et al. 2000; Coþkuner 2002; Forster et al. 2002; Nielsen 2002; Wagner and Loy 2002; Nielsen and

Nielsen 2002a; Kim et al. 2004). A great number of microorganisms have been isolated and the genera most frequently found are cited as follows: *Pseudomonas, Bacillus, Achromobacter, Enterococcus, Acinetobacter, Aeromonas, Alcaligenes, Arthrobacter, Escherichia, Salmonella, Proteus, Streptococcus, Staphylococcus, Micrococcus, Corynebacterium, Clostridium, Penicillium* (Mehandjiyska 1995) etc. Besides, many researchers report that the majority of bacteria in activated sludge belong to Gram-negative genera (Pick 1995; Sharifi-Yazdi et al. 2001).

2.2 Methods of isolation and identification

The current approaches for activated sludge analysis include: traditional microbial systematics, microscopic analysis, molecular analysis, microbial systematics using 16S ribosomal ribonucleic acid (rRNA), 16S rRNA oligonucleotide probe, 16S rRNA community analysis, designing diagnostic probes, and/or gene probes (Lajoie 1997; Blackall et al. 1998).

Traditionally, microbial communities in WWTPs have been analysed for decades either by light microscopic observation or by cultivation-dependent techniques, identificaton has been based on shape (e.g., rod, coccus), cell wall type (Gram-negative or Gram-positive) and a host of biochemical tests (e.g., oxidase-positive, oxidase-negative); moreover, the total number of bacteria was usually assessed by 4',6-diamidino-2-phenylindole (DAPI) staining and the living fraction as positive by either fluorescence *in situ* hybridization (FISH) with oligonucleotide gene probes staining all bacteria with a significant content of ribosomes, or the fraction able to take up radioactive substrates as measured by microautoradiography (MAR) (Nielsen and Nielsen 2002a).

Fluorescence in situ hybridization (FISH) is a powerful technique for detecting DNA or RNA sequences in cells. This molecular biological technique enables the localization of specific DNA sequences within genome of microorganisms and the identification of microorganisms under different environmental conditions. Direct quantification of bacterial strains is possible by manual counting of hybridized cells (epifluorescence and laser confocal microscope) or by image analysis of digital photos (both microscopes) or

automated counting with a flow cytometer. FISH is quickly becoming one of the most extensively used fluorescent staining techniques owing to its sensitivity and versatility, and with the improvement of current technology and cost effectiveness, its use will surely continue to expand (Lajoie 1997; Blackall et al. 1998).

DAPI is known to form fluorescent complexes with DNA, showing a fluorescence specificity with the observation using a fluorescence microscope. Because of this property DAPI is a useful tool in various cytochemical investigations (Nielsen and Nielsen 2002a).

The most sensitive technique for enumerating metabolically active bacteria that utilised single-cell method is microautoradiography (MAR) (Hoppe 1976). The bacteria that are actively engaged in substrate uptake are detected by this method and are measured by using a radiolabeled tracer such as typically thymidine (an amino acid) or a mixture of amino acids (Nielsen and Nielsen 2002a).

Modern systematics is based on the nucleotide sequence of the ribonucleic acid (RNA) found in the 16S subunit of the ribosome (16sRNA). All RNA is composed of four nucleotides; adenie (A), cytosine (C), guanine (G) and uracil (U). the sequence of these four bases in 16sRNA is used for the classification and identification of microorganisms. While so-called full-cycle rRNA approach involving the establishment of a 16S rRNA gene library and the subsequent design and use of clone specific probes for FISH analysis (Juretschko et al. 2002a).

The full cycle rRNA approach offers a cultivation independent alternative to the established techniques for the identification of new microorganisms. It should, however, be stressed that even a complete rRNA study cannot substitute for the isolation and characterization of microorganisms. Currently, only part of the applications of the rRNA approach encompass sequencing and probing. Not surprisingly, initial applications of the rRNA approach focused on identification of microorganisms in less complex samples.

Once its suitability had been demonstrated, it started to find widespread application (Juretschko et al. 2002b).

Anyway, it is impossible to decrypt the *in situ* microbial community composition due to the low percentage of activated sludge bacteria culturable on the routinely used cultivation media and the biases caused by the cultivation process (Wagner et al. 1993). Therefore, the only way to reveal their identity is by using molecular biological methods that are independent of cultivation. During the past decade, a variety of molecular approaches were developed and used to study bacterial diversity in WWTPs in a cultivation-independent manner (Michael et al. 1996; Khan and Hiraishi 2001; Dionisi et al. 2002). These studies presented that most of the suggested model organisms are of minor relevance *In situ* and that orther microorgansms, often not yet culturable, are reponsible for most key processes in WWTPs. The most comprehensive analyses of the bacterial diversity in an ecosystem can be achieved by the so-called full-cycle rRNA approach (Amann et al. 1995) involving the establishment of a 16S rRNA gene library and the subsequent design and use of clone specific probes for FISH analysis. The basic concept is extraction of nucleic acids from the sludge, amplification of the 16S-rDNA gene by PCR, construction of a clone library, sequencing of the clones and identification by using databases. This gives an overview of the species present in the sample, but is only qualitative or semi-quantitative. Therefore, it is important to use the full-cycle rRNA approach, which also includes design and application of gene probes by FISH so the growth form, number and location of dominant bacteria can be viewed directly in a fluorescence microscope. The entire full-cycle rRNA-approach is very time-consuming and requires expertise that is still limited to a few research groups working in wastewater treatment research. Hence no comprehensive identification of dominant bacteria in plants treating municipal wastewater exists. Only one single complete study on activated sludge from an industrial wastewater treatment plant has been conducted (Juretschko et al. 2002a). However, while several 16S rRNA gene clone libraries of mainly lab-scale wastewater treatment reactors have been published (Bond et al. 1995b; Snaidr et al. 1997a; Liu et al. 2001b), only a single full-scale municipal wastewater treatment plant has been analyzed by the full-cycle rRNA approach (Snaidr et al. 1997a).

Recently, first insights into the ecophysiology of these microorganisms were obtained by combing molecular methods for community composition analysis with techniques to infer the function of the detected microorganisms *in situ* (Wagner and Loy 2002). The isolation and cultivation of bacterial strains from sludge provide information on the roles and the niches of particular organisms in the community, whereas community analysis and probing methods are valuable in verifying that the isolated organisms are actually present in significant numbers within the community (Layton et al. 2000).

2.3 Microorganisms responsible for most key processes in activated sludge

2.3.1 Microorganisms for removal of soluble organic matter (SOM)

The removal of soluble organic matter (SOM) from wastewater has been the major process of biochemical treatment for many years. For typical domestic wastewater streams, which have a biodegradable chemical oxygen demand (COD) range between 50 - 4,000 mg/l, aerobic cultures of microorganisms are especially suitable. The removal is achieved by microorganisms using a portion of the carbon in the wastewater as a substrate, converting it to new biomass and converting the remaining into carbon dioxide (CO_2). The CO_2 is released as a gas, and the biomass is removed by sedimentation. The microorganisms are classified as heterotrophic because they derive their carbon from an organic source, such as the incoming wastewater, methanol, or ethanol.

A number of obligately anaerobic fermentative bacteria are also known to degrade a variety of organic substrates such as sugars, amino acids, and others, to products such as hydrogen, CO_2, acetate and higher fatty acids, and ethanol.

A summary of organic matter degradation by various bacteria is presented in Table 1.

2.3.2 Microorganisms responsible for enhanced biological phosphorus removal (EBPR)

Phosphorus can cause eutrophication (extraordinary growth of algae) when it is excessively discharged into natural water bodies. Therefore phosphorus removal from wastewater is important to prevent eutrophication. Activated sludge processes with alternating anaerobic and aerobic conditions have been successfully used for enhanced biological phosphate removal (EBPR) from wastewater (Mino et al. 1998; Liu et al. 2001b). The EBPR process is as follows: phosphate release occurs in the anaerobic stage followed by an excess of phosphate uptake in the aerobic stage. When wastewater enters the anaerobic phase, specialized organisms, called polyphosphate-accumulating bacteria (PAOs) accumulate carbon sources as internal polymer named (polyhydroxyalkanoate or PHA). The energy to store this polymer is obtained from breakdown of glycogen and hydrolysis of an energy rich internal phosphorus chain called poly-phosphate (poly-P). Since poly-P is broken down to ortho-phosphate for energy supply, the phosphate concentration in the anaerobic phase increased. Two different models were postulated for the production of the reducing equivalents for this anaerobic metabolism, the details of the two model were stated by Comeau et al. (1986) and Mino et al. (1987). The anaerobic phase needs to be followed by an aerobc phase. During this phase, the stored PHA is consumed, generating energy and carbon for replenishment of the glycogen and poly-P pool. Under these conditions, P in wastewater is assimilated by the biomass (sludge), and it is finally removed from the process through the wastage of sludge (Smolders et al. 1994a; Smolders et al. 1994b). Since phosphorus removal in these processes is achieved by the dominant growth of polyphosphate-accumulating bacteria in activated sludge, control of the composition of the microbial population in activated sludge is very important for maintaining a sufficient level of phosphorus removal activity.

Acinetobacter spp. were first proposed as the bacteria responsible for EBPR. Many researchers reported its predominance in EBPR processes based on culture-dependent identification methods (Lotter 1985; Wentzel et al. 1988). The identification of the *Acinetobacter* genus is often based on the results of simple biochemical tests, e.g. Analytical profile index (API) test kit. The API test kits are the best known biochemical tests for microbial identification. Furthermore, the use of a neural network approach for

identifying bacterial species appears promising in genospecies (Kim et al. 1997b). A neural network can correctly capture seemingly complex input and output patterns by mimicking the learning ability of the brain. The backpropagation neural network consisted of input (i neurons), hidden (j neurons), and output (k neurons) layers with a bias at the input and hidden layer. The detailed procedure of this approach was presented by Kim et al. (1997). Bacteria like *Pseudomonas* spp., *Lampropedia* spp. *Moraxella* spp., *Escherichia coli, Mycobacterium* spp., and some others also have the ability to accumulate phosphorus, a summary of microorganisms responsible for enhanced biological phosphorus removal (EBPR) is presented in Table 2.

2.3.3 Microorganisms responsible for nitrogen removal (nitrification and denitrification)

In wastewater, four types of nitrogen mainly exist: organic nitrogen, ammonia nitrogen, nitrite nitrogen, and nitrate nitrogen. These different forms constitute the total nitrogen content. The predominant forms of nitrogen in wastewater are organic nitrogen and ammonia (NH_3). In theory, the nitrogen in wastewater will be converted to harmless nitrogen gas and will be lost to the atmosphere by going through three major biological transformations during removal of nitrogen. The three biological transformations are ammonification, nitrification, and denitrificaion (Michael et al. 1996; Strous et al. 2002).

Organic nitrogen is converted to ammonia in the first step of the nitrogen cycle. In ammonification, microorganisms decompose the organic nitrogen and produce ammonia. In order to remove nitrogen from wastewater, the ammonia must be oxidized to nitrate (NO_3). This process is commonly referred to as nitrification. An oxic environment must be maintained for a sufficient period of time to promote nitrification. Removal of inorganic nitrogen compounds from wastewaters can be accomplished by a combination of the biological processes of nitrification and denitrification (Michael et al. 1996; Strous et al. 2002). Nitrification, the oxidation of ammonia to nitrate via nitrite, is an important step in the full treatment of wastewater. In the first step of nitrification, obligate autotrophic ammonia-oxidizing bacteria convert ammonia to nitrite, subsequently nitrite-oxidizing bacteria catalyze the oxidation of nitrite to nitrate. To avoid the limitation of

traditional microbiological methods, an *in situ* identification technique for ammonia- and nitrite–oxidizing bacteria was developed (Michael et al. 1996).

The anaerobic ammonium oxidation process (anammox) is a new process for ammonia removal from wastewater (Jetten et al. 2001; Strous et al. 2002). It is also a new approach in microbial physiology that was previously believed to be impossible. In the anammox process, ammonia is oxidized with nitrite as primary electron acceptor under strictly anoxic conditions. The reaction is catalysed by a specialized group of planctomycete-like bacteria. These anammox bacteria use a complex reaction mechanism involving hydrazine (N_2H_4) and hydroxylamine (NH_2OH) as intermediates. The basic equation is shown as: $NH_4^+ + NO_2^- = N_2 + 2H_2O$ (Jetten et al. 2001; Strous et al. 2002).

Denitrification steps are introduced into biological wastewater treatment in order to diminish the high load of inorganic nitrogen compounds in the secondary effluents contributing to eutrophication, in particular in estuarine environments. It is generally accepted that *Pseudomonas* spp. are the predominant heterotrophic bacteria involved in denitrification during activated sludge treatment. However, uncertainty still exists regarding other bacteria involved (Lemmer et al. 1997; GD Drysdale et al. 1999; Juretschko et al. 2002a).
A summary of microorganisms responsible for nitrogen removal is presented in Table 3.

2.3.4 Polyhydroxyalkanoates (PHA) producing bacteria

Because phototrophic bacteria can produce and accumulate polyhydroxyalkanoates (PHAs), a kind of biodegradable plastics, much attention has been focused on their use for PHA production.

Sawayama et al. (Sawayama et al. 2000) isolated two strains of phototrophic bacteria from the effluent of a lighted upflow anaerobic sludge blanket (LUASB) reactor. Their 16s rRNA gene sequences and phenotypic characteristics suggested that two isolates were *Rhodopseudomonas palustris* and *Blastochloris sulfoviridis* strains. Some strains of *R. palustris* have been reported to produce and accumulate poly-β-hydroxybutyrate

(PHB) under nutrient-limited conditions (Vincenzini et al. 1997). Since RN1 appeared to be the major strain in the phototrophic bacterial population of the effluent from the LUASB reactor, the PHB production in the reactor was likely to be by this strain *R. palustris* strain RNl. Considering that PHB accumulation by phototrophic bacteria occurs under organic acidrich and other nutrient-limited conditions, secondary incubation under these conditions could be necessary for efficient PHB production from wastewater using the LUASB method.

Two novel Gram-positive bacteria capable of accumulating poly(3-hydroxybutyrate-co-3-hydroxyvalerate) poly(3HB-co-3HV)] were isolated from an anaerobic-oxic activated sludge system fed with acetate (Liu et al. 2000b). Strains LphaS and Lpha7 were motile cocci, l-2 μm in diameter, occurring singly or in pairs with doubling times ranging from 0.4-1.7 d and could accumulate high levels of poly(3HB-co-3HV) (up to 44.7% of cell dry weight) when grown on complex media. Furthermore, these two strains exhibited the rapid substrate uptake and accumulation of storage granules as observed *in situ*. Under aerobic conditions, about 14.4% (cell dry weight) polyhydroxyalkanoate and 82% (carbon dry weight) cellular carbohydrate were produced from acetate and glucose, respectively. Under anaerobic conditions, poly(3HB-co-3HV) and cellular carbohydrate accumulated when glucose was fed. The result of analysis of 16S rRNA sequence revealed that both strains belonged to the Gram positive high-G + C group, but are significantly different from their closest phylogenetic relatives, *Dermatophilus* sp. and *Terrabacter* sp., to warrant classification as a new species (Liu et al. 2000b).

PHA-producing bacteria were successfully isolated from an Anaerobic/oxic (A/O) system fed with acetate and peptone. Accumulation of poly(3HB-co-3HV) by these isolates was observed under both anaerobic and aerobic conditions. Analysis of almost the entire 16S r RNA sequences of these isolates revealed them to be a new group of Gram-positive high-G+C (HGC) bacteria. Multiple ecological roles have been proposed for PHA metabolism in A/O systems. The stored PHA from acetate under anaerobic conditions may serve as an electron sink in a reductive process. Under subsequent aerobic conditions, these polymers may serve as a source of carbon and energy. At present, only a few bacterial

strains capable of accumulating PHA have been isolated from the A/O system (Vincenzini et al. 1997; Liu et al. 2000b; Sawayama et al. 2000). The most common isolate from the process is *Acinetobacter* sp.. *Acinetobacter* sp. could accumulate PHB up to 11.5% in cell dry weight basis after growth in a medium containing acetate as the carbon source (Vincenzini et al. 1997). However, *Acinetobacter* have not been shown to rapidly produce PHA during substrate uptake under anaerobic conditions. Another isolate from the A/O system belongs to the new genus *Amaricoccus* from the alpha subdivision of Proteobacteria (Liu et al. 2000b). Only *Lampropedia* sp. was shown to rapidly store acetate as PHA under anaerobic conditions. In addition, this isolate can accumulate polyphosphate, and has a distinct morphology like "glistening" tablets, as described in Bergey's Manual of Systematic Bacteriology, and is very different from the dominant microorganisms observed in A/O system (Liu et al. 2000b). Strains Lpha5 and Lpha7 isolated from the A/O system exhibited PHA formation. After aerobic cultivation in media containing balanced nutrients, both isolates rapidly accumulated poly(3HB-co-3HV). A number of bacteria were observed to produce poly(3HB-co-3HV) when cultivated with propionate or valerate as the sole carbon source. In addition, co-polymer of 3HB and 3HV can also be produced from substrates such as glucose and succinate via the propionyl-CoA-producing pathway. Some suggested propionyl-CoA-producing pathways leading to the 3HV production from the condensation of propionyl-CoA and acetyl- CoA, although not completely understood, include the methyl- malonyl-CoA and the acrylyol-CoA pathways, and threonineimethionine metabolism. Thus, this mechanism appears to be an important feature for the survival of those microorganisms in activated sludge processes. The production of PHA from acetate or glucose under anaerobic conditions is another distinct feature observed in the A/O system. Liu et al. (Liu et al. 2000b) presumed that the microbial populations in the A/O system were diverse in substrate metabolism and have only partially succeeded in isolating two representative members of the dominant groups. Comparative analysis of the partial 16s rRNA sequences of strains Lpha5 and Lpha7 showed that both isolates belong to the Gram-positive HGC group. The presence of Gram-positive HGC bacteria in activated sludge system was previously confirmed using 16s rDNA-based methods. The phylogenetic analysis shows that Lpha5 and Lpha7 are closely related to each other but distant from

other close relatives, warranting classification as novel species. Several studies have suggested that Gram-positive HGC bacteria are the micro-organisms responsible for the enhancement of biological phosphorus removal activity observed in A/O systems (Liu et al. 2000b).

2.3.5 The filamentous bacteria

The settling properties of activated sludge are vital to obtain good separation of the sludge from the treated wastewater. Since the activated sludge process was developed, several kinds of sedimentation problems have been observed, in particular sludge bulking which means the excessive growth of filamentous bacteria. The basis for understanding and characterising sludge bulking is generally thought to depend on a proper identification of the filamentous bacteria involved in the process (Liu et al. 2000a; Hugenholtz et al. 2001; Liu and Seviour 2001; Eikelboom and Geurkink 2002; Francis et al. 2002; Kim et al. 2002; Snaidr et al. 2002; Ramothokang et al. 2003; Martins et al. 2004).

Eikelboom (1975) developed the first identification key to identify filamentous bacteria in activated sludge systems. This identification is mainly based on morphological characteristics and on the response of the filamentous bacteria to a few microscopic staining tests. The procedures, techniques and identification keys were compiled in a microscopic sludge investigation manual that, together with a slightly different manual by Jenkins et al. (1993), have been used as world-wide references on filamentous bacteria identification. Anyway, this type of identification has its limitations. For instance, many filamentous bacteria (e.g., the *morphotypes Sphaerotilus natans*. *natans*, 1701, 0092 and 0961) can change morphology in response to changes in environmental conditions (Seviour EM 1997) and although some of them can look morphologically the same, they probably vary considerably in their physiology and taxonomy. For instance, the filamentous bacterial morphotype ‘Nostocoida limicola’ has several phylogenetically different bacteria, belonging to the following groups: low mol% G+C Gram-positive bacteria (Liu et al. 2000a; Liu et al. 2001a), high mol% G+C Gram-positive bacteria (Liu et al. 2001a; Seviour et al. 2002), Planctomycetes (Liu et al. 2001a; Seviour et al. 2002),

green non-sulphur bacteria (Schade et al. 2002) and alphasubclass of Proteobacteria (Snaidr et al. 2002). Similar conditions occur for the filamentous morphotype Eikelboom type 1863 (Seviour et al. 1997). A new genus, *Alisphaera*, has been proposed for the '*Nostocoida. limicola'* belonging to alphasubclass of *Proteobacteria* (Snaidr et al. 2002). Because this genus contains large and robust filamentous bacteria which were found to be dominant in many activated sludge process for industrial wastewater treatment (Eikelboom and Geurkink 2002). About 40 new morphotypes of filamentous bacteria were identified in a survey study in industrial activated sludge systems (Eikelboom and Geurkink 2002), making the identification of filamentous bacteria more complex.

Molecular methods based on analysing DNA or RNA of the bacteria have developed rapidly. The full rRNA cycle is considered to be the best approach to characterize the community structure of activated sludge (Snaidr et al. 1997b). A huge research effort, however, has to be undertaken in the development of more specific gene probes since from a total universe of about 80 different morphotypes of filamentous microorganisms, only less than 20 species can be currently identified with specific gene probes by FISH. Kim et al. (2004) described a tool, based upon the DNA microarray chip, for the identification of specific bacteria from activated sludge, using the hybridization of genomic DNA with random probes. This chip was developed using the genomic DNAs from *Gordonia amarae*, the natural filamentous actinomycete that causes sludge foaming and bulking, as well as a nonfilamentous floc forming bacterium (*Zoogloea ramigera*) and the skin pathogen *Mycobacterium peregrinum* without any sequence information. The sets of target probes on amine-coated glass were made from a genomic library, constructed with PCR products derived from randomly fragmented genomic DNAs extracted from pure cultures of the three strains. The results showed that the probes are specific, with only mild cross-hybridization occurring in a small number of cases. Furthermore, the chip clearly discriminated the presence of all three strains when they were present alone or together within mixed samples. Moreover, using the spot intensity and DNA hybridization kinetics, the starting genomic DNA concentrations could be estimated relatively well, which would make it possible to predict the number of specific bacteria present within the test samples. Therefore, the random genomic hybridization

approach, i.e., without any sequence information available for the probes, is a practical protocol for the identification of and screening for specific bacteria within any complex bacterial community from the environmental samples, such as in activated sludge, although the possibility of cross-hybridization may still exist (Kim et al. 2004).

Micromanipulation was used to obtain an isolate (BEN 52) of Eikelboom Type 1851 from a bulking activated sludge plant (Beer et al. 2002). Its 16S rDNA sequence reveals its closest relative is `Roseiflexus castenholzii', a member of the phylum `Chloroflexi', class `Chloroflexi', previously called the green non-sulfur bacteria. The 16S rRNA targeted oligonucleotide probe designed for fluorescence *in situ* hybridisation against this sequence successfully identified filamentous bacteria with the morphological features of Type 1851 in activated sludge samples from plants in several countries and different operational configurations (Beer et al. 2002). The summary of isolation and identification of filamentous bacteria frequently found in activated sludge was shown in Table 4.

2.4 Some important parameters affecting the microbial community

2.4.1 Temperature

Temperature is a fundamental factor that affects all living organisms. It influences the rates of enzymatically catalyzed reactions and affects the rate of diffusion of substrate into the cells (Grady et al. 1999). Due to the differences in their optimum growth temperatures, the temperature of the wastewater-microbial mixture (mixed liquor) strongly influences the population composition of the consortium. The effects of temperature on the efficiency and the kinetics of excess biological phosphorus removal (EBPR) systems have been under investigation, but the studies have yielded contradictory results. Early researchers (Barnard 1976; Ekama and Wentzel 1999) reported that EBPR efficiency was greater at lower temperatures than at higher temperatures over the range from 5 to 24 °C. Mamais and Jenkins (1992) showed that there was a wash-out SRT for all temperatures over the range from 10 to 30^{o}C. This introduces the paradox that, even though EPBR system performance becomes more efficient at lower temperatures, if the SRT-temperature combination is below a critical value, EBPR ceases before other

heterotrophic functions wash-out. A phylogenetically novel aerobic bacterium was isolated from an anaerobic–aerobic sequential batch reactor operated under EBPR conditions for wastewater treatment (Zhang et al. 2003). The isolate, designated strain T-27^T, was reported to grow at 25–35 °C with an optimum growth temperature of 30 °C, whilst no growth was observed below 20 °C or above 37 °C within 20 days incubation (Zhang et al. 2003).

The effects of temperature variations on aerobic biological wastewater treatment were evaluated by Morgan-Sagastume and Allen (2003) with respect to treatment efficiency, solids discharges, sludge physicochemical properties and microbiology. The effects of controlled temperature shifts (from 35 to 45°C; from 45 to 35°C) and periodic temperature oscillations (from 31.5 to 40 °C, 6-day period, for 30 days) were assessed in 4 parallel, lab-scale sequencing batch reactors (SBRs) that treated pulp and paper mill effluent. Overall, the temperature shifts caused higher effluent suspended solids (ESS) levels (25-100 mg/L) and a decrease (up to 20%) in the removal efficiencies of soluble chemical oxygen demand (SCOD). Lower ESS levels were triggered by a slow (2 °C/day) versus a fast (10 °C/12h) temperature shift from 35 to 45 °C, but the SCOD removal efficiencies decreased similarly in both cases (from 66±3% and 65±2% to 49±3% and 51±3%). Temperature oscillations caused an increased deterioration of the sludge settleability [high sludge volume indices (SVI); low zone settling velocities (ZSV)], high ESS levels and lower SCOD removals. The temperature transients were associated with poor sludge settleability (SVI>100 mL/g MLSS, ZSV<1 cm/min), more negatively charged sludge (up to -0.35±0.03 meq/g MLSS), increased filament abundance (approximately 4 to 4.5, subjective scale equivalent to very common), and decreased concentrations of protozoa and metazoa (25,000-50,000 microorganisms/mL sludge) (Morgan-Sagastume and Allen 2003).

The seasonal change of microbial population and activities in an existing building wastewater reuse system using membrane separation activated sludge process (MSAS) were investigated, and they were also compared with those in a municipal wastewater treatment plant using conventional activated sludge (CAS) process. The microfauna in

the MSAS process was unstable and changed a lot seasonally, but it would not affect the treatment efficiency. Moreover, the specific activities of nitrification, denitrification and organic removal fluctuated largely and seasonally, and were lower than those in the CAS process (Zhang and Yamamoto 1996).

Mehandjiyska (1995) found that the representatives of genus *Pseudomonas* predominated in quantitative aspect among the bacteria from municipal wastewater treatment plant. Actinomycetes were isolated only during the summer months and yeasts were not found. The percentage of *E. coli* was the biggest in *Enterobacteriaceae*. The presence of molds in the activated sludge during all seasons, regardless of their small amount showed that they played certain functions in the biodecomposing treatment processes.

2.4.2 Effects of Shock Loads of Salt on Protozoan Communities of Activated Sludge

The effects of wastewater salinity variations on communities of microorganisms taken from activated sludge were studied by Salvadó et al. (2001). Batch cultures were grown for 96 h at final salt concentrations of 3, 5, 10, 20 and 40 NaCl g/l. Protozoa and small metazoa was counted and ciliated protozoan species in these cultures were identified. An increase in salt concentration from 3 to 10 g/l gradually affected the microbial community and few protozoa and metazoa survived at 96 h. Ciliate abundance was species dependent: *Vorticella* spp. and Opercularia articulata resisted the high dosages of NaCl better than other ciliates. Total ciliate abundance and diversity fell drastically at 20 g/l, which would compromise reliability in activated sludge processes. At 40 g/l neither protozoa nor metazoa survived after 24 h.

2.5 Microorganisms analysis in a full scale wastewater treatment plant

Sharifi-Yazdi et al. (2001) examined a wastewater treatment plant in Iran. The comparison of results with Bergey Manual showed that out of the thirteen Gram-negative bacilli isolated, ten were identified as genus of *Flavobacterium* and three were identified as genus of *Alcaligenes.* High percentages of similarities were found with *Flavobacterium aquatile,* more than with other species of the genus, and with *Alcaligenes*

faecalis more than with other species of genus *Alcaligenes.* The other Gram-nagative rods were identified as genus *Pseudomonas.* High percentage similarities were found with *Pseudomonas stutzeri,* more than other species of genus *Pseudomonas.* Two strains of Gram-positive cocci were identified as genus of *Micrococcus.* High prercentage similarities were found with *Micrococcus luteu,* more than with other species of the genus (Sharifi-Yazdi et al., 2001). The results showed that Gram-negative bacilli with a yellow pigment was considered as a major group of the population. In this study the majority of the isolated Gram-negative bacteria belonged to the genus *Flavobacterium,* while 22% of isolated belonged to genus *Psendomonas.* The presence of Gram-positive bacteria has been reported by some workers. In this study the only Gram-positive found from the activated sludge belonged to the genus *Micrococcous.*

2.6 Some isolation examples from industrial wastewaters (WW)

Stefanie et al. (1998) studied the microbial population in granular sludge from a full-scale upflow anaerobic sludge blanket (UASB) reactor, using the 16S rRNA dot-blot hybridization method in combination with most probable number estimates, to gain more insight into the competitive and syntrophic interactions between sulfate-reducing and methanogenic consortia in sulfate-limited anaerobic reactors. The wastewater which was treated in the UASB reactor contained mainly starch, acetate, propionate, butyrate and formate, and had a chemical oxygen demand/sulfate ratio of 9.5. Evidence was obtained that acetate was mainly degraded by Methanosaeta-like microorganisms, while propionate was the preferred substrate for sulfate reduction. The Desulfobulbus-like propionate-degrading sulfate reducers in the sludge competed with Syntrophobacter-like bacteria for the available propionate. Hydrogen and formate were probably mainly degraded via methanogenesis by members of the order Methanobacteriales. Hydrogen, formate and butyrate-degrading sulfate reducers could not be characterized with the 16S rRNA probes available to date. The microbial population in granular sludge from a full-scale anaerobic bioreactor, was examined by using the 16S rRNA hybridization technique in combination with the conventional most probable number (MPN) enumeration technique, and microscopical examinations. From the results presented it was clear that the bacterial interactions in such sulfate-limited reactors are very complex. The results

indicated that *Methanosaeta* spp. were the dominant acetate degraders, and Methanobacterium spp. The dominant hydrogen- and formate-consuming methanogens. *Desulfobulbus* spp. and *Syntrophobacter* spp. were important for propionate degradation. However, new probes need to be developed to get a better picture of the sulfate-reducing and acetogenic sludge populations. For example, probes for Gram-positive sulfate reducers and syntrophic butyrate degraders would be very useful in this respect.

The bacterial population from activated sludge samples of a pulp and paper mill was investigated by Paris and Blondeau(1999). Analysis of colonies on plates count, including Gram stain, cells and colonies morphology, cyto-chrome oxidase and catalase reactions, revealed the presence of four bacterial groups, however, only one group was studied in detail and was identified as *Arthrobacter* sp.. The other three groups were only partially identified. The presence of this main population (24.90% to 64.80% of the total population) was followed during one year at the sampling rate of once in two-months. The taxonomic study of several isolates and use of rRNA-targeted oligonucleotide probes led to the identification of *Arthrobacter* sp., with a marked rod-coccus Gram-positive growth cycle, catalase-positive and oxidase negative reactions and obligately aerobic non-motile cells. L-lysine was identified in the cell wall peptidoglycan and the % G +C of the DNA was about 72.0 (Tm). Moreover, these bacteria were devoid of cellulolytic, amylolytic and proteolytic activities and possesed very low vitamin content. The maintenance of biofloc efficiency seemed to be the main function of these bacteria.

3. Soluble microbial products (SMP)

SMP are defined as soluble cellular components that are released during cell lysis, diffuse through the cell membrane, are lost ‘during synthesis, or are excreted for some purpose. They have moderate formula weights and are biodegradable. SMP are important because they are ubiquitously present and usually form the majority of the effluent chemical oxygen demand (COD) and biochemical oxygen demand (BOD) from biological treatment processes. SMPs have been classified into two groups: utilization associated products (UAPs) and biomass associated products (BAPs). UAPs are associated with

substrate metabolism and biomass growth and are produced at a rate proportional to the rate of substrate utilization, while BAPs are associated with biomass decay and are produced at a rate proportional to the concentration of biomass (Barker and Stuckey 1999; Barker et al. 2000; Laspidou and Rittmann 2002b).

3.1 Identification of SMP

SMP have been found to comprise a variety of organic compounds, such as humic acids, polysaccharides, proteins, nucleic acids, organic acids, antibiotics, steroids, exocellular enzymes, structural components of microbial cells, and metabolic products (Barker and Stuckey 1999; Lebrun et al. 1999). Several reaserches to identifiy individual compounds present in the effluents and their findings are summarised in the review by Barker and Stuckey (1999), Aquino and Stuckey (2002), Chipasa and Medrzycka (2004).

In general, a considerable portion of the effluents from wastewater treatment process is hardly defined chemically. Lebrun et al. (1999) stated that dissolved organic carbon (DOC) from a biological treatment can be divided into two main fractions shown in Figure 1: DOC coming from the influent, and SMP produced during the treatment. SMP are both biodegradable and non-biodegradable in proportions depending on the treatment plant efficiency (Lebrun et al. 1999). Schiener et al. (1998) tried to identify the high molecular weight (MW) fraction present in effluents from an anaerobic baffled reactor (ABR). MW fraction was identified as a hetero-polysaccharide consisting of monosaccharides and an amino sugar, although 9% of the hydrolysis products remained unidentified. Many industries produce large quantities of difficult to degrade material (``refractories'') and this has a significant effect on the final effluent concentration. In these cases there is no degradation or very low degradation of refractory compound consequently there may not be significant relation with SMP production.

In anaerobic systems, in particular, the residual COD may be comprised of residual nondegraded substrate, intermediate volatile fatty acids (VFA) and soluble microbial products (SMP). In well-operated systems only a small fraction of the effluent COD is usually due to VFAs, while SMP account for 85 to 100% of the residual COD, and up to

15% of the influent COD ends up as SMP (Kuo and Parkin 1996b; Barker and Stuckey 1999). These SMP may not be readily biodegradable, or may even be refractory, and comprise a wide variety of organic compounds distributed across a broad spectrum of MW (Aquino and Stuckey 2003).

Soluble microbial products (SMP) formation kinetics was investigated by employing a laboratory-scale biofilm reactor and naturally-grown oligotrophs. The results indicated that the majority of effluent soluble organic carbon (SOC) was SMP, while only a small fraction of the effluent SOC was the residual original substrate. Utilization-associated products (UAP), which were produced directly from substrate metabolism, were more important than biomass-associated products (BAP), which were produced by basic metabolism. The SMP contained mainly high-molecular-weight organic compounds, although the organic carbon source to a biofilm reactor was a low-molecular-weight compound. The steady-state concentrations of the effluent SMP and SOC were directly proportional to the influent substrate concentrations in this study. An extended steady-state biofilm model was developed by incorporating into the steady-state biofilm model an SMP formation model based on two types of SMP (i.e. UAP and BAP).

3.2 The characteristics of SMP

Parameters such as MW distribution, biodegradability and toxicity are of particular importance for the characteristics of SMP. MW distribution has been shown to be important in assessing the efficiency and suitability of the process and removal technology to be employed (Boero et al. 1996; Aquino and Stuckey 2002), biodegradability is important for the BOD assessment of effluent, while toxicity is important when considering the disposal of an effluent.

