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Développement d'un procédé de production de biofloculants à base de *Cloacibacterium normanense* utilisant les boues d'épuration comme matière première

Par

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AVANT -PROPOS

L'objectif principal de ce projet de thèse est de développer et optimiser un procédé de production de biofloculants à partir de cultures pures utilisant les boues secondaires comme milieu de culture et le glycérol brut comme source de carbone supplémentaire, pour le traitement de boues municipales de station d'épuration tel que la décantation et la déshydratation de boues. Le développement d'un procédé de production de biofloculants mettant en synergie la souche bactérienne productrice des SPEs dans les boues activées et la potentialité de méthodes d'extraction pourrait constituer une avenue potentielle pour le traitement à grande échelle de boues biologiques. Cette étude est constituée de 7 chapitres. Les expérimentations ont été entièrement réalisées au Centre de recherche Eau, Terre, Environnement (ETE) à l'institut national de la recherche scientifique (INRS).

Le premier chapitre correspond à la synthèse de ce travail, laquelle comprend la revue de littérature relative à la problématique de l'augmentation de la production de boues à la source (station d'épuration) et l'utilisation des polymères chimiques ainsi que le procédé de traitement à développer afin d'y remédier. Les hypothèses, les objectifs et la démarche méthodologique utilisée et enfin un bilan des résultats obtenus sont également présents dans ce chapitre.

Parmi les recherches scientifiques concernant les exo polymères cités dans la littérature, l'ingénierie des SPEs reste un sujet restreint dû aux résultats limités. Dans ce point de vue, le second chapitre correspond à une connaissance de processus et de mécanismes de biosynthèse permettra l'optimisation de la production des SPEs chez les bactéries. Ces connaissances pourraient constituer un modèle avantageux pour la compréhension de l'organisation et la régulation de la biosynthèse des SPEs bactériennes, lequel a fait l'objet d'un article synthèse soumis dans Critical Reviews in Environmental Science and Technology. **Klai Nouha, Balasubramanium, S., Tyagi R. D., (2016) Critical review on EPS production, synthesis and composition for sludge flocculation.**

Vu les exigences de plus en plus strictes afin d'assurer une bonne gestion des boues, les spécialistes s'orientent vers de procédés de traitement moins coûteux. C'est dans cette optique que la production des SPEs par des souches bactériennes isolées à partir des boues a été proposée afin d'améliorer la floculation et la décantation des boues en réduisant ainsi l'utilisation des polymères chimiques toxiques et le coût de traitement. Le troisième chapitre correspond à l'étude portant sur l'isolement, l'identification moléculaire et la caractérisation

biochimiques des souches bactériennes productrices des SPEs isolées à partir de boues, lequel a fait également l'objet d'un article recherche publié dans *Journal of Petroleum & Environmental Biotechnology,* 7, 255 : **Nouha K, Yan S, Tyagi RD, Surampalli RY (2015) EPS Producing Microorganisms From Municipal Wastewater Activated Sludge.**

En tenant compte de résultats du troisième chapitre, la meilleure souche bactérienne productrice de SPEs *Cloacibacterium normanense* a été sélectionnée pour être appliquée par la suite dans un procédé de production des SPEs, choisissant les meilleures conditions de fermentation. Ainsi, le quatrième chapitre traite le procédé optimisé de la production des SPEs par *Cloacibacterium normanense* et évalue leur potentialité dans la floculation, décantation et la déshydratation de deux types de boues; municipales et papetières. Cette étude scientifique a fait l'objet d'un article publié dans *Journal of Civil and Environmental Engineering*, 191, 5-7: **Klai Nouha, Hoang NV, Yan Song, Tyagi RD and Surampalli RY (2015) Characterization of Extracellular Polymeric Substances (Eps) Produced by *Cloacibacterium normanense* Isolated from Wastewater Sludge for Sludge Settling and Dewatering.**

Selon les résultats précédents, la centrifugation est la méthode utilisée pour l'extraction des SPEs. C'est une méthode simple et plus connue. Néanmoins, la méthode d'extraction est un des facteurs importants qui peut affecter les résultats à savoir la concentration de SPEs et leurs potentialités. Toutefois, le manque d'un protocole d'extraction standard pose le problème de la comparaison des résultats des différents travaux de la littérature. De ce point de vue, de nombreuses techniques (physiques et chimiques) ont été développées afin de déterminer le choix d'une méthode avec un minimum de lyse cellulaire et sans contamination apportée par les réactifs utilisés. Le cinquième chapitre présente les différentes méthodes d'extraction (chimiques et physiques et la combinaison de deux) utilisées pour extraire les SPEs produites naturellement dans les boues.

Ce chapitre a fait l'objet d'un article publié dans *Journal of Environmental Management,* 180, 344–350. **Viet Hoang Nguyen, Nouha Klai, Thanh Dong Nguyen, Rajeshwar Dayal Tyagi (2015) Impact of extraction methods on bio-flocculants (BFs) recovered from backwashed sludge of bio-filtration unit.**

L'efficacité de procédé de production des SPEs par *Cloacibacterium normanense* dans les boues biologiques (unité de biofiltration) stérilisées passe par l'optimisation des conditions de culture. Une méthode fréquente est l'apport plus important d'une source de carbone. Le glycérol brut pourrait améliorer la production de SPEs et diminuer le coût de la fermentation. L'étude relative à l'utilisation de glycérol brut à différentes concentrations comme source de carbone supplémentaire et son effet sur la production des SPEs ainsi que l'efficacité de ces

SPEs à éliminer les métaux lourds d'eaux usées primaire est présentée en détail dans le sixième chapitre. Les résultats de ce ces travaux ont été valorisés sous forme d'article publié dans *Journal Bioressource Technology,* 212, 120-129 : **Klai Nouha, Saurab Ram Kumar, Tyagi RD (2016) Heavy metals removal from wastewater using extracellular polymeric substances (EPS) produced by *Cloacibacterium normanense* in wastewater sludge supplemented with crude glycerol and study of EPS extraction by different methods.**

La caractérisation biochimique des SPEs produites par *Cloacibacterium normanense* est une étape très importante afin de déterminer leur composition et structure chimique. Différentes classes de composées biologiques dans SPEs ont été identifiées portant divers groupements fonctionnels qui leur confèrent des propriétés physicochimiques distinctes. L'étude de ces propriétés permet de mieux appréhender leur rôle au sein de biomasse et dans la floculation et la décantation de boues. Les résultats de cette étude ont été synthétisés dans le septième chapitre. Cette recherche a fait l'objet d'un article publié dans *Journal Material Sciences and Engineering, 5, 240 :* **Klai Nouha, Hoang NV, Tyagi RD (2016) Fourier transform infrared spectroscopy And Liquid chromatography-mass spectrometry study of extracellular polymer substances produced on secondary sludge fortified with crude glycerol.**

Dans l'optique d'étendre son spectre de capacité de production, *Cloacibacterium normanense* a été testé comme candidate potentielle pour l'accumulation de lipides couramment rencontrée chez d'autres bactéries ou levures. Ce procédé de production a été également étudié afin d'évaluer la potentialité des SPEs produites comme biofloculant remplaçant les polymères chimiques dans la station d'épuration, sur le plan commercial (coût) et efficacité. Le huitième chapitre correspond à l'étude portant sur la production simultanée de SPEs et des lipides par *Cloacibacterium normanense* dans les boues secondaires additionnées du glycérol brut comme source de carbone supplémentaire et de peptone comme source d'azote. Ce dernier chapitre fait référence à l'article soumis dans *Journal Biotechnology and bioengineering:* **Klai Nouha, Saurab Ram Kumar, Tyagi RD (2016) Simultaneous Extracellular Polymeric Substances (EPS) and Lipid Production by *Cloacibacterium normanense* via Fermentation of sterilized Activated Sludge fortified with crude glycerol.**

Résumé

Quel que soit le système d'épuration adopté, le traitement des eaux usées s'accompagne d'une production de quantités de boues non négligeables dont il faut se débarrasser. Plusieurs filières existent pour l'élimination de ces boues, mais le choix doit être tributaire du coût d'installation, de l'origine de boues, de la valeur ajoutée du produit qui en résulte et de l'impact que pourrait avoir la filière retenue sur l'environnement. La valorisation biologique ou agricole constitue des technologies qui permettent de transformer les boues en produits à haute valeur ajoutée en minimisant les risques de pollution. Néanmoins, l'épandage des composts obtenus ne doit pas se faire sans s'être assuré de leur hygéinisation et de leur stabilisation. De plus, le compost de boues doit être exempt de toxicité à cause des agents floculants (les polymères chimiques) utilisés pour le conditionnement de boues et des teneurs en micropolluants organiques (phtalates, PCB, HAP…) ou métalliques. Pour s'affranchir aux polymères chimiques, une attention scientifique particulière est portée sur l'utilisation des biofloculants et des bios polymères produits par des microorganismes. Ces sont des biopolymères qui représentent une excellente alternative en raison de leurs coûts économiques et de leurs biodégradabilités limitant ainsi les risques environnementaux. Des recherches antérieures sont intéressées aux substances polymériques extracellulaires qui sont considérées comme une approche économique et rentable pour améliorer la décantation et la déshydratation de boues.

Le présent travail œuvre pour explorer et découvrir de nouveaux biofloculants potentiels sous forme de substances polymériques extracellulaires (SPEs) qui limitent l'utilisation des agents floculant ; afin de favoriser et faciliter la décantation et la déshydratation de boues. L'objectif de ce projet est de découvrir un nouveau procédé mettant en synergie une nouvelle souche qui produit une concentration élevée de SPEs dans les boues et une potentialité maximale de biofloculation. Nous verrons la première étape qui est le screening des souches productrices des SPEs, ensuite les étapes de production et d'extraction des SPEs pour enfin terminer avec leur caractérisation et leur potentialité de floculation.

Dans ce contexte, différentes souches bactériennes productrices de SPEs ont été isolées à partir de boues secondaires (unité de biofiltration). Selon deux paramètres (concentration de SPEs et l'activité de biofloculation), *Cloacibacterium normanense* a été sélectionnée comme meilleure souche pour la production de SPEs qui produit 11,8 g/L de B- SPEs (Brute –SPEs) à 25 °C et 180 rpm pendant 48h. 92% d'activité de floculation est atteint utilisant 150 mg/L de Ca^{2+} et 2,3 mg/g kaolin de S-SPEs.

Dans l'optique d'optimiser les résultats précédents, les meilleures conditions de fermentation (pH, température, agitation et taille d'inoculum) ont été par la suite appliquées afin d'améliorer le procédé de production. 13,3 g/L de B-SPEs (13 g/L de S-SPEs et 0,3 g/L de C-SPEs) a été obtenu dans des boues stérilisées à pH 7, inoculées avec 3 % d'inoculum et incubées à 30°C et 180 rpm pendant 48 h avec une potentialité de floculation de 94,2% utilisant 1,3 mg S-SPEs/g Kaolin. Différentes méthodes d'extraction ont été utilisées, mais, aucune étude n'a évaluée l'effet de ces techniques d'extraction sur l'activité de biofloculation des SPEs. Dans ce contexte, dix méthodes ont été testées pour l'extraction de SPEs présentes naturellement dans les boues secondaires. Les méthodes chimiques sont les plus efficaces pour extraire la majorité de SPEs produites dans les boues secondaires. La méthode de la centrifugation et chauffage a présenté de faibles concentrations de SPEs (1,2 g/L) en comparaison d'autres méthodes (formaldéhyde, sonication, EDTA…). Par contre, aucune lyse cellulaire n'a été remarquée dans le cas d'EDTA et de sonication. L'utilisation de formaldéhyde a montré une protection des cellules et a minimisé le dommage cellulaire en combinaison que ce soit avec la méthode de sonication ou avec l'EDTA. 6,2 g/L et 6,8 g/L de SPEs ont été extraites par l'EDTA, et Formaldéhyde-sonication-EDTA, respectivement. Un taux d'extraction élevé a été remarqué en utilisant ces deux méthodes en comparaison de la centrifugation, par contre une lyse cellulaire a été accompagnée qui peut être expliquée par la variation de la teneur en acides nucléiques de 0.09 mg/L en cas de centrifugation au 1.1 mg/L en cas de l'EDTA (5 g/L). Par contre, ce dommage cellulaire n'a aucun effet négatif sur l'activité de biofloculation qui a atteint 95% evec l'EDTA (5 g/L).

Les différents facteurs inhérents à ce procédé tels que la source de carbone, le pH, la température, l'oxygène ont été pris en considération et particulièrement le type et la concentration de source de carbone. Ainsi, cette étude s'est orientée sur l'optimisation de la production de SPEs d'une part et la minimisation des coûts de la production d'autre part. Le glycérol brut est suggéré pour être utilisé comme substrat supplémentaire aux boues suite à son faible coût et sa disponibilité. Une caractérisation biochimique a été précédemment réalisée utilisant le test de Biolog afin de vérifier la possibilité de *Cloacibacterium normanense* d'assimiler le glycérol comme substrat. Différentes concentrations de glycérol (0,5, 1, 2% (v/v)) ont été utilisées. 21 g/L de B-SPEs à 72 h a été montré en utilisant 20 g/L de glycérol en comparaison au 5 g/L (15,8 g/L de B-SPEs) et 10 g/L (18 g/L de B-SPEs). La quantité de glycérol a été diminuée au cours de la fermentation de 20 g/L à 6 g/L. Trois méthodes d'extraction (EDTA, centrifugation et traitement thermique) ont été utilisées dans cette étude. L'EDTA a été sélectionné à partir de l'étude précédente et la centrifugation a été utilisée comme une méthode de contrôle. Une variation significative de la concentration de SPEs obtenue ; 21,3 g/L± 0.5 en cas de la centrifugation et 25,5± 2.5 g/L en cas de 5 g d'EDTA/L. Toutefois, utilisant 4,2 mg B-SPE/g Kaolin, les activités de floculation obtenues

par les trois méthodes d'extraction (Centrifugation, chauffage et EDTA) sont : 90,5 ; 77 and 77,5 %, respectivement. Dans cette étude, la centrifugation est la meilleure méthode qui offre à la fois une concentration élevée de SPEs et une haute potentialité de floculation avec un minimum de dose.

En tenant compte de résultats précédents, la concentration de SPEs est constante à partir de 72 h et la concentration de glycérol brut n'est pas totalement consommée. Une attention particulière est portée sur la variation du rapport carbone /azote au cours de la fermentation. Ce rapport qui peut favoriser la production de SPE lorsqu'il est faible contrairement à la production de lipides qui demande un rapport élevé pour stimuler l'accumulation de lipides. Dans ce sens, *Cloacibacteriunm normanense* semble une candidate d'une production de deux bioproduits, les SPEs et les lipides. De ce point de vue, cette étude est centrée sur l'ajout d'une source d'azote afin de favoriser l'accumulation de lipides et un environnement riche en carbone pour la production des SPEs. Les meilleurs résultats sont obtenus avec 22 g/L de SPEs et 27.6 % (p/p) de lipides accumulés pendant 72 h de fermentation utilisant un rapport C/N de 25.

La matrice polymérique des biomasses renferme un large et hétérogène panel de molécules qui sont différentes tant sur le plan structurel que sur le plan des propriétés. Toutefois, c'est l'ensemble de ces propriétés qui va engendrer leur rôle fondamental dans la formation de flocs, décantation et déshydratation de boues et adsorption de métaux. Les travaux ont d'abord consisté à caractériser la structure et la composition chimique des SPEs. Pour ce faire, le FTIR et le LC/MS ont été utilisés. Les résultats de cette étude ont montré que les SPEs produites par *Cloacibacterium normanense* sont riches en diverses classes de molécules (les protéines, les polysaccharides, les substances humiques, les acides uroniques, les acides nucléiques) portant des groupes fonctionnels variés tels qu'hydroxyle, carboxyle, amide, amine, sulfure, etc. Les SPEs ont de nombreuses fonctions dans la vie des biomasses, dues aux propriétés de ces molécules. Ces SPEs ont été étudiées dans la formation de flocs dans les boues avec une activité de 73% en comparaison de 63% d'activité utilisant le Zetag (un polymère chimique utilisé dans la station d'épuration de Québec). Le rôle de SPEs dans la décantation est clairement démontré suite à la diminution de l'indice du volume des boues IVB en comparaison de contrôle (sans addition de SPEs). Les SPEs ont été évalués dans la décantation et la déshydratation de deux types de boues (municipales et papetières) à des concentrationen matières en suspension (MES) variées (7, 5, 2 et 1 g/L). Un indice du volume des boues municipales et papetières avec 5 g/L MES est de 20 mL/g après 5 min de décantation en comparaison de 50 mL/g après 30 min dans le cas de contrôle (sans ajout de SPEs). De plus, une diminution de temps de succion capillaire a été enregistrée montrant une élimination d'une quantité importante d'eau par les SPEs avec un pourcentage de déshydratation de 37,6% utilisant seulement 0,02 g/L de S-SPEs.

De nombreuses études ont été menées sur les capacités de bio sorption des métaux par les SPEs. Celles-ci ont été appliquées pour le traitement des eaux usées primaires contaminées par des éléments métalliques. Une capacité d'élimination de 85% de Nickel (Ni), 70% de Fer (Fe) et Aluminium (Al) ont été montrés en utilisant 35 mg/L de SPEs. Avec tous les résultats obtenus sur le plan production, composition, structure et propriétés des SPEs, ces biopolymères représentent définitivement une excellente alternative à l'échelle laboratoire.

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ABRÉVIATIONS

APHA: American Public Health Association

Al: Aluminum

ADN: Acide désoxyribonucléique

ARN: Acide ribonucléique

B-EPS: Broth

BCA: l'acide bicinchonique

BS: bacterial strain

BSA: Bovine serum albumin

C/P ratio Carbohydrate to protein ratio

C-EPS Capsular EPS

Cd: Cadmium

CES : Chromatographie d'exclusion stérique

CFU: Colony forming units

C/N : Carbone/Azote

CST: Capillary suction time

Co: Cobalt

Cu: Cuivre

CUQ: Communauté urbaine à Québec

DLVO: Derjauguin, Landau, Verway, Overbeek

EDTA: Acide éthylène diamine tétraacétique

EPS: Extracellular polymeric substances or microbial polymers

FA: Flocculation activity

Fe: Fer

FTIR : Spectroscopie infrarouge à transformée de Fourier

G6P-DH : Le glucose-6-phosphate déshydrogénase

HAP: Hydrocarbures polyaromatiques

Lb: *Lactobacillus*

LB-EPS: Loosely bound EPS

MES: Matière en suspension

Mg EPS/g of kaolin: mg of EPS added per gram of kaolin suspension in water MWWS Municipal wastewater sludge

MWWS: Municipal wastewater sludge

WWTP: Wastewater treatment plant

Ni: Nickel

PAB: Produits associés à la biomasse

PAU : Produits associés à l'utilisation de biomasse

PCA: Plate count agar

PCB: Polychlorobiphényles

PEC: Polymères extracellulaires

PPS: Pulp and paper sludge

S-EPS: Slime EPS

SMP : Produits microbiens solubles

SPE: Substances polymériques extracellulaires

SS: Suspended solids

SVI: Sludge volume index

TB-EPS: Tightly Bound EPS

TSB: Typtic soy broth

VIB : Volume index de boues

XAD : Colonne de résine échangeuse d'ions

ζ: Zeta Potential

Zn : Zinc

Chapitre 1

Synthèse bibliographique

1. SYNTHÉSE

1.1 Introduction

Différents procédés d'épuration sont mis en place pour le traitement des eaux usées urbaines, industrielles et agricoles. Le principe est basé sur la capacité des bactéries présentes naturellement dans les eaux usées à dégrader la pollution organique qu'elles utilisent pour leur croissance. Les stations d'épuration s'appuient sur les technologies pour intensifier et améliorer ce phénomène naturel. Le traitement biologique des eaux usées permet de réduire et diminuer la pollution organique azotée carbonée et phosphorée. L'efficacité d'une station d'épuration dépend d'une part de l'adsorption des polluants et leurs dégradations par une population bactérienne adaptée et d'autre part de la bonne séparation solide -liquide de la biomasse épuratrice et de l'eau épurée. Cette étape de séparation est un point critique et important pour le bon fonctionnement d'une station d'épuration.

Certes, les procédés classiques de traitement des eaux usées par boues activées permettent d'arriver à un effluent de bonne qualité et de répondre aux exigences de rejet, cependant ceux-ci génèrent une grande quantité de boues excédentaires. Ces boues sont des déchets ou matières résiduelles qui ont un impact environnemental que celle des eaux usées. Face à des contraintes environnementales de plus en plus sévères conjuguées à des contraintes sociales, économiques et techniques, la gestion de ces résidus devient un problème réel qui nécessite une résolution rationnelle. Leur traitement et leur élimination représentent 50% des coûts des procédés de traitement des eaux usées (Leblanc, 2005).

Le procédé d'épuration à boues activées s'est basé sur la transformation de la pollution en biomasse qui est possible grâce à des microorganismes. La majorité des microorganismes préfèrent vivre en communauté et produisent naturellement des biopolymères appelés aussi des substances polymériques extracellulaires (SPEs) nécessaires à leur survie. Les bactéries réalisent la consommation /transformation de la pollution organique contenue dans les eaux usées et cette activité est accompagnée par la production de SPEs. L'étude de SPEs est donc un outil pour une meilleure compréhension du comportement microbien des boues.

Des recherches récentes montrent que les SPEs sont impliquées dans des mécanismes physicochimiques aux interfaces/interphases et donc dans la formation, la stabilité et la décantation de la biomasse (appelée boues biologiques). La communauté scientifique s'accorde sur le fait que les SPEs ont un rôle prépondérant sur la cohésion du floc bactérien et la formation de biofilm, leur structuration et leur charge à la surface et dans les procédés de décantation et de déshydratation des boues. Les bioflocs se constituent par des agrégats

microbiens, des souches bactériennes filamenteuses, des particules organiques et inorganiques, qui sont maintenus par les SPEs. Ces dernières sont variées et constituées principalement de polysaccharides, de protéines, d'acides nucléiques et d'autres composants cellulaires. Les SPEs interviennent dans les mécanismes de survie des bactéries (apport de nutriments, barrière de protection contre l'impact négatif extérieur, communication bactérienne…). Elles interviennent également dans le phénomène de rétention d'une quantité importante d'eau dans des boues produites. Jusqu'à ce jour, le développement de procédés de biofloculation des boues se heurte à plusieurs problèmes : (i) les interactions entre les SPEs et les boues ne sont pas bien connues (ii) le rôle des composants actifs des SPEs (protéines, hydrates de carbone) dans la formation des bioflocs est indéterminé (iii) l'étape de leur extraction est indispensable, toutefois, le manque de protocole standard pose le problème de la comparaison des résultats de différents travaux et (iv) la production de SPEs dans les boues activées est un processus non contrôlé. Selon les études précédentes, il est difficile de contrôler une communauté microbienne au cours du traitement des eaux usées pour produire des SPE spécifiques et/ou de favoriser la croissance d'une souche microbienne aux dépens des autres (Bala subramanian et al., 2010, More et al., 2012).

Dans ce cadre, les objectifs de cette étude sont poursuivis par d'une part un développement de procédé de production de SPEs basé sur un isolement de nouvelles souches productrices de SPEs et l'utilisation du glycérol comme source de carbone supplémentaire et d'autre part l'étude de différentes méthodes d'extraction et leur influence sur les propriétés de SPEs dans la bio floculation et la décantation de boues.

1.2 Synthèse bibliographique

Les procédés physiques, physico-chimiques et biologiques sont les différents procédés de traitement des eaux usées qui existent. Nous allons nous intéresser dans ce travail sur le traitement secondaire ou biologique. Le traitement biologique des eaux usées est avantageux et plus important en comparaison aux autres techniques de traitement. Il est économique et permet de traiter un débit important d'eau (Charackilis et Marshall, 1990). Cependant, le traitement biologique est très complexe, ce qui rend difficile la compréhension et l'analyse du procédé. En effet, chaque microorganisme répond de façon différente aux diverses conditions de milieu. De ce fait, le traitement biologique de l'eau usée est largement étudié pour élucider la réponse bactérienne face à différentes conditions. Malgré que la maîtrise des procédés biologiques d'épuration soit complexe et difficile, elle permettra d'améliorer les procédés existants et de créer de nouveaux procédés.

1.2.1 Procédés de traitement biologique des eaux usées

Les traitements biologiques utilisés en traitement de l'eau sont basés sur les activités métaboliques naturelles des organismes impliqués. Ces activités et réactions constituent le cœur des stations de traitement des eaux résiduaires. Ils font appel à une variété des microorganismes, principalement des bactéries et des archéobactéries. Ces bactéries sont ensuite exploitées à l'échelle de la station d'épuration dans différentes unités de traitement mises en œuvre de manière à retenir la biomasse pour pouvoir utiliser ses capacités épuratoires.

Deux formes des cultures bactériennes sont mises en œuvre. Les procédés dits à cultures libres et les procédés dits à cultures fixées. Dans les procédés à cultures libres, la culture bactérienne est développée sous forme de flocs dispersés au sein du l'eau à traiter avec une aération continue. Une formation d'agrégats a été mise en place dans le bassin biologique grâce à l'activité microbienne. Actuellement, les agrégats bactériens sont maintenus en suspension dans l'eau grâce à un brassage continu de la liqueur (Metcalf et Eddy, 2003). Ce procédé d'épuration a été appelé «boues activées ». Les procédés à culture fixée utilisent la capacité des microorganismes à produire des exo polysaccharides permettant leur fixation sur des supports pour former un biofilm. Le plus ancien est celui du lit bactérien. Le principe de fonctionnement d'un lit bactérien consiste à faire couler l'eau à traiter, sur un support où les microorganismes épurateurs sont attachés forment ainsi un film. Les matières organiques contenues dans l'eau, en présence d'oxygène, vont être dégradées par les microorganismes en passant à travers la barrière de film. Les biofiltres ont été utilisés aussi comme procédé à biomasse fixée.

1.2.2 Les substances polymériques extracellulaires

1.2.2.1 Définition

Les polymères sont des macromolécules formées par la répétition d'un ou plusieurs atomes ou groupes d'atomes liés les uns aux autres pour amener une variété de propriétés qui ne changent pas d'une manière significative par addition ou suppression de plusieurs unités (l'International Union of Pure and Applied Chemistry, 1960). Ils ont une structure linéaire, bi ou tridimensionnelle qui porte des groupements organiques ou inorganiques.

Cette étude se focalise sur les polymères extracellulaires (PEC) ou les substances polymériques extracellulaires (SPEs) produites pas des cultures pures dans les boues secondaires et extraites de boues activées. Elles ont la particularité d'être issues principalement de la matière vivante, ce sont des biopolymères. De plus par définition, les SPEs sont sécrétées à l'extérieur des cellules bactériennes. Elles constituent une matrice dans laquelle les micro-organismes sont plus ou moins immobilisés (Liu et Fang, 2003). Une

distribution hétérogène des SPEs autour des cellules bactériennes a été révélée par microscopie électronique (Jorand et al. 1998). Selon la littérature, la composition des SPEs varie en quantité et en qualité. L'abréviation anglaise EPS pour « Extracellular Polymeric Substances » est la plus communément utilisée dans la littérature. Parfois, EPS peut aussi désigner « Extracellular Polysaccharide », dans ce cas les polysaccharides sont les composants majeurs des EPS.

1.2.2.2 Contexte des SPEs

La majorité des micro-organismes vit et évolue en biofilms (Wingender et al., 1999). Dans ce contexte, on trouve des SPEs issues de boues activées, de biofilms ou directement produites par une souche pure bactérienne cultivée en laboratoire. Les SPEs forment la fraction organique en grande partie composant les flocs (Frolund et al., 1996). Ces mêmes auteurs considèrent que les SPEs issues de boues activées peuvent représenter jusqu'à 60% de la fraction organique d'une boue, alors que 20% de cette même fraction représente la biomasse cellulaire. En termes de pourcentage massique par rapport à la teneur en matières solides, Urbain et al., (1993) ont montré que les SPEs représentent environ 15% de la masse en suspension. Dans le cas de SPEs issues de biofilm peut varier entre 50 et 90% de la matière organique totale (Nielsen et al., 1997) et 2 % de matière organique des SPEs sont capables de retenir plus de 98 % d'eau (Christensen et Characklis, 1990).

1.2.2.3 Origines

Les SPEs peuvent avoir deux origines : une origine bactérienne détermine les molécules issues du matériel bactérien (lyse cellulaire) ou des molécules issues de la sécrétion bactérienne et une origine liée à l'adsorption de molécules présentes dans l'environnement (Wingender et al., 1999).

Tous les microorganismes ne produisent pas des SPEs. Ainsi, la composition des SPEs dans la biomasse dépend de leur origine et de l'espèce bactérienne présente. Différents mécanismes sont impliqués dans la diffusion des SPEs à travers la membrane vers la surface bactérienne ou dans le milieu environnant. Ces mécanismes ont été identifiés dans le métabolisme des protéines (Bhaskar et al., 2005) et des polysaccharides (Sutherland, 2001a). Durant leur croissance, les bactéries libèrent des vésicules membranaires composées de molécules issues de la membrane et de molécules péri plasmiques (lipopolysaccharides, protéines enzymatiques, phospholipides) (Li et al., 1998). Des acides nucléiques ont été identifiés dans les matrices des SPEs, le fait que les bactéries échangent des gènes en sécrétant des brins d'ADN chromosomique (Lorenz et Wackernagel, 1994).

La production de SPEs par les bactéries varie en fonction de leur phase de croissance avec une production maximale en phase stationnaire (Bhaskar et Bhosle, 2005). Les conditions

environnementales du milieu peuvent également influencer la production de SPEs par les microorganismes. Ainsi, les stress que ce soit biotique ou abiotique dus à la teneur en substrats (Hoa et al., 2003), à la présence d'éléments métalliques (Mikes et al., 2005) ou encore à la concentration en oxygène dans le milieu (Hsieh et al., 2006) affectent la sécrétion et la biosynthèse des SPEs. Plusieurs chercheurs ont essayé de modéliser l'influence générale de ces paramètres sur la production de SPE (Laspidou et Rittmann, 2002; Ni et al., 2009). Néanmoins, il est difficile d'élaborer un modèle devant le nombre de paramètres à prendre en compte et les réactions différentes suivant le type de microorganisme et le type de substrat. Au sein de la biomasse, lors de changement du milieu ou de conditions environnementales, les bactéries s'adaptent à ces changements en modifiant leur métabolisme de biosynthèse des SPEs. De plus, la communauté bactérienne s'adapte en favorisant ou diminuant la population microbienne capable de sécréter des SPEs (Liu et Fang, 2002).

Les SPEs peuvent également provenir de débris cellulaires issus de la lyse des bactéries (Wingender et al., 1999). En effet, les molécules intracellulaires de hauts poids moléculaires (exp : les acides nucléiques, protéines) et des membranes (peptidoglycanes, phopholipides) sont excrétées dans le milieu lors de la lyse et se retrouvent piégées dans la matrice polymérique. Ces macromolécules sont ensuite considérées comme des SPEs qui possèdent les mêmes rôles dans la biomasse que les SPEs synthétisées activement par les bactéries.

Enfin, les effluents traités biologiquement contiennent également des micropolluants ou des produits issus de la dégradation enzymatique de divers biopolymères réarrangés. Ce sont des substances humiques-like (Sponza, 2002). Ces molécules varient en fonction de l'effluent traité. Elles peuvent s'adsorber avec la matrice de SPE ou les bactéries elles-mêmes au cours du traitement et être intégrées à des SPEs, étant donné qu'elles ont les mêmes propriétés (Wingender et al., 1999).

1.2.3 Localisations et les types des SPEs

Selon leur définition, les SPEs sont situés à la surface ou à l'extérieur des cellules bactériennes. Cependant, certains auteurs (Nielsen et al., 1997 ; Wilen et al., 2003) assortirent une nuance dans la localisation des SPEs. Les SPEs sont la plupart du temps attachées à une phase solide donc ils sont insolubles. Par contre, l'association des SPEs avec une phase solide n'est pas toujours totale. Certains auteurs divisent alors les SPEs en deux fractions: les SPEs liées et les SPEs solubles. Les SPEs liées seraient autour des cellules bactériennes, parfois partiellement attachées aux cellules, leur composition contienne même des éléments constitutifs de la paroi des bactéries. Les SPEs solubles

seraient les SPEs libres (Figure1-1). Mais en fait, plusieurs théories et concepts ont été avancés dans la littérature.

La différence de composition chimique des SPEs pourrait expliquer la division des SPEs en SPEs liées et d'autres, solubles (Breedveld et al., 1990).

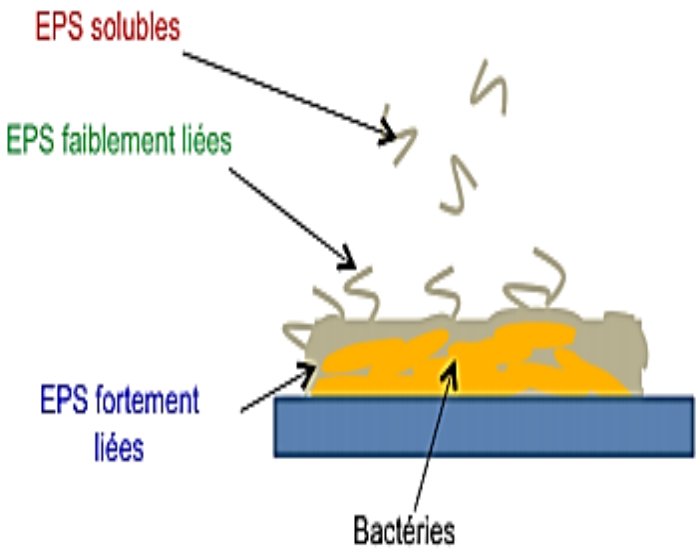


Figure 1-1 : Schéma théorique présentant la localisation des SPEs solubles/liées: trois types des SPEs sont différenciés, les EPS solubles libérées dans le milieu, les EPS faiblement liées au biofilm et les EPS fortement liées aux bactéries.

Le groupe des SPEs liées est raccordé par Wingender et al., (1999) à un glycocalyx qui correspond à des couches polymériques entourant la cellule bactérienne et associées aux protéines ou aux lipides de la membrane. Ces SPEs peuvent former une gaine qui contient des protéines qualifiées de lectine-like, ayant les mêmes propriétés que les lectines, capables de se lier spécifiquement aux oses (Higgins et Novak, 1997). Ainsi, ces protéines renforcent la structure et l'architecture de la gaine en se fixant fortement aux polysaccharides. Les SPEs liées comprennent également des composés capsulaires tels que des polysaccharides associés aux phospholipides de la paroi cellulaire par une liaison covalente (Roberts, 1996). Enfin, les SPEs liées peuvent être liées faiblement aux cellules et forment ainsi un gel (ou « Slime ») dans lequel les molécules sont dispersées et un peu organisées (Wingender et al., 1999). Ce gel est composé de biopolymères détachés de la surface cellulaire et de matériel organique issu de l'environnement. Toutefois, certaines études classent le « Slime » dans les SPEs solubles, même s'il contient des molécules sous forme non dissoute, car elles sont faiblement liées aux cellules.

La composition des SPEs solubles est beaucoup plus imprécise et n'est pas claire aussi leur définition, se diffère d'une recherche à une autre suivant les auteurs. Pour Wingender et al.,

(1999), les SPEs solubles regroupent les macromolécules dissoutes, les colloïdes non dissous et qui ne sont pas attachés aux cellules ainsi que les « Slimes ». D'après Hsieh et al., (1994), les substances solubles sont excrétées soit par dissolution ou soit par hydrolyse, à partir de la fraction de SPEs liées dans les boues vers la phase liquide.

Laspidou et Rittmann, (2002) suggèrent une autre explication des concepts de SPEs liées et SPEs solubles qui s'appuie sur la théorie des produits microbiens solubles (PMS) et sur l'origine des SPEs. Les PMS seraient des composants cellulaires solubles qui sont expulsés suite à une lyse cellulaire ou des composés qui diffusent à travers la membrane cellulaire pour différentes raisons (exemple : protection contre un agresseur telle que les métaux lourds). Ils existent en deux catégories : les produits associés à la biomasse (PAB) et les produits associés à l'utilisation de la biomasse (PAU). Les PAB représentent la fraction des PMS issue de la dégradation de la biomasse tandis que les PAU représentent la fraction des PMS issue du métabolisme bactérien et du développement de la biomasse. Dans ce modèle, les SPEs solubles et les SPEs liées sont définies par rapport à leur origine (Figure 1-2). Les SPEs solubles sont en fait les PMS constitués des PAB et PAU. Ils sont biodégradables. Les SPEs liées seraient synthétisées par les cellules bactériennes durant leur croissance proportionnellement à la quantité de substrat utilisé, ou seraient le résultat de l'association de différentes molécules telles que les résidus de PMS (ou SPEs solubles) biodégradés. Les SPEs liées seraient en partie attachées à la biomasse inerte et en partie en relation avec les cellules bactériennes actives. Les SPEs liées peuvent être hydrolysées et formeraient ainsi les PAB.

Cependant, Aquino et Stuckey, (2004) proposent une autre version à la théorie de Laspidou et Rittmann, (2002). D'après leurs résultats, les PMS seraient aussi constitués de SPEs liées. Pour ces chercheurs, les SMP seraient constituées de SPEs (solubles et liées), de produits issus de leur hydrolyse, de composés intracellulaires de haut poids moléculaire provenant de la lyse cellulaire, et aussi de composés excrétés par les microorganismes afin de neutraliser des métaux et des toxiques organiques présents dans l'environnement extérieur. Les PAB seraient issus de SPEs liées hydrolysées, mais seraient aussi composés de matériels intracellulaires de haut poids moléculaire provenant de dommage cellulaire.

Il faut ajouter le fait que le type et la composition des SPEs sont dépendant de plusieurs paramètres environnementaux tels que le pH et la présence de cations disponibles (Nielsen et al., 1997). Certains auteurs (Wingender et al., 1999 ; Liu and Fang, 2003) définirent les SPEs solubles et les SPEs liées à partir de leur protocole d'extraction. Ainsi les SPEs solubles sont les biopolymères qui nécessitent un traitement minimum afin de les séparer des cellules bactériennes tandis que l'extraction de SPEs liées exige l'emploi de méthodes plus drastiques, tenant ainsi compte du fait que les SPEs liées seraient difficiles à séparer

des cellules bactériennes. Pour cette étude, nous considérons la définition des SPEs en tant que « solubles » et « liées ».

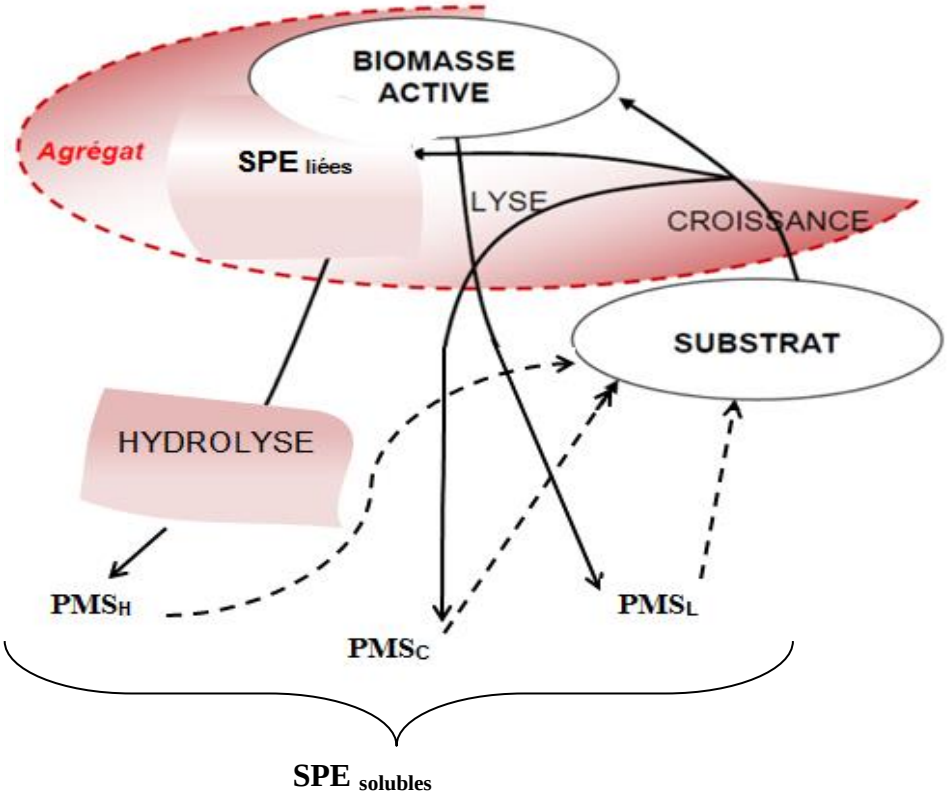


Figure 1-2: Schéma montrant les mécanismes de production et de relargage de PMS (Massé, 2004)

1.2.4 Caractérisation des SPEs

1.2.4.1 Composition chimique des SPEs

En termes de composition des SPEs, leur nature chimique est très hétérogène et dépend de plusieurs paramètres : la nature de la biomasse c'est-à-dire l'origine de SPEs (boues activées, biofilms, cultures de souches pures de bactéries), la composition du milieu (nature de l'effluent, propriétés physico-chimiques du milieu) ou le type de l'espèce présent. D'après la littérature, on divise les SPEs en six grandes classes de composés biologiques : les polysaccharides, les protéines, les substances humiques, les acides uroniques, les lipides et les acides nucléiques (Frølund et al., 1996). On peut également retrouver d'autres catégories des molécules suite à des combinaisons des grandes classes de molécules biologiques comme les glycoprotéines ou les lipopolysaccharides et les lipoprotéines (Garnier et al., 2005). Les principaux composés des SPEs sont les protéines et les polysaccharides.

Concernant les boues activées ou les biofilms épuratoires, les protéines sont les composés majeurs alors que pour des cultures pures de souches bactériennes, les polysaccharides sont souvent les molécules les plus abondantes (Higgins et Novak, 1997). Néanmoins, la composition du milieu de croissance ou le type de substrat est un facteur et paramètre important dans la composition des SPEs (Dignac et al., 1998).

1.2.4.1.1 Les polysaccharides

Les polysaccharides sont les molécules les plus étudiées des exopolymères, car ce sont souvent les composés les plus abondants des SPEs issues des biofilms et des cultures de souches bactériennes pures. C'est pour cette raison que dans la littérature, les exo polysaccharides sont souvent assimilés aux SPEs. La majorité des microorganismes sécrètent des polysaccharides (bactéries Gram positifs et négatifs, champignons, algues) (Sutherlant et al., 2001b). Les polysaccharides sont des composés de poids moléculaires très variés. On distingue les homopolysaccharides qui sont des molécules constituées d'une même unité monosaccharidique et les hétéropolysaccharides sont formés de 2 à 8 monosaccharides différents (sucres neutres et acides uroniques). Les sucres neutres les plus présents dans la composition des exo polysaccharides sont le glucose, le galactose, le mannose et le rhamnose (Dignac et al., 1998). Les polysaccharides peuvent être neutres ou chargés (anioniques) et les molécules peuvent posséder des groupes fonctionnels hydroxyles, acyles, sulfates ou phosphates (Sutherland, 2001b). La biosynthèse des polysaccharides (catabolisme de dégradation enzymatique des sucres + polymérisation) peut être intracellulaire ou extracellulaire (Kumar et al., 2007). Les exopolysaccharides rassemblent également des composés de la paroi cellulaire (lipopolysaccharides) et des composés intracellulaires tels que les riboses (sucres issus de l'hydrolyse de l'ARN) présents dans la matrice polymérique (Dignac et al., 1998). Les polysaccharides, au sein de la matrice de SPEs, présentent sous forme de capsules liées de manière covalente à la membrane cellulaire ou sous forme de « Slime » faiblement liées aux cellules (Kumar et al., 2007). La classe des polysaccharides regroupe ainsi des composés variés sur le plan de leur structure et de leurs propriétés.

1.2.4.1.2 Les substances humiques

Dans l'étude des SPEs, le terme « substances humiques » est abusif, car ces molécules de nature proviennent exclusivement des sols, ce qui n'est pas le cas dans la matrice polymérique ou le biofilm. Les auteurs utilisent donc souvent le terme de « humic-like substances » rassemblant les molécules issues du sol et celles issues de la biomasse. Les substances humiques-like sont des macromolécules variées tant que leur état physique que chimique. Ce sont des produits d'hydrolyse biologique ou chimique de résidus organiques de

l'environnement ayant subi une polymérisation ou réarrangement (Francioso et al., 2002). Les substances humiques-like peuvent se trouver en suspension ou sous forme dissoute en milieu aqueux (Chang Chien et al., 2007). Dans les biomasses, les substances humiques-like proviennent de l'adsorption ou l'association de ces molécules du milieu avec la matrice polymérique ou de la dégradation microbienne de biopolymères. De nombreux groupements fonctionnels sont présents dans la structure chimique de ces substances telles que des fonctions alcools, phénols, carboxyles, quinones, lactones, etc (Chang Chien et al., 2007).

1.2.4.1.3 Les protéines

Les SPEs issues des biomasses épuratoires et de certains biofilms sont formées en grande partie par des protéines comme composés principaux (Liu et Fang, 2002b; Comte et al., 2006c). Les protéines dans les SPEs regroupent des molécules ayant des fonctions hétérogènes telles que des enzymes, des protéines de structure comme les lectines, des protéines intracellulaires issues de la lyse cellulaire ou encore des polypeptides capsulaires (Higgins et Novak, 1997). Les protéines sont des chaînes plus ou moins longues d'acides aminés. Elles possèdent une variété de groupements fonctionnels tels que des fonctions amines, carboxyliques, hydroxyles ou thiols. La majorité des protéines des SPEs est d'origine intracellulaire. Leur synthèse se fait au niveau du cytoplasme pour être excrétée par la suite dans le milieu. Il existe de nombreux mécanismes de sécrétion des protéines qui varient selon les fonctions et les propriétés des protéines et le type de bactérie (bactéries Gram positive ou négative) (Binet et al., 1997). Les protéines peuvent être fixées à des sucres ou des lipides, on parle alors respectivement de glycoprotéines et de lipoprotéines. La classe des protéines au sein des SPE regroupe des molécules hétérogènes ayant des rôles structurels (lectines) ou enzymatiques nécessaires pour la structure et le développement de la biomasse.

1.2.4.1.4 Les acides nucléiques

Le dosage des acides nucléiques utilisé généralement dans l'étude des SPEs permet de mesurer les concentrations en ADN et ARN dans les échantillons. Donc le terme « acides nucléiques » détermine ces deux types de composés. Les acides nucléiques sont formés par un nucléotide qui comprend un sucre à cinq atomes de carbone (le désoxyribose pour l'ADN et le ribose pour l'ARN), un groupement phosphate et une base azotée purique ou pyrimidique. Une faible teneur a été révélée en acides nucléiques composant les SPEs extraites à partir de biomasses épuratoires (Frølund et al., 1996). À l'origine, les acides nucléiques extracellulaires servaient d'indicateur de lyse cellulaire. Toutefois, de nombreux microorganismes sont capables de sécréter de larges quantités d'ADN extracellulaire à des teneurs 50 % plus élevées que l'ADN intracellulaire (Steinberger et Holden, 2005). Les

acides nucléiques ont donc deux origines possibles : des produits de lyse cellulaire ou des sécrétions produites sous forme de vésicules libérées dans le milieu extracellulaire (Allesen-Holm et al., 2006). Des études ont montré que les acides nucléiques extracellulaires ont des similarités, mais également des différences par rapport au matériel génomique induisant ainsi une différenciation entre les acides nucléiques issus de la lyse et ceux sécrétés (Böckelmann et al., 2006). Dans les biofilms composés de plusieurs espèces de microorganismes, de fortes teneurs en acides nucléiques ont été révélées au niveau de la couche externe des microcolonies (Allesen-Holm et al., 2006). Ces résultats confirmeraient le rôle de ces composés aux interactions et la « communication » cellulaire.

1.2.4.1.5 Les acides uroniques

Le terme « acide uronique » est également exagéré et représente un ensemble de molécules dosées par une méthode définie par exemple le dosage par colorimétrie de Blumenkrantz et Asboe Hansen, (1973). Les acides uroniques sont des oses simples ayant subi une oxydation du carbone portant la fonction cétone. L'acide glucuronique par exemple provient du glucose oxydé en C6. Ils représentent 1 à 2 % de la masse des molécules identifiées composant les SPEs (Frølund et al., 1996; Bura et al., 1998). Les acides uroniques entrent dans la composition de certains polysaccharides, leur apportant une fonction ionisable (Sutherland, 2001b).

1.2.4.1.6 Les Lipides

Les lipides regroupent différentes classes de composés tels que les acides gras, des lipides neutres, des glycolipides ou encore les phospholipides (Conrad et al., 2003). Les lipides peuvent être associés à d'autres composés formant ainsi des lipoprotéines ou des lipopolysaccharides. 1à 2 % des lipides sont présents dans les SPEs issues de biomasses épuratoires (Conrad et al., 2003). Ils ont un rôle adhésif des biomasses aux surfaces (Sand et Gehrke, 2006). Les lipides sont formés de longues chaînes aliphatiques saturées ou insaturées (phospholipides) qui possèdent principalement des propriétés hydrophobes (Réveillé et al., 2003). Ces molécules possèdent diverses fonctions telles que des esters, alcools, amines, phosphates ou carboxyles…etc. Les lipides peuvent avoir trois origines : la sorption de lipides présents dans le milieu, la dissolution des composés membranaires, des matériels intracellulaires issus de la lyse cellulaire ou des constituants issus du métabolisme bactérien sécrétés dans le milieu (Conrad et al., 2003).

1.2.4.2 Caractérisation de la structure et de propriétés moléculaires

Les SPEs rassemblent donc une série de macromolécules aux structures et propriétés hétérogènes. Au sein de la matrice polymérique, différents types de SPEs sont identifiés de la littérature, qui auront donc divers rôles dans la formation et le développement des biofilms. Les différentes molécules composant les SPEs portent divers groupements fonctionnels qui leur confèrent des propriétés physico-chimiques et biologiques différentes. L'étude de ces propriétés permet de mieux appréhender leur rôle au sein des biomasses.

1.2.4.2.1 Les poids moléculaires

Les SPEs ont des tailles et des poids moléculaires différents suite à leur composition hétérogène. Des études se sont focalisées sur la détermination du poids moléculaire des SPEs utilisant la chromatographie d'exclusion stérique (SEC). Cette méthode permet de séparer les molécules des SPEs selon leur volume hydrodynamique permettant d'avoir des profils chromatographiques dans un temps limité (environ 40 minutes) évitant ainsi l'altération des molécules (Comte et al., 2007). La détection par infrarouge couplée à la séparation par SEC permet d'identifier les molécules composant les différentes fractions (Garnier et al., 2005). En revanche, comme il a été montré précédemment, la composition biochimique des SPEs est très hétérogène et variable suivant la biomasse étudiée et les profils par poids moléculaire des SPEs seront donc différents (Andersson et al., 2009). Par exemple, des polysaccharides ont été identifiés ayant des poids moléculaires inférieurs à 1 kDa jusqu'à 40kDa (Garnier et al., 2006) et des protéines ayant des poids moléculaires compris entre 10 et 700 kDa (Görner et al., 2003; Garnier et al.,2005).

Certaines données de la littérature se contredisent néanmoins. Ainsi, une étude a déterminé qu'un tiers des molécules composant les SPEs a un poids moléculaire compris entre 15 et 30 kDa (Liu et al., 2001), ce qui va dans le sens d'une autre étude ayant déterminé que 48% des SPEs ont un poids moléculaire supérieur à 25 kDa (Zhou et al., 2001). Il apparaît également que les SPEs fortement liées aux cellules ont des poids moléculaires plus élevés que les SPEs faiblement liées des « Slimes » (Andersson et al., 2009; Yu et al., 2009). En effet, Yu et al., (2009) ont effectué des profils de poids moléculaire compris entre 0,3 et 1200 kDa. Les SPEs fortement liées se trouvent dans toutes les fractions du profil, mais sont présentes en grande partie dans la fraction de poids moléculaire compris entre 330 et 1200 kDa et la majorité de ces molécules sont des protéines et des acides nucléiques. Les SPEs des « Slimes », ont un poids moléculaire qui varie de 0,3 à 20 kDa. Andersson et al., (2009) confirment ces résultats en montrant que les SPEs capsulaires (fortement liées aux cellules) ont des poids moléculaires plus importants que les SPEs faiblement liées. D'autres recherches ont montré que les protéines ont des poids moléculaires plus élevés que les

polysaccharides (Görner et al., 2003; Garnier et al., 2005). Les résultats révèlent que le poids moléculaire des protéines contenues dans les différentes SPEs varie de 10^3 kDa à 10^5 kDa tandis que le poids moléculaire de polysaccharides est inférieur à 10^3 kDa. Ainsi, les recherches de Görner et al., (2003) ont indiqué que les protéines dans toutes les fractions de poids moléculaire entre 45 et 670 kDa avec une majorité de molécules comprises entre 67 et 200 kDa. Concernant les polysaccharides, leur poids moléculaire est compris entre 0,5 et 1 kDa. Ces résultats ont par la suite été confirmés par Garnier et al., (2006). De plus, Görner et al., (2003) ont noté que certains polysaccharides étaient élués après le volume total de colonne. Dans ce contexte, deux hypothèses ont été mises : la présence de certaines protéines qui ont les mêmes propriétés que la lectine (15-20 kDa), par exemple les glycoprotéines. En effet, certaines protéines sont capables de se fixer fortement aux sucres (Higgins et Novak, 1997), la deuxième hypothèse pourrait être à l'origine du ralentissement de certains polysaccharides dans la colonne. Concernant les autres molécules (les lipides, les substances humiques-like, les acides nucléiques), il existe très peu de données relatives à leur poids moléculaire.

1.2.4.2.2 Les groupements fonctionnels

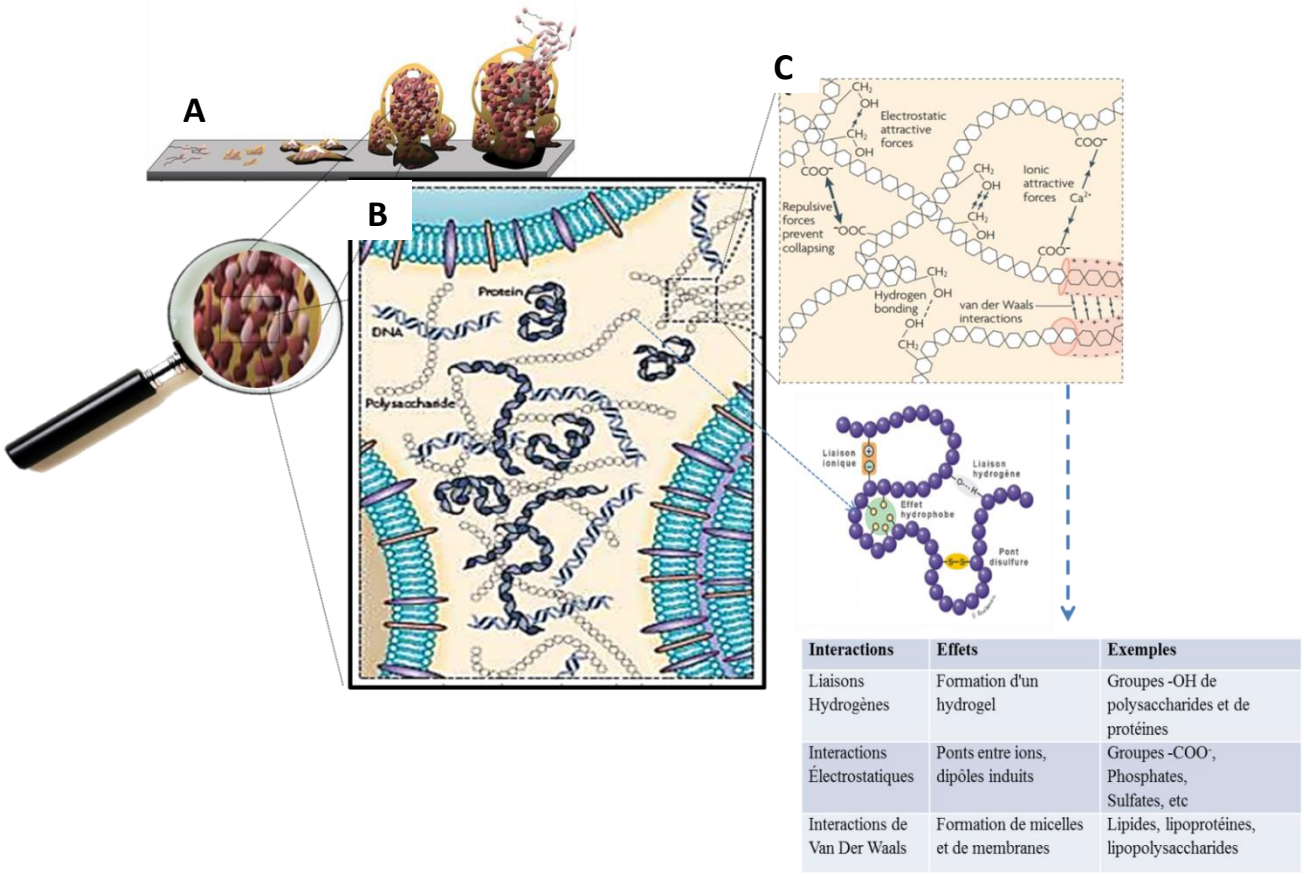
Les molécules composant les SPEs possèdent des groupements fonctionnels potentiellement ionisables en fonction du pH avec des structures variées (Figure 1-3: Représentation schématique de la matrice extracellulaire d'un biofilm (a et b).Les types de liaison entre les différents exopolymères sont représentés sur le schéma(c) (Flemming, 2010)). Les principaux groupements fonctionnels sont les groupements carboxyliques et hydroxyles, et en moindre teneur on trouve les groupements phénols, carbonyles, amines, amides, thiols ou encore phosphoriques (Huffman et al., 2003; Comte et al., 2006c; Sun et al., 2009). La détection de ces groupements se fait principalement par spectroscopie infrarouge. Ainsi, les groupements fonctionnels des SPEs sont identifiés en fonction de leur nombre d'ondes d'absorption. Néanmoins, les échantillons de SPEs sont un mélange très hétérogène de molécules variables en termes de compositions et structures pouvant également avoir des interactions et des liaisons entre elles. En spectrométrie infrarouge, les nombres d'ondes d'absorption peuvent être modifiés. De plus, les mêmes groupements fonctionnels se retrouvent dans diverses classes de composés, par exemple les groupements amines et amides sont présents dans la structure des acides nucléiques et des protéines (Figure 1-3: Représentation schématique de la matrice extracellulaire d'un biofilm (a et b).Les types de liaison entre les différents exopolymères sont représentés sur le schéma(c) (Flemming, 2010)). Dans ce cas, l'analyse des SPEs par spectrométrie infrarouge a donc plus un rôle qualitatif que quantitatif. Il permet par exemple d'identifier les structures

chimiques des composés de poids moléculaires différents après une séparation par SEC (Görner et al., 2003).

En fonction du pH, ces groupements fonctionnels peuvent être sous forme ionisée ou non. Ils ont alors des sites de fixation électrostatique potentiels pour d'autres éléments du milieu tels que des cations métalliques (Liu et Fang, 2002a). Quand le pH augmente, les fonctions peuvent se déprotoner si la constante d'acidité du groupement (pKa) est inférieure au pH.

Il existe très peu de données (Yee et al., 2004; Fein, 2006) concernant les pKa des SPEs. La concentration en nombre de sites montre que les groupements hydroxyles et carboxyliques sont les plus importants. D'autres études ont déterminé deux ou trois pKa dans les échantillons de SPEs (Comte et al., 2006b; Braissant et al., 2007), mais les groupements majoritaires restent identiques. Ces différences surviennent des compositions biochimiques variées des SPEs extraites, des techniques et des modèles utilisés pour les déterminer. Le pH de l'environnement des microorganismes est compris entre 6,5 et 7,5, les fonctions ionisées à ce pH seront donc principalement les groupements carboxyliques et phosphoriques des SPEs.

La propriété des SPEs ionisables en fonction du pH est un facteur important dans le développement des biofilms. En effet, les SPEs peuvent s'interagir avec leur environnement grâce à ces groupements fonctionnels. Ils sont capables de fixer les cations métalliques potentiellement toxiques pour les bactéries ou en se liant à des cations pontants tels que Ca^{2+} ou Mg^{2+} afin de renforcer et intensifier la structure du biofilm.



Interactions	Effets	Exemples
Liaisons Hydrogènes	Formation d'un hydrogel	Groupes -OH de polysaccharides et de protéines
Interactions Électrostatiques	Ponts entre ions, dipôles induits	Groupes -COO⁻, Phosphates, Sulfates, etc
Interactions de Van Der Waals	Formation de micelles et de membranes	Lipides, lipoprotéines, lipopolysaccharides

Figure 1-3: Représentation schématique de la matrice extracellulaire d'un biofilm (a et b).Les types de liaison entre les différents exopolymères sont représentés sur le schéma(c) (Flemming, 2010)

1.2.4.2.3 Charge de surface et hydrophobicité

Dans différentes études orientées sur les biofilms et le matériel bactérien, la charge de surface est souvent couplée à celle sur l'hydrophobicité. Néanmoins, il existe peu de données concernant les SPEs sur ces deux paramètres.

Dans les agrégats bactériens, la charge de surface est fortement affectée par la teneur et la composition des SPEs (Mikkelsen, 2003). En effet, les SPEs portent souvent des charges négatives dues à l'ionisation des groupements carboxyles et phosphoriques à un pH proche de la neutralité. La source principale de ces groupements anioniques serait les protéines et les acides nucléiques (Liao et al., 2002; Sponza, 2003). Cependant différents travaux ont montré que les ratios protéines/polysaccharides et protéines / (polysaccharides + acides nucléiques) se corréleraient avec la charge de surface des particules contenues dans les échantillons (Zhang et al., 2007.). Les méthodes existantes pour estimer la charge de

surface des SPEs sont au nombre de trois. On peut utiliser la détermination du potentiel zêta en mesurant la mobilité électrophorétique, la titration acide-base ou la titration par des colloïdes (Morgan et al., 1990). La mesure du potentiel zêta est basée sur la mobilité électrophorétique des SPEs soumis à un champ électrique (Forster, 1968). Les mesures du potentiel zêta ne peuvent être pratiquées que sur un nombre limité de particules et ne représentent pas que les SPEs.

La charge de surface et l'hydrophobicité sont liées. Si les agrégats bactériens ayant une charge de surface positive, elles ont un caractère hydrophobe, et inversement, si la biomasse porte une charge négative, elle aura un caractère hydrophile (Sponza, 2002). Les molécules hydrophobes sont des molécules incapables d'interagir avec les molécules d'eau alors que les molécules hydrophiles sont attirées (Garnier et al., 2005). L' hydrophobicité de la biomasse dépend et est souvent influencée par des SPEs (Sheng et Yu, 2006b). Elle correspond à la moyenne des propriétés hydrophobes et hydrophiles des SPEs (Liao et al., 2001; Zhang et al., 2007). De caractères hydrophobes et hydrophiles, les molécules composant les SPEs peuvent être des molécules qui portent des groupes hydrophobes ou hydrophiles et même des molécules amphilyophiles (Jorand et al., 1998). Liao et al., (2001) ont montré que l'hydrophobicité des SPEs est majoritairement marquée par la présence de fortes teneurs en protéines et en polysaccharides plutôt que par la teneur totale en SPEs. Ainsi, les protéines ont un effet positif sur l'hydrophobicité alors que les polysaccharides ont un effet négatif. Pour confirmer ces résultats, Jorand et ses collègues (1998) ont séparé les SPEs solubles des SPEs qui précipitent à pH acide (pH 2). Ensuite, des résines XAD ont été utilisées pour séparer les SPEs hydrophobes et hydrophiles au sein de la fraction soluble. Les SPEs hydrophobes sont fixées à la résine XAD8 alors que les SPEs hydrophiles sont les molécules sorbées à la résine XAD4 ou non sorbéés. La fraction précipitée représente 50% du carbone organique dissous, les composés hydrophobes 7 %, 13 % des composés hydrophiles acides sorbés sur XAD4 et 30 % des substances hydrophiles non sorbés. Les protéines sont les principales molécules composant la fraction hydrophobe. Ces auteurs notent également que cette fraction contient une grande quantité de fonctions aromatiques que l'on trouve majoritairement dans la structure des protéines et des substances humiques-like. La fraction hydrophile non sorbée est quant à elle composée quasi exclusivement de polysaccharides. Finalement, la fraction précipitée est composée de 77 % des protéines et de 16 % des polysaccharides. Cette présence simultanée dans la fraction est due en majorité aux glycoprotéines présentes dans l'échantillon de SPEs. Park et al., (2008) ont également détecté des composés polysaccharidiques dans la fraction hydrophobe résultante que les polysaccharides élués sont en grande partie sous forme de lipopolysaccharides, de glycoprotéines ou sont fixés et liés à d'autres molécules des SPEs. D'autres études confirment le rôle positif des protéines ainsi que le rôle négatif des polysaccharides dans

l'hydrophobicité (Arabi et Nakhla, 2008) montrant également la participation des acides uroniques et des substances humiques-like à l'hydrophobicité des SPEs (Wilén et al., 2003a). Ainsi, quand le ratio protéine/polysaccharide augmente proportionnellement à l'hydrophobicité des SPEs (Zhang et al., 2007). Les protéines sont composées d'acides aminés hydrophiles et hydrophobes. Higgins et Novak, (1997) ont déterminé dans des échantillons extraits à partir de différentes biomasses (boues, cultures de bactéries) que les acides aminés hydrophobes tels que l'alanine, la leucine ou la glycine représentent une grande quantité des acides aminés présentes dans les protéines, ce qu'explique le caractère hydrophobe de SPEs (Dignac et al., 1998).