3.2.1 MW distribution of SMP

It is difficult to compare results from different studies because there is no standardised procedure for determining the MW distribution of soluble organic compounds. This

means that different results serve only as a relative measure of MW distribution. Size distributions of soluble organic compounds are determined either as a continuous distribution using GPC or as a discrete distribution using UF membranes in stirred cells. The sizes of these soluble organics are always referred to as apparent MW (AMWs), since separations are calibrated with compounds of known MW, not size, but both techniques have several disadvantages. For example, during a chromatographic analysis, there should be no chemical interaction between the column packing, the solvent (effluent), or the organic components. This is because it has been found that some components pass through the column more rapidly than calibration standards due to ion exclusion or complex formation, resulting in an overestimation of the component MWs, while other components may be delayed by adsorption or electrostatic interaction with the column packing and their MWs underestimated. Also, chromatographic analysis usually requires concentration by evaporation or freeze-drying and these procedures may alter the sizes of dissolved components. In ultrafiltration, the diffusive and advective transport of organics through ultrafiltration membranes is influenced by a variety of factors, including membrane pore size distribution, water temperature, cell pressure, solution pH and ionic strength, as well as molecule size, shape and affinity for the different membrane materials. In addition, the performance of the membrane can be significantly affected if material is allowed to accumulate on the membrane surface (Boero et al. 1996; Huang et al. 2000; Aquino and Stuckey 2002; Laspidou and Rittmann 2002b; Aquino and Stuckey 2003; Aquino 2004; Aquino and Stuckey 2004).

Confer and Logan (1997a; 1997b; 1998) considered the macromolecular aspects of wastewater treatment effluents and in particular the MW distribution of hydrolytic fragments in solution during protein and polysaccharide degradation. They used ultrafiltration (UF) to follow the MW of the macromolecules during degradation and showed that small MW compounds (<1 kDa) accumulated in solution during both polysaccharide and protein degradation. Many workers have specifically investigated the MW distribution of SMP. Some of the recent results on distribution of MW are shown in Table 5. Boero et al. (1996) used 14C-labelled compounds to investigate the MW distribution of SMP in aerobic treatments of glucose and phenol. They split the

generation of SMP into three distinct regions based upon: the direct transformation of the original substrate (region I); the ``polymerisation'' of the lower MW SMP (region II) and the endogenous decay of cells (region III). They found that, on average, the trends were similar for both substrates (glucose and phenol) and indicated that the lower MW fraction (<1 kDa) SMP dominated region I but decreased with time (>46 h) as these compounds were ``polymerised'' to form the higher MW fractions. However, the phenol associated SMP tended to be of a higher MW than the glucose associated SMP. Kuo and Parkin (1996) performed work on the MW distribution of SMP in anaerobic chemostats. They found the distribution of SMP showed the same type of distribution as for aerobic systems, i.e. bimodal with the majority of SMP having a MW less than 1 kDa or greater than 10 kDa, while very little SMP had a MW between 1 and 10 kDa. Schiener et al. (1998) examined the MW distribution of SMP in an ABR and found that the SMP exhibited a bimodal distribution with 30% having MW<1 kDa and 25% having MW>100 kDa. They also showed that large MW compounds increased as a percentage of the total SMP down the reactor.

It was stated that the MW distribution was influenced by reactor type, and that the majority of the organics appeared in the low MW range. Although reactors with a high SRT and VSS tend to produce more high MW material, the low MW material is always the most abundant, and hence this material should be targeted for post-treatment removal, or its production minimized by manipulaing operating parameters (Barker and Stuckey 1999). This is the practical significance of these conclusions in the operation of a wastewater treatment plant.

3.2.2 Biodegradability of SMP

Schiener et al. (1998) and Barker et al. (1999) have investigated the biodegradability of residual COD. It was found that over 90% of the residual COD measured in batch or continuous flow reactors, while being the practical or engineering non-biodegradable residual with respect to secondary treatment processes, is subject to biological degradation. They used a semi-continuously fed batch reactor which was fed daily to

increase the liquid concentration by 1000 mg l^{-1} every day. During the first 32 days of feeding, 1570 mg COD l^{-1} of SMP built up. This build up represented about 5% conversion of glucose COD to SMP COD. However, the residual soluble COD declined to only 324 mg l^{-1} by day 66, even though new glucose was continually being fed. Thus, almost all of the previously formed SMP, as well as newly formed SMP, were degraded (Gaudy and Blachly 1985). Schiener et al. (1998) investigated the anaerobic biodegradability of various MW fractions from different compartments in an ABR. All the MW fractions in the compartments investigated were found to be between 65 and 82% degradable. Barker et al. (1999) studied the aerobic and anaerobic degradability of different MW fractions from various effluents from anaerobic treatment and found that generally the high MW material was more readily degraded aerobically and the low MW was more readily degraded anaerobically, although there were differences from effluent to effluent. Hejzlar and Chudoba (1986) studied the different polymers that are excreted into the cultivation medium. From the practical viewpoint it is very important to determine which are polymers readily degradable and which are refractory and they assumed that the polymers which are commonly present in most microbial cells (e.g. nucleic acids, peptidoglycans and phospholipids) are easily degradable by many micro-organisms but that, on the contrary, specific and unrepeatedly synthesised polymers (e.g. extracellular heteropolysaccharides or polysaccharidic components of lipopolysaccharides) are refractory. This work also quantified their refractoriness as having a BOD rate constant of 0.03-0.04 d^{-1}. Namour and Muller (1998) proposed a new physico-chemical technique based on the fractionation of organic material into hydrophobic and basic, acid and neutral hydrophilic entities, to quantify the refractory organic matter from sewage treatment works. This is an alternative to the highly sensitive bioassay techniques and allows the extraction of the major part of the refractory organic matter in order to generate fractions available for further physical, chemical or biological tests.

3.2.3 Toxicity of SMP

The toxicity can be generated in biological treatment process itself raises concern when considering the adoption of biological systems for post-treatment and the reuse of treated

wastewaters. SMP may actually be more toxic than the original organic compounds present in the wastewater (Aquino and Stuckey 2002). As SMP production is a concentration related phenomena, the problem becomes more severe with high strength wastewaters (Barker and Stuckey 1999). It was found that SMP with high MWs strongly adsorb onto carbon and has proposed using GAC as an effective tertiary treatment to reduce the toxicity of effluents. All this work has been with aerobic treatment systems and no data has been found concerning the toxicity of anaerobic SMP.

3.3 Effect of SMP on process performance

The formation of SMP influences the wastewater treatment process in many ways. Not only the treatment efficiencies, but also the kinetic activity, flocculation and settling properties of activated sludge as well as being inhibitory to nitrification (Chudoba 1985a).

In addition to contributing to the BOD and COD of the effluent and toxicity properties, SMP can have further implications on process performance (Barker and Stuckey 1999). It was observed that microbial waste products in high concentrations adversely affected the kinetic activity and the flocculating and settling properties of activated sludge micro-organisms (Gaudy and Blachly 1985). The growth kinetic of microorganism is affected through inhibition by SMP as described in the preceeding section, and consquently affectes the treatment efficiency. Further, accumulation of SMP have been found to decrease specific respiration rates (Washington et al. 1970) and specific rates of COD removal (Chudoba 1985a). However, at present how SMP disrupts the activated sludge flocs causing poor settling is not clear and require further research.

3.4 Process parameters (temperature, HRT, SRT etc) which affect the production of SMP

Many process parameters are known to affect SMP production. It has been demonstrated that SMP production is enhanced at high biomass concentrations, at low or high hydraulic retention time (HRT), at high sludge retention time (Boero et al. 1996; Kuo and Parkin 1996b), and at low temperatures (Schiener et al. 1998). It has also been shown that SMP production is proportional to the feed strength, and that different amounts of SMP are produced when different substrates are fed to the system (Barker and Stuckey 2001). Furthermore, SMP may be produced as a response to environmental stress imposed on microrganisms. It was suggested that severe limitation of nutrients N and P could lead to the release of a large flow of organic molecules to the environment (Barker and Stuckey 1999).

3.4.1 Effect of feed strength, HRT, OLR and SRT on SMP production

Several investigators have studied the effect of feed strength, HRT, OLR and SRT on SMP production and these studies are summarised. The main conclusions are as followings: 1). SMP production increases with the influent concentration (S_0) increases. 2) When normalised SMP production (SMP/S_0) is plotted against SRT, SMP/S_0 appears to decrease to a minimum and then increase again, indicating the existence of an optimal SRT for minimising the production of SMP. For aerobic systems this optimum appears to exist between 2 and 15 days (Pribyl et al. 1997), while for anaerobic systems the optimum appears at approximately 25 days (Kuo et al. 1996). 3). A certain optimum range of organic loads is possible to obtain minimum values of SMP. The optimum organic load is in the range of 0.3-1.2 g COD/g MLSS.d or 0.2-0.8 g BOD/g MLSS.d. A substantial increase or decrease above or below the optimum range results in an increase of SMP (Pribyl et al. 1997).

3.4.2 Effect of substrate type on SMP production

It was shown that systems fed with glucose produced higher SMP levels than those fed with acetate (Kuo et al. 1996). Whether the substrate affects the quantity and type of SMP produced is closely linked to whether bacterial type affects the quantity of SMP. When using a CSTR with phenol and glucose feeds showed that phenol resulted in more

SMP than glucose, but that the phenol related SMP was more biodegradable than glucose SMP (Barker and Stuckey 1999). Thus, it was indicated that the type of substrate and hence microbial population is another factor influencing the production of SMP. However, the significance of this is probably not so important as most of the literature indicates that biomass decay products account for most of the SMP rather than substrate metabolism products (Laspidou and Rittmann 2002a).

3.4.3 Effect of temperature on SMP

Schiener et al. (1998) and Aquino and Stuckey (2002) studied the production of SMP in an anaerobic baffled reactor (ABR), it was found SMP production increased with decreasing temperature. However, a decreasing temperature will also cause a decrease in the rate of degradation of SMP due to kinetic considerations. It was found that the decrease in performance at lower temperatures was due to a combination of decrease in catabolic rate, an increase in the Ks for VFAs and an increase in the production of SMPs (or a decrease in their rate of catabolism) (Barker and Stuckey 1999).

3.4.4 SMP in Aerobic and anaerobic process

The majority of work on SMP has been conducted on aerobic systems, few works were investigated SMP in anaerobic systems (Kuo and Parkin 1996b; Kuo et al. 1996; Schiener et al. 1998; Barker and Stuckey 1999), there is a need to further study SMP production in anaerobic systems. When comparing the results with the available literature reports on aerobic and anaerobic production of SMP, Kuo et al. (1996) found that the normalised production of SMP appears to be lower in anaerobic systems (0.2 to 2.5%) than in aerobic systems [3.1±0.4% for cultures fed on glucose and 14.7±3.7% for cultures fed on phenol (Barker and Stuckey 1999)]. from 15 to 25 mg g^{-1}, but that under anaerobic conditions the specific value of Kuo et al. (1996) used enrichment cultures to study the production of SMP during the anaerobic degradation of acetate and glucose to methane in chemostats. Significantly, as mentioned in the section concerning the definition of SMP, Kuo et al.(1996) did not consider the short chain volatile fatty acids produced by the bacterial consortia during the degradation to be part of the SMP. Production of SMP

increased as SRT increased for both acetate and glucose fed systems and systems fed glucose produced higher levels of SMP than those fed acetate. For both systems effluent COD was mostly SMP. Glucose fed systems had higher SMP/effluent soluble COD ratios than the acetate fed chemostats and the fraction of the effluent soluble COD made up of SMP increased with increasing SRT. This results in a higher bacterial yield and thus higher biomass levels, which should lead to higher levels of SMP. Additionally, glucose will support the growth of different groups of micro-organisms (i.e. fermenters, acetogens and methanogens), whereas acetate will support predominantly aceticlastic methanogens whereas Kuo et al. (1996) expect that the more diverse population would produce higher levels of SMP.

3.5 Treatment of SMP

Some SMP still remain in the effluent from biological wastewater treatment process, substantially reduce the efficiency of the treatment system. Therefore post-treatment process should target the removal of these compounds rather than the influent substrate. It is important to individually identify the SMP and to characterize the collective parameters such as MW distribution when assessing the employed post-treatment process.

There are a number of possible post-treatment processes available for further SMP removal, but the most favored ones are: solid/liquid separation (especially membrane filtration techniques); granular activated carbon and ozonation (Kallas and Munter 1994). It was stated that granular activated carbon (GAC) is the most effective method for the removal of SMP. Investigation of several methods (various oxidants, chemical coagulants, ion exchange and GAC) for the removal of soluble organic nitrogen (SON) from effluents showed that SON was most efficiently by GAC adsorption (85% removal) and chemical precipitation using high concentrations of ferric chloride (70% removal). Nearly 50% of SMP were adsorbed onto powdered activated carbon (PAC) in a powdered activated carbon treatment (PACT) system, but only 4% of the adsorbed SMP was biodegraded by the PACT sludge demonstrating the refractory nature of the SMP

(Barker and Stuckey 1999). Very little has been reported regarding the post treatment of anaerobic effluents with activated carbon. Low MW materials (i.e. MW<1 kDa) from anaerobic treatments were the most difficult to adsorb on GAC (Barker and Stuckey 1999). The anaerobic biodegradability was investigated by means of the biochemical methane potential (BMP) assay (Owen et al., 1979), it was found that various MW fractions from different compartments in the Anaerobic baffled reactor (ABR) were degradable between 65 and 82% w/w after a period of 60 days.

The collective parameters of MW distribution and biodegradability have been shown to be important in assessing the efficiency and suitability of a process and the removal technology to be employed in order to achieve discharge consent levels in order to achieve discharge consent levels.

MW distribution, biodegradability and activated carbon adsorption characteristics; to determine if any generic patterns emerge and to draw conclusions regarding the post-treatment of anaerobic effluents.

4. Conclusions

Based on the review of the literature, the following conclusions can be drawn:

- Complex microbial communities are directly responsible for the effectiveness and success of the wastewater treatment process, organic matter reduction, and nutrient removal depending on the differences influent of wastewater and the operation process.
- In the last few years several 16Sr RNA sequences have been published. These data are very important and form the basis of a phylogenetically based classification. The development of 16S RNA oligonucleotide probing and fluorescent antibody techniques has provided new tools for *in situ* identification of functional bacteria without the necessity for cultivation.
- A combination of new molecular methods, new microscopic techniques and improved cultivation, maintenance and physiological information about the bacteria

isolates may lead to detailed insight into the activated sludge process, allowing the treatment process to be controlled to improve the performance of WWTPs.

- SMP have been classified into two groups: utilisation associated products (UAP) and biomass associated products (BAP). UAP are associated with substrate metabolism and biomass growth and are produced at a rate proportional to the rate of substrate utilisation, while BAP are associated with biomass decay and are produced at a rate proportional to the concentration of biomass.
- SMP are produced across a wide range of MWs (<0.5 to >50 kDa) and the MW distribution has been shown to be significantly affected by the operating conditions with higher MW material becoming more evident at higher SRT (>15 days for anaerobic systems and >46 h for aerobic systems).
- SMP have been shown to be biodegradable and toxic in aerobic biological systems.
- The formation of SMP may affect treatment efficiency, the kinetic activity and the flocculating and settling properties of activated sludge.
- Process parameters such as feed strength, HRT, SRT, substrate type, biomass concentration, temperature and reactor type affect SMP production, but there is considerable evidence that through optimisation of the biological treatment process significant decreases in SMP that results in reduced residual COD in the effluent.
- The production of SMP is less in anaerobic systems (0.2 to 2.5%) than aerobic (3.1 to 14.7%) and it has been demonstrated that more SMP were produced during acidogenesis than methanogenesis.
- SMP can be removed from effluents using a variety of different technologies, but activated carbon appears to be the most effective one.
- Further research is needed to identify how the presence of SMP inffluences the stability of microbial communities and their diversity.

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Table 1. Summary of organic matter degradation by various cultures.

strains	Organic matter	process	efficiency	reference
Aspergillus terreus, Azotobacter chroococcum, Geotrichum candidum)	phenolic compounds	aerobic pretreatment	This was attributed to the fact that pretreatment was capable of reducing the COD and total phenols concentration as well as toxicity by about 63–75%, 65–95% and 59–87%, respectively, for the various cultures used	Borja et al. (1998)
Pleurotus ostreatus	phenolic	aerobic	Aerobic treatment for 21 days led to about 65% phenols removal,	Fountoulakis et al. (2002)
Pleurotus ostreatus	olive mill wastewater (OMW)	aerobic	nearly complete removal of phenols after 20 days in batch fermentor	Aggelis et al. (2003)
Phanerochaete chrysosporium, Aspergillus niger, Aspergillus terreus, Geotrichum candidum	olive mill wastewater (OMW)	aerobic	92% phenols, 100% o-diphenols and 75% COD removal after 6 days with *P. chrysosporium* in batch fermentor. Respective values were 76%, 82% and 73% after 4.8 days with *A. niger* and 64%, 76% and 63% after 4.7 days with *A. terreus*. No phenols and only 10% o-diphenols removal after 9.3 days with *G. candidum.*	Garcı´a et al. (2000)
Pleurotus	olive mill wastewater (OMW)	aerobic	69–76% removal of phenols after 12–15 days in shake flasks	Tsioulpas et al. (2002)
seven strains of *Penicillium*	COD and the phenolic content of olive mill wastewater (OMW)	aerobic	Best results were obtained by using strain P4,	Robles et al. (2000)
Rhodobacter shaeroide S (S) and R. shaeroides NR-3 (NR-3).	cooking oil from the domestic wastewater	anaerobic dark conditions	after 6 days of batch treatment, 74.2, 58.2 and 15.8% of oil was removed with the alginate-immobilized S, NR-3, and control, respectively.	Takeno et al. (2005)
Thiobacillus ferrooxidans	methanol	electrochemical–microbiological system	it decreases the energy cost by 35% when compared with the pure electrochemical system traditionally used.	Lo´ pez-Lo´ pez et al. (1998)
Pseudomonas putida	Phenolic industrial wastewater	Fluidized-bed bioreactor	Phenoal degradation efficiencies higher than 90%.	Gonzalez et al. (2001)

Table 2. Summary of the microorganisms responsible for enhanced biological phosphorus removal (EBPR).

strains	Method of isolation	**Cultivation medium**	**Identification method**	**Reference**
Acinetobacter spp.	Micromanipulation		culture-dependent identification methods	(Lotter 1985; Wentzel et al. 1988)
			fluorescent in situ hybridization (FISH) with an oligonucleotide probe specific for *Acinetobacter*	(Wagner et al. 1994b)
				(Tandoi et al. 1998)
			a neural network approach for identifying bacterial species appears promising in genospecies	(Kim et al. 1997a)
Microlunatus phosphovorus.	Micromanipulation		16S rRNA sequence	(Nakamura et al. 1995a)
Lampropedia spp.	Micromanipulation	Modified green top agar (mod. GTA) and a chemical defined medium (ADM)	culture-dependent identification methods	(Stante et al. 1997)
Rhodocyclus.			PCR cloning approach	(Bond et al. 1995a)
Rhodocyclus			fluorescence *in situ* hybridization (FISH) combined with microautoradiography (MAR)	(Pijuan et al. 2005)
Accumulibacter, Competibacter			fluorescence *in situ* hybridization (FISH)	(Pijuan et al. 2006)
Acinetobacter calcoaceticus var. *lwoffi, Aeromonas, hydrophila* *Pseudomonas fluorescens* *Staphylococcus aureus* *Enterobacter agglomerans* *Micrococcus spp.* *Staphylococcus spp.* *Pseudomonas testesteroni* *Staphylococcus epidermidis* *Bacillus cereus* *Pseudomonas acidovorans*	Micromanipulation		using biochemical tests, API 20E and 20NE	(Sidat et al. 1999b)

Aeromonas hydrophila				
Pseudomonas mendocina				
Pseudomonas putrefaciens				
Alcaligenes denitrificans				
Moraxella spp.				
Moraxella phenylpyruvica				
Streptococcus spp.				
Gemmatimonas aurantiaca (type strain T-27^T=JCM 11422^T=DSM 14586^T)	Micromanipulation	combining low-speed centrifugations and prolonged incubation on a low-nutrient medium	16S rRNA sequence	(Zhang et al. 2003)

Table 3. Summary of the microorganisms responsible for nitrogen removal.

strains	Method of isolation	**Identification method**	**Reference**
Nitrosomonas	Micromanipulation	identified *in situ*, bright hybridization signals with all *nitrobacter* specific probes	(Michael et al. 1996)
Proteobacteria *Planctomycetes*. The *Verrucomicrobia*, *Acidobacteria*, *Nitrospira*, *Bacteroidetes*, *Firmicutes* and *Actinobacteria*	Micromanipulation	full-cycle rRNA approach	(Juretschko et al. 2002a)
*Nitrosomonas oligotropha*like	Micromanipulation	the principle of competitive PCR	(Dionisi et al. 2002)
Brocadia anammoxidans	Micromanipulation	16S rRNA sequence	(Jetten et al. 2001)
Pseudomonas spp.	Micromanipulation	fluorescence *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes	(Nogueira et al. 2002)

Table 4. Summary of isolation and identification of filamentous bacteria frequently found in activated sludge.

Taxonomic group/microorganism	Method of isolation	**Cultivation medium**	**Identification method**	**Reference**
Alpha-subclass of *Proteobacteria*				
Alisphaera europea (EU24)	Micromanipulation	GS media	16S rRNA sequence	(Snaidr et al. 2002)
Alisphaera MC2			16S rRNA sequence	(Snaidr et al. 2002)
Alisphaera PPx3			16S rRNA sequence	(Snaidr et al. 2002)
Beta-subclass of *Proteobacteria*				
Leptothrix discophora and other members of the β1 group of *Proteobacteria*		R2A Rouf/Stokes GMBN	16S rRNA sequence	(Seviour et al. 1994) (Wagner et al. 1994a) (Kampfer et al. 1995) (Spring et al. 1996)
S. natans and other members of the β1 group of *Proteobacteria*			16S rRNA sequence	(Wagner et al. 1994a)
type				
Type 0803	Micromanipulation	R2A agar	16S rRNA sequence	(Bradford et al. 1996)
Type 1701	Micromanipulation	R2A Rouf/Stokes GMBN	16S rRNA sequence	(Seviour et al. 1994) (Wagner et al. 1994a) (Kampfer et al. 1995)

Gamma-subclass of *Proteobacteria*				
Acinetobacter spp., some Eikelboom type 1863	Micromanipulation	R2A	16S rRNA sequence	(Seviour et al. 1994; Blackall et al. 1996a)
Eikelboom type 021N		Stokes	16S rRNA	(Wagner et al.
Eikelboom type 021N group I			sequence	1994a)
Eikelboom type 021N group II (*T. eikelboomii*)		IAM/EGGC medium	16S rRNA sequence	(Kanagawa et al. 2000) (Kanagawa et al. 2000)
Eikelboom type 021N group III (*T. defluvii*)				(Kanagawa et al. 2000) (Kanagawa et al. 2000)

Taxonomic group/microorganism	Method of isolation	Cultivation medium	Identification method	Reference
Gamma-subclass of *Proteobacteria*				
T. nivea, T. unzii		Stokes	16S rRNA sequence	(Wagner et al. 1994a)
Leucothrix mucor		Stokes	16S rRNA sequence	(Wagner et al. 1994a)
T. fructosivorans, T. ramosa			16S rRNA sequence	(Kim et al. 2002)
Thiothrix sp. and type 021N *Beggiatoa* sp.		GMBN		(Kampfer et al. 1995)
			For *Thiothrix* spp. 16S rRNA sequences are available	(Polz et al. 1996)
Competitor against TNI and TFR			16S rRNA sequence	(Kim et al. 2002)
Cytophaga–flavobacterium–Bacteroides				
Haliscomenobacter spp.		stokes	16S rRNA sequence	(Wagner et al. 1994a)
***Cytophaga* subgroup of the *Flexibacter-Cytophaga-Bacteriodes* phylum**				
Type 0092	Micromanipulation	R2A	16S rRNA sequence	(Seviour et al. 1994; Bradford et al. 1996)
Type 0411	Micromanipulation	R2A	16S rRNA sequence	(Seviour et al. 1994; Bradford et al. 1996)
Haliscomenobacter hydrossis		GMBN	Physiological characterization	(Kampfer et al. 1995) (Kampfer 1995)

Green non-sulphur bacteria				
'Chloroflexi': Eikelboom type 1851 (BEN 52)	Micromanipulation	R2A	16S rRNA sequence	(Beer et al. 2002)
'*N. limicola* '- like bacteria	Micromanipulation	R2A	16S rRNA sequence	(Schade et al. 2002)
Herpetosiphon sp.	Micromanipulation	R2A	16S rRNA sequence	(Bradford et al. 1996)

Taxonomic group/microorganism	Method of isolation	Cultivation medium	Identification method	Reference
TM7, candidate division of the domain *Bacteria*				
Eikelboom type 0041/0675	Micromanipulation		16S rRNA sequence	(Hugenholtz et al. 2001)
High mol% G+C Gram-positive bacteria:*Actinomycetes*				
Corynebacterineae			16S rRNA sequence	(Davenport et al. 2000)
Genus *Gordona*	Micromanipulation		16S rRNA sequence	(de los Reyes et al. 1997)
Gordona amarae			16S rRNA sequence	(de los Reyes et al. 1997)
Gordona amarae group 1 strains	Micromanipulation			(de los Reyes et al. 1998)
Gordona amarae group 2 strains				(de los Reyes et al. 1998)
N. limicola II	Micromanipulation		16S rRNA sequence	(Liu and Seviour 2001)
M. parvicella	Micromanipulation		16S rRNA sequence	(Erhart et al. 1997)
Genus: *Gordona* (Nocardiaceae) (Actinomycetales) / *Gordona amarae* (formerly *Nocardia amarae*)	Micromanipulation	TYG medium, YG medium		(Bond et al. 1995b)
		TYG medium	Chemotaxonomic methods	(Klatte et al. 1994)
Genus: *Skermania* (Nocardiaceae) (Actinomycetales)/ *Skermania piniformis* (formerly *Nocardia pinensis*)	Micromanipulation	TYG medium, YG medium	16S rRNA sequence	(Bond et al. 1995b; Chun et al. 1997)

Low mol% G+C Gram-positive bacteria				
N. limicola I			16S rRNA sequence	(Liu and Seviour 2001)
Planctomycetes				
N. limicola III			16S rRNA sequence	(Liu and Seviour 2001)
Actinomycetesnew Gram-positive phylum				
Microthrix parvicella	Micromanipulation	R2A NTM medium	16S rRNA sequence	(Blackall et al. 1995) (Blackall et al. 1996b)

Table 5. Some of the results on MW distribution from different reactors:

Treatment process	substrate	GPC		UF		Refs.
		parameter characterising organic content	findings	parameter characterising organic content	findings	
anaerobic baffled reactor (ABR)	Peptone, meat extract, sucrose			COD	≤1 kDa : 7%, 1 kDa <MW ≤10 kDa: 2%, 10 kDa< MW ≤300 kDa: 1% >300 kDa: 10%	(Aquino and Stuckey 2002)
anaerobic chemostat (CSTR)	Glucose			COD	≤1 kDa : 47%, 1 kDa< MW ≤10 kDa: 0, 10 kDa <MW ≤300 kDa: 2% >300 kDa: 32%	(Aquino and Stuckey 2002)
anaerobic membrane bioreactor (MBR)	Peptone, meat extract			COD	≤1 kDa : 8%, 1 kDa< MW ≤10 kDa: 57%, 10 kDa< MW <300 kDa: 7% ≥300 kDa: 13%	(Aquino and Stuckey 2002)
anaerobic CSTR	Glucose	-	-	COD	concentration of SMP increased with increasing SRT; bimodal distribution; fraction of SMP with MW>10 kDa increased with increasing SRT;SRT of 15 days: 43% MW<1 kDa, 48% MW>10 kDa; SRT of 25 days: 27% MW<1 kDa, 62% MW>10 kDa; SRT of 40 days:16% MW<1 kDa, 76% MW>10 kDa	(Kuo and Parkin 1996b)
SBR	Papermill effluent	-	-	TOC	0.2 mm-0.45 mm: 11%, 30 kDa-0.2 mm: 6%, 10-30 kDa: 0%, 1-10 kDa: 73%, <1 kDa: 10%	(Franta J. 1994)
various anaerobic reactors	Various	absorbance at 210, 256 and 280 nm	majority of material in all effluents MW<1 kDa	COD	majority of material in all effluents MW<1 kDa	(Barker and Stuckey 1999)

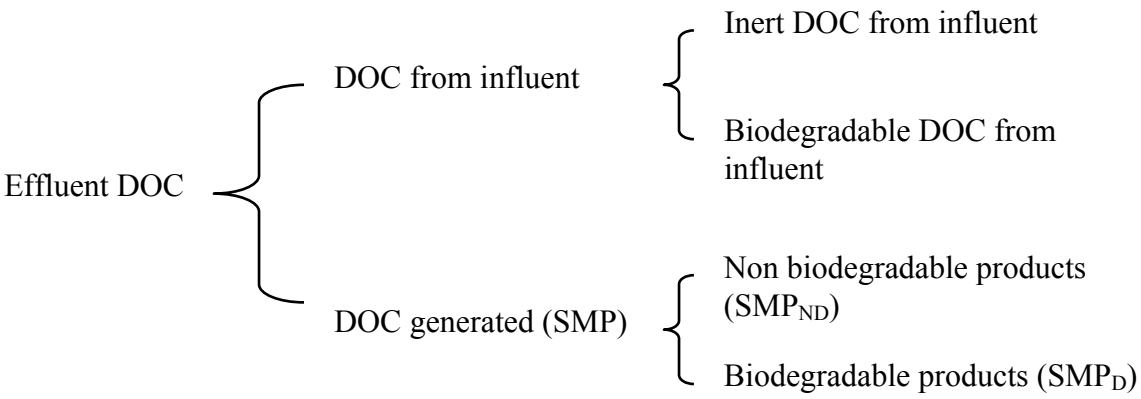

Figure 1. Fractions of dissolved organic carbon (DOC) from a biological treatment.

Part II

Polymer production by bacterial strains isolated from municipal activated sludge

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Résumé

Cetaines bactéries isolées à partir d'échantillons de boues activées recueillies auprès des usines de traitement des eaux usées municipales au Québec accumulent des Polyhydroxyalcanoates (PHA). Douze souches bactériennes ont été criblés pour la production de ces polymères en utilisant de l'acétate comme seule source de carbone. Les granules de PHAs présentaient de fortes colorations oranges en fluorescence sous microscope et avec du bleu de Nil bleu, comme colorant. Ces PHAs ont été analysés par chromatographie en phase gazeuse reliée à la spectroscopie de masse (GCMS) pour confirmer la présence et la concentration de ces biopolymères. Pour comparer les capacités des souches bactériennes accumulant les PHAs, elles ont été cultivées en erlenmeyers dans des milieux ne contenant que l'acétate comme source de carbone. Il a été constaté que ces isolats bactériens produisent et accumulent des PHAs. Les résultats ont aussi montré que ces souches bactéries isolées des boues possédent des aptitudes différentes pour accumuler les biopolymères en question. Un taux maximal en PHA de 27,5% a été obtenu avec la souche PHA - M3. Le rapport PHB / PHV des copolymères produits dans l'étude change en fonction des souches et de la durée d'opération.

***Mots-clés*:** boues activées; Polyhydroxyalcanoates (PHA); Stockage de polymères; plastiques biodégradables; isolement.

Abstract

Polyhydroxyalkanoates (PHAs) accumulating bacteria were isolated from activated sludge samples collected from municipal wastewater treatment plants in Quebec. Twelve bacterial strains were screened for PHA production with acetate as sole carbon source. PHA granules exhibited a strong orange fluorescence when stained with Nile blue A observed under microscope. PHA was also analyzed by Gas Chromatography Linked to Mass Spectroscopy (GCMS) to further confirm the presence and the concentration of PHA. To compare the abilities of these PHA accumulating bacterial strains, the synthetic media with acetate as carbon source was prepared to accumulate PHA in shake flask experiments with the isolated bacterial strains. The results showed that the bacterial strains isolated from sludge possess different abilities for accumulating PHA. The maximum PHA content of 27.5% was obtained by strain PHA-M3. The PHB/PHV ratio of the copolymer produced in the study changed in accordance with operating time and strains.

Key words: Activated sludge; Polyhydroxyalkanoates (PHAs); Storage polymer; Biodegradable plastics; isolation**.**

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are regarded as new environmental-friendly biodegradable plastics. Poly-β-hydroxybutyrate (PHB) and its copolymer poly-hydroxybutyrate-co-hydroxyvalerate (P(HB-co-HV)) are the common types of PHAs, and other forms also exist. A wide variety of micro-organisms accumulate PHA within cells as an intracellular storage material of carbon and energy (Reis et al. 2003; Akar et al. 2006; Dionisi et al. 2006).

Activated sludge used as a mixed culture for PHA production has also been studied by many researcher (Chua et al. 2003; Lemos et al. 2003; Reis et al. 2003). The novel technique to synthesis of PHA from activated sludge is beneficial in two ways. On the one hand, excess sludge generated from activated sludge processes needs further treatment such as anaerobic digestion and disposal by landfilling. This technique can reduce the quantity of excess sludge, therefore reducing the costs of sludge treatment. On the other hand, large quantities of biomass harvested from activated sludge wastewater treatment processes were induced to produce PHA instead of producing by pure culture fermentation. These significantly reduced the costs of PHA production.

Most of the published work concerning PHA production by activated sludge focused on understanding the storage mechanisms and the optimization of the PHA production process. Very little is known about the microorganisms in activated sludge system. it is worth trying to find new isolates with unique PHA production capabilities.

In the previous study (Yan et al. 2006), municipal activated sludge was used as a mixed culture for PHAs production in shake flask employing acetate as the sole carbon source, the PHA content reached 30.6%w/w. In order to have a better understanding of various microbial strains of activated sludge responsible for PHAs accumulation, in this study, the PHA-accumulating bacterial strains were isolated from municipal activated sludge. The capacity of PHA production of each isolate was evaluated and compared using a synthetic medium.

MATERIALS AND METHODS

Activated sludge

For screening of PHA accumulating bacterial strains from wastewater sludge, activated sludge samples were collected from municipal wastewater treatment plants "Communauté Urbaine du Québec" (CUQ), Quebec.

Isolation of Bacterial Strains

Isolation of bacterial strains was carried out by serial dilution of fresh activated sludge samples in 0.85% w/v sterile saline solution followed by plating of the samples on a modified agar E2 medium with Nile blue A containing acetate as sole carbon source. The composition of the medium was as follows (g/l): $NaNH_4HPO_4 \cdot 4H_2O$, 3.5; K_2HPO_4, 10.0; $MgSO_4 \cdot 7H_2O$, 0.2; citric acid, 2.0; agar, 15; acetate, 5.0. For routine analysis, 0.002 ml of a solution of 0.25 mg Nile blue A (Sigma) per ml dimethylsulfoxide (DMSO) was added to the sterilized medium to give a final concentration of 0.5 mg dye per ml of the medium. The plates were incubated at 25°C for 48–72 hrs.

Gram staining:

Preliminary identification was carried out based on the standard procedures of gram staining.

Screening of PHA accumulating strains

PHA accumulating bacterial strain was validated by exposing on ultraviolet light (312 nm) using UV transilluminator (Biometra) to detect the presence of intracellular PHA granules in the bacteria. A fluorescent bacterial isolate was considered a potential PHA producer (Ostle and Holt 1982). Further fluorescent bacterial strains were differentiated on the basis of colony characteristics and pure culture strains were maintained on suitable agar slants and stored at 4° C for further studies.

Microscopic analysis (confirmation) of PHA granules

Fluorescent bacterial strains isolated from the Nile blue-A incorporated plates were examined under fluorescent microscopy (Carl Zeiss Axiovert microscope with an episcopic fluorescence attachment) to confirm the presence of PHAs granules with in the bacterial cell. Morphological characteristics of the bacterial strains and PHA granules were also observed under the phase-contrast illumination microscope (Carl Zeiss), all digital images were captured. Only those bacterial isolates which found to accumulate PHAs were selected for further studies.

Production of PHA in shake flask

In order to further confirm and to compare the abilities of these PHAs accumulating bacterial strains, selected fluorescent PHA isolates were first grown in M9 medium supplemented with 0.02% (w/v) yeast extract and containing acetate (2 g/L) as carbon source. The composition of M9 medium was as follows : $Na_2HPO_4 \cdot 7H_2O$, 12.8 g; K_2HPO_4, 3.0 g; NaCl, 0.5g; NH_4Cl, 1.0g; 1ml of 1 M $MgSO_4$ stock solution, and 1 ml of 0.1 M $CaCl_2$ stock solution. The initial pH of the media was adjusted to 7 with 6N HCl. In all cases, acetate and rest of the media components were sterilized separately and mixed aseptically before inoculation (Hein et al. 1997). The inoculum was prepared by inoculating 10 mL of the sterile medium using 20 mL test tubes followed by incubation for 24 hrs in an orbital shaker. Subsequently 4% of inoculum (individual strain and consortium as well) was transferred in to 150 mL of M9 mineral salts medium (in 500mL Erlenmeyer) with acetate and yeast extract and incubated at 25°C for 48 hr at 220 rpm in an orbital shaker. Samples were drawn at predetermined time intervals to measure cell dry weight, PHA accumulation and acetate concentration.

Analytical techniques

Cell concentration was determined by measuring the cell dry weight of culture broth. Four-ml culture broth was sampled in a pre-weighed centrifuge tube and centrifuged at 5000 rpm for 10 minutes. The settled biomass was washed 3 times with distilled water and centrifuged, then lyophilized for at least 48 hours to a constant weight. The final weight of the tube was measured to calculate the cell dry weight (CDW). The extraction

of PHAs was made by the methanol and chloroform extraction method described by Comeau et al. (1988) using benzoic acid as an internal standard. The co-polymeric composition was measured with a Gas Chromatography Linked to Mass Spectroscopy (GC-MS) (Perkin Elmer, Clarus 500) equipped with a Capillary column Zebron ZB-5 and a Shimadzu C-R5A Chromatopac flame-ionization detector. Poly-(3-Hydroxybutyrate-co-3-hydroxyvalerate) (3HB-co-3HV) (PHV content 12%w/w, Aldrich Chemical Company, Inc.) was used as a standard.

The supernatant of the centrifuged samples was used to determine acetate (Ac^-) concentration using ICS-2000, Ion chromatography systems (Dionex Co.) equipped with the column of Ion Pac AS14 anion-exchange. The variation between replicates in all analytical determinations was less than 5%.

RESULTS AND DISCUSSIONS

Screening of PHA accumulating bacterial strains

After incubation, individual colonies were obtained from agar plates. Colonies capable of accumulating PHAs were identified first by examining in a fluorimeter, and the observed fluorescence was considered a potential PHAs producer.

Furthermore, microscopic investigation of the strain stained with Nile blue-A was conducted to confirm the presence of PHAs. PHAs granules fluoresced as bright orange, with individual granules often visible within a cell. The accompanying black-and-white photomicrographs showed these fluorescing orange granules as an intense bright image within the cells. The strains were found to accumulate granule inclusions by phase-contrast microscopy. These accumulated cellular granules were further confirmed to be poly(3HB-co-3HV) by GC-MS analysis. Twelve PHA-accumulating bacterial strains were selected from the isolates of the municipal wastewater activated sludge. The twelve strains were designated as PHA-M1 to PHA-M12 (M: municipal acrivated sludge). The purity of these twelve isolates was determined based on uniform colony morphology after

streaking them at least twice on agar plates. One example of the twelve isolates were shown in Fig. 1a, b.

The gram staining results (presented in Table 1) revealed that six out of twelve bacterial strains were identified to be gram-negative bacteria and the remaining six as gram-positive.

Accumulation of PHA by the isolated strains

In order to evaluate PHAs accumulation capacity of bacterial strains, the isolated strains were grown individually as pure cultures using synthetic medium in shake flasks. Figure 2 showed the profiles of all 12 strains for cell dry weight (CDW), PHA and acetate concentration and PHA content (%w/w of cell dry weight) with respect to cultivation time. The overall results of the twelve strains for PHAs accumulation are summarized in Table 2. Though in general, all isolated strains showed a notable capacity to use acetate to accumulate PHA, the PHA amounts produced and the time required accumulating maximum amount of PHA varied significantly among the twelve strains. The maximum PHA content of 27.50% (w/w) of cell dry weight was obtained by strain PHA-M3. Moreover, strains of PHA- M10, PHA- M9, PHA- M1 and PHA- M8 also showed a good PHA accumulation potential as they produced maximum PHA content of more than 25% of dry cell weight. However, strain PHA-M5 showed the minimal capacity of 3.25% (w/w) PHAs, the strains of PHA-SM2, PHA-M11, PHA-M12 showed a comparable low capacity of PHAs accumulation and the maximum PHA contents were less than 10% (w/w). Regarding the strain PHA-M3 with the best capacity of PHA accumulation, PHA content gradually increased and reached the maximum value of 27.5% (w/w) at 18 hrs. The cell dry weight (biomass) of this strain also reached maximum (0.3452 g/l) at 18 hrs of growth followed by a gradual decline. The maximum PHA concentration and PHA productivity with the strain PHA-M5 of poorest capacity were 0.03245 g/l and 0.00002 g/l/h, respectively at 48 hrs.

Acetate utilization

The profiles of acetate consumption of all bacterial strains are shown in Figure 2. It was noticed that the maximum PHAs accumulating strain (PHA-M3) consumed almost all the acetate at 18 hrs of growth. Further, eight out of twelve strains consumed all the acetate provided in the medium (almost 100% acetate consumed), however, the acetate concentration reached almost zero at different cultivation time. Only three strains PHA-M2, PHA-M5 and PHA-M11 poorly used the acetate. At the end of the experiment (48 hrs), Strain PHA-M2 used only 40% of the added acetate, whereas strains PHA-M11 and PHA-M5 used only 16% and 2% of the added acetate, respectively.

Copolymer composition

The copolymer composition, i.e., %PHB and %PHV, is primarily influenced by the substrate used (Choi and Lee 1999b). In this study, the same organic substrate was used during the experiments, yet the PHB/PHV ratio of the copolymer changed in accordance with operating time and strains (shown in Table 3) It was found that all isolated strains produced copolymers, PHB and PHV. At the time of maximum PHAs content, the strains PHA-M3, PHA-M5 PHA-M6 PHA-SB8 and PHA-M9 accumulated up to 50% PHV and the strains PHA-M2, PHA-M4 contained 100% PHB. The PHV/PHB ratios of the copolymers produced by each individual strain varied during 48 hrs of growth. Therefore, there was need for further studies on this aspect. The PHB/PHV ratios of the experiments were also summarized in Table 2.

In the study, the Initial pH for all the experiment was adjusted to 7, and final pH of the medium was about 8.0 or above (Table 1). It was observed that when the difference of pH between initial and final was large, the PHA production behavior also more distinctive This might explain the reason for highest accumulation of PHA (27.5% w/w) as pH of the strain 3 changed the most from initial to the end of the experiment (final). The trend of pH change was almost the same as the capacity of the bacterial strain accumulating PHA. It was suspected that the diffusion of undissociated acetic acid into the bacterial cells had suppressed the PHA production (Kasemsap and Wantawin 2007; Liu et al. 2007).

Like other isolates described in other studies in literature, our isolates were presumed to convert acetate to poly(3HB-co-3HV). However, different from other known PHA producing bacteria, our isolates could rapidly produce PHA under uncontrolled conditions of pH and dissolved oxygen concentration in shake flask, and generated PHA with varying composition (HB/HV ratio) (Shown in Table 3) at different growth stages or different cultivation time. The observation of the shift of PHA composition from 3HB to 3HV (or HB/HV ratio) during cell cultivation (batch growth) has not been reported earlier. Only one study reported this variation while using activated sludge as mixed culture for PHA production (Liu et al. 2000b). The change of HB/HV ratio with cultivation time has not been shown in pure culture. This warrants further study, However, for our isolates the ratio of HB/HV varied with cultivation time and this warrants further studies.

PHA productivity and PHA yield

The yield of PHA with respect to acetate consumed and specific growth rate of different strains is also presented in Table 2. The specific growth rate was calculated from the slope of the straight line between cell dry weight and time in exponential growth phase. It was found that the capacity of different isolated strains to produce PHA varied from $1x10^{-3}$ to $38x10^{-3}$ gPHA/g acetate consumed. The largest yield ($1x10^{-3}$ gPHA/gAcetate consumed) was observed for the strain PHA-M3. The specific growth rate of different strains was highly variable. There seems to be no relation between specific growth rate and PHA yield constant with respect to acetate consumed. The PHA yield constant with respect to acetate consumed for all strains appeared to be low than in general obtained by other researchers and could be attributed to the fact the all strains were grown in shake flask under uncontrolled conditions, as said before. It therefore, warrants testing these strains in fomenters under controlled condition to ascertain their real potential for PHA production.

An ideal organism for PHA production would be a culture that can store high PHA content and grow rapidly on an inexpensive substrate. These experiments have shown, however, that it is possible and potentially economical to develop a PHA production

system for biodegradable plastics using activated sludge to isolate the PHA-accumulating bacteria for PHA production.

It was known that different bacteria may utilize different carbon substrates (e.g. sugars, fatty acids) for PHA production; some have specific requirements for substrate and limiting nutrients in order to accumulate PHA. In this study, acetate was used as the carbon source in the synthetic medium for PHA-accumulating bacteria cultivation. Glucose was also used in this study (data not shown) for the screening of PHA-accumulating bacteria, the results showed the twelve bacterial strains could produce PHA with glucose as carbon source (less than those from acetate The results showed that in this activated sludge samples, the twelve strains accounted for the majority of the PHA-accumulating bacteria. However, the second-step screening process should be expanded to include carbon substrates other than acetate and glucose, and limiting medium other than nitrogen, to induce PHA accumulation in different isolates showing lipid inclusions. This way, a wider range of bacteria which produce different types of PHA may be selected.

It is also necessary to mention that PHA accumulation in this study was carried under uncontrolled environmental (pH and dissolved oxygen) conditions of shake flasks. Further, the C/N ratio may not be the optimum condition for the PHA production. However, PHA content reached up to 27.50%, it is certain that with the control pH, dissolved oxygen (DO) concentration and optimum C/N ratio, the PHAs content will further substantially increase. Thus, the effect of nitrogen limitation on cell growth and PHA synthesis needs further investigations by batch as well as fed-batch culture in a fermentor where pH and DO are well controlled.

In future, identification of microorganisms will be carried out using 16S rDNA gene sequences.

CONCLUSIONS

The following conclusions can be drawn from this work:

Twelve PHA accumulating bacterial strains were isolated from municipal wastewater activated sludge. Six of them were gram-negative, the remaining six were gram-positive. Though in general, all isolated strains showed a notable capacity to use acetate to accumulate PHA, the PHA amounts produced significantly varied among the twelve strains. The maximum PHA content of 27.50% was obtained by strain PHA-M3 under uncontrolled conditions of pH and dissolved oxygen concentration. Using same carbon source, the PHB/PHV ratios of the copolymers produced substantially varied for the twelve isolated bacterial strains. Also, the PHB/PHV ratio of the copolymer in individual strains changed during cultivation with operating time.

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Table 1. Microbiological staining results of isolates strains

Bacterial strain	Gram staining results	Final pH
PHA-M1	Negative	8.91
PHA-M2	Positive	8.01
PHA-M3	Negative	8.96
PHA-M4	Negative	8.84
PHA-M5	Positive	7.75
PHA-M6	Negative	8.94
PHA-M7	Positive	8.95
PHA-M8	Negative	8.87
PHA-M9	Positive	8.85
PHA-M10	Negative	8.87
PHA-M11	Positive	7.56
PHA-M12	Positive	8.70

Table 2. Comparison of the production of PHA using acetate as carbon source of isolate strains.

Bacterial strain	Time for Max.PHA (h)	Max.PHA conc.(g/l)	HB:HV at max PHA	Cell conc. at max.PHA (g/l)	Max PHA content (%)	$Y_{P/S}$: gPHA/gAc-	Specific growth rate	Productivity (g/l/h)
PHA-M1	30	0.025	86:14	0.0975	25.30	0.006	0.016	0.0008
PHA-M2	48	0.014	100:0	0.1650	8.36	0.001	0.002	0.0003
PHA-M3	18	0.119	50:50	0.3452	27.50	0.038	0.014	0.0066
PHA-M4	30	0.012	100:0	0.090	13.33	0.002	0.014	0.0004
PHA-M5	48	0.001	50:50	0.0325	3.25	0.0001	0.005	0.00002
PHA-M6	36	0.022	50:50	0.0945	23.54	0.004	0.015	0.0006
PHA-M7	24	0.012	100:0	0.0525	23.05	0.003	0.007	0.0005
PHA-M8	30	0.054	50:50	0.2175	24.67	0.010	0.03	0.0018
PHA-M9	36	0.034	50:50	0.1345	25.45	0.006	0.015	0.0009
PHA-M10	24	0.049	86:14	0.1900	25.63	0.012	0.058	0.0020
PHA-M11	36	0.0004	86:14	0.0075	5.67	0.0001	0.01	0.00001
PHA-M12	48	0.015	86:14	0.1950	7.87	0.002	0.031	0.0003

Table 3. Summary results of the HB/HA % with twelve bacterial strains during 48 h experiments.

time (h)	PHA-M1	PHA-M2	PHA-M3	PHA-M4	PHA-M5	PHA-M6	PHA-M7	PHA-M8	PHA-M9	PHA-M10	PHA-M11	PHA-M12
0												
6		0	87,	100		87,5	89,1	90,0	88,6	87,6	0	
12	88,0	0	87,6	100	88,8	0	88,0	100	100	87,6	100	87,6
18	87,4	0	50	0	87,5	100	89,8	100	88,1	87,5	87,6	100
24	87,6	87,5	0	87,5	0	87,5	100	100	100	86	87,3	0
30	86,0	86,9	87,3	100	0	87,6	100	50	100	87,6	100	100
36	100	87,5	100	100	87,5	50	87,0	100	50	100	86,1	87,9
42	87,5	100	87,6	86,9	0	0	100	87,5	100	0	87,6	0
48	100	100	0,0	0	50	86,9	86,8	100	100	0	87,7	86,0

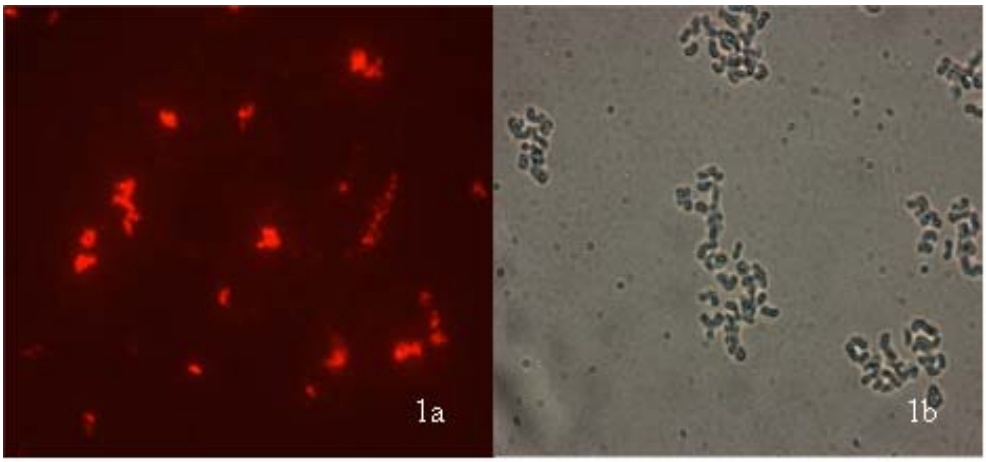
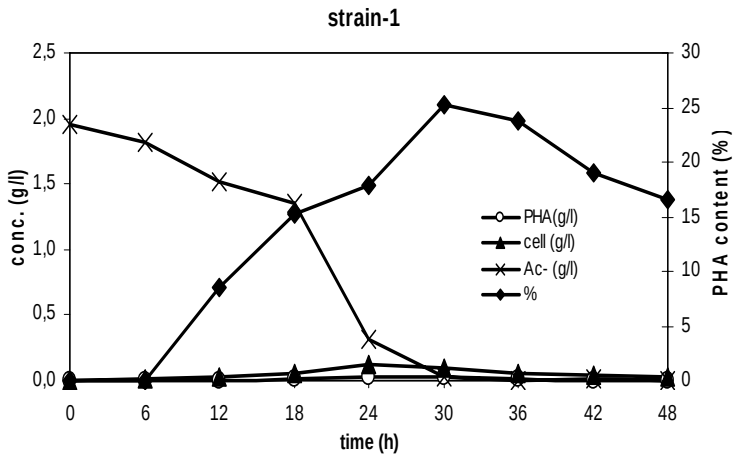


Figure 1. PHA granules in cell stained with Nile blue A and observed under simultaneous fluorescent (1a) and visible light (1b) (strain PHA-M9).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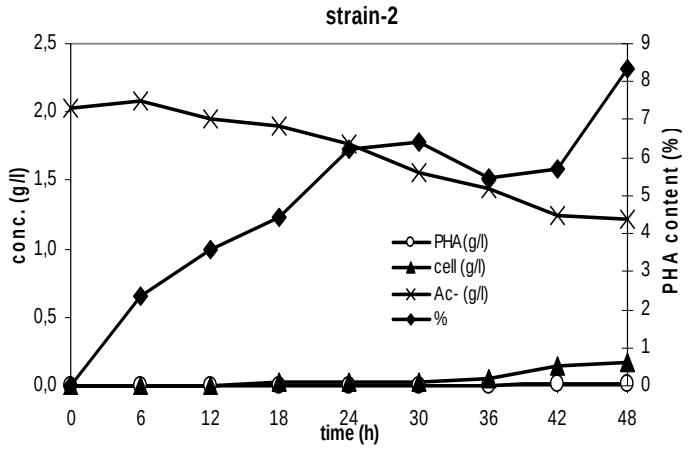
PHA content (%)
time (h)
PHA(g/l)
cell (g/l)
Ac- (g/l)
%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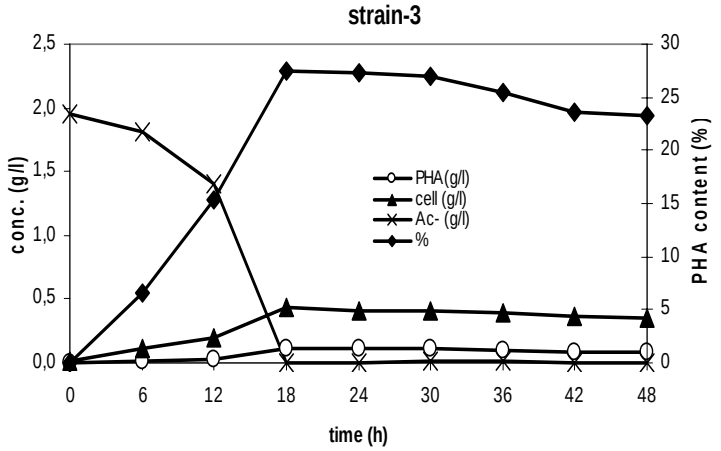
PHA content (%)
time (h)
PHA(g/l)
cell (g/l)
Ac- (g/l)
%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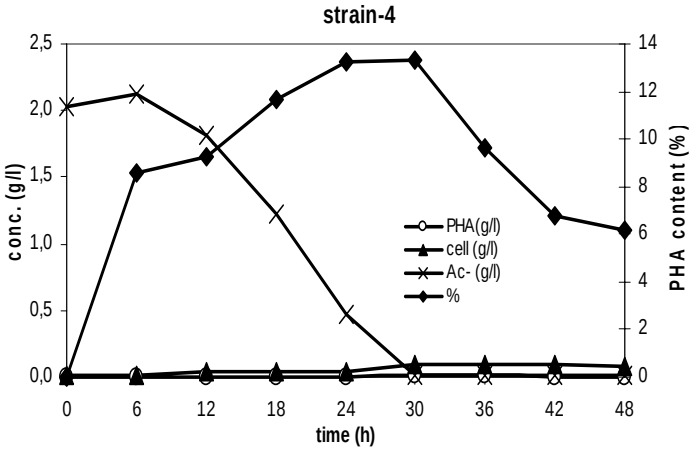
conc. (g/l)
PHA content (%)
time (h)
PHA(g/l)
cell (g/l)
Ac- (g/l)
%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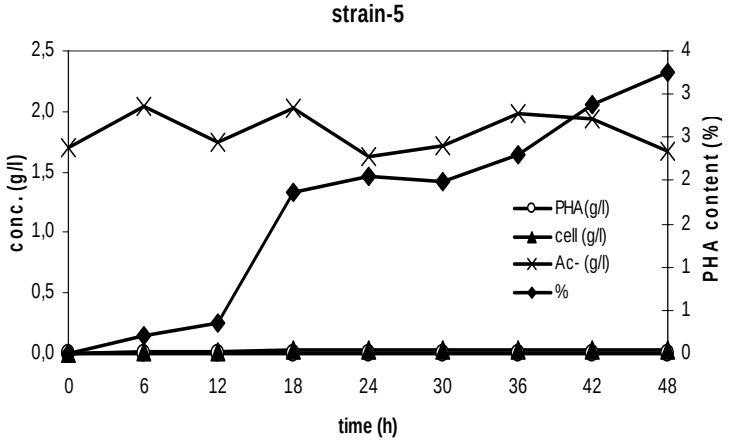
PHA content (%)
time (h)
PHA(g/l)
cell (g/l)
Ac- (g/l)
%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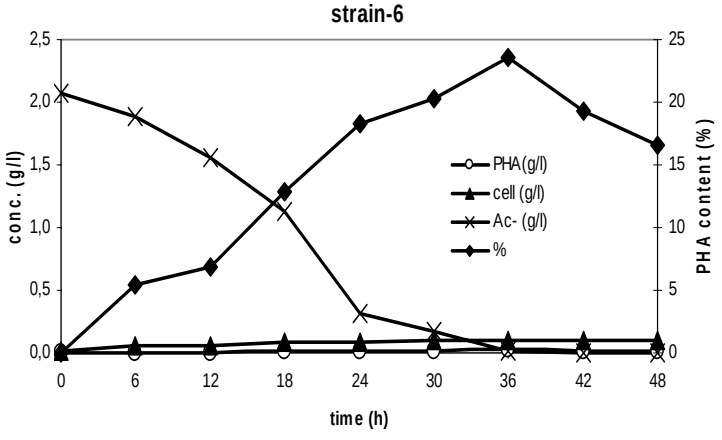
conc. (g/l)
PHA content (%)
time (h)
PHA(g/l)
cell (g/l)
Ac- (g/l)
%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cell (g/l)
Ac- (g/l)
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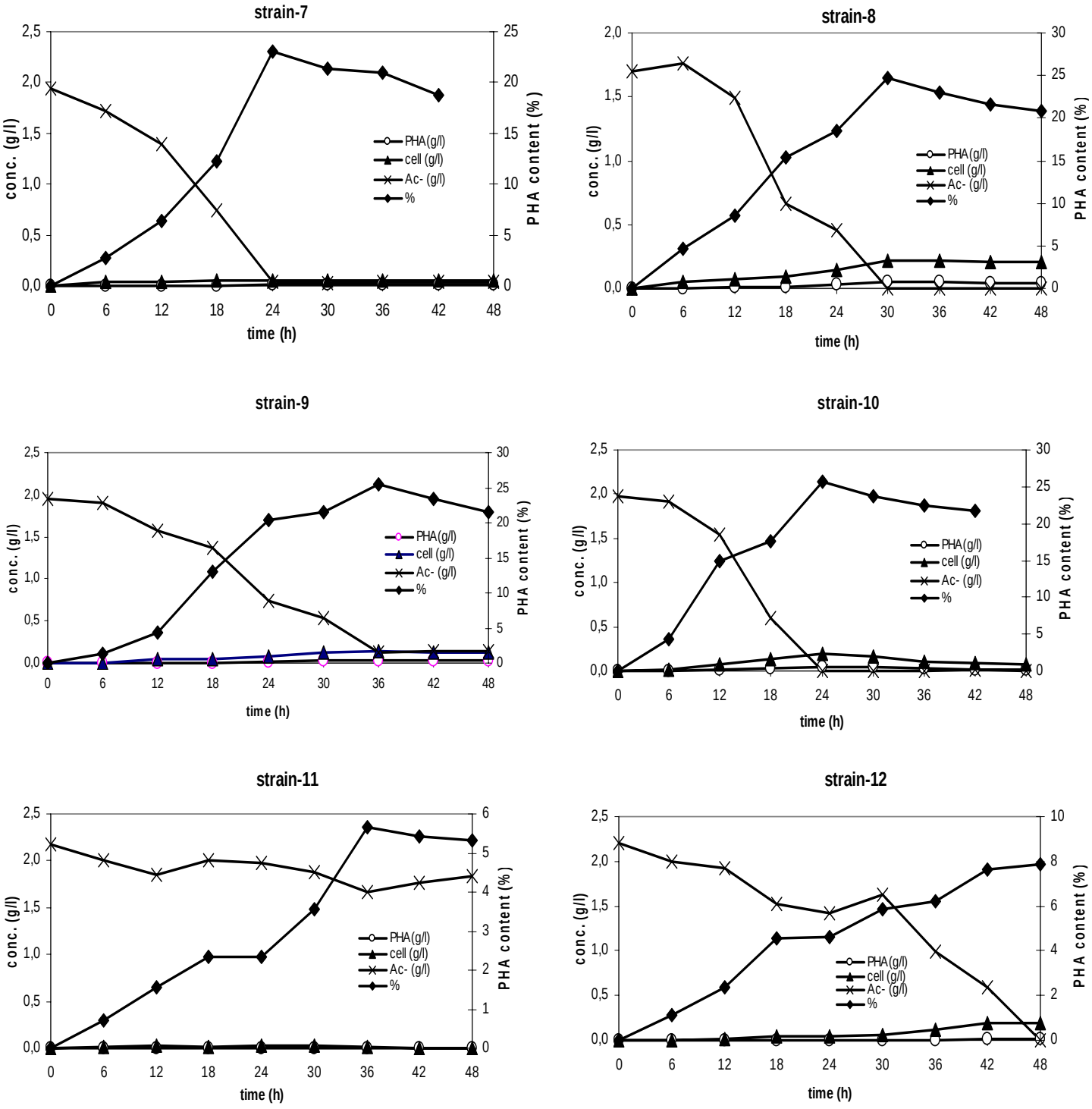

Figure 2. Profiles in cell dry weight (CDWs), PHA, acetate concentrations and PHA content (%) of the bacterial strains (PHA-M1 and 12) with respect to cultivation time.

Part III

Isolation of polyhydroxyalkanoate (PHA) accumulating bacterial strains from pulp and paper industry wastewater activated sludge.

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Résumé

Des boues activées provenant d'eaux usées de pâtes et papier ont montré un potentiel intéressant pour produire en fermenteur des PHAs en employant de l'acétate ainsi que différents types d'eaux usées comme sources de carbone ; La teneur maximale en PHAs atteignant jusqu'à 60% (p / p). Dans cette étude, les isolats bactériens de boues activées de pâtes et papier ont été cribblées pour l'obtention de PHAs. Initialement une coloration au bleu de Nil a été menée pour détecter des inclusions lipidiques cellulaires. Les isolats positifs, contenant donc ces inclusions, ont été cultivés dans un milieu contenant de l'acétate comme seule source de carbone afin de promouvoir l'accumulation de PHAs. Ceux-ci ont été analysés par chromatographie en phase gazeuse reliée à la spectroscopie de masse (GCMS) pour confirmer la présence et la concentration des PHAs. Il a été constaté que six souches bactériennes isolées des boues activées de pâtes et papier peuvent produire des PHAs (Les contenus maximaux obtenus variant de 5,22 à 35,5%) . Le taux maximal de 35,5% a été synthétisé par la souche PHA - P5.

***Mots-clés*:** boues activées; polyhydroxyalcanoates (PHA); Stockage polymère; isolement.

Abstract

Pulp and paper activated sludge showed a potential of producing PHA in fermentor employing acetate as well as different types of wastewater as the carbon sources, the maximum PHA content reached up to 60% (w/w). In this study, Bacterial isolates from the pulp and paper activated sludge were screened for polyhydroxyalkanoates (PHAs). Initially Nile Blue A staining was performed to detect lipid cellular inclusions. Lipid-positive isolates were then grown in a synthetic medium containing acetate as sole carbon source to promote accumulation of PHA. The PHA was analyzed by Gas Chromatography Linked to Mass Spectroscopy (GCMS) to further confirm the presence and the concentration of PHA. It was found that six bacterial strains isolated from pulp and paper activated sludge can produce PHA (PHA content obtained from 5.22 to 35.5%), The maximum PHA content of 35.5% was obtained by strain PHA-P5.

Key words: Activated sludge; polyhydroxyalkanoates (PHAs); Storage polymer; isolation.

INTRODUCTION

Bacterial polyhydroxyalkanoates (PHA) are polyesters produced intracellularly as storage material for carbon and energy. PHA has properties similar to petrochemical-based plastics, is biodegradable and is produced from renewable resources. Activated sludge used as a mixed culture for PHA production has been studied by many researchers (Chua et al. 2003; Lemos et al. 2003). As with most microbial products, the search for now producer organisms is a continuous process, necessitated by the desire for higher product yield, more efficient utilization of specific raw materials, and novel end-products.

In the previous study (Yan et al. 2006), pulp-paper activated sludge was used as a mixed culture for PHAs production in fermenter employing acetate as well as different types of wastewater as the carbon source, the maximum PHA content reached up to 60%w/w. pulp-paper activated sludge showed higher PHA producing potential (43.1% content) than municipal activated sludge (30.6%) under the same (sludge concentration, acetate concentration, pH and temperature, etc.) conditions. This showed the high potential of activated sludge to use as a source of microorganisms to accumulate PHA.

The objective of this work was to isolate PHA-accumulating bacterial strains present in pulp and paper wastewater activated sludge, and screening of the isolates for PHA-producing abilities. The bacterial isolated strains from pulp and paper activated sludge were also compared with isolates from municipal activated sludge.

MATERIALS AND METHODS

Activated sludge

Activated sludge samples were collected from pulp-paper industry wastewater treatment plants - Papiers Stadacona (Québec), Canada.

Isolation of Bacterial Strains

Bacterial strains isolation was carried out as described in earlier article (bacterial strain isolation from municipal wastewater sludge).

Gram staining:

Preliminary identification of bacterial strains was carried out using standard procedures of gram staining.

Screening for PHA-producing bacteria

PHA accumulating bacterial strains were screened as described in previous article.

Microscopic analysis

Presence of PHA accumulating granules in the bacterial strains were done as detailed in earlier article.

Production of PHA in shake flask

Shake flask fermentation was carried out to produce PHA as described in previous article. Except that 8% of inoculums and acetate of 5 g/l was used in this study.

Analytical techniques

PHA accumulating bacterial cell concentration (to measure cell dry weight [CDW]), extraction of PHA from bacterial biomass and PHA co-polymeric composition was carried out as described in earlier article. Acetate concentration in the supernatant of each centrifuged sample was determined using Ion chromatography as described earlier. Also the variation between replicates in all analytical determinations was less than 5%.

RESULTS AND DISCUSSIONS

Screening of PHA accumulating bacteria strains

After incubation, individual colonies were obtained from agar plates. Colonies capable of accumulating PHAs were identified first by examining in a fluorimeter, and the observed fluorescence was considered a potential PHAs producer.

Furthermore, microscopic investigation of the strain stained with Nile blue A was conducted to confirm the presence of PHAs. PHAs granules fluoresced as bright orange, with individual granules often visible within a cell. The accompanying black-and-white photomicrographs showed these fluorescing orange granules as an intense bright image within the cells. The strains were found to accumulate granule inclusions by phase-contrast microscopy. These accumulated cellular granules were further confirmed to be poly(3HB-co-3HV) by GC-MS analysis. Six PHA-accumulating bacterial strains were selected from the isolates of the pulp-paper industry wastewater activated sludge. The six strains were designated as PHA-P1 to PHA-P6. The purity of these six isolates was determined based on uniform colony morphology after streaking them at least twice on agar plates.

The gram staining results (presented in Table 1) revealed that five out of six bacterial strains were identified to be gram-negative bacteria and the remaining one as gram-positive. The results showed that sludge microorganisms are generally gram negative, which is in agreement with literature (Brown and Lester, 1980). One image of the gram-negative strain was shown in Fig. 1.

Accumulation of PHA by the isolated strains

In order to evaluate PHAs accumulating capacity of the isolated bacterial strains, they were grown individually as pure cultures using synthetic medium in shake flasks. The acetate concentration, cell dry weight (CDW), PHA concentration and PHA content (wt% of CDW) were measured for all six strains and all profiles are presente in Figure 2. The overall results of the six strains for PHAs accumulation are shown in Table 2. Though in general, all isolated strains showed a notable capacity to use acetate to accumulate PHA, the PHA amounts produced varied significantly among the six strains. The maximum PHA content of 35.45%(w/w) of dry cell weight was obtained by strain PHA-P5 which was much higher than the maximum PHA (27.5% of CDW) accumulated in strains isolated from municipal sludge. Moreover, strains of PHA-P3 PHA-P1 PHA-P2 also showed a good PHA accumulating potential as they produced maximum PHA content (31.8, 29.1, and 23.4% w/w, respectively). However, strain PHA-P4 showed the minimal capacity of 5.2% (w/w) PHAs, the strains of PHA-P6 showed a comparable low capacity of PHAs and the maximum PHA contents were 15.1% (w/w).