La matrice polymérique des biomasses renferme donc un large et hétérogène panel de molécules qui sont variables tant sur le plan structurel que sur le plan des propriétés. Toutefois, c'est l'ensemble de ces propriétés qui va procréer leur rôle potentiel dans la formation, le développement et le maintien des biomasses.

1.2.5 Les facteurs influençant la production de SPEs

II est important de noter que la production de SPEs dépend de différents facteurs. La production peut varier d'abord selon l'espèce bactérienne. Même si la majorité des bactéries peuvent produire des SPEs dans n'importe quel milieu, la production peut être influencée par certaines conditions des milieux bien définis (Dupont, 1998).

La structure, la composition et la viscosité des exopolysaccharides produits par les bactéries dépendent de plusieurs paramètres tels que la composition du milieu, la source de carbone et d'azote, le type de la souche et les conditions de fermentation (pH, température, la concentration d'oxygène et l'agitation) (Duta et col, 2006).

Tableau 1-1 : La production de SPEs et leurs caractéristiques chimiques présente les différentes conditions de culture influençant la production de SPEs.

1.2.5.1 Effet de la source de carbone, azote et le rapport carbone /azote

La source de carbone est facteur majeur qui peut affecter la biosynthèse de SPEs par les bactéries. Le type et la concentration du substrat (glucose, galactose, lactose, mannose, fructose, etc.) peuvent stimuler et optimiser la production (Cerning et al., 1995; Gamar et al., 1997). La production de SPEs par *Lactobacillus Delbrueckii* ssp. *bulgaricus* a été plus élevée dans un milieu additionné du glucose ou du lactose par rapport à un milieu contenant du fructose, tandis que la supplémentation en mannose a résulté en une production faible de SPEs (Grobben et al., 1996). Gancel et Novel, (1994) ont comparé le rendement en SPEs de *Streptococcus. salivarius* ssp*. thermophilus*. Le rendement avec le milieu contenant du glucose et du fructose a été plus fort en comparaison que celui avec du lactose ou le saccharose, tandis que la prolifération des bactéries a été moins rapide. Pour *Lb. rhamnosus*

C83, la production de SPEs a été favorisée utilisant le mannose ou une combinaison de glucose et de lactose comme substrat (Gamar et al., 1997). Une combinaison de glucose et de lactose dans un milieu de culture a augmenté la production de SPEs par *S. thermophilus* LY03 et *S. thermophilus* Sfi20, comparé avec un milieu de culture avec le glucose ou le lactose seul, malgré que la concentration totale des sucres ajoutée était la même (7.5%) (Degeest et de Vuyst, 2000).

Li et Yang, (2007), ont montré que la boue supplémentée de glucose présente une production de SPEs plus importante que celle additionnée d'acétate. Par contre, une floculation et une décantation avec de faibles valeurs d'IVB et SRT sont plus importantes utilisant les SPEs produites par l'acétate que celle obtenue en ajoutant le glucose. C'est expliqué par l'augmentation de la concentration de SPEs solubles produites qui ont un effet négatif sur la décantation et ainsi dans la déshydratation de boues.

Depuis maintenant plusieurs années, des études portaient une attention toute particulière à l'utilisation du glycérol brute comme source de matière première pour la production de biodiesel, polymères, etc. Des recherches sont en train de mettre au point des façons d'utiliser le glycérol brut pour produire davantage de SPEs. Freitas et al., (2009) ont utilisé le glycérol comme matière première pour la production des SPEs. *Pseudomonas oleovorans a produit* 8,1 g/L de SPEs après 96h utilisant 25 g/L de glycérol.

Selon la majorité des études, les matières azotées sont essentielles à la croissance bactérienne, mais elles ne participent pas directement au mécanisme de biosynthèse des SPEs (Torres et al., 2014). Un équilibre optimal entre la source de carbone et d'azote est nécessaire pour obtenir des productions accrues de SPEs (De Vuyst et Degeest, 1999.

Pour certaines bactéries comme des souches de *Xanthomonas, Pseudomonas et Rhizobium,* une limitation de l'azote résulte en une production élevée de SPEs. Des chercheurs ont montré qu'une limitation par l'azote stimule la production de SPEs neutres, mais pas celle de SPEs chargées. Un environnement riche en carbone et pauvre en azote (rapport carbone : azote élevé) favorise la production de SPEs (Shene et al., 2008).

Par contre, un faible rapport carbone/azote stimule la production de SPEs à faible poids moléculaire (Shene et al., 2008).

Dans ce contexte, il est important de souligner que l'apport carbone /azote joue un rôle indispensable dans la production de SPEs.

Des travaux précédents ont montré que les boues activées qui présentent un rapport C/N faible tend à produire de SPEs avec un fort rapport protéine/carbohydrate (Liu et Fang, 2003). La recherche de Liu et al. (2010) a révélé un rapport C/N optimal de 0.5. L'augmentation ou la diminution de rapport de 0.5 résulte en une réduction de la quantité de SPEs produites par *Bacterium Zunongwangia profunda* et par conséquent de l'activité de floculation. Alors que pour les études de Ye et al. (2011), les boues à rapport C/N 20 est

favorable pour la production de SPEs. Récemment, Torres et al. (2014) ont évalué l'impact de source de carbone et d'azote sur la production de SPE. Ils ont montré que la synthèse des SPEs est favorisée par l'augmentation de C/N ratio de 10 à 50, utilisant le glycérol comme source de carbone.

Il est également important de souligner que le C/N a un effet prépondérant dans la floculation et la déshydratation de boues. Avec une augmentation de rapport de 20 à 100, la teneur en protéines contenues dans les SPEs solubles est diminuée et résulte en une réduction de floculation et du taux de déshydratation de boues (Ye et al., 2011).

1.2.5.2 Effet du stade de croissance bactérienne

La production de SPEs dépend du développement des microorganismes. Certaines bactéries synthétisent des SPEs parallèlement à leur croissance tandis que d'autres ne les produisent que durant de phase exponentielle ou au stade stationnaire (Sutherland, 2001b; Ruas-Madiedo & de los Reyes-Gavilan, 2005). Le déclin est accompagné par une réduction de la production de SPEs suite à une hydrolyse enzymatique. La relation entre la croissance bactérienne et la production de SPEs est complexe et dépend de différents paramètres : du flux de carbone intracellulaire de la glycolyse, des conditions de culture ainsi que des espèces et des souches microbiennes et de leurs origines.

1.2.5.3 Effet de la souche et de l'espèce

Les souches et les espèces utilisées influencent d'une manière significative la quantité et la composition des SPEs (Ricciardi & Clementi, 2000). Une étude portant sur la comparaison de cent quatre-vingt-deux (182) souches de *Lactobacillus*, seulement soixante ont produit plus de 100 mg/L de SPEs (Van Geel- Schutten et al., 1998). Vingt-six souches de *Streptococcus thermophilus* ont été utilisées afin de déterminer la diversité et la quantité des SPEs (Vaningelgem et al., 2004a). Seulement quatre souches ont produit plus de100 mg/L de SPEs tandis que pour douze souches, la concentration de SPEs produites était comprise entre 20 et100 mg/L. Les mêmes résultats ont été investigués par Bala et al., (2010), 25 souches bactériennes de diverses espèces produisant différentes concentrations de SPEs dans les boues secondaires. Par contre, la production de plusieurs SPEs observée par certains auteurs pourrait être due à une dégradation de ces derniers suite à une incubation prolongée (Degeest & De Vuyst, 1999). De plus, une série des études ont investigué que certaines bactéries peuvent synthétiser plus d'une SPEs (Degeest & De Vuyst, 1999; Ricciardi & Clementi, 2000; Shene et al., 2008; Ariga et al., 1992; Vaningelgem et al., 2004b).

1.2.5.4 Effet du pH

Souvent les études sont réalisées sans contrôle du pH ou à un pH fixe de fermentation (Lin & Chang Chien, 2007; Looijesteijn et al., 2001). Néanmoins, il a été montré qu'un contrôle du pH de la culture augmente la production de SPEs comparé à des pH acide (De Vuyst et al., 1998; Gassem et al., 1997a; Grobben et al., 1998). En général, le pH idéal pour favoriser la production de SPEs est celui associé à la phase optimale de croissance pour les bactéries.

Un ajustement du pH à une valeur de 6.2 au cours des fermentations en mode batch avec *Lb. delbrueckii* ssp. *bulgaricus* a abouti à une augmentation de la production de SPEs, suite à une mesure de la viscosité du milieu (Gassem et al., 1997a). Lors d'une fermentation en monde continu (Gassem et al., 1997b), ce résultat a été confirmé avec un pH de 6.5 optimisant la production de SPEs (comparé à un pH de 5.2 et de 5.8 (Tableau 1-1 : La production de SPEs et leurs caractéristiques chimiques).

1.2.5.5 Effet de la température d'incubation

La température d'incubation a un effet important sur la production de SPE (Ruas-Madiedo & de los Reyes-Gavilan, 2005). Certaines études rapportent que la production de SPEs est favorisée à une température inférieure de la zone de température optimale de croissance (Ricciardi & Clementi, 2000). La croissance bactérienne étant ralentie, la production de composés pour le développement des cellules aussi. Conséquemment, 9.8 °C était la température optimale pour une concentration de SPEs maximale produite par la souche SM-A87 après 8 jours. Une augmentation de la température à 35°C (la température optimale de la croissance de la souche SM-A87) a diminué la production de SPEs (Satherland et al., 2001). Ceci peut être due aux transporteurs lipidiques sont moins sollicités et plus disponibles pour la synthèse de SPEs (Cerning, 1995). Toutefois, d'autres auteurs ont présenté des résultats contradictoires. Il a été observé que la production de SPEs est accru aux températures optimales de croissance (Looijesteijn et al., 2001; De Vuyst et al., 1998). L'impact de la température d'incubation dépend fortement de la souche et de l'espèce utilisée et des conditions de fermentation utilisés. Par exemple, 1.4 g/L de SPEs produite par *Bacillus firmus* à température de 35°C alors que *Bacillus subtilus* (E1) produit 6.3 g/L à 28°C (Tableau 1-1 : La production de SPEs et leurs caractéristiques chimiques).

1.2.5.6 Effet du temps de fermentation

Le temps de fermentation est un paramètre important peut influencer la quantité et le poids moléculaire des SPEs (Lin et Chang Chien, 2007). Selon l'étude menée par Lin et Chang Chien, (2007), une longue durée de fermentation (environ 60 h) permet une synthèse accrue de SPEs de deux souches de *Lactobacillus helveticus*. Parallèlement, la

population bactérienne totale diminue. Ces résultats concordent avec d'autres études démontrant qu'une production importante de SPEs est entraînée par les microorganismes afin de se protéger contre les conditions défavorables de l'environnement extérieur. Lin et Chang Chien, (2007) ont également révélé une augmentation du poids moléculaire des SPEs après une durée d'incubation de 32 à 60h comparativement à 12 à 24h. Par contre, une autre étude a démontré qu'une fermentation de 12 h apporte une diminution de la production de SPEs (Vaningelgem et al, 2004). Certains chercheurs ont également observé une diminution de la quantité de SPEs en fonction du temps probablement assignable à la dégradation des SPEs par des enzymes glyco-hydrolases (α-et β-glucosidase, β-glucuronidase) ou par une lyse cellulaire du à un manque de source de carbone (Petry et al., 2003; Degeest & De Vust, 1999) (Tableau 1-1 : La production de SPEs et leurs caractéristiques chimiques).

1.2.5.7 Effet de la quantité d'oxygène dissout

Les études portant sur la quantité d'oxygène essentielle pour la survie des microorganismes dans le milieu de culture sont discordantes. Certains auteurs ont remarqué une production élevée de SPEs lorsque le taux d'oxygène diminuait (De Vuyst et al., 1998) tandis que d'autres affirment que la présence d'oxygène favorise la production de SPEs (Cerning et al., 1990).

Par exemple, l'aération du milieu pendant la culture n'est pas nécessaire pour la production de SPEs. Au contraire, il apparaît qu'une faible quantité en oxygène stimule la production (De Vuyst et al., 1998; Looijesteijn et Hugenholtz, 1999). Gamar-Nourani et al., (1998) ont déterminé une concentration d'O_2 de 10% comme étant optimale pour la biosynthèse de SPEs par *Lb. rhamnosus* C83. Pham et al., (2000) ont étudié l'impact de l'oxygène sur la production de SPEs par *Lb. rhamnosus*. Des concentrations faibles en oxygène (jusqu'à 20%) ont augmenté la croissance et la production de SPEs. Les auteurs ont conclu que des conditions de stress ont induit la production de SPEs, D'autre part, ces conditions de stress peuvent affecter la croissance cellulaire induisant la lyse qui peut diminuer la concentration des SPEs produites.

Tableau 1-1 : La production de SPEs et leurs caractéristiques chimiques

Microorganismes	Milieu de culture	Conditions de fermentation	Concentration des SPEs (g/L)	Caractéristiques chimiques					Références
				Carbohydrates (%w/w)	Protéines (%,w/w)	Autres (%,w/w)	poids moléculaires Da	Groupes Fonctionnels	
Alcaligenes cupidus	Sucrose	200 rpm, 28°C, 96-120h	1,5	72,25 : Galactose, glucose et mannose de rapport 6,3:5,5:1			2* 106	Acetyl ester	Toeda et Kurane , (1991)
Klebsilla sp.	Glucose	200 rpm, 30°C, 120h	1	Les sucres neutres : 69 Sucres totaux:72,55 Galactose, glucose, mannose de rapport 5:2:1		Acide uronique 15,8	> 2*106	Methoxyl	Dermlim et al., (1999)
Citobacter	Acétate-propionate	120 rpm, 30°C, 72h	1,5						Fujita et al., (2000)
Bacillus sp.	Glucose et gélose	130 rpm, 28°C, 10-15h		Carbohydartes :83	17	Acide uronique: 11,4 acide pyruvique : 6,3			Salehizadeh et al., (2000)
Corymebacterium	Sucrose	120 rpm, 28°C, 48 h	2						He et al., (2002)
Bacillus firmus	Glucose	150 rpm, 35°C,72h	1,4	Sucres totaux : 87		Acide uronique : 38 Acide pyruvique : 6,3	2* 106	Carboxyl, Hydroxyl, Methoxyl,	Salehizadeh et al., (2002)

Bacillus mucilagiginosus	Amidon	150 rpm, 30°C, 84h		Sucres neutres : 47,4		Acide uronique : 19,1 Sucres amines : 2,7	2.6 106	Carboxyl Hydroxyl	Deng et al., (2003)
Vagococcus sp		120 rpm, 25 °C, 48-72h	2,3				2* 105	Hydroxyl carboxyl methoxyl	Gao et al., (2006)
Bacillus sp (MBFF19)	Glucose	200 rpm, 30°C, 48h	0,8	Sucres neutres : 3,6 Sucres amines : 0,5	16,4	Acide uronique : 37		Hydroxyl carboxyl methoxyl	Zheng et al., (2008)
Bacillus subtilis	Glycérol et Éthanol	150 rpm, 28°C, 48h	6,3	107 ± 7,5 µM	111 ± 3,7 µM	Acide uronique 27,31 ± 0,017 mM Sucres amines : 41±1,41 mM			Buthelezi et al., (2010)
Pseudomonas plecoglossicida A14	Glycérol et éthanol	150 rpm, 28°C, 48h	8,3	706 ± 12,2 µM	40 ± 1,6 µM	Acide uronique : 26,9 ± 0,018 mM Sucres amines: 5 ± 0,1 mM			Buthelezi et al., (2010)
Klebsilla terrigena (R2)	Glycérol et Éthanol	150 rpm, 28°C, 48h	27,7	356 ± 7,5 µM	4 ± 0,8 µM	Acide uronique : 2mM			Buthelezi et al., (2010)
Exiguobacterium acetylcum (D1)	Glycérol et Éthanol	150 rpm, 28°C, 48h	10,2	20 ± 0,9 uM	22 ± 0,22 µM	Acide uronique :			Buthelezi et al., (2010)

						26,36 ± 0,007 mM			
Staphylococcus aureus	Glycérol et Éthanol	150 rpm, 28°C, 48h	10,8	470 ± 7,5uM	9 ± 0,01 µM	Acid uronique: 26,18 ± 0,009 mM Sucres amines: 2 ± 0,08 mM			Buthelezi et al., (2010)
Proteus mirabilis (Tj-1)	Glucose	160 rpm, 30°C, 48h	1,3	63,1	30,91		1,2 *105		Buthelezi et al., (2010)
Azatobacter indicus (ATCC9540	Extrait de Modhuca latifolia	180 rpm, 30°C, 144h	6,1	97,7	2,3		2*106	Orcinol, Acetyl, Carboxyl Hydroxyle	Patil et al., (2011)
Chryspbacterium daeguense	Glucose tryptone	180 rpm, 30°C, 36h		13,1	32,4	Acide nucléique : 6,8		Carboxyle Hydroxyle Methaxyl	Liu et al., (2010)
Holomona sp	Molasse	180 rpm, 37°C, 10-15h		90	0,5	Acides nucléiques : 4-5			Sam et al., (2011)
Paenibacillus elgii	Glucose, peptone, Lactose, peptone Sucrose, extrait de viande	220 rpm, 30°C, 96h 220 rpm, 30°C, 96h 220 rpm, 30°C, 96h	12,12 11,49 10,15			Glucose, glucuronic acid, xylose et mannose : 1 :0,53 :1,15 : 0,46	3,5 *106		Liu et al., (2013)
Cloacibacterium normanense	Boues secondaires, Glycérol brut	180 rpm, 30°C, 72h	22,4	67		Glucose : 13 Xylose : 9 Sucrose :8 Lactose: 3		Carboxyl, Hydroxyl, Sulphur, phénol	Klai et al., (2016)

1.2.6 Extraction des SPEs à partir de boues et des cultures pures

Différentes méthodes d'extraction physiques (chauffage, centrifugation, sonication) et chimiques (utilisation de bases, d'acides, d'alcools) ont été utilisées principalement pour séparer les SPEs capsulaires du reste de la biomasse. Certains protocoles combinent également des méthodes chimiques et physiques. Dans la littérature, de nombreuses variantes sont mentionnées pour une même méthode, en jouant sur les différents paramètres tels que le temps de contact ou de la réaction, la puissance de sonication, la vitesse de centrifugation, la concentration des réactifs et la température d'incubation ou de chauffage…etc. En effet, la plupart des techniques étaient à l'origine adoptées pour l'extraction des SPEs à partir de biofilms ou de cultures de bactéries, et chaque auteur a sélectionné et ajusté ces méthodes en fonction de la biomasse étudiée.

Les SPEs sont fortement attachées aux cellules, piégées dans les flocs et constituent l'architecture de flocs reliés par des cations. De ce fait, leur analyse nécessite au préalable une étape d'extraction. Selon la littérature, une méthode d'extraction préférée est celle qui garantit un minimum de lyse cellulaire (pas de contamination des substances polymériques intracellulaires) ; ii) qui permet d'obtenir une quantité significative des SPEs extraites; iii) et qui ne doit pas contribué à une contamination aux échantillons extraits par les réactifs utilisés (Forlund et al., 1996).

On distingue différentes méthodes mentionnées dans les études antérieures, qui sont brièvement présentées dans le Tableau 1-2. Chaque méthode présente divers modes d'action pour dissocier les liaisons qui retiennent les SPEs dans la matrice biologique.

De nombreuses études ont été consacrées à la comparaison des méthodes d'extraction (Frolund et al., 1996 ; Park et Novak, 2007). Trois méthodes d'extraction différentes : l'extraction par la résine échangeuse d'ions, l'extraction basique (avec NaOH à pH 11) et l'extraction thermique (à 80°C) ont été évaluées par Frolund et al., (1996). Ils ont marqué un taux d'extraction élevé avec la résine échangeuse d'ions (temps d'extraction de 17 heures) par rapport à deux autres méthodes. La quantité de SPEs extraites augmente de façon significative avec le temps de la réaction. Cependant, les rendements d'extraction sont représentatifs aux différents composants constituant les flocs (protéines, polysaccharides, lipides, ADN). En ce qui concerne les protéines et les polysaccharides (composés majoritaires des SPEs), un temps d'extraction de plus de 6 heures n'améliore pas le taux d'extraction. Toutefois, l'augmentation de durée d'extraction provoque et augmente la lyse cellulaire. Pour une durée d'extraction inférieure à 2 heures, aucune lyse cellulaire n'est requise. En revanche, après 9 heures d'extraction, le pourcentage de la lyse cellulaire achève 60 à 70%.

Liu et Fang, (2002) ont comparé trois méthodes d'extraction utilisant l'EDTA, le formaldéhyde et l'extraction par la résine échangeuse d'ions. Du fait d'un faible rendement d'extraction avec le formaldéhyde, il s'est avéré nécessaire de suivre la méthode avec une extraction basique (NaOH) ou une sonication pour obtenir un taux d'extraction plus élevé par rapport à la résine échangeuse d'ions et l'EDTA. Malgré le rendement d'extraction plus important avec le formaldéhyde + NaOH, il est nécessaire d'effectuer des purifications de l'échantillon par dialyse afin d'éliminer les espèces chimiques rajoutées. Extraction avec l'EDTA a montré une forte teneur en ADN qui a été associée à la lyse cellulaire.

Les travaux de Comte et al., (2007) ont permis également de mettre en évidence l'hétérogénéité des SPEs. En effet, ces derniers ont mis en évidence un profil de tailles des molécules extraites en fonction de la méthode d'extraction utilisant la chromatographique de poids molécules de ces biopolymères. Les profils relatifs aux méthodes chimiques (EDTA, NaOH et formaldéhyde) ainsi que celui de la méthode thermique diffèrent considérablement entre eux et sont bien distincts d'autres techniques. Ces variations sont attribuées d'une part à la modification de la structure et de la composition chimique des polymères induites par les méthodes chimiques et d'autre part, à la sélectivité des espèces extraites par chaque méthode. Il est à noter que l'extraction utilisant le chauffage provoque l'hydrolyse et la dissociation des molécules.

Certaines études ont suggéré la combinaison chimique-physique des méthodes d'extraction afin d'obtenir un taux important des SPEs extraites. Les études de Ras et al., (2008) se sont orientées sur l'extraction des protéines des boues. Ils ont proposé une technique qui intègre trois types d'extractions différentes, l'extraction de type mécanique (ultrasonication 37W, 2min), de type ionique (résine échangeuse d'ions (70g/g MVS)) et de type hydrophobe (éthanol 20-50- % entre 1et 2h). D'après eux, ce protocole permet l'extraction entre 45 et 49% de protéines totales extractibles du floc. De plus, aucune lyse cellulaire n'a été identifiée.

Bien que de multiples efforts ont été effectués afin de trouver la (les) meilleure(s) méthode(s) d'extraction, aucune méthode n'était simplifiée ou sélectionnée comme une méthode standard afin de comparer les résultats de différentes études. La plupart des études (Wilén et al., 2003a; Jin et al.,2003) cherchent à examiner l'activité de boues biologiques dans les différents procédés de traitement .

Il existe donc une gamme des méthodes d'extraction des SPEs, mais les variétés des méthodes n'ont pas la même potentialité d'extraction et peuvent induire à une extraction spécifique de certains types de molécules des SPEs. Différentes études sont consacrées à comparer ces techniques d'extraction à partir de cultures de bactéries (Sheng et al., 2005; Metzger et al., 2009), de biofilms (Aguilera et al. 2008), de boues activées (Comte et al., 2006c; Ras et al., 2008)

Tableau 1-2 : Principe de la majorité des méthodes d'extraction des SPEs

Types d'extraction	Méthodes d'extraction	Description
Physique	Centrifugation	• Technique douce qui ne provoque pas la lyse cellulaire. • Rendements d'extraction, car elle ne nécessite pas d'ajout de réactifs chimiques. • La quantité des SPEs extraites est très faible comparée à d'autres méthodes (Liu et fang, 2002). • Conditions d'extraction : 20 000g, 20 min (Liu et Fang , 2002); 4000 g, 20 min (Comte et al., 2007)
	Thermique	• Technique provocante de la lyse cellulaire à cause de la dissolution des polymères de la membrane cellulaire. • Dénaturation des SPEs extraites • Conditions d'extraction : 80°C, 1 h (Frolund et al., 1996)
	Ultrasons	• Dissociation des agrégats • Détection de la lyse cellulaire même pour des temps de sonication faibles (Zhang et al., 2007) • Conditions d'extraction : 25 kHz, 0-30 min (Zhang et al., 2007)
	Résine échangeuse d'ions	• Très bonne extraction des SPEs avec un peu de lyse selon les conditions d'extraction (Frolund et al., 1996) • La méthode la plus utilisée actuellement • Conditions d'extraction : 70 g-résine / g MVS. 600 rpm, 1 h (Liu et Fang ., 2002)
Chimique	EDTA	• Agent chélatant, leur rôle est d'éliminer les cations multivalents qui forment de ponts entre les sites négativement chargés des SPEs et ainsi dissoudre les flocs bactériens • provoque la lyse cellulaire: Les SPEs extraites contiennent de l'ADN intracellulaire (Liu et Fang, 2002) • Contamination de l'échantillon par les réactifs chimiques ajoutés (Comtes et al., 2007). • Conditions d'extraction : EDTA 2% pendant 3 h à 4°C (Comtes et al., 2007; Liu et Fang ,2002)
	NaOH	• Augmente le pH, dissocie les groups acides et génère la répulsion des SPEs chargées négativement (Liu et Fang , 2002). • Conditions d'extraction : 1 N pendant 3 h à 4°C (Comte et al., 2007, Liu et Fang , 2002)

Formaldéhyde	• Le formaldéhyde fixe les cellules, car il établit des liaisons avec les protéines et l'ADN, et empêche la solubilisation des lipides (Gavrilov et Razin, 2009). De ce fait, il prévient la lyse cellulaire. • Conditions d'extraction avec formaldéhyde : 36,5% pendant 1 h à 4°C (Comte et al., 2007 ; Liu et Fang, 2002)

1.2.6.1 Effet sur la composition chimique des SPEs

Comme le montre le Tableau 1-3, pour la même méthode d'extraction, la quantité de SPEs extraites varie en fonction de la biomasse d'origine. Ainsi, des faibles teneurs en SPEs sont extraites à partir de boues granulaires en comparaison aux boues activées. En effet, pour le même protocole d'extraction, la dispersion de la biomasse est limitée pour les biomasses granulaires qui ont une structure dense (Fang, 2000) contrairement aux flocs. De plus, la quantité totale de SPEs extraites diffère suivant le protocole utilisé pour extraire les SPEs de la même biomasse. Ainsi, une adaptation du protocole d'extraction est nécessaire en fonction de la biomasse étudiée. De manière générale, les extractions chimiques sont des méthodes plus efficaces permettant l'extraction d'une plus grande quantité de biopolymères (Liu et Fang, 2002b ; Liu et Fang, 2003 ; Comte et al., 2006c).

Ces méthodes d'extraction modifient la quantité de SPEs extraites mais également la répartition des différentes teneurs en composés (protéines, polysaccharide, etc.) (Tableau 1-3). Dans ce contexte, les études qui ont comparé les protocoles d'extraction ont permis de mettre en évidence certaines tendances concernant l'impact de la méthode sur la composition biochimique des SPEs car la composition chimiques des SPEs peut affecter la potentialité de floculation.

Concernant les extractants chimiques, par exemple, l'utilisation de l'EDTA provoque une extraction des SPEs à de fortes teneurs en protéines et substances humiques-like. Néanmoins, cet agent chélatant est connu pour altérer le dosage des protéines, ce qui peut surestimer la quantité de protéines (Brown et Lester, 1982; Ras et al., 2008). Autre exemple, l'ajout d'hydroxyde de sodium comme extractant apporte de très fortes concentrations en acides nucléiques (Tableau 1-3) dues à un dommage cellulaire lors de l'extraction (Sheng et al., 2005). La combinaison du formaldéhyde et de la soude (NaOH) permet alors de limiter ou réduire cette lyse cellulaire (Liu et Fang, 2002b).

1.2.6.2 Le rendement de l'extraction

Par définition, le rendement d'extraction est la quantité de SPEs extraites en fonction de la quantité de SPEs présentes dans la biomasse (Wingender et al., 1999). Néanmoins, la concentration initiale des SPEs dans la biomasse est généralement inconnue et aucune

méthode n'est disponible qui permet de déterminer ce paramètre. Toutefois, Ras, (2008) propose une méthode qui combine des extractions répétées ou un modèle pour appréhender la teneur originale en SPEs dans la biomasse. Ce rendement d'extraction s'exprime en pourcentage, mais les variables diffèrent en fonction des études. Il correspond au rapport du poids sec de l'extrait de SPEs en fonction du poids sec de la biomasse.
Le rendement d'extraction peut aussi s'exprimer en fonction de carbone organique total et réfère alors au rapport du carbone organique de l'extrait sur le carbone organique de la biomasse.

1.2.6.3 Évaluation de la lyse cellulaire

Une contamination des extraits de SPEs par le matériel intracellulaire peut être accordée par une lyse cellulaire suite aux méthodes d'extraction utilisées. Dans la littérature, différentes techniques sont proposées pour estimer cette contamination.

- Les acides nucléiques sont des constituants des SPEs expulsés dans l'environnement extérieur suite à la mort naturelle des cellules de la biomasse ou excrétés par les cellules au cours de leur croissance. De faibles quantités ont été identifiées dans la matrice de SPEs. La centrifugation étant une méthode réputée non provocante de lyse des cellules. Elle a été utilisée dans de nombreuses études comme une méthode de contrôle. Dans ce contexte, la comparaison de la teneur en acides nucléiques des autres méthodes avec celle obtenue avec la centrifugation permet d'évaluer la lyse cellulaire. Par exemple, le chauffage ou la soude sont des méthodes entraînant une forte extraction des acides nucléiques possiblement due à la lyse de cellules.

- Le ratio protéines/polysaccharides est souvent utilisé comme indicateur. Si ce rapport est trop élevé, il est synonyme de lyse cellulaire. Ce rapport a été pratiqué pour les biomasses d'origine biofilms ou cultures pures de bactéries, car les polysaccharides sont les principaux constituants des SPEs de ces biomasses. Un ratio inférieur à 1 équivaut à une faible lyse cellulaire. Donc, la présence du matériel intracellulaire riche en protéines conduit à une augmentation de ce ratio. Toutefois, la quantité de protéines est naturellement plus abondante dans des biomasses de type boues activées ou boues granulaires. Ainsi, les ratios sans lyse cellulaire notée sont compris dans l'intervalle1-2 et 3-6 respectivement pour des boues activées et des boues granulaires (Adav et Lee, 2008). Il est donc nécessaire de prendre en compte le type ou l'origine de la biomasse et d'identifier ce rapport et de le comparer avec une méthode qui n'induit pas la lyse des cellules.

- Le glucose-6-phosphate déshydrogénase (G6P-DH) est une enzyme d'origine intracellulaire. La quantification de l'activité enzymatique de cette enzyme dans les extraits de SPEs est utilisée par certains chercheurs pour évaluer la lyse des cellules (Frølund et al. 1996; Ras et al. 2008).

Tableau 1-3 : Exemples de composition biochimique des échantillons de SPEs extraites à partir de différents types de boues par différentes méthodes d'extraction.

Cultures pures	Méthodes d'extraction	Composition des SPEs (mg/g DW)				Concentration des SPEs (mg/g- MS)	Prot/car	Références
		Prot	carbo	AH	ADN			
Rhodopseudomonas acidophilap		4.1	6.2	-	-	12.9	1.5	Sheng et al. (2005)
		6.5	58.5	-	-	70.3	9.0	
		7.7	126.5	-	-	159.2	16.4	
	Centrifugation	10.3	37.3	-	-	71.6	3.7	
	EDTA							
	NaOH							
	Chauffage							
Acidiphilium.sp		600	220	-	-	820	0.3	Tapia et al. (2009)
		550	200	-	-	750	0.3	
		430	170	-	-	600	0.4	
		570	200	-	-	770	0.4	
Cultures mixtes								
Aérobie		54.6	40.5	50.4	0.3	165	0.2	Liu and Fang, (2002)
		17.7	12.7	16.4	0.1	109	0.1	
		22.9	12.4	59.2	0.4	146	0.1	
		20.4	28.8	18.9	0.1	78	0.1	
	Formal + NaOH							
	CER							
	EDTA							
	Formal+ ultrasons							

Acidonique		110.9	25.8	0.1	15.1	179	4.3	
			38.7	6.2	0.1	58	6.2	
				3.0		105	4.6	
		41.7	6.5	0.2	15.9	100	6.6	
		71.6	10.8	5.0	0.05			
Boues mutagéniques	Formal + NaOH	19.1	42.1	23.3	0.19	102	2.2	
	CER	7.9	10.6	5.5	0.05	30	1.3	
	EDTA	6.8	12.0	24.3	1.20	73	1.7	
	Formal+ ultrasons	12.0	13.1	5.6	0.04	30	1.1	
Boues Activées								Zuriaga-Agustí et al. (2013)
	Sonication	140	343	62	46	200	2.4	
	Heating	166	378	126	17	369	2.3	
	CER	16	4	-	-	24	4.2	
	Triton X 100	81	10	-	-	100	7.9	
Biofilm	Centrifugation	57.0	-	-	-	57	-	Pan et al., (2010)
	Ultrasonication	22.3	56.6	-	-	79	0.4	
	EDTA	1.7	3.2	-	-	45	0.5	
	Formaldéhyde	8.0	25.2	-	-	33	0.3	
	Formal+NaOH	17.0	13.3	-	-	30	1.3	

1.2.7 Les méthodes de caractérisation des SPEs (dosage colorimétrique)

La littérature sur le contexte des SPEs fait partie de nombreuses techniques pour déterminer les teneurs des composés contenues dans les SPEs. La plupart de ces méthodes sont des dosages colorimétriques. Outre les plus anciennes, diverses autres méthodes se trouvent afin d'améliorer la fiabilité et la crédibilité du dosage et de corriger les interférences pouvant se présenter. Toutefois, bien que ces méthodes simples soient très utiles, elles présentent des inconvénients. En effet, si on prend le cas des protéines, trois méthodes sont souvent utilisées pour leur caractérisation : la méthode de Lowry et al., (1961), la méthode de Smith et al., (1985) et la méthode de Bradford, (1976). Concernant la méthode de Lowry, une modification de protocole est nécessaire afin de tenir compte l'interférence des substances humiques-like dans le dosage des protéines (Frølund et al., 1995). De plus Ras et al., (2008) ont montré que cette méthode tend à sous-estimer la réelle teneur en protéines. La méthode de Smith permet le dosage des protéines, mais également de certains polysaccharides (Wingender et al., 2001) utilisant l'acide bicinchonique (BCA) comme réactif. Elle entraîne une surestimation de la réelle teneur en protéines (Ras et al., 2008). Enfin, la méthode de Bradford permet de quantifier une partie des protéines. En effet, les petites protéines qui comportent moins de huit peptides ne sont pas dosées (Wingender et al., 2001).

Par contre, l'efficacité de ces méthodes est limitée dans le cas de caractérisation des SPEs issues de biomasses épuratoires, car ces dernières sont composées de molécules très hétérogènes et très diversifiées (Ras et al., 2008). De plus, différentes recherches ont mis en avant que les méthodes d'extraction peuvent également affecter le dosage colorimétrique des SPEs à cause de présence de réactifs chimiques (Brown et Lester, 1982 ; Ras et al., 2008). Ainsi, l'EDTA qui forme des complexes stables avec les protéines altère les résultats obtenus pour la caractérisation des protéines (Brown et Lester, 1982).

De même, Ras et al., (2008) ont montré que l'extraction en utilisant du Triton X ou un tampon Tris engendre une sous-estimation de la teneur en protéines déterminée. Néanmoins, les méthodes ne sont pas toutes affectées de la même manière par la présence d'un réactif défini. Il est donc judicieux de sélectionner la méthode de dosage en fonction de l'impact possible des réactifs chimiques utilisés en solution.

Concernant les polysaccharides, le mécanisme d'action est le même pour les deux méthodes utilisées pour leur quantification. Les polysaccharides sont hydrolysés à travers le chauffage par un acide fort (l'acide sulfurique). Ensuite, les saccharides réagissent avec le réactif spécifique à chaque méthode. La méthode de Debois (Debois et al., 1956) utilise le phénol. Ce réactif produit la même intensité de couleur pour tous les polysaccharides. L'anthrone est utilisé dans la méthode de Dreywood, (1964), l'intensité de la couleur produite par ce réactif varie en fonction du type de polysaccharide (Koehler, 1952). Selon l'étude

d'Herbert et al., (1971), l'Anthrone réagit plus fortement avec les hexoses et plus faiblement avec les pentoses et heptoses.

Dans les cas des eaux usées, les deux méthodes de dosage de polysaccharides (Dubois et Anthrone) ont été indistinctement utilisées dans la littérature. Il n'existe pas de choix privilégié. Certaines études ont employé la méthode de l'Anthrone (Frolund et al., 1996; Yu et al., 2008) et d'autres, ont fait appel à la méthode de Dubois (Li et Yang, 2007).

Pour la caractérisation des autres composées de SPEs comme les lipides, les acides nucléiques et les substances humiques, la littérature n'est pas consacrée pour leur caractérisation, car les protéines et les polysaccharides sont les constituants principaux de SPEs.

1.2.8 Rôles des SPEs dans les procédés de traitement des eaux usées

Différents types d'interactions ont été mis en place pour maintenir l'agrégation et de flocs bactériens et l'architecture de biofilm. Ces interactions correspondent aux forces définies par la théorie DLVO (les interactions van der waals) (Zita et Hermansson, 1994), aux interactions polymériques (Flemming et al, 2001), aux théories de pontage de cations multivalents (Ca^{2+} ,Mg^{2+}, Cu $^{2+}$ et Fe^{3+}) (Keiding et Nielson, 1997) et aux liaisons hydrophobes (Urbain et al., 1993). L'importance de la matrice polymérique sur les différentes étapes du procédé de traitement des eaux usées (biofloculation, décantation et déshydratation des boues) a été examinée dans différentes études.

L'influence des SPEs sur la structuration des flocs a été souvent étudiée. Les résultats ont été contradictoires due à la complexité de la nature des agrégats. Le type des agrégats varie en fonction de multiples facteurs dans les stations d'épuration. Les tendances générales rapportées dans la littérature sont bien récapitulées.

L'incidence des SPEs sur les procédés de floculation, décantation et déshydratation a été étudiée par Subramanian et al., (2010). Différentes souches bactériennes productrices des SPEs ont été isolées. Leurs potentialités de floculation ont été analysées dans la solution de kaolin et la décantation et déshydratation des boues municipales. La majorité des souches bactériennes produisent de SPEs solubles. Ce type de SPEs a montré une amélioration de la floculation des particules de kaolin suite à leurs poids moléculaires élevés. Aussi, il a notamment favorisé la décantation des boues urbaines avec des valeurs d'IVB inférieur à 150 mL/g obtenues ainsi que la déshydratation de boues. Ceci suggère que la variété des SPEs en termes de quantité et composition dépend principalement des espèces bactériennes. Cette diversité engendre des caractéristiques et des mécanismes de réaction différents dans la floculation, sédimentation et déshydratation des boues.

1.2.8.1 Décantation des boues

Dans les études de Liao et al., (2001), l'augmentation de la concentration de SPEs dans les boues a affecté négativement sur la qualité de la décantation des boues. La décantation dépend de la concentration ajoutée de SPEs. Il existe une concentration optimale pour une bonne décantation.

Le nombre relatif des microorganismes filamenteux et de biofloculants est l'un des facteurs les plus importants qui déterminent les caractéristiques physiques du floc et influencent les propriétés de décantation. Cette étude a été bien détaillée par Sezgin et al. (1978). À des conditions bien déterminées de bassin d'aération, les bactéries filamenteuses dominent les autres microorganismes et forment une structure en treillis dans le floc. Ainsi, ils s'étendent du floc créant une résistance de frottement très élevé, qui diminue la vitesse de sédimentation des boues. Dans ce cas, les boues présentent des problèmes de décantation et signalent ce qu'on appelle foisonnement des boues, qui pose des difficultés de séparation dans les stations d'épuration des eaux usées. L'indice de sédimentation ou est nommé le volume indice du volume des boues (IVB) est un paramètre mesurant la décantation des boues. Selon Dick et Vesilind, (1969), l' IVB est le volume en ml occupé par un gramme de boue après 30 min de décantation. C'est un moyen rapide qui donne des données brutes sur la décantation des boues, mais ne mesure pas la vitesse de sédimentation.

La relation entre les SPEs et la décantation des boues a été largement étudiée (Urbain et al., 1993). Mais, la majorité des recherches a montré des contradictions (Chao et Keinath, 1979). De ce fait, c'est souvent difficile de comparer les résultats de la littérature. De nombreuses études (Jorand et al, 1998; Liao et al., 2001) ont révélé qu' une quantité importante de SPEs entraîne une faible valeur de l'indice du volumes des boues (IVB) montrant ainsi une bonne décantation.

D'autres recherches (Bura et al., 1998) ont été également marquées que la composition chimique et les propriétés des SPEs peuvent plus affecter la décantation des boues que leurs quantités.

Sponza, (2005) a noté que la faible teneur en protéines est associée à des valeurs élevées d'IVB. De même, Higgins et Novak, (1997a) ont observé que les protéines contenues dans les SPEs ont un rôle primordial dans la sédimentation des boues par rapport aux autres constituants. Au contraire, accordant aux d'autres études, Wilén et al., (2008b), ont montré qu'une mauvaise séparation entre les boues et l'eau à traiter a été associée à une forte teneur en protéines. En contrepartie, Martinez et al., (2000) ont observé qu'aucune corrélation entre l'indice du volumes des boues et les teneurs en hydrates de carbone ni en lipides constituants les SPEs. De plus, Urbain et al., (1993) ont remarqué qu'une

augmentation de la concentration des hydrates de carbone contenus dans les SPEs entraîne une bonne amélioration de la décantation de boues. Tandis qu'une forte teneur en lipides provoque une détérioration des flocs. Bura et al., (1998) ont montré que la teneur en ADN est corrélée avec l'IVB.

Trois réacteurs en batch ont été utilisés pour étudier l'effet de débits d'air sur le rapport hydrates de carbone/protéines et sur la décantation des boues. Des résultats n'ont montré aucune relation entre la quantité de SPEs et l'index de décantation. Cela s'oppose aux recherches précédentes qui ont révélé le rôle principal de SPEs dans l'adhérence des cellules et dans la formation des flocs. En effet, au cours de la croissance bactérienne dans des conditions aérobies avec des concentrations d'oxygène élevées, les propriétés des SPEs ont été variées. À un débit d'air élevé, le rapport Carbone/Azote (C/N) augmente, la quantité d'hydrates de carbone constituant les SPEs a également augmenté provoquant ainsi une augmentation des SVI et une mauvaise sédimentation (Shin et al, 2001). Étant donné que la nature du substrat et par le type des micro-organismes dans les boues activées peuvent varier la composition des SPEs, les caractéristiques de décantation sont également affectées par l'environnement, y compris la biomasse et les matières organiques dégradables. Par exemple, les flocs constituants les boues activées contenant facilement des matières organiques dégradables, présentaient de bonnes propriétés de décantation avec des valeurs IVB faibles. Alors que les flocs contenant des substrats organiques dégradables comme produits chimiques et des colorants entraînent de mauvaises propriétés de décantation (Sponza, 2004).

La capacité de l'interaction des SPEs avec le métal est une autre propriété qui peut influencer les caractéristiques de sédimentation des boues (Jin et al., 2003). Biggs et al., (2000) ont montré une stabilité significative de la taille des flocs à des concentrations de calcium inférieures à 8 meq /L. Cependant, une concentration de calcium supérieur à 8 meq/L entraîne une bonne décantation. Higgins et Novak, (1997a) ont remarqué que l'addition excessive d'un cation monovalent, sodium, diminue la vitesse de sédimentation et provoque aussi une mauvaise déshydratation.

En conclusion, des tendances générales sur les rôles des SPEs et leurs implications dans la décantation de boues ne peuvent pas être posées et le sujet reste toujours à discuter (Liu et Fang ,2003).

1.2.8.2 La biofloculation

Les SPEs ont un rôle primordial dans la phase de croissance des biomasses, que ce soit concernant la formation des biofilms ou l'adsorption sur une surface solide (Vu et al., 2009)

ou la floculation des boues activées (Wilén et al., 2003b). D'après la littérature, l'agrégation des cellules se fait selon trois types d'interactions: les forces électrostatiques d'attraction ou de répulsion, les forces de Van der Waals selon la théorie DLVO (Derjauguin, Landau, Verway, Overbeek) (Hulshoff Pol et al., 2004) et les interactions des polymères (Azeredo et Oliveira, 2000) (Figure 1-4). Les forces d'attraction ont un effet positif alors que les forces de répulsion ont un effet contraire sur la floculation et l'agglomération des flocs. La présence de grandes quantités de SPEs à la surface cellulaire favorise l'adhésion des particules par les interactions polymériques et d'autres forces d'attraction (Tsuneda et al., 2003). Les cellules sont des particules chargées négativement, dans ce cas, deux composés de même charge se repoussent selon la théorie de DLVO. Mais différentes études ont montré que la production de SPEs par les cellules permet de réduire cette charge négative ainsi que les forces de répulsion et donc de stimuler la liaison entre les bactéries (Schmidt et Ahring, 1994). L'hydrophobicité est un autre facteur important dans la biofloculation et dans l'architecture de biofilm aussi bien dans la structure de biomasse (Liu et al. 2004a). Ainsi, les SPEs sont composés de molécules hydrophobes (protéines, acides uroniques…) et de molécules hydrophiles (polysaccharides) (Dignac et al. 1998). En effet, à un rapport protéines/polysaccharides élevé, l'hydrophobicité des cellules augmente, la charge de surface diminue, influençant ainsi la biofloculation (Liao et al., 2001 ; Wang et al., 2006). Outre la quantité des SPE produites par les bactéries, la composition des SPEs joue un rôle principal et est également à prendre en compte lors de l'initiation de l'agrégation des bactéries.

Les propriétés des SPEs jouent un rôle essentiel dans la formation de biofilm et l'agglomération de flocs permettant la liaison physique entre les biomasses: des interactions électrostatiques avec des composés organiques ou inorganiques du milieu et des liaisons polymériques définies en protéines-polysaccharides. Les SPEs présentent 20 à 30 fois plus de sites de fixation électrostatique que les membranes et les surfaces bactériennes (Liu et Fang, 2002). Elles possèdent des groupements ionisables qui présentent des sites de fixation d'autres molécules. Ces sites permettent d'intensifier et renforcer l'architecture de l'agrégat par le mécanisme de pontage à des cations divalents. Ces « cations pontants » favorisent la liaison entre les SPEs eux-mêmes ou entre les SPEs et les cellules (Yu et al. 2001). En effet, des études ont montré que la présence de cations divalents dans le milieu tels que Ca^{2+}, Mg^{2+} ou Fe^{2+} améliore la biofloculation (Higgins et Novak, 1997; Yu et al., 2000). Ainsi, la présence d'ions calcium augmente la stabilité des biofilms en permettant l'attachement des polysaccharides de charge anionique de type alginate (Körstgens et al., 2001). Higgins et Novak, (1997) ont noté la présence de protéines structurales dites « lectine-like » ayant la particularité de se fixer spécifiquement aux oses. Ainsi, les cations

pontants et les protéines lectine-like forment un réseau polymérique permettant un enchevêtrement des molécules de SPEs qui consolide la structure et l'architecture de flocs.

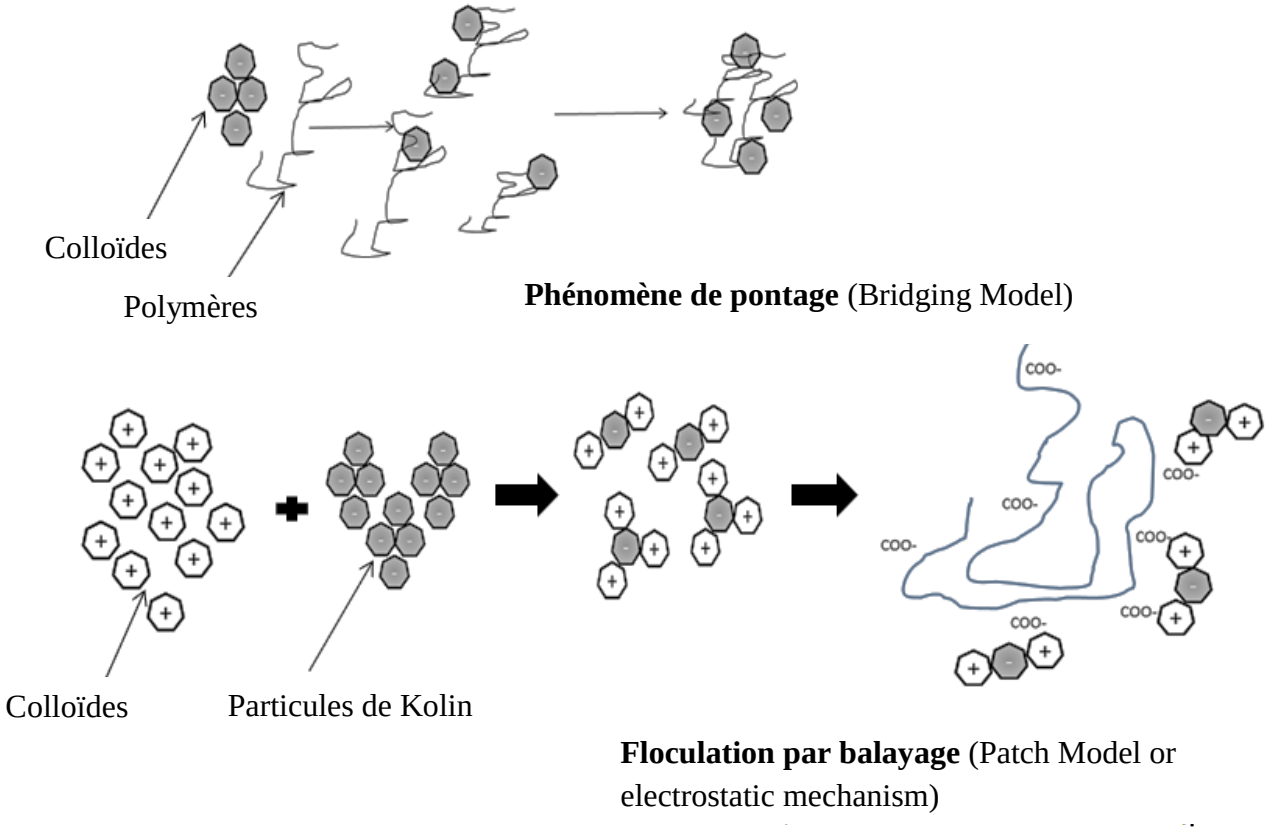


Figure 1-4 : Mécanisme de biofloculation

1.2.8.3 Déshydratation des boues

L'eau contenue dans les boues a été classée dans des catégories selon différents critères en ce qui concerne le procédé de déshydratation des boues (Vesilind, 1997). i) l'eau libre qui est facilement séparée par décantation; ii) interstitielle liée, une déshydratation mécanique peut l'enlever iii) vicinale, l'eau associée aux agrégats; iv) l'eau liée d'hydratation, l'eau associée chimiquement aux agglomérats qui peut être retirée que par séchage thermique (Colin et Gazbar, 1995).

L'épaississement des boues utilisant les cations inorganiques ou les polymères cationiques est une des stratégies fréquemment mises en œuvre des traitements physicochimiques pour la déshydratation des boues. Toutefois, cette technique consiste à enlever, au maximum, 50% de l'eau contenue dans les boues (United States Patent No. 3772191). Les problèmes de déshydratation sont souvent liés à la présence de SPEs. Ces substances polymériques sont capables de retenir une grande quantité d'eau grâce à leur composition et structure chimique (Sutherland, 2001). D'après Flemming, (1996), deux types d'interactions entre les

molécules de l'eau et les polymères SPEs existent : (i) les interactions actives électrostatiques résultent en présence des dipôles permanents des molécules de l'eau et des dipôles induits dans les groupes fonctionnels des SPEs. (ii) les liaisons hydrogènes : liaisons établies entre les molécules d'eau et les groupes hydroxyles des SPEs (particulièrement présents dans les polysaccharides et dans la structure tertiaire des protéines). La déshydratation de boues est une étape nécessaire, dans le procédé de station d'épuration concernant la gestion de boues pour être épandue ou incinérée (Spellman, 2009). La déshydratation des boues présente 50% de coûts totaux d'opération d'une station d'épuration (Baeyens et al., 1997).

Puisque les SPEs ont rôle primordial pour prévenir la dessiccation des cellules. Elles sont fortement hydratées, de ce fait, les méthodes mécaniques utilisées pour la déshydratation de boues sont dépendantes essentiellement de la quantité de SPEs (Keiding et al., 2001). Par exemple, une augmentation de la quantité de SPEs dans les boues provoque une détérioration de la déshydratation (Liu and Fang, 2003). Mikkelsen et Keiding, (2002) ont également observé que les boues dont la teneur en SPEs est faible, montrent une potentialité de déshydratation plus élevée. Les protéines constituant les SPEs ont été établies comme majeurs composés influençant le piégeage de l'eau dans la matrice du floc.

1.2.8.4 Biosorption des métaux

Les SPEs présentent de très bonnes propriétés de rétention des métaux avec des variations de spécificité et d'affinité (Figure 1-5).

La fixation de cations aux bios polymères bactériens se fait généralement par l'interaction électrostatique avec des groupes fonctionnels négativement chargés comme des acides uroniques, des groupes phosphoryles associés aux composants de membrane ou les groupes carboxyliques d'acides aminés. De plus, il peut aussi y avoir fixation de cations par des polymères positivement chargés par l'intermédiaire de groupements hydroxyles. Figure 1-6 présente les principaux constituants de SPEs et leurs sites de fixation potentiels des métaux.

Les SPEs des microorganismes agissent comme des poly anions dans des conditions naturelles par la formation de ponts avec les groupes carboxyles de polymères acides (polysaccharides contenant des acides uroniques) ou en formant des liaisons électrostatiques faibles avec des groupes hydroxyles sur des polymères contenant des glucides neutres (Tableau 1-4). Tandis qu'un grand nombre de métaux sont connus pour se lier aux polysaccharides, la partie protéique des EPS joue aussi un rôle majeur dans la complexation d'ions métalliques. Les protéines riches en acides aminés acides (notamment l'acide glutamique et aspartique) contribuent aussi aux propriétés anioniques des EPS. Les acides nucléiques sont polyanioniques en raison de la présence des résidus de phosphate

comme les composants négativement chargés des EPS, les acides uroniques, les acides aminés acides et les nucléotides contenant du phosphate sont suspectés d'être impliqués dans des liaisons électrostatiques avec les cations (Figure 1-6) (Pal and Paul, 2008).

Néanmoins, peu des données sont disponibles dans la littérature impliquant la fixation des SPEs aux métaux lourds. Geesey, (1994) ont montré la sécrétion abondante des SPEs de type acide uronique dans un environnement avec une forte teneur en cuivre. De ce fait, les groupes carboxyliques constituant les acides uroniques fixent le cuivre en protégeant ainsi la croissance des bactéries. De ce point de vue, Keevil, (2004) a révélé que dans un milieu riche en cuivre, les biofilms ont généralement un rapport cellules/SPEs élevé, suggérant que la production des SPEs participe à une stratégie de défense cellulaire contre la toxicité des ions cuivre. La synthèse et la structure des SPEs sont influencées par la présence du cuivre dans le milieu extérieur des bactéries.

Loaec et al., (1997) ont montré que la composition chimique des exopolysaccharides issus de bactéries *Alteromonas macleodii fijiensis* comprenant des sucres neutres, acides, des amino sucres et autres composants tels que des esters sulfate et des pyruvates favorisant la fixation des métaux. Ces auteurs interprètent par leurs recherches que la spécificité de la bio sorption des métaux par les SPEs mentionnée précédemment dépend principalement de leur composition. Cette composition des SPEs est liée à leur origine.

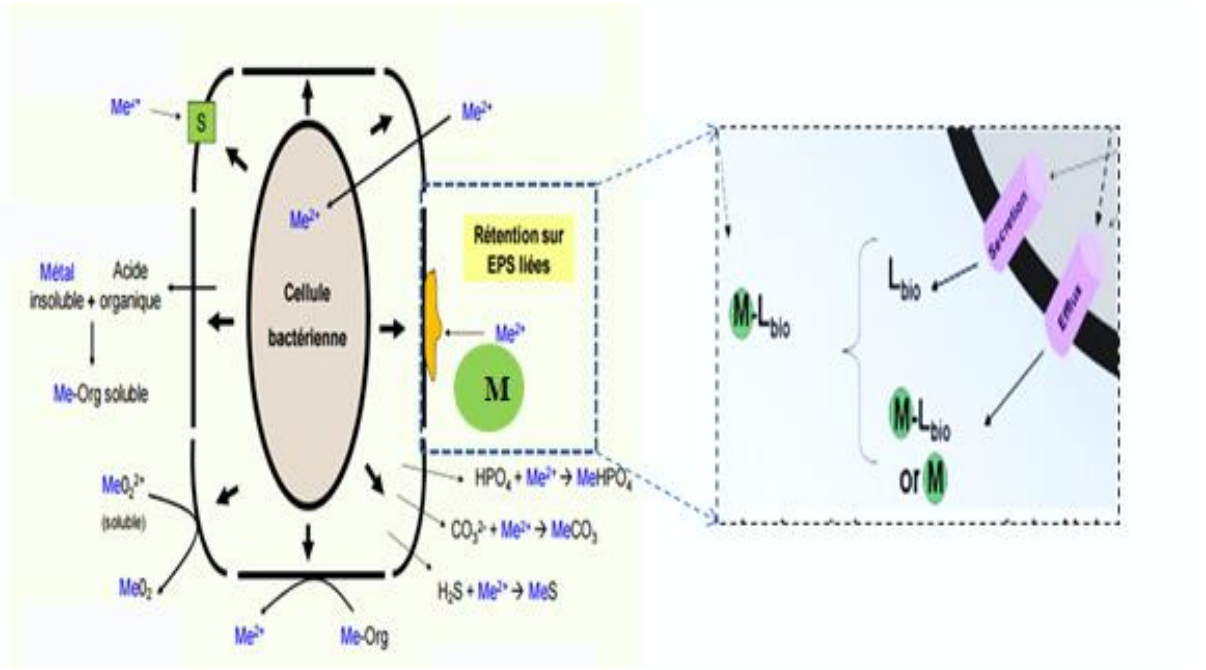
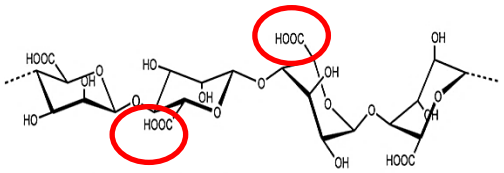
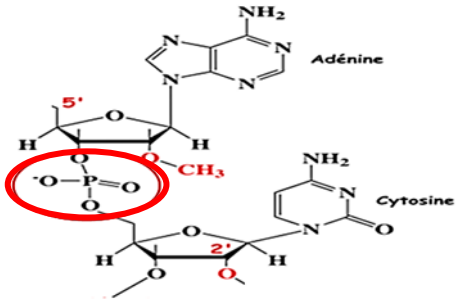


Figure 1-5 : Schématisation des interactions entre métaux et SPEs: L_{bio} correspond à un ligand biologique capable de fixer le métal M.

Les polysaccharides
Groupes Carboxyles



Les Acides nucléiques
Sites phosphodiester



Les protéines
Amines
Carboxyles

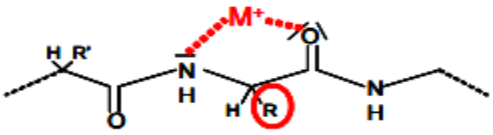


Figure 1-6 : Principaux constituants des substances polymériques Extracellulaires (SPEs) bactériennes et leurs sites de fixation potentiels des métaux

Aux pH proches de la neutralité, la complexation métal-SPEs peut être envisagée par des forces électrostatiques grâce aux groupements fonctionnels chargés négativement. Certains articles ont évalué la capacité des SPEs à fixer un métal et la variation de cette efficacité en fonction du pH. Pardo et al., (2003) ont étudié la biosorption de différents métaux par la biomasse de *Pseudomonas putida* et ont trouvé des affinités différentes de métaux aux SPEs en fonction du pH. À pH 4,5, l'ordre selon l'efficacité d'élimination est de Pb> Cu> Cd≈Zn alors dans des pH plus élevés, l'ordre devient Pb> Cu≈Zn> Cd. Par contre, selon les études de Savvaidis et al., (2003), la capacité de biosorption de *Pseudomonas cepacia* au cuivre était plus importante en comparaison aux autres métaux (Cu> Ni> Cd> Zn). En outre, il a été établi que non seulement le pH peut affecter la capacité de biosorption, mais aussi l'électronégativité du métal a une influence sur la capacité de complexation. Ils ont signalé que plus l'électronégativité est forte plus que la force d'attraction et de complexation est importante (Allen et Brown, 1995).

Donc, l'ordre de métal peut varier en fonction de l'électronégativité du métal (c'est à dire la spéciation des métaux) et la charge de biosorbant impliqué par les changements de pH.

Guibaud et al., (2004) ont aussi étudié la fixation de certains métaux (Zn, Cu, Ni) aux SPEs dans les boues activées. Leurs études statistiques ont montré que plus les taux de protéines,

d’acides humiques et de polysaccharides augmentent et plus les SPEs ne sont pas capables de fixer le Cu. Tandis que la complexation du Ni et les SPEs s’est révélée dépendantes de la concentration en acides uroniques.

Dans une récente étude, Kenney et Fein, (2011) ont montré que les EPS présentent des groupements fonctionnels similaires à ceux que l’on retrouve à la surface des cellules bactériennes. A masse équivalente, les parois bactériennes et les EPS présentent des concentrations de sites réactifs et des affinités similaires vis-à-vis des protons et du Cd.

Cependant, la possibilité des SPEs à fixer les métaux est difficilement comparable (Tableau 1-4: Comparaison de capacité d'affinité des métaux par SPEs dans quelques études.). En effet, chaque capacité de fixation est liée à la méthode utilisée pour mesurer cette capacité et de la spécificité des SPEs mises en jeu. Ainsi, aux vues des études précédentes, la potentialité de biosorption des SPEs en fonction d’un métal semble très variable.

Tableau 1-4: Comparaison de capacité d'affinité des métaux par SPEs dans quelques études.

Métal	Adsorbent	Concentration initiale (mg/L)	Concentration finale (mg/L)	Capacité d’élimination (%)	Références
Ni	SPE de *Cloacibacterium* sp	48.0	7.0	85	Klai et al., (2016)
	SPE de *Pseudomonas* EJ01	125	85	28	Chien et al., (2013)
Fe	SPE de *Cloacibacterium* sp	14.2	4.0	71	Klai et al., (2016)
Zn	SPE de *Cloacibacterium* sp	17.4	5.5	65	Klai et al., (2016)
	SPE de *Pseudomonas* EJ01	11.0	8.8	20	Chien et al., (2013)
	Egg Shell	18.0	6.84	62	Shaheen et al., (2013)
Al	SPE de *Clocibacterium* sp	26.9	7.2	73	Klai et al., (2016)
Cu	SPE de *Clocibacterium* sp	26.0	16.5	36	Klai et al., (2016)
	Bassillus sp	2.88	1.6	44	Rani et al., (2010)
	Egg Shell	553	150	73	Shaheen et al., (2013)
	Chitosan	-	-	79	Sikder et al., (2014)
	SPE de *Sinorhizobium meliloti*	-	-	40	Hou et al., 2013

1.3 Les problématiques de cette étude

1.3.1 Les problèmes de déshydratation et décantation des boues

Selon les données de Recyc-Québec, (2009) et du ministère du Développement durable, de l'Environnement et des Parcs (MDDEP), 218 389 tonnes sèches ou 772 565 tonnes humides de boues municipales ont été générées en 2008. Plus de 82% de ces boues ont été éliminées, dont 58% par incinération et 24% par enfouissement. De 1998 à 2008, la production de ces boues n'a cessé d'augmenter, une situation qui nécessite la mise au point de procédés de traitement. La réduction de la production de boues à la source (station d'épuration) présente une étape primordiale et un intérêt économique. Il détermine un processus de gestion durable des matières résiduelles.