Acetate utilizing

Figure 2 shows the profiles of acetate consumption for all bacterial isoaltes. The acetate concentration reached almost zero at different cultivation time reflecting the different capacity of different isolates to consume acetate and produce PHAs. It was noticed that the strains PHA-P2, PHA-P3, PHA-P5 and PHA-P6 consumed almost the all the acetate at 48 hrs of growth. Further, PHA-P6 consumed all the acetate provided (almost 100% acetate consumed) in about 36 hr. Only two strains PHA-P1 and PHA-P4 used acetate

poorly. At the end of the experiment (48 hrs), Strain PHA-P4 used only 10% of the initial acetate, whereas strains PHA-P4 showed the lowest PHA production.

Copolymer composition

It was found that acetate consumption leads to the production of a copolymer of HB and HV repeating units with HB being predominant (more than 85%). At the time of maximum PHAs content, the strains PHA-P4, PHA-P6 contained 100% PHB. The PHV/PHB ratios of the copolymers produced by each individual strain varied during 48 hrs of growth. The results showed that it was difficult to control the PHB and PHV content, as even with the same bacterial strain, during the cultivation time of 48 hrs, the percentage of PHB and PHV were changed. Therefore, there was need for further studies on this aspect. The PHB/PHV ratios for all isolates were also summarized in Table 2.

The copolymer composition, i.e. %PHB and %PHV, is primarily influenced by the substrate used (Doi, 1990). Furthermore, Renner et al. (1996) and Tsuge (2002) stated that different bacteria were able to produce PHAs with different compositions when growing on the same substrate. Doi *et al.* (1987) studied the copolymer composition produced by *R. eutropha* H16 grown in a medium containing sodium acetate and sodium propionate. The PHV content of the copolymer increased as the propionate concentration in the medium increased. They also found that copolymer was obtained even when propionate was used as the sole organic carbon compound. During the study reported herein, the same organic substrate was used during the experiments, yet the PHB/PHV ratio of the copolymer changed in accordance with operating time and strains.

In the study, the Initial pH for all the experiment was adjusted to 7, but the final pH (at the end of experiments) of the medium was substantially increased (Table 1). It was observed that higher the difference between initial and final pH, the PHA production behavior also was more distinct. When acetate was diverted into storage instead of being used for oxidation or growth, it was evident that, pH of the broth would increase concomitantly with PHA accumulation. This might explain the reason for a highest pH difference (between initial and final) of the strain PHA-P5 which also accumulated highest concentration of PHAs.

Like other isolates described in the aforementioned studies (Alias and Tan 2005), our isolates were presumed to use acetate to produce poly(3HB-co-3HV). However, different from other known PHA producing bacteria, our isolates could rapidly produce PHA under uncontrolled conditions (pH, dissolved oxygen, feed strategy), and could vary the PHA composition at different growth stages. To our knowledge, the observation of the shift of PHA composition from 3HB to 3HV during cell cultivation (batch growth) has not been reported in pure culture. However, the variation of HB/HV ratio during growth was reported while using activated sludge as mixed culture for PHA production (Liu et al. 2000b).

PHA productivity and PHA yield

The best PHA productivity reported to date was obtained from the study by Wang and Lee (1997). They obtained PHA productivity of 4.94 gPHB/L/hr from *A. latus*. PHA productivity obtained in this study was significantly lower than that reported by Wang

and Lee (1997). This was to a lower biomass concentration in the present system and the uncontrolled cultivation conditions used in shake flask. Concentrations of biomass and substrate used in commercial production are significantly higher than the concentrations of this study. For example, the cell concentration of *A. latus* was 76 g/L and 5-20 g/L of sucrose was applied in the study by Wang and Lee (1997), while the concentration of the biomass in this study is always lower than 500 mg/l. An ideal organism for PHA production would be a culture that can store high PHA content and grow rapidly on an inexpensive substrate. Pure cultures of selected bacteria are best suited for PHA production, but the cost of maintaining pure culture conditions and providing the suitable substrate has increased the cost of biodegradable plastics to the point that it is not competitive with synthetic plastics, in spite of the environmental benefits. These experiments have shown, however, that it is possible and potentially economical to develop a PHA production system for biodegradable plastics using activated sludge to isolate the PHA-accumulating bacteria for PHA production.

It was known that different bacteria may utilize different carbon substrates (e.g. sugars, fatty acids) for PHA production; some have specific requirements for substrate and limiting nutrients in order to accumulate PHA (Dawes & Senior 1973). In this study, acetate was used as the carbon source in the synthetic medium for PHA-accumulating bacteria cultivation. Glucose was also used in this study (data not shown) for the screening of PHA-accumulating bacteria; the results showed the six bacterial strains could produce PHA utilizing these types of carbon sources. The results shown that in this activated samples, the twelve strains accounted for the majority of the PHA-accumulating

bacteria. However, the second-step screening process should be expanded to include carbon substrates other than acetate and glucose, and limiting medium other than nitrogen, to induce PHA accumulation in isolates showing lipid inclusions. This way, a wider range of bacteria which produce different types of PHA may be selected for.

The most common isolate from the process is *Acinetobacter sp.* (Mino et al. 1998). Rees et al. (1993) reported that *Acinetobacter sp.* could accumulate PHB up 11.5% of cell dry weight after growth in a medium containing acetate as the carbon source. However, *Acinetobacter* has not been shown to rapidly produce PHA during substrate uptake under anaerobic conditions. Another isolate from the A/O system belongs to the new genus *Amaricoccus* from the alpha subdivision of *Proteobacteria* (Maszenan et al., 1997), however, the ability of this strain to accumulate PHA was not studied. Liu et al. (2000) isolated two novel PHA-accumulating bacteria from a laboratory-scale SBR that could accumulate poly(3HB-co-3HV) up to 44.7% of dry cell weight. However, the two isolates did not take up and convert acetate into PHA, but consumed glucose and produced cellular carbohydrate and poly(3HB-CO-3HV). The study of Stante et al. (1997) presented that *Lampropedia sp*. was shown to rapidly convert acetate into PHA under anaerobic conditions. These strains were isolated from the A/O system exhibited PHA formation. In this study, the activated sludge was from the biofilter process of a municipal wastewater treatment plant, but not the A/O process, the results showed that not only the A/O system, but also other conventional activated sludge system contained the PHA-accumulating strains.

It was presumed that the microbial populations in the A/O system are diverse in other process. In future, identification of microorganisms will be carried out using 16S rDNA gene sequences.

CONCLUSIONS

Six bacterial strains isolated from pulp paper industry wastewater activated sludge can produce PHA (PHA content obtained from 5.22 to 35.5%), five of them were gram-negative, the remaining one were gram-positive. Though in general, all isolated strains showed a notable capacity to use acetate to accumulate PHA, the PHA amounts produced significantly varied among the six strains. The maximum PHA content of 35.5% was obtained by strain PHA-P5.

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Table 1. Microbiological staining results of isolates strains

Bacterial strain	Gram staining results	Final pH
PHA-P1	positive	8.31
PHA- P2	Negative	8.69
PHA- P3	Negative	8.97
PHA- P4	Negative	7.69
PHA- P5	Negative	9.03
PHA- P6	Negative	8.60

Table 2. Comparison of the production of PHA using acetate as carbon source of isolate strains.

Bacterial strain	Time for Max.PHA (h)	Max.PHA conc.(g/l)	HB:HV at max PHA	Cell conc. at max.PHA (g/l)	Max PHA content (%)	$Y_{P/S}$: gPHA/gAc-	Specific growth rate	Productivity (g/l/h)
PHA-P1	48	0,201	99:1	0,693	29,061	0.017	0.007	0,0042
PHA-P2	42	0,202	85:15	0,865	23,370	0.030	0.254	0,0048
PHA- P3	36	0,224	92:8	0,705	31,806	0.032	0.153	0,0062
PHA- P4	48	0,032	100:0	0,607	5,220	0.004	0.124	0,0007
PHA- P5	30	0,227	95:5	0,640	35,446	0.042	0.052	0,0076
PHA- P6	36	0,077	100:0	0,513	15,100	0.013	0.187	0,0021

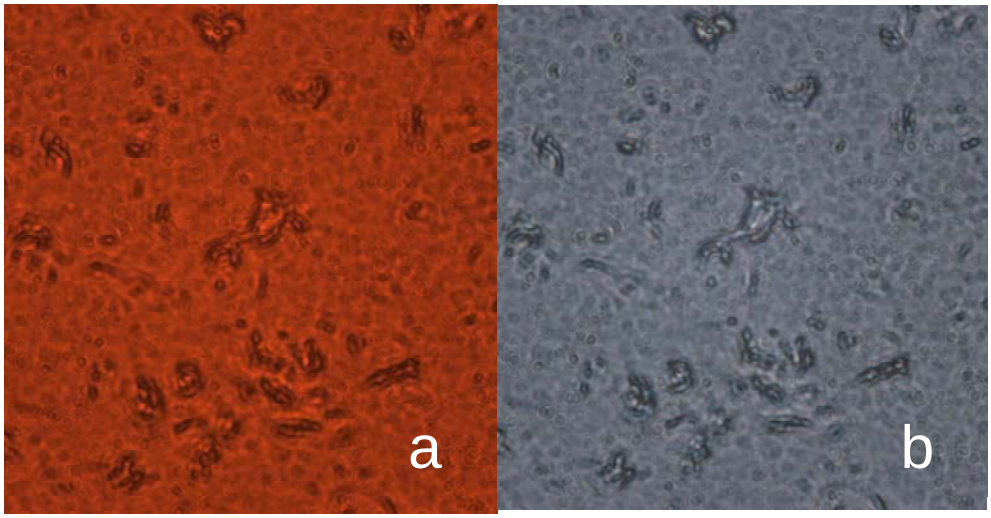

Figure 1. PHA granules in cell stained with Nile blue A and observed under simultaneous fluorescent (a) and visible light (b). (Strain PHA-P3)

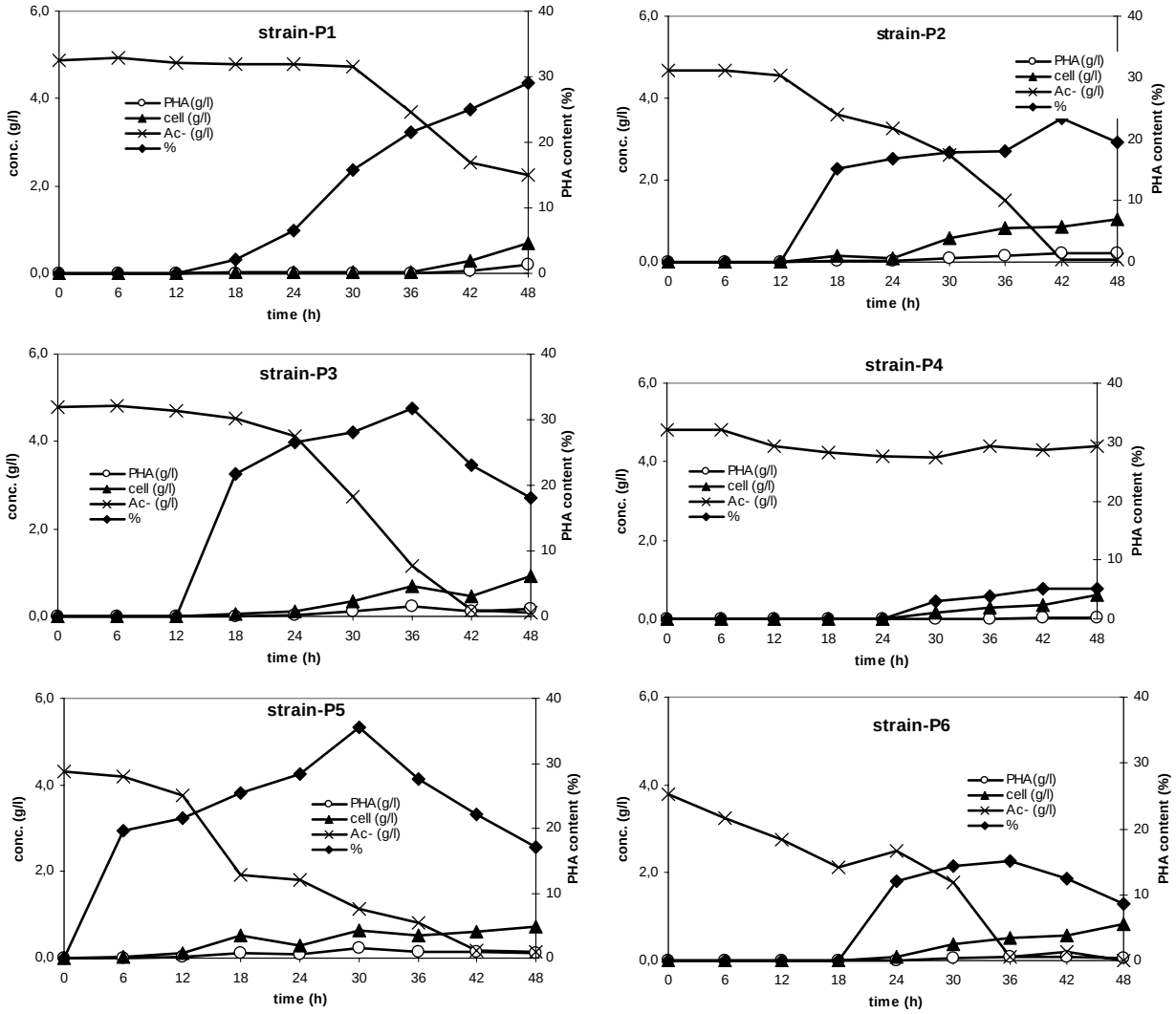

Figure 2. Profiles in cell dry weight (CDWs), PHA, acetate concentrations and PHA content (%) of the bacterial strains (PHA-P1 to PHA-P6) with respect to cultivation time.

Part IV

Molecular identification of PHA producing microbial strains isolated from municipal wastewater sludge

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(to be submitted)

Résumé

Il a été observé que les boues activées provenant d'une usine de traitement d'eaux usées municipales peut produire des PHAs à un taux maximal de 30,64% p / sem. En outre, douze souches bactériennes accumulant des PHAs ont été isolées de ces boues et leurs capacités de synthèse de ces bioplastiques ont été évaluées. Bien que de manière générale, toutes les souches isolées montrent une capacité remarquable d'utiliser l'acétate pour accumuler des PHAs, les quantités produites varient considérablement entre elles. Un contenu maximal de 27,50% m / m a été obtenu avec la souche PHA - M3, même dans des conditions non contrôlées du pH et de la concentration en oxygène dissous. Ces micro-organismes ont été identifiés sur la base de la séquence de leur ADNr 16S. Ils ont été identifiés respectivement comme *Bacillus lichineformis, Enterobacter sp., Microbacterium* sp., *Enterobacter* sp., *Serratia* sp, *Bacillus* sp., *Yersinia* sp., *Microbacterium, et Bacillus cereus;* L'isolat PHA - M3 synthétisant le plus de PHA étant été identifié comme *Citrobacter* sp.

Mots-clés: séquençage de l'ADN, micro-organismes, PCR, ARNr gène, production de PHA

Abstract

The mixed culture (activated sludge) from a municipal wastewater treatment plant was found to produce PHA with maximum content of 30.64% w/w. Furthermore, twelve PHA accumulating bacterial strains were isolated from the sludge and their capabilities of the PHA production had been evaluated. Though in general, all isolated strains showed a notable capacity to use acetate to accumulate PHA, the PHA amounts produced significantly varied among the twelve strains. The maximum PHA content of 27.50% w/w was obtained by strain PHA-M3 even under uncontrolled conditions of pH and dissolved oxygen concentration. These sludge microorganisms were identified based on their 16S rDNA sequences. The twelve bacterial strains were identified as *Bacillus. lichineformis, Enterobacter* sp.*, Microbacterium, Enterobacter* sp.*, Serratia* sp*, Bacillus* sp.*, Yersinia* sp*, Microbacterium,* and *Bacillus cereus,* respectively. And the PHA-M3 strain with the maximum PHA production was identified as *Citrobacter* sp.

Keywords: DNA sequencing, Microorganisms, PCR, rRNA gene, PHA production

Introduction

There are currently more than 300 different microbial species known to synthesize PHA. (Lee 1996) Little information is known about the microorganisms responsible for PHA accumulation of mixed culture (activated sludge).

At present, only a few bacterial strains capable of accumulating PHA have been isolated from the Anaerobic/oxic (A/O) system (Vincenzini et al. 1997; Liu et al. 2000b; Sawayama et al. 2000). The most common isolate from the process is *Acinetobacter* sp. *Acinetobacter* sp. could accumulate PHB up to 11.5% (w/w) on cell dry weight basis after growth in a medium containing acetate as the sole carbon source (Vincenzini et al. 1997). Another isolate from the A/O system belongs to the new genus *Amaricoccus* from the alpha subdivision of Proteobacteria (Liu et al. 2000b).

While Dionisi et al. (2006) stated that the dominant genera belongs to *Thauera*, this organism was not described as being able to accumulate PHA and its capacity for PHA storage has not yet been unequivocally demonstrated.

In a recent study (Dionisi et al. 2006), after confirming the speciation of the population for PHA accumulation by DGGE (Denaturing Gradient Gel Electrophoresis), a clone library was constructed from the total DNA extracted from the SBR sludge. The screening of the clones was performed using amplified ribosomal DNA restriction analysis (ARDRA). However, the isolates have not yet been associated with PHA accumulation. Further investigation regarding the aforementioned microbial groups is necessary to certify their status as PHA producers.

A different approach was employed by Serafim et al. (2006) to identify microbial community presents in sequencing biological reactor (SBR) using fluorescence in situ hybridization (FISH). FISH results showed a positive signal to the *Azoarcus* sp. probe as a predominant organism present in a sequencing biological reactor (SBR), which was operated under feast-and famine conditions. Using Nile blue staining, it was possible to

correlate the presence of PHA inclusions with the *Azoarcus* sp., and thus confirmed this organism as a PHA producer.

In our previous study, the PHA production by mixed culture (activated sludge) from a municipal wastewater treatment plant was found to reach a maximum PHA content of 30.64% w/w (Yan et al. 2006). Furthermore, 12 PHA accumulating bacterial strains were isolated from the sludge and their capabilities of PHA production were evaluated. Six of those strains were gram-negative, and the remaining six were gram-positive. Though in general, all isolated strains showed a notable capacity to use acetate to accumulate PHA, the PHA amounts produced significantly varied among the twelve strains. The maximum PHA content of 27.50% w/w was obtained by strain PHA-M3 under uncontrolled conditions of pH and dissolved oxygen concentration.

Therefore, the aim of this work was to identify these 12 PHA producing bacterial strains based on their 16S rDNA sequences.

Materials and Methods

Activated sludge

Wastewater sludge samples were collected from Communauté Urbaine du Québec (CUQ, Québec). Microbial isolation was attempted on Plate Count Agar (PCA) and Sabouraud's Dextrose Agar (SDA) media using serial dilution techniques (Yan et al. 2006). Plates were incubated at 25°C for 72 hrs.

Bacterial strains

Molecular identification of twelve PHA accumulating bacterial strains screened based on Nile blue – A staining was used in this study. The isolated pure culture bacterial strains were grown on Luria Bertani (LB) Agar (Difco, Canada.) for DNA isolation and PCR amplification of 16S rRNA gene for their molecular identification.

Genomic DNA isolation:

DNA was extracted from all 12 EPS producing bacterial strains (PHA-M1 to PHA-M12) isolated from municipal wastewater sludge. Genomic DNA isolation procedure was used as described in manual of molecular biology (Maniatis et al., 1989). Bacterial strains were grown in Luria Bertani broth for 8 hours at 37^{o}C. Bacterial cell pellet was obtained by spinning at 6700g (rotor SL-50T, Sorvall Super T21) at 4^{o} C for 15 min. Pellet was washed with Tris-Cl buffer by short spin at 6700 g at 4^{o} C for 5 min. Bacterial pellet was resuspended with Tris-Cl buffer, 10% SDS and Proteinase K (Amresco, Solon, USA) to break down the cell wall and incubated at 37^{o}C for 60 min. Cell soup was passed through 25G needle 3-5 times to shear the DNA. The supernatant was obtained by centrifugation and DNA was extracted with phenol, phenol: chloroform (1:1) mixture twice and chloroform to remove the protein molecules from DNA. DNA was precipitated in presence of 5M NaCl, 100% ethanol and incubated for 2 h at -20^{o}C. Tubes were vortexed briefly and centrifuged at 7630 g for 10 min, supernatant was decanted and the pellet was air dried. The pellet was resuspended in 100 µl TE buffer, treated with 5µl of RNase (5µg/ml, heat treated, Qiagen, GmbH, D-40724, Hilden.) and incubated at 37^{o}C for 30 min. DNA was further precipitated with 5M ammonium acetate and Isopropyl alcohol. This mixture was incubated at room temperature for 5 min and centrifuged at 17160 g for 10 min at 4 ^{o}C. Pellet was washed twice with 70% ethanol, dried in air and DNA was resuspended in appropriate volume of TE buffer and stored at -20 ^{o} C.

Agarose gel electrophoresis

Agarose gel (0.7%) was made in 0.5 X Tris Borate EDTA (TBE) buffer and run at 60 V for 1h at 10^{o}C in electrophoretic apparatus. The fractionated DNA bands were visualized under UV Transilluminator (Biometra, Gottingen.) and compared with known DNA molecular weight markers. Genomic DNA showed above 23kb band of λ DNA Hind III digest in agarose gel electrophoresis (Agagel electrophoresis system, Biometra, Made in Germany).

Polymerase Chain Reaction (PCR)

The genomic DNA isolated from 12 different PHA producing bacterial strains isolated from municipal wastewater sludge (PHA-M1 to PHA-M12) was used for direct amplification of 16S rRNA gene portions (Widmer et al 1998). Universal primers were used to amplify the full length 16S rRNA gene from rrn operon of 12 bacterial isolates (Weisburg et al., 1991). Primers were custom synthesized from Eurogen (Eurogen, San Diego, California, USA) and primers for 16S rDNA had following sequence: Forward primer 5' <agagtttgatcatggctcag> 3' and Reverse primer 5' <aaggaggtgatccarccgca> 3'. PCR performed for 100µl reaction volume contained 1 X PCR amplification buffer (MBI Fermentas Inc, Amherst, NY 14226), 200 micro moles of each deoxynucleotides, 100 picomoles of each oligonucleotide primers and template DNA (0.25µg of purified DNA from 1µg/µl of stock solution) of 10 different bacterial isolates [B1-B10]. Amplification was carried out in a thermalcycler (Eppendorf Mastercycler® ep Systems, NorthAmerica) with heated lid (104° C) facility and was run with block temperature control (thermal regulation by 6^{o} C/Sec). Denaturation of template DNA was done for 5 min at 94^{o} C. After hot start, 2U of Taq DNA polymerase (MBI Fermentas, Amherst, NY 14226) was added. PCR was performed for amplification of 16S rRNA gene under specific thermal profile as follows; denaturation at 94^{o}C for 60 sec, annealing at 54^{o}C for 2 min and polymerization at 72^{o}C for 3 min for 36 cycles followed by final extension at 72^{o}C for 10 min. Five µl of amplified PCR product was resolved by electrophoresis on 1.2% agarose gel (Amresco, Ohio, USA.) and observed on a UV transilluminator (UVP, 3UV benchtop transilluminator, Canada). The remaining PCR product was stored at – 20° C for further work.

Purification of Amplified products

Amplified product was fractionated on 0.7% low melting agarose gel (Amresco, Ohio, 44139). PCR fragments were gel cleaved and purified using spin columns from Qiagen gel extraction Kit (Qiagen#28704, Canada). Finally DNA was eluted with elution Buffer EB (10 mM Tris-Cl; pH-8.5) and the purified products were stored at - 20° C. The product purity was ascertained by the presence of a single amplified band in agarose gel

electrophoresis and subsequently used for sequencing (ABI 3730xl DNA Analyzer, Applied biosystems, USA).

Gene sequencing and Homology search

16S rDNA sequences were blasted into the internet (http://www.ncbi.nlm.nih.gov/BLAST/) for similarity search. Isolated bacterial strains 16S rRNA gene sequences to be submitted to GenBank, USA.

Results and Discussions

DNA characteristics

Twelve bacterial strains were screened and isolated from municipal wastewater sludge dwelling microbial community for PHA production. Isolated Bacterial DNA was visualized in agarose gel (0.7%) electrophoresis and compared with known molecular weight markers. All bacterial genomic DNA bands were seen $\geq$ 23 kb marker band (Figure 1. Lane 2-7 & 9-14). Genomic DNA was gel purified and used for the direct amplification of 16S rRNA gene sequences.

16S rRNA gene amplification

PCR amplified 16S rDNA product from all the PHA accumulating bacterial *strains* was nearly similar in size (Figure 2) approximately 1.4 kb.

DNA sequencing

Gel purified 16S rDNA products were sequenced. The obtained 16S rDNA sequence data was compiled using DNASIS bioinformatics software and later subjected to BLASTn search. The sequence homology search results of PHA-M1 to PHA-M12 bacterial strains resembling 96-99% with existing DNA sequence database. Based on 16S rDNA sequence homology the bacterial strains were identified and presented in Table 1.

Molecular identification of PHA-accumulating bacterial strains present in sludge showed that four bacterial strains belonged to *Enterobacter* species (PHA-M1, 4 , 6 & 7), three bacterial strains belonged to *Bacillus* sp (PHA-M 2, 9 & 12), two strains belonged to *Microbacterium* sp (PHA-M 5 & 11), one strain belonged to *Serratia* sps, one strain belonged to *Klebsiella* sps and one strain belonged to *Bacillus* sp.

Gram straining results of PHA accumulating strains PHA-M1 to PHA-M12 were showed six bacterial strains belonged to gram negative and remaining six were gram positive. This result was further confirmed and comparable with molecular identification results. Maximum PHA accumulation was observed from gram negative bacterial cultures, which was varied from 13.33 to 27.50 (% w/w). Gram positive bacterial strains showed lower % of PHA accumulation than gram negative bacterial strains, which was varied from 3.25 to 23.05 (%w/w). This showed that major % of PHA obtained from activated sludge microorganisms were contributed by these gram negative bacterial strains. This similar findings were reported in literature elsewhere (Lee, 1996, Lee et al., 1999; Alias Z., and Tan, 2005.). Further, for the interest of augmenting PHA accumulation using activated sludge as a microbial inoculum, we can selectively enhance the growth of these gram negative bacterial strains in sludge, particularly by adding some growth inhibitors for gram positive bacteria, in order to obtain higher quantity of PHA accumulation from gram negative strains.

Isolated microorganisms showed PHA accumulation variation 3.25 to 27.50 (%w/w). The reason for different PHA production capabilities is due to these bacterial strains are different from each other at species and/or strains level. It is well known that, each and every bacterial strain possesses different metabolic rate and activity depends on their PHA accumulating genes and their necessity for their expression. Hence, these bacterial strains showed different percentage of PHA accumulations among them.

In this study bacterial strain, PHA-M3 (*Citrobacter* sp..) showed maximum percentage of PHA accumulation (27.50 % w/w).

Conclusions

Based on this study sludge possesses more number of bacterial strains belong to genus *Enterobacter*, *Bacillus* and *Microbacterium.* PHA accumulation results revealed that PHA-M3 bacterial strain identified as *Citrobacter* species was found as best strain when compared to other bacterial strains.

Acknowledgements

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Table 1. Molecular identification of bacterial strains isolated from wastewater sludge for PHA production.

S.No	Bacterial code	Molecular identification results	Gram staining results
1	PHA-M1	*Enterobacter* sp.	Negative
2	PHA- M 2	*Bacillus* sp.	Positive
3	PHA- M 3	*Citrobacter sp.*	Negative
4	PHA- M 4	*Enterobacter* sp.	Negative
5	PHA- M 5	*Microbacterium* sp.	Positive
6	PHA- M 6	*Enterobacter* sp.	Negative
7	PHA- M 7	*Enterobacter* sp.	Positive
8	PHA- M 8	*Serratia* sp.	Negative
9	PHA- M 9	*Bacillus* sp.	Positive
10	PHA- M 10	*Yersinia* sp.	Negative
11	PHA- M 11	*Microbacterium* sp.	Positive
12	PHA- M 12	*Bacillus* sp.	Positive

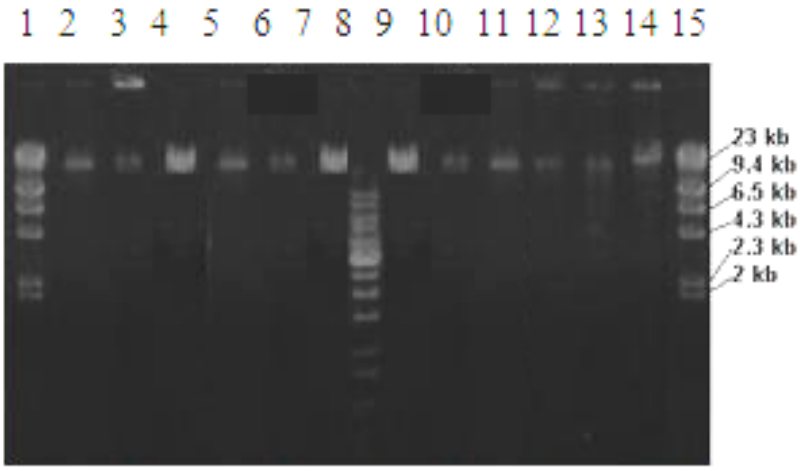

Figure 1. Genomic DNA isolated from the different PHA accumulating species. Lane (1 & 15) λ DNA Hind III digest marker, Lane (2-7 & 9-14) Bacterial genomic DNA from PHA-M1 to PHA-M12 and Lane (8) 1 Kb DNA molecular weight marker.

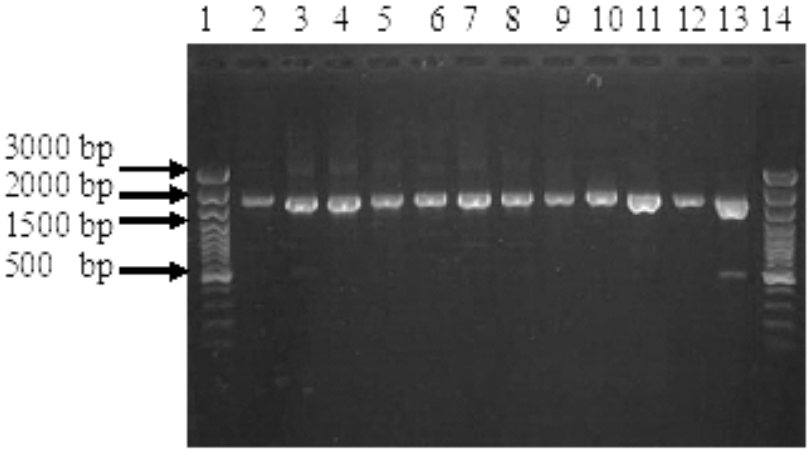

Figure 2. PCR amplification of 16S rDNA gene showing similar PCR product size (1.4 kb) in different PHA accumulating strains (PHA-M1 to PHA-M12). Left to Right lanes showing, Lane (1&14) 100 bp DNA marker and Lane (2-13) amplified PCR product from PHA-M1 to PHA-M12 bacterial strains.

Part V

Molecular screening of microbial strains from pulp and paper industry wastewater sludge for polyhydroxyalkanoate (PHA) production

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(to be submitted)

Résumé

Il a été constaté que des boues activées isolées d'une usine de traitement des eaux usées d'une industrie de pâtes et papier produit un taux de PHAs atteignant jusqu'à 60% du poids sec . En outre, six souches bactériennes accumulant des PHAs ont été isolées des boues et leurs capacités de la production de ces polymères ont été évaluées. De manière générale, toutes les souches isolées montrent une remarquable capacité d'utiliser l'acétate pour accumuler ces polymères; les quantités produites variant considérablement entre les six souches. Un contenu maximum de PHA de 35,45% a été obtenu avec la souche PHA - P5, même dans des conditions incontrôlées du pH et de la concentration en oxygène dissous. Ces micro-organismes ont été identifiés sur la base de la séquence de leur ADNr 16S. Ces dix souches bactériennes ont été identifiées comme *Microbacterium, Stenotrophomonas maltophilia, Acinetobacter* sp.*, Wautersiella falsenii et Comamonas* sp., respectivement, L'isolat PHA - P5 produisant le plus de PHAs étant identifié comme *Comamonas* sp..

Mots-clés: séquençage de l'ADN, micro-organismes, PCR, ARNr gène et production de PHA

Abstract

The mixed culture (activated sludge) from a pulp paper industry wastewater treatment plant was found to produce PHA of a maximum PHA content up to 60%. Furthermore, six PHA accumulating bacterial strains were isolated from the sludge and their capabilities of the PHA production had been evaluated. Though in general, all isolated strains showed a notable capacity to use acetate to accumulate PHA, the PHA amounts produced significantly varied among the six strains. The maximum PHA content of 35.45% was obtained by strain PHA-P5 even under uncontrolled conditions of pH and dissolved oxygen concentration. These sludge microorganisms were identified based on their 16S rDNA sequences and these ten bacterial strains were identified as *Microbacterium*, *Stenotrophomonas maltophilia*, *Acinetobacter* sp., *Wautersiella falsenii*, and *Comamonas* sps respectively. And the PHA-P5 with the maximum PHA production was identified as *Comamonas* sps.

Keywords: DNA sequencing, Microorganisms, PCR, rRNA gene and PHA production

Introduction

There are currently more than 300 different microbial species known to synthesize PHA. Little information is known about the microorganisms responsible for PHA accumulation of mixed culture (activated sludge) isolated from pulp and paper industry.

At present, only a few bacterial strains capable of accumulating PHA have been isolated from the Anaerobic/oxic (A/O) system (Vincenzini et al. 1997; Liu et al. 2000b; Sawayama et al. 2000). The most common isolate from the process is *Acinetobacter* sp.. *Acinetobacter* sp. could accumulate PHB up to 11.5% in cell dry weight basis after growth in a medium containing acetate as the carbon source (Vincenzini et al. 1997) Another isolate from the A/O system belongs to the new genus *Amaricoccus* from the alpha subdivision of Proteobacteria (Liu et al. 2000b).

While Dionisi and co-workers (Dionisi et al. 2006) stated that the dominant genera belong to *Thauera*, this organism was not described as being able to accumulate PHA and its capacity for PHA storage has not yet been unequivocally demonstrated.

In a recent study (Dionisi et al. 2006), after confirming the speciation of the population for PHA accumulation by DGGE, a clone library was constructed from the total DNA extracted from the SBR sludge. The screening of the clones was performed using amplified ribosomal DNA restriction analysis (ARDRA). However, the isolates have not yet been associated with PHA accumulation. Further investigation regarding the aforementioned microbial groups is necessary to certify their status as PHA producers.

A different approach was employed by Serafim et al. (2006). Using fluorescence in situ hybridization (FISH), the dominant organism present in a SBR operated under feast-and famine conditions gave a positive signal to the *Azoarcus* sp. probe. Using Nile blue staining, it was possible to correlate the presence of PHA inclusions with the *Azoarcus* sp., and thus confirm this organism as a PHA producer.

In our previous study, the PHA production by mixed culture (activated sludge) from a pulp paper industry wastewater treatment plant was found to reach a maximum PHA content up to 60%. Furthermore, six PHA accumulating bacterial strains were isolated from the sludge and their capabilities of the PHA production had been evaluated. Four of them were gram-negative, the remaining two were gram-positive. Though in general, all isolated strains showed a notable capacity to use acetate to accumulate PHA, the PHA amounts produced significantly varied among the six strains. The maximum PHA content of 35.45% was obtained by strain PHA-P5 even under uncontrolled conditions of pH and dissolved oxygen concentration.

Therefore, the aim of this work was to identify these six PHA producing bacterial strains based on their 16S rDNA sequences.

Materials and Methods

Activated sludge

Wastewater sludge samples were collected from Pulp-paper industry wastewater treatment plant -Papiers Stadacona (Québec). Microbial isolation was attempted on Plate Count Agar (PCA) and Sabouraud's Dextrose Agar (SDA) media using serial dilution techniques. Plates were incubated at 25°C for 72 hrs.

Bacterial strains

Bacterial strains used in this study are shown in Table 1. The obtained isolated pure culture bacterial strains were grown on Luria Bertani (LB) Agar (Difco, Canada.) for molecular identification studies.

Genomic DNA isolation

DNA was extracted from various PHA accumulating bacterial strains [PHA-P1 to PHA-P6] isolated from pulp and paper industrial wastewater sludge. Genomic DNA isolation procedure was used as described in earlier article (molecular identification of bacterial strains isolated from municipal wastewater sludge).

Polymerase Chain Reaction (PCR)

The genomic DNA isolated from six different PHA accumulating bacterial strains was used for direct amplification of 16S rRNA gene portions as described in earlier article.

Agareose gel electrophoresis and purification of Amplified products

Isolated genomic DNA and amplified PCR products were validated in agarose gel electrophoresis. Gel extraction of amplified products for DNA sequencing was carried out as described in previous article.

Gene sequencing and Homology search

16S rDNA sequences were blasted into the internet (http://www.ncbi.nlm.nih.gov/BLAST/) for similarity search. Isolated bacterial strains 16S rRNA gene sequences to be submitted to GenBank, USA.

Results and Discussion

Six PHA accumulating strains were screened from mixed microbial community dwelling in pulp and paper wastewater sludge. Isolated Bacterial DNA was visualized in agarose gel (0.7%) electrophoresis and compared with known molecular weight markers. All bacterial genomic DNA bands were seen $\geq$ 23 kb marker band (Figure-1. Lane 2-7 & 9-14). Genomic DNA was gel purified and used for the direct amplification of 16S rRNA gene sequences.

16S rRNA gene amplification

PCR amplified 16S rDNA product from all the PHA accumulating bacterial *strains* was nearly similar in size (Figure 2) approximately 1.4 kb.

DNA sequencing

Gel purified 16S rDNA products were sequenced. The obtained 16S rDNA sequence data was compiled using DNASIS bioinformatics software and later subjected to BLASTn search. The sequence homology search results of PHA-P1 to PHA-P6 bacterial strains resembling 96-99% with existing DNA sequence database. Based on 16S rDNA sequence homology the bacterial strains were identified and mentioned in Table 1.

Molecular identification of PHA-accumulating bacterial strains present in sludge showed that two bacterial strains belonged to *Microbacterium* species (PHA-P1 and PHA-P6), one bacterial strain belonged to *Stenotrophomonas maltophilia* (PHA-P2), one strain of *Acinetobacter* sp (PHA-P3), one strain of *Wautersiella falsenii* (PHA-P4), and one strain belonged to *Comomonas* sps (PHA-P5).

Out of six, four bacterial strains belonged to gram negative bacteria and remaining two were gram positive groups. These results were in accordance with that sludge microorganisms generally gram negative in nature as also indicated in literature (Brown and Lester, 1980). Gram positive bacterial strains showed percentage (%w/w) of PHA accumulation in a range of 15.10 to 29.06 (%w/w) and gram negative bacterial strains howed PHA accumulation in a range of 5.22 to 35.45 (% w/w). Also we found that gram negative bacterial strains were found to accumulate maximum percentage of PHA than gram positive organisms. The reason could be due to the fact that gram negative microorganisms commonly may possess PHA accumulating genetic machinery in their

genomes. These results were in accordance with our earlier findings in municipal wastewater sludge as a microbial inoculum to produce PHA.

We observed that PHA accumulation was higher while using pulp and paper activated sludge as microbial inoculum than the municipal wastewater sludge. And also the same trend was observed while using isolated individual pure bacterial strains from pulp and paper wastewater sludge which showed maximum PHA accumulation activity. The reason was due to well acclimatization of bacterial strains in sludge also the composition of sludge favoured the higher PHA accumulation in pulp and paper wastewater sludge.

Also we identified two new bacterial strains found to accumulate PHA such as *Stenotrophomonas maltophilia* and *Wautersiella falsenii* which was first reported in this study. More interestingly *Stenotrophomonas maltophilia* strain was reported to degrade the PHA, but in this study we found this strain to accumulate high quantity of PHA. This could be favourable action of sludge which provided suitable conditions for gene transfer for PHA accumulation in this particular strain.

Microorganisms isolated from pulp and paper wastewater sludge showed better PHA accumulation than microorganisms reported from literature (Table-1). This shows for maximum production of PHA we can use activated sludge as microbial inoculum and/or as isolated individual bacterial strains as inoculum.

Municipal wastewater sludge possesses more number of PHA accumulating bacterial strains (twelve strains) than the pulp and paper (six bacterial strains) wastewater sludge. But pulp and paper wastewater sludge microbial community found to accumulate maximum PHA content irrespective of their growth conditions, such as growing in a consortium and individual microorganisms.

In terms of individual microorganisms, municipal and pulp and paper activated sludge commonly possesses *Microbacterium* species only (*Microbacteriaceae* family - Actinobacteria class), remaining all bacterial strains are entirely different from each other. Also comparison of 16S rRNA gene sequences among these *Microbacterium* species showed significant different to be a different species and/or strains.

Most importantly municipal wastewater sludge microbial community mainly belongs to *Enterobacteriaceae* family - Gamma proteobacteria (*Enterobacter* sp., *Serratia* sp., *Klebsiella sp* and Yersinia sps), which is mostly originated from faecal matter. Hence these bacterial strains were not showing maximum PHA accumulation than the microbial community from pulp and paper wastewater sludge. Pulp and paper wastewater sludge possesses mainly *Moraxellaceae* family - Gamma proteobacteria (*Acinetobacter* sp), *Comamonadaceae* family - beta proteobacteria

Further to enhance maximum PHA production we can carry out fermentation under optimized conditions in a bioreactor, which could render even higher accumulation of PHA.

Conclusions

Gram negative bacterial strains showed maximum PHA accumulation. PHA-P5 strain was identified as *Comomonas* sp and found to accumulate maximum PHA than other individual bacterial strains isolated from activated sludge.

Acknowledgements:

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Table 1. Comparison of the isolates strains for PHA production.

S.No	Bacterial code	Molecular identification results	PHA content obtained in this study (%)	Reported PHA production %	Gram staining results	Ref.
1	PHA-P1	*Microbacterium sp*	*29.06*	*15.10*	Positive	
2	PHA-P2	*Stenotrophomonas* sp.	*23.37*	A few *Stenotrophomonas* strains are reported to degrade poly(3-hydroxybutyrate), copolymers with 3-hydroxyvalerate and other bacterial and synthetic plastics and polymers have been reported.	Negative	(SWINGS et al., 1993; MERGAERT and SWINGS, 1996)
3	PHA-P3	*Acinetobacter* sp.	*31.81*	% of PHA accumulation was not mentioned	Negative	
4	PHA-P4	*Wautersiella* sp.	*5.22*	*No reports available*	Negative	
5	PHA -P5	*Comamonas* sp	*35.45*	1-2% of cell dry weight	Negative	Nakamura S, Doi Y, Scandola M (1992)
6	PHA -P6	*Microbacterium* sp	*15.10*	% of PHA accumulation was not mentioned	Positive	Hollender et al. (2002)

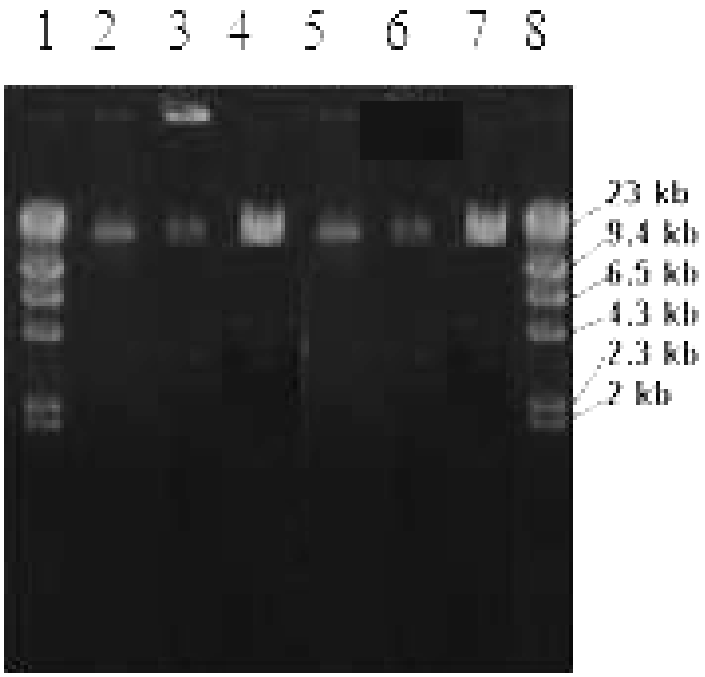

Figure 1. Shows the Genomic DNA isolated from the different PHA accumulating species. Lane (1 & 8) λ DNA Hind III digest marker, Lane (2-7) Bacterial genomic DNA from PHA-P1 to PHA-P6.

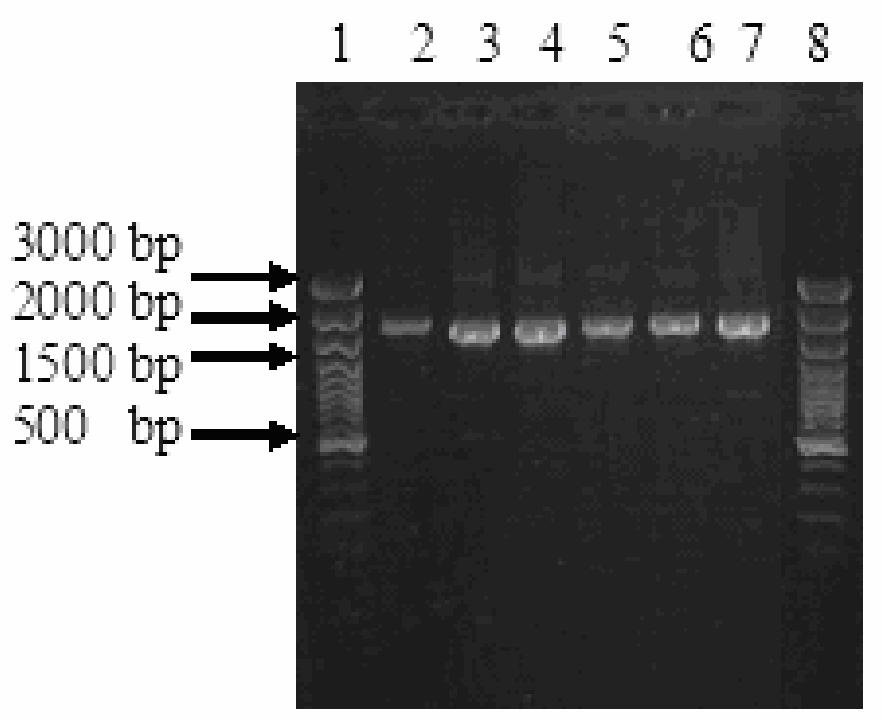

Figure 2. PCR amplification of 16S rDNA gene showing similar PCR product size (1.4 kb) in different PHA accumulating strains (PHA-P1 to PHA-P6). Left to Right lanes showing, Lane (1&8) 100 bp DNA marker and Lane (2-7) amplified PCR product from PHA-P1 to PHA-P6 bacterial strains.

CHAPITRE 5.

CONCLUSIONS ET RECOMMANDATIONS

5.1. Conclusions

Les travaux de R&D réalisés au cours de cette étude concernant la production de PHAs en utilisant des microorganismes présents dans des boues activées et des eaux usées comme substrats de fermentation, ont permis d'obtenir les résultats suivants :

5.1.1 Chapitre 2. Production de PHAs par des cultures pures ou des boues activées

Des biopolymères sont couramment accumulés comme réserves d'énergie par une grande variété de bactéries; Sous certaines conditions de culture, certaines pouvant en accumuler jusqu'à 70% en poids sec des cellules. Le polyhydroxybutyrate (PHB) dérivant de l'acétyl-CoA , représente le PHA le plus abondant dans la nature; Ces biopolymères partageant de nombreuses propriétés avec les plastiques conventionnels tel que le polypropylène. Afin de protéger l'environnement, certaines industries développent des stratégies pour produire des plastiques biodégradables. Dans ce contexte, de nombreuses méthodes sont utilisées pour développer ce secteur, en particulier l'isolement de nouvelles souches de microorganismes capables d'accumuler des concentrations plus élevées de polymère. Par ailleurs, afin de réduire le coût de production de PHAs, de nombreux déchets sont employés comme supports pour la croissance de ces bactéries.

La production de PHAs par des cultures mixtes en utilisant des matériaux moins onéreux et moins chers, des équipements non stériles permet de réduire significativement les coûts des processus d'obtention des bioplastiques. À ce propos , le procédé employant des cultures mixtes a un bon potentiel pour la production de PHAs et il sera encore meilleur si le taux de synthèse de ces polymères peut être augmenté. Dans cette optiuqe, les résultats de nombreuses études démontrent que les cultures mixtes produisant des PHAs ont une grande capacité de stockage de ces bioplastiques, des rendements élevés, et une haute productivité de PHAs spécifiques. La teneur maximale intracellulaire de PHA signalé est de 65% du poids sec des cellules, ce qui est comparable à ce qui est obtenu avec certaines cultures pures. Par contre, ce taux est inférieur à celui produit par des E.coli recombinants,; Cette teneur atteignant environ 90% du poids sec des cellules. En

utilisant des cultures mixtes, le rendement en polymère (0,56 g de PHB. G de - 1 substrat) et la spécificité de productivité (0,77 g de PHB. G de - 1 cellule de poids sec. H 1) obtenus à ce jour sont très prometteurs. Cette spécificité des cultures mixtes est d'environ dix fois plus élevée que celle des E.coli recombinants. Par contre, la productivité volumétrique en PHA obtenue avec des cultures mixtes est toujours plus faible que celle de la plupart des cultures pures. Cela est probablement dû aux difficultés d'atteindre des concentrations élevées de biomasse dans le procédé avec des cultures mixtes. Par conséquent, le principal défi en ce qui concerne les méthodes de culture est de développer des stratégies de croissance rapide des microorganismes qui sont, dans le même temps, capables d'accumuler une grande quantité de PHAs; Les propriétés physiques de ces polymères produits par des cultures mixtes issues de boues activées étant comparables aux bioplastiques synthétisés par des cultures pures. Par rapport aux données présentes dans la littérature concernant les cultures pures, celles relatives aux mixtes sont limitées en terme de croissance, de stratégies de production et de contrôle afin d'améliorer le taux de PHAs contenu dans les cellules. De nombreux défis de recherche et de développement doivent donc être encore relevés.

5.1.2 Chapitre 3: Obtentention de PHAs en utilisant des micro-organismes présents dans des boues activées et des eaux usées comme substrats en erlenmeyers ou en fermenteur.

Des boues activées provenant d'usines de traitement d'eaux usées (municipales, industrie de pâtes et papiers, fabrication d'amidon et fromageries) ont été employées comme sources de microorganismes pour produire en erlenmeyers, des plastiques biodégradables. L'acétate, le glucose et différentes eaux usées ont été utilisés comme sources de carbone. Il a été constaté que des boues d'épuration de pâtes et papier accumulent le plus de PHAs , soit à une concentration maximale de 43% en poids sec des matières solides en suspension avec de l'acétate comme source de carbone.

Les effets de différents modes d'alimentation (Batch, Fed-batch, alimentation continue) ont été examinés en utilisant des boues activées d'usines de traitement des eaux usées d'industries de pâtes et papier, en tant que sources de microorganismes pour produire des

PHAs, en fermenteurs contrôlés par ordinateur. Les résultats en mode Batch ont montré que l'accumulation maximale de PHAs (39,6% m / m de boues sèches) a été obtenue avec une concentration d'acétate de 15 g / l de SS et 10 g / l ; Une concentration plus élevée de ce substrat inhibant la croissance ainsi que l'accumulation de biomasse et de PHAs.

L'addition continue d'acide acétique a augmenté les contenus en PHAs et la productivité. Il a aussi été observé qu'un rapport de C: N de 144 permet d'atteindre le plus haut taux de production et de contenu en bioploymères. Ce taux maximal étant de 60,9% de la masse sèche des boues et une concentration de bioplsatiques de 16,23 g / l . Les eaux usées d'industries de pâtes et papier permettant le plus haut taux en PHA (54,9%) parmi quatre types d'eaux usées. Ce procédé est une méthode prometteuse pour réduire le coût de production de PHA ainsi que le recyclage de déchets organiques en produits à valeur ajoutée. Une quantité importante de boues pourrait être réduite, réduisant d'autant les rejets devant être éliminés. Des copolymères (polyhydroxybutyrates - PHB et polyhydroxyvalérates - PHV) et dont la concentration molaire varie ont été produits par batchs par des boues activées.

5.1.3 Chapitre 5. Isolement et identification des souches bactériennes présentes dans des boues activées et produisant des PHAs.

Six souches bactériennes accumulant des PHAs ont été isolées des boues de pâtes et papiers et leurs capacités de synthèse de ces biopolymères ont été évaluéss. Les quantités produites variaient considérablement d'un isolat à l'autre. Un maximum de PHA de 35,45% en poids sec a été obtenu avec la souche PHA - P5, même sans contrôle du pH et dela concentration en oxygène dissous. Ces micro-organismes présents dans les boues ont été identifiés sur la base de la séquence de leur ADNr 16S et l'isolat PHA - P5 avec le maximum de production de PHA a été identifié comme Comamonas SPS.

Par ailleurs,douze souches bactériennes accumulant des PHAsont été isolées des boues municipales et un taux maximal de 27,5% en pHA (en poids sec des cellules) a été obtenu avec l'isolat PHA - M3. Ces micro-organismes des boues ont également été identifiés sur

la base de la séquence de leurADNr 16S séquences et PHA - M3 synthétisant le maximum de PHA a été identifié comme Klebsiella sps.

Ce procédé actuel utilisant les eaux usées et des boues activées pour l'obtention de PHAs est un moyen prometteur pour réduire le coût de production de ces bioplastiques ainsi que pour recycler des déchets organiques en produits à valeur ajoutée.

5.2. Recommandations

Le présent travail de recherche a été mené en vue de développer un processus économiquement réalisable pour produire des bioplastiques en utilisant des microorganismes présents dans des boues activées et des eaux usées comme substrat de fermentation. Gràce aux résultats obtenus, il est possible de recommander ce qui suit :

1. Les effets combinés des paramètres du procédé tels que le pH, la température et la concentration d'oxygène dissous devraient être étudiés pour atteindre une production de PHAs la plus élevée possible.

2. L'emploi d'une grande variété de déchets comme substrat pour produire des PHAs doit faire l'objet d'une étude exhaustive, en particulier les rejets qui contiennent de fortes concentrations d'acides gras volatils.

3. Une étude systématique à une échelle pilote de fermentation est absolument requise pour une évaluation économique précise des coûts d'obtention des PHAs en employant des boues activées et des eaux usées.

4. L'efficacité et les rendements de production de PHAs par des isolats de cultures pures et des isolats provenant de boues activées municipales et industrielles doivent être étudiés sytématiquement, compte tenu de leur fort potentiel d'obtention de PHAs.

ANNEXES

Annexe – I

Données

Chapitre 3 Étude de la production de polyhydroxyalkanoate (PHA) en utilisant les eaux usées comme source de carbone et les boues activées comme source de microorganismes

Part II Polyhydroxyalkanoates (PHA) production using activated sludge from a pulp-paper industry wastewater treatment plant-batch experiments

Figure 1. Profiles of acetate concentrations during batch process of PHA production at different sludge solids and acetate concentrations.

Figure 2. Profiles of PHA concentrations during batch process at different sludge solids and acetate concentrations

Figure 3. Profiles of PHA content (w/w%) of dry sludge SS during batch experiments at different sludge solids and acetate concentrations.

Figure 4. Profiles of sludge dry weight (biomass) (g/l) change during batch process of PHA production at different sludge SS and acetate concentrations

Bioreactor experiments (batch experiment)

5 g/l PAS+5 g/Ac-/l total at beginning

time (h)	PHA content(%)	sludge dry weight(g/l)	PHA con. (g/l)	Ac-(g/l)
0	4,6	4,322	0,197	5,70
3	7,8	4,727	0,368	4,75
6	12,5	4,752	0,596	4,38
9	14,7	5,029	0,738	3,86
12	21,0	5,276	1,108	3,55
21	24,5	5,147	1,262	2,63
24	27,7	5,252	1,453	1,78
27	28,7	5,332	1,530	0,76
30	28,6	5,244	1,500	0,51
36	29,7	5,801	1,723	0,00
45	21,8	6,277	1,368	0,00
48	20,8	6,347	1,319	0,00
51	16,9	6,277	1,060	0,00
57	13,7	6,270	0,856	0,00
60	12,7	6,265	0,792	0,00

5 g/l PAS+7.5 g/Ac-/l total at beginning

time (h)	PHA content(%)	sludge dry weight(g/l)	PHA con. (g/l)	Ac-(g/l)
0	6,52	5,062	0,330	7,824
3	8,91	5,262	0,469	7,221
6	13,13	5,309	0,697	6,658
9	15,06	5,340	0,804	4,874
12	22,76	5,385	1,226	4,322
21	25,23	5,565	1,404	3,974
24	28,16	5,577	1,570	2,734
27	30,72	6,044	1,857	2,284
30	30,97	6,410	1,985	1,274
36	32,07	6,327	2,029	1,034
45	31,80	6,257	1,990	0,674
48	25,78	6,250	1,611	0
51	22,18	6,245	1,385	0
57	19,43	6,241	1,213	0
60	13,74	6,228	0,856	0

5 g/l PAS+10 g/Ac-/l total at beginning

time (h)	PHA content(%)	sludge dry weight(g/l)	PHA con. (g/l)	Ac-(g/l)
0	10,0	4,5425	0,4543	10,20
3	11,0	4,9475	0,5442	9,50
6	18,0	4,9725	0,8951	8,76
9	20,0	5,2500	1,0500	7,10
12	25,0	5,4975	1,3744	6,50
21	28,0	5,3675	1,5029	5,26
24	32,5	5,2725	1,7136	5,01
27	27,9	5,5525	1,5491	4,20
30	25,4	5,2650	1,3352	3,56
36	26,4	6,0225	1,5899	4,20
45	25,6	6,4975	1,6634	1,00
48	21,3	6,3150	1,3451	0,90
51	19,8	6,2450	1,2365	0,49
57	20,1	6,2375	1,2526	0,00
60	19,2	6,2325	1,1966	0,00

Bioreactor experiments (batch experiment)

10 g/l PAS+10 g/Ac-/l total at beginning

time (h)	PHA content(%)	sludge dry weight(g/l)	PHA con. (g/l)	Ac-(g/l)
0	12,0	10,423	1,25	10,03
3	14,0	10,828	1,52	9,23
6	16,0	10,953	1,75	8,23
9	17,0	11,130	1,89	7,95
12	21,0	11,377	2,39	7,02
21	20,0	11,248	2,25	6,31
24	23,0	11,153	2,57	5,20
27	25,0	11,433	2,86	4,01
30	29,0	11,145	3,23	3,25
36	32,1	11,902	3,82	3,05
45	33,1	12,378	4,10	2,01
48	34,1	12,195	4,16	0,59
51	32,0	12,125	3,88	0,02
57	29,8	12,118	3,61	0,00
60	28,2	12,433	3,51	0,00

10 g/l PAS+15 g/Ac-/l total at beginning

time (h)	PHA content(%)	sludge dry weight(g/l)	PHA con. (g/l)	Ac-(g/l)
0	9,8	10,1075	0,99	15,27
3	11,1	10,1580	1,13	13,85
6	16,2	10,2450	1,66	11,54
9	17,8	10,5200	1,87	10,47
12	18,8	10,6275	1,99	9,94
21	18,0	11,2850	2,03	8,75
24	18,5	11,2325	2,08	7,34
27	22,5	11,3300	2,55	5,89
30	27,9	12,0075	3,35	5,01
36	30,2	11,2475	3,40	5,06
45	33,2	12,2975	4,08	4,01
48	32,4	12,9450	4,20	3,50
51	32,4	12,3600	4,01	3,30
57	28,0	12,6825	3,55	2,00
60	26,2	13,0425	3,42	1,28

10 g/l PAS+20 g/Ac-/l total at beginning

time (h)	PHA content(%)	sludge dry weight(g/l)	PHA con. (g/l)	Ac-(g/l)
0	8,6	10,245	0,88	20,19
3	9,1	10,313	0,94	18,74
6	14,2	10,465	1,49	16,32
9	15,3	10,120	1,55	15,36
12	17,5	10,195	1,78	14,87
21	17,9	11,610	2,08	14,67
24	18,2	10,953	1,99	12,26
27	21,3	11,113	2,36	10,82
30	29,5	12,800	3,77	9,93
36	28,93	10,995	3,18	9,99
45	28,14	12,678	3,57	8,93
48	28,16	12,973	3,65	8,43
51	23,71	12,590	2,99	8,23
57	21,93	13,215	2,90	7,92
60	21,62	13,418	2,90	7,20

15 g/l PAS+10 g/Ac-/l total at beginning

time (h)	PHA content(%)	sludge dry weight(g/l)	PAH con. (g/l)	Ac-(g/l)
0	11,0	14,5550	1,6011	9,84
3	16,0	14,8605	2,3777	8,51
6	19,0	14,8850	2,8282	6,92
9	20,0	15,2625	3,0525	6,41
12	22,0	15,5105	3,4123	6,35
21	23,0	15,3805	3,5375	4,51
24	23,6	15,2850	3,6075	4,41
27	29,0	15,5650	4,5139	3,75
30	33,2	15,2775	5,0721	3,37
36	35,2	16,0350	5,6443	1,13
45	38,0	16,5105	6,2740	0,00
48	39,6	16,3275	6,4657	0,00
51	32,1	16,2575	5,2187	0,00
57	29,8	16,2505	4,8426	0,00
60	29,5	16,2565	4,7957	0,00

15 g/l PAS+15 g/Ac-/l total at beginning

time (h)	PHA content(%)	sludge dry weight(g/l)	PHA con. (g/l)	Ac-(g/l)
0	9,4	14,325	1,34	15,97
3	12,4	15,335	1,91	13,39
6	14,5	15,588	2,26	11,08
9	15,5	15,308	2,37	9,82
12	17,5	15,325	2,68	9,29
21	19,5	15,330	2,99	8,09
24	22,1	15,500	3,43	6,68
27	25,6	15,553	3,98	5,23
30	28,9	15,553	4,49	4,35
36	32,9	16,065	5,28	4,41
45	38,2	15,662	5,98	3,35
48	36,3	15,717	5,71	2,85
51	35,7	15,043	5,38	2,65
57	34,4	15,333	5,28	1,05
60	31,1	15,288	4,76	1,05

15 g/l PAS+30 g/Ac-/l total at beginning

time (h)	PHA content(%)	sludge dry weight(g/l)	PHA con. (g/l)	Ac-(g/l)
0	10,4	14,608	1,52	30,87
3	11,2	16,510	1,86	29,42
6	14,4	16,950	2,44	27,00
9	14,5	16,345	2,38	26,03
12	18,1	16,900	3,07	25,52
21	19,4	16,668	3,24	25,33
24	20,1	16,240	3,26	22,94
27	25,4	17,098	4,34	21,47
30	29,6	16,515	4,89	20,61
36	31,6	16,743	5,29	20,66
45	34,4	16,385	5,64	19,60
48	35,1	16,628	5,84	19,10
51	32,9	16,538	5,43	18,94
57	26,3	16,440	4,32	18,60
60	25,9	16,398	4,25	17,88

20g/l PAS+10 g/Ac-/l total at beginning

time (h)	PHA content(%)	sludge dry weight(g/l)	PHA con. (g/l)	Ac-(g/l)
0	9,8	20,5545	2,01	10,15
3	10,2	20,9595	2,14	7,54
6	12,3	20,9845	2,58	6,78
9	15,6	21,2620	3,32	5,31
12	19,0	21,5095	4,09	4,53
21	22,1	21,3795	4,72	3,21
24	24,6	21,2845	5,24	3,10
27	28,1	21,5645	6,06	2,68
30	30,1	21,2790	6,40	1,25
36	32,1	22,0365	7,07	0,00
45	31,0	22,5115	6,98	0,00
48	28,2	22,3290	6,30	0,00
51	27,3	22,2590	6,08	0,00
57	26,5	22,2515	5,90	0,00
60	26,1	22,2265	5,80	0,00

20 g/l PAS+20 g/Ac-/l total at beginning

time (h)	PHA content(%)	sludge dry weight(g/l)	PHA con. (g/l)	Ac-(g/l)
0	5,35	18,768	1,00	20,90
3	5,67	17,755	1,01	16,28
6	7,37	18,480	1,36	13,87
9	10,05	17,905	1,80	12,90
12	12,80	17,735	2,27	12,41
21	16,32	17,300	2,82	11,21
24	23,34	16,738	3,91	9,60
27	25,18	17,668	4,45	8,84
30	29,30	16,730	4,90	7,77
36	28,42	17,510	4,98	7,73
45	26,53	18,710	4,96	6,67
48	24,26	18,800	4,56	5,97
51	21,53	17,340	3,73	5,77
57	19,88	17,895	3,56	5,47
60	19,56	17,650	3,45	4,75

20 g/l PAS+30 g/Ac-/l total at beginning

time (h)	PHA content(%)	sludge dry weight(g/l)	PHA con. (g/l)	Ac-(g/l)
0	3,22	19,3	0,622	31,45
3	3,54	18,3	0,647	26,83
6	5,24	18,4	0,965	24,41
9	7,92	17,9	1,418	23,44
12	10,67	18,1	1,928	22,95
21	13,18	17,5	2,308	21,75
24	15,21	17,2	2,618	20,15
27	22,05	18,2	4,011	19,38
30	24,67	17,4	4,291	18,32
36	25,89	19,1	4,934	18,27
45	24,40	18,5	4,519	18,21
48	22,13	17,3	3,832	17,51
51	21,40	18,3	3,913	16,31
57	21,75	17,5	3,812	16,01
60	21,43	17,8	3,813	15,29

Figure 5. Profiles of mol HB/HA ratio during batch process of PHA production at different sludge SS and acetate concentration 7

5 g/l PAS+5 g/Ac-/l total at beginning		10 g/l PAS+10 g/Ac-/l total at beginning	
time (h)	HB/HA%	time (h)	HB/HA%
0	24,5	0	26,8
3	27,7	3	34,5
6	30,1	6	36,2
9	33,7	9	42,7
12	42,1	12	45,3
21	50,0	21	50,3
24	53,4	24	59,0
27	54,6	27	62,5
30	56,9	30	62,5
36	64,6	36	67,4
45	66,7	45	68,6
48	73,0	48	76,8
51	68,1	51	72,1
57	62,9	57	65,8
60	61,8	60	62,0

5 g/l PAS+7.5 g/Ac-/l total at beginning		10 g/l PAS+15 g/Ac-/l total at beginning	
time (h)	HB/HA%	time (h)	HB/HA%
0	24,4	0	28,0
3	37,7	3	30,6
6	44,9	6	35,8
9	54,4	9	37,2
12	58,9	12	38,8
21	66,0	21	47,5
24	69,9	24	52,9
27	70,0	27	53,0
30	69,4	30	53,5
36	72,9	36	54,5
45	80,0	45	55,8
48	82,6	48	55,9
51	85,5	51	57,7
57	100,0	57	59,9
60	86,1	60	64,2

5 g/l PAS+10 g/Ac-/l total at beginning		**10 g/l PAS+20 g/Ac-/l total at beginning**	
time (h)	HB/HA%	time (h)	HB/HA%
0	20,0	0	24,5
3	30,8	3	27,0
6	36,5	6	36,4
9	38,8	9	36,2
12	50,5	12	38,3
21	52,0	21	47,7
24	54,2	24	48,0
27	54,7	27	49,3
30	65,5	30	49,6
36	67,1	36	51,5
45	79,0	45	51,5
48	100,0	48	56,8
51	83,2	51	56,9
57	75,6	57	60,9
60	71,4	60	63,1

15 g/l PAS+10 g/Ac-/l total at beginning		**20g/l PAS+10 g/Ac-/l total at beginning**	
time (h)	HB/HA%	time (h)	HB/HA%
0	22,7	0	36,1
3	22,7	3	40,3
6	34,2	6	54,8
9	37,8	9	60,5
12	45,5	12	70,9
21	46,2	21	74,9
24	58,2	24	95,0
27	64,1	27	95,0
30	71,9	30	95,0
36	69,9	36	95,0
45	68,9	45	95,0
48	57,7	48	87,0
51	56,7	51	87,7
57	53,0	57	85,0
60	50,1	60	84,0

15 g/l PAS+15 g/Ac-/l total at beginning		**20 g/l PAS+20 g/Ac-/l total at beginning**	
time (h)	HB/HA%	time (h)	HB/HA%
0	25,8	0	24,4
3	26,8	3	32,5
6	39,0	6	42,4
9	43,9	9	52,4
12	47,7	12	56,9
21	51,3	21	67,0
24	62,3	24	69,9
27	67,2	27	70,0
30	70,1	30	72,4
36	74,1	36	72,9
45	75,1	45	82,0
48	64,8	48	82,6
51	61,8	51	95,0
57	57,1	57	95,0
60	54,2	60	88,1

15 g/l PAS+30 g/Ac-/l total at beginning		**20 g/l PAS+30 g/Ac-/l total at beginning**	
time (h)	HB/HA%	time (h)	HB/HA%
0	23,5	0	22,7
3	26,0	3	23,7
6	36,4	6	31,6
9	39,2	9	33,0
12	39,3	12	40,5
21	47,7	21	45,2
24	50,0	24	56,2
27	50,3	27	62,1
30	50,6	30	72,6
36	51,5	36	75,8
45	52,5	45	67,4
48	57,8	48	55,5
51	58,9	51	54,6
57	62,9	57	52,0
60	67,1	60	52,0

Part III impact of feeding pattern on polyhydroxyalkanoates (PHA) production using activated sludge from a pulp-paper industry wastewater treatment plant