La déshydratation et la décantation des boues dans les usines de traitement des eaux usées (UTEU) présentent des problèmes rigoureux dus au phénomène de foisonnement en particulier qui s'expliquent par une présence excessive des organismes filamenteux et non filamenteux (Zoogloea) dans les boues (Martins et al. 2004; Peng et al. 2003). En effet, les microorganismes peuvent se développer selon trois types de croissance: (i) croissance dispersée dans le cas de procédé de boues activées (bactéries libres), (ii) croissance floculée dans le cas de biofiltration (bactéries regroupées en amas ou fixées sur un support organique ou minéral), et (iii) croissance filamenteuse (multiplication cellulaire incomplète). En effet, lors de la croissance filamenteuse, la séparation de cellules mères et filles n'a pas lieu en totalité, les cellules-mères et filles restent en contact, partage ainsi une paroi cellulaire forme un filament. Dans ce cas, les boues sont mal décantées à cause d'une mauvaise compaction des flocs.

De même, la production excessive de substances polymères extracellulaires (SPEs) produites par les souches bactériennes non filamenteuses peut être également responsable du phénomène de foisonnement. Elles sont capables de retenir l'eau grâce à leur composition chimique. Lajoie et al. (2000) n'ont observé qu'aucune corrélation entre les flocs obtenus de *Zoogloea* et la déshydratation des boues. En effet, une production élevée de SPEs microbiennes pouvant contenir plus de 90% d'eau est susceptible d'empêcher la déshydratation des boues (Houghton et al. 2001).

La présence des populations bactériennes libres ou fixées entraîne souvent une bonne décantation des boues. Ainsi, *Achromobacter, Alcaligenes, Arthrobacter, Citromonas, Flavobacterium, Pseudomonas* sont des bactéries qui jouent un rôle important dans la floculation et la décantation des boues. Par contre, la production abusive des SPEs peut provoquer une mauvaise déshydratation.

En plus, d'autres facteurs peuvent provoquer ce phénomène de foisonnement. Le fonctionnement d'une station d'épuration dépend souvent des différentes propriétés physicochimiques des boues (Liao et al. 2001; Vogelaar et al. 2005). L'excès de nutriments, le taux d'alimentation, le rapport C/N et la température sont des paramètres associés aux procédés de traitement. Ils affectent la croissance bactérienne ainsi que les caractéristiques et la structure de flocs, et par conséquent, limitent la floculation, la décantation et la déshydratation des boues.

1.3.2 Utilisation des polymères chimiques pour la floculation et la décantation des boues et d'autres applications industrielles

Des polymères chimiques ont été utilisés généralement pour la décantation et la déshydratation des boues dans le procédé de traitement des eaux usées. Les boues sont généralement chargées négativement. Une neutralisation de charge est nécessaire pour faciliter la floculation et la décantation des boues. Il utilise souvent des polymères cationiques synthétiques (CSP) ou des coagulants (Al^{3+}, Fe^{3+}) (Higgins et Novak, 1997a). Ces polymères sont actifs à différents pH et favorisent rapidement la formation de flocs. Néanmoins, ils sont coûteux (Wang et al. 2005; Chu et Lee 2001). En 2002, en province de Québec, 3 à 7 kg de polymères/tonne de boue ont été consommés pour traiter 218 000 tonnes de boues générées. Un coût allant de 7,6 à 10,6 millions $CAD/an est enregistré. Aux USA, la consommation de polymères était entre 25 et 50 million kg par an qui correspond à peu près 130 millions de dollars/an (Hébert, 2004).

De plus, les polymères synthétiques (PS) sont connus également pour leurs effets polluants pour l'environnement et leurs effets indésirables sur la population microbienne du sol. En effet, les boues après traitement sont souvent valorisées en agriculture (utilisées dans la fertilisation des terrains agricoles) (Chang et al., 2002). De ce fait, ils peuvent affecter la flore microbienne présente naturellement dans de nombreux environnements, tels que : le compost et les terreaux agricoles (Gagnon et Ziadi, 2004). Par conséquent, l'utilisation des PS pour la déshydratation des boues doit être réduite au maximum, afin de minimiser le coût de traitement des boues et atténuer leurs effets toxiques (Leem et Liu, 2000 ; Chang et al., 2002). L'alternative pour minimiser l'utilisation des PS est le recours à une approche biologique utilisant les biofloculants.

D'une part, plusieurs études (Zang et al., 2002; Deng et al., 2003) ont mis en œuvre l'isolement d'une série des microorganismes du sol producteurs de SPEs (*Rhodococcus erythropolis, Klebsiella pneumoniae, Bacillus firmus; Sorangium cellulosum et B. mucilaginosus*) à fin de les utiliser dans les procédés de biofloculation. De même, en 2004,

Kumar et ses collègues ont découvert des biofloculants produits par les espèces de *Bacillus* isolées à partir des échantillons d'eau de mer (Corée) (Kumar et al., 2004).
De même, BalaSubramanium et al. (2010), ont isolé une gamme de microorganismes à partir de boues secondaires. Ces microorganismes sont capables de croître dans les boues et produisent une quantité importante de SPEs. Des tests de floculation, et décantation de boues ont été évalués en montrant une décantation avec IVB 60 mL/g.

Des problèmes de déshydratation des boues chimiques (tannerie) ont été résolus par l'addition des boues activées à un rapport de 1:4 et de 1:8 (v/v) (Chang et al., 2001). Cette étude a montré que la déshydratation des boues chimiques pourrait être améliorée par l'ajout de boues activées grâce à la présence de substances polymériques extracellulaires (SPEs). Cette étude a révélé que les SPEs des boues sont impliquées dans le processus naturel de biofloculation. Dans ce contexte, More et al. (2012b), ont montré un taux de déshydratation de 52.2% dans la solution de kaolin, utilisant des SPEs capsulaires produites par les boues.
D'autre part, d'autres intérêts des SPEs ont été illustrés dans plusieurs études. Kaplan et al. (1987) ont montré la complexation des métaux lourds aux SPEs produites par *Chlorella* spp. Pinotti et al. (1997) ont, de leur côté, étudié le rôle de chitosane comme poly électrolyte dans la floculation de déchets d'industries alimentaires. De même, Mohan et al., (2002) ont analysé la coagulation et la biosorption de colorants azoïques en utilisant des espèces de *Spirogyra*. En 2003, Yoon et ses partenaires ont utilisé des floculants bactériens dans le but d'accroître les algues. Alors que les études de Natarajan et Das, (2003) sont basés sur l'utilisation d'*Acidithiobacillus* pour la floculation de minéraux.

D'autres recherches sont orientées sur les rôles des SPEs dans d'autres applications industrielles. L'utilisation de SPEs dans l'industrie laitière est basée principalement sur les capacités épaississantes, gélifiantes, stables et émulsifiantes de ces polymères. Ils jouent donc un rôle principal dans la formation de la texture des aliments. Par exemple, le curdlane produit par *Agrobacterium* et *Rhizobium* est utilisé au Japon pour améliorer et modifier la constitution des aliments comme les pâtes de poisson et les gels de fèves (Sutherland, 1998). Aussi, le pullulane est un polysaccharide, synthétisé par *Aureobasidium pullulans*. Il est utilisé comme film d'emballage alimentaire. Une solution du polymère peut être appliquée directement sur l'aliment et former une couche sans odeur et sans goût (Sutherland, 1998). Le xanthane est sans doute le polymère le plus fameux et connu parmi les SPEs commercialisés et il est accordé comme additif alimentaire aux États Unis et en Europe depuis plusieurs années. Il est produit par *Xanthomonas campestris*. Il est compatible avec la plupart des autres ingrédients alimentaires (les protéines, les lipides et les autres polysaccharides), peut être généralement utilisé dans une variété d'aliments. Il est utilisé

dans la fabrication de yaourt suite à leurs stabilités dans des conditions acides. Le dextrane est synthétisé par *Leuconostoc mesenteroides*. Il est appliqué dans la récupération assistée de pétrole, utilisé comme couche protectrice pour les graines de céréales et comme agent stabilisant des sirops (Cerning, 1995).

1.3.3 Utilisation des milieux synthétiques pour la production de SPEs

Pour la production à grande échelle de SPEs, une grande variété de milieux (sucrose, glucose, glycérol, saccharose, galactose, etc.) a été utilisée pour la croissance de différentes bactéries productrices des SPEs (Cerning et al., 1994). La source de carbone est l'un des facteurs majeurs qui peuvent influencer la croissance des microorganismes et par conséquent, la production de SPE. Néanmoins, le prix et la disponibilité de ces substrats peuvent affecter généralement le coût de procédé de production.
Différentes études (Cerning et al., 1994; Gamar et al., 1997) ont mis en œuvre l'impact de la concentration et le type de source de carbone utilisés pour la production de SPEs. En effet, la biosynthèse de SPEs par *Lb. delbrueckii ssp. bulgaricus* a été plus élevée dans un milieu avec du glucose ou du lactose que dans un milieu avec du fructose, alors qu'une supplémentation en mannose a résulté en une croissance bactérienne faible accompagnée d'une production minimale de SPEs (Grobben et al., 1996).

Cerning et al., (1994) ont étudié la production de SPE par *Lb. casei* en utilisant une gamme vaste de sources de carbone (galactose, glucose, lactose, saccharose, maltose,). Ils ont montré qu'avec le lactose et le galactose, la production de SPE a été la plus faible, tandis que, le glucose a présenté la source la plus efficace. Dans le cas de *Lb. rhamnosus*, le mannose ou la combinaison de glucose et de fructose ont simulé la production de SPE (Gamar et al., 1997). En fait, l'utilisation de ces milieux de culture en milieu industriel est limitée surtout par le coût élevé. Des recherches ont été déclenchées, afin de mettre au point des milieux de culture économiques et capables de soutenir la croissance des bactéries ainsi que la biosynthèse des SPEs.

La valorisation des déchets constitue une solution bénéfique pour assurer un environnement de qualité. Ainsi, le défi pour les usines de traitement des eaux usées est de trouver des moyens pour réduire les coûts de la disposition des rejets. Le traitement et la gestion des boues constituent une part importante de ce défi. L'augmentation de la production des boues a fourni une raison importante pour intensifier les recherches sur leur disposition finale. Généralement, les boues provenant du traitement des eaux usées industrielles et municipales peuvent être utilisées pour le compostage. Elles contiennent certains éléments nutritifs utiles à la croissance des microorganismes, dont l'azote, le carbone, le phosphore, le potassium et le magnésium. Il est toutefois possible d'exploiter la richesse des boues en

source d'énergie peu coûteuse utilisables pour la croissance des microorganismes particulièrement celles productrices des SPEs. La valorisation des boues d'épuration municipales et industrielles en les utilisant comme milieu de culture pour la production de SPEs ; peuvent être présentées. Un nombre restreint d'études (BalaSubramanian et al., 2010; More et al., 2012a) ont mis en évidence l'utilisation de boues comme matière première et source de carbone pour la production de SPEs à partir de culture pure ou mixte. Par contre, les résultats ont montré des faibles concentrations obtenues des SPEs (1,5 – 4,9 g/L) en comparaison aux autres études utilisant les milieux synthétiques (8 – 25 g/L).

Toutes ces études nous amènent à optimiser ces concentrations obtenues dans les boues soit par d'autres souches isolées productrices des SPEs ou par ajout des sources de carbone supplémentaires de faible coût.

1.3.4 Les rôles de méthodes d'extraction sur les propriétés des SPEs la floculation et décantation des boues.

Plusieurs facteurs peuvent affecter la biofloculation, tels que : la concentration, la charge à la surface, l'hydrophobicité des SPE et leurs propriétés fonctionnelles ainsi que leurs méthodes d'extraction. Il a été constaté que les SPEs pouvaient être sécrétées avec des concentrations élevées au cours du procédé de production des boues activées. La quantité de SPEs dans les boues varie particulièrement en fonction de la nature et l'origine des boues, de l'espèce bactérienne ainsi que de la méthode d'extraction utilisée. Une comparaison des données de la littérature des rendements en SPEs extraites par ces différentes méthodes doit être faite avec précaution, car des techniques d'extraction différentes ont été exploitées. Ceci peut avoir une forte influence sur les résultats. De nombreuses méthodes ont été développées pour extraire les SPEs des agrégats biologiques de boues activées, de biofilms ou de cultures pures. Ainsi, des techniques plus ou moins efficaces, comme la centrifugation à haute vitesse (Novak et Haugan, 1981), l'utilisation de l'ultrasonication (Urbain et al., 1993a), le chauffage (Zhang et al., 1999), mais également des méthodes chimiques comme l'ajout d'EDTA (Liu et Fang, 2002), de soude (NaOH), de formaldéhyde (Azeredo et al., 1998), des résines échangeuses de cations (Nielsen et jahn, 1999) ont été développées. D'autres méthodes associent des méthodes physiques et chimiques comme les ultrasons avec une résine échangeuse de cations (Cadoret et al., 2002), la centrifugation et l'utilisation de l'éthanol (Wase et Balasundaram, 1980) ou l'ajout de formaldéhyde et de soude (Liu et Fang, 2002). En terme de coût, ces méthodes sont variées selon le coût de réactifs et l'appareil utilisé ainsi que le temps d'extraction. Ces paramètres sont très importants et peuvent influencer le coût de la production de SPEs et devraient être tenus en compte afin de sélectionner les meilleurs techniques d'extraction.

L’extraction des SPEs est une étape indispensable. Toutefois, le manque de protocole d’extraction standard pose le problème de la comparaison des résultats des différents travaux de la littérature. À l'heure actuelle, aucune méthode globale n'est reconnue par les chercheurs (Nielsen et Jahn, 1999) et, quelle que soit la méthode d'extraction, le rendement d'extraction des SPEs n'est malheureusement jamais déterminé. Des estimations ont été tentées pour déterminer ce rendement d'extraction en se basant par exemple sur la quantité totale de protéines mesurée dans les SPEs et la quantité théorique de protéines bactériennes (Nielsen et Jahn, 1999). Cette approche reste difficilement applicable, car le volume des bactéries et la quantité de protéines et de polysaccharides dans une bactérie varient au cours du temps, en fonction des conditions de culture et de la souche bactérienne. De fait, la quantité de polymères extraits dépend fortement de la méthode d'extraction engagée.

De plus, il est impossible d'apprécier correctement si les SPEs sont contaminées par des composants du domaine intracellulaire libérés par la lyse cellulaire. La lyse bactérienne est importante à déterminer, car elle induit directement une surestimation des SPE en comptabilisant des constituants intracellulaires.

La majorité des études (Liu et al., 2002; Comte et al., 2006) se sont basées sur le choix d’une méthode d’extraction avec un minimum de lyse cellulaire, un rendement d’extraction élevée et sans contamination apportée par les réactifs utilisés. Ces études ont illustré l’effet des méthodes d’extraction sur la concentration et la composition de SPEs et n’ont pas évalué leurs potentialités dans la biofloculation. Les différentes molécules composantes les SPEs ont des structures variées et portent divers groupements fonctionnels et différents poids moléculaires qui leur confèrent des propriétés physico-chimiques et biologiques. Dans ce contexte, la lyse cellulaire peut permettre d’extraire des polymères intracellulaires comme (ARN, ADN et les acides nucléiques) qui peuvent avoir les mêmes propriétés et des rôles similaires dans la biofloculation aux SPE (Wilen et al., 2003).

1.4 Les hypothèses

1.4.1 Hypothèse I : Gestion et valorisation des boues

La valorisation des résidus et des déchets pose actuellement un grand défi. Les boues d'épuration constituent un exemple de ses résidus dont leur gestion et leur disposition constituent un majeur défi auquel doit faire face la société. Plusieurs travaux sont dirigés vers la production des fertilisants ainsi que la production des biopesticides, des biofertilisants, et du bioplastique. Ces travaux portant sur la production de ces produits à valeur ajoutée offre une nouvelle voie pour la production de bio polymères à partir des boues. Les boues activées sont des sources potentielles de microorganismes et de substances polymériques extracellulaires (SPEs). Ces derniers sont impliqués dans la décantation et la déshydratation naturelles des boues. Donc, l'extraction et la production de SPEs produites à partir des nouvelles souches bactériennes isolées à partir de boues pourraient aboutir à une biofloculation améliorée ou de même efficacité par rapport à celle observée dans les boues activées en réduisant ainsi l'utilisation de polymères chimiques toxiques et le coût de traitement.

1.4.2 Hypothèse II : Gestion et valorisation du glycérol brut

La production de SPEs dépend des micro-organismes et des sources de carbone. Les boues activées sont une source de carbone, d'azote, du phosphore et de nombreux autres éléments nutritifs nécessaires à la croissance des micro-organismes et au processus de production de SPEs. Aujourd'hui, l'intérêt pour le carburant biodiesel a entraîné la production de grandes quantités de piètre qualité, le glycérol comme sous-produit majeur qui peut être utilisé dans plusieurs applications. Une autre approche pour la revalorisation de glycérol brut peut être envisagée durant cette étude étant donné que ce substrat peut être utilisé comme source de carbone et d'énergie. Par conséquent, l'utilisation de glycérol brute comme substrat peut diminuer d'une part le coût de la fermentation et d'autre part, elle peut améliorer la production de SPEs.

1.4.3 Hypothèse III: Optimisation de méthodes d'extraction

La composition et la concentration des SPEs sont variables suivant la biomasse d'origine, le substrat, l'espèce, mais aussi suivant le protocole utilisé pour les extraire de ces biomasses. En effet, il n'existe pas une méthode standard d'extraction des SPEs, d'où la difficulté de comparer les résultats obtenus avec la littérature. De plus, le rendement et la nature des EPS extraites dépendent pleinement de la méthode utilisée. Par conséquent, pour départager les protocoles d'extraction, la détermination de la quantité des SPEs obtenue, leur composition, leurs propriétés de floculation comme reflet de leur dispersion et la lyse

cellulaire pourraient contribuer à obtenir des échantillons de SPEs suffisantes en termes de quantité et de qualité.

1.4.4 Hypothèse IV : Accumulation de lipides : Cloacibacterium normanense Est-il une candidate potentielle ?

Selon la littérature, les résultats illustrent bien le fait que les cinétiques de formation des produits peuvent bien être associées de façon positive que de façon négative avec la croissance cellulaire, voire même avec une combinaison des deux. De ce fait, dans les cas où la formation des produits n'est pas corrélée avec une croissance cellulaire élevée, il est judicieux de maîtriser le taux de croissance cellulaire. En général, la croissance bactérienne est contrôlée par la maîtrise des conditions de fermentation (Température, pH...) et surtout par l'apport en source de carbone et d'azote. Il est important de souligner que la production de SPEs est favorisée lors d'un rapport carboné et azoté suffisant. Contrairement, il est ainsi connu que l'accumulation de lipides se produit lors d'un apport carboné suffisant, mais dans des conditions de carence en azote. Cela suppose que la variation du rapport carbone /azote au cours de la fermentation peut favoriser la production de SPE lorsqu'il est faible comme il peut stimuler l'accumulation de lipides lorsqu'il est élevé. Par conséquent, on choisit d'adapter la source carbonée et d'azote à la bactérie étudiée en fonction du métabolite produit. Ainsi, le développement de procédé de production de SPEs par *Cloacibacterium* sp, dont la capacité d'accumulation de lipide serait amplifiée, constitue un élément clé pour valoriser plus efficacement le procédé étudié.

1.5 Objectifs de recherche

1.5.1 Objectif global

Développement de procédé de production de biofloculants à partir de cultures pures dans les boues secondaires (unité de biofiltration), utilisant le glycérol brut comme source de carbone supplémentaire et évaluation de différentes méthodes d'extraction des SPEs obtenues pour la biofloculation, la décantation de boues et dans des autres applications (l'élimination de métaux lourds).

1.5.2 Objectifs spécifiques

Pour répondre à cet objectif général, nous avons développé une approche subdivisée en cinq parties : le premier objectif est d'isoler et identifier des souches bactériennes productrices des SPEs à partir de boues secondaires. La détermination de la concentration de SPEs et leurs caractérisations chimiques permettra de sélectionner la meilleure souche bactérienne à étudier. Le second objectif est d'optimiser les conditions de fermentation, à l'échelle de banc d'essai en laboratoire, par une évaluation de différents facteurs opérationnels (pH, Température, Agitation, source de carbone) dans le but d'atteindre les meilleures conditions d'un procédé de production développé. Dans cette optique, le glycérol brut à différentes concentrations a été utilisé pour améliorer la production de SPEs en déterminant l'effet de ses composées à savoir le méthanol, le savon, les acides gras libres, etc. Le troisième objectif vise l'impact de différentes méthodes d'extraction sur la composition chimique et la potentialité de biofloculation de SPEs extraites par *Cloacibacterium normanense* et de proposer un meilleur protocole d'extraction. Le quatrième objectif est de caractériser la structure chimique des SPEs par IRTF (spectroscopie infrarouge à transformée de Fourier). Le cinquième objectif est de valider les performances et les potentialités des SPEs produites sur la biofloculation, la décantation des boues et sur l'élimination de métaux lourds à partir d'eaux usées municipales.

1.6 Originalité de la recherche

L'originalité de cette étude se situe à plusieurs niveaux. Si le procédé de production des biopolymères par divers microorganismes (bactéries, levures) dans différents milieux nutritifs a été étudié par plusieurs auteurs, la combinaison de boues secondaires et le glycérol brut comme milieu de culture et la découverte de nouvelle souche bactérienne productrice de SPE à forte concentration n'a pas été amplement utilisée et constitue la pierre angulaire de ce projet de recherche. L'intérêt de développer un nouveau procédé de production de substances polymériques extracellulaires profitant de *Cloacibacterium normanense* comme majeure candidate réside dans sa capacité de synthèse de deux métabolites : de produire les SPEs et d'accumuler les lipides dans les boues ainsi que la potentialité des SPEs dans la décantation et floculation de boues. Bien que des études récentes aient exploité la production de SPEs dans les boues ou des déchets industriels, ce procédé a contribué une production simultanée avec une technologie économiquement viable.

1.7 Démarche méthodologique

Les premiers travaux ont consisté en l'isolement et l'identification de nouvelles souches productrices de SPEs à partir de boues biologiques. Par la suite la performance des souches isolées a été évaluée par la forte concentration de SPEs produites et leurs potentialités de floculation. La partie suivante portait sur l'optimisation du procédé de production de biopolymères mettant en synergie la source de carbone et les conditions de fermentation. Subséquemment, des méthodes d'extraction des SPEs ont été comparées en choisissant le meilleur protocole grâce à une stratégie basée sur un rendement d'extraction élevé, un minimum de lyse cellulaire et sans contamination apportée par les réactifs utilisés. Par la suite, la bactérie étudiée a été testée pour l'accumulation des lipides en prenant compte l'apport carbone /azote au cours de la fermentation. Finalement, dans l'optique d'évaluer la performance de ce procédé, les SPEs ont été testées pour la floculation, la décantation, la déshydratation des boues et pour l'élimination de métaux lourds couramment rencontrés dans les eaux usées municipales en estiment aussi bien le coût de ce procédé de production. Une description schématique de la démarche méthodologique suivie est présentée à la Figure 1-7.

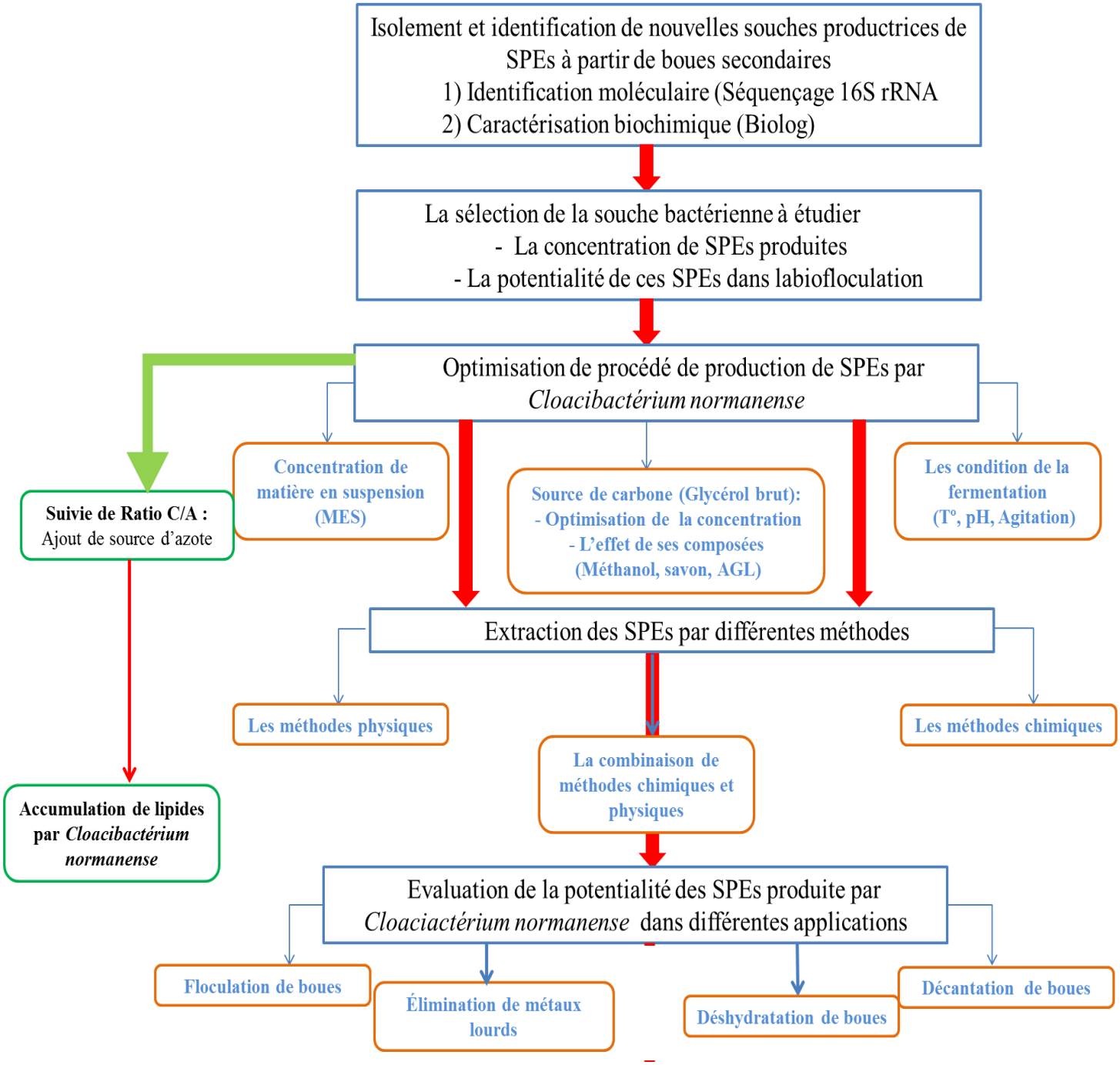


Figure 1-7 : Description schématique de la démarche méthodologique suivie

1.7.1 Les souches bactériennes étudiées

1.7.1.1 Screening des souches productrices de SPEs

Plusieurs méthodes simples et rapides existent pour déterminer la capacité d'une souche bactérienne à produire des SPEs. L'observation du phénotype des colonies cultivées sur boîte de Pétri donne déjà une information. Les souches productrices de SPEs forment des colonies brillantes, bombées et visqueuses ; on dit alors qu'elles ont un phénotype mucoïde (Dupont, 1998). L'une des caractéristiques de ce phénotype est que lorsque l'on touche et étire la colonie avec un cure-dent, celle-ci forme un long filament (Figure 1-8). Dans ce contexte, les bactéreis ont été isolées par selecton

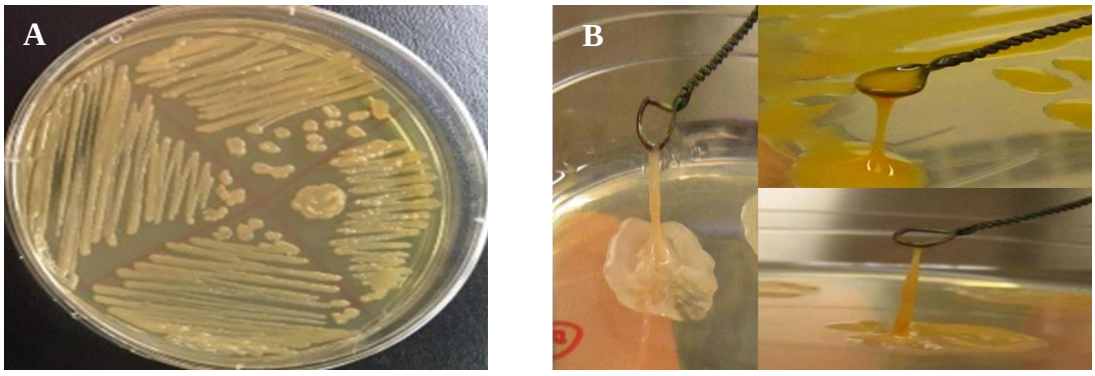
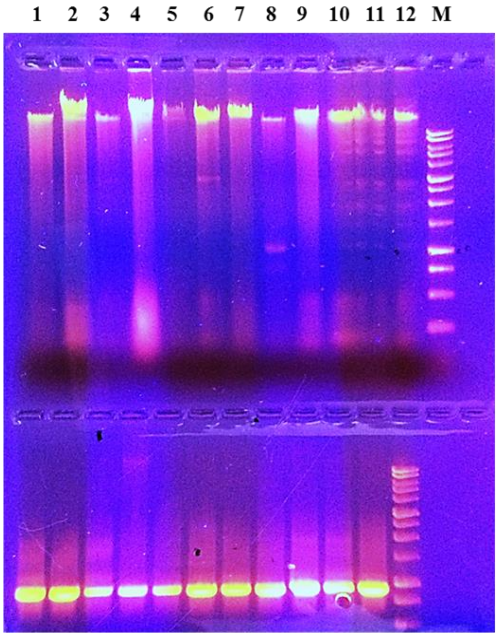


Figure 1-8: Photos de colonies mucoides. A) colonies bactériennes ayant un aspect mucoide. B): filament formé au contact d'une anse avec une colonie bactérienne mucoide.

1.7.1.2 Identification moléculaire et caractérisation biochimique des souches bactériennes isolées à partir de boues secondaires

Des échantillons de boues secondaires de la station d'épuration (CUQ, Québec) ont été collectés pour isoler des nouvelles souches bactériennes productrices de SPEs. L'isolement a été réalisé par épuisement, en étalant l'échantillon des boues après une dilution dans Nacl 10^3à 10^5) à la surface d'un milieu solide approprié. Elles ont été identifiées par la suite par 16S rDNA séquençage. Les bactéries isolées ont été identifiées par le système BIOLOG utilisant de plaques GEN III. Elles permettent de vérifier simultanément la réaction métabolique des bactéries en regard de 94 réactions phénotypiques (71 sources de carbone et 23 tests de sensibilité aux inhibiteurs chimiques). À la suite de l'inoculation d'une plaque GEN III avec une bactérie inconnue, un profil métabolique est obtenu. L'inoculation de la plaque GEN III se fait en déposant 100 µl de la suspension bactérienne dans chacun des puits. L'incubation se fait à 30°C pour une durée de 24 à 48 heures (Figure 1-9).

A)



1-12 Genomic DNA/PCR amplica, M 1Kb DNA Marker

Genomic DNA isolation –via Qiagen DNA extraction kits

PCR

16S rRNA primers

9F: 5'-gagtttgatcctggctcag-3'
1512R: 5'-acggtaccttgttacgac-3'

Reaction mix

Genomic DNA	-1µL
Buffer 5X	-5µL
dNTP	-0.75µL (10µM)
FP	-1µL (0.4µM)
RP	-1µL (0.4µM)
$MgCl_2$	-1µL (10µM)
Taq DNA polymerase	-0.02 (1U)
MilliQ Water	-15.23µL
Total	-25µL

Reaction Condition
95° C for 5 minutes
95° C for 30 seconds
51° C for 30 seconds
72° C for 1 minutes 30 seconds
72° C for 10 minutes

B)

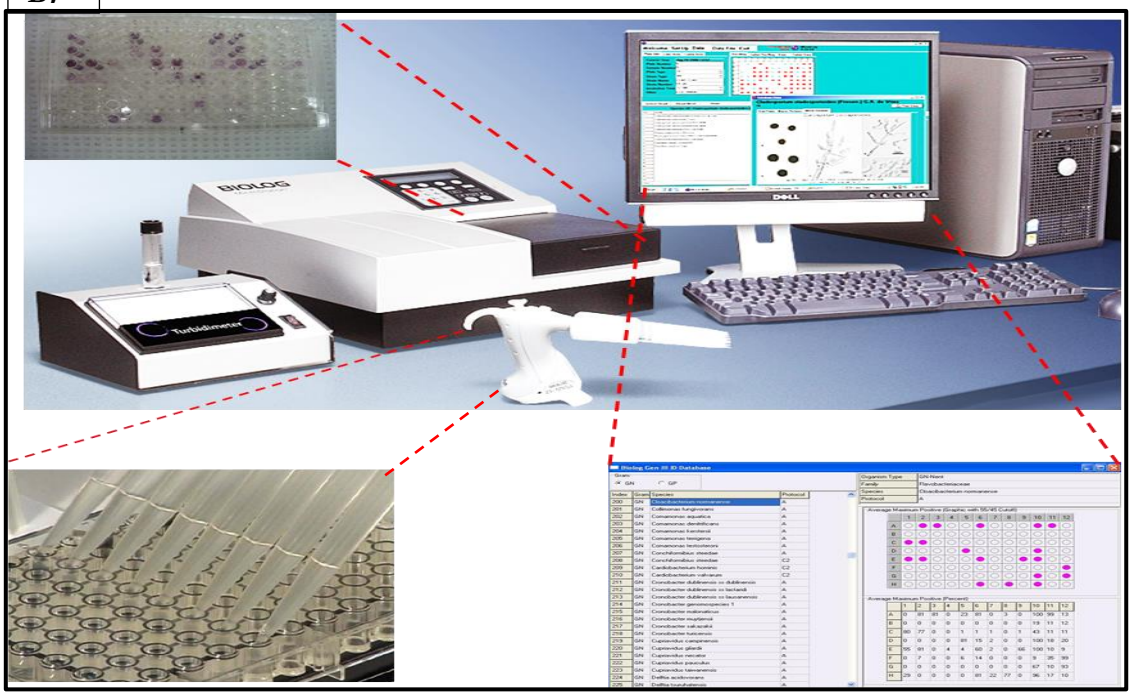


Figure 1-9 : A) Identification moléculaire des souches bactériennes par séquençage 19S rRNA et B) Caractérisation biochimique par le test Biolog.

1.7.2 Milieux de culture

Des boues activées ont été échantillonnées de l'unité de biofiltration de la station d'épuration (CUQ, Québec). Ces effluents proviennent de la station municipale des eaux usées de la ville de Québec. La filière de traitement de cette station se compose d'un prétraitement, d'un traitement primaire caractérise par une décantation gravitaire suivie d'un traitement secondaire par biofiltration. La Figure 1-10 présente une description détaillée de la filière de traitement secondaire des eaux usées de la station municipale de la ville de Québec. Les effluents de l'unité de biofiltration ont été utilisés comme milieux de culture et une source de carbone pour la production de SPEs après les concentrer à une concentration de 25 g/L. Les effluents échantillonnés se caractérisent par une matière en suspension (MES)= 120 mg/L, une DCO= 250 mg/L, une DBO5= 110 mg/L , un COT=80 mg/L un azote total (NT)= 20 mg/L et un pH =6,9.

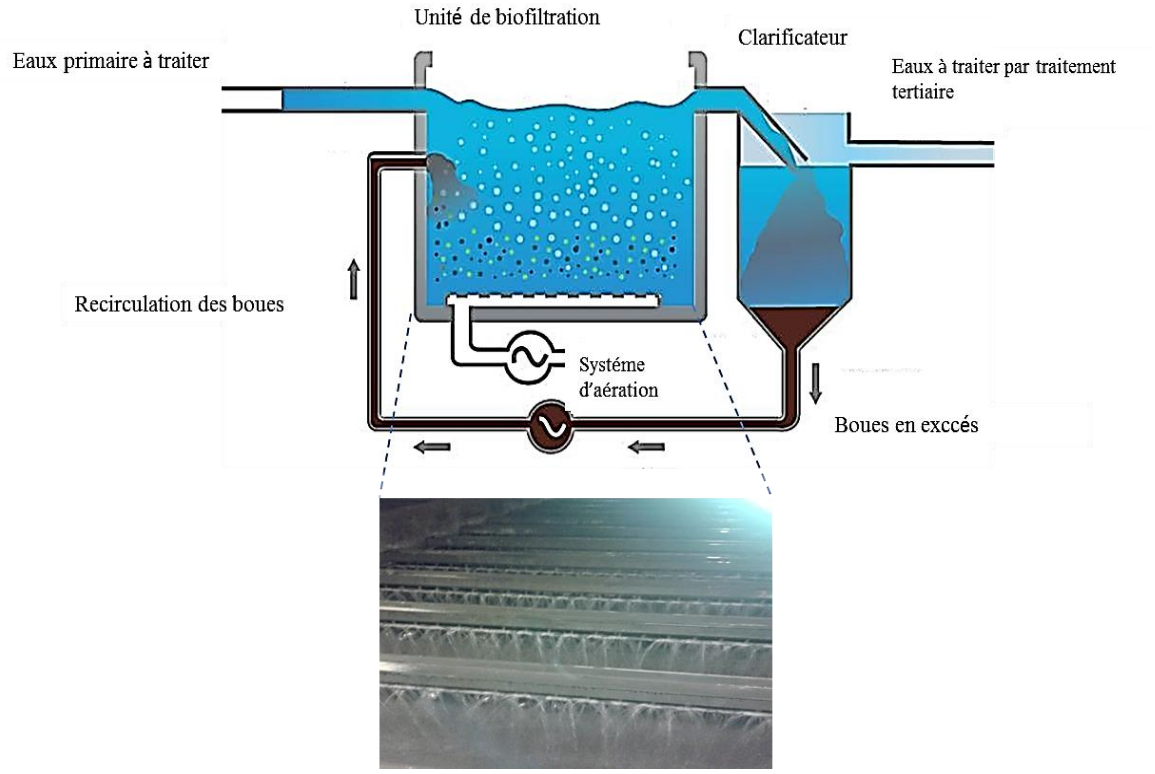


Figure 1-10 : Unité de biofiltration de traitement biologique des eaux usées de la station d'épuration de la ville de Québec (Secteur EST), Canada.

1.7.2.1 Préparation de milieux de culture pour la fermentation

- Production de SPEs

La boue a été prétraitée par une stérilisation à 120 °C pendant 15 min. Le pH a été maintenu à 7. Huit bactéries isolées ont été cultivées dans la boue secondaire stérilisée utilisant 3% (v/v) d'inoculum. Le temps de la fermentation est 96 h. Un prélèvement a été effectué chaque 12 h pour le dénombrement (UFC/mL) et chaque 24 h pour estimer la concentration de SPEs produites. Selon les résultats obtenus dans cette étude, *Cloacibacterium normanense* a été sélectionnée pour développer le procédé de production. Une optimisation de conditions de fermentation (concentration de la matière en suspension, taille d'inoculum, T°, agitation, temps d'incubation) a été effectuée. Le glycérol brut a été additionné à différentes concentrations (0.5 ; 1 et 2 % (v/v)) comme une source de carbone supplémentaire pour améliorer le procédé de production des biopolymères. Une description détaillée de procédé de production est présentée dans la Fiagure 1-11.

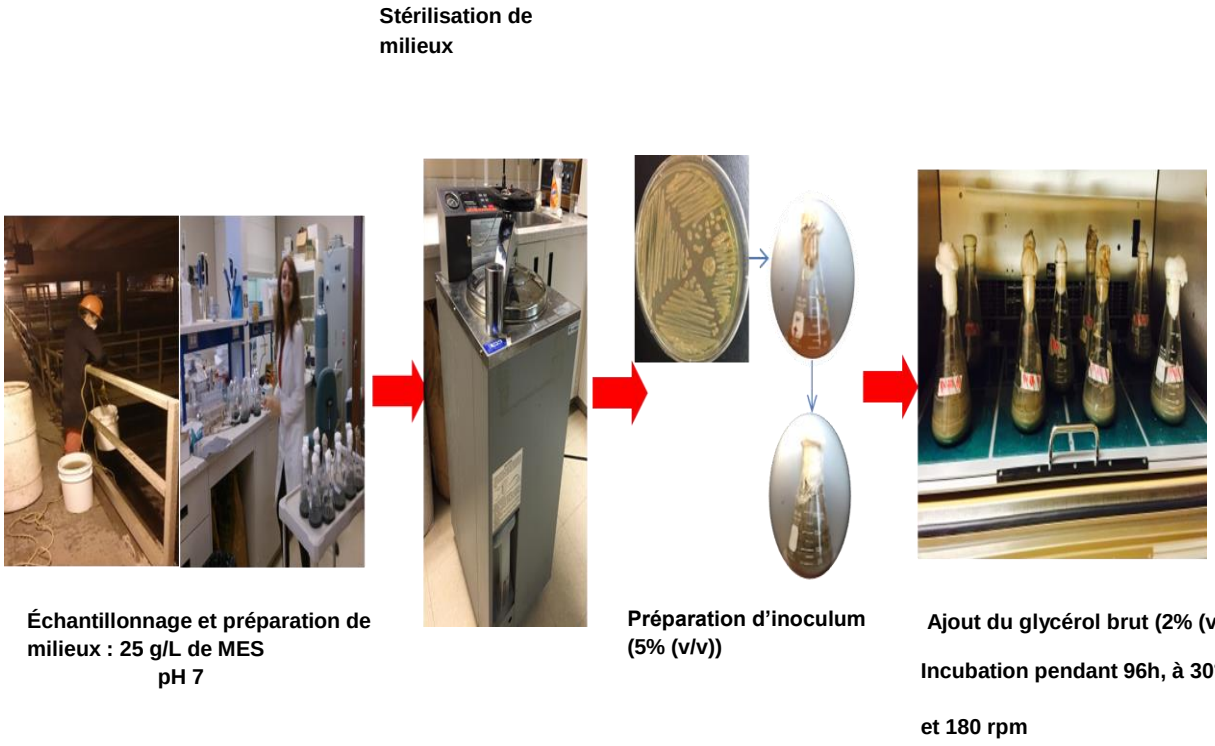


Figure 1-11: Description du procédé de production de SPEs par *Cloacibacterium normanense* dans les boues secondaires suivie.

❖ Accumulation des lipides

Le même procédé de production de SPEs a été suivi que pour l'accumulation des lipides. Dans cette étude, la peptone a été ajoutée à différentes concentrations comme une source d'azote pour varier le rapport C/N et favoriser l'accumulation des lipides. 18, 25, 30, 50 et 100 sont les C/N utilisés. Le glycérol brut utilisé contient des concentrations importantes de savon, méthanol et acides gras libres (AGL). Cependant, le rapport C/N désiré a été déterminé en prenant compte de ces composants. Tableau 1-5 et Tableau 1-6présentent la composition chimique du glycérol brut employé et les mesures de C/N mises en jeu.

Tableau 1-5: La composition chimique du glycérol brut

Paramètres	**Poids (w/v) %**
Densité	0.845
Glycérol	13.4
Cendre	1.5
Savon	23.5
Méthanol	4.6
Autres	57.0
pH	6.8

Tableau 1-6: Les conditions de fermentation pour l'accumulation de lipides et la production de SPEs en mettant en jeu le ratio C/N.

	C/N 18			C/N 25			C/N 30			C/N 50			C/N 100		
	Savon	**Glyc**	**MeOH**	**Savon**	**Glyc**	**MeOH**	**Savon**	**Glyc**	**MeOH**	**Savon**	**Glyc**	**MeOH**	**Savon**	**Glyc**	**MeOH**
Les composés du glycérol brut dans glyc 44.77 mL (g/L)	10.5	6.0	2.0	10.5	6.0	2.0	10.5	6.0	2.0	10.5	6.0	2.0	10.5	6.0	2.0
Gramme de Carbone	7.8	2.3	0.8	7.8	2.3	0.8	7.8	2.3	0.8	7.8	2.3	0.8	7.8	.2.3	0.8
Gramme de Carbone Totale		10.9			10.9		10.9				10.9			10.9	
Teneur en Azote Peptone (g/150 mL)	6.04			4.3			3.6			2.2			1.1		
Concentration initiale des composes de glyc (g/L)	70.0	40.0	13.6	70.0	40.0	13.6	70.0	40.0	13.6	70.0	40.0	13.6	70.0	40.0	13.6

Note : Glyc =Glycérol

MeOH : Méthanol

1.7.3 Méthodes d'Extraction

1.7.3.1 Extraction de SPEs

Suite à une étude bibliographique des techniques d'extraction des SPEs existantes, 10 protocoles couramment utilisés ont été sélectionnés : 3 méthodes physiques, dont une méthode de « contrôle », 2 méthodes chimiques et 5 combinaisons de méthodes physiques et chimiques. Chaque protocole a été réalisé en duplicats pour chaque boue.

1.7.3.1.1 Extraction physique

Trois protocoles d'extraction physiques ont été testés :

- Extraction par centrifugation : centrifugation à 6000 g, 4°C pendant 20 min pour extraire les S-SPEs, et une étape de chauffage à 60°C pendant 15 min à fin d'extraire de C-SPEs (Wingender et al., 1999).

- Chauffage : Les échantillons ont été chauffés pendant 20 min à 80°C dans le bain marin (Comte et al., 2006).

- Sonication : les échantillons ont été traités par sonication à 40 W pendant 2 min utilisant Utrasonic processor –Cole Parmer (Comte et al., 2006).

1.7.3.1.2 Extraction chimique

Deux protocoles d'extraction chimique ont été testés :

- EDTA de 20 g/L a été ajouté dans 70 mL d'échantillon. L'échantillon a été incubé pendant 3 h à 4°C (Liu et al., 2002).

- Formaldéhyde + NaOH : 0,4 ml of formaldéhyde (368 g/L) est additionné dans 70 ml de boues fraîches et incubées pendant 1 h à 4°C. Après une heure d'incubation, 3 mL de NaOH de 10 M est ajouté et incubé pendant 3 h à 4°C (Liu et al., 2002).

1.7.3.1.3 Combinaison physique et chimique

Une combinaison de méthodes d'extraction chimiques et physiques a été aussi testée.

- Formaldéhyde suivi de chauffage : 0,4 ml of formaldéhyde (368 g/L) est additionné dans 70 ml de boues et incubées pendant 1 h à 4°C. Après une heure d'incubation, Le mélange est chauffé au bain-marie 10 min à 80 °C.

- Formaldéhyde suivi de sonication et chauffage : 0,4 ml of formaldéhyde (368 g/L) est additionné dans 70 ml de boues et incubés pendant 1 h à 4°C. Après une heure d'incubation, l'échantillon est traité par sonication à 40 W pendant 2 min puis il a été chauffé au bain-marie 20 min à 80 °C (Comte et al., 2006).

- Formaldéhyde suivi d'EDTA : 0,4 ml of formaldéhyde (368 g/L) est additionné dans 70 ml de boues et incubés pendant 1 h à 4°C. Après une heure d'incubation, 5 g/L d'EDTA a été ajouté dans l'échantillon et a été incubé pendant 3h à 4°C (Liu et al., 2002)

- Formaldéhyde suivi d'une sonication et EDTA : 0,4 ml of formaldéhyde (368 g/L) est additionné dans 70 ml de boues et incubés pendant 1 h à 4°C. Après une heure d'incubation, l'échantillon est traité par sonication à 40 W pendant 2 min et par 5 g/L d'EDTA pendant 3h à 4°C (Comte et al., 2006).

- Formaldéhyde, sonication et chauffage : 0,4 ml of formaldéhyde (368 g/L) est additionné dans 70 ml de boues et incubés pendant 1 h à 4°C. Après une heure d'incubation, l'échantillon est traité par sonication à 40 W pendant 2 min et chauffé au bain-marie 20 min à 80 °C (Comte et al., 2006).

Après les différentes étapes d'extraction, toutes les SPEs (biofloculants (BF)) ont été récupérées par centrifugation (6000g pendant 20 min à 4°C).

L'étape de centrifugation est commune à tous les protocoles et permet de séparer les SPEs (Slime et capsulaires) de la biomasse. Le bouillon fermenté est centrifugé avec 6000 g pendant 20 min à 4°C pour obtenir les SPEs solubles (Slime –SPEs). Le culot a été redissous dans un volume initial de l'eau distillée, et chauffé dans un bain-marie pendant 15 min à 60°C déterminant ainsi les SPEs capsulaire (C-SPEs) (Wingender et al., 1999; Yang, 2007). Le surnagent extrait que ce soit pour les S-SPEs et les C-SPEs est précipité dans deux volumes d'éthanol (2 :1) et incubé à – 20°C pendant 24 h (APHA, 2005).

1.7.3.2 Extraction de lipides

Initialement, les cultures cellulaires sont centrifugées à 5000 g pendant 10 min, après élimination du surnageant, les suspensions cellulaires sont lavées deux fois avec l'eau distillée. Le prérequis pour l'extraction de lipides est qu'il existe deux types de lipides : les lipides polaires et les lipides neutres. L'utilisation de solvants de différentes polarités est donc nécessaire. Parmi les méthodes couramment utilisées, méthode de Folch, (1957) modifié est procédé durant le travail qui permet d'extraire la totalité des lipides (neutres, phospholipides, glycolipides) grâce à une mixture du chloroforme et méthanol (2 :1). En effet, les différents lipides sont connus par leur variation de solubilité dans les solvants d'où la variation de la proportion méthanol et chloroforme pour extraire la totalité des lipides. Le chloroforme est utilisé pour les composés organiques non polaires et polaires comme les triglycérides TG alors que le méthanol est couramment utilisé pour les composés organiques polaires comme phospholipides. L'extraction est répétée 2 fois avec la proportion chloroforme et méthanol (2:1) et (1 :1). Trois différents surnageant sont récupérés.

L'extraction est menée dans des tubes pyrex placés dans une agitation douce pendant 12h. On a ajouté dans cette tape les billes de zirconium de silicate pour la lyse cellulaire comme une fore mécanique. Après, le solvant contenant l'extrait lipidique est récupéré suite à une centrifugation (1500 rpm, 20 min, 4oC). Les tubes sont ensuite pesés pour avoir le contenu lipidique à 60°C. Figure 1-12 présente la méthodologie d'extraction des lipides.

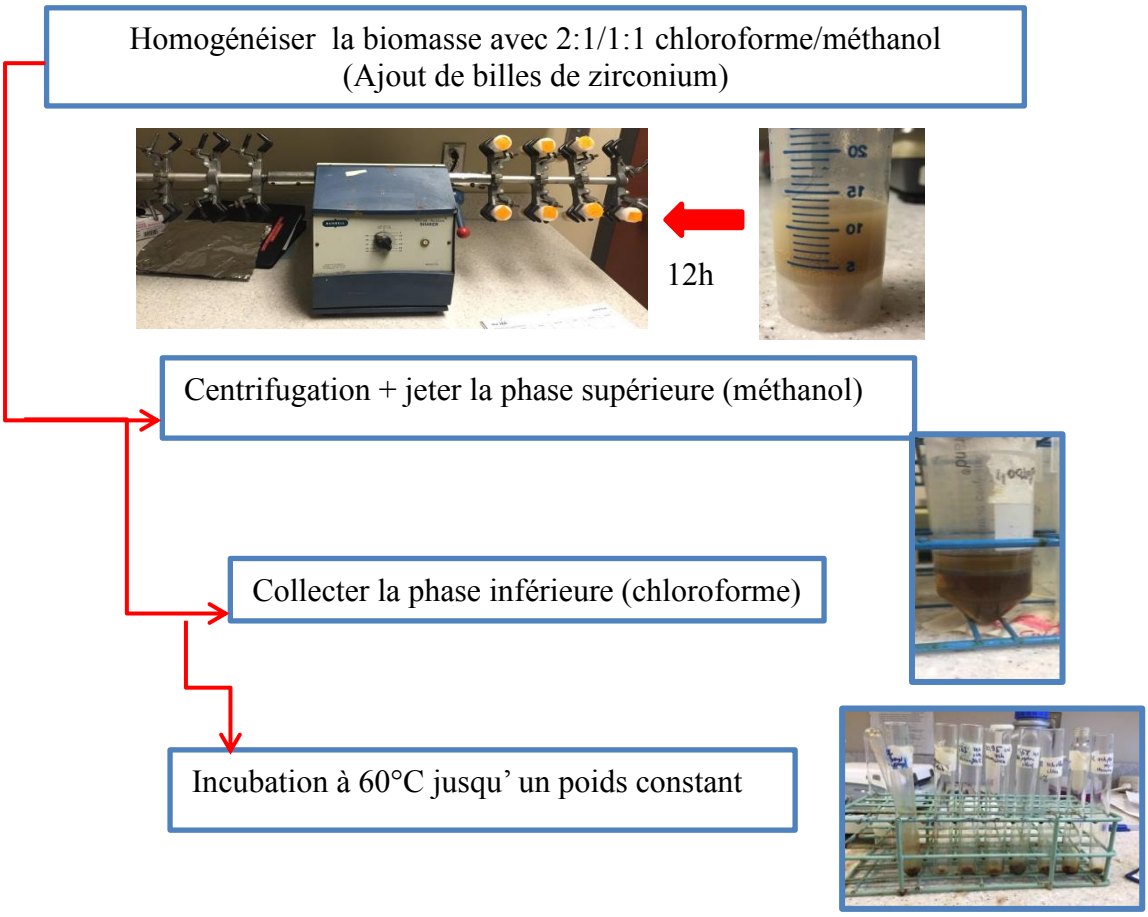


Figure 1-12: Description schématique de la méthodologie d'extraction de lipides.

1.7.4 Méthodes analytiques

1.7.4.1 Mesure des paramètres opératoires

Lors de différentes expérimentations, des mesures de pH, de biomasse, de dénombrement microbiologique (UFC/mL) ont été effectuées afin de suivre le bon fonctionnement du processus de production, de comprendre les différents mécanismes mis en jeu et d'établir certaines conclusions en terme d'efficacité de production. Le pH a été déterminé en utilisant un pH-mètre (Fisher Acumet, modèle 915) équipé d'une électrode Cole palmer à double jonction (référence Ag/AGgCl).

La biomasse sèche (notée X), exprimée en g/L, est mesurée à l'aide de la méthode gravimétrique (Rodier, 1975). Un volume précis de la culture cellulaire est préalablement filtré à l'aide d'une pompe à vide sur une membrane, ensuite placée à l'étuve à 105 pendant 24 heures. La masse de la culture cellulaire est égale à la différence de masse du filtre avant et après filtration.

La croissance bactérienne était suivie au cours du temps par mesure de dénombrement des bactéries (UFC/mL). Pour cette expérience, les cultures ont été réalisées dans des Erlenmeyers de 250 ml contenant 50 mL de bouillon fermenté. Les mesures ont été faites toutes les 6 heures. Ainsi, 0,5 mL de culture a été collecté et mélangé à une solution de NaCl 0,85 % (v/v). Plusieurs dilutions en cascade au 1/10ème ont été faites dans des tubes à essai puis 100 µL de chacune de ces dilutions ont été déposés sur milieu gélosé TSA en boîtes de Pétri. Après 24 à 48h d'incubation à 30°C, les colonies (UFC) ont été comptées. Ces mesures ont été répétées trois fois.

1.7.4.2 Mesure de composées de glycérol brut

- Mesure de glycérol

Le glycérol est mesuré au cours de la culture par une méthode spectrophotométrique (Bondioli et Bella, 2005; Lee et Park, 2006). Son principe repose sur la détection de formaldéhyde généré par une réaction entre le glycérol et le periodate de sodium et l'acétyle acétone donnant naissance au 3,5- diacetyl-1,4 dihydrolutidine ayant une coloration jaunâtre mesurée à 410 nm (Bondioli et Bella, 2005).

- Mesure de savon

La détermination de la concentration de savon a été effectuée en suivant une méthode d'acidification utilisant la solution acide 85% H_3PO_4. 50 g d'échantillon a été ajusté à pH égal à 1. L'échantillon est centrifugé à 5000 g pendant 15 min. La phase supérieure corresponds aux acides gras libres a été collectée en mesurant ainsi le poids enregistré de savon (Liang et al. 2010). La concentration de savon est calculée suivant l'équation suivante :

Savon % (w/w)= (304*M/282)/Wg*100

Avec : 304 : la masse équivalente d'oléate de sodium

282 : la masse de l'acide oléique

Wg : le poids d'échantillon utilisé

M : le poids de la phase des acides gras libres obtenue après centrifugation.

- Mesure de méthanol

La teneur en méthanol a été évaluée par l'évaporateur «Heidolph Laborota 4011 digital evaporator». 100 g (W1) d'échantillon brut est déposé dans un évaporateur rotatif pendant 15 min à 60 °C. Le méthanol évaporé est ainsi collecté et noté W2. Le contenu méthanolique est déterminé par l'équation ci-dessous :

Méthanol % (w/w) $=W_2/W_1 \times 100$.

- Mesure des acides gras (AGs)

La mesure des AGL a été déterminée en utilisant la méthode de titration (Harper et col, 1956). Deux gouttes de phénolphtaléine ont été ajoutées à l'échantillon lipidique. La phénolphtaléine est un indicateur utilisé qui en général est incolore, mais vire au rose (pink) en milieu basique. Le contrôle présente 10 mL de mélange chloroforme: méthanol 2:1 v/v avec deux gouttes de phénolphtaléine. La titration s'opère à l'aide d'une micro burette de 25 mL remplie d'une solution de 0.01 N KOH. 0.01 N KOH est ajouté à l'échantillon goutte par goutte avec une agitation continue. La titration se termine lorsque la couleur rose apparaît et persiste au moins 5s.

Le volume de la solution KOH ajouté est déterminé afin de calculer le contenu en AGL à l'aide de l'équation suivante :

$$AGL = 282 * N * (V - B) / W_{lipide}$$

Avec V = le volume en mL de la solution 0.01 N KOH utilisé; B = le volume en mL du contrôle;

N = la normalité de la solution de titration ; W= le poids d'échantillon lipidique en gramme.

1.7.4.3 Mesure de la concentration de SPEs

Après 24h d'incubation à – 20°C des échantillons extraits par éthanol. Les précipités sont ainsi collectés par une centrifugation (6000 g, 4°C). Ils ont été par la suite séchés dans un incubateur à 60°C pendant 24 h déterminant ainsi le poids sec de SPEs extraites qui estime laconcentration de SPE produites. Figure 1-13 présente les SPEs solubles et capsulaires précipitées dans l'éthanol et incubées à 60 °C.

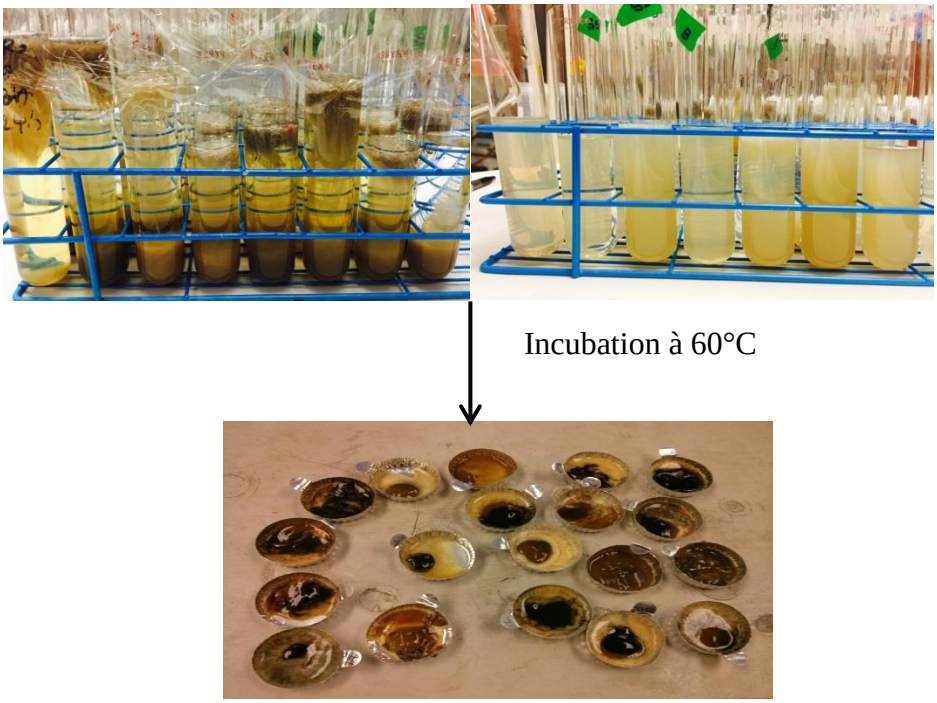


Figure 1-13 : Détermination de la concentration de SPEs par incubation à 60° C

1.7.4.4 Mesure des acides gras libres (AGL) par trans estérification

20 mg de lipide a été solubilisé dans 1 mL d'Hexane. L'hexane est un solvant non polaire qui va solubiliser ou extraire seulement les molécules non polaires comme les lipides. C'est pour cela il a été utilisé comme cosolvant dans l'étape de trans estérification. 5 % (v/v) de méthanol / NaOH a été utilisé pour la réaction de Trans estérification comme un alcool (méthanol) dont les huiles réagissent avec un alcool (éthanol ou méthanol) afin d'obtenir des mono esters éthyliques ou méthyliques, et un catalyseur pour la réaction (NaOH) Le flacon a ensuite été bouché et chauffé pendant une nuit à 50o C. Ensuite, 5 mL de 5 % (v/v) de NaCl a été ajouté pour émulsifier l'émulsion de l'huile avec et les FAME (Acides gras libres esters méthyliques) ont été extraites dans la phase de l'hexane. La phase d'hexane supérieure a été lavée avec 2 % (w/v) de bicarbonate de sodium pour purifier d'autres impuretés comme les protéines et séchée à 60 °C. L'expérience a été réalisée en double exemplaire. Un petit volume de la phase hexane a été utilisé pour les analyses sur GC-FID (Figure 1-14) afin de déterminer le profil lipidique et la composition en acides gras estérifiés ou libres dans les lipides totaux. Les principales

caractéristiques de l'appareil chromatographiques et les conditions opératoires sont comme suit. Préalablement, les échantillons sont méthylés au niveau de fonction carboxylique afin de rendre les acides gras (libres ou estérifiés) volatiles pour une analyse en phase gazeuse. L'analyse des esters des acides gras est employée sur le chromatographe, muni d'un détecteur à ionisation de flamme (FID) et une colonne Varian Factor Four. Les standards utilisés sont, respectivement, les acides gras (C16:1, C17:0, C18:0, C18:1, C18:2, et C18:3).