Figure 1. Profiles of acetate, PHA, sludge SS concentrations and PHA content (w/w% of SS) during fed-batch experiments at sludge SS concentration of 15 g/l with different acetate feeds

fed-batch mode; 15 g/l PAS+15 gAc-/l total

(10gAc-/l at beginning+5 g/l Ac- at 12hr]

time (h)	PHA content(%)	sludge dry weight(g/l)	PHA con. (g/l)	Ac- (g/l)
0	13,4	14,918	2,001	10,51
3	15,9	15,080	2,401	10,42
6	24,8	15,083	3,736	9,79
9	25,8	15,068	3,882	8,98
12	26,7	15,120	4,033	8,49
12	27,7	15,120	4,187	12,93
21	32,9	15,338	5,051	10,65
24	32,3	15,583	5,035	10,03
27	35,3	15,965	5,629	8,57
30	34,6	16,512	5,720	7,25
36	40,4	16,375	6,616	6,89
45	44,2	15,223	6,728	6,87
48	39,5	15,179	5,996	6,86
51	37,3	15,074	5,622	6,85
57	34,1	14,943	5,095	6,85
60	33,2	14,911	4,950	6,84

fed-batch mode:15 g/l PAS+17.5 gAc-/l total

[10gAc-/l at beginning+5 g/l Ac- at 12hr+ 2.5 g Ac-/l at 24hr]

time (h)	PHA content(%)	sludge dry weight(g/l)	PHA con. (g/l)	Ac- (g/l)
0	10,3	14,725	1,509	10,46
3	15,4	16,474	2,531	8,06
6	17,5	16,499	2,886	7,79
9	23,7	16,708	3,960	6,68
12	23,8	16,725	3,979	6,49
12	24,7	16,730	4,136	11,15
21	27,9	16,900	4,714	9,67
24	26,7	16,953	4,535	9,29
24	27,6	16,953	4,677	12,42
27	27,1	17,465	4,725	12,03
30	27,2	17,306	4,699	11,64
36	36,5	17,117	6,241	10,71
45	39,6	17,044	6,743	6,49
48	44,5	16,733	7,448	6,04
54	49,5	16,688	8,260	4,01
60	46,6	16,280	7,590	2,42
69	43,6	17,158	7,483	2,79

four feeds: 15 g/l PAS+20 gAc-/l total [adding 10, 5, 2.5, and 2.5 gAc-/l at 0,12,24,36 hrs, respectively)

time (h)	Ac-(g/l)
0	10,00
3	6,78
6	6,42
9	4,93
12	4,68
12	10,25
21	8,27
24	7,77
24	10,41
27	9,89
30	9,37
36	8,12
36	10,58
45	4,92
48	4,31
54	1,60
60	0,00

time (h)	sludge(g/l)	PHA(g/l)	PHA(%)
0	15,4171	1,033	6,7
3	15,7144	1,943	12,4
6	15,7796	2,760	17,5
9	16,3052	4,692	28,8
12	16,3139	5,186	31,8
21	17,5051	6,108	34,9
24	17,9220	6,962	38,8
27	17,9262	7,763	43,3
30	18,3498	8,236	44,9
36	19,0131	8,241	43,3
45	19,0603	8,598	45,1
48	19,0614	9,180	48,2
54	19,9760	10,048	50,3
60	19,7599	8,498	43,0

Figure 2. Profiles of acetate, PHA, sludge SS concentrations and PHA content (w/w% of SS) during fed-batch experiments at sludge SS concentration of 20 g/l with different acetate feeds

20 g/l PAS+17.5 gAc-/l total [adding 10gAc-/l at T=0, add 5 Ac- at T=12h, and add Ac- of 2.5 at T=24, respectively)

time(h)	Ac-	time (h)	PHA(%)	biomass (g/l)	PHA(g/l)
0	9,824	0	11,3	18,370	2,07
3	9,521	3	13,6	18,475	2,50
6	9,400	6	15,7	18,500	2,90
9	9,000	9	17,9	18,541	3,31
12	8,300	12	19,6	18,590	3,65
12	13,700	21	21,3	18,650	3,97
21	12,541	24	22,5	18,862	4,24
24	11,146	27	23,9	18,964	4,53
24	15,581	30	26,6	18,976	5,06
27	15,164	36	31,9	19,057	6,07
30	14,218	45	30,6	18,852	5,77
36	12,654	48	29,3	18,653	5,47
45	11,397	51	27,8	18,577	5,16
48	9,888	54	26,9	18,570	5,00
51	7,334	60	26,6	18,538	4,94
54	4,073				
60	1,192				
69	0,000				

20 g/l PAS+20 gAc-/l total [adding 10, 5, 2.5, and 2.5 gAc-/l at 0,12,24,36 hrs, respectively)

time(h)	Ac-
0	9,273
3	7,031
6	6,825
9	6,597
12	6,157
12	12,066
21	10,964
24	10,613
24	13,902
27	13,825
30	14,044
36	13,179
36	16,196
45	14,408
48	13,695
51	11,167
54	6,434
60	1,269

	time(h)	PHA(%)	biomass (g/l)	PHA(g/l)
0	0	12,0	18,590	2,22
3	3	13,1	18,700	2,45
6	6	19,3	18,780	3,63
9	9	20,0	18,816	3,77
12	12	22,1	18,854	4,16
21	21	25,6	18,874	4,83
24	24	27,1	18,864	5,10
27	27	29,6	18,965	5,61
30	30	33,0	19,055	6,29
36	36	35,8	19,248	6,89
45	45	42,0	19,253	8,08
48	48	35,3	19,200	6,77
51	51	34,0	19,123	6,50
54	54	27,1	18,835	5,11
60	60	25,5	18,720	4,78

Figure 3. Profiles of acetate, PHA, sludge SS concentrations and PHA content (w/w% of SS) during continuous feed experiment at sludge SS concentration of 15 g/l.

15 g/l PAS+10gAc-/l at beginning+HAc- at 24hr continuing+HAc till 60 h

Time(h)	PHA(%)	Biomass(g/l)	PHA(g/l)	Ac-(g/l)
0	14,71	15,632	2,300	9,651
3	16,48	15,837	2,610	9,369
6	19,87	15,883	3,156	8,413
9	24,32	16,245	3,951	7,458
12	26,14	16,130	4,216	6,369
21	29,78	17,185	5,118	3,100
24	31,52	17,073	5,381	0
27	45,00	17,360	7,812	0
30	51,98	16,905	8,787	0
36	57,65	17,655	10,178	0
45	36,00	18,113	6,521	0
48	32,19	18,145	5,840	0
51	29,61	17,838	5,281	0
57	29,67	18,628	5,526	0
57	29,55	18,628	5,504	0
60	30,85	18,505	5,709	0

Figure 4. Profiles of HB/HA(mol%) during fed-batch experiment at sludge SS concentration of 15 and 20 g/l.

15 g/l PAS+15 g/Ac-/l with two feeds	
time (h)	HB/HA%
0	25,9
3	27,3
6	37,4
9	46,0
12	48,5
21	49,9
24	62,2
27	65,3
30	76,3
36	76,8
45	73,4
48	62,5
51	58,9
57	57,1
60	53,6

15 g/l PAS+17.5 g/Ac-/l with three feeds	
time (h)	HB/HA%
0	22,6
3	24,5
6	41,0
9	42,1
12	48,6
21	53,6
24	66,5
27	66,8
30	78,6
36	78,1
45	78,1
48	80,5
51	71,8
57	67,1
60	64,2

15 g/l PAS+20 g/Ac-/l with four feeds

time (h)	HB/HA%
0	28,5
3	35,0
6	38,4
9	44,2
12	51,3
21	53,7
24	62,0
27	66,0
30	69,6
36	72,5
45	72,5
48	75,8
51	77,9
57	80,9
60	79,1

20g/l PAS+17.5 g/Ac-/l with three feeds

time (h)	HB/HA%
0	24,2
3	37,7
6	44,9
9	54,4
12	58,9
21	66,0
24	69,9
27	70,0
30	69,4
36	72,9
45	80,0
48	82,6
51	85,5
57	80,0
60	80,1

20 g/l PAS+20 g/Ac-/l with four feeds	
time (h)	HB/HA%
0	24,4
3	36,0
6	39,4
9	43,0
12	51,4
21	59,3
24	62,7
27	63,9
30	66,2
36	73,9
45	76,0
48	77,3
51	89,3
57	95,0
60	88,1

Figure 5. Profiles of HB/HA(mol%) during continuous feed experiment at sludge SS concentration of 15 g/l.

continuous feed at sludge SS of 15 g/l	
time (h)	HB/HA%
0	20,3
3	27,7
6	24,7
9	28,4
12	32,2
21	40,1
24	49,3
27	50,0
30	50,1
36	50,9
45	52,0
48	52,6
51	55,5
57	60,0
60	66,1

Figure 6. Profiles of acetate, PHA, sludge SS concentrations and PHA content (w/w% of SS) during continuous feed experiments with different C/N ratios at sludge SS concentration of 15 g/l.

C/N=48 Ac-=1 g/l at T=0

time(h)	PHA(%)	Biomass(g/l)	PHA(g/l)	Ac- (g/l)
0	9,9	15,359	1,514	0,82
3	14,5	16,211	2,344	0,01
6	17,3	17,968	3,101	0,01
9	18,1	19,158	3,472	0,02
12	23,2	20,037	4,652	0,02
21	26,1	24,295	6,337	0,05
24	29,2	24,670	7,197	0,14
27	30,0	25,327	7,598	0,30
33	32,1	25,486	8,173	0,32
36	36,9	25,795	9,518	0,35
45	39,4	25,957	10,227	0,34
48	41,5	25,924	10,758	0,38
51	40,5	25,919	10,487	0,53
57	37,0	26,019	9,627	0,54
60	36,0	26,342	9,483	0,58

C/N=96 Ac-=1 g/l at T=0

time(h)	PHA(%)	Biomass(g/l)	PHA(g/l)	Ac- (g/l)
0	8,5	14,6475	1,243573	1,0479
3	12,5	16,0215	2,009096	0,2299
6	18,1	17,6850	3,206291	0,004
9	19,2	19,1155	3,677822	0,0098
12	24,3	19,5505	4,758162	0,0054
21	26,3	23,8295	6,267159	0,0083
24	29,6	24,6117	7,275204	0,0106
27	32,8	24,6965	8,100452	0,3477
30	36,7	25,2485	9,2662	0,4432
36	42,4	25,2715	10,71512	0,5014
45	43,3	25,3925	10,99495	0,3887
48	44,2	25,0905	11,09	0,4088
54	43,3	25,1985	10,90087	0,4101
60	39,3	25,0255	9,835022	0,4087

C/N=120 Ac-=1 g/l at T=0

time(h)	PHA(%)	Biomass(g/l)	PHA(g/l)	Ac- (g/l)
0	11,86	14,475	1,72	0,9027
3	17,64	15,793	2,79	0,0093
6	24,70	17,505	4,32	0,0097
9	28,12	18,662	5,25	0,0252
12	27,17	19,520	5,30	0,0224
21	28,83	23,668	6,82	0,0569
24	34,73	24,033	8,35	0,1526
27	35,00	24,673	8,64	0,4527
33	36,00	24,223	8,72	0,6664
36	39,00	24,155	9,42	0,6028
45	38,00	25,288	9,61	0,6783
48	40,00	24,588	9,84	0,6439
51	48,00	24,537	11,78	0,633
57	34,00	24,538	8,34	0,6981
60	30,00	24,688	7,41	0,6439

C/N=144 Ac-=3 g/l at T=0

time(h)	PHA(%)	Biomass(g/l)	PHA(g/l)	Ac- (g/l)
0	12,0	14,483	1,73	3,007
3	15,7	14,458	2,27	2,887
6	18,1	14,948	2,71	3,016
9	18,2	14,905	2,71	3,130
12	20,3	15,378	3,12	3,168
21	23,0	16,307	3,75	3,169
24	24,8	16,495	4,09	3,105
27	27,8	16,473	4,58	3,110
33	29,3	16,828	4,94	3,130
36	30,8	17,425	5,36	3,002
45	32,8	18,538	6,08	3,011
48	33,3	18,175	6,06	2,912
51	33,5	18,455	6,17	3,077
57	35,5	18,535	6,57	2,771
60	34,3	18,978	6,51	2,667

C/N=144 Ac-=1 g/l at T=0

time(h)	PHA(%)	Biomass(g/l)	PHA(g/l)	Ac- (g/l)
0	18,37	14,568	2,68	1,0479
3	19,73	15,443	3,05	0,2299
6	20,81	17,377	3,62	0,004
9	24,00	19,335	4,64	0,0098
12	25,14	19,685	4,95	0,0054
21	35,68	24,440	8,72	0,0083
24	39,00	24,825	9,68	0,0106
27	47,60	25,448	12,11	0,3477
30	48,00	25,793	12,38	0,4432
36	53,00	26,193	13,88	0,5014
45	60,88	26,660	16,23	0,3887
48	56,00	26,580	14,88	0,4088
54	48,00	27,198	13,05	0,4101
60	35,00	27,170	9,51	0,4087

C/N=168 Ac-=1 g/l at T=0

time(h)	PHA(%)	Biomass(g/l)	PHA(g/l)	Ac- (g/l)
0	15,0	16,002	2,40	0,8764
3	16,5	16,013	2,64	0,2395
6	23,7	17,130	4,07	0,0053
9	23,2	18,558	4,30	0,0236
12	26,0	18,960	4,93	0,0491
21	27,7	22,215	6,14	0,1886
24	28,6	21,980	6,29	0,2005
27	31,2	23,605	7,36	0,3785
30	32,5	24,143	7,83	0,4434
36	37,0	24,603	9,10	0,5188
45	33,2	25,538	8,47	0,7373
48	34,1	25,875	8,81	0,7335
54	32,3	26,518	8,57	0,7683
60	32,3	25,760	8,32	0,7650

C/N=192 Ac-=1 g/l at T=0

time(h)	PHA(%)	Biomass(g/l)	PHA(g/l)	Ac- (g/l)
0	11,8	15,8670	1,87	0,9672
3	14,5	15,8775	2,31	0,2341
6	21,8	15,9950	3,48	0,0118
9	21,2	16,4225	3,48	0,0112
12	24,0	16,8250	4,04	0,0346
21	25,7	20,0800	5,16	0,1204
24	25,4	20,6350	5,24	0,2216
27	28,0	22,2600	6,23	0,2957
30	29,3	22,7975	6,67	0,3124
36	33,8	23,2575	7,86	0,3225
45	35,1	23,2925	8,18	0,4327
48	32,8	24,5300	8,05	0,4616
54	31,4	25,1725	7,90	0,5085
60	31,1	24,4150	7,59	0,5126

Figure 7. Profiles of HB/HA(mol%) during continuous feed experiment at different C/N ratios.

Results HB/HA% for continuous feed at different C/N ratio results HB/HA%

C/N=	48 Ac-=1g/l at T=0	120 Ac-=1g/l at T=0	144 Ac-=1g/l at T=0
time (h)	HB/HA%	HB/HA%	HB/HA%
0	36,4	46,8	39,6
3	53,3	57,1	54,6
6	60,1	66,2	54,9
9	62,2	67,4	57,5
12	63,7	67,5	60,8
21	63,5	67,6	67,2
24	64,1	67,4	69,3
27	63,5	67,4	70,4
30	63,6	67,5	71,5
36	65,2	67,4	67,2
45	64,6	68,2	66,5
48	66,5	75,3	65,6
51	67,0	73,5	64,3
57	66,2	66,2	62,3
60	61,5	60,7	62,1

C/N	96 Ac-=1g/l at T=0	144 Ac-=1g/l at T=0	168 Ac-=1g/l at T=0	192 Ac-=1g/l at T=0
time (h)	HB/HA%	HB/HA%	HB/HA%	HB/HA%
0	33,2	34,5	32,4	30,2
3	43,4	51,5	42,1	37,2
6	54,1	51,7	44,2	42,8
9	57,3	56,4	52,2	47,5
12	60,8	56,4	51,7	49,2
21	61,1	56,4	51,7	49,5
24	61,9	56,4	51,7	49,7
27	62,0	56,8	51,7	49,6
30	63,1	56,4	51,7	49,2
36	65,2	60,8	51,7	52,6
45	65,5	60,9	58,9	52,7
48	65,8	63,1	56,1	54,9
54	64,1	56,9	56,1	52,2
60	64,3	56,1	52,3	50,8

Part IV. Bioplastics production using activated sludge from pulp-paper wastewater treatment plant with continuous feed of carbon source

Figure 1: Profiles of acetate, PHA, sludge SS concentrations and PHA content (w/w% of SS) during continuous feeding experiments at sludge SS concentration of 15 g/l with different wastewater and acetic acid as the substrate at T=0 (PWW, pulp and paper wastewater; MWW, municipal wastewater; SWW, starch industry wastewater; and DWW, dairy industry wastewater).

Acetic acid at T=0

Time(h)	PHA(%)	PHA(g/l)	sludge dry weight(g/l)	COD(g/l)
0	10,2	1,55	15,230	1,280
1	15,3	2,34	15,300	0,520
3	23,9	3,76	15,742	0,132
6	24,6	3,92	15,950	0,135
9	25,3	4,24	16,772	0,193
12	35,2	6,24	17,727	0,095
21	43,9	9,21	20,987	0,420
24	46,8	10,69	22,851	0,324
27	48,9	11,29	23,080	0,163
30	50,2	11,34	22,591	0,152
36	56,9	13,23	23,256	0,126
45	60,2	14,30	23,751	0,072
48	54,6	12,86	23,545	0,065
51	55,2	12,85	23,277	0,134
57	49,2	11,36	23,087	0,125
60	47,2	10,86	23,015	0,160

PWW at T=0

Time(h)	PHA(%)	PHA(g/l)	sludge dry weight(g/l)	COD(g/l)
0	7,8	1,21	15,532	0,731
1	8,6	1,33	15,422	0,246
3	15,2	2,40	15,817	0,202
6	19,3	3,24	16,763	0,154
9	22,5	3,85	17,095	0,143
12	28,9	5,20	18,000	0,084
21	37,1	7,10	19,125	0,524
24	38,1	7,85	20,603	0,120
27	41,5	9,08	21,870	0,201
30	47,9	10,73	22,405	0,143
36	54,9	12,70	23,125	0,103
45	53,6	12,65	23,593	0,025
48	52,4	11,83	22,585	0,120
51	53,1	11,83	22,288	0,213
57	50,1	11,07	22,088	0,135
60	46,5	10,22	21,985	0,150

MWW at T=0

Time(h)	PHA(%)	PHA(g/l)	sludge dry weight(g/l)	COD(g/l)
0	5,2	0,77	14,821	0,281
1	6,5	0,99	15,167	0,078
3	10,3	1,61	15,602	0,090
6	12,3	1,95	15,839	0,082
9	15,4	2,57	16,679	0,072
12	23,1	4,08	17,674	0,065
21	31,0	5,62	18,114	0,102
24	32,4	6,16	19,007	0,083
27	44,3	8,83	19,940	0,103
30	46,9	10,43	22,239	0,126
36	47,9	10,41	21,735	0,101
45	52,3	11,73	22,420	0,130
48	50,2	11,52	22,947	0,081
51	48,6	10,82	22,264	0,087
57	47,2	10,40	22,037	0,086
60	43,1	9,83	22,805	0,051

SWW at T=0

Time(h)	PHA(%)	PHA(g/l)	sludge dry weight(g/l)	COD(g/l)
0	5,3	1,47	27,821	5,500
1	6,2	1,75	28,168	4,520
3	9,2	2,63	28,605	4,102
6	10,3	2,92	28,394	4,231
9	16,5	4,73	28,681	4,560
12	19,1	5,48	28,676	4,322
21	31,4	9,39	29,915	5,102
24	33,4	9,96	29,809	4,560
27	40,2	11,96	29,742	3,852
30	45,2	13,58	30,040	4,021
36	48,3	14,27	29,536	3,570
45	51,4	15,53	30,222	4,023
48	49,8	15,31	30,748	4,510
51	44,5	13,38	30,065	4,624
57	47,2	14,08	29,838	4,752
60	45,3	13,86	30,607	4,581

DWW at T=0

Time(h)	PHA(%)	PHA(g/l)	sludge dry weight(g/l)	COD(g/l)
0	5,1	0,761	14,923	0,354
1	7,8	1,185	15,188	0,089
3	11,2	1,751	15,637	0,075
6	14,2	2,264	15,942	0,1023
9	19,1	3,190	16,702	0,1305
12	26,6	4,514	16,969	0,1201
21	36,2	6,491	17,932	0,4501
24	37,2	7,000	18,818	0,2013
27	45,1	9,042	20,049	0,1043
30	46,2	9,955	21,549	0,1352
36	48,5	10,781	22,229	0,1026
45	50,2	11,419	22,748	0,1006
48	50,6	11,546	22,818	0,0721
51	48,9	10,732	21,947	0,0912
57	47,6	10,817	22,726	0,0134
60	43,9	9,932	22,624	0,0781

Figure 2. Profiles of HB/HA(mol%) during continuous feeding experiment at sludge SS concentration of 15 g/l with different wastewater and acetic acid as substrate at T=0.

time (h)	Acetic acid at T=0 HB/HA%	PWW at T=0 HB/HA%	MWW at T=0 HB/HA%	SWW at T=0 HB/HA%	DWW at T=0 HB/HA%
0	32,3	23,1	34,5	23,1	18,6
1	43,2	34,6	51,5	33,2	30,1
3	50,6	36,9	51,7	33,9	32,4
6	56,6	46,5	56,4	41,2	42,0
9	56,7	50,8	56,4	45,8	46,3
12	56,7	57,1	56,4	53,1	51,5
21	58,6	59,3	56,4	59,7	53,7
24	58,8	60,4	56,8	60,1	54,8
27	58,6	61,5	56,4	61,1	55,9
30	58,8	61,4	60,8	61,0	55,8
36	62,9	62,4	60,9	62,8	56,8
45	62,1	65,2	63,1	63,2	59,6
48	57,9	64,1	56,9	63,1	60,4
51	56,5	62,3	56,1	62,1	58,6
57	56,5	62,1	56,0	61,1	58,4
60		62,1	56,0	61,1	58,4

During my research, detailed techno-economic analysis of PHA production by fermentation using wastewater and activated sludge in comparison with different synthetic substrates and pure culture was carried out. The analysis was based on experimental results obtained from the study and considered other process scenarios (Table 1). The study demonstrated that, the principal advantages of this new process include: high PHA content in very low process time; concomitant reduction of sludge mass (50% w/w) hence low disposal cost; the process reduces substantial quantity of green house gas because carbon is sequestered in PHA and hence positive impact on climate change problem and low cost of bioplastics production (1$/kg versus 6$/kg in a conventional process) (Table 2). This work will be documented in a separate report.

Table 1. Different scenarios for the design and cost estimation of PHA production (production 10,000t/year).

Scenario (s)	baterium	Carbon source	Culture time	Cell concentration (g/l)	PHA concentration (g/l)	PHA content (%)	reference
1	*A. eutrophus*	glucose	50	164	121	76	Kim et al.(1994)
2	Recombinant *E.coli*	glucose	39	110	85	77.3	Wang and Lee, 1997
3	Activated sludge	Acetic acid	45	26.6	16.2	60.9	Our lab
4	Activated sludge	Glucose	45	26.6	16.2	60.9	Our lab
5	Activated sludge	Acetic acid	45	35	27.48	78.5	The best result could be reached
6	Activated sludge	Acetic acid	Based on scenario 5, but the fermentors exit already in plant can be used.				
7	Activated sludge	wastes	Based on scenario 5, but use wastes as carbon source.				
8	Activated sludge	wastes	Based on scenario 6, but use wastes as carbon source. That means no cost on fermentors as well as carbon source.				

Table 2. Techno-economic analysis of PHA production from activated sludge (in US$)

	scenario 1	scenario 2	scenario 3	scenario4	scenario 5	scenario 6	scenario 7	scenario 8
Total direct costs	**61857534**	**63343777**	**37214276**	**37214276**	**32602788**	**17970517**	**32602788**	**17970517**
Total indirect costs	**24492723**	**25081207**	**14735132**	**14735132**	**12909196**	**7115494**	**12909196**	**7115494**
Total fixed-capital investment, CFC	**86350257**	**88424984**	**51949408**	**51949408**	**45511984**	**25086011**	**45511984**	**25086011**
Working capital, CWC	**15238280,65**	**15604409**	**9167543**	**9167543**	**8031527**	**4426943**	**8031527**	**4426943**
Total capital investment, CTC	**101588538**	**104029392**	**61116951**	**61116951**	**53543511**	**29512954**	**53543511**	**29512954**
Annual total product cost (CTC)	**57193298**	**67239855**	**30601096**	**28091811**	**28374290**	**21344622**	**16913801**	**9884133**
Total product cost, $ per Kg	**5,72**	**6,72**	**3,06**	**2,81**	**2,84**	**2,13**	**1,69**	**0,99**

Annexe – II

Données

Chapitre 4. Isolement et identification des contraintes bactériennes d'PHA-accumulation de la boue.

Figure 2: Profiles in cell dry weight (CDWs), PHA, acetate concentrations and PHA content (%) of the bacterial strains (PHA-M1 to 12) with respect to cultivation time.

	time(h)	cell (g/l)	PHA(%)	PHA (g/l)	acetate(g/l)
strain 1	0	0,000	0,0	0,0000	1,9593
	6	0,020	0,0	0,0000	1,8221
	12	0,033	8,5	0,0028	1,5132
	18	0,050	15,2	0,0076	1,3495
	24	0,120	17,8	0,0214	0,3137
	30	0,098	25,3	0,0247	0,0241
	36	0,053	23,8	0,0125	0,0040
	42	0,035	19,0	0,0067	0,0082
	48	0,025	16,6	0,0042	0,0040
strain 2	0	0,005	0,0	0,0000	2,0315
	6	0,006	2,4	0,0001	2,0770
	12	0,007	3,6	0,0002	1,9547
	18	0,021	4,4	0,0009	1,9043
	24	0,023	6,2	0,0014	1,7689
	30	0,025	6,4	0,0016	1,5595
	36	0,055	5,5	0,0030	1,4431
	42	0,140	5,7	0,0080	1,2371
	48	0,165	8,4	0,0138	1,2199
strain 3					
	0	0,020	0,0	0,0000	1,9577
	6	0,113	6,5	0,0073	1,8119
	12	0,195	15,3	0,0298	1,3999
	18	0,433	27,5	0,1189	0,0040
	24	0,413	27,4	0,1129	0,0037
	30	0,403	26,9	0,1084	0,0207
	36	0,388	25,4	0,0983	0,0140
	42	0,360	23,6	0,0848	0,0040
	48	0,350	23,2	0,0813	0,0040

strain 4	time(h)	cell (g/l)	PHA(%)	PHA (g/l)	acetate(g/l)
	0	0,007	0,0	0,0000	2,0360
	6	0,018	8,6	0,0015	2,1247
	12	0,035	9,3	0,0033	1,8159
	18	0,035	11,7	0,0041	1,2186
	24	0,043	13,2	0,0056	0,4745
	30	0,090	13,3	0,0120	0,0149
	36	0,091	9,7	0,0087	0,0195
	42	0,088	6,8	0,0059	0,0118
	48	0,083	6,2	0,0051	0,0106
strain5					
	0	0,000	0,0	0,0000	1,7033
	6	0,019	0,2	0,0000	2,0432
	12	0,020	0,4	0,0001	1,7453
	18	0,028	1,9	0,0005	2,0247
	24	0,032	2,1	0,0007	1,6253
	30	0,031	2,0	0,0006	1,7136
	36	0,032	2,3	0,0007	1,9857
	42	0,032	2,9	0,0009	1,9308
	48	0,032	3,3	0,0011	1,6779
strain 6					
	0	0,009	0,0	0,0000	2,0750
	6	0,055	5,5	0,0030	1,8825
	12	0,061	6,9	0,0042	1,5582
	18	0,085	12,9	0,0110	1,1351
	24	0,093	18,3	0,0169	0,3086
	30	0,093	20,3	0,0190	0,1659
	36	0,095	23,5	0,0222	0,0072
	42	0,094	19,4	0,0182	0,0040
	48	0,094	16,6	0,0156	0,0053

strain 7	time(h)	cell (g/l)	PHA(%)	PHA (g/l)	acetate(g/l)
	0	0,000	0,0	0,0000	1,9398
	6	0,035	2,7	0,0009	1,7166
	12	0,038	6,3	0,0024	1,3990
	18	0,048	12,3	0,0059	0,7365
	24	0,053	23,0	0,0121	0,0378
	30	0,053	21,4	0,0112	0,0284
	36	0,053	21,0	0,0110	0,0332
	42	0,052	18,7	0,0096	0,0354
	48	0,049	18,2	0,0088	0,0455
strain 8					
	0	0,000	0,0	0,0000	1,7005
	6	0,053	4,7	0,0025	1,7636
	12	0,075	8,5	0,0064	1,4931
	18	0,093	15,3	0,0142	0,6678
	24	0,140	18,6	0,0260	0,4554
	30	0,218	24,7	0,0537	0,0042
	36	0,213	23,0	0,0488	0,0040
	42	0,208	21,7	0,0449	0,0040
	48	0,203	20,8	0,0421	0,0040
strain 9					
	0	0,000	0,0	0,0000	1,9471
	6	0,005	1,3	0,0001	1,9030
	12	0,045	4,4	0,0020	1,5789
	18	0,053	13,0	0,0068	1,3656
	24	0,076	20,3	0,0153	0,7377
	30	0,123	21,5	0,0263	0,5298
	36	0,135	25,5	0,0342	0,1317
	42	0,133	23,5	0,0311	0,1378
	48	0,133	21,6	0,0286	0,1366

strain 10	time(h)	cell (g/l)	PHA(%)	PHA (g/l)	acetate(g/l)
	0	0,000	0,0	0,0000	1,9790
	6	0,018	4,4	0,0008	1,9094
	12	0,073	15,0	0,0109	1,5369
	18	0,138	17,6	0,0242	0,6036
	24	0,190	25,6	0,0487	0,0073
	30	0,170	23,7	0,0402	0,0040
	36	0,105	22,5	0,0236	0,0036
	42	0,088	21,7	0,0190	0,0118
	48	0,078	20,4	0,0158	0,0049
strain 11					
	0	0,000	0,0	0,0000	2,1814
	6	0,020	0,7	0,0001	2,0019
	12	0,022	1,6	0,0003	1,8522
	18	0,015	2,3	0,0004	2,0085
	24	0,027	2,3	0,0006	1,9720
	30	0,030	3,6	0,0011	1,8789
	36	0,008	5,7	0,0004	1,6637
	42	0,005	5,4	0,0003	1,7637
	48	0,005	5,3	0,0002	1,8371
strain 12					
	0	0,000	0,0	0,0000	2,1984
	6	0,003	1,1	0,0000	1,9929
	12	0,015	2,3	0,0004	1,9210
	18	0,040	4,6	0,0018	1,5177
	24	0,050	4,6	0,0023	1,4206
	30	0,055	5,9	0,0032	1,6270
	36	0,113	6,2	0,0070	0,9932
	42	0,193	7,7	0,0147	0,5898
	48	0,195	7,9	0,0153	0,0037

Part III. Isolation of polyhydroxyalkanoate (PHA) accumulating bacterial strains from pulp and paper industry wastewater activated sludge

Figure 2: Profiles in cell dry weight (CDWs), PHA, acetate concentrations and PHA content (%) of the bacterial strains (PHA-P1 to PHA-P6) with respect to cultivation time.

	time (h)	biomass(g/l)	PHA (g/l)	PHA(%)	Ac-(g/l)
strain P1	0	0,0000	0,00	0,00	4,884
	6	0,0000	0,00	0,00	4,929
	12	0,0000	0,00	0,00	4,804
	18	0,0200	0,00	2,18	4,798
	24	0,0225	0,00	6,54	4,799
	30	0,0350	0,01	15,80	4,739
	36	0,0425	0,01	21,61	3,686
	42	0,2775	0,07	25,06	2,542
	48	0,6925	0,20	29,06	2,257

	time (h)	biomass(g/l)	PHA (g/l)	PHA(%)	Ac-(g/l)
strain P2	0	0,0000	0,00	0,00	4,667
	6	0,0000	0,00	0,00	4,669
	12	0,0000	0,00	0,00	4,564
	18	0,1575	0,02	15,21	3,610
	24	0,1025	0,02	16,92	3,261
	30	0,5875	0,11	17,90	2,603
	36	0,8400	0,15	17,97	1,496
	42	0,8650	0,20	23,37	0,053
	48	1,0350	0,20	19,52	0,051

	time (h)	biomass(g/l)	PHA (g/l)	PHA(%)	Ac-(g/l)
strain P3	0	0,0000	0,00	0,00	4,800
	6	0,0000	0,00	0,00	4,819
	12	0,0000	0,00	0,00	4,694
	18	0,0525	0,01	21,82	4,525
	24	0,1175	0,03	26,60	4,126
	30	0,3600	0,10	28,08	2,745
	36	0,7050	0,22	31,81	1,166
	42	0,4650	0,11	23,00	0,138
	48	0,9100	0,17	18,16	0,083

	time (h)	biomass(g/l)	PHA (g/l)	PHA(%)	Ac-(g/l)
strain P4	0	0,0000	0,00	0,00	4,801
	6	0,0000	0,00	0,00	4,800
	12	0,0000	0,00	0,00	4,385
	18	0,0000	0,00	0,00	4,221
	24	0,0000	0,00	0,00	4,138
	30	0,1620	0,00	2,98	4,116
	36	0,2995	0,01	3,76	4,394
	42	0,3675	0,02	5,04	4,308
	48	0,6070	0,03	5,22	4,390

	time (h)	biomass(g/l)	PHA (g/l)	PHA(%)	Ac-(g/l)
strain P5	0	0,0000	0,00	0,00	4,299
	6	0,0325	0,01	19,69	4,196
	12	0,1050	0,02	21,56	3,743
	18	0,5150	0,13	25,45	1,912
	24	0,3000	0,09	28,41	1,816
	30	0,6400	0,23	35,45	1,131
	36	0,5350	0,15	27,52	0,819
	42	0,6025	0,13	22,22	0,168
	48	0,7300	0,12	17,10	0,134

	time (h)	biomass(g/l)	PHA (g/l)	%	Ac-
strain P6	0	0,0000	0,00	0,00	3,793
	6	0,0000	0,00	0,00	3,234
	12	0,0000	0,00	0,00	2,753
	18	0,0000	0,00	0,00	2,121
	24	0,1000	0,01	12,10	2,502
	30	0,3750	0,05	14,34	1,794
	36	0,5125	0,08	15,10	0,074
	42	0,5825	0,07	12,37	0,202
	48	0,8200	0,07	8,62	0,000

Annexe – III

Synthèse (Version anglaise)

Synopsis (English version)

1.1 Introduction

The non-biodegradable plastics can cause harmful effects during the production, the transformation and the utilisation. Moreover, its discharge in the environment causes an ecological problem (Fiechter, 1990). In order to confirm the new environmental legislations, industries are encouraged to reduce the utilisation of the non-biodegradable plastics and develop the production of biodegradable plastics. Because, bioplastics present desirable properties and can be used for human, a lot of biodegradable plastics are incorporated in many materials including packaging material, coating of fibrous materials and also in pharmaceutical or agrochemical agents. Hence, the world consumption of biodegradable plastics is estimated at 1.3 billion Kg per year (Lindsay, 1992).

In 1926, polyhydroxyalkanoate (PHA) was observed as a constituent of *Bacillus megaterium* and identified as biopolymer (Lemoigne, 1926). After that the poly-β-hydroxybutyrate (PHB) which is the most frequently found members of PHAs and other PHAs were discovered and more than 40 PHAs were characterized (Steinbuchel, 1991). PHB is accumulated by a large number of bacteria, generally, under conditions of nutrient limitation and in the presence of excess carbon source.

1.2 Production conditions of biopolymers

Generally, polymers are produced by bacteria under specific growth conditions. For example, PHAs and PHBs are accumulated if a carbon source is abundantly available but another essential growth component such as nitrogen is limiting or missing in the medium (Hrabak, 1992). Generally, Bacteria produce PHAs under some specific conditions. Some bacteria (*Alcaligenes eutrophus, Protomonaas extorquens, Pseudomonas olevorans,* etc) need limitation of some essential nutrient such as N, P, Mg, K , O or S. The presence of an excess carbon source permits an efficient PHA synthesis. However, other bacterial strains (*A. latus*, a mutant strain of *Az. Vinelandii*, and recombinant *E. coli.* etc) do not require nutrient limitations (Lee, 1996a). Moreover, bacteria produce PHB while growing in mineral medium containing metals in trace (such as Co, Cr, Cu, and Zn) (Repaske and Repaske, 1976). According to Findlay and White

(1983), the PHBs are produced in large quantity in ground water (oligotrophic environment). However, the production can be inhibited by Mg and SO_4^{2-}. A. eutrophus, can accumulate 80% of its dry weight while growing under an excess supply of carbon (glucose) and limitation of P or N. Accumulated polymer acts as a carbon reserve and electron sink for the bacteria (Anderson and Dawes, 1990). When limiting conditions are alleviated (addition of P or N), PHB will be catabolized to acetyl-CoA (Yves et al., 1995). Generally, PHAs are accumulated in the form of granule form (Page et al., 1992). These granules are visible under an electron microscope (Lageveen et al., 1988; Hrabak, 1992; Vincent et al., 1962).

1.3 Biosynthesis, structure and proprieties of biopolymers

1.3.1. Biosynthetic pathways

The majority of data on the production mechanism of PHA are obtained on the basis of A. eutrophus. In this bacterium, PHB is synthesized from acetyl-CoA in series of three reactions catalyzed by three enzymes (thiolase, reductase and synthase). The PHB pathway is shown in Figure 1. At the beginning, the reversible condensation of two molecules of acetyl-CoA, by the action of the 3-ketothiolase, produces the Acetoacetyl-CoA. After that, the acetoacetyl-CoA reductase enzyme reduces the acetyl-CoA into R-(-)-3-hydroxybutyryl-CoA. The reductase enzyme is NADPH-linked (Figure 1). Finally, R-(-)-3-hydroxybutyryl-CoA is polymerized to form PHB under the action of PHA synthase. PHA is formed by the polymerisation of 103 to 104 monomers and accumulate in cells as inclusions of 0.2 width 0.5□m in diameter (Yves et al., 1995; McCool and Cannon, 2001; Fukui and Doi, 1997). Actually, nucleotide sequences are known for more than 30 PHA synthases and they are arranged into three classes (Class I : catalyse polymerisation of short-chain length HACoAs; Class II : catalyse polymerisation of medium-chain length HACoAs and class III : catalyse polymerisation of short-chain length HACoAs) (Rehm and Steinbuchel., 1999). Therefore, the nature of PHAs produced, by A. eutrophus, is related to the structure of the source of carbon used in the growth medium. PHAs produced are composed with different monomers having 3 to 5 carbon atoms (C3 to C5) (Steinbuchel, 1991). The addition of propionic acid or valeric

acid to the growth media containing glucose permits the accumulation of copolymer (P(3HB-3HV): copoly(3-hydroxybutyrate-3-hydroxyvalerate)) (Yves et al., 1995). However, the PHA biosynthetic system allows the incorporation of saturated 3-, 4-, and 5-hydroxyalcanoic acids with up to five carbon atoms. Hence, in this case, hydroxyalkanoic acids with six or more carbon atoms were not detected in PHA produced by this bacterium (Yves et al., 1995; Liebergesell et al, 1991; McCool and Cannon, 2001; Fukui and Doi, 1997). However, large number of bacteria including Pseudomonas olevorans accumulate polymer composed of monomers having 6 to 14 carbon atoms (C6 to C14) (Steinbuchel, 1991). Therefore, the flexibility of the PHA pathway will help the design of a new biopolymer having potentially specific physical proprieties (Yves et al, 1995).

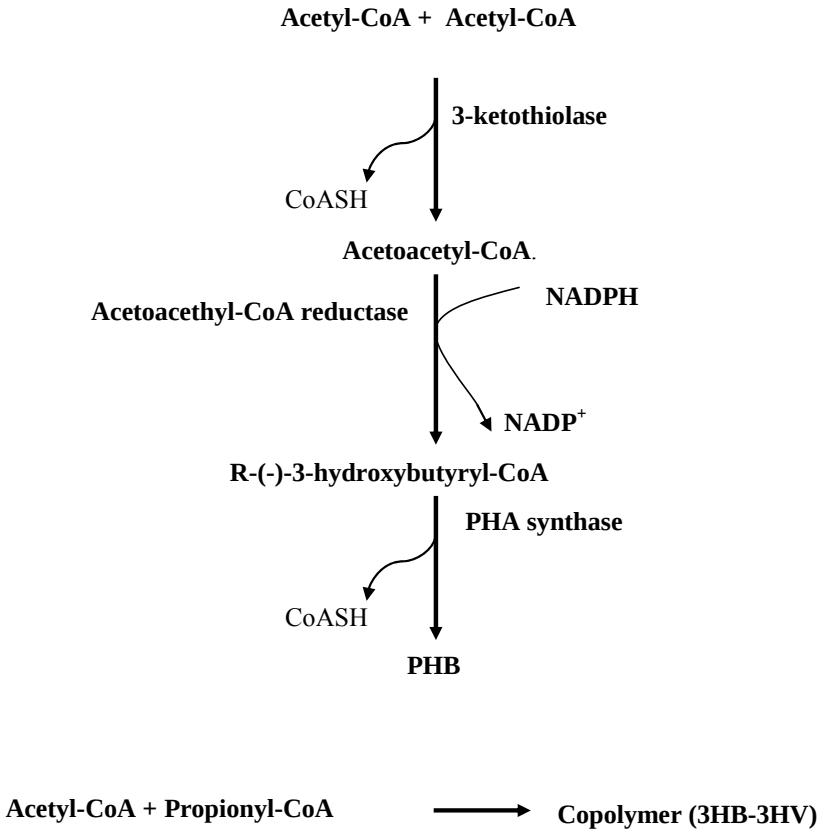

Figure 1. Pathway of PHA biosynthesis

1.3.2 Structure of PHB

PHAs polymer has a number of interesting characteristics and can be used in various ways similar to many conventional synthetic plastics. Generally, PHAs are formed by polymerisation of R-(-)-hydroxyalkanoic acid monomers having 3 to 14 carbons (C3 to C14). PHB is the most interesting and most known polymer (Budwill et al., 1992). A large number of monomer units have been identified in various bacterial species as constituents of PHAs (more than 90 monomers) (Steinbuchel, 1995). Example of few these monomer units are: 3-hydrxyalkanoates (having 3 to 12 carbon atoms), 4-hydroxypentanoate (having 4 to 8 carbon atoms), 5-hydroxypentanoate, 5-Hydroxyhexanoate and 6-Hydroxydodecanoate. However, only a few of these PHAs have been produced and characterized.

1.3.3 Proprieties

The proprieties of PHBs are comparable to conventional plastics (polypropylene). They are natural biopolymer which is 100% resistant to water and moisture, thermoplastic and 100% biodegradable (Hrabak, 1992). However, it has some specific proprieties such as low permeability to gases.

The homo-polymer PHB is a stiff and relatively brittle thermoplastic (Holmes, 1988). It has a melting point of 175 ^{o}C and a relatively poor resistance to reagents (acids and bases). However, it has a good UV resistance. Compared to some other biodegradable plastics, PHB is insoluble in water and relatively resistant to hydrolytic degradation. It is characterized also by a good oxygen permeability (Holmes, 1988).

The use of PHBs is limited because higher melting point and a slow rate of degradation. These proprieties can be modified by changing the configuration of the PHB polymer which can give different PHAs composition such as the copolymers (Cox, 1994). For example, the incorporation of 3HV (3-hydroxyvalerate) monomers into PHB polymer resulted in a decrease in the crystallinity and in the melting point compared to the homopolymer PHB (Holmes, 1988). Hence, the copolymer represents a decrease in stiffness and an increase in toughness. Therefore, the copolymer P(3HB-3HV) has

suitable proprieties for many commercial application. The polyhydroxyoctanoate has the proprieties of an elastomer such as low melting point, low crystallinity, poor tensile and higher extension to break (Gagnon et al, 1992).

1.3.4 Biodegradability

Many micro-organisms such as bacteria and fungi degrade PHA polymers and use the by-products as carbon sources (Matavulj and Molitoris, 1991; Jendrossek et al., 1993). PHAs can be degraded rapidly in anaerobic sewage and slowly in sea water (Luzier, 1992). The final products of biodegradation are CO2 and water under aerobic conditions. However, in anaerobic conditions, PHBs can be transformed to CH4 and CO2. (Budwill et al, 1992; Lee, 1996b; Cox, 1994).

The biodegradation occurs by the attack of biopolymer by bacteria which secrete an extracellular enzyme called depolymerases. Some extracellular depolymerases (which hydrolyse PHB, P(3HB-3HV) and PHO: Poly 3-hydroxyoctanoate) have been recognized and their specific genes are cloned (Schirmer et al., 1993; Saito et al., 1989). Also, a PHV depolymerase has been isolated and characterized from Pseudomonas Lemoignei (Muller et al., 1993). Moreover, PHB is known as a biocompatible material. The degradation in vivo is slow (Fiechter, 1990) compared to in vitro degradation (in soil and in sludge) and its byproduct is R-(-)-3-hydroxybutyric acid (Yves et al., 1995).

The biodegrdation of PHB based plastics were studied in different types of compost and by different composting technologies (Hrabak, 1992). A PHB weight loss of 18 to 40% was reported after 12 weeks of exposure in green manure compost. However, compacting the shape of PHB products was shown to be unfavourable for degradation (Hrabak, 1992). Composting under higher temperature and mechanical disruption permit a rapid degradation of bottles made of copolymer (P(3HB-3HV)) and, after 15 weeks, 80% weight lost was observed (Yves et al., 1995). However, a weight loss of about 50% for the same material was observed under simulated managed landfill condition at 35^0C and after a period more than 40 weeks (Yves et al., 1995). Generally, the rate of bioplastic biodegradation is controlled by some environmental factors (pH, temperature, nutrients, and moisture) and by material parameters (co-monomer HV: 3-hydroxyvalerate) content,

molecular weight, crystallinity degree, surface area and the formulation). Therefore, the material parameters can be controlled, during synthesis, in order to optimise the biodegradation rate. For example, increasing the HV content of P(HB-HV) copolyesters can enhance the biodegradability rate (Cox, 1994).

1.4. Diversity of micro-organisms and substrates for producing biopolymers

The accumulation of PHA at higher concentration has been observed in a variety of micro-organisms such as *Clostrodusim, sytrophomonas, Pseudomonas* and *Alcanegene*. However, some cyanobacteria produce PHB at lower level (Fiechter, 1990).

Alcaligenes eutrophus produces short-chain PHAs (polymerisation of C3 to C5 monomers) (Anderson and dawes, 1990; Steinbuchel, 1991). *A. eutrophus* is the most used strain because it produces PHB and P(HB-HV) at higher rate (Ramsay et al, 1992). A. eutrophus can accumulate about 80% of its dry weight (Byrom, 1987). A. eutroophus is a chimiolithotrophe bacterium grows in mineral medium. The enrichment medium contains (in g/l) yeast extract :10; peptone :10; meat extract : 5 and $(NH_4)_2SO_4$: 5 (Yoshiharu et al, 1988). Growth of *A. eutroophus* in this medium can not produce polyester. In order to produce polymers, these bacteria should be transferred into another medium under nitrogen limitation (Repaske and Repaske, 1976) and in the presence of an excess of carbon at pH 7, 30^0C and under oxygenation.

The nature and the proportion of polymers produced are influenced by the carbon source used (Steinbuchel, 1991). Propionic and valeric acid permit the production of copolymer P(3HB-3HV) (mixture of 3HB and 3HV). However, while growing the same strain only on valeric acid based-medium, the poroprtion of 3HV reached 90% of PHA produced (Doi et al., 1988; Steinbuchel et al., 1991).

A. eutrophus is inhibited while growing in propionic acid (0.1%) (Byrom, 1987), however, it sustains the accumulation of P(3HB-3HV). The use of glucose (at 2%) and starch (2%) are the ideal condition of higher production of PHBs. Generally PHBs are produced under limited concentration of ammonium (Heinzle and Laffery, 1980).

Pseudomonas olevorans is a chimiolithotrophic bacterium which produces medium-chain of PHAs by incorporation of C6 to C14 monomers (Anderson and dawes, 1990; Steinbuchel, 1991) and grows in a mineral medium under oxygenation at 30°C and at pH 7.

A number of bacterial strains are able to accumulate the copolymer PHA containing both the short and the medium-chains. Among these strains *P. aeruginosa* produces a copolymer composed of C5 to C10 monomers while growing on valerate (Timm and Steinbushel, 1990). In the same way, *P resinovarans* cultivated on hexanoate accumulates the PHA from C4, C6, C8 and C10 monomers (Ramsay et al, 1992).

Azotobacter vinelandii produced a large quantity of PHB while growing on a variety of sugar sources (Page et al, 1992) without requiring limited growth conditions. This bacterium is used as an alternative for PHB production (Page and Knops, 1989). It accumulates a maximum of PHB without nitrogen and under an excess of carbon (glucose). The use of other substrates (methanol, acetate, butyrate) have been evaluated by Yamane (1992). The UWD Azotobacter vinelandii strain was able to produce PHA efficiently while cultivated in beet molasses, cane molasses and core syrup (Page, 1989; 1990); while using molasses as a substrate it accumulated PHA more than 80% of its weight. The production of a copolymer by this strain was also studied by Page et al. (1992). Growth on glucose as a principal source of carbon added by other secondary sources (valerianate, pentanoate, etc) and the effect of the addition of fish peptone was studied by growing Azotobacter vinelandii in two type of cultures (batch and fed- batch). The use of fish peptone enhanced the PHB synthesis. It seemed to be used as a nitrogen source wich allowed a rapid accumulation of PHB (Page and Cornish, 1993).

Other bacterial strains are also known for their PHA producing potential, however, their application is limited. Some studies showed that the rhizobia strains are able to accumulate PHB at a significant level (Hayward et al, 1959; Vincent et al, 1962). The Methylobacterium organophilum strain showed an optimum growth at 0.5% (w/v) of methanol. Methanol concentration of 4% (w/v) and lack of divalent cations reduced the bacterial growth. However, under potassium limited condition and with methanol as carbon source, the PHA (P(3HB)) content reached 52% of the cell dry weight. (Kim et

al., 1996). The growth of chromobacterium violaceum on valeric acid based-medium gave a polymer composed of 100% of the monomer 3HV (Doi et al., 1988; Steinbuchel et al., 1991). However, Rhodococcus rubber grown on glucose produces copolymer with 75 mol % 3HV and 25 mol % 3HB (Haywood et al., 1991).

1.5. Industrial production of biopolymers

1.5.1. Technologies for the production

Generally, fed-batch and continuous cultivation techniques have been used for accumulation of higher concentration of PHB. Fed-batch culture is mostly employed for bacteria (Alcaligenes eutrophus, Protomonas extorquens, Protomonas. oleovorans, etc) requiring limiting factors (N, P, Mg, K, O or S) and an excess carbon source for an efficient PHA production (Lee, 1996a). In the beginning, cells are grown to reach a desired concentration without nutrients limitation. After that, an essential nutrient is eliminated to allow higher PHB accumulation in the cell. A complete elimination of P or N in the case of A. eutrophus significantly increases the polymer accumulation (up to 80% of the cell weight for P(3HB) and P(3HB-3HV)) (Byrom, 1987; Kim et al., 1994). In addition to that, for other bacteria (P. extorquens and P. oleovorans) a total elimination of N or P did not permit an efficient accumulation of polymers (Preusting et al., 1993; Suzuki et al., 1986). A two-stage chemostat should be employed for this type of microorganisms – 1st stage to generate cell mass and the 2nd stage to accumulate PHB. However, for some strain such as P. oleovorans, one stage process could be used (Preusting et al., 1993).

The application of Fed-batch culture strategy, for bacteria that does not require nutrient limitating conditions (A. latus, a mutant of Az. Vinelandii, etc), is important for fermentation success (Lee, 1996a). Nitrogen sources (corn steep liquor, yeast extract, fish peptone, etc) were added to increase the cell growth and consequently PHA accumulation (Lee and Chang, 1994, Page and Cornish, 1993). However, continuous cultivation gave the highest PHA accumulation. A latus and P. oleovarans were successfully cultivated in continuous process for producing PHA (Preusting et al., 1993; 199). For these type of bacterial strains (that did not require conditions of nutrients limitation) single stage

chemostat could also be used. However, it is very important to optimize continuous and fed-batch processes in order to compare the productivity of different strains depending on the nature of the media used. Moreover, economic analysis should be conducted in order to choose an appropriate process.

In 1975, the ICI (Imperical Chemical Industry, United Kindom) started the production of PHBs in response to the increased oil price (Yves et al., 1995). A mutant strain of A. eutrophus (Byrom, 1987; 1990), grown in glucose and propionic acid based medium, was used to produce copolymer (P(3HB-3HV)). In order to avoid toxicity and to control the composition of the copolymer (proportion of HV unit), the addition of propionic acid was controlled. The final PHA contents reached 70 to 80 % of the cellular dry weight. Depending on the substrate used, HV represents 0 to 30 % of the copolymer. At the end of fermentation, the PHA was purified by solvent extraction. However, the use of solvents is replaced by enzymes and detergents to solubilize the cellular compounds and to extract the polymers (Byrom, 1987; 1990). The copolymer P(3HB-3HV) is marketed under a commercial name BIOPOL by Zeneca Bio. The polymer is produced by fed batch culture and the production is about 1000 tonnes per years (Byrom, 1994).

Another company (Austrian Company Chemie Linz GmbH) produced homopolymer PHB by a process of continuous fermentation of A. latus strain in sucrose-based medium (Hrabak, 1992). The strain used (A. latus btF-96) permit the production of more than 100 kg of PHB in a week in a 15-m3 fermenter. After fermentation, cells should be harvested, washed with tap water followed by the extraction of the polymer. During this process, cell suspension is treated by solvent (methylene chloride) and PHB is separated from cell debris by decantation. Then PHB is precipitated (by addition of water) and dried. The use of this process permit to get a PHB purity of 99% (Hrabak, 1992; Lafferty and Braunegg, 1984a; 1984b).

1.5.2. Applications

The proprieties of PHB are comparable to conventional used plastics (polypropylene). Therefore, it can be processed in the same way as polypropylene. Hence, bacterial homo and copolymers are suitable for some specialized application, such as slow release

encapsulation and biomedical devices (Seebatch, et al., 1987). The monomer unit (3-hydroxybutyric acid) can be used for the production of complex chiral pharmaceutical or agrochemical agents (Seebatch, et al., 1987). Moreover, the application of PHB as a matrix material for slow release of pharmaceuticals has been investigated (Lafferty et al., 1988; Korsatko and Wabnegg, 1983).

The useful proprieties of the copolymer P(3HB-3HV) (known under the commercial name of BIOPOL) such as the water resistance and oxygen impermeablity make it suitable for large applications including packaging material (films and bottles), coating of fibrous materials such as paper and board (Cox, 1994; Holmes, 1988; Marchessault et al., 1990).

However, PHB application is limited for some use because of the thermal degradation during melt processing, relatively slow crystallization and stiffness. These proprieties can be controlled by changing the PHB polymer chain configuration, producing different PHA composition or using formulations containing coproducts (Cox, 1994). In order to overcome these problems (thermal degradation and relatively slow crystallization), the use of P(HB-HV) copolyesters with nucleants has been considered as the most successful approach. Moreover, it permit the reduction of stiffness which enlarge the range of applications.

1.6. Cost of bioplastics production

The most important factors to consider for economical production of PHBs are product yield (Yp/s - g of PHB produced per g of substrate consumed), productivity (Pr – g PHB produced/L/h), cell content of PHB (Yp/x - g of PHB/g dry cell) and cell yield (Yx/s – g cell formed per g of substrate consumed). Yp/s and Yx/s determines the substrate or raw material requirement; higher values of these yield factors mean low reuirement for substrate. Productivity has a direct impact on the fixed (equipment) capital cost. The factor Yp/x has an impact on the recovery cost of PHB through solvent extraction, a step after fermentation. Efforts have been spent in optimizing the PHB production process and reducing the cost of production in the following directions: strain development,

optimization/improvement of fermentation and separation processes, utilisation of cheap carbon sources.

As indicated in the preceeding text, a large number of substrates such as carbohydrates, oils, alcohols, organic acids and hydrocarbons can be used by PHA-producing microorganisms. On the bases of carbon source, the theoretical yield of P(3HB) has been estimated (Yamane, 1993). The theoretical yield was used to determine the cost of the production. Based on the biochemical stoichiometry, values of theoretical yield varied between 0.32 PHB/Kg (for glucose) and 1.16 Kg PHB/Kg (for butanol) (Yamane, 1992). The Substrate costs and yields affect the production cost. Generally, the cost of synthetic plastic (polypropylene and polyethylene) is 1 $/kg less than biodegradable plastics (Chemical marketing reporter, 1994). Moreover, the cost of production depends on the raw material cost, operation cost, polymer extraction process and purification methods used in large-scale production.

Yield of PHAs has been improved through the development of high yielding recombinant microorgnisms (Lee et al., 1994) or through process improvement in fed batch culture (Shirai et al., 1994). Another method to obtain high yield of PHAs is to optimize the growth phase (to enhance biomass yield, Yx/s) and the production phase (to enhance the PHAs yield, Yp/x) or to conduct two phases (growth phase and production phase) separately (Shimizu et al., 1992). Despite these efforts, the cost of production of PHAs remains high in comparison to the production cost of synthetic plastics (Choi and Lee, 1997).

Broader use of biodegradable plastics in packaging and disposable products as a solution to environmental problems would heavily depend on further reduction in costs and discovery of novel biodegradable plastics with improved properties. In PHB production, about 25-29% of the total production cost is for direct fixed capital cost and 40-50% of the total production cost is for raw material (Choi and Lee, 1999). Thus, the use of cheaper carbon source (beet molasses, cheese whey, plant oils, hydrolystates of starch, etc) is required in order to substantially reduce the high production cost of PHB (Brandl et al., 1990). Development of new bacterial strain for large scale production on cheaper and complex substrate can also help in reducing the cost of production.

1.7 New strategies for PHA production

One of the major problems preventing the commercial application of PHAs is their high production cost. Much effort has been made to reduce the production costs. The new approach involves the use of renewable carbon resources derived from agriculture or industrial wastes as substrate and/or excess activated sludge from a wastewater treatment plant as a source for PHA accumulation (Chua et al., 2003; Khardenavis et al., 2007; Kumar et al., 2004). These approaches have the advantages of saving cost on PHA production and volume reduction of waste activated sludge by extracting PHA. The saving on waste activated sludge disposal cost following volume reduction could reduce PHA production cost thereby attributing economic advantage to the process (Khardenavis et al., 2007). This review focuses on the recent researches on use of waste materials and/or activated sludge from wastewater treatment plant for PHA production.

1.7.1 Waste materials as carbon substrates for PHA production using pure culture

From an economical point of view, the cost of substrate (mainly carbon source) contributes most significantly to the overall production cost of PHA. It has been estimated to be about 40% of the total PHA production costs (Choi and Lee, 1999; Dias et al., 2006). In the past decade, a wide variety of low-cost carbon substrates (e.g., renewable carbon sources), such as wastewater, municipal wastes, agricultural and industrial residues or by-products (e.g., starch, tapioca hydrolysate, whey, xylose, molasses, malt, and soy wastes), crude carbon substrates (food wastes or by-products) have been tested for PHA production by pure cultures due to their low price and potential availability (Lee and Gilmore, 2005). Both cheap carbon sources and cheap nitrogen sources are available from industrial or agricultural waste and surplus materials and make a substantial contribution for minimizing PHA production costs (Koller et al., 2005). This way, municipal, industrial or agricultural waste and problematic surplus materials that until today have to be disposed are converted into high-value and sustainable bioplastics (Koller et al., 2005). Therefore, Waste materials offer the greatest promise as a source of carbon for bacterial growth, their use can solve the environmental problem of waste disposal and concomitantly help to reduce the cost of PHAs production.

It has been reported that *Methylobacterium* sp. ZP24 and *Pseudomonas cepacia* could produce PHB from whey or its major component, lactose (Yellore and Desai, 1998; Young et al., 1994). *P. cepacia* was also evaluated for PHB production from xylose, a major hemicellulosic sugar of hardwoods (Ramsay et al., 1995; Young et al., 1994). Besides *A. chroococcum*, *Haloferax mediterranei* was used to produce PHB from starch as a carbon source (Garcia Lillo and Rodriguez-Valera, 1990). The high PHB productivity was obtained by fed-batch culture of *Ralstonia eutropha* from tapioca hydrolysate (Kim Beom and Chang Ho, 1995). *Azotobacter vinelandii* UWD was used to produce relatively high concentration of PHB (22 g l^{-1}) from molasses as a carbon source (Page and Cornish, 1993).

Whey is the major byproduct from the cheese manufacturing industry, representing 80 - 90% of the volume of milk transformed. Since whey is available in large amounts and only partially used as animal feed, its high production claims for an alternative way of disposal or for enhancing the added value of this material. Whey has been the most extensively studied waste for PHA accumulation by a variety of microorganisms (Khardenavis et al., 2007; Park et al., 2002; Povolo and Casella, 2003). Lactose is the major component of whey and many *E. coli* strains can utilize lactose for their growth (Khardenavis et al., 2007; Park et al., 2002; Povolo and Casella, 2003).

Povolo and Casella (2003) found that sweet whey contains approximately 5% lactose, 0.2% lactic acid and 1% proteins as well as fats, minerals and vitamins, and *Hydrogenophaga pseudoflava* DSM 1034 was a good candidate for production of PHAs. However, the P(3HB) concentration and P(3HB) content obtained from waste products were considerably lower than those obtained using purified carbon substrates. Therefore, Recombinant strains utilizing a cheap carbon source and corresponding more efficient fermentation strategies have been developed for efficient production of PHA. Lee et al. (1997) examined various recombinant *Escherichia coli* strains for their ability to accumulate a large amount of PHB in whey-based medium. The highest PHB concentration and PHB content obtained were 5.2 g/L and 81% of dry cell weight, respectively.

Wong and Lee (1998) studied the recombinant *Escherichia coli* strain GCSC 6576, harboring a high-copy-number plasmid containing the *Ralstonia eutropha* genes for polyhydroxyalkanoate (PHA) synthesis and the *E. coli* ftsZ gene, was employed to produce poly-(3-hydroxybutyrate) (PHB) from whey. They obtained high cell concentration of 87 g l^{-1} with 80% PHB in 49 h by fed-batch culture of strain selected (Wong and Lee, 1998).

Waste edible oils exhausted from the food industry and the food service industry are largely destroyed by incineration or lost into the environment – both routes being unacceptable from a green chemistry perspective. PHA production was conducted by *Ralstonia eutropha* with waste edible oils and fats as carbon sources (Taniguchi et al., 2003). The efficiency of this interesting process is high and the polymer can be easily extracted. Thus a waste is converted by an environmentally benign method to a valuable product (Taniguchi et al., 2003).

Plant oils and fats are renewable and inexpensive agricultural co-products, and thus, waste oils would be one of the most suitable candidates for microbial production of PHAs. Several pseudomonades produce PHA from plant oils and tallow, and *Aeromonas caviae* also accumulates PHBHHx from olive oil, although the productivities of them are quite low in all the cases (Doi et al., 1995; Fuchtenbusch et al., 2000; Taniguchi et al., 2003). By using *R. eutropha*, renewable and inexpensive agricultural fats/oils and their waste products are potentially useful feedstocks for the PHA fermentation processes (Taniguchi et al., 2003).

Agro-industrial oily wastes was also used for production of poly(3-hydroxyalkaonates) (PHA) by *Pseudomonas aeruginosa* 42A2 (Fernandez et al., 2005). A 54.6% PHA accumulation was obtained when technical oleic acid (TOA) was used as carbon source. PHA accumulation ranged between 66.1% when waste-free fatty acids from soybean oil (WFFA) were used as carbon substrate, 29.4% when waste frying oil (WFO) was used and 16.8% when glucose was used. Depending on the substrate supplied a wide range of components was observed (Fernandez et al., 2005). It was found that the residual waste

frying and other oily wastes are suitable substrates for PHA production (Fernandez et al., 2005).

Large amounts of homopolymers containing β-hydroxybutyrate (PHB) and copolymers containing β –hydroxyvalerate (P[HB-co-HV]) are produced by *Azotobacter chroococcum* strain H23 when growing in culture media amended with alpechìn (wastewater from olive oil mills) as the sole carbon source. Copolymer was formed when valerate (pentanoate) was added as a precursor to the alpechìn medium, but it was not formed with the addition of propionate as a precursor. *A. chroococcum* formed homo- and copolymers of polyhydroxyalkanoates (PHAs) up to 80% of the cell dry weight, when grown on NH_4^+-medium supplemented with 60% (v/v) alpechı´n, after 48 h of incubation at 100 rev min^{-1} and 30 °C. Production of PHAs by strain H23 using alpechı´n looks promising, as the use of a cheap substrate for the production of these materials is essential if bioplastics are to become competitive products (Pozo et al., 2002). The The polymer content (88.6% of dry cell mass) reported in this study is the highest one obtained from organic wastes and is comparable with the PHA content from pure sucrose fermentation (Wang and Lee, 1997).

Organic solid wastes, however, are usually in complex form that cannot be directly digested and utilized by PHA-producing microbes such as *Ralstonia eutropha*, a representative bacterium for PHA synthesis (Du et al., 2004; Yu, 2001)To enhance the PHA production from the organic wastes, a pretreatment stage may be favorable. Hydrolysis and acidogenesis are the first step to convert biodegradable solids into short-chain volatile fatty acids such as acetic, propionic, and butyric acids that can be further utilized by PHA-producing bacteria.

Yu (2001) studied the production of PHA from a starchy wastewater in a two-step process of microbial acidogenesis and acid polymerization. The starchy organic waste was first digested in a thermophilic upflow anaerobic sludge blanket (UASB) reactor to form acetic (60–80%), propionic (10–30%) and butyric (5–40%) acids. The acid effluent, after microfiltration, was introduced into a second reactor where bacterium *A. eutrophus*

took up the acids to form PHA. Polyhydroxybutyrate was formed from butyric acid and poly(hydroxybutyrate-hydroxyvalerate) formed from propionic acid with 38% hydroxyvalerate (Yu, 2001). The PHA was a copolymer of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) with 2.8 mole % of hydroxyvalerate (Du et al., 2004).

If waste product/stream can be used as a substrate for the production of P(3HB), combined advantages of reducing disposal cost and production of value-added products can be realized.

1.7.2 Mixed cultures in activated sludge used as the microorganisms for PHA production

A novel PHA production strategy, which is to utilize the mixed bacterial culture in activated sludge for PHA production has been proposed. Many workers have shown that the activated sludge could produce bioplastics (Dionisi et al., 2004; Lemos et al., 2004; Lemos et al., 2003; Lemos et al., 1998; Reis et al., 2003; Satoh et al., 1998a; Serafim et al., 2004a; Serafim et al., 2006; Yan et al., 2006)

It is well-known that PHA serves as an important carbon storage material in microorganisms in activated sludge, especially in the anaerobic –aerobic activated sludge process or the Enhanced Biological Phosphorous Removal (EBPR) process (Mino et al., 1998). In EBPR process, microorganisms in activated sludge consume polyphosphate as an energy source for anaerobic uptake of carbon substrates, the carbon substrates taken up are temporarily stored as PHA. When the condition turns aerobic, PHA is utilized for growth and polyphosphate regeneration, the microorganisms in EBPR process should therefore possess the characteristic of phosphate removal and PHA accumulation. For these reasons, the idea of PHA production by using activated sludge was ignited. When compared with pure culture fermentation processes, the merits of PHA production system by activated sludge will have following advantages: cost reduction in cultivating PHA producing bacterial cultures, lowering the PHA production cost, simpler facility construction, reuse of excess sludge from wastewater treatment plants (WWTPs), contribution to the reduction of excess sludge production from WWTP and to supply of a

new way of carbon recycle by the conversion of organic pollutants into valuable biodegradable plastics (Chua et al., 2003; Satoh et al., 1998a).

1.7.2.1 Biosynthesis PHA by mixed culture in activated sludge

PHA production by anaerobic-aerobic sludge under anaerobic condition

There are two known microbial populations capable of anaerobic utilization of organic substrate in EBPR processes: (i) the polyphosphate accumulating organisms (PAO) and (ii) the glycogen accumulating organisms (GAO) (Cech and Hartman, 1993). Several metabolic model for the stoichiometry of acetate uptake under anaerobic conditions by mixed culture of PAO and GAO were developed (Filipe et al., 2001a, b; Hesselmann et al., 2000; Pereira et al., 1996). Biological phosphorus removal is based on the enrichment of activated sludge with polyphosphate-accumulating organisms (PAO). Under anaerobic conditions PAO take up organic substrates (preferably volatile fatty acids) and store them as PHA. In the subsequent aerobic or anoxic phase, the internal pool of PHA is oxidized and used for growth, phosphate uptake, glycogen synthesis and maintenance (Smolders et al., 1994b). It has been reported that glycogen-accumulating non-poly-P organisms (GAO, also called G bacteria) may appear when biological P removal deteriorates (Cech and Hartman, 1993; Liu et al., 1994; Matsuo, 1994; Satoh et al., 1994). These organisms are also capable of anaerobic utilization of organic substrates that are converted and stored as PHA, while the energy and reduction equivalents are provided only by glycogen degradation without involvement of poly-P. In the absence of glycogen, anaerobic substrate uptake by PAO or GAO can not occur (Brdjanovic et al., 1998; Mino et al., 1996).

Acetate is one of well-studied substrates for PHA production by mixed cultures. When acetate is the only carbon source available in the anaerobic phase, the 3-hydroxybutyrate unit is the major unit in the PHA formed (Satoh et al., 1992). 3-Hydroxyvalerate is formed by conversion of glycogen to propionyl-CoA via the succinate-propionate pathway and subsequent reaction with acetyl-CoA (Pereira et al., 1996).