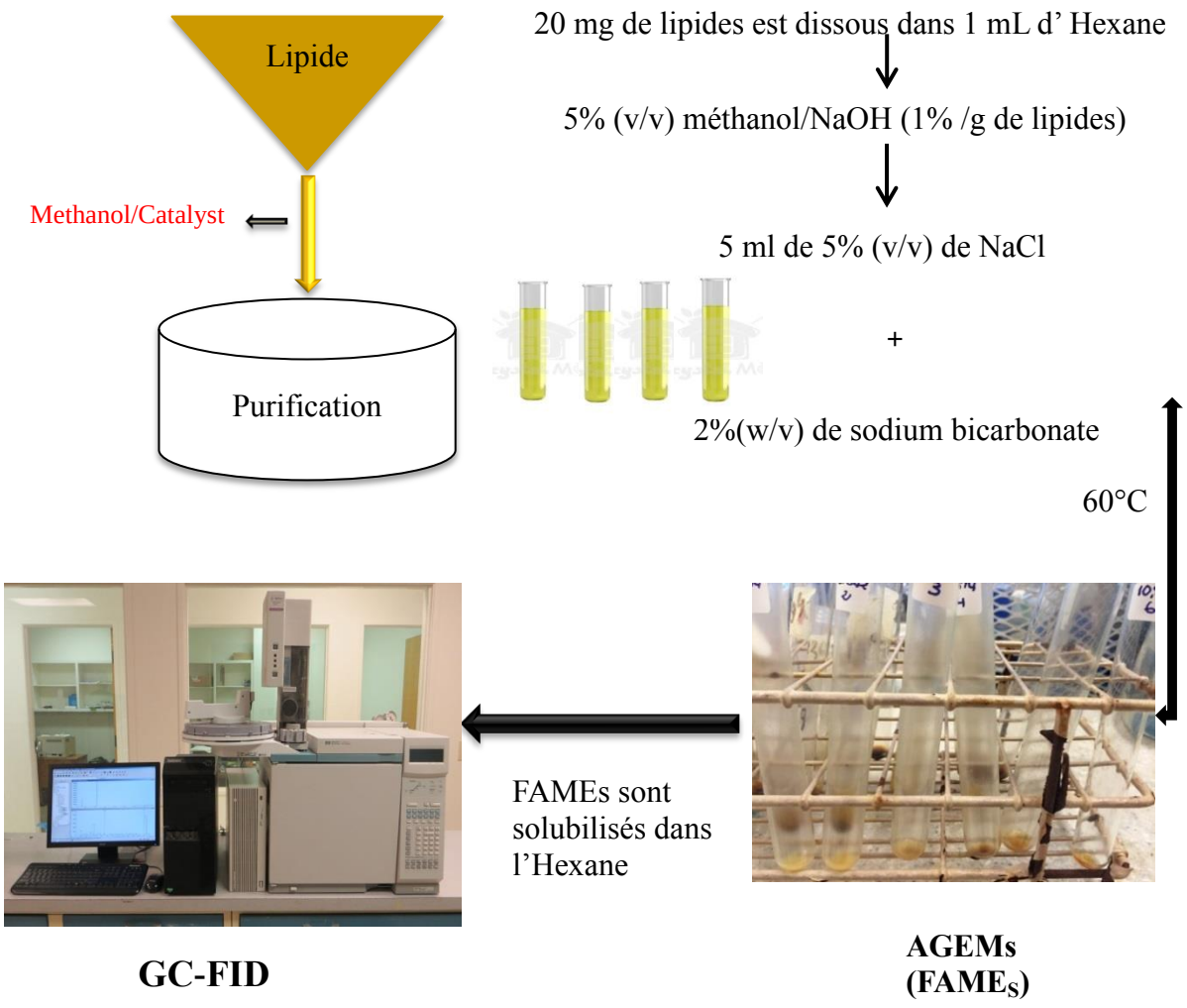


Figure 1-14 : Étape de trans estérification et la détermination de types des acides gras ester méthyliques (AGEM) par GC-FID

1.7.4.5 Caractérisation des SPEs

Le suivi de contenu en protéines, polysaccharidique et en acides nucléiques des SPEs a été réalisé par une technique spectrophotométrique utilisant la spectrométrie UV–visible. Il consiste à mesurer l'absorbance ou la densité optique d'une solution contenant des espèces capables d'absorber certains rayonnements UV ou visibles. La concentration d'une espèce est déterminée par analogie entre la mesure de la densité optique et la droite d'étalonnage effectuée préalablement au maximum d'absorbance de ce composé (λmax).

- Dosage de protéines

La méthode de Bradford est une méthode d'analyse spectroscopique utilisée pour mesurer la concentration des protéines en solution. La méthode de Bradford est un dosage colorimétrique, basé sur le changement d'absorbance (la mesure se fait à 595 nm), se manifestant par le changement de la couleur du bleu de Coomassie après liaison (complexification) avec les acides aminés basiques (arginine, histidine, lysine) et les résidus hydrophobes des acides aminés présents dans la ou les protéines (Bradford, 1979). Le standard utilisé est le sérum albumine bovine (Figure 1-15).

- Dosage d'hydrate de carbone

Les hydrates de carbone totaux ont été dosés par la méthode phénol sulfurique utilisant le glucose comme standard (Debois et al., 1956). L'absorbance est mesurée à 490 nm. Dans ce procédé, l'acide sulfurique concentré va décomposer tous les polysaccharides, les oligosaccharides, les disaccharides et les monosaccharides. Les pentoses (composés 5-carbone) sont ensuite déshydratés en furfural, et hexoses (composés 6-carbone) à hydroxyle méthyle furfural. Ces composés réagissent ensuite avec le phénol pour produire une couleur jaune-or à brun (Figure 1-15).

- Dosage des acides nucléiques

Les acides nucléiques sont dosés par la méthode de Burton, (1956), qui fait intervenir la réaction de Dische (réaction à la diphénylamine) après hydrolyse des acides nucléiques à chaud en milieu acide. Une solution de diphénylamine est préparée dans un mélange d'acide acétique et d'acide sulfurique : 10 g de diphénylamine sont dissous premièrement dans 25 mL d'acide sulfurique et dans 975 mL d'acide acétique. 4 ml de cette solution sont ajoutés à 1 ml d'échantillon.

Immédiatement après agitation au Vortex, les tubes hermétiquement fermés sont placés à 36°C pendant une nuit.

L'absorbance est lue à 595 nm contre un blanc réactif (Figure 1-15).

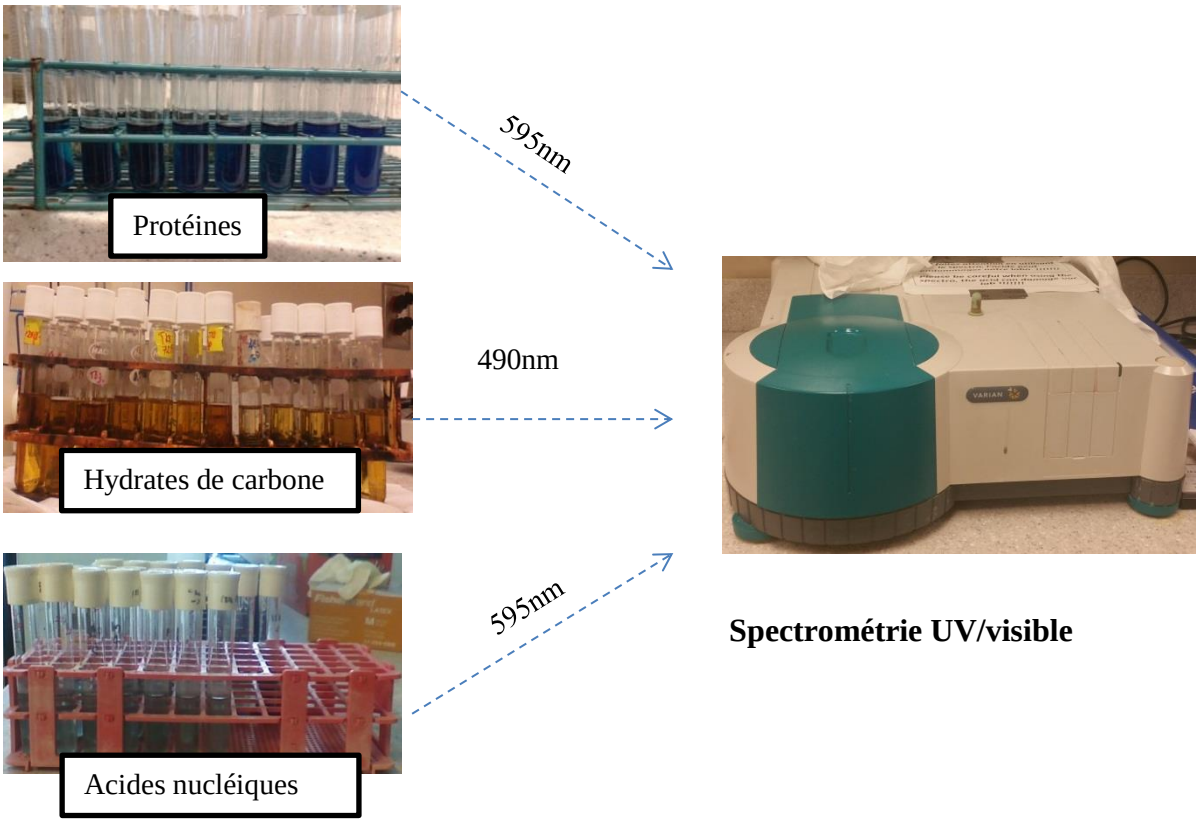


Figure 1-15: Spectrométrie UV/Visible utilisé pour analyser les protéines, les hydrates de carbone et les acides nucléiques.

- ❖ Viscosité et le Zeta potentiel

La viscosité a été mesurée pour chaque type de SPEs extraites (bouillon, S-SPEs et C-SPEs) utilisant le viscosimètre (DV-II PRO, Brookfield). La charge de différents types de SPEs produites a été aussi déterminée utilisant le Zeta photomètre (Zetaphotometer IV, Zetacompact Z8000, CAD Instrumentation, France) (Liao et al., 2001). Le potentiel zêta est un bon indicateur des interactions entre particules et donc de la stabilité des colloïdes. Il représente la charge des particules.

La valeur de cette charge de surface permet de comprendre et de prédire les interactions entre particules en suspension.

1.7.4.6 Caractérisation chimique des SPEs au LC-MS/MS

Le suivi de la composition chimique des SPEs a été réalisé par analyse au LC-MS/MS (Thermo TSQ Quantum Access) (Figure 1-16). Les analyses à la LC ont été effectuées en utilisant une colonne chromatographique Hypersil Gold column C18 ayant une taille de particule de 3 µm et un diamètre intérieur de 2,1 mm x 100 mm). Un flux isocratique de 0,4 mL/min d'éluant constitué de A : 85% d'eau et 0.1% d'acide formique et B : 15% acétonitrile (ACN) et 0.1% d'acide formique a été appliqué. La détection a été conduite par un système MS utilisant une interface électrospray (ESI) en mode ionisation positive.

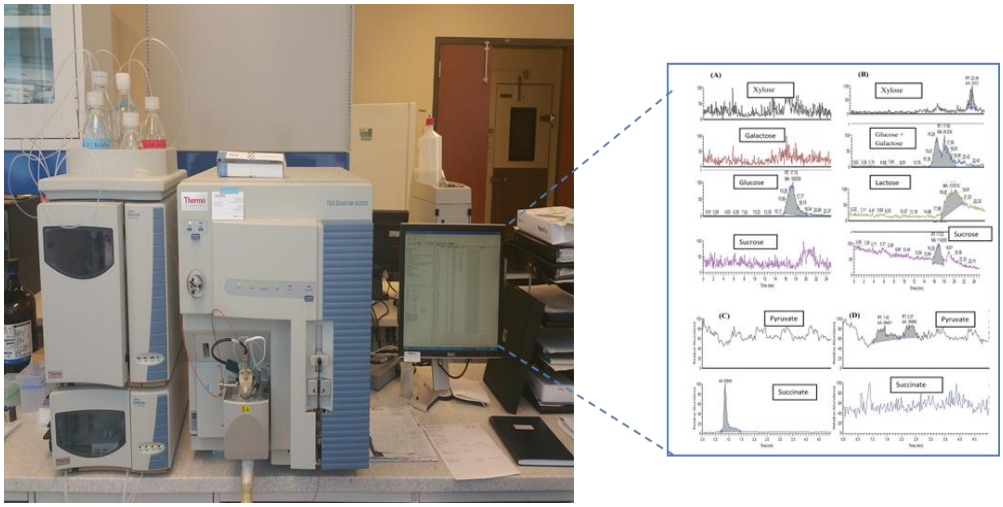


Figure 1-16 : Chromatographie liquide couplée à spectrométrie de masse utilisée pour l'analyse de la composition chimique de SPEs.

1.7.4.7 Identification de la structure chimique de SPEs par IR-TF

La technique de spectroscopie infrarouge permet d'une part, la caractérisation qualitative de l'échantillon par l'identification de groupes fonctionnels et d'autre part, la quantification en suivant l'intensité des bandes d'absorption caractéristiques à chaque groupe fonctionnel. En effet, l'intensité de la vibration/rotation de molécules est proportionnelle à la quantité de molécules présentes, c'est-à-dire proportionnelle à la concentration et à l'épaisseur de l'échantillon. 0,1 à 0,2 mg des SPEs purifiées ont été mélangés et broyés finement avec 100 mg de bromure de potassium KBr déshydraté. Ce mélange a été ensuite compacté 1 min à 5

tonnes pour obtenir une pastille. Les mesures ont été faites entre 4000 et 400 cm-1 en accumulant 16 scans et en ayant soustrait le bruit de fond. L'appareil utilisé était un appareil IR-TF Perkin Elmer (Figure 1-17).

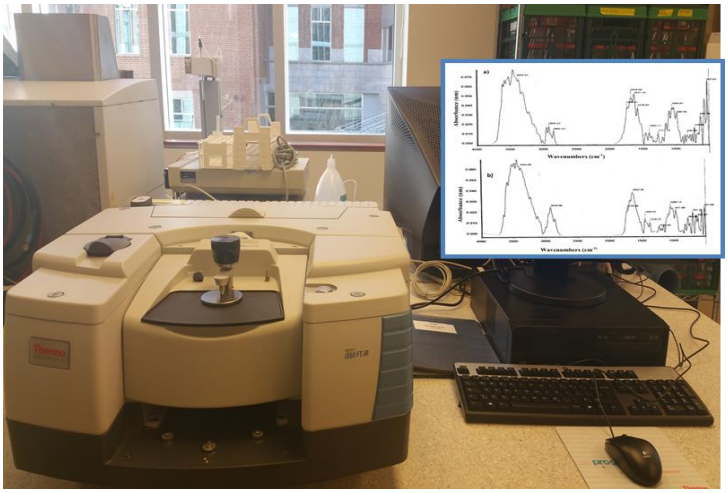


Figure 1-17 : La spectrométrie infrarouge à transformée de Fourier ou spectroscopie IRTF utilisée pour l'identification structurale des SPEs.

1.7.4.8 Les propriétés des SPEs

1.7.4.8.1 L'activité de biofloculation

L'activité de floculation des SPEs a été déterminée par la méthode JAR test (Yakoi et al., 1995). Une solution de Kaolin de concentration 5g/L est préparée. Un mélange rapide à 175 rpm pendant 3 min en ajoutant immédiatement une concentration de Ca^{2+} (150 mg/L), suivie d'un ajustement de pH à 7. Durant cette étape, Le coagulant (Ca^{2+}) déstabilise les particules colloïdales en compensant les forces de répulsion, engendrant le rassemblement des colloïdes et la formation de microflocs. La deuxième étape consiste à ajouter les différents types de SPEs extraites et collectées, à différentes concentrations en diminuant le mélange à 70 rpm pendant 30 min. Après 30 min de mélange, les échantillons sont ensuite transférés dans des cylindres pour décanter encore 30 min à fin de déterminer la turbidité de surnagent utilisant le turbidimètre (Micro 100 turbidimètre, Scientific Inc). Le contrôle présente la solution de kaolin avec le coagulant Ca^{2+}(sans ajout de bio polymères). Le même protocole a été utilisé pour évaluer la biofloculation de SPEs dans les boues municipales et papetières (Figure 1-18). L'activité de floculation est mesurée selon l'équation suivante:

FA(%)= (Contrôle-Échantillon /Contrôle)*100

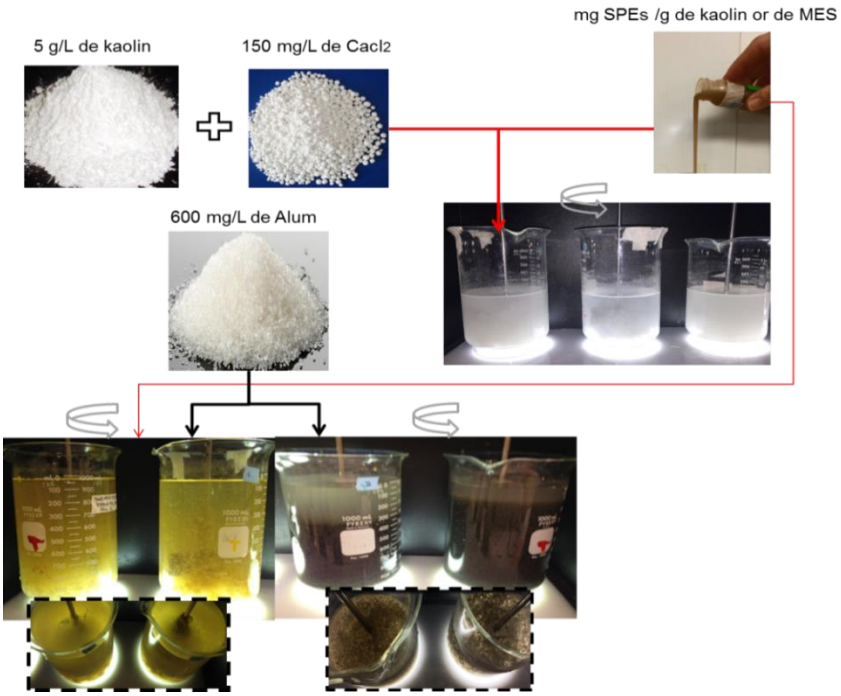


Figure 1-18: Mécanisme de floculation des SPEs dans la solution de kaolin et dans les boues utilisé dans cette étude.

1.7.4.8.1.1 Digestion enzymatique

Une diminution de la concentration de SPEs à la fin de fermentation due probablement à une dégradation des SPEs par une des enzymes sécrétées et produites par la souche bactérienne (*Cloacibacterium normanense* dans notre cas). Cette dégradation résulte une diminution de l'activité de floculation qui peut être due à une dégradation de protéines ou des polysaccharides. Dans cette expérience, on a évalué le rôle de protéines et de polysaccharides (l'un de deux composants majeurs des SPEs) dans la floculation. Les traitements enzymatiques consistent en des digestions de polymères et ciblent les liaisons intrachaines des SPEs. Dans ce contexte, des digestions enzymatiques ont été faites utilisant la protéinase K pour dégrader les protéines. Elle hydrolyse les protéines de toutes origines avec une préférence pour les liaisons peptidiques situées après les acides aminés hydrophobes (leucine, par exemple). Elle a été choisie, car elle n'a aucun effet d'hydrolyse sur les acides nucléiques. La cellulase (β-glucosidase) qui agit sur la liaison β (1-4) permettant d'hydrolyser des chaînes polysaccharidiques de glucose en une unité de glucose. L'activité enzymatique β- glucosidase est détectée pour *Cloacibacterium normanense* (Allen et al., 2006), c'est pour cela elle a été choisie dans cette expérience.

La protéinase K et la cellulase ont été ajoutées dans 200 mL de bouillon de la culture pure (48 h), qui a montré une concentration élevée de SPEs, avec une concentration de 80 unités et 150 unités, respectivement. Les cultures sont incubées pendant 36 h, à 30°C et pH 7.

1.7.4.8.1.2 Microscopie électronique à balayage (MEB)

La microscopie électronique à balayage s'appuie sur la capacité de produire des images en haute résolution de la surface d'un échantillon en utilisant le principe des interactions. Les échantillons ont été récupérés sur une lame de verre après la digestion enzymatique.

Après trois rinçages au NaCl à 0,9 %, l'échantillon a été fixé 20 min dans une solution aqueuse de glutaraldéhyde à 3 % (v/v) puis séché progressivement en étant trempé dans un gradient d'éthanol (30 %, 60 % puis 95 %, 10 min chacun). Enfin l'échantillon a été posé sur un support en aluminium puis métallisé à l'or. La fine couche de métal ainsi formée permet de rendre les échantillons conducteurs. L'analyse microscopique s'est fait grâce à un microscope électronique à balayage (Model Carl Zeiss EVO 50 SMART) (Ajila et al., 2008) (Figure 1-19).

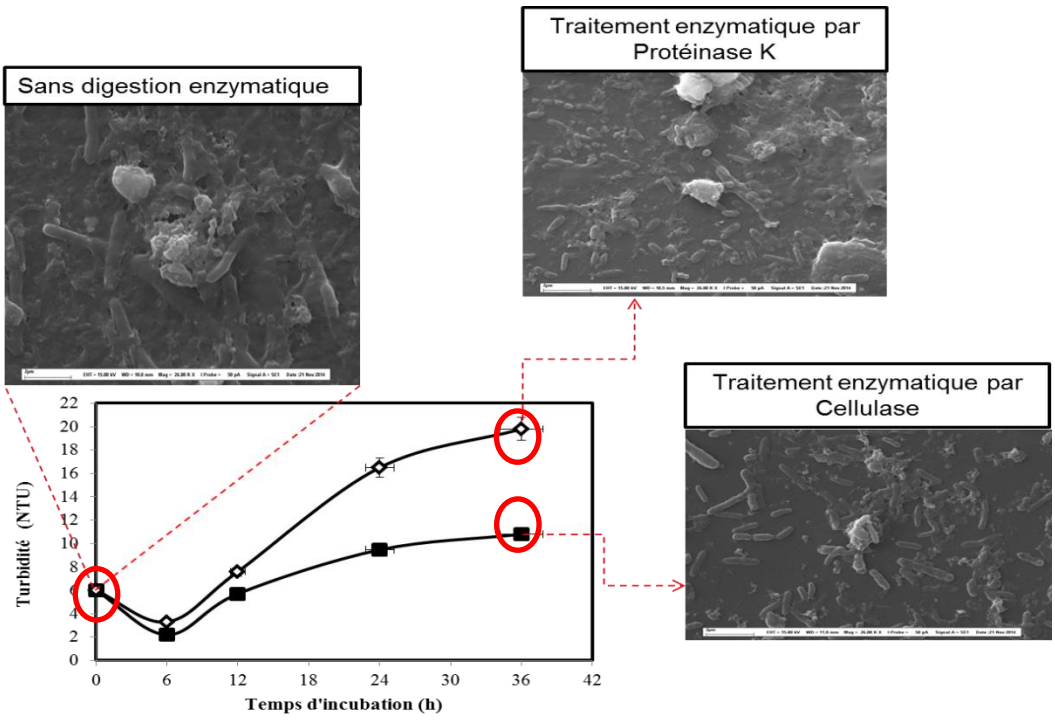


Figure 1-19 : Observations par microscopie électronique à balayage : Image (MEB) de biofilm (*Cloacibacterium normanense*) après digestion par cellulase et protéinase K

1.7.4.8.2 Décantation de boues (Indice du volume des boues)

L'indcie deu vlolumes des boues (IVB) est le volume de boue décantée en un temps donné (t) rapporté à la masse de MES. Il s'exprime en mL de boue par g de MES. Ce paramètre est mesuré pour différents temps de décantation. On considère que les VIB5 et VIB30 (pour des temps t de décantation de respectivement 5 et 30 minutes) sont représentatifs des caractéristiques des agrégats. 1L de boues maintenues sous agitation est versée dans une éprouvette graduée. Elles sont ensuite laissées décantées pendant le temps t. Le volume V occupé par la boue est mesuré. Et l'IVB est déduit directement par la formule suivante :

$$\mathrm{IVB\ (mL/g)} = \frac{\text{Volume après décantation de 1 L de boues (mL /L)}}{\text{MES (mL/L)}})$$

Le (IVB) a été mesuré pour évaluer la capacité de SPEs produites à décanter les boues. Deux types de boues (municipales et papetières) à différentes concentrations de solide en suspension (7, 5, 2, 1 g/L) ont été utilisés. 600 mg d'Alum ont été ajoutés comme agent coagulant suivi d'un ajustement du pH à 7. Les SPEs collectées durant la fermentation ont été évaluées. Le VIB est contrôlé chaque 5, 10, 20 et 30 min. La capacité des SPEs a été déterminée utilisant un contrôle (sans ajout de SPE) à chaque expérience (Balasubramanian et al., 2008) (Figure 1-20).

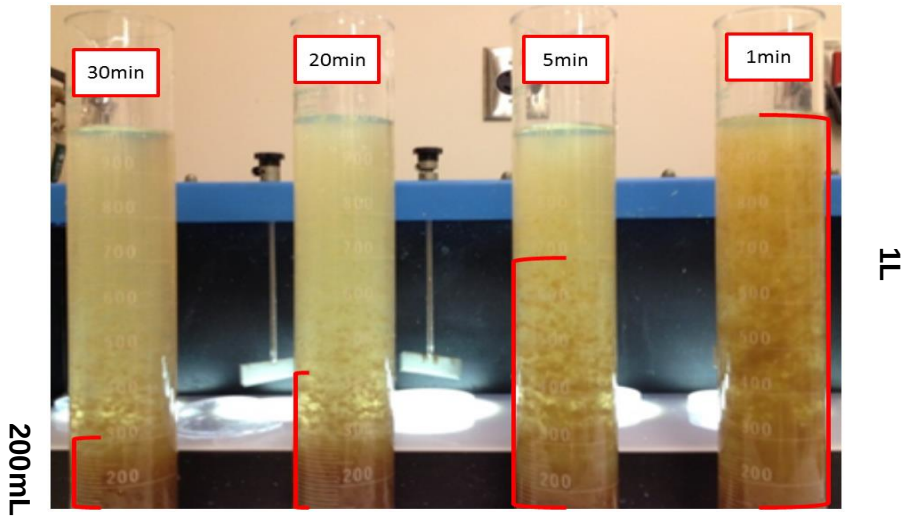


Figure 1-20: Mesure de l'index décantation des SPEs sur les boues papetières.

1.7.4.8.3 La déshydratation de boues

La déshydratation de boues a été déterminée en mesurant le temps de succion capillaire (TCC). Cette technique s'appuie sur la pression de succion générée par un papier filtre est utilisée pour aspirer l'eau des boues. La mesure de filtrabilité des boues est obtenue par calcul du temps nécessaire au front d'eau (le CST) pour passer entre deux électrodes placées à distance de l'entonnoir (Scholz et al., 2005). C'est une méthode rapide et fiable qui ne dépend pas du volume de boue testée. À la suite de la décantation de boues, le culot a été récupéré pour tester la déshydratation et la filtrabilité de boues.

1.7.4.8.4 Interactions SPEs-éléments métalliques (EM)

Cette étude se base sur les capacités de sorption des EM présentes dans les eaux usées municipales primaires par les SPEs utilisant la spectrométrie à plasma à couplage inductif (ICP-AES). Cette méthode consiste à ioniser les échantillons en l'injectant dans un plasma d'argon permettant de transformer les atomes de la matière à analyser en ions par une flamme chaude. Une fois les SPEs extraits des biomasses, la solution de SPEs est mélangée avec les eaux usées étudiées. Parmi les facteurs étudiés, la concentration en SPEs (35 et 50 mg/L) (Prado Acosta et al. 2005) et le pH 7 ont été utilisés. Les échantillons sont incubés à 30 °C, 250 rpm pendant 12 h. Le contrôle utilisé est les eaux usées municipales sans ajout des SPEs. Pour l'analyse des métaux, 40 mL d'échantillon a été prélevé et centrifugé à 3000 g pendant 5 min pour éliminer les complexes SPEs-EM. Les métaux (le surnageant) ont été dosés par spectrométrie (ICP-AES) (Figure 1-21) après digestion des échantillons à l'acide nitrique (HNO_3) concentré (6N) pendant 24h.

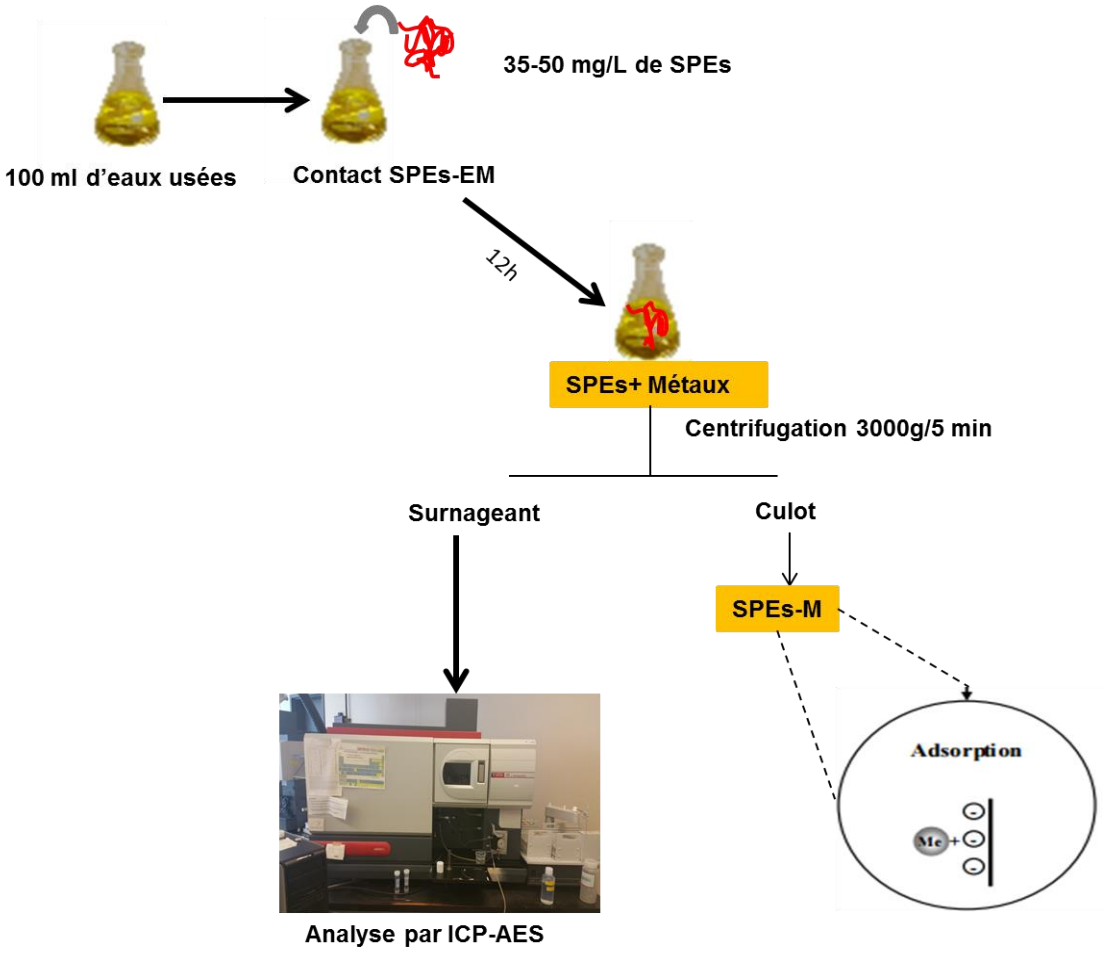


Figure 1-21 : Procédé de biosorption des métaux aux SPEs.

1.8 Principaux résultats et discussion générale

Les résultats de la présente étude ont permis d'évaluer l'efficacité d'un procédé de production de SPEs par *Cloacibacterium normanense* utilisant les boues secondaires et le glycérol brut comme une source de carbone pour différents traitements tels que la floculation et la décantation de boues et pour le traitement d'effluents contaminés par des métaux. Cette section consiste en une discussion générale des différents résultats obtenus.

1.8.1 Isolement de souches bactériennes productrices de SPEs à partir de boues municipales (Chapitre 3)

Huit souches bactériennes productrices de SPEs ont été isolées à partir de boues secondaires collectées de la station d'épuration de Québec. Les souches bactériennes isolées ont été identifiées puis caractérisées. L'identification des souches a été faite par comparaison des séquences de l'ADN 16S aux banques de données et par le test de Biolog. Ces résultats ont pu être complétés par la morphologie des bactéries utilisant la coloration de Gram. Il est intéressant de constater que les souches isolées regroupent 7 genres différents. Les genres fréquemment isolés des eaux usées municipales, tels que *Bacillus, Pseudomonas, Acitonobacter et Flavobacterium*, sont majoritairement présents dans notre étude. Une nouvelle espèce, *Cloacibacterium normanense* (NK6) a été isolée pour la première fois dans cette étude. La majorité des bactéries isoloées est à Gram négatif (excepté la souche *Brevibacillus parabrevis* NK5). La concentration de SPEs par les différentes souches isolées a été déterminée. Le séquençage de gènes montre une différence significative de cluster de gêne entre les bactéries isolées ce qui explique la variation de leur concentration de SPEs. Trois types de SPEs (solubles, capsulaire et bouillon) de chaque souche bactérienne ont été extraits par centrifugation. Leur composition chimique en termes de protéines et de polysaccharides, leur caractérisation physicochimique (potentiel zêta, viscosité) ainsi que leurs activités de biofloculation ont été évaluées. *Cloacibacterium normanense* a produit une meilleure concentration de SPEs (11,8 g/L) dans les boues secondaires stérilisées avec une activité de floculation de 90,2% utilisant les SPEs solubles (S-SPEs) dans la solution de kaolin suivie de *Brevibacillus parabrevis* avec 3,52 g/L et 83,2% de biofloculation.

Les résultats de cette partie des travaux (incluant les aspects matériaux) ont fait l'objet d'un article publié dans la revue internationale Journal **Petroleum and Environmental Biotechnology**

1.8.2 Caractérisation des SPEs produites par Cloacibacterium normanense isolée à partir des boues municipales et leurs potentialités dans la décantation de boues et déshydratation de boues (Chapitre 4)

À partir des résultats du chapitre précédent, nous avons présélectionné la souche *Cloacibacterium normanense* sur les critères suivants : la concentration de SPEs et un critère supplémentaire ont été pris en compte pour notre sélection à savoir la capacité de ces SPEs dans la floculation. Cette souche apparaît intéressante de par le simple fait qu'elle est différente des autres souches isolées dans les études précédentes (Balsubramanian et al., 2010). Ce chapitre est consacré à l'optimisation de la production des SPEs par *Cloacibacterium* sp et la caractérisation des SPEs produites par cette souche. Ceci passe, par la mise au point des conditions de production de SPEs à savoir la taille d'inoculum, la concentration de MES, durée d'incubation pH, Température. Dans la mise au point d'un protocole de production de bio polymères, la durée d'incubation étant importante, il faut trouver une durée qui permet une production suffisante de SPEs tout en évitant une mortalité trop importante des bactéries qui, en se lysant, contamineraient les SPEs avec des composés intracellulaires. De plus, la composition du milieu de culture joue elle aussi un rôle primordial sur la quantité de SPEs produites mais aussi sur la composition des SPEs. La concentration de boues en matières en suspension (MES) est fréquente pour améliorer la production d'exo polysaccharides (Chalkiadakis et al., 2013). Dans cette étude, 25 g/L de MES, 3% (v/v) d'inoculum, 30°C, 180 rpm sont les meilleures conditions de fermentation favorisant une forte concentration de SPEs (13,3 g/L) avec S-SPEs (13 g/L) et C-SPEs (0,3 g/L) pendant 48h. Ces SPEs ont été caractérisées montrant une forte teneur en polysaccharides en comparaison aux protéines. La composition de ces derniers est également intervenue sur la potentialité de floculation, la décantation et la déshydratation de boues. 94,2% d'activité de biofloculation dans la solution de kaolin ont été montrées utilisant 150 mg Ca^{2+} et 1,3 mg/L des SPEs solubles en comparaison au 86,8% et 79% utilisant 2,6 mg B-SPEs/g Kaolin et 0,5 mg –SPE/g kaolin, respectivement. Ceci peut être expliqué par la forte teneur en protéines (219 mg BSA/ g S-SPE) et de polysaccharides (128,5 mg d'hydrate de carbone /g S-SPE) de S-SPE par rapport à celles de C-SPE (145,6 mg BSA/g C-SPE ; 104,0 mg d'hydrate de carbone/g C-SPE), respectivement. Selon les résultats obtenus concernant l'activité de floculation, les S-SPEs ont été par la suite testées pour la décantation et la déshydratation de boues de types municipales et papetières de différentes concentrations (7,5, 2 et 1 g/L).

Selon le type de boues, la concentration de SPE ajoutée et la concentration de MES de boues, l'indice du vlumes des boues est varié. Une meilleure décantation a été enregistrée dans les deux types de boues utilisant 5 g/L avec une (20 mL/g) en comparaison au contrôle (sans ajout de S-SPEs) est de 50 mL/g. Une capacité de 37,6% de déshydratation a été révélée avec 2 g/L de matière sèche de boues municipales combinées de 600 mg/L d'Alum et 0,02 g/L de S-SPEs. Un article portant sur ces résultats a été publié dans le Journal **Civil and Environmental Engineering.**

1.8.3 Effet de méthodes d'extraction sur les biofloculants présents dans les boues "Backwash" (l'unité de biofiltration) (Chapitre 5)

En permettant de les étudier en solution, l'extraction des SPEs directement de boues peut apporter de nombreuses informations sur la matrice des agrégats bactériens étudiés. En effet, à condition d'obtenir un échantillon représentatif des polymères de la matrice, cela peut nous permettre de mieux connaitre la nature des SPEs et leurs propriétés chimiques.

Comme nous l'avons vu dans la partie étude bibliographique, la plupart des protocoles d'extraction permettent de séparer les SPEs selon leurs liens avec les bactéries au sein du biofilm : les SPEs solubles qui sont libres dans le milieu de culture, les SPEs faiblement liées et les EPS fortement liées pour lesquels le protocole d'extraction est plus complexe que celui permettant d'isoler les SPEs solubles et les EPS faiblement liées (Liang et al., 2010 ; Aires et al., 2011 ; Pellicer-Nàcher et al., 2013). Ces dernières sont en effet plus faciles à séparer des cellules et des méthodes physiques (centrifugation ou sonication) suffisent pour les isoler. Les SPEs qui sont fortement liées aux bactéries sont, quant à elles, plus difficiles à isoler, en raison de la cohésion élevée de ces SPEs aux membranes cellulaires. Les méthodes utilisées sont soit trop peu efficaces et ne permettent pas d'extraire une quantité représentative de SPE, soit trop intense, et présentent un risque trop élevé de lyse cellulaire ce qui a pour conséquence de mêler aux SPEs, des molécules intracellulaires qui vont perturber les analyses. C'est pourquoi notre objectif a été de mettre au point une méthode d'extraction qui ne présenterait pas ces problèmes, c'est-à-dire présentant un rendement d'extraction relativement élevé, mais sans contamination des SPEs par des composés intracellulaires.

Différentes méthodes d'extraction ont été testés que ce soit physique, chimique ou la combinaison de deux. Une étape mécanique permet de déstabiliser les liens intermoléculaires de manière spécifique, puis une première étape chimique cible les liaisons hydrophobes et une seconde étape chimique déstabilise les interactions ioniques. La stratégie d'extraction des SPEs

que nous avons utilisée a bien montré l'effet de ces méthodes sur la composition chimique des SPEs (évitant au max la lyse cellulaire et la contamination par les composés intracellulaires) et leur potentialité de floculation.

Toutefois, si l'on s'intéresse plus précisément aux différents résultats obtenus pour les différentes méthodes, les méthodes de centrifugation et chauffage présentent une faible concentration de SPEs (1,2 g/L) et une activité de floculation (40%), mais aucune lyse cellulaire n'a été contribuée. L'utilisation de formaldéhyde a montré une protection importante des cellules en combinaison avec la méthode de sonication et EDTA.

Nous avons poursuivi l'étude de ces EPS en évaluant la teneur en protéines, glucides et acides nucléiques dans chaque extrait afin d'en connaître globalement la composition. La composition de presque la moitié des échantillons n'est pas déterminée. Les substances humiques et les lipides peuvent représenter une part importante des SPEs. Les glucides sont globalement majoritaires (dans les méthodes de Centrifugation, chauffage et Formaldéhyde-Chauffage), tandis que les acides nucléiques sont bien présents, en particulier dans les SPEs extraites par F/NaOH, F-Sonic-NaOH et EDTA. Nous pouvons alors nous demander si cela ne refléterait pas une lyse cellulaire due au protocole d'extraction ou si cette souche produit effectivement autant d'ADN extracellulaire. Les protéines sont largement majoritaires dans les SPEs extraites par F-Sonic-Chauffage et Sonication.

L'étude des différentes méthodes d'extraction a montré que 5 g d'EDTA/L est la méthode la plus appropriée avec une concentration de SPEs extraites (6,2 g/L). Par contre, une lyse cellulaire a été remarquée en mesurant la teneur des acides nucléiques. Ce dommage cellulaire n'a aucun effet négatif sur l'activité de biofloculation qui achève 95 % utilisant 2.4 mg SPE/g kaolin.

Les résultats de cette partie des travaux ont fait également l'objet d'un article publié dans la revue de calibre international Journal of **Environmental Managment**

1.8.4 Traitement des eaux usées municipales contaminées par les métaux utilisant les SPEs produites par Cloacibacterium sp dans des boues additionnées du glycérol brut (Chapitre 6)

Dans l'optique d'élargir le spectre de production des exo polymères, le glycérol brut a été utilisé comme une source de carbone supplémentaire. Plusieurs scénarios ont été étudiés. Initialement, la concentration de glycérol brut a été optimisée, la concentration la plus appropriée est de 2%, au-delà de 2%, une baisse de croissance est observée, toutefois, le glycérol brut est considéré comme une source potentielle de carbone même que le glycérol utilisé dans cette partie ne contient pas de teneurs en savon et méthanol si importantes. Ceci peut être dû au contenu remarquable de glycérol brut en macroéléments calcium, potassium, magnésium (Johnson et Taconi, 2007), qui peut accentuer le potentiel nutritif de glycérol brut (Cuelik et al., 2008). Une forte concentration de SPEs a été enregistrée avec 21,3 g/L pendant 72 h utilisant 2% (v/v) de glycérol. Pour cette étude, nous avons aussi opté pour trois protocoles basés sur des traitements physiques et chimiques. La centrifugation est souvent utilisée en raison de son minimum de lyse cellulaire, le chauffage en raison de sa capacité à solubiliser la cohésion de la matrice tout en préservant l'intégrité des cellules (Comte et al., 2006) et finalement l'EDTA est l'agent chélatant qui permet en effet d'éliminer les cations multivalents qui forment de ponts entre les sites négativement chargés des SPEs permettant ainsi la dissociation de la matrice. En plus, cette méthode a été précédemment testée montrant à la concentration de 5 g/L d'EDTA un bon rendement d'extraction avec un minimum de lyse cellulaire.

Les SPEs extraites sont par la suite caractérisées en déterminant leurs compositions chimiques. Une forte teneur en protéines et en hydrates de carbone a été remarquée dans les SPEs extraites par EDTA en comparaison de celle extraites par centrifugation et par chauffage. Nous avons vu que le faible pourcentage en acides nucléiques dans les SPEs extraites par centrifugation et chauffage est un gage du maintien de l'intégrité cellulaire pendant l'extraction. Alors qu'une forte teneur en acides nucléiques a été montrée avec l'EDTA, ce qui peut être expliqué par une contamination par des protéines intracellulaires. Les SPEs ont été par la suite testés pour évaluer leur potentialité de floculation et leur capacité d'éliminer les métaux des eaux usées municipales. On a constaté que les doses de SPEs utilisées pour assurer une bonne floculation dépendent généralement de protocole d'extraction. Pour atteindre un maximum d'activité de floculation (95,3 %), 23,1 mg SPE/g kaolin extraite par EDTA a été ajouté, qui est 11 fois plus la dose des SPEs utilisée en cas de centrifugation. Cela pourrait expliquer la variation de la composition et de la structure chimique des SPEs extraites par différentes méthodes d'extraction.

4,2 mg SPE/g de kaolin, utilisant la centrifugation comme méthode d'extraction, ont contribué à une activité de floculation de 90% en comparaison de 77,5% et 77% respectivement dans le cas de chauffage et EDTA, utilisant la même dose de SPEs (4,2 mg SPE/g de kaolin).

En termes d'efficacité d'adsorption aux métaux, selon les résultats précédents, les SPEs extraites par centrifugation ont été utilisées pour déterminer le pourcentage d'adsorption aux éléments métalliques. Les résultats obtenus ont montré que pour les deux concentrations d'étude (35 et 50 mg/L), les SPEs étaient capables de biosorber jusqu'à 85 % du Nickel et 73 % d'Aluminium. Nos résultats ont également montré que les mécanismes de rétention des métaux par les SPEs s'avèrent être contrôlés par la concentration métallique, la concentration de SPEs et le temps de rétention. En effet, les quantités de métaux fixés sont plus importantes à plus faible concentration de SPEs (35 mg/L). En revanche, on a pu remarquer que la capacité d'élimination dépend du type de métal, cela correspond proportionnellement à l'affinité de métal vis-à-vis les SPEs. Dans la littérature, de nombreuses études ont révélé des affinités variables des SPEs avec les éléments métalliques (Comte et al. 2006, Joshi et Juwarkar 2009).

Toutefois, la composition chimique des SPEs en termes des protéines, polysaccharides et substances humiques-like joue un rôle indispensable dans la propriété de biosorption de SPEs. La forte teneur en protéines et en polysaccharides qui caractérise les SPEs étudiées peut expliquer la capacité de biosorption en favorisant le nombre de fixations des SPEs et ainsi l'accessibilité des métaux aux sites de fixation.

Un article, portant sur ces résultats originaux a été publié dans la revue internationale **Bioressource Technologie.**

1.8.5 Caractérisation chimique des SPEs produites par Cloacibacterium normanense par analyse chromatographie liquide couplée à spectrométrie de masse et par spectroscopie infrarouge à transformée de Fourier (Chapitre 7)

L'analyse en CL-SM a fait l'objet de la composition chimique des SPEs (avec et sans ajout du glycérol) en monosaccharides. Les SPEs (avec ajout de glycérol) contiennent majoritairement du galactose 67 %mol, 13%mol du glucose, 9,8 et 3%mol de xylose, sucrose et du lactose. Tandis que les SPEs (sans ajout du glycérol) ne contiennent que du glucose au lieu de galactose. Cela pourrait expliquer que l'addition du glycérol a stimulé l'activation des enzymes nécessaire pour la biosynthèse et l'assemblage de nucléotides contribuant ainsi à une composition chimique différente. Toutefois, les profils chromatographiques CL-MS des SPEs (avec ajout de glycérol) a montré que les échantillons sont riches en pyruvate tandis que les SPEs (sans ajout de glycérol) sont riches en succinate.

L'analyse Infra-Rouge (IR-TF) des deux types de SPEs (avec et sans ajout du glycérol) a permis de révéler la présence de groupes fonctionnels caractéristiques en accord avec les résultats obtenus par analyse LC-MS. Nous constatons en effet, pour les deux types de SPEs, la présence d'une large bande intense, autour de 3400 cm^{-1} caractéristique des groupements hydroxyles. De faibles bandes de vibrations de valence des liaisons C-H entre 2800 et 2900 cm-1 et autour de 1454 cm^{-1} concernent les CH_2 aliphatiques des sucres, des protéines et lipides. Les bandes caractéristiques des liaisons peptidiques des protéines sont visibles, comme les deux bandes à 1724 et 1645 cm^{-1} (resp. amide I et amide II). Ces mêmes bandes pourraient spécifier la présence d'osamines N-acétylées (Chalkiadakis et al., 2013).

Par contre, une diversité entre profils IR-TF de deux types SPEs a été remarquée en présence de la bande 2855 cm^{-1} et de la bande 1578 cm^{-1} dans le cas des SPEs produites par addition de glycérol. Ceci peut engendrer l'amélioration de l'activité de floculation enregistrée utilisant les SPEs (avec ajout de glycérol). Ces bandes concernent le groupe carboxyle qui peut lier aux cations Ca^{2+} formant ainsi des flocs par mécanisme de pontage. Ce phénomène peut s'expliquer par un pontage par cations divalents entre polymères anioniques qui stabiliserait la structure de la matrice de SPE. Ce pontage peut avoir lieu entre des protéines possédantes des acides aminés chargés négativement (acides glutamiques et aspartiques), ou entre polysaccharides contenant des oses anioniques.

Suite aux résultats obtenus précédemment (la composition et la structure chimique des SPEs), des digestions enzymatiques ont été utilisées afin de déstabiliser certaines interactions

chimiques afin d'évaluer leur importance dans la formation de flocs. Étant donné le nombre de molécules différentes pouvant entrer en jeu dans la cohésion des agrégats, il a été nécessaire, dans le cas de notre étude, de sélectionner quelques enzymes en fonction de leur spécificité par rapport au substrat. Cette spécificité se définit bien sûr en fonction de la nature chimique de la molécule substrat, mais aussi en fonction du mécanisme de digestion qui peut être endogène (hydrolyses en milieu de chaîne) ou exogène (hydrolyses aux extrémités des chaînes). Les protéines étant très largement représentées dans la matrice des granules, il est vraisemblable qu'elles jouent un rôle dans les propriétés physiques de ces agrégats. Protéinase K a été retenue pour hydrolyser les diverses protéines de la matrice. En revanche, dans le cas des polysaccharides, la cellulase a été utilisée. Les SPEs ont été mis en incubation avec ces deux enzymes (protéinase K et cellulase) pendant 36 h, à 30 °C sous agitation. Une première estimation de l'effet des différentes enzymes sur la structure de biofilm est effectuée à l'aide de la mesure de la turbidité. Dans le cas de la protéinase K, on remarque que contrairement aux cellulases, la présence de l'enzyme dans le milieu provoque en elle-même une augmentation de la turbidité due à hydrolyse de protéines consécutive à la digestion enzymatique. Par conséquent, la cellulase a montré une augmentation de la turbidité, mais pas si importante que celle provoquée par protéinase K. Ces résultats ont été confirmés par une observation microscopique électronique à balayage. Cette expérience nous permet de déduire la présence importante de protéines et de polysaccharides dans le biofilm puisque leur digestion provoque une destruction de flocs.

Les résultats de cette partie des travaux ont fait également l'objet d'un article publié dans la revue de calibre international **Journal of Material Science & Engineering.**

1.8.6 Développement d'un procédé de production simultanée de SPEs et de lipides par Cloacibacterium normanense (Chapitre 8)

Le rapport Carbone sur Azote (C/N) a sans doute un impact direct sur la production de SPEs. À un rapport faible, la production des SPEs est favorisée (Ye et al., 2010). Par ailleurs, une alimentation faible en Azote (et donc un rapport C/N fort) semble avantager les organismes à accumuler les lipides (Zhang et al., 2014). Par conséquent, ce scénario laisse donc penser à la nécessité de maîtriser ce paramètre pour la mise en place d'un mécanisme de compétition entre la production de SPEs et l'accumulation de lipides par *Cloaibactérium normanense*. Ce mécanisme de compétition a été étudié afin de tenter de prévoir dans quelles conditions données *Cloacibacterium normanense* est une potentielle candidate de SPEs ou de lipides. Afin de maintenir ce paramètre, le glycérol brut et la peptone ont été utilisés. Les résultats obtenus dans ce chapitre ont montré qu'à un rapport C/N 25, la production de SPE est favorisée par *Cloacibacterium* sp (22,4 g/L). En revanche, le rendement global de la production de SPEs est inversement modifié par l'augmentation de C/N à 100. La productivité de lipides rp (g/L.h) augmente significativement de 0.08 to 0.2 g/L h pendant 72 h et la productivité de SPEs est diminué de 0,3 à 0,07 g/L h. La peptone a été totalement consommée par *Cloacibacterium normanense* dans les différents C/N étudiés. Une augmentation de biomasse a été constatée dépendamment du C/N. Durant la fermentation, la concentration du glycérol et ses impuretés (savon et le méthanol) ont été également suivies. On a remarqué que le glycérol, le méthanol et le savon ont été utilisés comme une source de carbone que ce soit pour produire des SPEs ou pour accumuler les lipides. Le savon a été particulièrement utilisé à partir de 24h en augmentant le C/N en favorisant ainsi l'accumulation de lipides. Selon les résultats obtenus, on a pu supposer que le C/N 25 est le rapport optimal pour la production simultanée de SPE et de lipides avec une concentration 22 g/L de SPEs et un continu lipidique de 27,6% (p/p).

Les lipides accumulés sont constitués principalement de triacylglycérols. Ces triacylglycérols étant des esters de trois acides gras, le profil en acide gras constitue une caractéristique importante des lipides accumulés. Le profil en acide gras est suivi dynamiquement par analyse chromatographique des extraits lipidiques. Les principaux acides gras présents dans la biomasse sont dans l'ordre décroissant de la quantité totale des acides gras: l'acide élaidique (C18:1n9t) l'acide linoléique (C18:2n6c), l'acide palmitique (C15:0), l'acide stéarique (C 18:0) et le palmitoleate (C16:1n7). L'acide élaidique peut représenter jusqu'à 53% (p/p) de la quantité totale des acides gras.

Les résultats obtenus concernant l'activité de biofloculation de S-SPE dans les boues ont montré que les SPE ont une capacité de floculer les boues de 72% utilisant 13 mg SPE/g de boues. Cette activité est élevée en comparaison de celle obtenue par le Zetag (66%) en ajoutant 10 mg zetag/g de boues. Ces résultats semblent être avantagés par le fait que les SPE sont des biopolymères non toxiques par rapport au Zetag (polymères chimiques et coûteux).

Il est nécessaire de signaler que ce chapitre a montré des résultats originaux s'appuient d'une part sur la production simultanée de deux bioproduits par *Cloacibacterium normanense* et d'autre part ce procédé a offert de biopolymères avec une forte capacité de floculation et un contenu lipidique qui peut servir comme un substrat pour la production de biodiesel. Ce chapitre a fait l'objet d'un article soumis dans la revue internationale **Biotechnolgy and bioengineering.**

1.9 Conclusion générale et recomandations

1.9.1 Cloacibacterium normanense candidate à deux potentialités : Productrice des SPEs et accumulatrice de lipides……

La littérature concernant les polymères extracellulaires est riche. La synthèse bibliographique a montré un intéressement croissant des recherches au cours des dernières années dû à l'importance de ces molécules organiques dans les biomasses. Les bactéries produisent par leur métabolisme les SPEs qui assurent leur adaptation et leur survie dans l'environnement. Ces SPEs traduisent le comportement bactérien ou le bien-être de la biomasse dans un milieu donné. Toutefois, les études se sont concentrées sur les SPEs issus de boues activées, de granules et de biofilms. Devant le peu de données concernant les SPEs provenant de cultures bactériennes pures cultivées dans des boues stérilisées, nous avons voulu apporter notre contribution à ce domaine en développant les recherches sur les SPEs issus d'un nouvel isolat à partir des boues.

Cette thèse nous a permis de développer un procédé de production des SPEs par une nouvelle souche isolée (*Cloacibacterium normanense*) à partir de boues. L'optimisation de condition de fermentation et l'ajout d'une source de carbone supplémentaire à faible coût (le glycérol brut) a stimulé la biosynthèse des SPEs avec une concentration élevée de 22,5 g/L.

En nous attardant sur l'évolution des quantités de SPEs produites durant la fermentation, nous avons également remarqué la variation de l'apport carbone/azote. A faible C/N ratio la biosynthèse est favorisée. En revanche, à un ratio plus fort, la concentration de SPEs est diminuée. Ce scénario nous a permis d'accéder à une avancée scientifique la possibilité de production simultanée de SPEs et de lipides par *Cloacibacterium* sp en mettant en jeu l'apport carbone/azote. Les résultats de cette étude ont montré qu'à faible C/N 18, la production de SPEs est favorisée par la souche avec une concentration de 22,4 g/L. En augmentant ce rapport à 100 l'accumulation de lipides est induite par *Cloacibacterium* sp avec un contenu lipidique de 14,1 g/L. Dans cette étude, le C/N 25 est favorisé pour refléter la potentialité simultanée de *Cloacibacterium normanense* à produire de SPEs et accumuler de lipides. Dans ces conditions (C/N 25), une production de SPEs allant jusqu'à 22 g/L et une accumulation lipidique allant jusqu'à 27,6% (p/p) a été enregistrée.

Notre étude a également montré que, comme dans les études précédentes, les SPEs contiennent principalement des polysaccharides, protéines, et des substances humiques-like et en moindre mesure, des acides nucléiques.

L'analyse par chromatographie liquide couplée à la spectrométrie de masse (CLSM) et par TR-IF nous a permis de caractériser la composition chimique des SPEs étudiées (avec et sans l'ajout de glycérol) (Galactose, glucose, xylose, sucrose) et d'identifier les groupes fonctionnels (les groupes hydroxyles et carboxyles, phénols, amides).

Ces résultats nous ont permis de faire le lien entre les SPEs produites par la nouvelle souche étudiée et leur composition, structure chimique à la floculation et décantation de boues. Ainsi, cette étude nous a permis de comprendre partiellement la contribution de l'apport C/N à l'amélioration de ce procédé (accumulation de lipides). Ces résultats nous ont permis de confirmer l'hypothèse I, II et IV.

Toutefois, il est essentiel d'évaluer la performance de ce procédé à l'échelle pilote afin d'améliorer les résultats obtenus.

En ce qui concerne, le coût, cette étude met en avant la nécessité de l'estimer et de l'améliorer. Il apparaît intéressant d'optimiser le processus d'extraction en utilisant un minimum de dilution de biomasse pour l'extraction de C-SPEs, cela pourrait épargner le coût de chauffage. La détermination de la dilution optimale est donc importante afin de diminuer le volume d'éthanol utilisé pour un maximum de précipitation de SPEs permettant par conséquent d'économiser le coût élevé de la matière première, centrifugation, le chauffage et le refroidissement.

1.9.2 … Extraction …

La seconde tâche que nous nous sommes assignée, a été la mise au point d'un protocole d'extraction des SPEs permettant la récupération d'une quantité significative de polymères avec moindre lyse cellulaire et contamination par les réactifs. L'analyse des produits d'extraction que nous avions à disposition a permis de mettre en évidence certaines caractéristiques chimiques des SPEs produites. De plus, la répartition quantitative des SPEs extraites lors des différentes méthodes d'extraction a permis d'établir le profil ou « empreinte du protocole d'extraction». Ainsi, les SPEs de biofilm se distinguent de celles extraites de *Cloacibacterium normanense* par le même protocole par un plus fort rendement d'extraction par l'EDTA et un plus faible par centrifugation. L'EDTA (0,5 %) a permis une extraction poussée de SPEs directement à partir de boues (biofilm). Par contre, cette méthode a affecté le profil chimique des SPEs produites par *Cloacibacterium normanense*. La concentration ajoutée des SPES extraites par EDTA est 11 fois plus élevée que celle ajoutée utilisant la centrifugation pour avoir la même activité de floculation. De plus, cette technique a permis d'extraire une teneur en acides nucléiques élevée en comparaison à d'autres méthodes (centrifugation, chauffage, etc), ce qui pourrait concevoir la contamination des SPEs par des substances intracellulaires. Dans ce cas, il serait

très important d'effectuer des travaux supplémentaires en mesurant l'activité de la G6P-DH, une enzyme intracellulaire témoin de la lyse bactérienne.

Cette étude a également identifié le protocole d'extraction adéquat des SPEs particulièrement produites par *Cloacibacterium normanense* et des SPES extraites directement de boues. L'effet des méthodes d'extraction sur la composition chimique des SPEs et leur propriété (comme biofloculants) a été bien démontré.

Cette étude nous a confirmé notre hypothèse (III) dont le choix de la méthode d'extraction dépend de l'origine de SPEs (biofilm ou cultures bactériennes).Ainsi, ces résultats ont mis en évidence la sensibilité des SPEs (leur composition et propriété) aux méthodes d'extraction utilisées.

1.9.3 …… et Rôle dans la floculation, décantation des boues et dans la fixation des éléments métalliques

En premier lieu, ce travail a permis de révéler le potentiel floculant des fractions des SPEs enrichies en polysaccharides et protéines synthétisées par la souche *Cloacibacterium normanense.* 72 % d'activité de floculation a été déterminé utilisant (13 mg S-EPS/gm de boues) en comparaison de 66% en ajoutant 10 mg de Zetag/g de boues. Une décantation systématiquement bonne (40 mL/g) a pu être associée à une activité bactérienne intense : concentration importante en SPEs après une vingtième de minutes en comparaison de 50 ml/g (pendant 30 minutes) utilisant le Zetag.

Dans un deuxième temps, à l'aide d'incubation dans des solutions contenant diverses enzymes, nous avons cherché à cibler plus spécifiquement les protéines ou polysaccharides afin de déterminer quelles étaient les SPEs sources de cohésion dans les boues. Ces expériences nous ont permis de mettre en évidence deux types de digestion enzymatique (protéinase K et cellulase) permettant d'avoir deux structures cohésives distinctes. D'après nos résultats, la première structure obtenue suite à la digestion par protéinase K résulte une augmentation de la turbidité. En revanche, la deuxième structure issue d'un traitement enzymatique utilisant la cellulase contribue également à une augmentation de la turbidité, mais moins importante que celle obtenue par protéinase K. Nous avons pu déduire de ce dernier point que la cohésion établie consiste en une matrice majoritairement protéique. Elle était basée sur l'établissement de liaisons ioniques et en particulier sur l'existence d'un pontage par les ions calcium entre les protéines. Au travers de cette étude, nous avons donc abouti à une vision plus précise des mécanismes de cohésion dans les flocs obtenus en montrant que l'établissement de ponts

ioniques entre protéines et la présence d'une gangue compacte de polysaccharides étaient des facteurs assurant la cohésion.

La dernière partie de nos travaux est l'étude des capacités de sorption des EM par les SPEs. La synthèse bibliographique a montré que cette sorption est liée aux interactions possibles entre les groupements fonctionnels des molécules organiques des SPEs et les EM. L'analyse infrarouge a confirmé la présence dans les extraits de SPEs de groupements carboxyles, carbonyles, amines, amides, hydroxyles et phosphoriques souvent évoqués dans la littérature comme impliqués dans ces interactions. Notre étude a donc permis de montrer que les SPEs représentent des biosorbants forts des métaux lourds tels que le Nickel, l'Aluminium et le Fer.

Il serait intéressant de se rapprocher des conditions naturelles en évaluant la capacité des SPEs à biolixivier des métaux retenus dans des sols naturels contaminés à forte dose. Il est également important d'évaluer la potentialité de SPEs dans l'élimination de la couleur.

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CHAPITRE 2

CRITICAL REVIEW ON EPS PRODUCTION, SYNTHESIS AND COMPOSITION FOR SLUDGE FLOCCULATION. A REVIEW.

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2 CRITICAL REVIEW ON EPS PRODUCTION, SYNTHESIS AND COMPOSITION FOR SLUDGE FLOCCULATION. A REVIEW

2.1 Abstract

Extracellular polymeric substances (EPS) produced by microorganisms represent biological macromolecules with unfathomable potentials and they are required to be explored further for their potential application as a bioflocculant in various wastewater sludge treatment. Although several studies already exist on biosynthetic pathways of different classical biopolymers like alginate and xanthan, no dedicated studies are available for EPS in sludge. This review highlights the EPS composition, functionality, and biodegradability for its potential use as a carbon source for production of other metabolites. Furthermore, the effect of various extraction methods (physical and chemical) on compositional, structural, physical and functional properties of microbial EPS has been addressed. The vital knowledge of the effect of extraction method on various important attributes of EPS can help to choose the suitable extraction method depending upon the intended use of EPS. The possible use different molecular biological techniques for enhanced production of desired EPS were summarized.

2.2 Keywords

Extracellular polymeric substances, metabolic pathways, Extraction methods and flocculation properties.

2.3 Introduction

In general, sludge settling is improved by the addition of synthetic polymers, but they are known to be expensive and may further pollute the environment (Deng et al. 2003). To minimize the use of synthetic flocculants in sludge settling an alternative and novel approach will be to use eco-friendly bio coagulants/bioflocculants. The role of extracellular polymeric substances (EPSs) produced by sludge microorganisms during the wastewater treatment process have been extensively studied (Balasubramanian et al., 2008 and 2010; More et al., 2014; Hay et al., 2010). Recently, a demand of biopolymers for various industrial, biotechnological and environmental applications like flocculation, settling, dewatering of sludge, dyes and metal removal from wastewater has rekindled the interest in EPS production (Nontembiso et al., 2011; Zhang et al., 2012; More et al., 2014).

The main characteristic of EPS is to enhance aggregation of bacterial cells and suspended solids (SS). Adhesion and cohesion occur between EPS and the biomass along with suspended solids by complex interactions such as London forces, electrostatics interactions and hydrogen bonding, which leads to the formation of flocs. These EPS properties make them suitable for many applications such as sludge flocculation, settling, dewatering, metal binding ability and removal of toxic organic compounds (Solis et al., 2012; Nouha et al., 2015; Jia et al., 2011; Chien et al., 2013).

Microbial EPS biosynthesis promotes the attachment of the cells to a solid support. It helps in establishment and continuation of microbial colonies to a mature biofilm structure and protects from environmental stress. In 2010, Rehm et al. reported on critical EPS biosynthesis and metabolic pathways. EPS biosynthesis pathway depends on the type of EPS being produced i.e. homopolysaccharides or heteropolysaccharides. Three major steps involved in EPS synthesis are (i) assimilation of a carbon substrate, (ii) intracellular synthesis of the polysaccharides and (iii) EPS exudation out of the cell (Vandamme et al., 2002). However, these EPS production steps depend on multiple factors like the microbial species (genes involved in EPS synthesis), medium composition (carbon and nitrogen source, C/N ratio), and operating conditions (pH, temperature, dissolved oxygen).