Satoh et al. (1992) and Satoh et al. (1996) demonstrated the formation of very unique PHA containing monomeric units 3HV, 3H2MB (3-hydroxy-2-methyl butyrate), and 3H2MV (3-hydroxy-2-methyl valerate). Inoue et al. (1996) showed that the PHA produced by anaerobic-aerobic activated sludge is a mixture of P(3HB-co-3HV-co3H2MB-co-3H2MV) of different compostions. The presence and relative proportion of different PHAs is dependent on the type of carbon substrate available. When acetate is the only carbon source available in the anaerobic phase, the 3HB unit is the major unit in the PHA formed (Satoh et al., 1992; Smolders et al., 1994a), the composition of PHA formed from some other carbon sources can be predicted theoretically based on the stoichiometries developed by Mino et al. (1994) and Satoh et al. (1996).

In wastewater treatment processes microorganisms can accumulate storage materials as PHA are generally exist under a feast and famine regime though the PHA storage in EBPR process is only widely accepted (van Loosdrecht et al., 1997). Not only accumulated under anaerobic condition, the PHA can also be produced under aerobic or anoxic conditions. In the last decade, many researchers reported the PHA production by mixed culture under dynamic conditions in activated sludge processes. The growth of biomass and storage of polymers occur simultaneously when there is an excess of external substrate (feast period), when all the external substrate is consumed, stored polymer can be used as a C- and energy source (famine period).

It is important to select an appropriate substrate in the optimization of PHA production. The nature of the substrate not only determines the PHA contents of cells but also its composition, which subsequently affects the final polymer properties. Furthermore, the cost of the carbon source contributes significantly to the overall production cost of PHA (Yamane, 1992, 1993); the cost of the carbon sources may account for 70–80% of the raw materials, raw materials may account for 40–50% of the total operating costs (Choi and Lee, 1997), the price of PHAs can be substantially reduced if cheap organic substrates, such as waste materials from agriculture and food industries (e.g. whey and molasses) and other industries are used.

Acetate is the most used single carbon source for PHA production in mixed cultures or activated sludge. Only a few studies consider other volatile fatty acids (VFAs) like propionate, butyrate or their mixtures (Beccari et al., 1998; Lemos et al., 1998) and even less investigated the effects of other carbon compounds like lactate, succinate, pyruvate and malate (Satoh et al., 1992), ethanol (Beccari et al., 1998; Majone et al., 2001), aspartate and glutamate (Satoh et al., 1998b) and glucose (Dircks et al., 2001).

Hollender et al. (2002) presented that the highest PHA accumulation using activated sludge during the anaerobic phase was observed with acetate as carbon source (20 mgC as PHAs/g dry matter, DM) and smallest with glucose (6 mgC as PHAs /g dry matter, DM). This is in accordance with the phosphate release in anaerobic/aerobic process because the energy for PHA formation is produced by poly-P hydrolysis.

During the PHA accumulation, the highest substrate consumption rate was observed for acetate followed by propionate and finally butyrate (Lemos et al., 1998) using activated sludge. The yield of polymer produced per carbon consumed ($Y_{p/s}$: mg polymer/mgCOD) obtained when using acetate, propionate, and butyrate were 0.97, 0.61, and 0.21, respectively. These results showed that in this system, acetate is the best substrate for PHA production by the phosphorus-accumulating bacteria.

Lemos et al. (1998) noticed that when using mixed substrates, the $Y_{p/s}$ value is the sum of the individual contributions of each substrate. With a carbon concentration of 320 mgCOD/l, identical amounts of HB and HV units were produced. The utilization of more concentrated carbon substrate leads to the consumption of propionate primarily, the P(HB-co-HV) being enriched in HV units.

1.7.2.2 Effect of operation parameters

DO

Although activated sludge acclimatized under anaerobic–aerobic conditions accumulates PHA, there is no guarantee that anaerobic–aerobic operation of the activated sludge process is best for enrichment of PHA accumulating microorganisms. Satoh et al. (1998)

found that sludge accumulated more PHB under aerobic conditions than under anaerobic conditions when using acetate as the substrate. The accumulation of PHA increased linearly with the increase in oxygen supply rate, but the maximum PHA accumulation remained at 33% of sludge dry weight (at oxygen supply rate of 8 mgO_2/min/gMLVSS). In order to further increase the PHA content of activated sludge, Satoh et al. (1998) introduced a new activated sludge process tentatively named 'microaerophilic–aerobic' activated sludge process. The anaerobic–aerobic activated sludge process enriches PHA accumulators that are capable of glycogen and/or polyphosphate accumulation, because glycogen and/or polyphosphate are required for anaerobic substrate uptake. Satoh et al. (1998) expected that introduction of small amounts of oxygen into the anaerobic zone of the anaerobic–aerobic process may promote the accumulation of PHA accumulators that are not capable of accumulating glycogen and/or polyphosphate. They achieved 62% of PHA accumulation by using activated sludge acclimatized in the microaerophilic–aerobic process by incubation with acetate for 30 h. The results from Third et al. (2003) presented that the yield of PHA on acetate was 0.49 g PHA. g^{-1} substrate when oxygen was limiting and 0.34 g PHA g^{-1} substrate under excess oxygen.

Temperature

The influence of temperature (15, 20, 25, 30 and 35^oC) on the accumulation of PHB by activated sludge in sequencing batch reactor cultures fed with acetate has been studied by Krishna and Van Loosdrecht (1999). The PHB formation rate was shown to decrease with increasing temperature because of an increase of the anabolic rate at higher temperatures, the PHB level reached the highest (0.21 Cmmol PHB/Cmmol biomass) at 15^oC and the lowest (0.06 Cmmol PHB/Cmmol biomass) obtained at 30^oC.

Chinwetkitvanich et al. (2004) also investigate the effects of temperature on PHA production and storage by activated sludge biomass. The two-stage operation approach, i.e. a growth phase followed by a nutrient limitation phase, was applied to induce PHA accumulation. The pre-selected temperatures of 10, 20 and 30°C were investigated using fully aerobic SBR systems. It was found that PHA production was greater in the 10°C system than in the 20°C and 30°C systems but there was little difference between the two

higher temperatures. The maximum PHA fractions of the sludge were 52, 45 and 47%TSS for the three temperatures from low to high. This result partly agreed with that from Krishna and Van Loosdrecht (1999), PHA production increased with the decrease of the temperature, anyway, in the higher temperature, the trends showed differently.

pH

Chua et al., (2003) investigated the PHA production behaviour of activated sludge using acetate as carbon source under different pH conditions. As pH increased from 6 to 9, PHA content of sludge increased as well. At pH 6 and 7, there was very little PHA accumulation, and PHA content was less than 5% of sludge dry weight. At pH 8 and 9, PHA accumulation was stimulated, and PHA content reached 25-32% of sludge dry weight. The results indicated that pH control is essential in optimizing the PHA production process and pH≥8 is recommended.

Sludge retention time (SRT)

Chua et al. (2003) studied sludge retention time (3 and 10 days) on PHA production with acetate as the carbon source, it was noticed that the longer the SRT, the higher the biomass concentration in the reactor. Microorganisms in reactor with lower MLSS had a chance to take up about more organic substrate than those in higher MLSS. This might have led to the higher PHA production capability of activated sludge in lower MLSS (or lower SRT, i.e. 3 days). In addition, activated sludge process with longer SRT normally contains higher amount of inert biomass and this might also contribute to the lower PHA content.

C:N ratio

Chua et al. (1997) studied the effect of C:N ratio on PHB production.. The polymer content of cells increased to a maximum of 37.4% (cell dry weight) when C:N ratio was increased from 24 to 144, whereas the cell yield (g of cells formed per g of TOC used) decreased with increased C:N ratio. An optimum C:N ratio of 96 provided the highest polymer yield 0.093 g of polymer per g of carbon substrate consumed The C:N ratio in

activated sludge process must be kept around 24 in order to enable normal microbial cell synthesis.

Nitrogen and/or phosphorus limitation

PHA accumulation in bacteria can be stimulated under conditions of unfavorable growth such as the deprivation of oxygen, nitrogen, phosphate, sulfur, magnesium or potassium in the presence of excess carbon (Chinwetkitvanich et al., 2004). Chinwetkitvanich et al. (2004) found that all experiments with P limitation resulted in large accumulations of PHA, expressed as % of TSS in the biomass, the PHA yields (mg PHA/mg CODu) and productivities (mg/l-d) were significantly lower than those obtained during the nitrogen limitation experiments (Chinwetkitvanich et al., 2003).

Processes using activated sludge for PHA production

In recent years the interest for PHA production by mixed cultures has increased and many studies addressed this subject. Cultivation strategies to improve the PHA content, yield and productivity in pure cultures are very well defined. However, for mixed cultures or activated sludge this knowledge is still very limited.

Among the different processes described for PHA production by mixed cultures, the feast and famine process is the most promising because of the high sludge PHA content and productivity. The substrate is fed during a short period of time, followed by a longer period of substrate lack. This period of famine is needed to stimulate the PHA storage capacity of cells. The feast and famine process is commonly carried out in a sequencing batch reactor (SBR). SBRs are ideal reactors for a selection of robust populations with high ability of PHA storage, because biomass grows under transient (unsteady) conditions. Furthermore, this kind of reactor is easy to control and is highly flexible, allowing for a quick modification of the defined process conditions (length of feed and cycle length).

Serafim et al. (2004) found that sludge submitted to aerobic dynamic feeding could accumulate high amounts of PHA by manipulating feeding concentrations and the reactor operating parameters. The maximum PHB content, 67.5%, was obtained for 180 Cmmol/l of acetate supplied in one pulse. However, such high substrate concentration proved to be inhibitory for the storage mechanism, causing a slowdown of the specific PHB storage rate. In order to avoid substrate inhibition, 180 Cmmol/l of acetate was supplied in different ways: continuously fed and in three pulses of 60 Cmmol/l each. In both cases the specific PHB storage rate increased and the PHB content obtained were 56.2% and 78.5%, respectively. The latter value of PHB content is similar to that obtained by pure cultures and was never reported for mixed cultures.

Dionisi et al. (2005) studies the operation of a new process for the production of biopolymers (polyhydroxyalkanoates, PHAs) at different applied organic load rates (OLRs) applied to an SBR. The process is based on the aerobic enrichment of activated sludge to obtain mixed cultures able to store PHAs at high rates and yields. As a consequence the best performance of the process was obtained at an intermediate OLR (20 gCOD/L/day) where both biomass productivity and PHA storage were high enough.

If a continuous system for PHA production is desired, then a possible configuration could include two reactors in series in which a plug flow reactor (PFR) is followed by a continuous stirred tank reactor (CSTR), coupled to a settler or membrane filter. Beccari et al. (1998) used a CSTR operated with intermittent feeding. This process selects and produces sludge with high storage capacity. The PHB storage or accumulation yield ranged from 0.06 to 0.5 g COD/g COD and the sludge stored up to 40–50% of the total dry weight.

Most of the studies on PHA production by mixed culture or activated sludge were conducted with activated sludge acclimatized with synthetic wastewater (Beun et al., 2000a; Beun et al., 2000b; Chua et al., 2003; Lemos et al., 1998; Satoh et al., 1998a; Satoh et al., 1998b; Satoh et al., 1996) in order to assess the feasibility of PHA

production by activated sludge, the capability of activated sludge from full scale wastewater treatment plants had been hardly reported.

The limited data shows that the homo and copolymers obtained by mixed cultures bear average molecular weights, in the same range of variation as those obtained by the use of pure cultures (Lemos et al., 1998).

1.7.3 PHA production by using activated sludge as microorganisms and wastes as carbon sources

The process for the production of biodegradable plastic material (polyhydroxyalkanoates, PHAs) from microbial cells by mixed-bacterial cultivation using readily available waste (renewable resources) is the main consideration nowadays (Chua et al., 2003; Khardenavis et al., 2007; Md Din et al., 2006; Rhu et al., 2003).

Chua et al. (2003) investigated the production of biodegradable plastics polyhydroxyalkanoates (PHA) by activated sludge treating municipal wastewater. It was found that sludge acclimatized with municipal wastewater supplemented with acetate could accumulate PHA up to 30% of sludge dry weight, while sludge acclimatized with only municipal wastewater achieved 20% of sludge dry weight.

In the further study, Rhu et al. (2003) attempted the PHA (polyhydroxyalkanoate) production with SBRs from food waste. Seed microbes were collected from a sewage treatment plant with a biological nutrient removal process, and acclimated with synthetic substrate prior to the application of the fermented food waste. The maximum content of 51% PHA was obtained with an anaerobic/aerobic cycle with P limitation.

PHA production from activated sludge has also been investigated with synthetic wastewater (65.8% of dry cell weight) and anaerobic wastewater (58%) (Khardenavis et al., 2005) (Khardenavis et al., 2007). Anyway, the PHB producing bacterial biomass was enriched by aeration in synthetic medium in lab-scale.

Khardenavis et al. (2007) evaluated waste activated sludge generated from a combined dairy and food processing industry wastewater treatment plant for its potential to produce biodegradable plastic, PHA. The results showed that, deproteinized jowar grain-based distillery spentwash yielded 42.3% PHB production (w/w), followed by filtered rice grain-based distillery spentwash (40% PHB) when used as substrates. Addition of di-ammonium hydrogen phosphate (DAHP) resulted in an increase in PHB production to 67% when raw rice grain-based spentwash was used. Same wastewater, after removal of suspended solids by filtration and with DAHP supplementation resulted in lower PHB production (57.9%). However, supplementing other wastes with DAHP led to a substantial decrease in PHB content in comparison to what was observed in the absence of DAHP. Though the PHB content achieved in this study (67%) was lower than that obtained with pure cultures (up to 80%), the results were amongst the highest obtained so far with activated sludge using wastewater as substrate (Khardenavis et al., 2007).

Md Din et al. (2006) studied the production of PHA by using single fed-batch mixed cultures and renewable resources. A sequencing batch reactor (SBR) was used in the study. The initial mixed culture was developed using 10% activated sludge from the sewage treatment plant and 90% from palm oil mill effluent (POME). The cultivation was kept maintained in a single fed-batch reactor and operated in two steps: growth and accumulation stage. Firstly, the system will allow an extensive growth (using nutrient medium) and then the limiting nutrient (no nutrient medium adaptation) will be introduced in the next step. The average production of PHA could only reach up to 44% of cell dry weight. However, the favourable factors (e.g. temperature and harvesting time) have been made in the next stage to induce the PHA production (Md Din et al., 2006).

1.8 Isolation of the PHA-accumulating bacterial strains from municipal and industrial wastewater sludge

Activated sludge process is the most widely used technology for municipal and industrial wastewater treatment. The microbial community named "activated sludge" is a mixed population of microorganisms containing many species of viruses, bacteria, protozoa,

fungi, metazoa and algae. Population shifts within the microbial community may result from changes in the plant operating conditions and cause sludge quality problems such as poor sludge settling, compaction, and dewatering (Chipasa and Me˛drzycka 2004; Nielsen and Nielsen 2002a; Nielsen et al. 2004; Wagner and Loy 2002). Today, most modern wastewater treatment processes rely on the composition and activity of their microbial community in activated sludge. Therefore, the analysis of the microorgansms in activated sludge is of considerable interest. Determination of the dominant role of the microorganisms in the ecosystem of the activated sludge allows the treatment process to be controlled to improve the performance of WWTPs (Nielsen et al. 2004).

Many different bacterial groups are present in activated sludge systems. The bacteria are present either as single cell microcolonies or as filamentous bacteria. The bacterial count in activated sludge is in the range of 1–10 × 10^{12}/g VSS. Of these, typically 80% are active or alive (Nielsen 2002; Nielsen et al. 2004). In a nutrient removal treatment plant, many bacterial strains having different functions may be present including phosphorus accumulating organisms (PAOs) (Ansa-Asare et al. 2000; Carucci et al. 2001; Crocetti et al. 2000; Das et al. 2004; Kawaharasaki et al. 1999; Lina et al. 2003; Nakamura et al. 1995b; Nielsen et al. 1999; Oehmen et al. 2005; Pijuan et al. 2005; Reddy and Bux 2002; Sidat et al. 1999a; Wong et al. 2005; Zhang et al. 2003); ammonia-oxidizing bacteria (Purkhold et al. 2000); nitrite-oxidizing bacteria (Nogueira et al. 2002); nitrate-reducing bacteria (Dionisi et al. 2002; Drysdale et al. 1999; Etchebehere et al. 2001; Jetten et al. 2001; Juretschko et al. 2002b; Khan and Hiraishi 2001; Nogueira et al. 2002); glycogen accumulating organisms (GAO) (Liu et al. 1996; Seviour et al. 2000); filamentous micro-organisms (Blackall et al. 1996b; Francis et al. 2002; Kämpfer 1997; Kanagawa et al. 2000; Kitatsuji et al. 1996; Rossett et al. 1997); Fe(III)-reducing bacteria; sulfate-reducing bacteria (Kjeldsen et al. 2004); and methane-producing bacteria (Nielsen and Nielsen 2002a; Nielsen et al. 2004). Iron reducers, sulfate reducers and methanogens groups are usually considered less important in the normal activated sludge processes. Under some conditions (e.g. when bulking problems appear), domination of sulfide and useful oxidizing bacteria can be observed. It is important to note that up until now, the majority of the bacteria in activaed sludge are just roughly known as aerobic heterotrophs

or denitrifiers, and very little is known about their main functions in the sludge (Nielsen et al. 2004; Strous et al. 2002; Wagner and Loy 2002). These groups of bacteria can be observed in most types of treatment plants. However, the direct taxonomical identity on a species level is presently almost unknown except a few bacterial groups. Most of these groups and other bacterial species in the sludge are still uncultured so that they can only be detected by molecular methods independent of cultivation (Coþkuner 2002; Forster et al. 2002; Kim et al. 2004; Nielsen and Nielsen 2002a; Nielsen 2002; Radajewski et al. 2000; Wagner and Loy 2002). A great number of microorganisms have been isolated and the genera most frequently found are cited as follows: *Pseudomonas, Bacillus, Achromobacter, Enterococcus, Acinetobacter, Aeromonas, Alcaligenes, Arthrobacter, Escherichia, Salmonella, Proteus, Streptococcus, Staphylococcus, Micrococcus, Corynebacterium, Clostridium, Penicillium* (Mehandjiyska 1995) etc. Besides, many researchers report that the majority of bacteria in activated sludge belong to Gram-negative genera (Pick 1995; Sharifi-Yazdi et al. 2001).

1.8.1. Methods of isolation and identification

The current approaches for activated sludge analysis include: traditional microbial systematics, microscopic analysis, molecular analysis, microbial systematics using 16S ribosomal ribonucleic acid (rRNA), 16S rRNA oligonucleotide probe, 16S rRNA community analysis, designing diagnostic probes, and/or gene probes (Blackall et al. 1998; Lajoie 1997).

Traditionally, microbial communities in WWTPs have been analysed for decades either by light microscopic observation or by cultivation-dependent techniques, identificaton has been based on shape (e.g., rod, coccus), cell wall type (Gram-negative or Gram-positive) and a host of biochemical tests (e.g., oxidase-positive, oxidase-negative); moreover, the total number of bacteria was usually assessed by 4',6-diamidino-2-phenylindole (DAPI) staining and the living fraction as positive by either fluorescence *in situ* hybridization (FISH) with oligonucleotide gene probes staining all bacteria with a

significant content of ribosomes, or the fraction able to take up radioactive substrates as measured by microautoradiography (MAR) (Nielsen and Nielsen 2002a).

Fluorescence in situ hybridization (FISH) is a powerful technique for detecting DNA or RNA sequences in cells. This molecular biological technique enables the localization of specific DNA sequences within genome of microorganisms and the identification of microorganisms under different environmental conditions. Direct quantification of bacterial strains is possible by manual counting of hybridized cells (epifluorescence and laser confocal microscope) or by image analysis of digital photos (both microscopes) or automated counting with a flow cytometer. FISH is quickly becoming one of the most extensively used fluorescent staining techniques owing to its sensitivity and versatility, and with the improvement of current technology and cost effectiveness, its use will surely continue to expand (Blackall et al. 1998; Lajoie 1997).

DAPI is known to form fluorescent complexes with DNA, showing a fluorescence specificity with the observation using a fluorescence microscope. Because of this property DAPI is a useful tool in various cytochemical investigations (Nielsen and Nielsen 2002a).

The most sensitive technique for enumerating metabolically active bacteria that utilised single-cell method is microautoradiography (MAR) (Hoppe 1976). The bacteria that are actively engaged in substrate uptake are detected by this method and are measured by using a radiolabeled tracer such as typically thymidine (an amino acid) or a mixture of amino acids (Nielsen and Nielsen 2002a).

Modern systematics is based on the nucleotide sequence of the ribonucleic acid (RNA) found in the 16S subunit of the ribosome (16sRNA). All RNA is composed of four nucleotides; adenie (A), cytosine (C), guanine (G) and uracil (U). the sequence of these four bases in 16sRNA is used for the classification and identification of microorganisms. While so-called full-cycle rRNA approach involving the establishment of a 16S rRNA

gene library and the subsequent design and use of clone specific probes for FISH analysis (Juretschko et al. 2002b).

The full cycle rRNA approach offers a cultivation independent alternative to the established techniques for the identification of new microorganisms. It should, however, be stressed that even a complete rRNA study cannot substitute for the isolation and characterization of microorganisms. Currently, only part of the applications of the rRNA approach encompass sequencing and probing. Not surprisingly, initial applications of the rRNA approach focused on identification of microorganisms in less complex samples. Once its suitability had been demonstrated, it started to find widespread application (Juretschko et al. 2002a).

1.8.2. Bacterial strains isolated from activated sludge for PHA production

At present, only a few bacterial strains capable of accumulating PHA have been isolated from the Anaerobic/oxic (A/O) system (Liu et al., 2000; Sawayama et al., 2000; Vincenzini et al., 1997). The most common isolate from the process is *Acinetobacter* sp.. *Acinetobacter* sp. could accumulate PHB up to 11.5% in cell dry weight basis after growth in a medium containing acetate as the carbon source (Vincenzini et al., 1997) Another isolate from the A/O system belongs to the new genus *Amaricoccus* from the alpha subdivision of Proteobacteria (Liu et al., 2000).

In a recent study (Dionisi et al., 2006), after confirming the speciation of the population for PHA accumulation by DGGE, a clone library was constructed from the total DNA extracted from the SBR sludge. The screening of the clones was performed using amplified ribosomal DNA restriction analysis (ARDRA). However, the isolates have not yet been associated with PHA accumulation. Further investigation regarding the aforementioned microbial groups is necessary to certify their status as PHA producers.

A different approach was employed by Serafim et al. (2006). Using fluorescence in situ hybridization (FISH), the dominant organism present in a SBR operated under feast-

andfamine conditions gave a positive signal to the *Azoarcus* sp. probe. Using Nile blue staining, it was possible to correlate the presence of PHA inclusions with the *Azoarcus* sp., and thus confirm this organism as a PHA producer.

1.9. Conclusions

Base on the literature review, the following major problems of this study can be summarised:

1.9.1 Problem of conventional plastics:

The use of conventional plastics is an increasing environmental problem because of the increase of the production and the disposal of waste plastics. It can cause harmful effects during the production, the transformation and the utilisation. Moreover, its discharge in the environment causes an ecological problem because of their non-degradable characteristics. This kind of plastics can be replaced by one of the biodegradable plastics-PHA.

1.9.2. High production cost of biodegradable plastics

PHA's widespread applications have been limited by high production costs, which is more than 10 times higher compared to conventional petrochemical plastics using pure culture and pure carbon sources.

1.9.3. Wastewater and waste activated sludge management and reuse

The management of municipal and industrial waste (sludge and wastewater) also represents an increasing environmental problem. Wastewater discharges required an efficient treatment process in order to satisfy the environmental regulations. Moreover, wastewater treatment plants generate sludge, the sludge handling and disposal cost represents about 50% of the operating costs of a typical wastewater treatment facility.

The fact that activated sludge contains mixed culture for produce PHA, and wastewater contains biodegradable carbon, nitrogen, phosphate and other nutrient.

1.10. Objectives and hypotheses

1.10.1 Hypotheses

The PHA production involved two parts: microorganisms and carbon sources. Pure culture and activated sludge (i.e. mixed culture) could be used as microoganisms, pure substrate and wastes materials used as carbon sources. All the studies and industrial production of PHA currently in use are three combined systems: pure culture and pure carbon sources, pure culture and wastes, and activated sludge (or mixed culture) and pure carbon sources. As activated sludge has the capacity to accumulate PHA to varying degrees and depends on the types of substrates used. Wastewater contains biodegradable carbon, nitrogen, phosphorus and many other nutrient required for the growth and the product formation process of the microorganisms, so it could be used as a raw material for accumulation PHAs. Therefore, the production of PHA by using activated sludge as a source of microorganism and wastewater as a source of carbon could be established and optimum conditions must be figured out. By this approach, it can convert the organic pollutants in wastewater into biodegradable plastics (e. g. PHA), reduce COD load by to be treated in wastewater treatment plant, substantially reduce the treatment cost, as well as the sludge production and sludge disposal cost.

1.10.2 Major objectives

The main research objective is to produce PHA using waste activated sludge as the source of microorganisms and wastewaters as the carbon sources. The specific research objectives are as follows:

Study the possibilities of using different municipal and industrial wastewater activated sludge and/or wastewater for PHA production

The activated sludge with best PHA production capacity was selected for the further study of process development.

Effect of different activated sludge concentrations of activated sludge were test for PHA production.

Batch, fed-batch and continuous feeding strategy experiments were conducted, respectively to evaluate the produce PHA.

Effect of different C:N ratios on PHA production was studied.

Activated sludge bacteria in a municipal wastewater treatment plant were induced to accumulate storage polymers (PHAs). The isolation of the PHAs accumulating bacteria from activated sludge as well as the comparison of their abilities of accumulating PHAs were conducted.

Activated sludge bacteria in an industrial wastewater treatment plant were induced to accumulate storage polymers (PHAs). The isolation of the PHAs accumulating bacteria from activated sludge as well as the comparison of their abilities of accumulating PHAs were conducted.

The isolated PHA-accumulating bacterial microorganisms are identify based on their 16S rDNA sequences.

1.11. Originality

From the foregoing review of literature it was deduced that PHA production using industrial wastewater activated sludge as microorgansms and wastewater as the substrate still remains to be explored. The activated sludge concentration higher than 10 g/l has never been used for PHA production. Therefore, the process development will be unique. Furthermore, the isolation and identification of the PHA-accumulaing bacterial strains from the activated used will provide inspring results for the PHA production.

1.12 Summary of contents of the thesis

1.12.1. Chapter 2: PHA production by pure culture and mixed culture (activated sludge)

The chapter comprises of two parts, first which provides exhaustive information about the PHA requirement nowadays. Bioplastics are energy reserve polyester commonly accumulated by a large variety of bacteria. Some bacteria can accumulate as much as 70% of cell dry weight under certain cultivation conditions. Polyhydroxybutyrate (PHB) is derived from acetyl-CoA and represents the most abundant PHAs in nature. These biopolymers share many proprieties with the conventional plastics (polypropylene). In order to protect the environment, industries are encouraged to develop the production of biodegradable polymers. Many strategies are used to develop this sector through development of new strains which could accumulate higher polymer concentration. Also, in order to reduce the cost of PHAs production, many wastes have been used as growth media for bacteria. Therefore, the second part of chapter 1 presents the new approach of PHA production by waste materials and/or mixed culture (activated sludge). PHA production by mixed cultures may allow use of cheaper substrates and cheaper nonsterile equipment for an important reduction on the process operating costs. Therefore, if the PHA content of mixed cultures can be improved, the mixed-culture process has high potential for PHA production. The results of many studies demonstrate that mixed cultures for the PHA production have high PHA storage capacity, high PHA yields, and high specific PHA productivity. The maximum intracellular PHA content reported so far (around 70% on cell dry weight) (Serafim et al., 2004b) is comparable with that of some pure cultures, howevere; it is lower than that obtained in recombinant E. coli (about 90% on cell dry weight).

1.12.2. Chapter 3: PHA production by using different municipal and industrial activated sludge and /or wastewater in shake flask and fermentor experiments.

In the present work, activated sludge from different full-scale wastewater treatment plants (municipal, pulp and paper industry, starch manufacturing and cheese manufacturing wastewaters) was used as a source of microorganisms to produce biodegradable plastics in shake flask experiments. Acetate, glucose and different wastewaters were used as

carbon source. Activated sludge from full-scale pulp-paper industrial wastewater treatment plants was used as a source of microorganisms to produce biodegradable plastics (PHA) in bioreactors. Acetate was used as carbon source. Effect of different activated sludge concentrations of activated sludge were test for PHA production. Batch, fed-batch and continuous feeding strategy experiments were conducted, respectively to produce PHA. Activated sludge from full-scale pulp-paper industrial wastewater treatment plants was used as a source of microorganisms to produce biodegradable plastics (PHA) in bioreactors (15 L). Four types of wastewaters from municipal and industrial wastewater treatment plants, respectively and acetic were used as carbon source. The continuous feed strategy experiments were conducted, respectively.

1.12.3. Chapter 4: Isolation and identification of PHA-accumulating bacterial strains from the activated used in the experiments.

This chapter consists five parts. The first part focuses on the recent advances in microbiology of the activated sludge process. The bacterial population in activated sludge system is examined. The standard procedure, medium used, analytical methods and biochemical characterization techniques required for isolation, and identification of bacteria responsible for the key process of wastewater treatment systems (nutrient removal, aerobic, anaerobic, etc.) are discussed in the review. Effect of seasonal (winter and summer) temperature variations and salinity variation on the bacterial species for wastewater treatment is examined. The second part and the third part are the isolation of the PHA-accumulating bacterial from municipal and industrial activated sludge, respectively, the fourth and fifth parts are the isolates bacterial strains identification based on their 16S rDNA sequences from the two wastewater sludge, respectively.

5.1. Conclusions

This study was conducted to the PHA production using activated sludge as microorganisms and wastewater as the substrates, the following results could be drawn and listed as follows;

5.1.1 Chapter 2. PHA production by the pure culture and activated sludge

Bioplastics are energy reserve polyester commonly accumulated by a large variety of bacteria. Some bacteria can accumulate as much as 70% of cell dry weight under certain cultivation conditions. Polyhydroxybutyrate (PHB) is derived from acetyl-CoA and represent the most abundant PHAs in nature. These biopolymers share many proprieties with the conventional plastics (polypropylene). In order to protect the environment, industries are encouraged to develop the production of biodegradable polymers. Many strategies are used to develop this sector through development of new strains which could accumulate higher polymer concentration. Also, in order to reduce the cost of PHAs production, many wastes have been used as growth media for bacteria.

PHA production by mixed cultures may allow use of cheaper substrates and cheaper nonsterile equipment for an important reduction on the process operating costs. Therefore, if the PHA content of mixed cultures can be improved, the mixed-culture process has high potential for PHA production. The results of many studies demonstrate that mixed cultures for the PHA production have high PHA storage capacity, high PHA yields, and high specific PHA productivity. The maximum intracellular PHA content reported so far (65% on cell dry weight) is comparable with that of some pure cultures, howevere, it is lower than that obtained in recombinant E. coli (about 90% on cell dry weight). Using the mixed culture, the polymer yield (0.56 g PHB. g-1 substrate) and the specific productivity (0.77 g PHB. g-1 cell dry weight . h-1) obtained so far are very promising. The specific productivity of mixed cultures is approximately tenfold that of recombinant E. coli cultures. Anyway, the volumetric PHA productivity reported for mixed cultures are still lower than that by most pure cultures. The reason for this is due to the difficulty in reaching high biomass concentrations in the mixed-culture process. Therefore, the main challenge regarding the mixed culture process is the development of

culture selection strategies of fast-growing organisms that have, at the same time, a high PHA storage capacity. The physical properties of the PHAs produced by mixed culture of activated sludge were comparable to PHAs produced by pure cultures. Compared to the pure culture, the knowledge of cultivation strategies and the process control to improve the PHA content, yield and productivity in mixed cultures are limited. There are many research challenges that need to be further studied.

5.1.2 Chapter 3. PHA production using activated sludge as microorganisms and wastewater as substrate in shake flask and fermentor.

Activated sludge from different full-scale wastewater treatment plants (municipal, pulp and paper industry, starch manufacturing and cheese manufacturing wastewaters) was used as a source of microorganisms to produce biodegradable plastics in shake flask experiments. Acetate, glucose and different wastewaters were used as carbon source. Pulp and paper wastewater sludge was found to accumulate maximum concentration (43 % of dry weight of suspended solids) of polyhydroxy alkanoates (PHA) with acetate as carbon source.

Effect of different feed patterns (batch, fed-batch and continuous feeding strategies) was conducted using activated sludge from full-scale pulp-paper industry wastewater treatment plants as a source of microorganisms to produce PHA in computer controlled fermentors. Batch results showed that maximum accumulation of PHA (39.6% w/w of dry sludge SS) was observed at 15 g/l SS and 10 g/l acetate concentration. Higher concentration of acetate was found to be inhibitory for PHA accumulation and biomass growth.

The continuous feeding of acetic acid further enhanced the PHA content and productivity. C:N ratio of 144 was found to furnish the highest PHA production rate as well as PHA content. Maximum PHA content (%) reached 60.9% of the dry sludge weight and PHA concentration of 16.23 g/l. Pulp and paper wastewater showed the highest PHA content (54.9%) among the four types of wastewater. The present process was a promising way to reduce the cost of PHA production as well as the conversion of

organic pollutants in waste into valuable products. A substantial amount of sludge could be reduced and therefore less sludge was left for final disposal. Copolymers (polyhydroxybutyrates - PHB and polyhydroxyvalerates - PHV) were produced by the sludge whose molar composition varied with the batch process time.

5.1.3 Chapter 5. Isolation and identification of PHA production bacterial strains from activated sludge.

Six PHA accumulating bacterial strains were isolated from the pulp and paper sludge and their capabilities of the PHA production had been evaluated. The PHA amounts produced significantly varied among the six strains. The maximum PHA content of 35.45% was obtained by strain PHA-P5 even under uncontrolled conditions of pH and dissolved oxygen concentration. These sludge microorganisms were identified based on their 16S rDNA sequences and the PHA-P5 with the maximum PHA production was identified as *Comamonas* sps.

Twelve PHA accumulating bacterial strains were isolated from the municipal sludge and the maximum PHA content of 27.5% was obtained by strain PHA-M3. These sludge microorganisms were also identified based on their 16S rDNA sequences and the PHA-M3 with the maximum PHA production was identified as *Citrobacter* sp.

The present process using wastewater and activated sludge for the PHA production was a promising way to reduce the cost of PHA production as well as the conversion of organic pollutants in waste into valuable products.

5.2. Recommendations

The present research work was carried out to develop the economically feasible process for the production using activated sludge as microorganisms and wastewater as substrate. The recommendations are as follows;

1.Combined effect of the process parameter such as pH, temperature and oxygen concentration should be investigated for the high PHA production based on this process.

2.More waste materials as the substrate for PHA production should be studied. Especially those contain high concentration of volatile fatty acid.

3.Pilot-scale fermentation study is required for the precise economic consideration of PHA production by activated sludge and wastewater.

4.The pure cultures isolates from municipal and industrial activated sludge should be further studied for their high potential of PHA production.