Many EPS extraction methods have been used to extract EPS produced by pure microbial cultures (laboratory conditions) and mixed culture (activated sludge) (Nouha et. al. 2016;

Nguyen et al., 2016). Chemical, physical and combination of both methods were used for EPS extraction (Nouha et. al. 2016; Comte et al., 2006a; Nguyen et al., 2016). The efficiency of EPS extraction by different methods have been compared (Comte et al. 2006a; Liu and Fang, 2002) based on the quantity and the composition of extracted EPS. EPS is mainly composed of carbohydrates and proteins. Carbohydrate was mainly observed in EPS produced from pure cultures, whereas proteins were found in higher quantities in the sludge-EPS of many wastewater treatment plants (Liu and Fang, 2002). However, the EPS chemical structure (functional group), molecular weight and its effect on bioflocculant activity were greatly limited by extraction methods, which were never reviewed.

Scientific findings on general metabolism required for EPS precursor biosynthesis and different metabolic engineering strategies for EPS overproduction in some bacterial strains are reported in this review. Secondly, the significant recent developments concerning the impact of extraction methods on EPS composition, chemical structure and molecular weight was critically reviewed and discussed in the ambit of sludge flocculation.

2.4 Composition of EPS

The chemical structure of polymeric substances secreted by the microbial cells depends upon different environment and they are highly diversified. The best investigated components of EPS are polysaccharides and proteins (Balasubramanian et al., 2010; More et al., 2012; Nouha et al., 2016). The presence of humic substances and nucleic acids as part of EPS were also reported in some of the previous studies (Sutherland et al., 2001; Nguyen et al., 2016; Nouha et al., 2016).

2.4.1 Polysaccharides (Carbohydrates)

Most EPS produced by microorganisms contains carbohydrate or polysaccharides. Microbial exopolysaccharides are comprised of either homopolysaccharides or heteropolysaccharides (Monsan et al., 2001). Homopolysaccharides are composed of only simple sugars and heteropolysaccharides contain repeated units of various monosaccharides such as D-glucose, D-galactose, L-fructose, L-rhamnose, D-glucuronic acid, L-guluronic acid and D-mannuronic acid. For example, Alginate is a heteropolysaccharide produced by Pseudomonas aeruginosa and Azotobacter vinelandii, which is composed of D-mannosyl and L-glucuronosyl residues. However, Dextran, a homopolysaccharide consisting only dextrose (glucose) units, is produced

by Leuconostoc sp. and Streptococcus sp. (Rehm et al., 2010). The carbohydrate content of EPS can get affected by various factors during the production and extraction of EPS. The major factors that significantly affect the carbohydrate content of EPS are the microorganism, carbon substrate, nutrients (N, P) and the extraction method utilized for extraction.

The microbial species is also one of the main factors that define the composition of EPS produced based on their genetics and metabolic pathways. Although, the same strain can also produce EPS with different concentration and compositions when fed with various carbon or nitrogen source in the cultivation media. It was reported that Lactobacillus delbrueckii produced 175 mg/L of EPS using glucose as carbon source whereas only 69 mg/L of EPS was obtained from fructose (Yuksekdag et al., 2008).

The use of different carbon sources had a considerable change in EPS concentration and composition. Ye et al. (2011) reported that the polysaccharide content in loosely bound EPS (LB-EPS) produced in the activated sludge using acetate was lower than grown in starch or glucose. The possible cause of this phenomenon may be due to the different metabolic pathways employed by the microorganism to metabolize glucose and sodium acetate. Sodium acetate can enter the citric acid cycle directly, but glucose and starch have to be degraded to pyruvate and then oxidized to form acetyl- CoA before it enters the citric acid cycle (Ye et al., 2011).

Furthermore, the effect on the content of EPS components was evaluated by varying nitrogen and phosphorus ratio by Hoa et al. (2003). The content of total EPS produced in AS (activated sludge) media ranged from 24.4 to 89.9 mg/g SS with 16 to 94% carbohydrate component of the total EPS. It was reported that phosphorus had a more significant effect on the carbohydrate content of EPS than nitrogen (Hoa et al., 2003).

Shin et al. (2001), reported that maximum EPS concentration observed by physical extraction methods were 166 mg/g DW (dry weight) of EPS and 183 mg/g DW of EPS from sludge A and B, respectively (Comte et al., 2006a). However, a low content of 24–53 mg polysaccharides/g EPS DW was observed when chemical extraction methods were used. Thus the carbohydrate content of extracted EPS varied widely as a function of sludge origin and the extraction conditions or the method used.

Therefore, the variation in carbohydrate content of EPS can be attributed to factors like media composition (carbon and nitrogen source), extraction methods and growth conditions, which in turn can affect the EPS bioflocculant property.

2.4.2 Protein

Ton-That et al. (2004) stated that the protein was the principal component of the EPS matrix in the activated sludge and EPS (protein) production was not hugely affected by the type of substrates used for microbial growth. These results were in agreement with the observations of Frolund et al. (1996) and Li and Yang (2007) who also reported a consistent protein content (in activated sludge EPS), when microbe was supplied with different types of carbon sources (glucose, sodium acetate). Hoa et al. (2011) investigated the effect of nitrogen supplementation and reported that the protein content of EPS could be affected by nitrogen (NH_4Cl) limiting situations, which result in an increase of protein content of EPS (1.25 to 8.56 mg protein/g SS). It was found that the protein content of EPS was inversely proportional to nitrogen content in the activated sludge, while it remains unaffected by phosphorus.

2.4.3 DNA and humic like substances

DNA or nucleic acid is an intracellular component once released by cell lysis, which could be adsorbed to EPS matrix. Humic substances are components which are present naturally in activated sludge from hydrolysis of organic residues. The humic substances get adsorbed to EPS matrix (biofilm) by different functional groups like a carboxylic and phenolic group. A biofilm is defined as an aggregation of bacteria enclosed in a matrix consisting of a mixture of polymeric compounds (Vu et al., 2009)

Nucleic acids and humic substances have been reported to influence the rheological properties and stability of biofilms (Neu, 1996). The extracellular DNA (eDNA) is required for the initial establishment of biofilms by *Pseudomonas aeruginosa*. The eDNA helps in bacterium-surface adhesion by modulating charge and hydrophobicity interactions between the microbe and the abiotic surface (Nguyen et al., 2016). Similarly, the biofilm is formed by many other bacteria that specifically release DNA in stress conditions or due to cell lysis (Marvasi et al., 2010).

As evident from the discussion above, EPS biochemical composition is affected by many factors like microbial species, carbon source, nutrient supplementation and the downstream extraction methods. The composition of the EPS molecule is very important as it determines ultimately the functional property of the molecule as bioflocculant. The chemical composition of the EPS thus produced can determine its suitability for various kinds of applications. Among the novel applications that EPS can be used for metal removal. EPS as

carbon substrate has drawn the significant attention of researchers and the following section is dedicated to these two applications of EPS.

2.5 EPS adsorbent

Heavy metals removal from a polluted environment is a major challenge in bioremediation, which has been studied extensively using microbes. EPS produced by many microorganisms are of particular significance to the bioremediation process because of their metal ions binding ability from solutions (Kachlany et al., 2001). The use of purified biopolymers in biosorption phenomenon is a cost-effective and useful approach than using methods like precipitation, coagulation, ion exchange, electrochemical and membrane processes used for metal removal (Gutnick and batch, 2000). EPS and other biopolymers exhibit excellent metal-binding properties with varying degrees of specificity and affinity (Gutnick and batch, 2000). The effect of and EPS bridging occur by electrostatic interactions with negatively charged functional groups such as uronic acids and phosphoryl groups of carbohydrates or carboxylic groups of amino acids in protein moiety. Besides, there may also be anionic binding by positively charged polymers or coordination with hydroxyl groups. EPS were able to chelate some metals (like Th4+ and Al3+) and bind them to the cell surface (Santamaria et al., 2003).

Polysaccharides and protein moieties of EPS, rich in negatively charged amino acids, including aspartic and glutamic acid, contribute to their anionic properties, which play a major role in complexation of metal ions (Mejare et al., 2001). DNAs are anionic in nature due to the presence of phosphate groups available in sugar-phosphate backbone of the molecule. The uronic acids, acidic amino acids and phosphate-containing nucleotides, act as negatively charged components of EPS, which are known to bind with multivalent cations by electrostatic interactions (Beech et al., 2004). Therefore a change in EPS composition will affect the availability of the functional groups which are responsible for metal binding and consequently may result in to decrease in the metal binding efficiency.

Numerous bacteria have been shown to produce exopolymeric substances. Several studies (Prado et al., 2005; Foster et al., 2000) have compared and evaluated the metal binding potential of microbial biofilms obtained from activated sludge and purified exopolysaccharides (Table 2.1). Over 90% of metal adsorption was achieved at an EPS concentration (67 and 160 mg/L) using different bacterial strain such as Rhizobium etli M4 and Paenibacillus polymyxa (P13), respectively. The cells and EPS showed a strong ability to bind Mn, Pb and Cu ions (Prado et al., 2005; Salehizadeh et al., 2003; Foster et al., 2000; Nouha et al., 2016).

Sludge EPS exhibited greater metal complexation, which suggests that the carboxylic and phosphoric groups in carbohydrate moiety of EPS might have played a major role in the complexation of metals (Singh et al., 2000). Few researchers (Ran et al., 2013; Adagalla et al., 2009; Yuncu et al., 2006; Comte et al., 2006b) investigated different factors affecting the metal binding ability to EPS. The metal sorption capacity of the activated sludge was dependent upon the C/N ratio and composition of sludge. An increase in C/N ratio (by supplying a carbon source such as glucose) resulted in an increase in Cd^{2+} but decrease in Cu^{2+} sorption capacity. The sorption capacity could be explained by the variation of EPS concentration and composition by using different C/N ratio. However, the adsorptive capacity of Zn and Ni was independent of C/N ratio.

EPS hydrophobicity is another significant factor, which favors the sludge flocculation and settling. EPS hydrophobicity can be rendered by EPS-proteins produced by the microbial communities. According to Geyik et al. (2016), higher protein content or protein to carbohydrate (P/C) ratio gives higher EPS hydrophobicity which is correlated with the substrate provided to the microbial communities. EPS hydrophobicity is significantly affected by the functional groups in its protein fraction. The hydrophobicity is an important factor when EPS is intended to use in organic pollutant removal (Flemming and Wingender 2002). A strong correlation was demonstrated by Zita and Hermansson (1997) between sludge particle adhesion and EPS hydrophobicity.

pH of the surrounding environment affects the deprotonation state of the side residues of the protein fractions present in EPS. At lower pH, the acidic residues are protonated to have higher hydrophobicity. Thus this allows dense intramolecular hydrogen bonding between flocculating particles and further improve sludge compactness and settling efficiency (Wang et al. 2013)

Table 2- 1: Metal-binding potential of bacterial EPS and wastewater sludge EPS

EPS producer	EPS concentration (mg/L)	Metal biosorbed	References
Methylobacterium	184.2	Pb(II)	Kim et al., 1996
Organophilum	200.3	Cu(II)	
Pseudomonas	320	Cu(II)	Kazy et al., 2002
aeruginosa Cur			
Rhizobium etli M4	67	Mn(II)	Pulsawat et la., 2003
Enterobacter cloaceae	8.3	Cr(VI)	Iyer et al., 2004
AK-I-MB-71a			
Paenibacillus	160	Cu(II)	Prado et al., 2005
polymyxa P13			
Paenibacillus jamilae	228	Pb(II)	Morillo et al., 2006
CECT 5266	55	Cd(II)	
	40	Cu(II)	
	37	Zn(II)	
	15	Ni(II)	
	10	Co(II)	
Dried activated sludge	294	Cu(II)	Gulnaz et al., 2005
used in batch			
systems, particle size			
<0.063 mm, pH 4.0, 20^{o}C.			
Powdered waste sludge	116	Cu(II)	Pamukoglu et al.,

(PWS) from paint industry used in batch kinetics, particle size 64 µm, pre-treatment with 1% H_2O_2			2006
EPS produced by anaerobic sludge of sulfate-reducing bacteria used	272	Cd(II)	Zhang et al., 2006

2.6 EPS as carbon source

Microorganisms often live and exposed to stressful conditions caused by natural environments, thus the production of EPS augments the survival capacity (Patel and Gerson, 1974). The bacterial EPS was found to be utilized either by self or neighboring microbes during carbon deficiency, using extracellular enzymes and this enzyme complex can be utilized for complete degradation of EPS (Patel and Gerson., 1974; Pirog et al., 1997; Zhang and Bishop 2003; Wu et al. 2007). The study by Pirog et al. demonstrated a successful utilization of EPS as a carbon source by isolated *Acinetobacter* sp. from a soil sample.

The EPS biodegradability studies were performed by various authors (Wu et al., 2007). Zhang and Bishop, (2003) observed that carbohydrate fraction of the supplemented EPS as carbon source was consumed more rapidly than the protein fraction. Pannard et al. (2015) investigated and confirmed the biodegradability of EPS by bacteria for growth under nitrogen (or phosphorus) and carbon limiting conditions.

The susceptibility of EPS towards degradation depends on the hydrolyzing agent, which leads to breaking the polymer chains, and also depends on the chemical nature of the polymer (Wingender et al., 1999). Many reports (Neyens et al., 2003; Watson et al., 1987) have been presented on the influence of sludge treatment in EPS degradation.

Watson et al. (1987) found that protein and carbohydrates, the main component of EPS in activated sludge are degraded or hydrolyzed by heat treatment. Neyens et al. (2003) revealed that heat treatment (120 °C) alters the structure of the sludge in term of proteins

and carbohydrate moieties of EPS and transforming some of EPS from less degradable to easily degradable. Acid-thermal and alkaline-thermal hydrolysis were also used by Neyens et al. (2004). They indicated an increase in the sludge filtration rate (capillary suction time (CST)-values). Extreme pH (acidity or alkalinity) also causes EPS proteins to lose their natural shapes thus improved their degradability. Polysaccharides and the other component of EPS, are unstable in strong acids, which lead to acid hydrolysis of the glycosidic linkages (Figure 1) (Wingender et al. 1999). The strong alkali may solubilize gels not only because of chemical degradation but also because of the ionization of the carboxylic groups, which leads to subsequent solubilization of EPS (Wingender et al., 1999). As the EPS is a complex molecule, different treatments could interfere with their chemical structure and even could form a novel compounds. Figure 1 presents alginate as an example of polymers and their transformation due to several different treatment methods.

Apart from being an excellent bioflocculant, EPS can also act as an adsorbent for heavy metal removal application and source of carbon substrate for various biotechnical applications. To attain more scientific knowledge and potential industrial applications of EPS needs to be explored. It is also important to have proper EPS production processes with higher yield and properties for desired applications. The first pre-requisite for any process improvement is to understand the underlying biosynthesis mechanism at molecular level. This information can significantly improve and enhance the EPS concentration and quality during a production process by using advanced techniques, which are discussed in the following sections.

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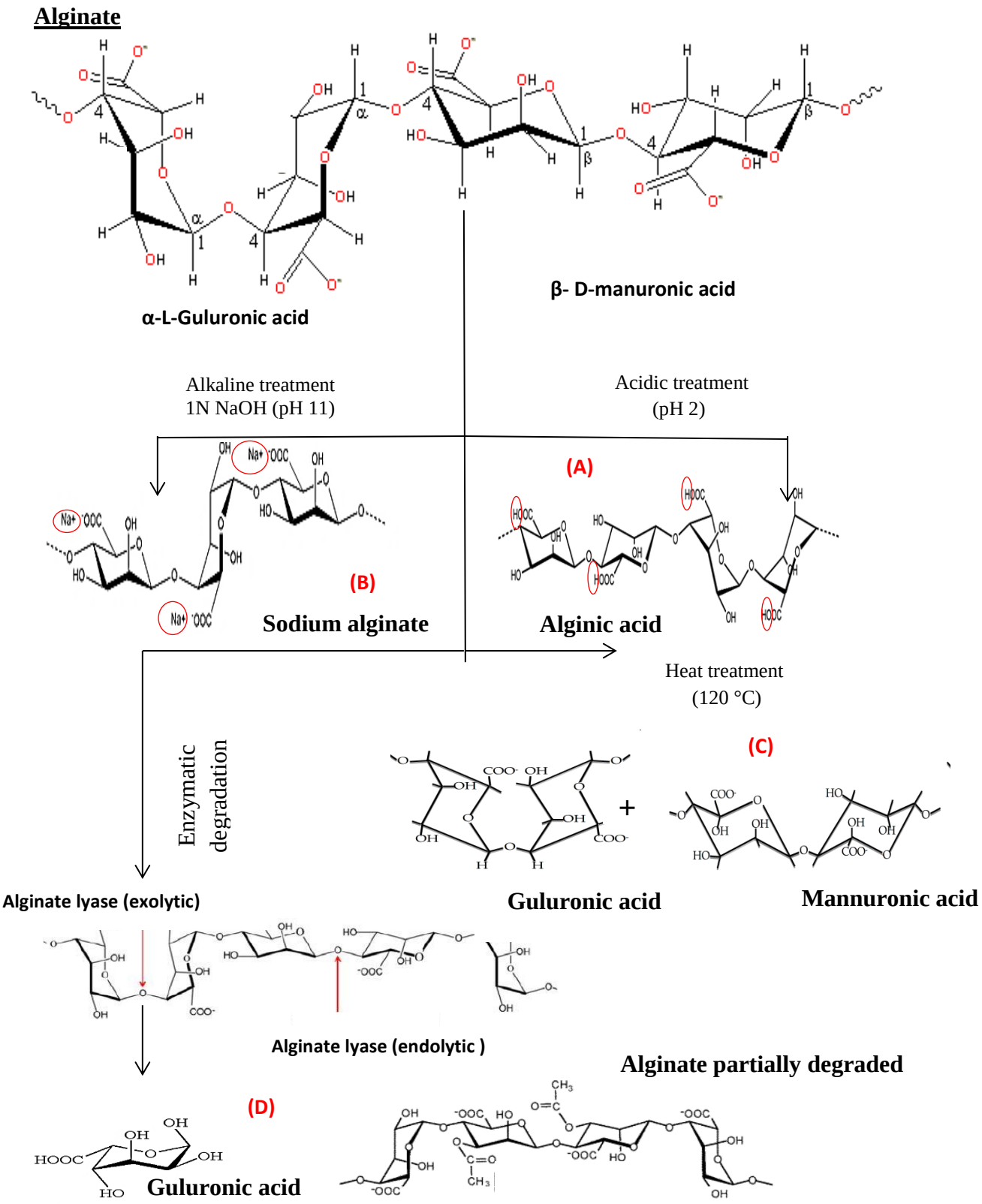

Figure 2-1: Alginate degradation: A) Acidic treatment; (B) Alkaline treatment; (C) Heat treatment and (D) enzymatic degradation

2.7 EPS biosynthesis

Extracellular polysaccharide synthesis by microorganisms is accomplished by a specific secreted enzyme (polymerization and precursor synthesis enzymes), and synthesis can occur either outside the cell or within the cell wall (Roger, 2002). Table 2-2 present the several classes of polymers and their diverse characteristics.

The EPS biosynthesis pathway can be divided into three major steps: (i) synthesis of precursor substrate, (ii) polymerization and cytoplasmic membrane transfer and (iii) export through the outer membrane (Figure 2-2). These three steps vary with carbon source used, from one microorganism to the other and specifically depends on polymers classes.

2.7.1 Synthesis of precursor substrate

This step involved in the conversion of intermediate sugar metabolites into the EPS precursor, such as nucleoside diphosphate sugars [for example Guanosine diphosphate (GDP)-sugar] corresponding to substrate or carbon source assimilated. Sugar nucleosides (nucleoside diphosphate sugars) provide an active form of the monosaccharides and also provide the bacterial cell with a means of interconversion of various monosaccharides through epimerization, dehydrogenation and decarboxylation reactions.

Polymer-specific enzymes are required for biosynthesis of the active polymer precursor, which is the first committed step and has been targeted by metabolic engineers to enhance polymer production and to allow the synthesis of tailor-made polysaccharides. In this context, for each type of polymers (dextran, xanthan, and alginate) specific precursors and specific enzymes were involved in their biosynthesis (Lin and Hassid, 1966). For example, uridine diphosphate (UDP)-glucose is the direct precursor of cellulose synthesis by *Acetobacter xylinum* and pullulan production by *Aureobasidium pullulans,* using uridine diphosphate glucose (UDPG) pyrophosphorylase and glucosyltransferase activity, respectively (Youshinaga et al., 1997; Duan et al., 2008). Similarly, every polymer has a dedicated precursor and enzymes which vary from organism to organism.

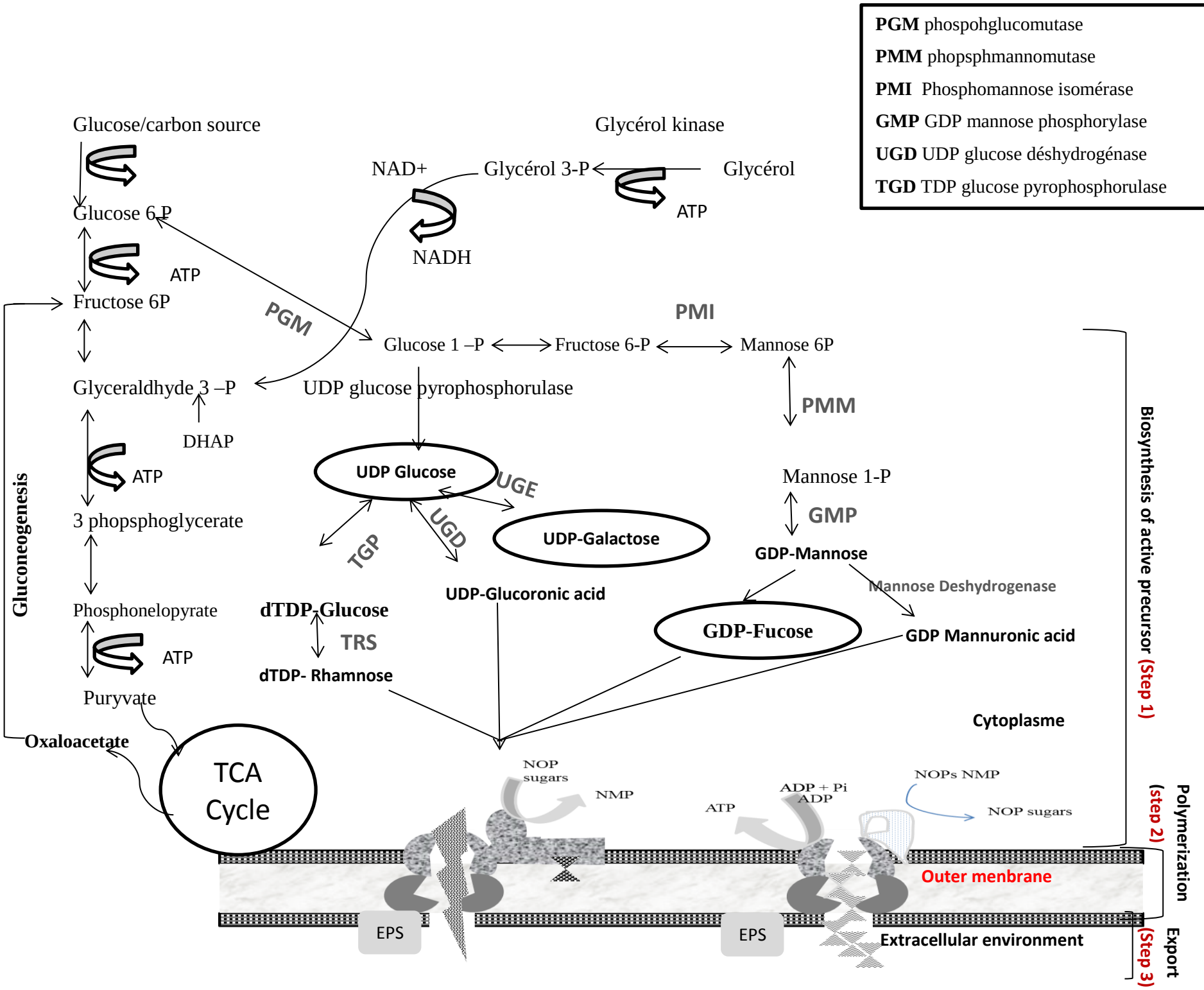

Figure 2-2: Biosynthesis pathway steps of bacterial polymers

2.7.2 Polymerization and cytoplasmic membrane transfer

The second step in EPS biosynthesis involves the transfer of the precursor nucleoside diphosphate polymerization of the monomers to polymer. Monosaccharides activation by the formation of sugar nucleotides complex is followed by sequential addition of the sugars on to an isoprenoid lipid and simultaneous addition of acyl groups. Highly specific sugar transferase enzymes facilitate a transfer of the monosaccharides and acyl groups to isoprenoid lipid acceptors (bactoprenol, C55-isoprenoid lipid) located in the cytoplasmic membrane. The oligosaccharide repeating units with acetyl, pyruvyl and other acyl adornments are then polymerized. After polymerization of the repeating units, the polysaccharide is excreted through the outer cytoplasmic membrane, which might be coordinated via the formation of a multi-protein complex involving cytoplasmic and outer membrane proteins as well as periplasmic proteins.

The biosynthetic pathway of xanthan (Figure 2-2 as an example of polymer biosynthesis) has been explored by Rosalam & England, (2006). The synthesis of Xanthan starts with the assembly of repeating pentose units (GDP–mannose and UDP–glucoronate). These units are then polymerized by GumE, which is the catalytic subunit of the xanthan polymerase, localized in the cytoplasmic membrane and then produces the macromolecule xanthan (Figure 2-2). Once xanthan is synthesized, it is exuded into the extracellular environment.

In the case of Alginate, this step requires the transfer of the cytosolic precursor GDP-mannuronic acid across the cell membrane and the polymerization of the monomers to polymannuronate using Glycosyl- transferase (Alg8) (Figure 2-2 and 2-3) (Rehm et al., 2010).

2.7.3 Export through the outer membrane

The final stage of EPS is the secretion from the cytoplasmic membrane. It involves passage across the periplasm, outer membrane and finally excretion into the extracellular environment. AlgE is the gene which produces the enzyme involved for Alginate export and GumJ is the gene producing the enzyme responsible for Xanthan excretion (Figure 2-3).

In EPS synthesis, lipid transporters provide an anchor to the extracellular membrane, which facilitates the precise and orderly formation of the carbohydrate chain proceeded by the transport of the chain through the cell membrane. Polysaccharides are polymerized on the inner side of the cytoplasmic membrane and then directly exported through the intermediary of a lipid transporter. These transporters are long-chain phosphate esters and isoprenoid

alcohols, identical to those described in the biosynthesis of lipopolysaccharides and peptidoglycans (Sutherland, 1999). They play an important role in heteropolysaccharide synthesis, which is combined with the EPS excretion. After excretion, the intervention of an enzyme specific to the EPS may liberate the polymer. Table 2-2 shows further categorization dividing the polysaccharides into repeated unit polymers and non-repeating polymers, presenting their main compounds, precursors and polymerizing enzymes.

In conclusion, the three dedicated steps of EPS synthesis requires an array of dedicated genes working in a much-regulated manner. These genes are translated to yield the proteins, which eventually perform the tortuous task of EPS synthesis. Molecular biologist and genetic engineers have targeted these genes and proteins in order to engineer the strains to have EPS of desired quality and quantity. It will be interesting to understand and overlay different molecular engineering approaches in the ambit of overproduction of EPS.

Table 2-2: Metabolic characteristic of Bacterial polymers and their fermentation conditions

EPS	Polymer localization	Precursors	Polymerization enzyme	Fermentation conditions	Microorganisms	EPS (g/L)	Références
Cellulose	Extracellular	UDP–d-glucose	Cellulose synthase (BcsA)	Glucose/fructose pH = 4–5; 30 ◦C; 40 h	*Acetobacter xylinum*	7- 23.9	Hwang, et al., (1999),
Dextran	Extracellular	Saccharose	Dextransucrase (DsrS)	Sucrose pH = 5.5; 35 ◦C; 1 bar; 5 days	*Leucomostoc sp.*	8 - 17	Santos, Teixeira, and Rodrigues (2000)
Xanthan	Extracellular	UDP–glucose, GDP–mannose and UDP–glucuronate	Xanthan polymerase (GumE)	Molasse pH = 7; 28 ◦C; 1 bar; 24 h	*Xanthomonas campestris*	50	Kalogiannis et al. (2003)
Alginate	Extracellular	GDP–mannuronic acid	Glycosyl-transferase (Alg8)	Glycérol+ ethanol pH = 5.8-6.5; 28 ◦C;150 rpm ; 48h	*Pseudomonas sp*	15.2	Hay et al., 2010)
Pullulan	Extracellular	UDP–d-glucose	-	Sucrose pH = 4–4.5; 30 ◦C; 100 h	*Aureobasidium pullulans*	1.3 –5 2.5	Jiang (2010), Ravella et al. (2010)
Curdlan	Extracellular	UDP–glucose	Curdlan synthase (CrdS)	Glucose/sucrose pH = 5.5; 22–26 ◦C; 3–4 days	*Rhizobium spp*	1–5	Pavlova et al., (2005)

Others EPS	Glycérol/glucose 6–18 pH = 7; 30 ◦C, 4 days	*Enterobacter sp.*	6-18	Alves et al. (2010)
	Sucrose/maltose pH = 6.8–9.8; 54–87 ◦C	*Geobacillus sp.*	0.1- 14	Kambourova et al. (2009)
	Sucrose/glucose pH = 7; 32–37 ◦C	*Halomonas sp.*	1.6 - 4.5	Bejar et al. (1998) and Poli et al. (2009)

2.8 Engineering strategies of Bacterial Polysaccharides

Several studies were performed to genetically engineer the EPS-producing microbes to produce novel polymer variants while improving the production. As presented in the previous section (2.7), various enzymes are involved in all three stages of the metabolic pathway of EPS biosynthesis. Gene-encoded enzymes regulate the formation of nucleotide sugar metabolite, chain length determination, repeat unit assembly, polymerization and export of polymers (Figures 2-2 and 2-3) (Broadbent et al., 2003).

Recently intensive researches have been performed by focusing on the underlying mechanisms behind bacterial exopolysaccharide biosynthesis pathways, on different operons, promoters and the expression of regulatory gene segments. The variability of sugar precursors, protein structure analysis, and new bioinformatics tools provide new avenues to enhance the EPS biosynthesis and understand the principal engineering strategies of EPS formation.

One of the primary goals of EPS production engineering to increase the volumetric productivity of EPS in a very cost effective manner. In this context, we focus on the recent advances in potential engineering strategies for better EPS production. Vorholter et al., (2008) attempted to increase the pool of sugar nucleotides (EPS precursors) to enhance the carbon flux toward the final polymer yield. Guo et al. (2014) studied xanA gene producing phosphoglucomutase (PGM) and phosphomannomutase (PMM) enzyme, which is involved in the conversion of glucose-6-phosphate to glucose-6-phosphate. They suggested that xanA is a regulator gene and it has a key role in precursor metabolite overexpression.

Huang et al., (2013), Schatschneider et al., (2013) and Wu et al. (2014) studied *Xanthanmonas campestris* EPS production in detail and found that it possess a series of 12 genes embedded in tandem. This operon includes seven genes needed for monosaccharide transfer and acylation of lipid intermediate to form the completely acylated repeating unit (Figures 2-2 and 2-3). It has been suggested that alteration in promoters related to this operon can yield higher precursor metabolites (Galindo et al., 2007).

Vojnov et al., (1998) studied the gum-protein operon containing gumBCDEFGHIJKLM (Figure 2-3) gene fragment. They tested a simple idea of whether inclusion of an additional promoter as upstream gumC may improve xanthan biosynthesis. It was found that promoter insertion to upstream of gumC resulted in enhanced yields and overexpression of the transcription of an operon and eventually increased the xanthan biosynthesis from 66 mg cell mass to 119 mg/g cell mass (Vojnov et al., 1998).

Schatschneider et al. (2013) studied most sensitive gene segments that significantly affect EPS synthesis. The study demonstrated that the overexpression of gumD is the key enzyme involved in the EPS assembly construction and precursor conversion (Figure 2-3). Thus, it was suggested to clone the entire gumD gene cluster of a 16kb chromosomal fragment with high copy number in *X.campestris*. The

results indicated elevated expression of all biosynthetic eps gene, which could be achieved by cloning them on a high copy number plasmid (Boelis et al., 2003; Janczarek et al., 2009).
In another strategy, the idea was to engineer the EPS molecules at the molecular level for having the desired behavior and material characteristics of the final polymer while improving the property as bioflocculant. For example, this molecular alteration can be deleting substituents or monomeric sugar residue from the side chain.

Deactivation of the GT GumI gene resulted in a truncated tetramer xanthan version, as a consequent of deletion of the terminal mannose, it was found to have much lower viscosity. Similarly, inactivation of GumK (a GT), causes the removal of glucuronic acid side chains resulted in an enhanced viscosity of the EPS as compared to wild type EPS (Hassler and Doherty, 1990). The gene deactivation is performed by homologous insertion of foreign genes within the operon segment at active gene locus (GT GumI and GT GumK). They have reported that transgenes can suppress their expression and that of endogenous homologous genes. This phenomenon has been called co-suppression (Hassler and Doherty, 1990).

Many efforts were done in engineering the degree of acetylation and pyruvylation of various polymers (alginate and xanthan), to control their rheological properties (Donati and Paoletti, 2009). The level of O-acetylation and pyruvylation can be controlled reasonably well using specific strains/mutants or altering the growth media and controlling cultivation conditions such as aeration, pH and temperature (Pena et al., 2006; Gaytan et al., 2012).
Interesting insights were given by Rehm, (2010) and Galván et al. (2013) regarding the general structure and functional relationships. The extent of acetylation and pyruvylation has antagonistic effects on viscosity. The GumL enzyme incorporates pyruvyl residues to the external β-mannose residues, while the acetyl residues are incorporated into internal α-mannose units by GumF, and external β-mannose by GumG (Becker et al., 1998). A high degree of pyruvylation by increasing the transcription of gumL (cloning an additional promoter upstream gumL) resulted in higher viscosity, whereas more acetyl group decreased the viscosity of the resulting EPS (Gaytan et al., 2012; Rehm, 2015).

Taxonomically different microbes can produce the same types of extracellular compounds with different concentration. *Pseudomonas aeruginosa* and *Azatobacter vinelandii*, despite having most of the genes involved in the biosynthesis, their organization, regulations and genetic switch clusters identical have differences at transcriptional and functional level expression of EPS.
In *Pseudomonas aeruginosa* and *A. vinelandii* a cluster of 13 structural and five regulatory genes (Hay et al., 2010) involved in EPS biosynthesis (Figure 2- 3). In *P. aeruginosa* the transcriptome is regulated

by algR, algB, algC and algD gene segment that are algT dependent whereas in *Azatobacter vinelandii,* these genes were independent of algT. The regulator gene algT encodes for the regulatory expressions of sigma factor (σ^{22}) which could explain the variation in alginate concentration (Muhammadi and Ahmed, 2007).

Though literature exists for genomic and proteomic level engineering to overproduce classical biopolymers, recombinant molecular engineering techniques are near to inexistent for EPS over production. Molecular techniques can be applied for transcriptional overexpression of RNAs involved in biosynthetic pathways, translational overexpression of the proteome involved in EPS biosynthesis. The most generic approach is an in-frame insertion of a strong promoter upstream of EPS operon to have more than basal level of constitutive or inductive expression of genome and proteome level.

Overexpression of EPS can be induced when the carbon flux channeling is streamed favorably toward the generation of precursor molecules like *nucleotide diphosphate-Glucose* conjugates by gene silencing, which divert carbon flux away from EPS synthesis without compromising the survival of the organism. Similarly, overexpressing enzymes involved in irreversible synthesis is a key mechanism and can be used as control points to induce diversion of this excess carbon pool toward EPS biosynthesis.

Bacteria produce a wide range of exopolysaccharides, which are synthesized via different biosynthesis pathways. A better understanding of basic biochemistry and genetics involved in exopolysaccharides biosynthesis and the regulatory mechanisms is critical for protein engineering approaches to produce novel polymers. At large scale production process having highly efficient downstream extraction is as important as enhancing the upstream process. Choice of downstream extraction method should be made diligently to obtain the maximum product yield without hindering its natural properties. In the following sections, the impact of various operational process parameters and downstream extraction process on EPS quantity and quality were discussed.

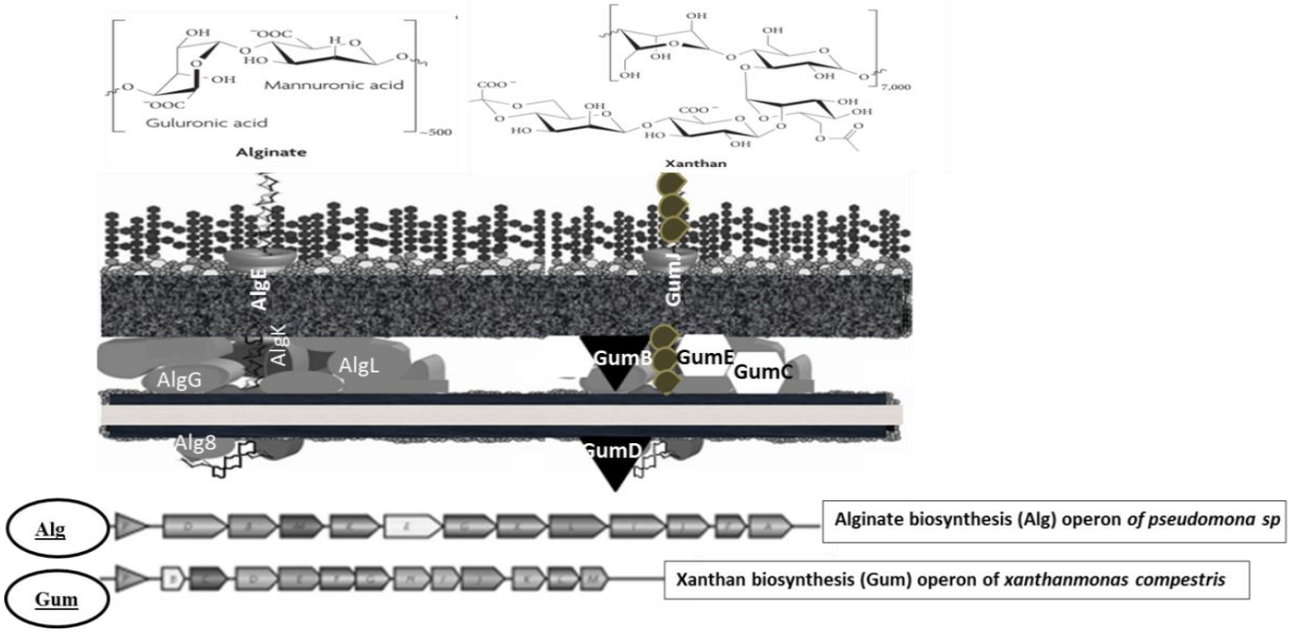

Figure 2-3: Model of Alginate and Xanthan biosynthesis and secretion mechanisms

2.9 EPS production methodology

2.9.1 EPS extracted directly from sludge

EPS has been successfully extracted directly from sludge by Urbain et al., (1993). They indicated that the EPS proteins extracted directly by ultra-sonication from municipal wastewater sludge were higher (97.8 mg/gVSS) as compared to 15.6 mg/g VSS of carbohydrate content. Liu and Fang (2002) efficiently performed EPS extraction from acidogenic and methanogenic sludge. The results revealed that carbohydrate was a major component in acidogenic sludge (62% w/w of EPS), while protein was a powerful component in methanogenic sludge (41% w/w of EPS).

Besides, Sponza, (2003) examined the EPS extracted directly from activated sludge treating various types of wastewaters. They found that the protein content of EPS is higher in the sludge treating winery and municipal wastewater than that of sludge produced in treating pulp-paper, textile and petrochemical wastewaters. Thus the EPS concentration varied accordingly to the type of wastewater treated in WWTPs.

Simon et al. (2009), reported that the nucleic acid content of EPS could be affected by the type of sludge from different municipal WWTP. High nucleic acid (7 mg/g EPS DW) concentration was observed in

Eerbeek municipal WWTP sludge as compared to 4 and 1 mg/g EPS DW in Emmtec and Revico WWTP, respectively.

2.9.2 EPS production using pure carbon sources

Although some researchers are convinced that the nature of the substrate cannot influence the composition of the EPS produced, however, many others have stated that medium composition either carbon source or nitrogen sources are important parameters in EPS biosynthesis and production (Bennama et al., 2012).

Li and Yang, (2007) reported that the activated sludge fed with glucose exhibited more EPS concentration than that fed with acetate. On the contrary, Ye et al. (2011) revealed that sludge fed with acetate was more favorable for high EPS production than that fed with glucose. Their results were also studied recently by Geyik et al., (2016), which explained how the type of organic substrate in a wastewater affects the production and composition of EPS. The activated sludge reactors were operated with three different feeds composed of various organic compounds, first of peptone, glucose, and acetate; then the second feed was only using only peptone and third feed with only glucose. They proved that the type of substrate affected the relative proportion of protein and polysaccharide content of EPS.

The effect of substrate and its suitability depends on upon the organisms. Different bacteria may produce different bioflocculants in composition and structure. For example, *Bacillus licheniformis* (Shih et al. 2001), *Nocardia amarae* (Takeda et al. 1992) produce protein bioflocculants whereas *Bacillus subtilis* (Yokoi et al. 1996) produce polysaccharide bioflocculant, and *glycoproteins* were produced by *Arcuadendron* sp. and *Arathrobacter* sp. (Wang et al. 1995). Shutten et al., (1998) reported that the *Lactobacillus* strains produce varying amounts of EPS with different sugar compositions when they are grown on sucrose, raffinose and lactose as a carbon source. The EPS produced by *Lactobacillus casei* with glucose as carbon source was reported to be different from that produced on lactose (Pahm et al., 2000). Vijayendra et al. (2003) investigated the effect of different hexose sugars (glucose, sucrose and lactose) on EPS production by *Agrobacterium, Alcaligenes, Pseudomonas* and *Xanthomonas*. Lactose was found to be the best carbon source for EPS production by *Pseudomonas* sp. whereas other bacterial strains favored sucrose.

Yuksekdag et al. (2008) studied the impact of various carbon sources on EPS production by *Lactobacillus delbrueckii, L. bulgaricus* and *Streptococcus thermophiles*. For all the strains, glucose was the most efficient carbon source and EPS concentration of 211 mg/L produced by *Lactobacillus delbrueckii sub sp.* was the highest concentration. The effect of carbon source on EPS synthesis by the

marine bacterium *Saccharophagus degradans* was also studied by Yolanda et al. (2015). *S. degradans* was able to grow in the mineral medium while producing EPS concentration depending on the carbon source: with glucose or starch, EPS 1.5 g/L; with galactose, sucrose, or xylose, EPS 0.7 g/L and with fructose, EPS 0.3 g/L. The results were in agreement with the recent studies (Qin et al., 2015; Mane et al., 2015). Lactose gave the maximum EPS concentration of 6.9 g/L as compared to 0.9 g/L when glucose was used (Qin et al., 2015). According to Mane et al. (2015), glucose gave maximum EPS concentration of 750 mg/L as compared to other substrates (lactose 390 mg/L; sucrose 670 mg/L) used.

Modification in feed media affects the sugar composition of EPS produced. The EPS composition characterized by 61.7% of galactose and 33% of glucose and 5.3% of mannose was produced by *L. bulgaricus* using milk as media. However, 39.7% of galactose, 57.9% of glucose and 1.8 % of mannose of total carbohydrate composition was found when the glucose was used as a carbon source (Petry et al., 2000).

In another case, the biosynthesis of extracellular polysaccharides in *Acetobacter xylinum* was improved by using galactose and xylose as carbon source comparing to fructose, sucrose and starch in the medium (Sutheland et al. 2001).

Recently, EPS from *Pseudomonas fluorescens* was produced using different concentrations of sucrose and sugarcane molasses as the carbon substrates (Sirajunnisa et al., 2013). Maximum EPS concentration of 2843 mg/L was obtained at 5% (w/v) sugarcane molasses concentration in the media. The sugarcane molasses as carbon source gave a higher EPS concentration than sucrose with 1389 mg/L of EPS concentration (Sirajunnisa et al., 2013).

It has been observed that selection of microbial source and growth substrates type, concentration and composition have a significant effect on EPS yield.

2.9.3 Use of Sludge as Nutrient source for EPS Production

Recently EPS production using sterilized sludge as nutrient and carbon source using pure bacterial strain isolated from wastewater sludge have been reported by Balasubramanian et al., (2010) and More et al., (2012). They indicated that different pre-treatment (heat, alkaline and acidic treatment) of sludge could vary the EPS production of pure cultures. More et al. (2014) also used a consortium of pure bacterial strains to improve the EPS production. Nouha et al. (2016) studied the sterilized sludge as a culture media using *Cloacibacterium normanense* for EPS production, and the sterilized sludge inoculated with pure culture also fed with crude glycerol as extra carbon source. High EPS concentration up to 13.3 g/L was recorded using only sludge as a growth medium, and 21.3 g/L of EPS was produced when the media was supplemented with 20 g/L of crude glycerol.

In addition to the carbon source, the extraction methods can also influence the EPS yield, chemical structure, molecular weight and their role as bioflocculant in wastewater treatment. Furthermore, the EPS produced by varying different optimized operating conditions has a different composition. Consequently, appropriate methods of extraction should be chosen to obtain desired EPS properties, which are required for specific applications. For this reason, different characteristics of EPS must be considered, including identification of the component monosaccharides and their relative proportions and the physicochemical properties of the final EPS.

2.10 EPS extraction methods

Several methods (centrifugation, sonication, heating, EDTA and CER) have been applied in different studies to extract EPS from pure cultures or undefined mixed cultures, mainly related to activated sludge and biofilms. The EPS extraction methods include various physical, chemical methods or their combinations. The extraction procedure must be selected considering the efficiency of the method to extract EPS in high yield. The best extraction method should not disturb the interactions that keep the EPS components together in the matrix.

2.10.1 Physical methods

Many physical methods have been tested to evaluate their extraction efficiencies and compare them to select the best technique of extraction. Comte et al. (2006a) studied three extraction techniques (centrifugation, sonication and heating). EPS was extracted with very high efficiencies by using heating method (82 mg/g VSS). High extraction of protein and polysaccharides content was obtained with a heating method as compared to others physical methods used (centrifugation and sonication). These results were in agreement with Tapia et al. (2009) studies. The concentration and composition of EPS extracted by heating (813 mg/g DW) were higher to that obtained by centrifugation (735 mg /g DW) method. Pan et al. (2010) compared two physical extraction methods (centrifugation and ultrasonication). They also observed that protein content was low in EPS samples prepared by centrifugation, as compared to the protein content in EPS sample extracted by ultra-sonication.

In this context, each researcher explored the reason for the variation in extraction efficiency while employing different EPS extraction methods, such as the physical methods. Comte et al., (2006a) proposed a hypothesis to explain these variations in results, that the proteins and polysaccharides moieties of EPS could be hydrolyzed during the extraction procedure by heating. According to Tapia et al. (2009) the heating extraction procedure allowed to extract the strongly bound EPS to flocs. However, some studies (Frolund et al., 1996; Liu and Fang, 2002) proposed that the high EPS extraction efficiency

by the heating method may be caused by significant cell lysis and disruption, which may reveal results into high protein content in EPS.

2.10.2 Chemical methods

As per literature, many methods have been proposed and applied to extract EPS from pure or undefined mixed cultures. The extraction efficiencies of chemical methods, such as cation exchange resin (CER), EDTA (Ethylene diamine tetra-acetic Acid) and NaOH methods have been studied (Frolund et al., 1996; Sheng et al., 2005; Liu and Fang, 2002).

Cation exchange resin along with a high concentration of NaCl has been used for the extraction of adhesive exopolymers from *Pseudomonas putida* and *Pseudomonas fluorescens* (Christensen et al. 1990). The CER method has become the most widely accepted EPS extraction method, largely because the resin used for selective extraction of EPS can be removed and recycled easily.

In recent study Zuriaga-Agustíetal et al. (2013) used CER and Triton X-100 as efficient EPS extraction methods to extract EPS from two different municipal MBRs. The protein and carbohydrates content was determined. This study was performed to understand the problem of membrane fouling of the MBR process due to EPS. Although EPS has effect fouling, it is not clear which EPS fraction (SMP or EPS) or component (proteins or carbohydrates) exert the most important contribution to membrane fouling. To elucidate the solution, activated sludge samples from two municipal MBRs were processed for extraction protocols comparison. They demonstrated that the proteins and carbohydrate content using Triton X-100 extraction method was higher (81.64 ±12.98 mg BSA/g VSS and 10.30 ±1.42 mg Glucose/g VSS, respectively) than cation exchange resin method (16.49 ±9.37 mg /g VSS and 3.93 ±2.47 mg /g VSS, respectively). They observed that CER protocol achieved lower extraction efficiency for the EPS than Triton X-100. The different values obtained between CER and Triton X-100 could be attributed to the floc composition. In fact, some researchers (Liu et al., 2010, Yu et al., 2009) asserted that the presence of two types of polymers in sludge flocs. One type of EPS is tightly bound within microcolonies of cells, and another is loosely bound in the floc matrix or at outer peripheries of the flocs. In this way, results may point that CER protocol withdraws mostly loosely bound EPS, whereas Triton X-100 extracts both types of EPS. The study of Meng et al. (2010) found that the EPS extracted by CER protocol was low comparing to Triton X-100.

Comparative study of chemical extraction methods has been exhaustively investigated. Tapia et al. (2009) conducted a comparative study of chemical and physical methods to understand the principal role of the different extraction methods on EPS composition and concentration. The concentration of EPS from bacterial culture of *Acidiphilium* sp. was higher when extracted by centrifugation (600mg

carbohydrate/g DW, 220 mg protein /g DW, 820 mg EPS/g DW) than extracted with NaOH (430mg carbohydrate/g DW, 170 mg protein/g DW, 650 mg EPS/g DW). However, in the study of Sheng et al. (2005) the concentration of EPS extracted by NaOH was higher 159.2 mg/g-DW in comparison to centrifugation and ion exchange resin (Table 2-3). These results have also been confirmed by using more complex microbial consortia such as activated sludge (Table 2-3). Liu and Fang, (2002) reported extractions of EPS ranging between 25.7 mg EPS/g volatile suspended solids (VSS) by centrifugation and 164.9 mg EPS/g-VSS by chemical methods (NaOH and formaldehyde). A significant difference (>60%) of EPS extracted was observed between the two methods when EPS was extracted from the same culture. However, it appears that the chemical extraction becomes more effective when it is combined with physical methods. Liu and Fang (2002) investigated high proteins, carbohydrates, humic acid and DNA content extracted by combined methods of formaldehyde and ultra-sonication compared to that obtained by only formaldehyde. Comte et al. (2006a) also proved that sonication and CER as combined EPS extraction method were more effective to extract high proteins, carbohydrate and nucleic acid content comparing to CER or sonication alone.

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Table 2-3: Comparaison of total EPS (carbohydrate and protein) extracted from various cultures and extraction methods

Pure Culture	Extraction methods	EPS composition (mg/g DW)				EPS concentration (mg/g- DW)	Prot/car ratio	References
		Prot	carbo	HA	DNA			
Rhodopseudomonas		4.1	6.2	-	-	12.9	1.5	Sheng et al.
acidophilap		6.5	58.5	-	-	70.3	9.0	(2005)
		7.7	126.5	-	-	159.2	16.4	
	Centrifugation	10.3	37.3	-	-	71.6	3.7	
	EDTA							
	NaOH							
	Heating							
***Acidiphilium*.sp**		600	220	-	-	820	0.3	Tapia et al.
		550	200	-	-	750	0.3	(2009)
		430	170	-	-	600	0.4	
		570	200	-	-	770	0.4	
Mixte culture								
		54.6	40.5	50.4	0.3	165	0.2	Liu and Fang. 2002
Aerobic		17.7	12.7	16.4	0.1	109	0.1	
		22.9	12.4	59.2	0.4	146	0.1	
	Formal + NaOH	20.4	28.8	18.9	0.1	78	0.1	
	CER							
	EDTA							
Acidonic	Formal+	110.9	25.8	0.1		179	4.3	
	ultrasound	15.1	38.7	6.2	0.1	58	6.2	
		3.0				105	4.6	
		41.7	6.5	0.2		100	6.6	
		15.9						
		71.6	10.8	5.0				
		0.05						

Mutagenic sludge								
		19.1	42.1	23.3	0.19	102	2.2	
		7.9	10.6	5.5	0.05	30	1.3	
		6.8	12.0	24.3	1.20	73	1.7	
		12.0	13.1	5.6	0.04	30	1.1	
Acitivated sludge								Zuriaga-Agustíetal.
	Sonication	140	343	62	46	200	2.4	et al., (2013)
	Heating	166	378	126	17	369	2.3	
	CER	16	4	-	-	24	4.2	Comte et al.,
	Triton X 100	81	10	-	-	100	7.9	(2006a)
Biofilm	Centrifugation	57.0	-	-	-	57	-	Pan et al., (2010)
	Ultrasonication	22.3	56.6	-	-	79	0.4	
	EDTA	1.7	3.2	-	-	45	0.5	
	Formaldehyde	8.0	25.2	-	-	33	0.3	
	Formal+NaOH	17.0	13.3	-	-	30	1.3	

DW=Dry Weight, HA=Humic acid, DNA=Deoxyribonucleic acid

2.10.3 Chemical methods Vs Physical methods

The type of EPS extraction method further influences its composition, nucleic acid content, protein content, and various functional properties. Comte et al. (2006a) investigated the effect of extraction methods from two different sludge on EPS composition. This study demonstrated that the protein content was higher (343-337 mg proteins/g EPS DW) with physical method (sonication), whereas low protein content was observed, 73–107 mg proteins/g EPS DW, with chemical extraction methods (Glutaraldehyde, Formaldehyde and NaOH).

Liu and Fang, (2003) and Comte et al., (2006a) indicated a constant nucleic acid content of EPS using different physical extraction methods (Cation-exchange resin, centrifugation, sonication and heating). This study indicated that the physical methods resulted in lower extent of cell lysis than chemical extraction methods (Table 2-4).

Further, Simon and his colleagues in 2009 have found the humic substances content in EPS was different using different extraction methods (CER, centrifugation and heating). The highest humic acid concentration was 224 mg/g DW when heating was used as extraction method in case of anaerobic granular sludge obtained from Eerbeek municipal WWTP. However, the lowest humic content was 5 mg/g DW in the case of anaerobic granular sludge from Revico municipal WWTP by applying centrifugation as an extraction method.

In case of metal absorption Cd^{2+} sorption capacity of EPS extracted using sonication was 245 ± 46 µmol/ g of EPS for Chau's model and 336 ± 22 µmol/ g of EPS for Rezic's model. Although the metal binding capacity of sludge EPS extracted by physical methods was consistently identical (except by heating); however, the metal complexation capacity was significantly improved when EPS was extracted by chemical methods (Comte et al., 2006b).

Moreover, the effect of EPS extraction method from activated sludge on metal binding ability was evaluated (Comte et al., 2006b), EPS extracted by physical and chemical methods showed a greater affinity for Pb ions than Cd ions. The EPS extracted by a physical method (sonication) had a Pb^{2+} adsorption capacity of 2135 ± 55 µmol/g of EPS and 2184 ± 27 µmol/g of EPS as analyzed using two adsorption models, Chau and Rezic, respectively.

Recently, many other factors such as variation of pH, temperature and mixing speed have been reported to affect the structure of EPS, and its metal removal efficiency (Addagalla et al., 2009; Ruan et al., 2013).

Table 2-4: Avantages and disavantages of different extraction methods used.

Extraction methods		Mecanism and Extraction conditions	Limitation	Avantages	References
Physical	Centrifugation	EPS separates from cell surface and dissolve to solution under the centrifugal force • 20000 / 600g in 20 min	(-) Low extraction efficiency (-) Bound EPS cannot be extracted. (-) Depend of shear rate	(+) Simple method (+) No cell lysis (+) No chemical addition	Liu and Fung, (2002) Comte et al., (2006a)
	Heating	Accelerates the EPS solubilisation • 80°C, 1h	(-) Cells disruption (-) Denaturation of EPS	(+) Extraction of bound EPS	Frolund et al., (1996)
	Ultrasonication	Act of applying sound energy or mechanic pression to agitate particles in a sample • 40 W, 0-30 min	(-) High cell lysis in less time (-) High energy	(+) Dissociation of aggregates (+) Break the hydrogen bound	Comte et al., (2006)
Chemical	CER	Removes the divalent cations, causing the EPS to fall apart • 70 g resin /g MVS, 600 rpm, 1h	(-) Extract only the proteins and carbohydrates coupled to cations	(+) Avoid EPS pollution by chemical reagent	Liu and Fang, (2002)
	EDTA	Remove the multivalent cations forming the bond between the charged compounds of EPS.	(-) EPS contamination by intracellular compounds (-) Interfere the proteins	(+) No modification of EPS structure	Liu and Fang, (2002) Comte et al., (2006a)

			analysis		
		• 150 mL of 2% EDTA added for 3 h at 4°C	(-) Cost of chemical		
	NaOH	Ionisation of carboxylic group • 1N of NaOH, 3h at 4°C	(-) Severe disruption in EPS composition (-) high damage of cell (-) Many charged groups results repulsion between the EPS (-) Alkaline hydrolysis of many polymers may take place	(+) Break the covalent disulfide binding in glycoproteins	Liu and Fang, (2002) Comte et al., (2006a)
	Formaldheyde	Fix the cell and denaturise the EPS, linking the proteins and carbohydrate • 36% , 1h , 4°C	(-) Modify EPS characteristics (-) Interferences in carbohydrates content (-) Cost of chemical reagents	(+) Prevent the cell lysis	Liu and Fang, (2002) Comte et al., (2006a)
Combined chemical and physical methods	NaOH-Heating CER-centrifugation Formaldehyde-sonication	-	(-) Extraction time (-) EPS need purification (-)Not economical	(+) High Extraction efficiency	D'Abzac et al., (2010)

2.10.4 Combination of different methods

According to D'Abzac et al. (2010), a combination of formaldehyde and heating leads to the highest EPS quantity extracted. Humic-like substances and the nucleic acids are more readily extracted using formaldehyde method than the heating method alone. By using a combination of sonication and CER methods the protein and polysaccharide content were found to be higher than obtained by only sonication or CER.

As discussed earlier, principally physical methods, are simply mechanical which can explain the low extraction yield and it has been a common observation that the chemical methods were always having higher yields than physical methods. Only handful of methods has been thoroughly evaluated and optimized to obtain high extraction efficiencies without cell lysis and reagent contamination. Most of the chemical extraction methods cause various problems either in the extraction or EPS analysis. For instance, in the case of alkali extraction methods, an addition of NaOH causes anionic groups to lose their protons, such as carboxylic groups in proteins and polysaccharides, The deprotonation causes a strong repulsion between EPS molecules within the EPS gel and provides a higher water solubility of the compounds. Similarly the EDTA method has high extraction efficiency, however, causes a high degree of cell lysis and possibly also contamination with cellular macromolecules interfering in the protein determination (Comte et al., 2006a).

Each and every method has their advantages and disadvantages (Table 2-4). Although the physical methods cause less cell lysis, it also has low EPS extraction efficiency. The chemical methods generated high protein content, and these proteins can be originated from extracellular enzymes and or intracellular materials contaminations. A combination of two methods could affect the production cost and the efficiency of EPS extraction during an industrial application (Dominguez et al., 2010). There is no simple and single method exist to extract 100% of total or complete EPS components from the microbial cell or activated sludge flocs. Each technique extraction efficiency depends on many factors mainly the origin of EPS. It is recommended that extraction is only performed after running a comparative study of various methods to select the best one for desired application. Furthermore, an extraction technique must be chosen and optimized for each case, taking into account many parameters (such as extraction time, cost and dosage of chemical used

and evaluation of cell lysis), which could affect the cost and the properties of EPS (Table 2-4)

2.11 Effect of extraction methods on functional group and molecular weight of EPS

The complex composition of EPS makes it difficult to analyze the conformation, chemical structure (their functional groups) and distribution of EPS. However, progress in analytical chemistry has led to the development of new instruments and techniques for characterization of EPS, which has generated a significant amount of information on the structural and functional properties of EPS as well as their molecular weight. Chromatography, Mass Spectrometry and their combination have been used to qualitatively and quantitatively analyze the EPS composition (Dignac et al., 1998).

Many researchers (Sheng et al., 2006; Tapia et al., 2009) proved the effect of the extraction methods applied on structural properties of EPS and their molecular weight (Table 2-5). Fourier transform infrared spectroscopy (FTIR) was mostly used to identify the functional group of extracted EPS (Omoike and Chorover, 2004; Sheng et al., 2006). However, quantitatively this aspect is not reviewed.

Tapia et al. (2009) compared two FTIR spectra of EPS obtained from EDTA and centrifugation method. Significant peaks were visible in both the spectra corresponding to hydroxyl, carbonyl and peptide group bonding. However, the spectrum of EPS extracted with EDTA shows specific bands in the fingerprint region, especially the thick band at 1717 cm^{-1}. This band corresponds to the C = O asymmetric stretching vibration of carboxylic acids of EDTA. It has been a general observation by many studies that during extraction with a chemical method the final EPS gets contaminated by the chemical reagent (Compte et al., 2006a). Similar contaminations were observed in other methods like NaOH-formaldehyde (Compte et al. 2006a; Sheng et al., 2005, Pervaiz et al. 2012)(Table 2-5).

In another analysis done by Lee et al. (2013), it was demonstrated that the FT-IR results of CER extracted EPS contained lower content of protein to carbohydrate, fewer acidic functional groups (i.e., COOH or OH groups) as compared to formaldehyde-NaOH technique. The same results were recorded for the EPS extracted from anaerobic granular sludge with physical methods which displayed very similar IR spectra (D'Abzac et al., 2010). Humic-like substances were hardly identifiable in physical extraction techniques and adsorption bands were intensively present at 2930 and 1650 cm^{-1}.

The molecular weight range of EPS varies from 10^3 to $2.5x10^6$ kDa (Yokoi et al., 1995). The molecular weight of the EPS reported in different studies has been presented in Table 2-5. To determine molecular weight, size exclusion chromatography (SEC) was frequently used in many studies (Simon et al., 2009; Comte et al., 2006a). These researchers investigated differences appeared in the peak corresponding to the biggest and lowest molecules.

Large additional peaks appeared in the chromatograms recorded for EPS extracted from sludge by cation exchange resin (CER), heat treatment and centrifugation methods. The EPS extracted by CER contains more polysaccharides and uronic acid 210 nm, which indicated that better carbohydrate content could be extracted by this method. For the EPS extracted by centrifugation, the highest peak observed had a lower absorbance at 210 nm representing a lower polysaccharide and uronic acid content.

Simon et al. (2009) studied EPS extraction from anaerobic granular sludge with different methods (heating and centrifugation) and proved that the extracted molecules of EPS were insignificantly affected. According to SEC analysis, two kinds of differences could be observed on the EPS fingerprints: variation of the number of detected peaks and significant evolutions of peak absorbance which corresponds to the high or low molecular weight.

Dominguez et al. (2010) conducted a comparative study between EPS extracted by physical and chemical methods to compare their molecular weight (MW) distribution using High-Pressure Size Exclusion Chromatography (HPSEC). The EPS extracted using chemical methods did not have any effect on the molecular weight (MW) distribution (fingerprints) of EPS or their average MW. Nevertheless, different physical extractions showed different behavior of EPS fingerprints. These results were in agreement with the results of Alasonati et al. (2012). The study revealed the effect of the extraction methods (centrifugation, EDTA and formaldehyde-NaOH) on the size distribution of EPS. According to Lee et al. (2013), the EPS obtained from aerobic sludge using CER method were made up of more aromatic and compact structures possessing higher molecular weight than those extracted using formaldehyde–NaOH extraction method.

Despite extensive efforts to analyze qualitatively the EPS chemical structure and size distribution, little is known about the effect of these parameters on EPS properties, functions and structure, which are essential for understanding the role of EPS in biofilms and floc formations. In this review, we performed compositional analyses of the EPS obtained by different extraction techniques. We also highlighted the effect of extraction methods on EPS molecular weight. In next section on how the functional group and molecular weight could interfere and vary their properties as bioflocculant.

Table 2-5: Effect of extraction methods on functional group of EPS

Extraction methods	Wave number (cm^{-1})	Transmittance (%)	Vibration type	Functional type	Molecular weight range	References
EDTA	1550-1600	50	Stretching vibration of C=O	Proteins (peptidic bond)	8 kDa 150–200 kDa	Comte et al., (2006a)
	1300	55	C–N stretching		5 kDa	Simon et al.,(2009)

NaOH + Formaldehyde	2450	60	Specific band corresponding to a product of a formaldehyde and EPS	Carboxylic acids	-	Comte et al., (2006a)
	1750	40	Stretching vibration of C=O			
	1400	10	Stretching vibration of C=O	Carboxylates		
	800	30	Several bands visible	Phosphore/sulphur functional group		
Gluraldehyde	1950 1500	75 70	Specific band corresponding to a product of a glutaraldehyde and EPS reaction	-	-	Comte et al., (2006a)
Centrifugation	3200–3420	80 85	Stretching vibration of OH	OH into polymeric compounds	Low molecular weight	Comte et al., (2006a)

	2935				0.16 – 0.3 kDa	Simon et al.,
	1630-1660	73	Stretching vibration of C=O and C–N (Amide I)	Proteins (peptidic bond)	0.7– 2.7 kDa 4.6 – 6 kDa	(2009)
	1550–1560	83	Stretching vibration of C–N and deformation vibration of N–H (Amide II)			
				Carboxylates		
	1450–1460	82	Deformation vibration of CH_2	Polysaccharides	High molecular weight 16 – 190 kDa 270 – 275 kDa	
	1400–1410	85	Stretching vibration of C=O			
	1060–1100	75	Stretching vibration of OH, of polysaccharide			
	<1000		Several bands visible	Phosphor/sulphur functional group		
Sonication	3200–3420	60	Stretching vibration of OH	OH into polymeric compounds	Low molecular weight	Comte et al., (2006a)
	2935	70	Stretching vibration of C=O and C–N (Amide I)	Proteins (peptidic bond)	0.16–0.3 kDa	Simon et al., (2009)
	1630-1660	52				

				0.7–2.7 kDa
1550–1560	60	Stretching vibration of C–N and deformation vibration of N–H (Amide II)		4.6–6 kDa
1450–1460	71	Deformation vibration of CH_2	Carboxylates Polysaccharides	High molecular weight
1400–1410	65	Stretching vibration of C=O		16–190 kDa
1060–1100	63	Stretching vibration of OH, of polysaccharide	Phosphore or sulphur functional group	270–275 kDa
<1000		Several bands visible		

Sonication + Resin	3200–3420	60	Stretching vibration of OH	OH into polymeric compounds	Low molecular weight 0.16 – 0.3 kDa 0.7 – 2.7 kDa 4.6 – 6 kDa	Comte et al., (2006a) Simon et al., (2009)
	2855	70				
	1630-1660	52	Stretching vibration of C=O and C–N (Amide I)			
	1550–1560	60	Stretching vibration of C–N and deformation vibration of N–H (Amide II)	Proteins (peptidic bond)		
	1450–1460	71	Deformation vibration of CH_2	Carboxylates	High molecular weight 16 – 190 kDa 270 – 275 kDa	
	1400–1410	65	Stretching vibration of C=O			
	1060–1100	63	Stretching vibration of OH, of polysaccharide	Polysaccharides		
	<1000			Phosphore/ sulphur functional group		
			Several bands visible			

CER	3200–3420	60	Stretching	OH into polymeric compounds	Protein molecular weight 45 to 670 kDa	Comte et al., (2006a) Simon et al., (2009)
	1630-1660	70	vibration of OH			
	1550–1560	52	Stretching vibration of C=O and C–N (Amide I)	Proteins (peptidic bond)		
	1450–1460 1400–1410 1060–1100	60	Stretching vibration of C–N and deformation vibration of N–H (Amide II)			
		71	Deformation vibration of CH_2	Carboxylates	Polysaccharides <1 kDa	
	<1000	65		Polysaccharides		
		63	Stretching vibration of C=O Stretching vibration of OH, of polysaccharide			
			Several bands visible	Phosphore/sulphur functional group		

Heating	3200–3420	30	Stretching	OH into polymeric compounds	Low molecular weight	Comte et al., (2006a)
	2935	40	vibration of OH		0.16 – 0.3 kDa	Simon et al., (2009)
	1630-1660	20	Stretching vibration of C=O and C–N (Amide I)	Proteins (peptidic bond)	0.7 – 2.7 kDa 4.6 – 6 kDa	
	1550–1560	25	Stretching vibration of C–N and deformation vibration of N–H (Amide II)			
	1450–1460	45	Deformation vibration of CH_2	Carboxylates	High molecular weight	
	1400–1410	33	Stretching vibration of C=O	Polysaccharides	16 – 190 kDa 270 – 275 kDa	
	1060–1100	41	Stretching vibration of OH, of polysaccharide			
	<1000		Several bands visible	Phosphore or sulphur functional group		

2.12 Effect of Functional group and Molecular Weight on flocculation activity

The flocculation ability of EPS has been one of the key properties for biopolymer application. Different studies are available which have investigated the important structure-function relationship between EPS functional composition and flocculation abilities. The flocculation activity has been modeled by various mechanisms, and the flocculant activity of high-molecular weight EPS has been explained by the bridge formation model. In the case of Patch Model, flocculation of the bacteria with the negatively charged cell surface, is a result of binding of the positively charged macromolecules to the surface of particles Coulomb forces, resulting in neutralization of part of the surface charge (patch model). Reduced electrostatic repulsion leads to agglomeration of particulate matter and formation of flocs by bridges between negatively charged particles (Zhou et al., 2006). Figure 2-4 illustrates the different mechanism of flocculation.

No consensus exist on the role of (importance) of carbohydrate and protein content of EPS for flocculation. Deng et al. (2005) concluded in his study that EPS containing 76.3% of sugar and 22.6% of protein gave high flocculating abilities of 98.1%.

Freitas et al. (2009) studied the monosaccharides in carbohydrates and concluded that 82.6% flocculation ability was achieved by EPS whose 70% (mol/mol) of carbohydrate was galactose and 23% (mol/mol) was mannose. When monosaccharide % decreased in carbohydrate fraction, the flocculation abilities were seen to be decreasing as observed by Kavita et al. (2014) only 40% flocculation ability was achieved when mannose (47.8%) and glucose (29.7%) were present in lower quantity. Li et al. (2008) emphasized the importance of acetyl groups on flocculation ability. The study showed that EPS with acetyl group shows a good flocculation (49.3%) comparing to deacetylate EPS (27.8%).

A more functional level analysis of flocculating abilities was performed by various studies (Kavita et al., 2011; Deng et al., 2005) to understand the importance of functional groups on flocculation ability. They investigated that cations stimulate flocculation by neutralization and stabilization of residual negative charges of the carboxyl group of a bioflocculant forming bridge that binds kaolin particles to each other. Further, the negatively charged carboxyl group (COO^-) of the bioflocculant could bind with the positively charged site of the suspended kaolin particles.

Although flocculation ability of EPS seems to be sensitive to the carbohydrate content (Shin et al., 2000), a study conducted by Yu et al. (2009) concluded that protein fraction of EPS is the most important parameter for flocculation activity. Researchers advocating for protein suggest that negatively charged amino acid contribute to flocculation abilities. The hydrogen bonds are present frequently in proteins and they could affect the capacity of bioflocculant to agglomerate.

The relationship between molecular weight and flocculation activity of bioflocculant remains unclear until now. Flocculation with high molecular weight bioflocculant involves more adsorption points, stronger bridging, and higher flocculating activity than the flocculation with a low-molecular-weight bioflocculants. Larger molecular weight flocculant usually has a sufficient number of free functional groups, to form bridges to bring many suspended particles together, and hence produce a larger floc size in the flocculating reaction (Shih et al., 2001). These results were in agreement with the findings of many researchers (He et al., 2002; Deng et al., 2005; Wu et al., 2007).

High molecular weight ($2.6x\ 10^6$ Da) bioflocculant produced by *Bacillus mucilaginosus* revealed high flocculation activity (99%) than compared to low molecular weight (MW 10^5 Da) obtained from *Corynebacterium glutamicum* (80% of flocculation activity) (Deng et al., 2003; He et al., 2002). Furthermore, the bioflocculant produced by *Bacillus subtilis* DYU500 ($3.20x10^6$ Da) in the study of Wu et al. (2007) seems to favor the performance of flocculation (97%) comparing to 90% obtained by *Gyrodinium impudicum* KG03 (MW $1.58x10^6$ Da) (Yim et al., 2007).

Recently, Tang et al. (2014) discovered a new bioflocculant produced by *Enterobacter* sp. ETH-2. The MWs of ETH-2 ranges from 603 to 1820 kDa, which is within the high MWs range with high flocculation ability of ETH-2 (94%) as compared to other strains. For example, *Bacillus megaterium* TF10 produced 2.5×10^3 kDa with 95.5% capacity to flocculate (Xiong et al., 2010) and 90% of bioflocculation was obtained by *Bacillus licheniformis* with 1.8×10^3 kDa molecular weight (Yuan et al., 2011).

In summary, bioflocculant produced by the various isolated microorganism are very diverse corresponding to their chemical structure (functional group) and their molecular weight as discussed earlier. These parameters affect their properties of EPS as a bioflocculant agent. High MW bioflocculant possess more adsorption points for bridging thus larger and stable flocs are obtained. The concentration of EPS used to obtain the highest flocculation activity is important from an economic standpoint.

Figure 2-4 illustrates the different mechanism of flocculation.

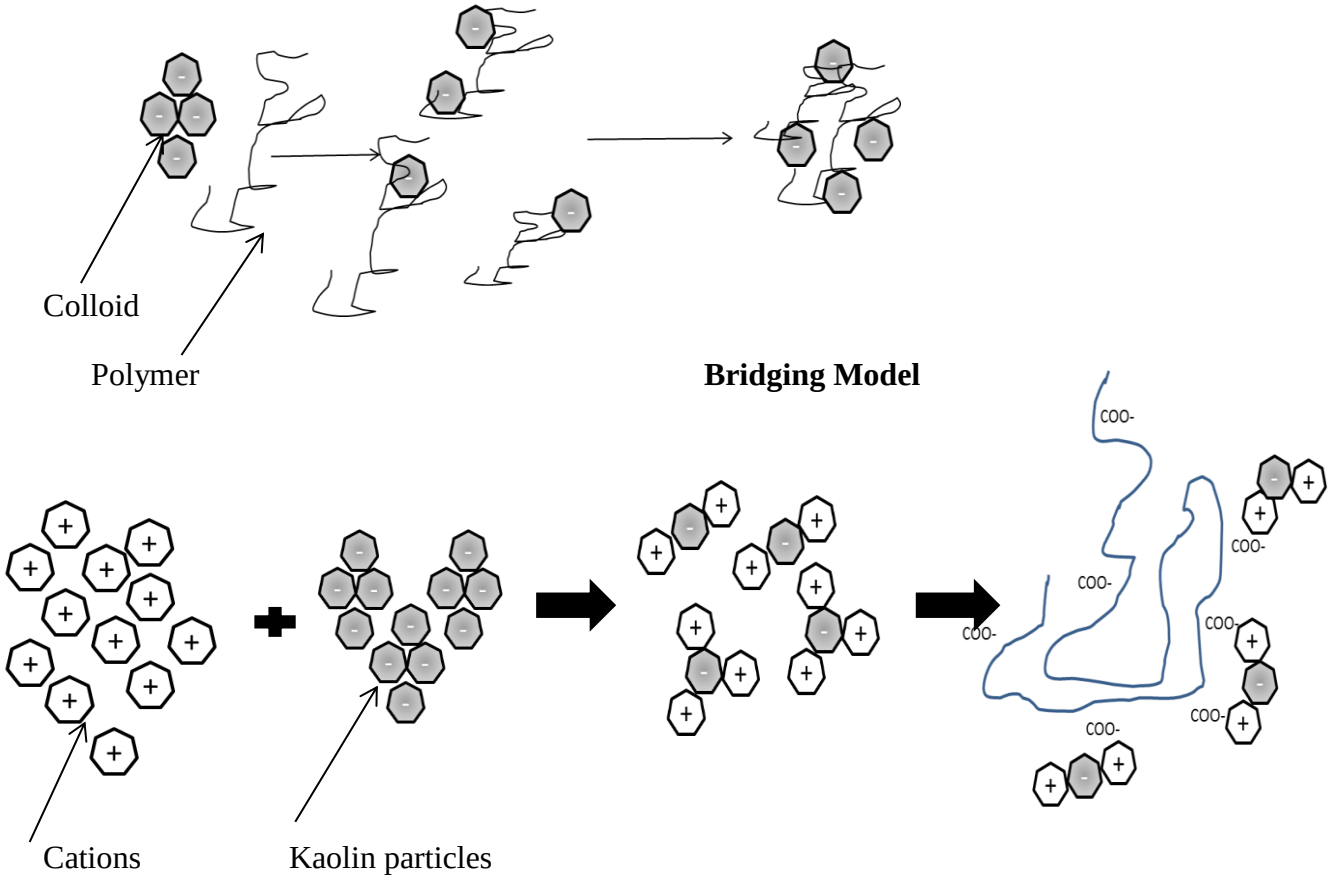

Figure 2-4: Flocculation mechanism of bioflocculant.

2.13 Conclusion and Future work

As evident from the review that sludge EPS remains to be an unexplored field of study with a plethora of research opportunities for many industrial and eco-friendly applications. EPS is composed of mainly carbohydrates and proteins, and they play very significant role in determining their functionality. The presence of nucleic acid and humic acid substances as

a resultant of cellular lysis during post-production processing can further contribute to enhance the functional properties of EPS. The microorganism, carbon substrate, and other growth conditions play very significant role in determining EPS composition. EPS has great potential to be used as metal removing agent in mining industries, as a flocculating agent in WWTP for sludge dewatering and as a carbon source for biotechnological production of other metabolites.

Progress in molecular level knowledge about EPS production, its genetics, and enzymology have been very limited. The limited knowledge has restricted the application of various engineering techniques to enhance biological production of EPS. A huge scope of research and development lies in developing mutant strains to have a higher titer of EPS with novel properties. There are no dedicated industrial production processes for EPS production. This technical development has been limited by the optimization of EPS production using mixed cultures and pure cultures in combination with various kinds of carbon substrate which significantly affect the functional properties. More dedicated studies are required toward optimization of EPS production processes using novel and cheaper carbon substrates like sludge. A dedicated process development and simulation are required to have high upstream production coupled with efficient downstream extraction. Proper characterization and documentation of the effect of extraction processes on EPS concentration, composition and functional groups (functionality) are required to realize a large scale EPS production process.

The research on sludge EPS still lacks the clarity on the mechanism of EPS production, proteome involved in EPS biosynthesis, mechanistic knowledge of role and effect of various components of EPS toward its functionality is still missing and very limited research is available. Currently many studies are going on EPS production, but there is a lot more to know about EPS than what has been known to make EPS a successful commercial product of application.

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CHAPITRE 3

EPS producing microorganisms from municipal wastewater activated sludge

Le chapitre 3 est constitué de l'article publié suivant:

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3 EPS producing microorganisms from municipal wastewater activated sludge

3.1 Abstract

Bacterial exopolysaccharides (EPSs) are produced by many bacteria. Nowadays there is an increasing interest in the isolation and exploitation of these polymers for industrial purposes. In general, biosynthesis of EPS varies from one strain to other strain and it depends on genes and enzymes involved in EPS production and secretion. In this study, EPS producing strains were isolated from municipal wastewater treatment plant and their potential of EPS production was evaluated. The role of EPS in sludge flocculation was also studied. Three types of EPS (LB-EPS (Loosely bound EPS), TB-EPS (Tightly bound EPS) and B-EPS (broth EPS)) were harvested and their characteristics were studied. Bio-flocculation capacity of the obtained EPS was evaluated by measuring the kaolin clay flocculation activity. The LB-EPS was better than TB-EPS and B-EPS for bioflocculation.

3.2 Keywords

Strain producing EPS isolation, EPS production, bioflocculation

3.3 Introduction

Activated sludge is the most commonly used biological process for wastewater treatment. Normal practice in wastewater treatment plants is to employ the use of synthetic polymers for enhancement of sludge settling. These synthetic polymers have many disadvantages; they are expensive, toxic and can pollute the environment (Abu-Orf et al., 2001). Thus, high flocculation potential, low cost, environment friendly and sustainable biopolymers (e.g., chitosan, sodium alginate and microbial flocculants) are being searched by many researchers (More et al., 2012 a,b). Extracellular polymeric substances (EPSs) are of considerable importance in the removal of pollutants from wastewater, in bioflocculation and settling and in the dewatering of activated sludge (More et al., 2012a). EPSs are located outside the cell surface and can be subdivided into bound and soluble EPSs. The bound EPS are closely bound with cells, due to many functional groups (carboxyl, phosphoric, sulfhydryl, phenolic and hydroxyl group) and apolar groups (e.g., aromatics, aliphatics in proteins, and hydrophobic regions in carbohydrates and proteins). The soluble EPSs are weakly attached to cells or dissolved into the solution that can be explained by the hydrophilic fraction mainly consisted of carbohydrates.

Activated sludge is comprised of a wide variety of microbial community, containing many types of viruses, bacteria, protozoa, fungi, metazoa and algae. In this complex environment, bacteria, which usually make about 95 % of the total microbial population, play a key role in efficiently eliminating the organic material and other pollutants from wastewater under highly oxygenated conditions. Majority of these microbial species were immobilized in EPS or matrices made of polymers of necessary protein, polysaccharides, humic substances, and lipids (Nielsen et al., 1996). EPS producing bacterial species are very efficient in converting nutrients into EPS. Considering the tendency in nature to conserve rather than to waste, this expenditure of energy is likely to hold benefits to the producers of EPS, as well as those organisms associated with them. Therefore, the EPS synthesis by microbial cells depends upon availability of the carbon and nitrogen in the culture medium and environmental conditions. The organisms differ in their carbon and nitrogen source utilization, mineral requirements, temperature and pH, which are the critical factors for maximum EPS production (Wingender et al., 1999). To understand the EPS production abilities of bacterial species growing in wastewater treatment plant and accumulated in wastewater sludge, it is necessary to have information or knowledge of their metabolic pathway, their genes, biochemical properties, and vulnerability to certain chemical substances and anti-microbial agents.

Various research on EPS biosynthesis have been conducted (Sobeck and Higgins, 2002), however, only few were directed towards the production of EPS using low-cost medium. Moreover, few studies investigated the variation of microbial community in WWTP with respect to seasonal changes due to the variation of environmental factors particularly the temperature and the composition of wastewater. There were few researches (Péant et al., 2005) comparing the EPS production among the strains based on their genetics. This study presented three parts: Isolation and identification of new strains, which could produce high EPS concentration. Secondly, the effect of environmental factors, specifically, the temperature on EPS production and on the total variation of EPS producing microbial community. Finally, the EPS production variation among the strains, the difference in their physicochemical characteristics (polysaccharides and proteins content) and the improvement of their activities as bio-flocculants in comparison to previous studies.

3.4 Materials and Methods

3.4.1 Isolation of EPS producing strains from wastewater sludge

EPS producing bacteria were isolated from wastewater sludge (WWS) collected from Communauté Urbaine du Québec (CUQ, Québec, Canada). Microbial isolation was carried out by incubating appropriate serial dilutions of WWS on plate count agar (PCA) at 30 °C for 48 - 72 h. Among the Colony forming unit (CFU) formed on PCA, EPS producing microorganisms were selected based on their ability to form string (indicating EPS production) while touching with inoculating loop. Congo red was employed to identify the capsular EPS (TB-EPS) (Cain et al., 2009).

3.4.2 Molecular identification and sequences analysis

Potential EPS producing bacterial isolates were identified genetically by adopting 16S rRNA Sangers sequencing technology. Total genomic DNA of all the bacterial strains was extracted by using Qiagen DNA extraction kit according to manufactures instructions. Polymerase chain reaction of these genomic DNA was carried out to amplify the 16S rRNA region using a primer set of 9F:5'-gagtttgatcctggtcag-3' and 1512R:5'-acggtaccttgttacgac-3' (Lee et al., 2009). Primers were synthesized from Integrated DNA technologies USA. PCR reaction mix of 50 µL reaction volume contains 1X PCR master mix from promega, USA and 100 pmol of each oligonucleotide primers and template DNA (0.25 mg of purified DNA from 1 mg/mL of stock solution) of 8 different bacterial isolates (NK2-NK9). PCR reaction cycle includes; denaturation at 95 °C for 5 min, 30 s, annealing at 51 °C for 30 s and polymerization at 72 °C for 1 min, 30 s for 35 cycles followed by

final extension at 72 °C for 10 min. PCR amplica of 1503 bp were eluted, purified and suspended in Milli Q water for sequencing.

PCR amplica were subjected to Sanger's sequencing using ABI 3730xl DNA Analyzer, Applied biosystems, USA. Thus obtained sequencing data were aligned using DNASIS bioinformatics software (Hitachi Software Japan, Tokyo) and were blasted against 16S rRNA sequences (BLSTn) for sequence homology followed by submission in NCBINational Center for Biotechnology Information) gene bank via with accession numbers from KF675198 to KF675205. Using the gene sequence information, the multiple alignment of gene sequences, phylogenetic tree (neighbourhood joining method) were predicted using DNASIS software, version4.

3.4.3 Biochemical identification

EPS producers were subjected to biochemical identification via BioLog substrate utilization profile. For BioLog identification of bacterial strains, according to manufactures protocols BioLog Gen III plates were incubated with 100 µL of the bacterial suspensions in inoculation fluid (IF) provided at 30 °C for 24 - 48 h. Substrates utilization is indicated when a purple colour appears in micro titre wells and no reactions was recorded if remains colour less. Growth of the bacterial strains at different pH, carbon sources, amino acids/proteins, carboxylic acids, and esters, fatty acids and presence of antibiotic or reducing power was determined by the change in colour towards purple.

3.4.4 Sludge as bacterial source and as growth medium

The sludge was concentrated on spot by removing the supernatant after gravity settling for 1 h. Concentrated sludge was stored at 4 ºC for less than 24 h before use to ensure that there was no sludge degradation during storage. Characteristics of the sterilized sludge, such as pH, total suspended solids (TSS), and volatile suspended solids (VSS), total carbon and total nitrogen were determined using Standard Methods (APHA, 2005).Viscosity was measured by viscometer (DV-II + PRO, Brookfield Engineering Laboratories, Inc., Stoughton, MA, USA), at constant higher shear rate of 20 s-1 and at room temperature. Zeta potential (ζ) was measured using Zetaphoremeter (Zetaphoremeter IV, Zetacompact Z8000, CAD Instrumentation, France).

3.4.5 EPS production and harvesting

Inoculum of all the bacterial strains was prepared in sterilized sludge (121 ºC for 30 min) at pH 7 by incubating at 30 °C and at 180 rpm for 24 h. Fermentation for EPS production was performed by incubating 150 mL of sterilized sludge in 500 mL Erlenmeyer flasks at 180 rpm, 30 °C for 72 h which was inoculated with 3 % inoculum. Standard plate count method was adopted to determine

the bacterial strain growth and was reported as CFU/mL. EPS produced were estimated after 48 h and at the end of the cultivation (72 h). After fermentation, broth samples were centrifuged at 6000 g for 20 min at 4 °C to obtain supernatant (containing loosely bound EPS; termed as LB-EPS) and pellets (containing tightly bound EPS or TB-EPS) with bacterial cells along with residual sludge material and termed as TB-EPS) (More et al., 2012a). LB-EPS in the supernatant was precipitated by incubating with 2 times volume of absolute ethanol at -20 °C for 24 h. The precipitates (containing pure LB-EPS) were collected by centrifugation at 6000 g for 20 min at 4 °C. Precipitates of LB-EPS were dried at 60° C for 24 h and dry weight of the precipitates was measured and denoted as LB-EPS (APHA, 2005). To determine TB-EPS, the pellet (crude TB-EPS) was re-suspended in deionized water, first heated in water bath at 60 °C for 30 min to release TB-EPS followed by centrifugation at 6000 g for 20 min at 4 °C (More et al., 2012 b). The supernatant (containing TB-EPS) was used to precipitate TB-EPS using same procedure as for LB-EPS. Dry weight of precipitates was measured and denoted as the concentration of TB-EPS produced. Sum of dry weights of LB-EPS and TB-EPS (measured above) was denoted as total broth EPS (B-EPS). The protein content in LB-EPS and TB-EPS was determined by Bradford, (1979) method using bovine serum albumin (BSA) as a standard. The carbohydrate content of the total EPS was determined by the phenol–sulphuric acid method using glucose as the standard (Dubois et al., 1956).

3.4.6 Characterization of EPS

EPS extracted from different isolates was characterized by analysing its viscosity, Zeta potential and flocculation activity.

3.4.6.1 Viscosity

Viscosity was measured for all different types of EPS (broth, slime and capsular) produced by bacteria after 3 days of fermentation. 20 mL of the culture (bacterial) broth was used to measure the viscosity using ULA S 34 spindle (Digital Viscometer, DV-II+ Pro, Brookfield), at 60 rpm and room temperature.

3.4.6.2 Zeta potential

The charge of each type of EPS was determined by adding 50-1000 µL of individual EPS sample to 100 mL of deoinzed water. The zeta potential of different EPS (Broth, slime and capsular EPS) was determined from individual bacterial grown media (non-centrifuged media) after 3 days of fermentation. The surface charge or zeta potential (ζ) measurements were carried out using three types of EPS obtained from all bacterial strains isolated. Characterization of charge (zeta

potential) was implemented using Zetaphoremeter (Zetaphoremeter IV, Zetacompact Z8000, CAD Instrumentation, France) with the application of the Smoluckowski equation. Surface charge of the wastewater sludge was also measured. The zeta potential values were obtained from an average of around 10 measurements, the average values are presented with its half-width confidence interval at 95 % confidence level.

3.4.6.3 Kaolin flocculation activity of different EPS

Flocculation activities of the EPS were based on a decrease in turbidity of the standard kaolin suspension after jar tests. The jar tests were performed using kaolin suspension (5 g/L) prepared in deionized water. Divalent cation (Ca^{2+}) (150 mg/L dissolved in deionized water) was added to the kaolin suspension of 500 mL by rapid mixing (180 rpm for 3 min) and then pH was adjusted to 7 by adding NaOH (1 M) (Zhang et al., 2010). The samples collected from bacterial isolates at 48 h and 72 h were added in different volumes (corresponding to desired concentrations of EPS, which was calculated through dry weight and the volume of EPS solution was added accordingly) to kaolin suspension and rapidly mixed at 100 rpm for an initial 5 min then slowly mixed for an additional 30 min at 70 rpm. After 35 min of mixing, samples were transferred to a 500 mL cylinder where they settled for 30 min and supernatants were then collected to measure the turbidity using turbidimeter (Micro 100 turbidimeter, Scientific Inc.). Flocculation activity was calculated according to the equation: Flocculation activity = [100*(C-S)/C] (%), where C is control turbidity and S is sample turbidity

3.5 Results and discussion

3.5.1 Isolation and characterization of EPS producing bacterial strains

Eight (named NK2 to NK9) bacterial strains were isolated from the municipal wastewater sludge. These microorganisms were selected based on their mucoidal colony formation on the PCA agar plates and string forming ability by touching with inoculating loop (Figure 3-1a). All these bacterial strains were screened to study their potential for EPS production. All of the bacterial strains were identified as gram negative except *Brevibacillus parabrevis* and *Acinetobacter parvus* (Table 3-1, Figure 3-1a, 3-1b). Whereas all the isolated strains were capable of producing LB-EPS, the strain NK6 was the best which could form an EPS string longer than 3 cm among all strains (Figure 3-1).

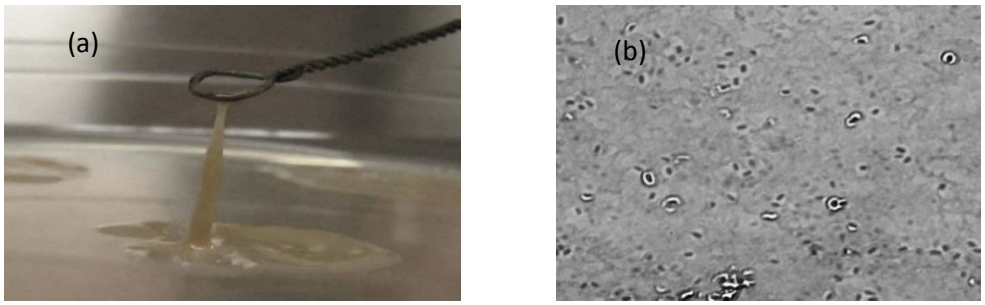


Figure 3-1: a) Slime EPS producing bacterial strain (NK6) showing string of secreted biopolymer on PCA agar plate, (b) Capsular staining results revealed the presence of capsular extracellular polymeric substances in isolated strains (NK5)

The 16S rDNA sequence data were subjected to a BLASTn search tool of NCBI and the homology search results of NK2–NK9 bacterial strains resembling (96–99%) with existing DNA sequence database were identified. The accession numbers of isolated strains submitted to genbank are provided in Table 3-1.

Table 3-1: Molecular identification and microbiological characterization of EPS producing bacterial strains isolated from municipal wastewater sludge

Bacterial strains code	Staining			Bacterial Identifiacation	Viscosity of fermented Broth (mPa)	Accession number
	Slime EPS	Capsular EPS	Gram staining			
NK2	+	+	Negative	*Chryseobacterium gregarium*	10.04 ± 0.9	KF675198
NK3	+	+	Negative	*Staphylococcus epidermidis*	8.96 ± 0.5	KF675199
NK4	+	+	Negative	*Stenotrophomonas acidaminiphila*	10.86 ± 1.1	KF675200
NK5	+	+	positive	*Brevibacillus parabrevis*	15.8 ± 0.8	KF675201

NK6	+	+	Negative	*Cloacibactéruim normanense*	85.7 ± 1.9	KF675202
NK7	+	+	Negative	*Pseudomonas veronii*	7.84 ± 1.4	KF675203
NK8	+	+	Negative	*Acinetobacter soli*	10.08 ± 0.9	KF675204
NK9	+	+	Positive	*Acinetobacter parvus*	8.96 ± 0.5	KF675205

3.5.2 DNA characteristics and sequences analysis of isolated strains

EPS production by different strains varied greatly. The EPS concentration (11.8 ± 1.2 g/L) produced by *Cloacibacterium normannese* is the highest among all EPS producing strains isolated from wastewater sludge in this study and the previous studies (More et al., 2012 a,b and Subramanian et al., 2010). The difference in EPS concentration could be explained by the genetics organisation of the EPS biosynthesis gene cluster among strains. The difference in gene structure, sugar precursor biosynthesis and regulatory elements among the strains could be the reason for the diversity in EPS production levels, their composition, their charge, which displayed different patterns in kaolin flocculation.

The gene sequences analysis and its relation to the EPS production has attracted the attention of many researchers (Hay et al., 2013; Péant et al., 2005 and Subramanian et al ., 2010). In general, genetic elements required for EPS production include genes encoding regulation, chain length determination, repeat -unit assembly, polymerisation and export.

The similarity analysis among all eight EPS producing bacterial strains was carried out by drawing a dendrogram using the neighbourhood joinging method. Multiple alignments of gene sequences was done followed by aligned sequences which was treated for phylogenetic tree construction (Figures 3-2 A,B), which revealed the relatedness among the microbial strains. The multiple alignments of gene sequences were conducted for all bacterial strains (NK2-NK9) isolated from wastewater sludge (Figure 3-2A). Each strain apparent in specific genes clusters. The conservation of gene sequences varied which confirmed that each strain was significantly different from each other.

There was little variation between the strains having same genus and species such as *Acinetobacter parvus* and *Acinetobacter soli* which is apparent in one cluster (Figure 3-2B). It was observed that *Cloacibacterium normanense* presented in first cluster in phylogenetic tree, belonging to different strains which indicated distance from other strains, proofing by their

high capacity for EPS production. However, *Chryseobacterium gerganium* and *Cloacibacterium normanence* apparent in the same cluster accounts for common Cytophaga- Flavobacterium group but different genus and species. This could explain the variation in genes sequences, different metabolic pathway involved in EPS biosynthesis and difference in the amount of EPS produced.

Each EPS producing strain has a chromosome, which includes the cluster of genes necessary for biosynthesis of EPS. The EPS cluster is continent of genes sequences. The EPS cluster includes genes for synthesis of the precursors of EPS biosynthesis, and the gene for one of the enzymes in this pathway. In addition, there are genes with homologous predicted functions in regulating EPS processing and polymerisation, these genes are found in many EPS gene clusters.

Figures 3-2 A and B present the multiple alignments of gene sequences of different strains and phylogenetic tree. Less conservation in genes sequences among the strains was observed. Thus, a variation in glycosyltransferase genes products was responsible for the structural organisation of EPS repeating unit. This explained that the genetics organisation and transcription of the EPS genes clusters in *Cloacibacteruim normanense* showed significant differences from those in others strains such as *Brevibacillus*, or *Acinetobacter*. sp. Then, the variation in EPS production levels observed among the strains studied here may originate from the difference in central metabolism and the availability of sugar precursors as well as difference in the activity of enzymes of central metabolism and precursors biosynthesis.

The genetics organisation and transcription of the gene clusters from *Lactobacillus rhamnaosus* differ from those implicated in the synthesis of expolysaccharides in *Streptococcus thermophilis*. Furthermore, it was investigated that the Cytosine/Guanine (CG) content in DNA sequence may affect the EPS biosyhthesis. It was revealed a variation of CG content from 41.6±0.5 to 45.1±0.2 mol% for strain *Lb. rhamnaosus* R and *Lb. rhamnaosus* RW-9595M, respectively. Nevertheless, it showed the differences in amino acid sequences of ORFs (open reading frame) of the gene clusters in different strains. Among the four strains exanimined, there are few amino acid differences in the glycosyltransferrase sequences (Péant et al., 2005). The strain *Lb. rhamnosus* R showed one amino acid difference in two glycosyltransferase gene products (Asp (Aspartic acid) to Asn (Asparagin) in WelG, and Glu (Glutamic acid) to Gly (Glycine) in WelJ) while *Lb. rhamnaosus* RW- 6541M, showed one amino acid change (Asp to Gly in WelI) in comparison to the corresponding sequences from the others three strains (*Lb. rhamnosus* ATCC 9595, *Lb. rhamansus* RW-9595m and *Lb. rhamnosus* R). In this context, comparing to the present study, the CG content and the change in conserved amino

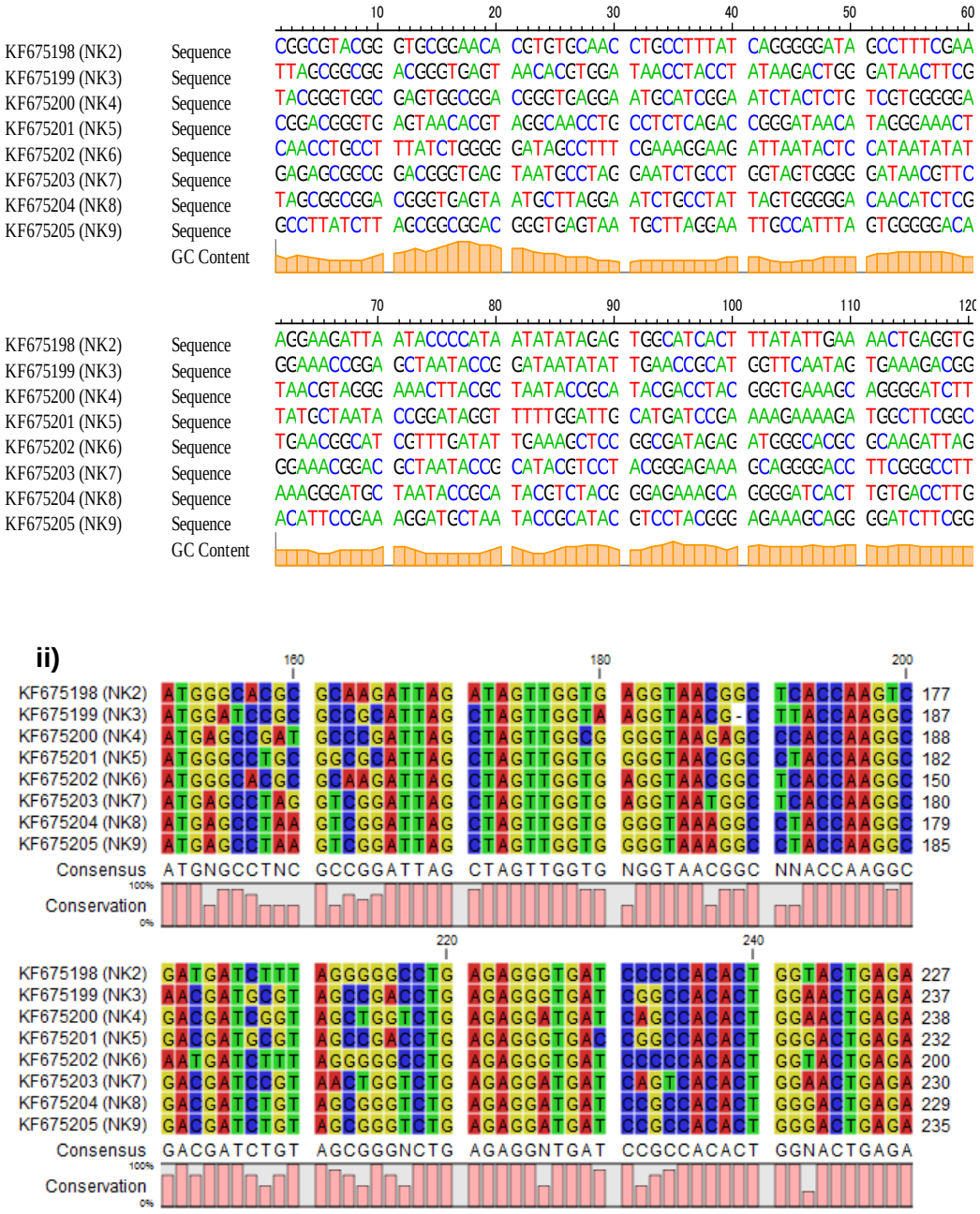
acid among the different strains could be one of the reasons that explained the difference in EPS concentration and composition (Figure 3-2(i)).

Most of the colanic acid (CA) genes have a high GC content (52 to 57%) compared with that usual one (50%) for *E. coli* (*Escherichia. coli*) gene, indicating that they were acquired by lateral gene transfer from another species (Stevenson et al., 1996). Interestingly though, a block of three genes (wcaD, wcaE, and wcaF), have a lower GC content. This suggests that these genes have a different evolutionary history to the other genes of the cluster.

Also, the genes implicated in nucleotide sugar precursor biosynthesis may have a role in EPS biosynthesis (Péant et al., 2005). These genes could not be present in the EPS synthesis genes clusters and seems to be controlled by the promoter of the cps (capsular polysaccharide synthesis) clusters. Exceptionally or in some strains, it could be present and its transcription could be controlled by different promoters. Then the expression of these genes might be activated independent of the EPS biosynthesis genes to form the cell wall polysaccharides, but the co- transcription of these genes with the EPS biosynthesis genes cluster might increase the sugar precursor pool available for biosynthesis of EPS. In fact, the variation in the EPS production level among the isolated strains (in present study) may be due the difference in the availability of sugar precursor and the enzymes responsible for EPS biosynthesis for the other strains. The research by Péant et al., (2005) revealed that four genes (rmlA-rmlD) exhibited significant similarity with rml gene products involved in the anabolism of dTDP- L-rhamnose from α-D-glucose 1-phosphate. EPS biosynthesis gene clusters of LAB do not generally contain these four strains. They found that its transcription is controlled by two different promoters (P2 and P3). They investigated that the location of genes involved in rhamnose precursor biosynthesis within this locus, and their coordinated expression suggest the importance of this monosaccharide for biosynthesis of *Lb. rhamnosus* EPS structure, where four out of seven monosaccharaides of the repeat unit are rhamnose.

Comparative analysis of EPS biosyhthesis genes clusters from strains that produce different EPS concentration is the first step to provide new insight into this industrially relevant phenotype. Many researches and present study accord that the presence of the genes encoding potential glycosyltransferases responsible for the structural organisation could be correlated well with the assembly of the EPS repeating structure. Furthermore, any variation or the difference in genes clusters involved in biosynthesis of EPS could be correlated to the variation in the amount of EPS produced among different strains.

i)



ii)

Figure 3-2 : A) Allignement of all strains isolated in present study.

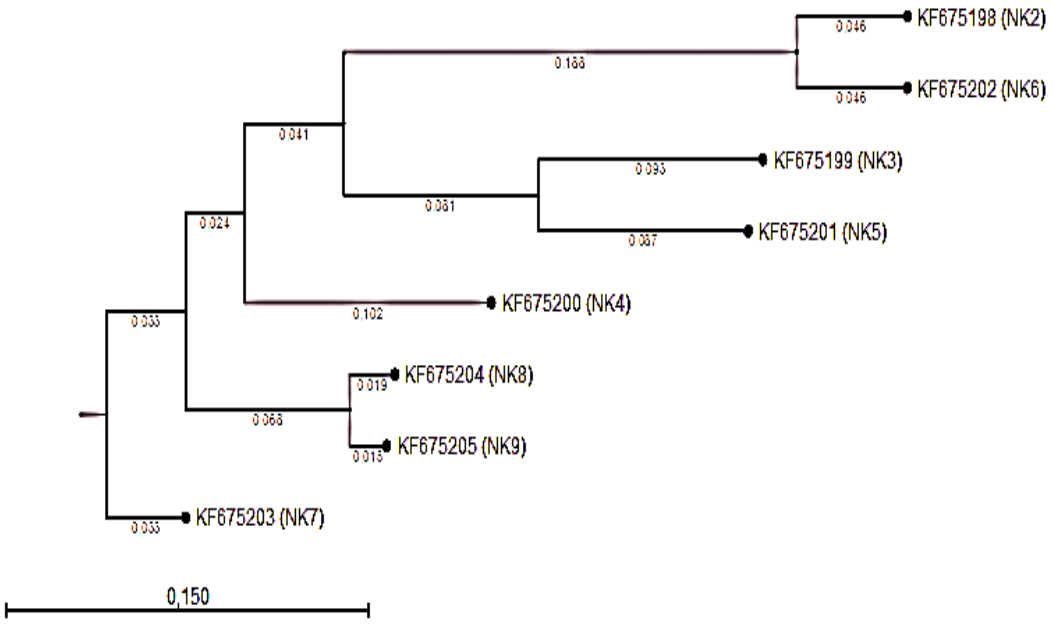


Figure 3-2 B: Phylogenetic tree of all the isolated strains in present study

3.5.3 Biochemical characteristics of EPS producers

The extracellular biopolymers synthesis by microbial cells depends on the carbon and nitrogen availability in the culture medium and fermentation conditions. Most of exopolymer-producing microorganisms utilize carbohydrates as their carbon and energy source and either ammonium salts and/or amino acids as their source of nitrogen. Therefore, the difference in EPS concentration produced by various isolated strains could be explained due to their different enzymatic machinery and their capacity to utilize different available carbon and nitrogen sources present in the sludge. Tables 3-2 and 3-3 present the carbon and nitrogen substrate utilization profiles of different strains obtained from the BioLog biochemical profiling.

All the strains isolated, particularly, *Brevibacillus parabrevis* (NK5) and *Cloacibacterium normanense* (NK6) revealed a broad spectrum of carbon and nitrogen substrate utilization profile as compared to other strains isolated in previous studies (More et al., 2012a). This is due to the difference in structure of strains, their capacity to assimilate carbon and nitrogen source available in the sterilized sludge, which in turn resulted in high EPS synthesis than others (Subramanian et al., 2010). The synthesis of high EPS concentration by *Cloacibacterium*

normanense (NK6) occurred in presence of some carbon source in the growth medium (Sutherland et al., 2001). The availability of carbohydrates, such as: glucose, maltose, mannose and lactose in the medium contributes to high efficiency or high concentration of EPS production in the medium (Table 3-2). The wastewater sludge contains a variety of organic compounds including monomers, oligomers and polymers (of different molecular weights) in the form of particulate or dissolved fractions. Major chemical fractions in municipal wastewater are proteins, complex sugars, volatile fatty acids, lipids and others (humic acid, DNA-RNA, tannic acid, fibers), which could serve as raw material for bacterial strains. Utilization and the availability of glucose could have role in EPS biosynthesis. In general, EPS production increased where growth was extended by high glucose content in the medium (Huang et al., 2010).

BioLog study present that *Brevibacillus parabrevis* (NK5), *Cloaciabacterium normanense* (NK6) and *Pseudomonas veronii* (NK7) have high glucose assimilating capacity for EPS production. However, the concentration of EPS produced by Pseudomonas *veronii (NK7)* was lower than obtained by the other two strains, this could be due to the fact that it might have specific metabolic pathways to utilize complex carbon source present in the sludge to produce EPS.

For example, there are different metabolic pathways between glucose and sodium acetate used as carbon source. In this case, the citric acid cycle which plays an important role in the metabolism of organic compounds and the biosynthesis of microbial products could be investigated. Sodium acetate can enter the citric cycle directly, but glucose and starch have to be degraded to pyruvate and then oxidized to form acetyl- CoA before it enters the citric acid cycle (Madigan et al., 1997). The degradation and uptake of readily biodegradable organic substrates probably resulted in a high level of exoenzymes in the EPS matrix (Nielsen et al., 1996)

Low nitrogen content in the growth environment also influences extensively the microbial synthesis of extracellular biopolymers (Sleytr et al., 1997). The high content and availability of nitrogen sources in the medium induce extracellular protein production by microbial cells. *Cloacibacterium normanense* (NK6) and *Brevibacillus parabrevis* (NK5) utilized almost all nitrogen sources such as gelatin, glycyl-L- proline, L-glutamic acid and L- serine compared to other strains, which explain the high protein content of EPS produced by these strain as compared to the EPS of other strains (Table 3-3).

Table 3-2: Carbon sources profiles of EPS producing

carbon source	*Chryseobacterium gregarium*	*Staphylococcus epidermidis*	*Stenotrophomonas Acidaminiphila*	*Cloacibacterium normanense*	*Brevibacillus parabrevis*	*Pseudomonas veronii*	*Acinetobacter soli*	*Acinetobacter parvus*
Dextrin	+	+	+	+	+	-	-	-
D-Maltose	+	+	+	+	+	-	-	-
D-Trehalose	-	+	-	-	+	-	-	-
D-Cellobiose	-	+	-	-	+	-	-	-
Gentiobiose	+	+	-	+	+	-	-	-
Sucrose	-	+	-	-	+	+	-	-
D-Turanose	-	+	-	-	+	-	-	-
Stachyose	-	-	-	-	-	-	-	-
D-Raffinose	-	-	-	-	-	-	-	-
a-D-Lactose	-	+	-	-	-	-	-	-
D-Melinoise	-	+	-	-	-	-	-	-
b-Methyl-DGlucoside	-	+	-	-	+	-	-	-
D-Salicin	-	+	-	-	+	-	-	-
N-acetyl-Dglucosamine	-	+	+	-	+	-	-	-
N-acetyl-b-Dmannosamine	-	+	+	-	-	-	-	-

N-acetyl-Dgalactoseamine	-	+	+	-	+	-	-	-
N-acetyl neuraminic acid	-	+	-	-	-	-	-	-
a-D-Glucose	-	+	-	+	+	+	+	-
D-Mannose	-	-	+	+	-	-	-	-
D-Fructose	-	+	-	-	-	-	+	-
D-Galactose	-	+	-	-	-	-	-	-
3-Methyl glucose	-	+	-	-	-	-	-	-
D-Fucose	-	+	-	-	-	-	+	-
L-Fucose	-	+	-	-	-	+	-	-
L-Rhamnose	+	+	-	-	-	-	-	-
Inosine	-	+	-	-	+	-	-	-
Glycérol	-	+	-	+	+	+	-	-
Pectin	+	-	-	-	-	-	-	-
D-Galacturonic Acid	+	+	+	-	-	-	-	-
L-Galactonic acid lactone	-	-	-	-	-	-	-	-
L-Gluconic acid	-	+	+	-	+	+	+	-
Glucuronic acid	-	+	+	-	-	-	-	-
	-	+	+	-	+	-	+	-

Glucuronamide	-	+	-	-	-	-	-	-
Mucic acid	-	+	-	-	-	-	+	-
Quinic acid	-	+	-	-	-	-	-	-
D-Saccharic acid	-	+	-	-	-	-	-	-
P-Hydroxyphenyl acetic acid	-	-	-	-	+	-	+	-
Methyl pyuvate	-	+	-	-	-	-	+	+
D-Lactic acid methyl ester	-	+	+	-	-	+	+	-
L-Lactic acid	-	+	-	-	+	+	+	-
Citric acid	-	+	+	-	+	-	+	-
a-Keto-glutaric acid	-	+	-	-	-	-	+	-
D-Malic acid+A72	-	+	-	-	-	+	+	-
L-Malic acid	-	+	-	-	-	-	+	-
Bromo-succinic acid	-	-	-	-	-	-	+	-
c-Amino-butyric acid	-	+	-	-	-	-	+	-
a-Hydroxy butyric acid	-	+	-	-	+	-	+	-
b-Hydroxy-D, Lbutyric acid	-	-	-	-	+	-	+	-
a-Keto-butyric acid	-	+	-	-	+	-	+	-

Acetoacetic acid	+	+	-	+	+	-	-	+
Propionic acid	-	+	+	-	+	-	+	-
Acetic acid	+	+	+	+	+	-	+	+
Formic acid	-	+	-	-	+	-	-	-

Table 3-3 : Nitrogen utilization profiles of all strains isolated.

Nitrogen sources	*Chryseobacterium gregarium*	*Staphylococcus epidermidis*	*Stenotrophomonas Acidaminiphila*	*Cloacibacterium normanense*	*Brevibacillus parabrevis*	*Pseudomonas veronii*	*Acinetobacter soli*	*Acinetobacter parvus*
Gelatin	+	+	+	+	+	-	-	-
Glycyl-L-proline	-	+	+	+	+	-	-	-
L-alanine	-	+	+	-	+	-	+	-
L-arginine	-	+	-	-	+	-	+	-
L-aspartic acid	-	+	-	-	+	-	+	-
L-glutamic acid	+	+	+	+	+	+	+	-
L-Histidine	-	+	+	-	+	+	+	-
L-Pyroglutammic acid	-	+	-	-	-	-	+	-
L-Serine	-	+	+	+	+	-	+	-
L-Aspartic acid	-	+	-	-	-	-	-	-
D-Serine	-	+	-	-	+	+	-	-

The strains isolated in the present work are totally different than those presented by More et al. (2012a). More et al. found that the *bacillu*s species had high spectrum of carbon utilization except *bacillus* sp. 1 and *Bacillus* sp.9 which utilized few carbon sources compared to other strains. In present study, it was revealed that some of the strains such as *Cloacibacterium normanense, Acinetobacter parvus and Chryseobacterium gregarium* utilized very few carbon sources; they still produced high EPS concentration compared to *bacillus sp.* This could be due to the fact that they had specific metabolic pathways which helped to utilize complex nutrients and carbon sources available in the sludge. Sometimes BioLog systems are insensitive to certain bacterial structure due to their metabolic redundancy. Species composition could change with or without a shift in the profile of the positive responses. In addition, every species has specific genetic elements and their genes clusters encode for EPS production. Their high expression could increase the availability of precursors and the enzymes which utilize specific and different substrates involved in the anabolism of sugar nucleotide precursors responsible for EPS biosynthesis. That's why sometimes the EPS produced by bacterial strains displays a great variety of structure, and many are heteropolysaccharides composed of different sugar moieties (glucose, galactose, rhamnose, mannose, N-acetyl glucosamine).

3.5.4 Factors affecting microbial community

In present work, the isolated strains were totally different from those isolated by Subramanian et al. (2010) even from same activated sludge. This could be explained by considering environmental factors that affected the bacterial community. Some of these factors are called modulators (Balser et al., 2001). Examples of modulators are temperature, pH and salinity. The intrinsic property of the microbial community is genotypically determined through the adaptation of the populations present to the selective pressure. The strains (NK2- NK9) isolated in this study in July 2013 were totally different from the strains (BS1-BS25) isolated by Subramanian et al. (2010) in January, 2010. In response to changes in season (summer/winter), the microbial community composition and biomass can change. For example, microbes will change the composition of their membrane fatty acids after a change in temperature (Finlay et al., 1997). A change in the environment will therefore induce a selection pressure, which will gradually shift the growth optima of the community. If the environment changes to a situation far from the initial optimum conditions for the bacterial community, a shift will occur in the composition of the community. In this case, the variation of temperature could be a reason for the diversity and change of microbial community in present study comparing to bacteria isolated by Subramanian

et al. (2010). Firstly, it is suggested that the optimal temperature growth of strains isolated by Subramanian was less than 10 °C according to the data required by WWTP, CUQ. The temperature of WWTP in winter was almost between 4 and 10°C. However in summer, it was higher than 10°C and can reach to 15°C. Secondly, it could be another reason that there were organisms better suited to the new environmental conditions which would grow and compete other organisms, or existing organisms will undergo evolutionary adaptation. Species of the microbial community can rapidly adapt even to momentary changes in the local environment (Finlay et al., 1997). The sensitivity to environmental change and disturbance differs among the bacterial communities and it depends on the species.

On the other hand, comparison with to the previous studies (More et al., 2012a), some of bacillus strains were isolated in this study, but they were different species.

It could be suggested that *Brevibacillus parabrevis* adapted to the change in temperature and it could be a mutation in its gene clusters which introduce another phenotype or genotype of bacillus sp. However, the total diversity and change in microbial community in present study could be explained by part of microbial community died and replaced by other microbial community species and genus. Davis and Shaw, (2001) suggested that adaptive responses of vegetation to climate change occurred at many levels, including phenotypic plasticity, genetic adaptation, succession and migration processes. If there is a drastic change in the environment, part of the community will die preparing for more tolerant species to take over. If a strong selection pressure is exerted, this can favour certain genotypes or mutants and thus introduce new genotypes in the community. A less dramatic change in the environment can lead to phenotypic changes in the population.

Many researchers reported seasonal variation correlating environmental factors with bacterial community and function. The seasonality of microbial communities in different ecosystems has been well documented, with little attention being paid to the activated sludge (AS) system (Kim et al., 2013), in which the seasonal dynamics of microbial communities greatly affects the performance and stability of pollutants removal. As reported by Ju et al. (2103), microbial communities in AS were predominated by bacteria. The abundance of bacteria in summer was 87.8 ± 2.8 %, which was lower than that in winter (91.0 ± 1.6 %). On one hand, abundance of Actinobacteria (phylum) was usually higher in winter 26.0 ± 3.1% than in summer 17.9 ± 6.9 %, with abundance ratio of summer to winter of 0.7. Actinobacteria sp. decreased with increasing salinity.

Changes in salinity affect bacterial cell by changing their osmotic environment, causing stress on the membranes and proteins structures. Taking consideration into that, the salts could be a reason causing stress and high EPS production for some bacteria as well as a disadvantage to some others for their growth. Here, the high salinity of water in winter season could be explained by the high quantity of salt added in the winter season used to melt ice. In present study, most of strains isolated have shown growth only in presence of 1% of NaCl as *Acinetobacter* sp, *Pseudomonas veronii* and *Chryseobacterium gregarium*, some of them resist 4% of NaCl to grow as *Brevibacillus parabrevis* and some others no growth in NaCl medium as *Cloacibacterium normanense*. This could be a reason to do not isolate this type of bacteria in previous studies (Subramanianet al., 2010). However, most of the *Bacillus* spp. isolated in winter by Subramanian et al., (2010), and characterized by BioLog test by More et al., (2012a), have shown growth in presence of 1%, 4% and 8% w/v NaCl, except *Bacillus* sp.1 which could not grow at 8% w/v NaCl. EPS producing Bacillus spp. were resistant to high salt stress conditions which were also reported by Upadhyay et al. (2011).

Further, in engineered systems, e.g. in wastewater treatment plants, it has recently been suggested that the diversity of specific bacterial groups in activated sludge influences the functioning of the reactor. The presence of many microorganisms able to conduct a specific process increases the probability that a change of environmental conditions does not worsen the effectiveness of wastewater treatment, since one of the species will adapt and maintain the specific metabolic pathway (LaPara et al., 2002). Taking this into consideration, the operational parameters of the wastewater treatment plant should be selected to favour the development of a highly diversified bacterial community. Cydzik- Kwiatkowska et al. (2012) reported the impact of operational parameters on bacterial community. Firstly, it was found that richness of microorganisms was correlated with organic load rate (B_C) of the system. During the year of sampling B_C varied from 0.09 to 0.21 g COD .g TSS-1.d-1 and bacteria richness increased with increasing B_C. At a very high BC, the diversity tends to decrease because a large quantity of organic carbon in wastewater stimulates dynamically growing bacteria with a very short generation time and these compete out other species and dominate the bacterial population. Similar results were obtained by Xia et al. (2008) investigated the microbial community structure in response to different ratios of carbon to nitrogen (C/N of 3:1, 5:1, and 10:1) in wastewater sludge. For sure that the carbon content as well as C/N ratio of activated sludge collected in 2010 by Subramanian et al., (2010.) was different from those collected in 2013 in present research, Secondly, Valentin et al., (2012) reported that biochemical oxygen demand (BOD), influent total suspended solid (TSS), solid retention time (SRT), food /microorganisms (F/M), seem to have the

strongest influence on the bacterial communities composition in the bio-reactors. The very low values of F/M in the wastewater treatment plant suggest that the microbial community was starving contributing to high mortality rate among the microbial populations. Similarly, some of these factors could be the stress causing the change and the diversity on microbial community between the present and previous study (More et al., 2012 a,b).

3.5.5 EPS production and characteristics

All the bacterial strains were individually cultivated in sterilized sludge under similar growth conditions to ascertain their capacity of EPS production. The sludge had total solids (TS) concentration of 11.3 ± 0.6 g/L, suspended solid (SS) concentration of 9.7 ± 1.2 g/L and pH of 6.2 ± 0.2. 1.6 ± 0.2 - 11.8 ± 1.2 g/L was the range of the maximum concentration of EPS harvested from different strains after 48 h of fermentation (Figure 3-3A). The control sample of the sterilized sludge (without inoculation) contained EPS concentration of 1.9 ± 0.2 g/L, whereas the control sludge without sterilization incubated for 48 h revealed 0.9 g/L ± 0.1 EPS concentration.The concentration of EPS produced by *Cloacibacterium normanense* (NK6) (11.8 ± 1.2 g/L) and by *Brevibacillus parabrevus* (3.5 ± 0.5 g/L) was higher than the other bacterial strains reported in this work (Table 3-4) as well as that of Serratia sp. (3.4 g/L) reported in previous work (More et al., 2012 a,b). Further, the production of EPS in sterilized sludge was higher the maximum concentration of 3.2 g/L obtained in synthetic media (Subramanian et al., 2010).

On the other hand, Figure 3-3b revealed that the EPS production by all the isolated strains while growing in the sterilized activated sludge closely related to the bacterial cell concentration (Figure 3-3A and B). *Cloacibacterium normanense strain* (NK6) attained the highest cell count of 7.5 x 108 CFU/mL at 48 h followed by *Brevibacillus parabrevis* (NK5) (Figure 3-3B) with cell count of 1.29 x 108 CFU/mL in 24 h. The pH increased in case of NK6 from 7 to 9 whereas in for the other strains the pH decreased from 7 to 4 (Figure 3-4). This difference in pH could be explained by the chemical nature of EPS which varies in terms of carbohydrates, protein, nucleic acid, lipids, and humic substances.The presence of uronic acids (such as glucuronic, galacturonic and mannuronic acid) or common substitutes like acetate ester, pyruvate ketals, formats, succinates and inorganic (phosphate ans sulfate) decides the nature (neutral, anionic or cationic) of EPS macromolecules with affect the pH (Marvasi et al., 2010; Sheng et al., 2010).

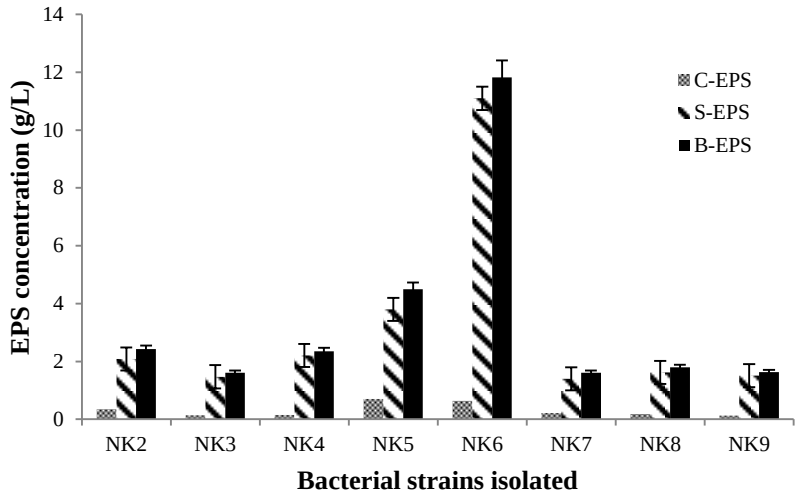


Figure 3-3-A : EPS concentrations produced by 8 bacterial strains and the control at the end of 3 days of fermentation at initial pH 7.0.

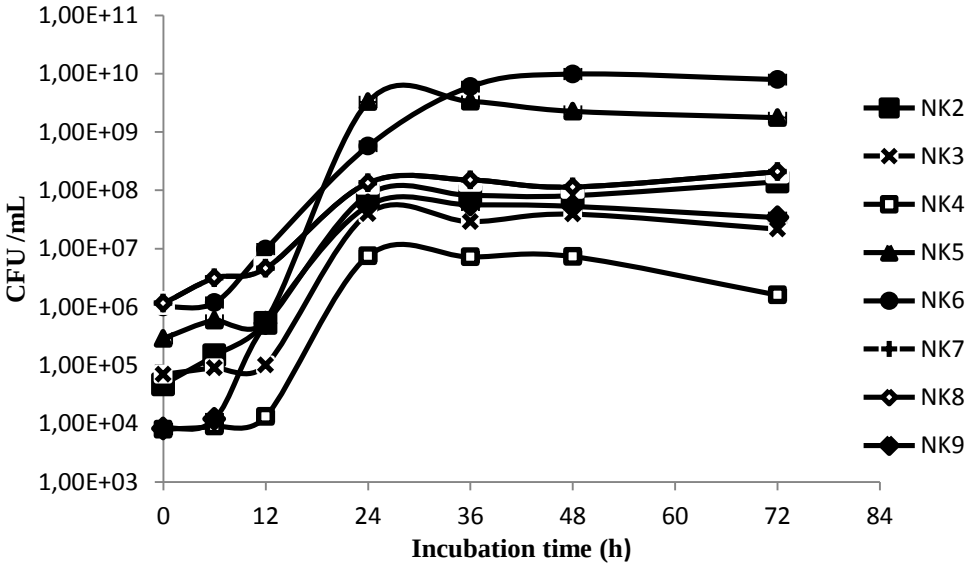


Figure 3-3-B: Growth profiles of different strains isolated.

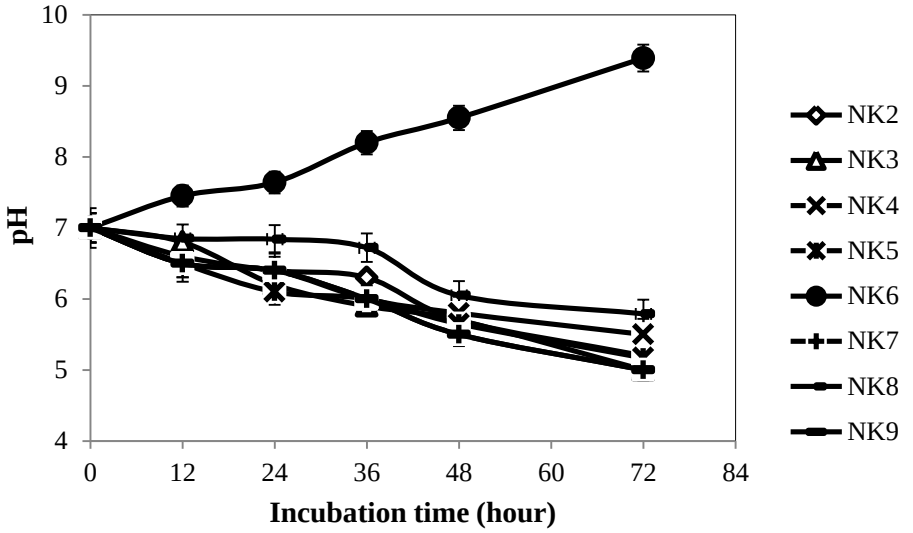


Figure 3-4 : Variation of pH during the fermentation

3.5.6 Flocculation activity

Flocculation activity (FA) of B-EPS, LB-EPS and TB-EPS for all isolated bacteria was studied and results are presented in Table 4. The general trend of flocculation activity for all strains is an increase of FA with an increase in EPS concentration (ranged 0.2 - 7.0 mg/g Kaolin) (for B-EPS and LB-EPS) and up to 0.03 mg/g kaolin (for TB-EPS) *Cloacibacterium normanense, Brevibacillus parabrevis, Stenotrophomonas acidaminiphila and Chryseobacterium gregarium* showed higher flocculation activities, of 89.8 ± 1.2, 79.4 ± 3.2 %, 77.0 ± 1.6 % and 74.0 ± 1.3 % respectively in concentration range between 1.2 – 2.4 mg B-EPS/g kaolin. For other strains, the flocculation activity of B-EPS ranged between 61- 69 %. Lower flocculation activity for some bacterial strains may be due to lower EPS concentration added which ranged between 0.6 ± 0.1 – 1.2 ± 0.2 mg B-EPS/g Kaolin. In present study, the highest flocculation activity was 90.2 ± 2.3 % in case of *Cloacibacterium* normanense (NK6) with the addition of 2.4 ± 0.3 mg LB-EPS/g kaolin. The highest flocculation activity 83.2% in case of *Brevibacillus parabrevis* (NK5) using 0.4 mg TB-EPS /g kaolin was also recorded. EPS concentration required to attain maximum flocculation activity was 2.30 mg LB-EPS/g kaolin and 2.36 mg B-EPS/g kaolin in case of *Cloacibacterium normanense* (NK6); following with 0.36 LB-EPS/g Kaolin and 1.20 mg for *Brevibacillus parabrevis*. The flocculation activity of 90.2 ± 2.3 % and 83.2 ± 2.3 % reported in the present study was higher than 81.7 % (B-EPS) produced by *Bacillus* sp.7 (More et al. 2012 a,b).

Table 3-4: Kaolin clay flocculation activity obtained for different types of EPS for isolated strains.

	Flocculation Activity (FA) %					
	TB-EPS		Broth EPS		LB-EPS	
Strains	EPS (mg/g kaolin)	FA (%)	EPS (mg/g kaolin)	FA (%)	EPS (mg/g kaolin)	FA (%)
Chryseo-bacterium gregarium	0.4 ±0.02	30.4 ± 3.4	0.4 ± 0.02	24.1 ± 0.6	0.04 ± 0.005	44.5 ± 3.4
	0.8 ± 0.09	59.5 ± 2.5	0.6 ± 0.09	19.5 ± 2.6	0.06 ± 0.010	46.3 ± 1.9
	1.4 ± 0.11	57.3 ± 1.9	0.8 ± 0.10	38.5 ± 3.3	0.08 ± 0.009	53.6 ± 4.6
	1.8 ± 0.13	74.0 ± 1.3	1.0 ± 0.14	72.8 ± 2.3	0.12 ± 0.002	57.8 ± 3.8
Staphylococcus epidermidis	0.2 ± 0.04	8.2.1 ± 2.8	0.4 ± 0.02	7.5 ± 6.3	0.02 ± 0.01	13.1 ± 4.2
	0.6 ± 0.10	13.7 ± 4.6	0.6 ± 0.09	11.2 ± 3.9	0.04 ± 0.02	15.7 ± 5.1
	1.0 ± 0.06	17.2 ± 1.9	0.8 ± 0.11	15.3 ± 3.7	0.08 ± 0.04	44.9 ± 3.9
	1.2 ± 0.10	31.4 ± 2.2	1.0 ± 0.14	29.4 ± 2.8	0.12 ± 0.065	51.9 ± 4.1
Stenotrophomonas acidaminiphila	0.4 ± 0.2	32.6 ± 1.4	1.2 ± 0.2	61.1 ± 2.9	0.2 ± 0.05	24.8 ± 0.5
	0.8 ± 0.9	68.8 ± 2.4	2.4 ± 0.3	61.9 ± 1.7	0.3 ± 0.1	24.9 ± 2.3
	1.4 ± 1.2	48.5 ± 2.3	3.6 ± 0.9	53 ± 2.4	0.4 ± 0.09	39.4 ± 2.8
	1.8 ± 1.3	77.2 ± 1.6	4.8 ± 0.6	57 ± 2.5	0.5 ± 0.02	53.3 ± 4.1
Cloacibacterium normanense	0.1 ± 0.05	69.9 ± 3.0	1.18 ± 0.1	81.0 ± 1.9	1.1 ± 0.02	85.1 ± 1.5
	0.2 ± 0.10	79.4 ± 3.1	2.36 ± 0.2	89.4 ± 2.3	2.3 ± 0.09	90.4 ± 0.9
	0.4 ± 0.10	77.5 ± 2.0	4.72 ± 0.1	86.2 ± 2.5	4.6 ± 0.10	88.2 ± 1.4
	0.5 ± 0.10	68.5 ± 2.3	7.08 ± 0.4	82.3 ± 3.1	6.9 ± 0.20	82.3 ± 2.3
Brevibacillus	0.4 ± 0.1	83.2 ± 2.3	0.6 ± 0.1	46.9± 3.4	0.02 ± 0.01	32.7 ± 1.2

parabrevis	0.6 ± 0.2	77.6 ± 1.6	1.2 ± 0.1	79.4 ± 3.2	0.04 ± 0.01	47.2 ± 1.3
	1.0 ± 0.2	71.9 ± 1.9	1.8 ± 1.3	71.5 ± 2.3	0.06 ± 0.01	51.7 ± 2.1
	1.4 ± 0.2	69.5 ± 2.1	2.4 ± 0.9	61.7 ± 1.9	0.08 ± 0.01	69.8 ± 1.8
Pseudomonas Veronii	0.32 ± 0.04	35.5 ± 1.3	0.28 ± 0.05	44.9 ± 3.7	0.032 ± 0.03	60.7 ± 1.7
	0.72 ± 0.08	55.1 ± 1.8	0.56 ± 0.04	46.2 ± 3.5	0.063 ± 0.05	64.4 ± 2.5
	1.04 ± 0.06	47.2 ± 2.1	0.84 ± 0.18	27.6±1.85	0.096 ± 0.03	61.6 ± 2.7
	1.36 ± 0.13	48.8 ± 2.4	1.12 ± 0.11	33.5 ± 2.3	0.12 ± 0.04	58.3 ± 1.8
Acinetobacter soli	0.03 ± 0.04	49.8 ± 1.9	0.2 ± 0.12	50.1 ± 0.8	0.036 ± 0.01	45.1 ± 1.5
	0.06 ± 0.01	52.5 ± 1.3	0.4 ± 0.03	55.3 ± 1.4	0.072 ± 0.02	46.9 ± 1.3
	0.1 ± 0.01	65.5 ± 0.5	0.6 ± 0.02	50.7 ± 1.5	0.100 ± 0.03	40.8 ± 0.9
	0.12 ± 0.01	43.1 ± 2.4	0.8 ± 0.12	53.2 ± 2.2	0.121 ± 0.02	45.3 ± 2.1
Acinetobacter parvus	0.03 ± 0.04	28.5 ± 1.1	0.2 ± 0.02	17.7 ± 1.7	0.04 ± 0.01	20.4 ± 2.9
	0.06 ± 0.01	34.5 ± 2.3	0.4 ± 0.03	27.4 ± 2.6	0.08 ± 0.08	33.9 ± 1.6
	0.10 ± 0.01	62.5 ± 3.4	0.6 ± 0.09	10.5 ± 2.3	0.12 ± 0.06	34.7 ± 3.5
	0.12 ± 0.01	40.3 ± 1.5	0.8 ± 0.06	17.7 ± 1.8	0.20 ± 0.04	48.5 ± 1.9
Control	0.2 ± 0.03	32.1 ± 2.3	0.2 ± 0.05	2.90 ± 0.8	0.02 ± 0.002	32.2 ± 1.9
	0.4 ± 0.04	35.7 ± 4.2	0.4 ± 0.05	16.6 ± 1.7	0.04 ± 0.003	37.2 ± 1.6
	0.6 ± 0.02	32.6 ± 1.7	0.6 ± 0.12	19.4 ± 4.5	0.06 ± 0.010	42.8 ± 2.5
	0.8 ± 0.03	30.1 ± 3.5	0.8 ± 0.10	32.5 ± 2.65	0.08 ± 0.004	40.2 ± 2.5

Furthermore, the concentration of B-EPS added was between 1.12 - 2.70 mg EPS/g Kaolin to attain high flocculation activity (>75%) (More et al., 2012a), whereas EPS produced in the synthetic media by the same bacterial strain required 250 - 2500 mg EPS/g Kaolin to attain similar flocculation activity (Subramanian et al., 2010). The lowest concentration of S-EPS to achieve maximum FA in present work was 2.3 mg/g kaolin. The most efficient EPS produced by different strains are compared in Table 3-5. A lower concentration of EPS is required to achieve higher FA with the new strains isolated in this study which is advantageous from an economic standpoint.

Table 3-5: Comparison of FA (%) results between present work and previous studies

This Work	EPS (g/L)	FA (%) (B-EPS)	FA (%) LB-EPS	Strains of More et al., (2012)	EPS (g/L)	FA (%) (B-EPS)	FA (%) (LB-EPS)
Chryseobacterium gregarium	2.41	74.0	72.8	*Bacillus sp.*2	1.27	69.0	65.0
Staphylococcus epidermidis	1.60	31.4	29.4	*Bacillus sp.*5	1.24	63.0	45.4
Stenotrophomonas acidaminiphila	2.35	77.2	61.9	*Bacillus sp.*6	1.45	76.5	56.0
Cloacibacterium normanense	11.80	89.9	90.2	*Bacillus sp.*7	1.56	81.7	63.5
Brevibacillus parabrevis	3.52	79.4	83.2	*Bacillus sp.*8	1.65	55.0	68.1
Pseudomonas veronii	1.61	55.1	44.9	*Bacillus sp.*9	1.23	56.0	53.0
Acinetobacter soli	1.80	65.5	55.3	*Serratia sp.*1	1.51	57.0	56.0
Acinetobacter parvus	1.63	62.5	27.4	*Serratia sp.*1	1.51	57.1	56.3
Lowest ESP for Max FA (%)	0.09 - 0.1 mg/SS				1.12 - 2.7 mg/SS		

3.5.6.1 Effect of EPS composition on flocculation activity

Three bacterial strains (NK2, NK3 and NK4) displayed an increased flocculation activity with the increased EPS concentration whereas five strains (NK5, N6, N7, NK8 and NK9) showed a decreasing trend with increasing B-EPS concentration (Table 3-4). B-EPS contains both EPS (LB-EPS & TB-EPS) and residual material of fermentation medium as well as other unknown metabolites. Therefore, flocculation activity might be affected by these factors and by the variation of protein and carbohydrate content of the EPS. Protein and carbohydrate content of EPS varies with the type of bacterial strain.

The carbohydrate content of TB-EPS was high in case of NK2, NK7, NK8 and NK9 comparing to NK5 and NK6 (Figure 3-5B). However, the carbohydrate content of S-EPS was almost similar in case of all strains. For the protein content, it was high in LB-EPS and TB- EPS for all the strain, except the LB-EPS of NK6 which contain less protein content (Figure 3-5A). According to these results, high flocculation activity (83.2 ± 2.3 %) of TB-EPS was achieved in case of NK5. However, the highest flocculation of LB-EPS was 90.2 ± 2.5 % in case of NK6. This may be due to the chemical composition of protein and carbohydrate content in LB-EPS which contain more groups with positive charge that helps good flocculation (Yu et al., 2009). Furthermore, the high FA could also be possible due to the fact that LB-EPS was long polymeric chain biopolymers which may also possess more active sites to bind with the colloidal particles and other microorganisms comparing to TB-EPS (Subramanian et al., 2010).

In present study, it can be suggested that the low carbohydrate content and high protein content increase the flocculation activity. On the other side, low flocculation activity had been presented by other bacterial strains (NK2, NK3, NK4, NK8, NK9) with high protein content. This could be due to different structures and types of protein produced by these strains (More et al., 2012 a,b).

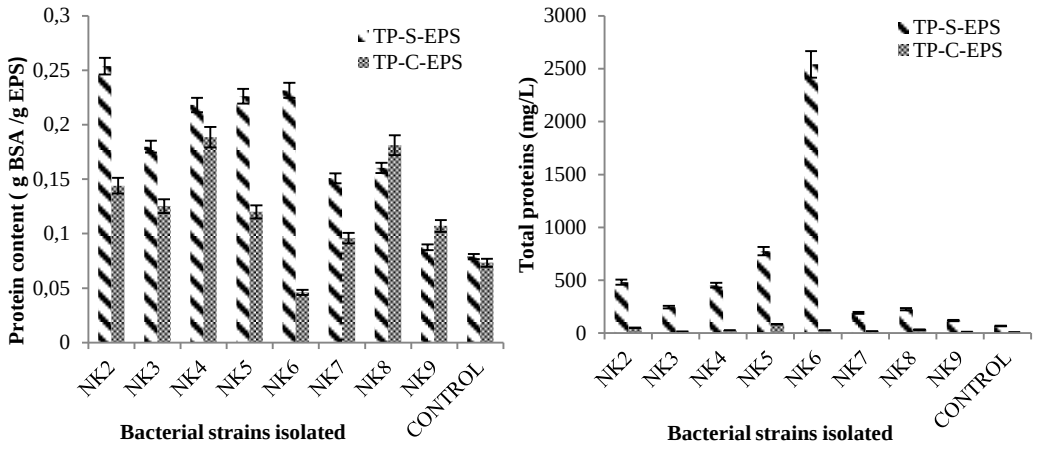


Figure 3-5 A: Extracted EPS total proteins (TP) content from bacterial strains isolated, and sterilized sludge

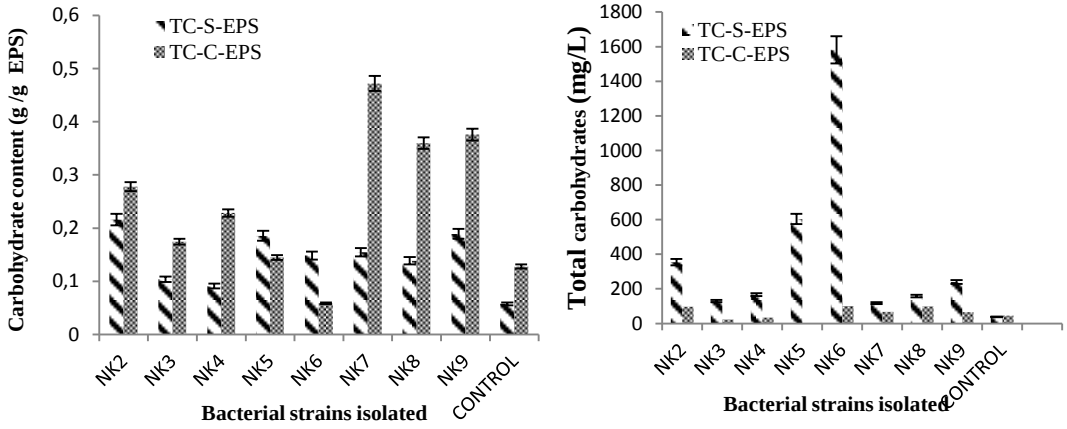


Figure 3-5-B: Extracted EPS total carbohydrate (TC) content from bacterial strains isolated, and sterilized sludge.

3.5.6.2 Effect of zeta potential on FA

The zeta potential value at pH 7 of LB-EPS, TB-EPS and B-EPS for all the strains isolated ranged between -47.8 ± 0.4 and -129.2 ± 9,1 (Figure 3-6). The control was -126.5 ± 5.1, -111.5 ± 6.6 and -103.1 ± 4.2 mV, respectively, revealed a higher negative charge than the EPS samples (EPS from bacterial strains).

The zeta potential of LB-EPS produced by bacterial strain NK6 was the highest (- 47.8 ± 0.4 mV), which revealed its better kaolin flocculation activity. These values of zeta potential were approximately in the same range (from -54.2 to -35.6 mV) as obtained by Subramanian et al. (2010) of extracted LB-EPS from selected six bacterial strains. Thus, higher value of zeta potential correlates to higher flocculation activity. Cells with higher negative surface charge would have lower floc strength and poorer flocculating properties. The reason is that the increased negative surface charge would lead to increased repulsive electrostatic interactions between approaching surfaces according to the DLVO theory, and therefore cause a weaker bonding between the cells. However, a better kaolin FA as well as one of the lowest CST value (48.4 s) for bacterial strain (BS8 or Serratia sp.) with the lowest zeta potential (-54.2 mV) was reported by Subramanian et al. (2010). Thus, the EPS charge is not related to floc formation, which is concurrent with the reported literature (Subramanian et al., 2010; More et al., 2012 a,b).

The variation of charge, on different EPS (slime, capsular and broth EPS) produced by different strains was due to different microorganisms may produce EPS of different chemical composition (different functional groups) and properties. The differences in the properties of polysaccharides and proteins of EPS can lead to different charges. For example, higher quantity of basic amino acids in EPS gives higher positive charge while the existence of higher acidic amino acids in the protein and hydroxyl group in polysaccharides (part of EPS) gives higher negative charge. In this case, the pH also plays an important role, when the pH increases; the carboxyl group in protein is ionized furnishing a negative charge. Moreover, the presence of capsular EPS (TB-EPS) on the cell surface can reduce the negative surface charge of microorganisms to a certain extent. Surface charge of the fresh sludge before inoculation (-79.5 ± 4.7 mV) used in this study revealed a higher negative charge than the EPS produced by all the strains (Figure 3-6) and it was higher than reported by Subramanian et al. (2010) (-35.0 mV). The possible reason for higher sludge-surface charge may be due to the fact that the sludge, in addition to EPS (proteins, carbohydrates, lipids, DNA and RNA), is also composed of various other components including microorganisms, humic substances and solids, which may contribute to higher anionic charge.

The EPS produced by 8 bacterial strains, in bacterial broth at pH 7 are also anionic in nature (Figure 3-6). The zeta potential value of LB-EPS produced had relatively less negative charges compared to B-EPS and TB-EPS. In this context, this can be argued that the S-EPS contains higher positively charged groups than other type of EPS (Broth and capsular). The EPS can be composed by many charged groups such as carboxyl, phosphoric, sulfhydryl, phenolic and hydroxyl groups. Many apolar groups (e.g., aromatics, aliphatics in proteins, and hydrophobic regions in carbohydrates) were characterised the EPS.

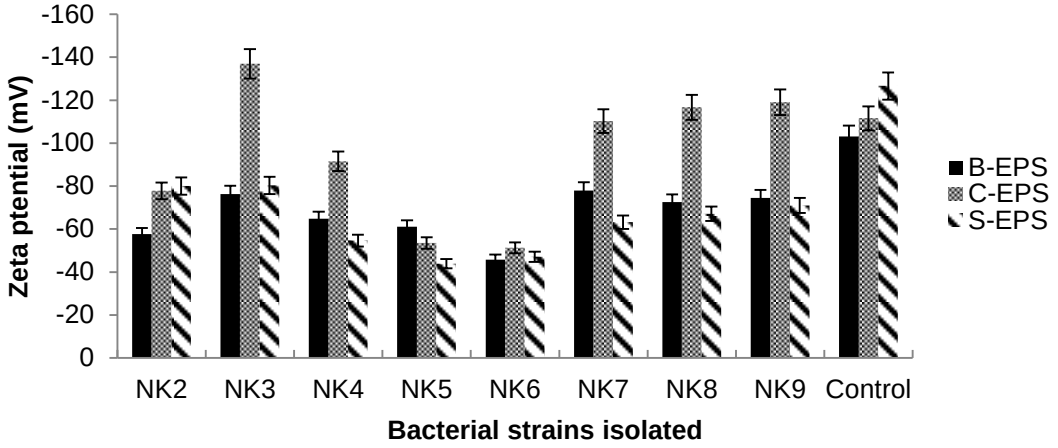


Figure 3-6: Zeta potential values of LB- EPS, TB- EPS and bacterial broth (B-EPS) produced by bacterial strains isolated at initial pH 7.

Moreover, the hydrophobicity is very important property of the EPS. Hydrophobicity results from the behaviour of EPS particles or molecules, which are incapable of interacting electrostatically or establishing hydrogen bonds with water, induce hydrophobic properties into the EPS matrix. This causes the EPS matrix or parts to aggregate together and separate from the water. Then, the presence of hydrophilic and hydrophobic groups in EPS molecules indicate the relative ratio of two groups is related to the composition of EPS.

According to the study of Yuan et al. (2013), the hydrophobicity values were all below 50% for the LB- EPS, while a hydrophobic part of approximately 71% was observed in the corresponding protein or polysaccharide component of the TB-EPS, respectively. Therefore, the high flocculation activity observed in this study could be because of the high hydrophobicity values of proteins present in LB-EPS.

Moreover, decrease in flocculation activity has been shown, after attaining maximum value (Table 3-4). This could be explained by an over dosage of the polymer that might have caused the re-suspension or instability of kaolin particles, which would lead to the high turbidity. EPS concentration is directly related to sludge flocculation (Houghton et al., 2001).
In case of sludge also an optimum EPS concentration is required for maximum flocculation. Excess EPS concentration is detrimental to sludge dewatering.

3.6 Conclusions

Eight bacterial strains capable of producing EPS using sterilized sludge as raw material were isolated and identified. The EPS production variation among the strains was revealed. The EPS, produced by isolated bacterial strains were characterized in terms of proteins and carbohydrates. *Cloacibacterium normanense* produced the highest concentration (11.8 ± 1.2 g/L) of EPS. S- EPS exhibited kaolin flocculation activity of 90 % using very low concentrations (2.36 mg of EPS/g Kaolin following by *Brevibacillus parabrevis*. The variation of EPS concentration produced could be due to many reasons. The initial information to address the metabolic cause of the low production levels of EPS suggest that increased EPS precursor availability, in combination with elevated enzyme activity levels involved in the specific EPS biosynthesis pathway, might enhance EPS production. Then the recent advances in analysis of physiology and genetics of strain producing EPS allow control of their metabolic activity not only by changing the fermentation conditions but also by applying genetic technique that result in the overexpression of existing or novel genes.

3.7 Acknowledgements

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CHAPITRE 4

Characterization of Extracellular polymeric substances (EPS) produced by *Cloacibacterium normanense* isolated from wastewater sludge for sludge settling and dewatering

Ce chapitre est constitué de l'article suivant:

Nouha K, Hoang NV, Song Y, Tagi RD, Surampalli RY (2015) Characterization of Extracellular Polymeric Substances (Eps) Produced by *Cloacibacterium normanense* Isolated from Wastewater Sludge for Sludge Settling and Dewatering. *J Civil Environ Eng* 5: 191. doi:10.4172/2165-784X.1000191

4 Characterization of Extracellular polymeric substances (EPS) produced by *Cloacibacterium normanense* isolated from wastewater sludge for sludge settling and dewatering

4.1 Abstract

In this study, extracellular polymeric substances (EPSs) producing strain was isolated from municipal wastewater sludge (MWWS). Growth profile and the EPS production by *Cloacibacterium normanense* using wastewater sludge as raw material in shake flask fermentation for 96 h were investigated. The highest concentration of S-EPS (13.0 ± 0.8 g/L) and C-EPS (0.3 ± 0.1 g/L) were attained at 48 h of fermentation. S-EPS revealed higher flocculation activity (94.2%) and dewaterability (59.9%) than other types of EPS in kaolin suspension. The dewaterability of MWWS with 2 g suspended solids (SS)/L was improved by 37.6% using 0.02 ± 0.01 g/L of S-EPS and 600 mg/L of $Al_2(SO_4)_3$. The study showed a promising approach of new isolated strain to produce high concentration EPS in sludge with high flocculation activity as well as good settling.

4.2 Keywords

Bioflocculants; Bacterial polymers; Extracellular polymeric substances; Wastewater; Sludge; Pollution control.

4.3 Introduction

Sludge settling and dewatering are the most important steps of wastewater treatment and sludge management. Better dewaterability leads to a sludge economical disposal, reuse as a soil conditioner in agriculture, bricks for construction, and raw material for growing industrial microorganisms (Tyagi et al., 2002).

In recent years, researchers have been venturing into bioflocculation of sludge using microorganisms (More et al., 2014). Bioflocculation is defined as an aggregation of bacterial flocs and it is utmost important for efficient separation of microorganisms from the treated effluent. A typical floc is formed by different types of bacteria together with other microorganisms (protozoa, fungi, filamentous microorganisms etc) and viruses along with some abiotic suspended materials. Flocs are held together in a polymeric network of extracellular polymeric substances (EPSs). The microbial EPS plays an important role in bioflocculation by interacting with the sludge solids (Garnier et al., 2005). The bacterial growth is often accompanied by the production of EPS, which has ecological and physiological functions (Flemming and Wingender, 2001).

EPSs are organic macromolecules that are formed by polymerization of similar or identical building blocks that may be arranged as repeated units within the polymer. The major organic fractions of EPS are carbohydrates and proteins (Nielsen and Jahn, 1999). EPSs also act as excellent emulsifying agents and this property is attributed to the diversity in bacteria (Keene and Lindberg, 1983). The bacterial EPSs are usually acidic heteropolysaccharides possessing different functional groups (e.g. hydroxyl, carboxyl and phosphoric acid), which exhibit high affinity towards certain metal ions. Many physical and chemical properties of microbial EPSs have led to a wide range of field applications, e.g. adhesion, chelation of heavy metals, coagulation and flocculation, detoxification of toxic compounds, nutrient sequestration, protection against osmotic shock, stabilizers, thickeners, gelling, film-forming and water-retention capability (in detergents, textiles, adhesives, paper, paint, food and beverage industries), oil recovery, mining industry and petroleum industries (More et al., 2014). A wide range of bacteria from various environmental habitats are known to produce complex and diverse EPS occurring as capsular polysaccharides (C-EPS, strongly associated with the cell surface) or as slime polysaccharides (S-EPS, loosely associated with the cell).

Recently, there has been growing interest in the isolation and characterization of microbial EPS owing to their practical importance. Different microorganisms produce various types of EPS with diverse characteristics and concentration. For economic reasons it is essential to

find a high EPS yielding microbial strain with high flocculation activity per unit weight of EPS. Therefore, the present study aimed at: i) isolation and identification of high concentration EPS producing bacteria, ii) chemical and physical characterization of the EPS produced by the strain and iii) to evaluate the potential of produced EPS with respect to flocculation activity and dewaterability.

4.4 Materials and Methods

4.4.1 Bacterial strain isolation and Identification

Wastewater sludge samples were collected from Communauté Urbaine du Québec (CUQ, Québec, Canada). EPS producing strain *Cloacibacterium Normanense* (NK6, accession number KF675204) was isolated from sludge samples using standard plate Count Agar (PCA). The strain was identified based on 16S rDNA sequencing. Isolated genomic DNA from the individual bacterial strains was subjected to PCR amplification of 16S rDNA using universal primers (Weisburg et al., 1991). Amplified products were purified using the Qiagen gel extraction kit and subsequently were sequenced (Bala subramanian et al., 2007). The obtained 16S rDNA gene sequences were submitted into the internet (http://www.ncbi.nlm.nih.gov/BLAST) for similarity search.

4.4.2 EPS production

The sludge was first settled by gravity for 1 h and the settled (concentrated) sludge was collected by discarding the supernatant. *Cloacibacterium normanense* was inoculated in Tryptic soy broth (TSB) (100 mL sterilized TSB in 500 mL flask) and incubated for 48 h. After 48 h incubation, the culture broth was used as inoculum to inoculate the sterilized (121 °C for 30 min) sludge (25 g suspended solids-SS/L, pH 7, 150 mL sludge in a 500 mL capacity flask). The flask was incubated in a shaking incubator at 180 rpm and 30 °C for 24 h. This culture (with an approximate cell concentration 6.7 x 10^6 colony forming units-CFU /mL) was used to inoculate (3% v/v) flask containing sterilized sludge (25 g/L SS, 150 mL sludge, pH 7). The flasks were incubated in a shaker at 180 rpm and 30 °C for 96 h for EPS production. Samples were withdrawn at each 12 h interval to measure the cell concentration, and each 24 h to measure the EPS concentration, flocculation activity and dewaterability.

All the samples were serially diluted with saline solution and the cell concentration was measured as CFU employing standard agar-plate technique. All the measurements were carried out in triplicates and the average of the results was presented.

4.4.3 Extraction of EPS

After incubation, the fermented broth was centrifuged at 6000 g, 4 °C for 20 min to obtain supernatant (containing Slime- EPS (S-EPS) and the biomass pellet was re-suspended in deionized water to the initial volume (containing Capsular (C-EPS)) (Bala subramanian et al., 2010).

To determine dry weight of S-EPS, the supernatant and ethanol (95% v/v) were mixed in 1:2 ratio and kept at -20 °C for overnight to precipitate the EPS. The precipitated EPS was collected (as a pellet) by centrifugation (6000 g, 4 °C, 20 min). The dry weight of the pellet corresponding to the EPS concentration (S-EPS) was measured by drying the precipitates at 60 °C to a constant weight (APHA, 2005).

To determine dry weight of Capsular- EPS (C-EPS), the re-suspended biomass pellets were first heated at 60 °C in a water bath for 30 min to release C-EPS into the liquid phase followed by centrifugation at 6000 g, 4 °C for 20 min (Li and Yang, 2007). The supernatant containing C-EPS was used to measure dry weight using the same procedure as for S-EPS.

The EPS concentration was estimated by the following formula:

$$[\mathrm{EPS}](\mathrm{g/L}) = \frac{W2 - \mathrm{W1}}{V}$$

Where, *W1*: Initial dry weight of the empty aluminium dish without a sample in g

W2: End dry weight of the aluminum dish with dried sample in g

V: volume of the sample in L

The total EPS (B-EPS) contained in the broth was calculated as sum of S-EPS and C-EPS.

All the measurements were carried out in triplicates and the average was presented.

4.4.4 Chemical characterization of EPS

After precipitation, the extracted C-EPS and S-EPS were dissolved in deionized water to the initial volume (100 mL of the broth) and protein and carbohydrate content were measured. Protein was determined using bovine serum albumin as the standard (Bradford et al., 1976). The carbohydrate content was measured by Phenol-Sulfuric acid method (Dubois et al., 1956) at 490 nm and glucose was used as the standard.

The sample concentration was calculated by the linear equation between absorbance and concentration of the standard solution. All measurements were carried out in triplicates and the average was presented. The protein and carbohydrate ratios were calculated for B-EPS, C-EPS and S-EPS and the control sample (EPS extracted from sterilized sludge) by dividing the protein content by the carbohydrate content of each EPS.

4.4.5 Flocculation activity of different EPS

The flocculation activity of EPS was determined by jar test method (Kurane et al., 1986) with minor modification. Kaolin solution (5 g/L) was suspended in distilled water, 150 mg/L of Ca^{2+} (CaCl2) was added to the kaolin suspension and pH was adjusted to 7. The desired concentration of different types of EPS was added (in terms of volume of the sample range from 0.25 mL to 4 mL collected at different times of fermentation) to kaolin suspension and rapidly mixed at 180 rpm for an initial 5 min then slowly mixed at 70 rpm for an additional 30 min. After mixing, samples were transferred to a 500 mL cylinder where they were allowed to settle for 30 min. The supernatant of each sample was then collected to measure the turbidity using turbidimeter (Micro 100 turbidimeter, Scientific Inc.). Flocculation activity was measured using the formula [100*(B-A)/B] where '*A*' is the turbidity of the sample (treated with S-EPS, C-EPS or B-EPS) and '*B*' is the turbidity of the control sample (in which equal volume of EPS solution was replaced with distilled water). All the tests were conducted in triplicates and the average values were presented.

4.4.6 Sludge volume index (SVI) study using S-EPS

SVI was measured to determine the kaolin and sludge settling efficiency using the produced EPS. S-EPS was selected because of high concentration obtained during production and a very good flocculation activity observed in kaolin solution. Sludge samples (24 h stored at 4 °C) collected from the municipal wastewater treatment plant and pulp and paper industry sludge (PPS) were used. SVI was measured using different sludge SS concentrations (1 g/L, 2 g/L, 5 g/L and 7 g/L). The well mixed sludge samples (1 L) were transferred into beakers followed by the addition of 600 mg/L of $Al_2(SO_4)_3$ as well as S-EPS and another beaker served as the control (no addition of EPS). After addition of EPS, the samples were mixed at 120 rpm for first 5 min (which enables the biopolymers to mix and bring them in contact with the sludge particles) followed by mixing at 50 rpm for 25 min (that enables flocs formation). Each mixed sludge sample was then transferred into 1 L graduated measuring cylinder for SVI measurement (Bala subramanian et al., 2008). Sludge settling efficiency in each cylinder was monitored at 5, 10, 20 and 30 min. Similarly, the sample of kaolin was prepared; 150 mg of Ca^{2+} was added in place of 600 mg/L of $Al_2(SO_4)_3$.

4.4.7 Sludge dewaterability

The capillary suction time (CST) was used to evaluate the dewaterability of the flocs in kaolin solution, MWWS and PPS using S-EPS produced by *Cloacibacterium Normanense*. The CST of the control (without addition of the S-EPS) was also measured. The samples were prepared similar to SVI measurement. The sediment from each flocculation activity test was used to measure the CST by a CST instrument (Triton, Model 304 M, UK) (APHA, 2005). A high value of CST usually implies a poor filterability and dewaterability.

4.5 Results and discussion

4.5.1 EPS production and characterization

Growth and EPS production profiles of *Cloacibacterium normanense sp.* are presented in Figure 4-1. Exponential growth phase was observed between 12 h and 24 h of fermentation. The maximum cell concentration (7.5×10^8 CFU/mL) reached at 48 h. The concentration of B-EPS increased with the fermentation time and reached maximum (13.3 ± 0.9 g/L) at 48 h where the cell concentration was also maximum (Figure 4-1). The EPS concentration increased from 1.9 to 2.8 g/L during lag phase (from 0 to 12 h). The EPS production occurred mainly during the exponential growth phase but significant EPS production was also observed during declining phase i.e. between 36 h and 48 h of fermentation (Figure 4-1). The research of More et al. (2014) investigated the effect of fermentation time on EPS production in activated sludge and found that the EPS content was proportional to the bacterial growth. A decrease in B-EPS concentration (from 13.3 to 11.4 g/L) was observed between 48 and 96 h. This decrease could be due to the fact that the bacteria may have consumed EPS when carbon limitation occurred in the medium. This phenomenon was also observed by other researchers using pure or mixed culture in sludge or synthetic medium (More et al., 2014; Sheng et al., 2010).

According to More et al. (2012b), the highest EPS concentration achieved was 3.4 g/L in 72 h with sterilized sludge as a growth medium employing *Serratia sp*., which was much lower than the B-EPS concentration (13.3 g/L) observed in the present study (Table 1). The EPS synthesis by microorganisms depends on the carbon and nitrogen availability in the culture medium. Most of the EPS producing microorganisms use carbohydrates as their carbon and energy source and either ammonium salts or amino acids or both as their nitrogen source (Gandhi et al., 1997). Therefore, the higher EPS concentration obtained in this work than those reported in the literature can be due to the fact that *Cloacibacterium normanense* strain

may have a wider range of carbon and nitrogen utilization ability that eventually helped it to use available complex carbon and nitrogen sources present in sludge for its growth and EPS production.

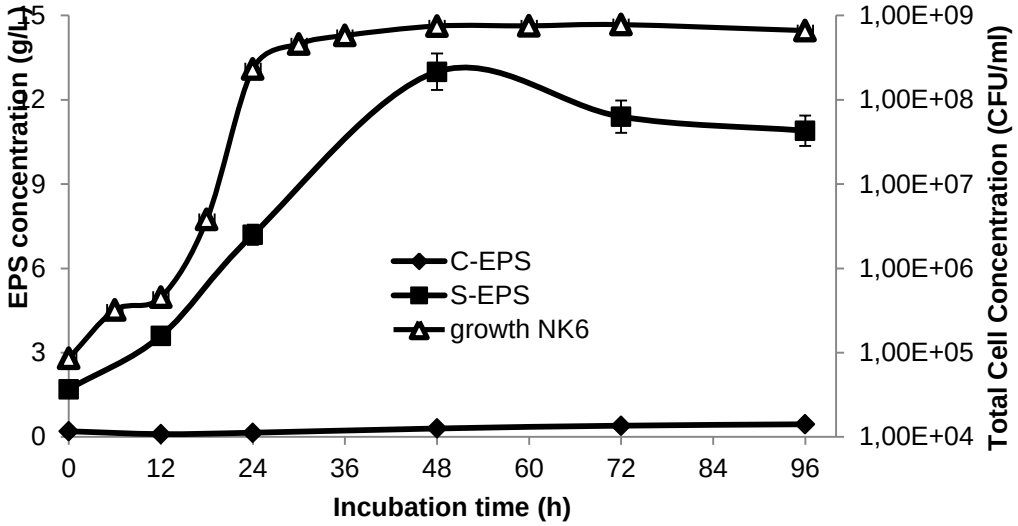

Figure 4-1 : Growth and EPS (C-EPS and S-EPS) production profiles of *Cloacibacterium normanense* (NK6) using wastewater sludge as sole raw material. Primary Y-axis - dry weight of EPS (C-EPS and S-EPS); and Secondary Y-axis - semi log plot for CFU.

Table 4-1 : Production of B-EPS by different strains in present study and reported in the literature.

Strains Name	Medium type	Maximum B-EPS (g/L)	Fermentation condition	References
***Bacillus* sp.3**	Sludge (10 g/L)	1.68	250 rpm, 25 °C, 72 h	More et al., (2012a)
***Bacillus* sp.6**	Sludge (10 g/L)	1.45	250 rpm, 25 °C, 72 h	More et al., (2012a)
***Bacillus* sp.7**	Sludge (10 g/L)	1.56	250 rpm, 25 °C, 72 h	More et al., (2012a)
***Bacillus* sp.8**	Sludge (10 g/L)	1.65	250 rpm, 25 °C, 72 h	More et al., (2012a)
***Serratia* sp.1**	Sludge (17 g/L)	3.4	250 rpm, 25 °C, 72 h	More et al., (2012b)
Pseudomonas	Mineral medium (25 g/L glucose, 0.2 g/L $MgSO_4$)	2.3	250 rpm, 25 °C, 72 h	Bala subramanian et al., (2010)
***Bacillus* sp BS9**	Mineral medium (25 g/L glucose, 0.2 g/L $MgSO_4$)	2.4	250 rpm, 25 °C, 72 h	Bala subramanian et al., (2010)
***Yersinia* sp BS11**	Mineral medium (25 g/L glucose, 0.2 g/L $MgSO_4$)	2.5	250 rpm, 25 °C, 72 h	Bala subramanian et al., (2010)
***Microbacterium* (BS15)**	Mineral medium (25 g/L glucose, 0.2 g/L $MgSO_4$)	2.1	250 rpm, 25 °C, 72 h	Bala subramanian et al., (2010)
***Staphylococcus aureus* (A22)**	Glycerol and Ethanol	10.8	150 rpm, 28 °C, 48 h	Buthelezi et al., (2010)
***Pseudomonas plecoglossicida* (A14)**	Glycerol and Ethanol	8.3	150 rpm, 28 °C, 48 h	Buthelezi et al., (2010)
Cloacibacterium normanense	Sludge (25g/L)	13.3	180 rpm, 30 °C, 48 h	Present work

4.5.2 Protein and carbohydrate content

Carbohydrate and protein content of S-EPS (LB-EPS) and C-EPS (TB-EPS) and their concentrations in the broth are presented in Figure 4-2 (a-d). The total protein (TP) and the total carbohydrate (TC) content of the EPS and their concentration in the medium increased with fermentation time and reached maximum at 48 h. The protein content (219.9 ± 5.3 mg BSA/g of extracted EPS) and carbohydrate content (128.5 ± 6.3 mg carbohydrate/g of extracted EPS) of S-EPS was higher than the protein content (145.6 ± 4.7 mg of BSA/g of extracted EPS) and carbohydrate content (104.0 ± 5.5 mg carbohydrate/g of extracted EPS) of C-EPS at 48 h fermentation. The protein and carbohydrate content of the EPS produced by the strain was higher than the control sample or the EPS extracted from the sterilized sludge (72.9 ± 1.1 mg BSA/g of S-EPS and 28.0 ± 2.1 mg carbohydrate/g of S-EPS).

The protein and carbohydrate content of the EPS increased in exponential and declining phase (i.e. until 48 h) followed by a decrease during the stationary phase (after 48 h). The decrease in B-EPS concentration after 48 h (Figure 4-1) was due to degradation of proteins and carbohydrate of EPS by the bacterial strain. The stationary phase corresponded to the beginning of nutrient depletion in the medium and the accumulation of waste products limiting the growth. The culture grew exponentially followed by a slow growth until the maximum cell density was reached (at 48 h), and eventually the growth ceased due to the cell lysis caused by the decrease in the integrity and stability of the cell surface. This process leads to a reduction or even complete cessation of extracellular product synthesis by microorganisms. Under this condition, the microorganism shift to use the carbohydrates and proteins of EPS to fulfill the demand of carbon and nitrogen sources. Zhang and Bishop (2003) performed a comparative study to examine the biodegradability of EPS by microorganisms from the original biofilm (its own producers) and it was found that the cells consumed the newly produced EPS and microbial activity gradually stopped. Similarly, the present study also suggested that the EPS (protein and carbohydrate) could be used as a substrate.

The protein content of the EPS was higher than the carbohydrate content (Figure 4-2) as observed by other researchers (Bura et al., 1998). According to these authors, the protein was the main component and polysaccharides or carbohydrates were the secondary component of the EPS matrix in sludge. The bacterial strain used in this study was isolated from sludge and also grown in sterilized sludge (as raw material), therefore, EPS contained higher protein similar to that observed in activated sludge process.

The total protein/total carbohydrate ratio of B-EPS varied from 1.48 to 1.85 with fermentation time (Figure 4-2e). This variation can explain the distinct nature of B-EPS produced at

different times of fermentation. The total protein/total carbohydrate ratio of B-EPS observed in this study was higher than reported in previous studies (0.34) in case of *Serratia* sp.1 (More et al., 2012b) (Table 4-2). Thus, the EPS composition (i.e. protein and carbohydrate content) synthesised by the new strain was different than those reported by others (More et al., 2012b). In general, the composition of EPS is heterogeneous and varies based on many factors such as bacterial strain, growth phase, the EPS extraction method and different EPS production process parameters (temperature, pH, agitation speed, cultivation time, medium composition, medium pre-treatment) (Sheng et al., 2010).

Table 4-2: Characterization of extracted EPS in terms of total protein and carbohydrates.

Strains	B-EPS (g/L)	Medium	TC (%)[a]	TP (%)[b]	Car/Protein ratio[c]	References
Bacillus sp.7	1.6	Sludge (10 g/L)			0.89	More et al., (2012a)
Serratia sp.1	3.4	Sludge (25 g/L)	12.3	40	0.3	Bezawada et al., (2013)
*Yersinia*sp.1 (BS11)	2.5	Synthetic medium	6	4.1	1.46	Bala subramanian et al., (2010)
Serratia sp.1 (BS8)	3	Synthetic medium	5.2	2.8	1.89	Bala subramanian et al., (2010)
Cloacibacterium Normanense	13.3	Sludge (25 g/L)	12.7	21.8	0.58	Present study

Note: [a]TC- total carbohydrate; [b]TP- total protein. [c]Car- carbohydrates

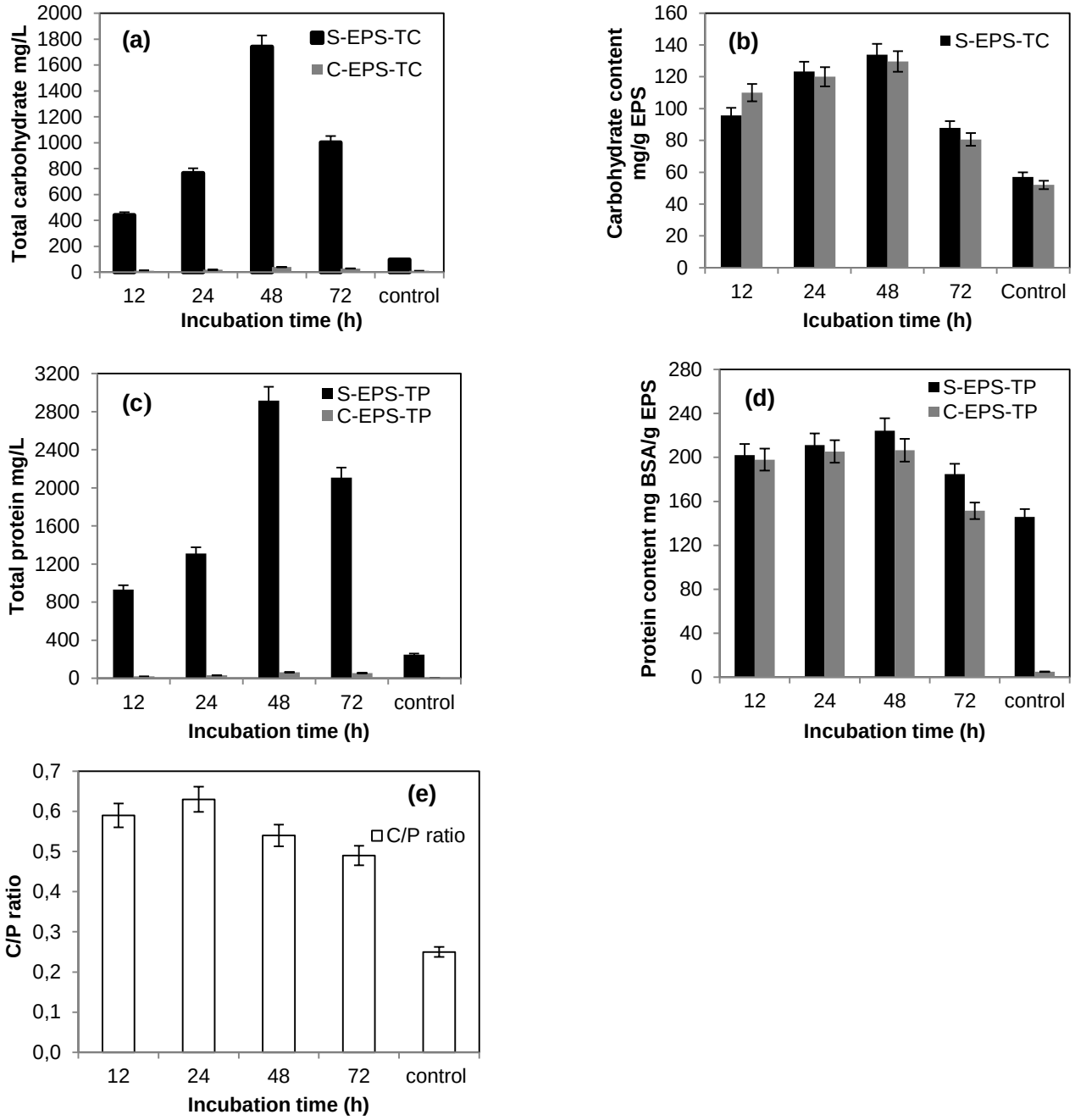

Figure 4-2 : EPS composition in terms of protein and total carbohydrates at different incubation time: (a) Total carbohydrates (mg/L) in the medium; (b) Carbohydrate content of EPS (mg BSA/g EPS), (c) Total protein mg/L in the medium; (d) Protein content of EPS (mg BSA/g EPS) and (e) carbohydrates/protein ratio of B-EPS.

4.5.3 Flocculation Activity

The results of flocculation activity (FA) of B-EPS, S-EPS and C-EPS are presented in Figure 4-3. The flocculation activity for B-EPS and S-EPS decreased with an increase in EPS concentration. The highest FA (48 h sample) was 94.2% ± 1.3 for S-EPS (1.3 ± 0.1 mg S-EPS/g kaolin, Figure. 4-3b), 86.8% ± 3.5 for B-EPS (2.6 ± 0.2 mg B-EPS/g kaolin, Figure 4-3c) and 79.4% ± 1.4 for C-EPS (0.50 ± 0.02 mg C-EPS /g of kaolin, Figure 4-3a). After attaining maximum value, a decrease in FA with EPS concentration was due to an over dosage of the polymer that caused re-suspension or instability of kaolin particles (flocs) leading to a high turbidity (Dermlim et al., 1999). An equal volume of the samples taken at different fermentation time exhibited different FA because of the variation in EPS concentration (Figuer 4-3). The maximum FA of 86.8% ± 3.5 using 2.6 ± 0.2 mg B-EPS/g Kaolin observed in the present study was higher than the maximum FA (79.1%) obtained using 0.7 mg B-EPS/g kaolin in case of *Serratia* sp.1 (More et al., 2012b)

Higher FA achieved using S-EPS in the present study is due to the specific structure of EPS. The difference in results of FA for different types of EPS (B-EPS, S-EPS and C-EPS) could be due to the presence of diverse nature of proteins and carbohydrates. Proteins and carbohydrates are complex materials and may contain structurally different components (or functional groups), which may change with fermentation time (Figure 4-2) and thus affecting the FA of the EPS (Higgins and Novak, 1997). The protein content and type could play a dominant role in flocculation through hydrophobic interactions and polyvalent cations bridging, which increases the floc binding strength and hence enhancing the stability of the biopolymer network. Moreover, the hydrogen bonding capacity of carbohydrates also helps in flocculation (Dignac et al., 1998).

The flocculation activity of B-EPS was lower than S-EPS. The difference in flocculation activity between S-EPS and B-EPS could be due to the fact that B-EPS contains both the C-EPS and S-EPS. C-EPS could hamper the efficiency of the S-EPS when two EPS were present together in the broth. C-EPS contained abundant hydrophilic compounds (hydroxyl group) that interacted with molecules of water hindering the combination of S-EPS (which contain hydrophobic compounds) with C-EPS or other hydrophilic particles (Yu et al., 2006). Moreover, B-EPS have the negative surface charge and contains both EPS (S-EPS & C-EPS) as well as other substances such as colloidal and residual matter (cells, organic and inorganic material etc.). Increase in volume of the B-EPS in kaolin solution (the assay solution) also increases the

negative surface charge due to increase in colloidal content, which could destabilize the flocs and thus decreases the flocculation activity.

The charge of EPS can affect the flocculation activity. The zeta potential of S-EPS (-47.9 ± 0.4 mV) is higher than that of C-EPS (-62.7 ± 0.9 mV) and that of B-EPS (-71.9 ± 1.2 mV). The higher zeta potential of S-EPS implies the degree of repulsion between the EPS molecules is less, which tend to improve the bio-flocculation.

The zeta potential of fresh sludge was -89.1 ± 0.8 mV, with the addition of Ca^{2+}, zeta potential increased to -45.9 ± 1.4mV. The zeta potential of kaolin suspensions (5 g/L) without Ca^{2+} and EPS was -38.4 ± 1.5 mV, and it was increased to -17.1 ± 0.5 mV by the addition of 150 mg of Ca^{2+}/L. The addition of EPS, after Ca^{2+}, to kaolin suspension had very small change in the charge. Therefore, charge neutralization of the kaolin particles was achieved mostly by the addition of Ca^{2+}. However, the EPS addition after Ca^{2+} revealed high flocculation activity and enhanced dewaterability (discussed in the next section). These results suggest that the specific interactions of EPS and calcium with kaolin particles can be supported by adsorption and bridging mechanism**.**

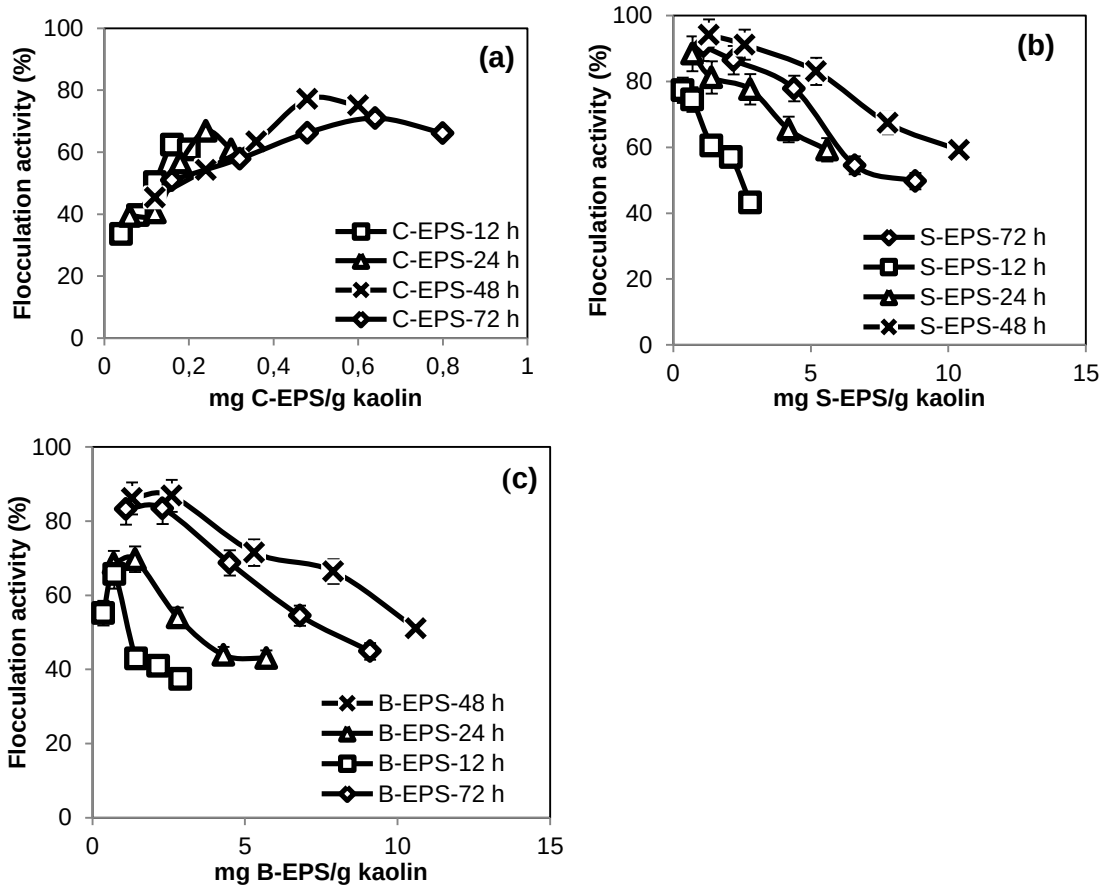

Figure 4-3 : Effect of EPS concentration (mg EPS/g kaolin) on flocculation activity (a) C-EPS, (b) S-EPS and (c) B-EPS, respectively.

4.5.3 Sludge Settling

S-EPS exhibited better kaolin FA than other types of EPS. Therefore, S-EPS was used to estimate the sludge settling characteristics of pulp and paper industry activated sludge (PPS) (Figure 4-4) and municipal wastewater secondary sludge (MWWS) (Figure 4-5) at different suspended solids concentrations (1, 2, 5 and 7 g/L). The SVI was below 100 ± 1.5 mL/g after 30 min settling. For a good sludge settling, SVI ≤100 is required (APHA, 2005). The addition of cations (600 mg/L of $Al_2(SO_4)_3$) without EPS slightly improved the SVI value of the control samples (SVI decreased from 140 to 110 mL/g in case of PPS with 7 g SS /L; and SVI decreased from 200 to 40 mL/g in case of MWWS with 5 g SS /L). The reduction of SVI in control is due to the coagulation effect of Al_2 $(SO_4)_3$ in combinations with the native EPS of sludge (1.6 ± 0.3 g EPS/L in fresh PPS and of 1.2 ± 0.5 g EPS/L in MWWS). Further, SVI was substantially improved by the addition of S-EPS (Figures 4-4 and 4-5).

The SVI varies with the concentration of sludge SS, the type of sludge and the added concentration of S-EPS. In this study, different volumes of S-EPS were used to obtain different concentrations of EPS. It was found that 1.5 mL (or 0.02 ± 0.01 g S-EPS/L) of S-EPS revealed the best settling compared to a lower or higher concentration of the EPS. An increase in SVI value with EPS concentration greater than 0.02 g/L (4 mg of S-EPS/g SS) was due to bound water increase into the aggregates, which produced highly porous flocs with low density (Yang and Li, 2009). Therefore, 0.02 g/L S-EPS concentration was used to evaluate the variation of SVI at different SS concentrations.In case of PPS at SS 5 g/L, the lowest SVI value was 20 ± 2 mL/g with an optimum concentration of EPS (1.5 mL or 0.02 ± 0.01 g S-EPS/L) and SVI of the control was 50 ± 1 mL/g. In case of MWWS at SS 5 g/L and same concentration of the EPS, the SVI value (20 ± 2 mL/g) was similar to the SVI value of the PPS; however, SVI of the control was different (40 ± 2.5 mL/g). The different SVI of the control of PPS and MWWS could be because of the difference of organic matter present in different sludges, which affected the bioflocculation process. These results of SVI are better than those reported by Bala subramanian et al., (2010) (Table 4-3). In case of MWWS at SS 5 g/L, the lowest SVI was 60 mL/g using S-EPS concentration of 2 g/L (400 mg S-EPS/g SS) produced by the bacterial strain BS8 (*Serratia* sp.). Thus, the biopolymer produced by *Cloacibacterium normanense* strain (present study) was more effective in sludge settling at a very low concentration (0.02 g/L or 4.0 ± 0.5 mg S-EPS/g SS) than the biopolymer produced by BS8 (*Serratia* sp.) (Balasubramanian et al., 2010).

The carbohydrate and protein contents of EPS were found to have a positive relationship with SVI (Bura et al., 1998). The protein probably is more important than the carbohydrates; the high protein content of S-EPS would improve bioflocculation and settling property of activated sludge (Hoa et al., 2003). The higher protein content of the EPS produced by the present strain than that produced by Serratia sp. (More et al., 2012b) could explain the lower value of SVI at lower concentration of S-EPS.

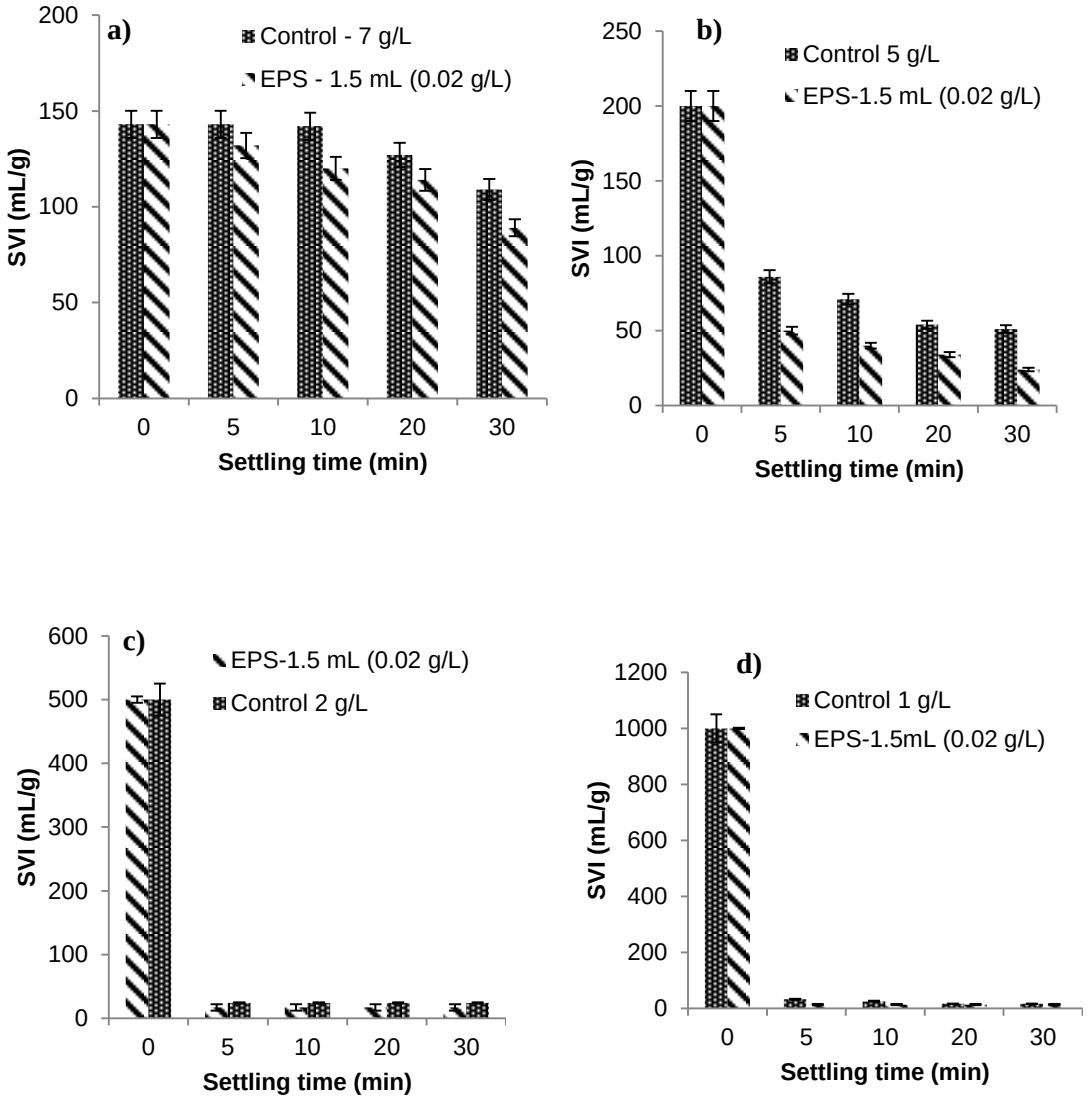

Figure 4-4 : Effect of EPS on pulp and paper sludge settling (SVI) at different solids (SS) concentrations; (a) SS: 7 g/L, (b) SS: 5 g/L, (c) SS: 2 g/L and (d) SS: 1 g/L.

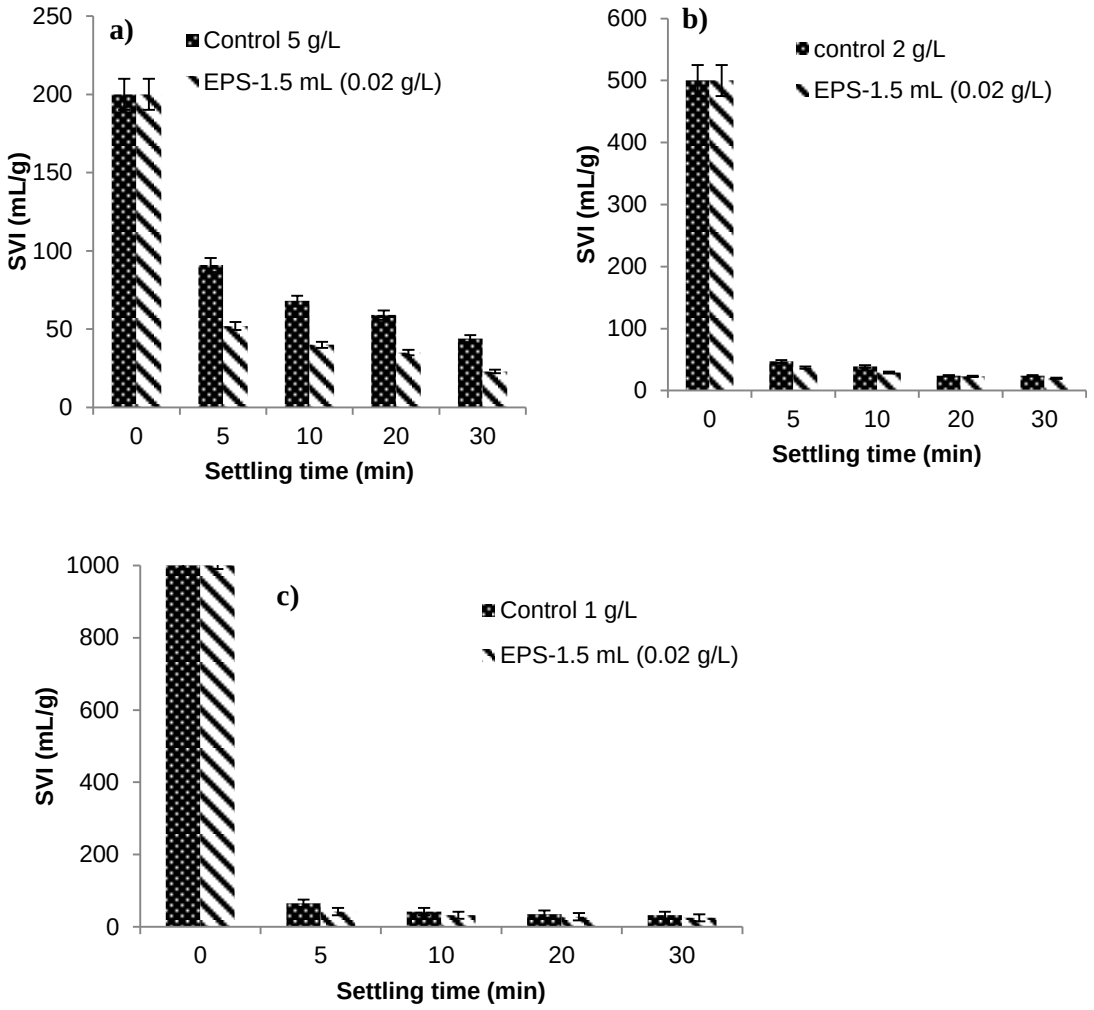

Figure 4-5 : Effect of EPS on MWWS settling (SVI) at different solids concentrations of (a) 5 g/L, (b) 2 g/L and (c) 1 g/L.

4.5.4 Sludge dewaterability

The minimum CST value of kaolin solution was 23.2 ± 0.3 s (ΔCST = 16.7 ± 0.4 s), 21.4 ± 2.4 s (ΔCST =18.5 ± 0.9 s) and 22.7 ± 0.7 s (ΔCST= 17.2 ± 1.2 s) with the addition of 2.6 ± 0.2 mg B-EPS/g Kaolin, 1.3 ± 0.1 mg S-EPS/g Kaolin and 0.03 ± 0.01 mg C-EPS/g Kaolin, respectively. These values were lower compared to the control sample (without addition of EPS) (39.9 ± 0.8 s).

This result is better than that reported by other researchers (Bezawada et al., 2013) who found that the minimum CST value of the kaolin solution was 23.7 s (ΔCST = 6.8 s) and 24.5 s (ΔCST = 8.1 s) with the addition of B-EPS and S-EPS, respectively with a dose of 3.44 ±

0.05 B-EPS/g kaolin and 1.70 ± 0.05 S-EPS/g kaolin, respectively. However, the required CST value for a good dewaterability is 20 s (Bala subramanian et al., 2010).

The CST value increased with the increase in EPS concentration. The CST value did not vary much (changed from 21.4 ± 0.3 s to 21.9 ± 0.1 s) by increasing the S-EPS concentration from 1.3 ± 0.1 mg S-EPS/g Kaolin to 3.9 ± 0.5 mg S-EPS/g Kaolin; whereas the CST value increased from 23.2 ± 0.2 s to 29.4 ± 0.3 s by increasing the B-EPS concentration from 2.6 ± 0.2 mg B-EPS/g kaolin to 4.0 ± 0.5 mg B-EPS/g kaolin. Thus, S-EPS was more efficient than B-EPS. Poor dewaterability was observed in case of C-EPS. The CST value increased from 22.7 ± 0.5 s to 26.2 ± 0.4 s by increasing C-EPS concentration from 0.03 ± 0.01 mg C-EPS/g Kaolin to 0.09 ± 0.02 mg C-EPS/g Kaolin.

Excessive EPS concentration might deteriorate cell attachment and weaken the floc structure, which in turn lead to a poor sludge settling and dewaterability (Li and Yang, 2007). In this work, S-EPS and B-EPS exhibited higher dewaterability than C-EPS in kaolin solution. This was due to the formation of bigger flocs caused by higher protein content of S-EPS and B-EPS (sample collected at 48 hr) compared to C-EPS. It was widely reported that an increase of EPS concentration would lower the sludge dewaterability (Mikkelsen and Keiding, 2002; Yang and Li, 2009). Houghton et al. (2001) found that an increase in dewaterability with EPS (at low concentration of EPS) was due to the enhancement of flocculation. The increase in flocculation resulted in an increase in floc size and thus improved the sludge dewaterability. In the present case, an increase in EPS concentration above 1.3 ± 0.1 mg EPS/g of kaolin increased the amount of surface bound water by EPS, and thus decreased the kaolin dewatrability. Contrary to these findings, Jin et al. (2004) found that the concentration of the individual polymers and total EPS had negative correlations with CST.

The results obtained for dewaterability on municipal and pulp and paper secondary sludge using S-EPS were presented (Figures 4-6a, b). At 2 g/L SS of MWWS, the CST value was decreased from 130 ± 2 s (in the control sample) to 81 ± 1 s with the addition of 0.02 ± 0.01 g/L of S-EPS (Figure 4-6b). A good dewaterability of PPS was achieved at 5 g/L SS; the CST value decreased from 10.0 ± 0.4 s (control) to 8.0 ± 0.1 s (with S-EPS dose of 0.02 ± 0.01 g/L) (Figure 4-6a). The EPS produced by *Cloacibacterium normanense* strain (this work) was more effective in lowering the CST value than the EPS present in sludge (1.6 g/L of PPS and 1.2 g/L MWWS). In this work, reduction in CST was more effective at low EPS concentration compared to the results reported by Bala subramanian et al. (2010), where the CST value decreased from 130 s (control) to 36.4 s after addition of 400 mg S-EPS /g SS produced by BS8 strain (Table 4-3).

The dewaterability improvement was higher in MWWS than in PPS (Figure 4-6). This might be due to the difference in characteristics of organic matter in MWWS and PPS as well as the structure of protein and carbohydrate of EPS and their content in sludge.

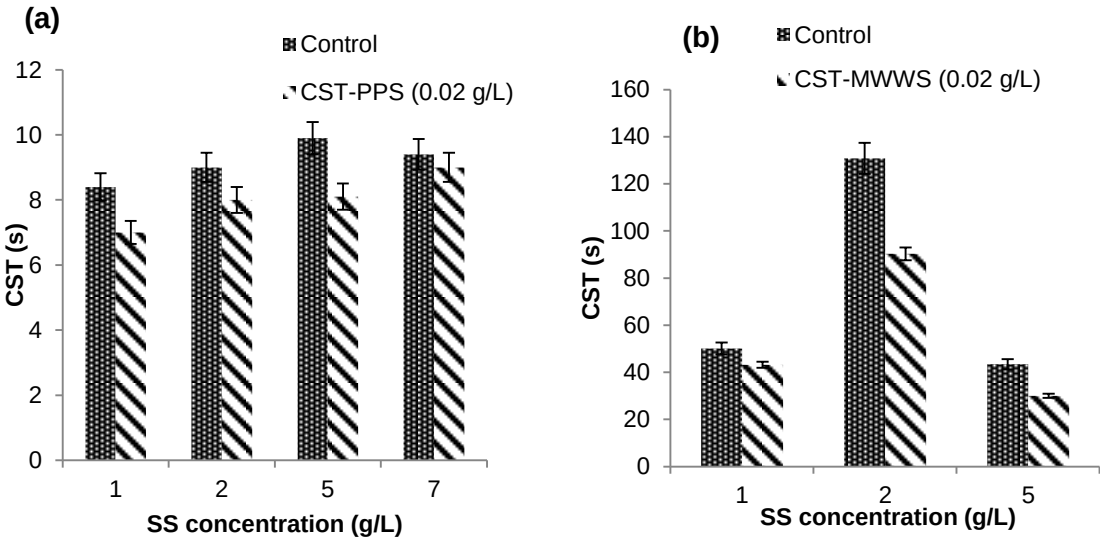

Figure 4-6 : Effect of S-EPS on sludge dewaterability at different sludge suspended solids concentration; (a) pulp and paper sludge and (b) municipal wastewater sludge.

Table 4-3: Comparison of flocculation activity, dewaterability and settling results

Strains	B-EPS (g/L)	Raw material	Flocculation activity		Dewaterability of Kaolin		Dewaterability of sludge		Settling of Sludge		References
			FA (%)	EPS Added (S-EPS mg/g kaolin)	CST(%)	EPS added, (S-EPS mg/g Kaolin)	CST(%) (MWWS)	EPS Added (S-EPS mg/g SS)	SVI (mL/g) (MWWS)	EPS Added (S-EPS mg/g SS)	
Bacillus sp.7	1.6	Sludge (10 g/L)	81.7	1.12 – 2.7	65	1.12-2.7	-	-	-	-	More et al., (2012a)
Serratia sp.1	3.4	Sludge (25 g/L)	79.1	2.7	34.7	2.7	-	-	-	-	More et al., (2012b)
BS11	2.5	Synthetic medium	85.7	250	-	250	77.38	250	63	250	Bala subramanian et al., (2010)
BS8	3	Synthetic medium	81.4	400	-	400	63.6	400	60	400	Bala subramanian et al., (2010)
Cloacibacterium Normanense	13.3	Sludge (25 g/L)	94.2	1.3	59.9	5.2	37.6	9.75	20	3.9	Present Study

4.6 Conclusion

High concentration (13.3 g/L) of extracellular polymeric substances (EPSs) was produced by *Cloacibacterium normanense* in sterilized sludge with 25 g/L of suspended solids. EPS combined with Ca^{2+} demonstrated to be a good bioflocculant. Slime EPS had shown higher flocculation activity (94.2%) and better dewaterability (59.9%) in kaolin solution compared to capsular EPS and broth EPS**.** The maximum dewaterability in 2 g/L of municipal wastewater sludge achieved was 37.6% with a combination of 0.02 ± 0.01 g/L of slime-EPS and 600 mg/L of $AlSO_4$. The study showed a promising approach of new isolated strain which produced high concentration of EPS in sludge with high flocculation activity as well as good settling, competing with other EPS producing strains in synthetic medium.

4.7 Acknowledgements

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CHAPITRE 5

Impact of extraction methods on bio-flocculants recovered from backwashed sludge of bio-filtration unit

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5 Impact of extraction methods on bio-flocculants recovered from backwashed sludge of bio-filtration unit

5.1 Abstract

Effect of ten extraction methods on flocculation activity and chemical composition of bio-flocculants (BFs) recovered from backwashed sludge of bio-filtration unit was studied. The results showed that the chemical method was better than physical method with respect to the extracted BFs weight and its flocculation activity. Cell lysis did not affect to the flocculation activity of BFs. Among ten extraction methods, EDTA (20 g/L) was the best one with extracted BFs dry weight of 6242 mg/L and flocculation activity of 83%. Optimization of EDTA concentration showed that 5 g EDTA/L (or 0.2 g EDTA/g SS) was suitable for recovery of BFs from backwashed sludge. The flocculation activity of BFs was 94% when using 2.4 mg of BFs/g of kaolin. The outcome of this study suggested that backwashed sludge of the bio-filtration unit was a potential source for exploiting bio-flocculants**.**

5.2 Keywords

Bio-flocculants; recovery; flocculation activity; wastewater sludge

5.3 Introduction

The chemical flocculants are popularly used in wastewater treatment plant to increase the size of flocs in coagulation step prior to settling and increases dewaterability of waste sludge prior to dewatering process. However, using of chemical flocculants might have a negative impact on the environment and health (Kunle et al., 2016). Bio-flocculants (BFs) is an urgent need to replace chemical flocculants, as it is biodegradable and less harmful to the environment. Bio-flocculants (BFs) produced by bacteria have been studied and shown high ability in the flocculation of kaolin solution. Bio-flocculants also have been applied in treating of various types of pollutants present in wastewater such as humics in leachate (Zouboulis et al., 2004), color and dyes in textile wastewater (Gao et al., 2011), heavy metals (Guibaud et al., 1999), suspended solids and organic compounds (Gong et al., 2008). Many bacterial strains have been isolated to produce bio-flocculants (BFs) in both synthetic growth medium (Dermlim et al., 1999; Watanabe et al., 1998; Yang et al., 2012) and low-cost growth medium such as wastewater (Aguilera et al., 2008; Wang et al., 2007), wastewater sludge (Bezawada et al., 2013; More et al., 2012) and industrial waste and byproducts (Banik et al., 2007). However, production of bio-flocculants in industrial scale is still limited due to the high cost of growth medium, complex fermentation and bio-flocculants extraction process and maintenance of bacterial strains.

Wastewater sludge is an increasing environmental issue. Treatment and disposal of wastewater sludge represent approximately 50% of wastewater treatment cost. Produced sludge need to be reused or recycled to prevent the environmental problems and to improve the value of sludge. Wastewater treatment plants generate many million tons of bio-sludge per year around the world and contains varying concentration of polymeric substances (both extracellular and intracellular), which could be potentially used as bio-flocculants (Comte et al., 2006; Yu et al., 2009; Zhang et al., 2012). However, in bio-sludge or activated sludge, the polymeric substances are tightly attached with microbial cells and most of the functional groups are occupied by linking with cell membrane, therefore, they exhibit low flocculation activity (Yu et al., 2009). By chemical/physical means, polymeric substances can be detached from the cell, free the functional groups and thereafter can be collected as active bio-flocculants.

So far, many methods were proposed and applied to extract BFs from activated sludge and bacterial broth. However, these methods were used as an analytical tool to determine the composition of BFs in sludge or BFs produced by the isolated bacteria (Bezawada et al., 2013; Liu and Fang, 2002; More et al., 2012; Sun et al., 2012; Zhang et al., 2012) and to study the effect of extraction method on cell lysis (D'Abzac et al., 2010; Sun et al., 2012). The extraction methods have also been used to examine how BFs correlated to bio-flocs structure, sludge

dewaterability and efficiency of the biological treatment unit such as bio-filtration, bio-membrane filtration (Duan et al., 2013; Jiao et al., 2010; Neyens et al., 2004; Zhang et al., 1999) or the presence of filamentous bacteria in activated sludge system (Al-Halbouni et al., 2008; Martins et al., 2004). There is no report on how the extraction methods affect the flocculation activity of the extracted BFs. Therefore, the aim of this study is to determine the effect of different bio-flocculants extraction methods on both quantities extracted and flocculation activity of the obtained BFs and thus proposes the most efficient method to recover BFs from backwashed wastewater sludge of bio-filtration unit.

5.4 Materials and methods

5.4.1 Sludge sample

Backwashed sludge was collected from the bio-filtration unit of CUQ (Communauté Urbain de Québec) wastewater treatment plant (Quebec, Canada). The sludge was washed twice with distilled water and then concentrated to 25g SS (suspended solids)/L by centrifugation. Concentrated sludge was stored at 4 ^{o}C for further studies.

The CUQ wastewater treatment plant is located in Quebec City in Canada. It treats domestic wastewater using bio-filtration technology with designed capacity of 231000 m^3/day. Food per microorganism ration (F/M) was around 0.4. Concentration of main pollutants in the influent wastewater was presented in table 5-1.

Table 5-1: Influent characteristics of CUQ wastewater treatment plant

Parameter	Concentration (mg/L)
pH	6.9
Total solids (TS)	390
Total dissolved solids (TDS)	270
Suspended solids (SS)	120
Biochemical oxygen demand (BOD_5)	110
Chemical oxygen demand (COD)	250
Total organic carbon (TOC)	80
Total nitrogen (Ntot-N)	20
Kjeldahl nitrogen (TKN-N)	20
Organic nitrogen (Norg-N)	8
Ammonia nitrogen (NH4-N)	12
Nitrite (NO_2-N)	0
Nitrate (NO_3-N)	0
Total phosphorus (Ptot-P)	4
Inorganic phosphorus (PO4-P)	3
Chlorides	30
Alkalinity (as $CaCO_3$)	50
Sulfates	20

5.4.2 Extraction methods

The following ten different extraction methods were used to extract BFs from the sludge sample. The volume of the fresh sludge (25 g SS/L) used for extraction was 70 mL.

1) Centrifugation: Fresh sludge was centrifuged at 6000 × g, 4 ^{o}C for 15 min to release BFs to the supernatant.

2) Heating: Fresh sludge was heated in a water bath at 60 ^{o}C for 30 min.

3) Sonication: Fresh sludge sonication was performed at 40 W for 2 minutes using Ultrasonic Processer – Cole Parmer. The condition for extracting BFs by sonication was adopted from Comte et al. (2006).

4) EDTA: EDTA was used with concentration of 20 g EDTA/L of sludge with 25 g SS/L. After adding EDTA to fresh sludge, the sample was mixed well and incubated for 3 h at 4 ^{o}C (Liu and Fang, 2002).

5) Formaldehyde and NaOH (F/NaOH): 0.42 mL of formaldehyde (36.8% w/w or 368 g/L) was added to 70 mL of sludge (25 g SS/L) and incubated for 1 h at 4 ^{o}C. Thereafter, 3 mL NaOH 10 M was added and left for 3 h at 4 ^{o}C to complete the reaction (Liu and Fang, 2002).

Further, a combination of several methods was used to extract BFs from fresh sludge; each step was conducted under similar conditions as described previously. The following combinations were employed:

6) Formaldehyde followed by heating (F-Heat);

7) Formaldehyde followed by sonication and the NaOH treatment (F-Sonic-NaOH);

8) Formaldehyde followed by EDTA extraction (F-EDTA);

9) Formaldehyde followed by sonication and EDTA (F-Sonic-EDTA) and

10) Formaldehyde followed by sonication and heating (F-Sonic-Heat).

The use of formaldehyde in combined methods is to fix the cell and prevent cell lysis and sonication was used to reduce the floc size and therefore, increase the extraction rate and extraction efficiency. After completion of reaction, all samples were centrifuged at 6000 × g for 15 min to remove the pellet. Supernatant of each sample was considered crude BFs. To purify, the crude BFs was mixed with cold ethanol (98%) in a ratio of 1:2 (v/v) and precipitated at -20 ^{o}C overnight. The precipitated BFs was collected by

centrifugation (6000 × g, 4 ^{o}C, 20 min) as a pellet. The pellet was dissolved in distilled water to initial volume and the resulting solution was considered purified BFs.

5.4.3 Chemical composition of BFs

For measuring dry weight, one volume of crude BFs was mixed in two volumes of cold ethanol (98%) and precipitated at -20 ^{o}C overnight. After precipitation, the sample was centrifuged at 6000 × g for 20 min. The pellet was dried at 50 ^{o}C until constant weight.

To determine the chemical composition, the purified BFs solution was used to determine the protein (PN), polysaccharide (PS) and nucleic acid concentration. Soluble protein was determined according to the method of Bradford (Bradford, 1976). Polysaccharide was analyzed by Phenol-Sulfuric acid method (DuBois et al., 1956) and nucleic acids were measured by Diphenylamine method (Burton, 1956). The results were presented as an average of the triplicate samples.

5.4.4 Flocculation activity test

Kaolin clay was used as a test material to measure the flocculation activity of BFs as kaolin has a negative surface charge (-32 mV), which is similar to the charge of particulate matter in wastewater and wastewater sludge (Bezawada et al., 2013). The flocculation activity of BFs was determined in Jar-test with 5 g/L of kaolin solution. Both crude and purified BFs were used for the flocculation activity test. Procedure of the flocculation activity test was as described briefly below.

CaCl2 was added to the kaolin suspension to obtain a final concentration of 150 mg Ca2+/L and pH was adjusted to 7.5 by NaOH 0.1 M. BFs solution was added into kaolin suspension and rapidly mixed at 100 rpm for an initial 5 min, then slowly mixed for an additional 30 min at 70 rpm. Thereafter, each sample was transferred to a 500 mL cylinder for settling during 30 min. Supernatant was then collected to measure the turbidity using a turbidity meter (Micro 100 turbidimeter, Scientific Inc.). Turbidity of the samples was measured three times to take the average. Flocculation activity (FA) was measured using the formula [100*(To-T)/To] where ‘To’ is the turbidity of the control (without addition of BFs) and ‘T’ is the turbidity of the sample.

5.5 Results and discussion

5.5.1 Effect of extraction method on dry weight and chemical composition of BFs

Table 5-2 summarizes dry weight, protein and polysaccharide content in the purified BFs, which were extracted by different methods. The extracted BFs concentration was low with centrifugation (430 ±14 mg/L), F-Heat (830±20 mg/L) and heating methods (910±12 mg/L). Sonication, F-Sonic-Heat, F/NaOH and F-EDTA methods gave higher BFs concentration, which was in the range of 3196 – 4214± 150 mg/L. F-Sonic-NaOH, F-Sonic-EDTA and EDTA methods had BFs extracted concentration range of 5270 – 6816 ± 103 mg/L. In general, chemical methods revealed higher BFs extraction efficiency than the physical methods and the efficiency obtained was similar to those reported by other researchers (D'Abzac et al., 2010; Zhang et al., 2012; Zhang et al., 1999).

Centrifugation and mild heating processes to extract BFs have been popularly used in many studies because they were considered less harmful to the bacterial cells (i.e. cell integrity was kept intact). Low concentration of nucleic acid content in BFs (Table 5-2) indicated clearly that centrifugation and heating methods did not affect the cell structure and BFs extracted by these methods were extracellular polymeric substances (EPS).

Sonication (40W, 2 min) enhanced BFs extraction efficiency when combined with F/NaOH (BFs concentration increased from 3196 to 5370 ± 120 mg/L) or F-EDTA (BFs concentration increased from 4214 to 6816 ± 101mg/L). Sonication decreased the floc size, therefore, increased the specific surface area, which increased the reaction rate and lead to a higher extraction efficiency. Higher nucleic acid content in BFs extracted by methods combined with sonication (0.11 mg/g in the case of the F-Sonic-Heat compared with 0.01 in case of F-Heat and 0.33 mg/g in the case of the F-Sonic-EDTA compared with 0.13 mg/g in the case of the F-EDTA) suggested that sonication disrupted the cell structure and released the intracellular polymeric material such as protein, nucleic acids, and storage polysaccharides. These intracellular polymeric materials contributed to a substantial increase in weight of the extracted BFs. However, sonication generated more colloidal particles due to cell disruption, which were difficult to remove by centrifugation and therefore, they might lead to an error in analyzing the dry weight of the BFs, soluble protein and polysaccharide. The presence of colloidal particles also leads to a decrease in flocculation activity (Bezawada et al., 2013). Sonication associated with F/NaOH and F-EDTA lead to an increase in ratio of protein to polysaccharide from 0.58 to 1.29 and 0.47 to 1.16, respectively. It indicated that higher quantities of tightly bound EPS were extracted; the

tightly bound EPS contains higher protein than loosely bound EPS (Bezawada et al., 2013; Chen et al., 2007).

A decrease in release of nucleic acids while combining the heating or EDTA process with formaldehyde resulted because of reduced cell lysis (Table 5-2). This is because aldehyde group of formaldehyde can combine with nitrogen and some other atoms of proteins, forming a cross-link -CH_2- called a methylene bridge. This reaction leads to the hardening of cell membrane; therefore, prevent the cell lysis (Helander, 1994). Further, the use of formaldehyde also reduced the amount of BFs extracted and protein content in the BFs due to less disruption of cells and less extraction of protein rich polymeric substances. The latter react with formaldehyde and get fixed on the cell membrane. The extracted BFs dry weight and protein content decreased from 910 to 830 mg/L and from 0.10 to 0.09 (g/g), respectively, when the formaldehyde treatment was combined with the heating process. The formaldehyde treatment combined with the EDTA process lead to a decrease of BFs extracted and protein content from 6244 to 4214 mg/L and 0.10 to 0.08 (g/g), respectively.

EDTA was one of the most effective methods with respect to BFs extraction efficiency (6244 mg/L of EPS extracted). The BFs extraction by EDTA takes place through chelation with divalent cations such as calcium, magnesium, etc., which is considered as the major binding mechanism between BFs and the bacterial cell. EDTA caused a strong damage to the cell resulting in high nucleic acid concentration (Table 5-2). These results were similar to those reported by previous workers (D'Abzac et al., 2010; Liu and Fang, 2002). Harmful effect of EDTA on cell could be explained due to: (1) EDTA chelation with divalent cations leads to the detachment of BFs from cell the membrane and therefore, the cell is less protected and easier to break; (2) high concentration of EDTA in solution leads to an increase of osmotic pressure between inside and outside of the cell membrane and thereby increases the cell lysis.

From overall results, it could be concluded that the highest BFs concentration was obtained with extraction methods, which had a harmful effect on cell or liberate high nucleic acid content in BFs. BFs extracted by chemical methods contain both extracellular and intracellular polymeric substances. The use of formaldehyde treatment combined with EDTA and heating reduced both the cell lysis and BFs weight.

Table 5-2 : Dry weight and chemical composition of BFs extracted by different methods

Extraction method	BFs dry weight		Concentration			Content in BFs			PN/PS (g/g)
	Concentration (mg BFs/L)	Content (g BFs/g SS)	PN (mg/L)	PS (mg/L)	Nucleic acids (µg/L)	PN (g/g)	PS (g/g)	Nucleic acids (mg/g)	
Centrifugation	430±15	0.02	18	43	9	0.04	0.10	0.02	0.42
Heat	910±20	0.04	92	148	17	0.10	0.16	0.02	0.62
F-Heat	830±14	0.03	74	129	10	0.09	0.15	0.01	0.58
F-Sonic-Heat	3540±10	0.14	500	387	381	0.14	0.11	0.11	1.29
Sonication	3548±15	0.14	981	444	761	0.28	0.13	0.21	2.21
F/NaOH	3196±25	0.13	233	494	1642	0.07	0.15	0.51	0.47
F-Sonic-NaOH	5370±40	0.21	1043	896	1090	0.19	0.17	0.20	1.16
EDTA	6244±73	0.25	623	790	1971	0.10	0.13	0.32	0.79
F-EDTA	4214±44	0.17	323	361	558	0.08	0.09	0.13	0.89
F-Sonic-EDTA	6816±16	0.27	615	399	2233	0.09	0.06	0.33	1.54

5.5.2 Effect of extraction method on flocculation activity of BFs

A comparison of the flocculation activity of BFs (crude and purified) extracted by different methods is presented in Fig.1. Volume of BFs used for flocculation was 3 mL (both crude and purified BFs). The BFs extraction method is considered a good method if it can extract a high amount of BFs from sludge and the extracted BFs possesses high flocculation activity. Therefore, the same volume of the BFs extracted by different methods was added to the kaolin solution to determine the most suitable extraction method.

The results clearly show that the purified BFs exhibited higher flocculation activity (FA) than the crude BFs (Fig. 1). It was because the crude BFs contained colloidal matter, excess extracting reagent and unknown materials that can exert a negative effect on the flocculation activity.

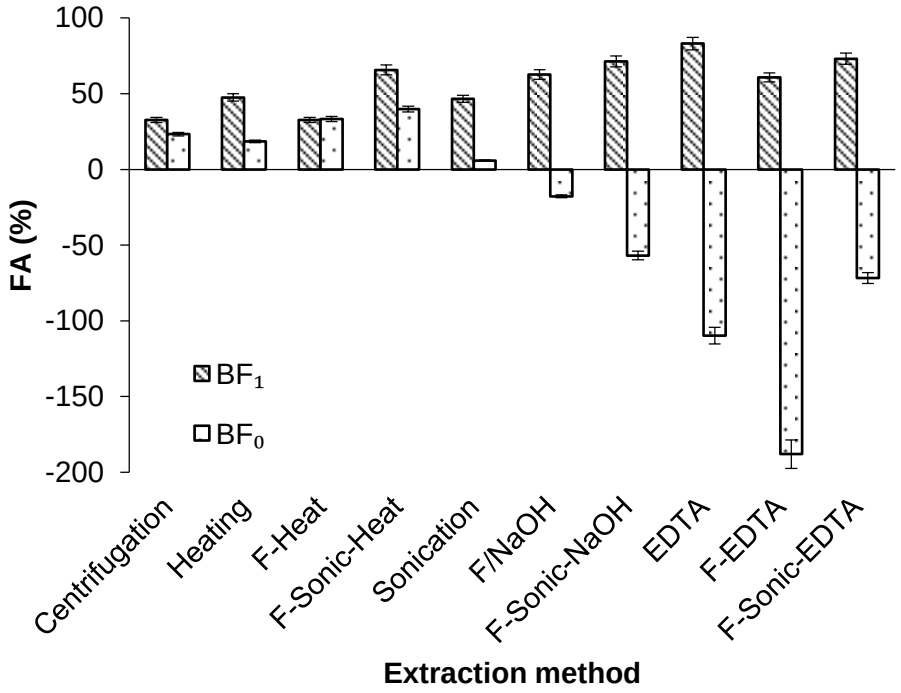

Figure 5-1: Effect of extraction method on flocculation activity of BFs (BF1: purified BFs; BFo: crude BFs)

The negative flocculation activities of crude BFs extracted by various methods that used NaOH and EDTA were due to the effect of Na^+ and EDTA on flocculation. EDTA present in crude BFs chelated with calcium, which made the calcium unavailable to neutralize the negative charge of kaolin and to form the bridge between BFs and kaolin particles. The presence of sodium in solution can displace the divalent cations from the bio-flocculants due to ion exchange reaction (Higgins and Novak, 1997). Therefore, it leads to a decrease of FA.

The flocculation activity of the purified BFs is in the following order: (centrifugation, F – heating) < (Heat, Sonication) < (F – sonication – heat, F/NaOH, F-Sonication-NaOH, F-Sonication-EDTA, F-EDTA) < EDTA. The order of the flocculation activity was similar to the order of BFs dry weight (as described in section 3.1). The purified BFs, which was extracted by centrifugation, heating or F-heat exhibited FA of less than 50% (Figure 5-1). The low flocculation activity of BFs extracted by these methods was due to low extraction efficiency, as indicated earlier.

Sonication extracted 3248 mg BFs/L and it was three times higher than the BFs extracted by the heating process. However, the purified BFs had low flocculation activity (47%) and it was similar to the flocculation activity of BFs extracted by the heating process. The low flocculation activity was due to the fact that the BFs extracted by sonication possessed high colloidal content, which lead to an increase of turbidity during the flocculation process.

Formaldehyde has been commonly used in bio-flocculants extraction studies because it is able to prevent the cell lysis and enhances the purity of the extracted bio-flocculants. However, the use of formaldehyde for BFs extraction exhibited different effects on FA. When formaldehyde treatment was combined with the sonication process, the FA of the purified BFs was significantly increased. It could be seen in the case of F/NaOH (FA-63%) vs F-Sonic-NaOH (FA-71%); F-Heat (FA-33%) and Sonication (FA-47%) vs F-Sonic-Heat (66%); F-EDTA (FA-61%) vs F-Sonication-EDTA (FA-73%). As discussed previously, sonication increases the BFs extraction efficiency as it could extract tightly bound EPS and intracellular polymeric substances, which contain high protein. High protein content in BFs is believed to be responsible for increased flocculation activity (Bezawada et al., 2013; Dermlim et al., 1999). The disadvantage of sonication process was creation of high concentration of colloidal particles in BFs solution, which reduced the flocculation activity. The use of formaldehyde could reduce the cell breakage during sonication reducing the colloidal particles and enhancing the flocculation activity.

Conversely, combination of formaldehyde treatment with heating and EDTA processes reduced the flocculation activity of the purified BFs. The negative effect of formaldehyde treatment on flocculation activity could be seen clearly in the case of EDTA (FA-83%) vs F-EDTA (FA-73%) and heating (FA-48%) vs F-Heating (FA-33%). Reduction of FA in these cases was due to the reaction of formaldehyde with polymeric proteins, which fixed them on cell membrane, reduced the amount of extracted BFs as well as reduced the protein content in the extracted BFs.

Purified BFs extracted by EDTA exhibited highest flocculation activity (Figure 5-1) in comparison with the other methods. The advantage of EDTA mediated EPS extraction over other chemical and physical methods is that EDTA selectively cut down the binding of BFs – divalent cations - EDTA by chelation with divalent cations (Sanin and Vesilind, 2000) and does not oxidizes or break the chain of high molecular substances. Therefore, it might not affect the quality of the BFs.

The above results indicated that the methods which can break the cell and prevent the formation of colloidal are favorable for extracting BFs with high flocculation activity. These methods include EDTA, F-Sonic-NaOH, F-Sonic-heat or F-Sonic-EDTA. It suggested that BFs might include both extracellular and intracellular polymeric substances. Inside bacterial cell, there are intracellular polysaccharide, intracellular protein, nucleic acids, DNA, etc. which have high molecular weight varying from thousands to millions kDa. These substances might play a definite role in the flocculation activity of BFs.

5.5.3 Optimization of EDTA concentration for extracting BFs from wastewater sludge

EDTA was used in many studies to extract BFs from bacterial broth for analyzing the composition and dry weight of the extracted BFs. However, the flocculation activity of the extracted BFs was not measured. The standard protocol suggested a use of 20 g EDTA/L of sludge sample for highest BFs extraction efficiency (Comte et al., 2006; D'Abzac et al., 2010; Zhang et al., 2012; Zhang et al., 1999). The relation between sludge solids concentration and EDTA was not considered. However, 20 g EDTA/L might not meet the economic aspect when applied to extract and reuse the extracted BFs from wastewater sludge. Therefore, effect of EDTA concentration on BFs extraction efficiency (or concentration extracted) and its flocculation activity was studied to reduce the extracting reagent consumption and hence enhance the economic benefits of extraction method.

In this study, different concentrations of EDTA were used to extract BFs from fresh wastewater sludge (25 g SS/L), include 20; 10; 5; 2.5; 1.25 and 0.6 g EDTA/L of sludge or 0.8; 0.4; 0.2; 0.1; 0.05 and 0.024 g EDTA/g SS. BFs dry weight and chemical composition of purified BFs were presented in Figure 5-2. The flocculation activity of the purified BFs was measured (Figure 5-3).

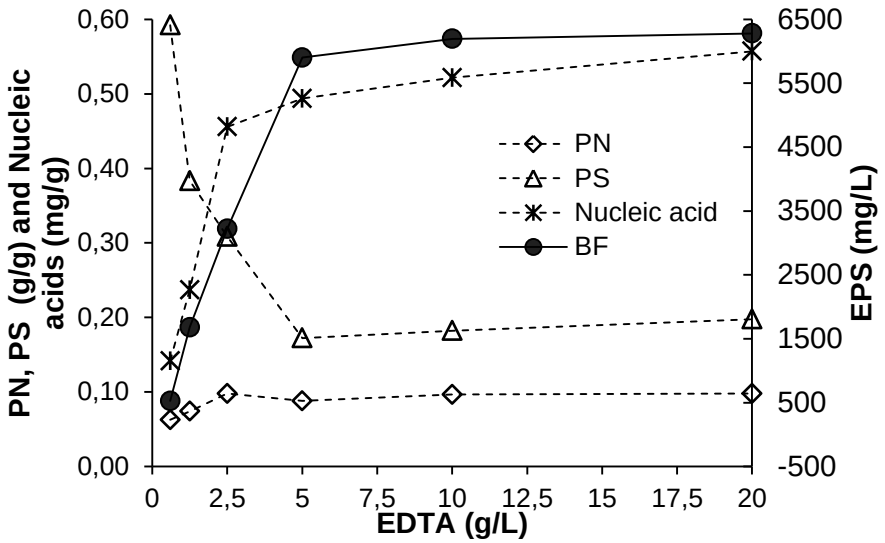

Figure 5-2 : Chemical composition and dry weight of the purified BFs extracted using different EDTA concentrations

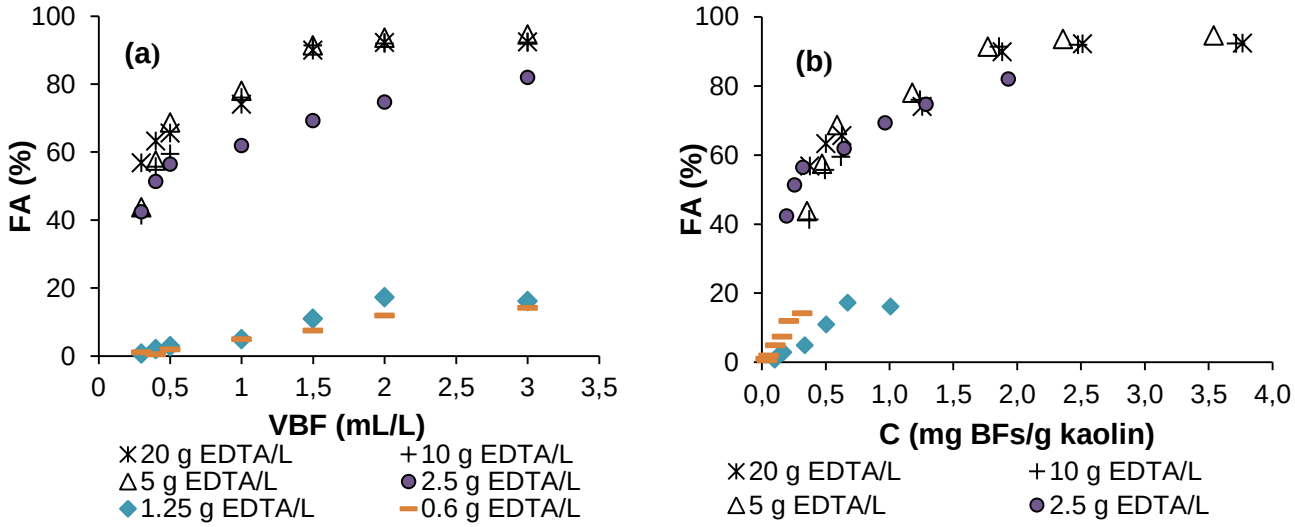

Figure 5-3 : Flocculation activity of the purified BFs extracted using different EDTA concentrations (a) FA vs volume of BFs (VBF) and (b) FA vs concentration (C)

The results indicated that EDTA was not effective when concentration was below 1.25 g/L (or 0.5 g EDTA/g SS). The extracted BFs dry weight was 527 and 1680 mg/L when using 0.6 g EDTA/L (0.024 g EDTA/g SS) and 1.25 g EDTA/L (0.05 g EDTA/g SS), respectively. The extracted BFs contained low protein (0.06 – 0.07 g/g), low nucleic acids (0.14 – 0.24 mg/g) and high carbohydrate (0.59 – 0.38 g/g). High carbohydrate contents indicated that BFs were extracellular polymeric substances (Salehizadeh and Yan, 2014). Flocculation activities of the extracted BFs by 0.6 and 1.25 g EDTA/L were low. At 3 mL of BFs/L (or 1 mg BFs/g kaolin), flocculation activity was less than 20%, which was much lower than flocculation activity of BFs extracted with EDTA concentration from 2.5 to 20 g/L when using the same volume or the same concentration of BFs (as shown in Figure 5-3).

The BFs dry weight extracted increased significantly to 3220 mg/L when 2.5 g EDTA/L (or 0.1 g EDTA/g SS) was used for extraction. Protein (0.10 g/g) and nucleic acids (0.46 mg/g) contents also increased compared with those of BFs extracted from 0.6 and 1.25 g EDTA/L. Extracted BFs (at 2.5 g EDTA /L) showed high flocculation activity (82%) when using 3 mL of purified BFs or 1.93 mg BFs/g kaolin. When using a similar concentration of BFs (mg BFs/g kaolin), it was comparable to the flocculation activity of BFs extracted by higher concentrations of EDTA (as shown in Figure 5-3b).

The extracted BFs dry weight, composition (protein, polysaccharide, nucleic acid content) and the flocculation activity was not significantly different when EDTA concentration varied from 5 to 20 g/L or 0.2 to 0.8 g EDTA/g SS (Figures 5-2 and 5-3). The amount of BFs extracted from sludge increased from 5900 to 6280 mg/L. Protein, carbohydrate and nucleic acid content were 0.09 – 0.10 (g/g), 0.17 – 0.20 (g/g) and 0.49 – 0.56 (mg/g), respectively. High protein and nucleic acid content indicated that the BFs contained a high fraction of tightly bound EPS and intracellular polymeric substances. The purified BFs revealed high flocculation activity and attained 92 – 95% FA using concentration around 2 mg BFs/g kaolin (Figure 5-3b). After settling, the remaining turbidity in the supernatant was around 5 – 6 NTU.

Although, concentration of BFs extracted by 5 – 20 g EDTA/L was higher than that extracted by 2.5 g EDTA/L, protein and nucleic acid content in BFs extracted with EDTA from 2.5 to 20 g/L were similar (Figure 5-2). Several authors argued that the protein and the nucleic acid content play an important role in the flocculation activity of BFs (Bezawada et al., 2013; Dermlim et al., 1999; Watanabe et al., 1998; Wilen et al., 2003). Moreover, EDTA disrupts the cells and releases the intracellular substances such as intracellular polysaccharide, intracellular protein, lipid etc. These components might enhance the flocculation activity of BFs

due to their high molecular weight. This was the reason why flocculation activity of BFs extracted by 2.5 – 20 g EDTA/L was comparable when using same BFs concentration (Figure 5-3b).

Optimization of EDTA concentration revealed that for extraction of bio-flocculants from backwashed sludge, 5 g EDTA/L needed to use as it exhibited high BFs extraction efficiency and high flocculation activity.

Table 5-3: Comparison of flocculation activity of different bio-flocculants.

BFs producing bacterium	**Growth medium**	**Cation added**	**FAmax (%)**	**BFs conc. (mg/g kaolin)**	**References**
Serratia ficaria	Synthetic	Ca^{2+}	96	0.4 (*)	(Gong et al., 2008)
Klebsiella sp. N10	Synthetic	Ca^{2+}	87	6.8	(Yang et al., 2012)
K. mobilis	Dairy wastewater	Ca^{2+}	95	1.04	(Wang et al., 2007)
Serratia sp.1	Wastewater sludge	Ca^{2+}	79	0.7	(More et al., 2012)
Serratia sp.1	Waste water sludge	Ca^{2+}	70	1.38	(Bezawada et al., 2013)
BFs of raw sludge extracted by 5 g/L of EDTA	-	Ca^{2+}	94	2.4	This study

(*) 0.4 ml of broth culture per little of 5 g kaolin solution.

Table 5-3 compared results of this study with other studies. More et al., (2012) reported that EPS produced by *Serratia* sp.1 in sterilized sludge medium were 2.3 g/L and maximum flocculation activity obtained was around 79% when using 0.7 mg EPS/g kaolin. Bezawada et al., (2013) reported that 4.34 g/L of EPS was produced by *Serratia* sp.1 in sterilized sludge medium at 48 h. Maximum flocculation activity obtained was 70% when using 1.38 mg EPS/g kaolin. In comparison with these two studies, both dry weight and maximum flocculation activity of BFs extracted from the fresh sludge by EDTA were remarkably higher.

Several authors reported flocculation activity of 87 – 96% (Gong et al., 2008; Wang et al., 2007; Yang et al., 2012), which is similar to the flocculation activity observed in this study. However, the flocculation activity of the BFs extracted using 5 g EDTA/L might be much higher than that of other studies because they used different settling condition. In other studies, the flocs were settled in 500 or 1000 mL beaker. In this study, after flocculation step, kaolin solution was transferred to a 500 mL cylinder for settling of flocs. Smaller settling area and higher settling depth of 500 mL cylinder compared to those of beaker lead to a lower settling efficiency and lower flocculation activity.

The outcome of this study is not only to reveal a method with high extracted BFs quantity and quality (high FA) but it also proved that wastewater sludge is a potential source of bio-flocculants. It offers a new approach to bring the bio-flocculants to application in wastewater treatment plant as wastewater sludge is a low-cost material and is available in large quantity in almost all wastewater treatment plants.

5.6 Conclusions

Ten different methods were studied to extract bio-flocculants (BFs) in backwash sludge of the bio-filtration unit. The results indicated that chemical methods and sonication revealed higher extraction efficiency than the centrifugation and the heating processes. Cell lysis did not affect the flocculation activity of BFs. Among ten extraction methods, EDTA was the best one with extracted BFs dry weight of 6242 mg/L and flocculation activity of 83%. EDTA concentration optimization showed that 5 g EDTA/L (or 0.2 g EDTA/g SS) was enough for BFs extraction (5900 g/L). Flocculation activity can be achieved up to 94% at 2.4 mg BFs/g kaolin.

5.7 Acknowledgements

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CHAPITRE 6

Heavy metals removal from wastewater using extracellular polymeric substances (EPS) produced by C*loacibacterium normanense* in wastewater sludge supplemented with crude glycerol and study of EPS extraction by different methods

Ce chapitre est constitué d'un article suivant:

Klai Nouha, Saurab Ram Kumar, Tyagi RD, (2016) Heavy metals removal from wastewater using extracellular polymeric substances (EPS) produced by *Cloacibacterium normanense* in wastewater sludge supplemented with crude glycerol and study of EPS extraction by different methods. *Bioressource technology.212, 120-129*

6 Heavy metals removal from wastewater using extracellular polymeric substances (EPS) produced by *Cloacibacterium normanense* in wastewater sludge supplemented with crude glycerol and study of EPS extraction by different methods

6.1 Abstract

Extracellular polymeric substances synthesis by *Cloacibacterium* was affected by different concentrations of glycerol in the medium. The concentration of EPS in 72h fermentation was increased from 13 g/L with no external carbon supplementation to 21.3 ± 0.7 g/L with 2% (w/v) crude glycerol addition. Physical and chemical extraction methods (heating, centrifugation and ethylene diamine tetra-acetic acid (EDTA)) were used in this study and their performa nce to extract EPS was compared. A significant variation in concentration of extracted B-EPS (broth-EPS) by heating (20.8 ± 0.5 g/L) and centrifugation (21.3 ± 0.7 g/L) extraction methods was not observed. However, in case of extracti on with EDTA (5 g/L), the BFs concentration extracted was 25.5 ± 0.9 g/L, which exhibited high flocculation activity of 95.3 ± 0.5% at optimum dose of 23.1 mg B-EPS/g kaolin. Moreover, Ni removal efficien cy of 80% from primary treated wastewater was achieved using 35 mg/L of B-EPS extracted by centrifugation method.

6.2 Keywords

Wastewater sludge, Crude glycerol, Extracellular polymeric substance, Flocculation activity, Heavy metal removal.

6.3 Introduction

The ability of microorganisms to synthesize extracellular polymeric substances (EPS) is widely found among bacteria, yeast, fungi and algae. They excrete EPSs as soluble and insoluble polymers. EPSs results from active bacterial secretion, cell lysis or can be extracted from activated sludge (Satpute et al., 2010). In the recent past, many studies have been reported on the isolation and identification of high EPS producing microbial strains. EPS biosynthesis in microorganisms is stimulated by the excess carbon availability and concomitant nitrogen or oxygen limitation (Sutherland et al., 2001). However, the high cost of carbon source (such as glucose, sucrose) used for the production of EPS (Kumar et al., 2007) lead to their limited market potential. Therefore, it is important to search for low cost or waste carbon sources such as crude glycerol, an abundant and cheaply available by-product of biodiesel production processes.

The EPS produced by the cell is either loosely bound or tightly bound to it. In order to use the EPS, they must be separated from the cell. The different components of EPS and their concentration obtained during extraction process depends on the extraction method employed (Liu and Fang, 2002). There are many methods proposed for extracting EPS (Comte et al., 2006). The quantity of EPS obtained can fluctuate from 165 mg (ethylenediamine tetra-acetic acid (EDTA) extraction) to 26 mg (centrifugation alone) of EPS per gram of activated sludge (Liu and Fang, 2002). The lack of a standardized EPS extraction procedure prevents any comparison and interpretation of the published results. Among the known methods, only thermal, centrifugal and EDTA methods were investigated in this study. Thermal and centrifugation methods are simple, economical and they do not use toxic chemicals. On the other hand, for comparison EDTA was selected, which reportedly gave maximum EPS extraction efficiency.

In this study, sterilized activated sludge with or without supplementation of crude glycerol was investigated as a carbon and nutrient source to produce a novel microbial biopolymer by employing a bacterial strain (*Cloacibacterium normanense*) isolated from wastewater sludge. Different EPS extraction methods (centrifugation, heating and EDTA) were studied and a preliminary polymer characterization in terms of proteins and polysaccharides composition was conducted. The role of the EPS components in bio flocculation and their efficiency in the removal of heavy metals was also investigated.

6.4 Material and methods

6.4.1 EPS production

Activated sludge was collected from bio-filtration unit of Communauté Urbaine du Québec (Municipal wastewater treatment plant, CUQ, Québec, Canada). A single colony of *Cloacibacterium normanense* (NK6, accession number KF675204) was revived and grown in 5 mL of Tryptic soy broth (TSB) for 48 h. To produce pre-culture, 5 mL of the TSB pre-grown broth was transferred to 100 mL of sterilized sludge (25 g/L suspended solids or SS) and incubated for 24 h. The pre-culture was added (5% v/v) to each flask containing 150 mL sterilized sludge (25 g/L SS, pH 7). The flasks were incubated in a shaker at 180 rpm and 30°C for 96 h. After 24 h of incubation, different concentration (0.5 %, 1 % and 2 % w/v (weight /volume)) of non-sterilized crude glycerol was added to each flask. During the experiments, samples of the broth were collected every 24 h to determine EPS concentration, glycerol concentration and cell population.

6.4.2 EPS extraction

Three different EPS extraction methods (centrifugation, heating and EDTA) were used to extract the EPS. In the centrifugation method, the culture broth was centrifuged at 9000 g, 4 °C for 20 min to obtain supernatant (containing slime EPS, termed as S-EPS). The biomass pellet was re-suspended in deionized water equal to the initial volume and then heated at 60°C for 20 min to extract capsular EPS (C-EPS) (Bala Subramanian et al., 2010a). Broth EPS (B-EPS) consists of both S-EPS and C-EPS, or the fermented broth is named as B-EPS.

In heating method, the sample was heated at 80°C for 1 h and then centrifuged at 9000 g and 4°C for 20 min.

In the third method, EDTA was added to the fermented broth up to a final concentration of 0.2 g EDTA/g SS, which is the optimal concentration (Liu and Fang, 2002). The sample was mixed well and then incubated at 4°C for 3 h and then centrifuged (9000 g, 4°C, 20 min).

6.4.3 Dry weight and EPS characterisation

For measuring dry weight, one volume of supernatant obtained after centrifugation (crude S-EPS) was mixed with two volumes of chilled ethanol (95% v/v (volume /volume)) and precipitated at -20°C overnight. After precipitation, the sample was centrifuged at 6000 g for 20 min to obtain the precipitated pellets. The pellet was dried at 60°C until constant weight.

To determine the chemical composition of the precipitated EPS (C-EPS or S-EPS), the heat or chemically treated sample or non-treated sample was centrifuged at 6000 g for 20 min. The

collected pellet was dissolved in distilled water to the initial volume. Then, the solution was used to determine soluble protein (Bradford, 1976) and carbohydrate (DuBois et al., 1956) content of the EPS. TC-C-EPS represents the total carbohydrate content of C-EPS and TC-S-EPS denotes the total carbohydrate content of S-EPS. Similarly, TP-C-C-EPS expresses the total protein content of C-EPS and TP-S-EPS indicates the total carbohydrate content of S-EPS.

The nucleic acid and humic substances content were determined by Burton et al. (1956) and Lowry et al. (1951), respectively.

Crude glycerol solution also contains other components, which can act as carbon source. The composition of crude glycerol was determined according to Hu et al. (2012) (Table 1).

6.4.4 Flocculation Activity (FA)

The flocculation activity of crude and purified EPS was determined by standard Jar-test with 5 g/L of kaolin solution (Bala Subramanian et al., 2010a). $CaCl_2$ was added to the kaolin suspension to get final concentration of 150 mg Ca2+/L and pH was adjusted to 7.5. S-EPS (20.3 g/L) was collected from centrifugation extraction method. It was added in different volumes (0.5, 1, 1.5, 2, 3 mL) to kaolin suspension. The mixture was rapidly mixed at 120 rpm for an initial 5 min, and then slowly mixed at 70 rpm for an additional 30 min. Thereafter, samples were transferred to a 500 mL cylinder for 30 min settling. Supernatant of the settled solution was then collected to measure the turbidity using a turbidity meter (Micro 100 turbidimeter, Scientific Inc.). Flocculation activity was measured using the formula [100*(B-A)/B] where 'A' is the turbidity of the sample (added with different types of EPS i.e. slime, capsular or broth-EPS)) and 'B' is the turbidity of the control in which equal volume of EPS solution was replaced with distilled water. Turbidity of the samples was measured three times to take the average.

6.4.5 Heavy metal removal capacity of EPS from metal contaminated solution.

B-EPS was added (35 mg/L or 50 mg/L) to six replicated Erlenmeyer flask each containing 100 mL of primary treated wastewater. The flasks were incubated at 250 rpm and 30°C for 12 hours. For sampling, each time a flask was withdrawn and used for residual metal analysis. Wastewater without adding EPS was used as a control to compare and calculate the heavy metals removal efficiency of the EPS. Heavy metal concentration of secondary wastewater effluent was also measured to compare the heavy metals removal efficiency of the EPS to chemical polymers used in wastewater treatment plant. For metal analysis, samples (40 mL from each flask) were transferred into a centrifuge tube and centrifuged at 3000 rpm for 5 min, to remove suspended particles containing EPS-metal complexes. Supernatant (10 mL) was transferred to 100 mL

volumetric flask and acidified with 90 mL nitric acid (HNO3) of 6 N (Normal concentration). These samples were stored in a freezer to terminate the reaction (Kelly et al., 2004). The concentrations of different heavy metals present in wastewater were determined by inductively coupled plasma Atomic emission spectroscopy (ICP-AES) (model DRE, Leeman Labs Inc). Experiments were conducted in triplicates and average values are presented.

6.5 Results and discussion

6.5.1 EPS production using glycerol as carbon source

The composition of crude glycerol solution is presented in Table 6-1. *Cloacibacterium normanense* (Gene bank - NK6 KF675204) was grown in sterilized wastewater sludge (suspended solids concentration 25 g/L) supplemented with crude glycerol as a carbon source, which was added at 24^{th} h of the fermentation. The results of B-EPS production and glycerol concentration in shake flask experiments at various concentrations of crude glycerol are presented in Figure 6-1. Growth of *C. narmanense* sp. is presented in Figure 6-2. During the initial adaptation phase (or lag phase), which continued until 12 h, there was a little cell growth (Figure 6-2a). The culture entered exponential growth phase at 12^{th} h and continued until 60^{th} h with maximum cell concentration reaching at 1.5 x 10^9 CFU/mL (Colony forming units/mL). In exponential phase, both the cell number and the total cell mass started to increase rapidly and the bacterial population was growing and dividing at a maximum rate.

The EPS concentration in the sludge fortified with 2 % crude glycerol during the lag phase (from 0 h to 24 h) increased from 1.9 ± 0.3 g/L to 6.0 ± 0.5 g/L (Figures 6-1 and 6-2). The EPS production actively started during the exponential phase of bacterial growth commencing at 12^{th} hour and continued until the 60^{th} hour (Figure 6-2a), but significant EPS production was observed after the culture has entered the mid log phase from 24^{th} hour (Figure 6-2b) and continued until late stationary phase at the 72^{nd} hour displaying the sigmoidal nature of the production. The highest EPS concentration (21.3 ± 0.7 g/L B-EPS) was observed at 72 h with the addition of 2 % (w/v) of crude glycerol to sludge. The EPS production almost stopped at 96 h in spite of the presence of 5.5 g/L of residual glycerol (Figure 6-1). It may be explained by *C. normanense* growth inhibition due to intrinsic metabolic aging of the culture, which enters in stationary phase at 96^{th} hour. At stationary phase bacterial cultures are reported to exhibit slow metabolism and enter stationary phase (Kolter et al., 1993) in response to exhaustion of other necessary trace growth nutrients like minerals and vitamins.

The final B-EPS concentration obtained in this study was higher compared to *Pseudomonas oleovorans (P. oleovorans*) NRRL B- 14682 grown in synthetic media supplemented with purified

glycerol (20 g/L) (Freitas et al., 2009). The EPS concentration produced using purified glycerol as a carbon source by *P oleovorans* was 7 - 18 g/L in 96 to 144 h.

Table 6-1: The crude Glycerol composition

Components	Concentration (w/w%)	Concentration (g/L)
Glycerol	86.45	1037.81
Methanol	0.12	1.44
Water content	18.5	222.10
Soap	0.09	1.08
pH	8.25	8.25

Note: Density of crude glycerol is 1.2 g/mL

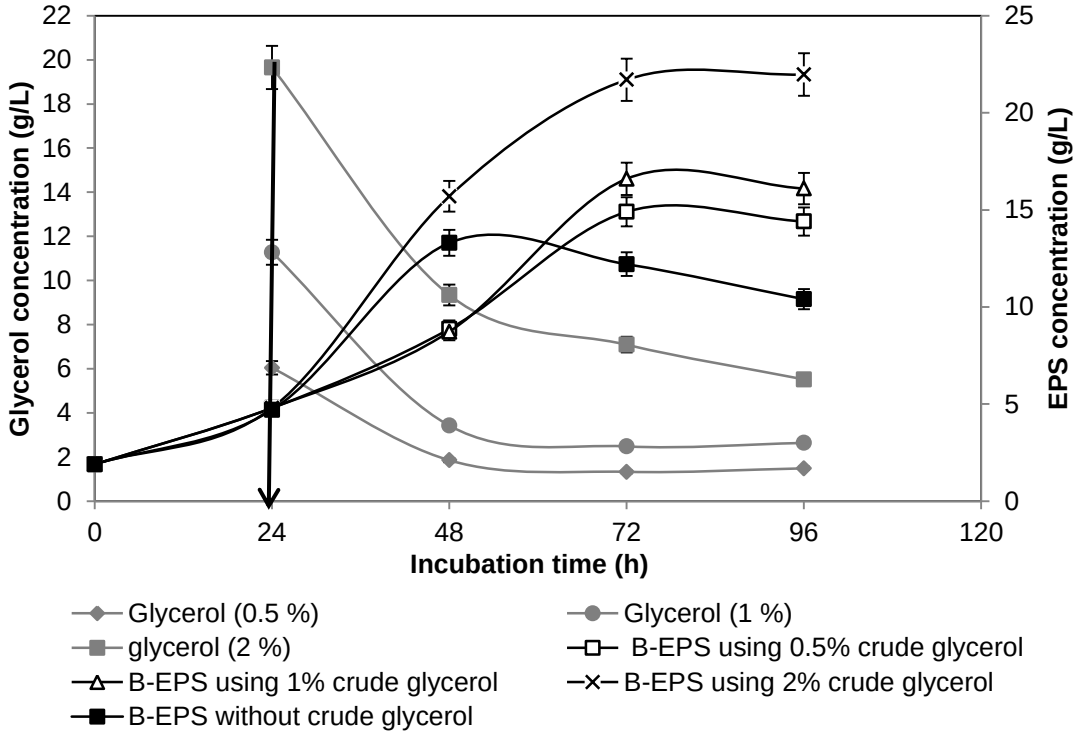

Figure 6-1 : Variation of glycerol consumption and B-EPS production by *Cloacibacterium normanense* in sludge fortified with different crude glycerol concentration (0.5, 1 and 2 % (w/v). The EPS was determined using centrifugation method. (Arrow represents the addition of glycerol at this point).

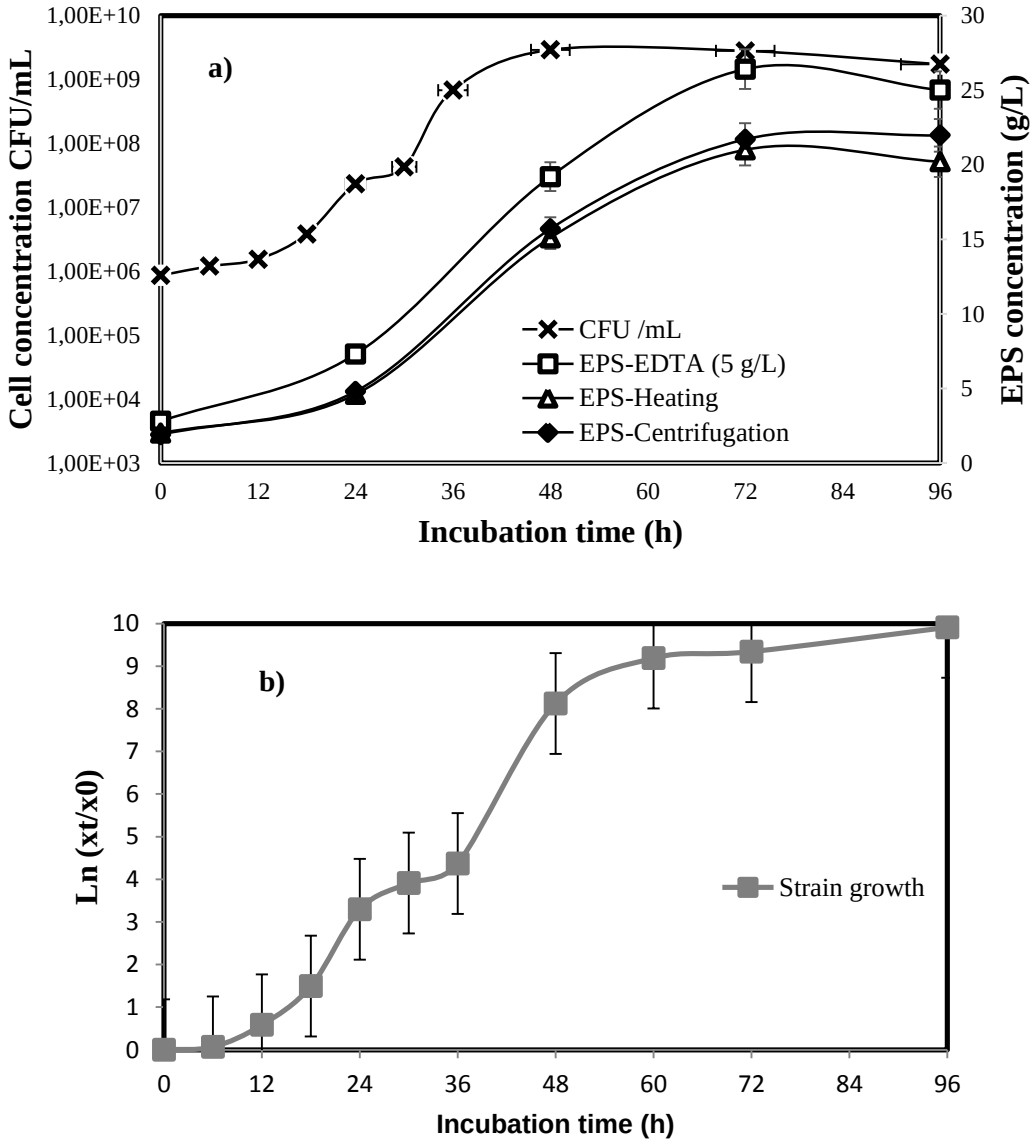

Figure 6-2a and b: Growth and B-EPS production by *Cloacibacterium normanense* in sludge supplemented with 2 % (w/v) of crude glycerol. The EPS was extracted using centrifugation, EDTA and heating methods.

The glycerol concentration decreased from 19.8 g/L to 5.5 g/L during 72 h (Figure 6-1 and Table 6-2). C. *normanense* assimilated 75 % of the added glycerol during the fermentation and produced 21.3 ± 0.7 g/L B-EPS (20.3 ± 0.6 S-EPS and 1.0 ± 0.1 g/L of C-EPS). The addition of 0.5 % (w/v) or 1 % (w/v) crude glycerol resulted in 90 and 95 % glycerol consumption and 13.9 ± 0.5 and 16.6 ± 0.5 g/L of B-EPS production, respectively.

The higher EPS production during these experiments using sludge supplemented with crude glycerol could be in part due to the consumption of methanol and soap, constituents of crude glycerol solution, as carbon sources. However, methanol and soap concentration in the medium at the beginning of fermentation (at t = 0) was 0.036 g/L and 0.027 g/L, respectively. Thus, very low soap and methanol concentration in the medium clearly established that these carbon sources did not contribute to high B-EPS production.

Table 6-2 : Summary of the results in the production of B-EPS in sludge fortified with different concentration of glycerol.

***Initial glycerol concentration (g/L)**	**Inocolum volume (mL)**	**Glycérol concentration at 72 hours (g/L)**	**EPS concentration at 72 h (g/L)**
5.8	7.5	1.5	14.9
11.5	7.5	2.6	16.6
19.8	7.5	5.5	21.3
Control (without crude glycerol)	7.5	-	13.3

**In all cases glycerol was added at 24h.*

6.5.2 Effect of glycerol as carbon source on EPS concentration and composition

Carbohydrate and protein content of S-EPS and C-EPS is depicted in Figures 6-3a, b. The total protein and the total carbohydrate content of S-EPS, C-EPS and their concentration in the medium increased with fermentation time and reached maximum at 72 h concomitant to the EPS concentration (Figures 6-3a and b). The EPS was extracted by centrifugation.

The carbohydrate content of EPS increased with glycerol concentration in the medium. In 72 h fermentation, the carbohydrate content of S-EPS produced in sludge with the addition of approximately 0.5, 1 and 2 % (w/v) crude glycerol was 126 ± 6, 218 ± 9 and 400 ± 24 mg/g S-EPS, respectively, whereas the carbohydrate content of C-EPS was 35 ± 6, 60 ± 11 and 84 ± 4 mg/g C-EPS, respectively. The carbohydrate content of the EPS produced in control (sterilized sludge without glycerol addition) was 12.0 ± 0.9 mg/g S-EPS and 15.0 ± 3.5 mg/g C-EPS, which was lower than the values obtained in the case of sludge fortification with crude glycerol. The B-EPS carbohydrate content reached maximum at 72 h fermentation. Wang and Yu, (2007) also observed first an increase in carbohydrate content of B-EPS from 10 to 40 mg/L when the medium was supplemented with 1%, 2% 3% and 4%) of glucose as carbon source.

The total protein content 342 mg/g S-EPS was higher than 310 mg/g C-EPS produced in sludge with the addition of 2% (w/v) crude glycerol (Figure 6-3a). The protein content increased with fermentation time. However, no significant difference in protein content of B-EPS was observed for different concentration of crude glycerol (0.5%, 1% and 2%) in the medium. Similar observations were made by (Wang and Yu, 2007); where there was no significant variation in protein content of B-EPS (200 mg/g B-EPS) obtained with different concentration of glucose (1%, 2% 3% 4%) as substrate. The centrifugation (4000 g, 5 min) was used for EPS extraction.

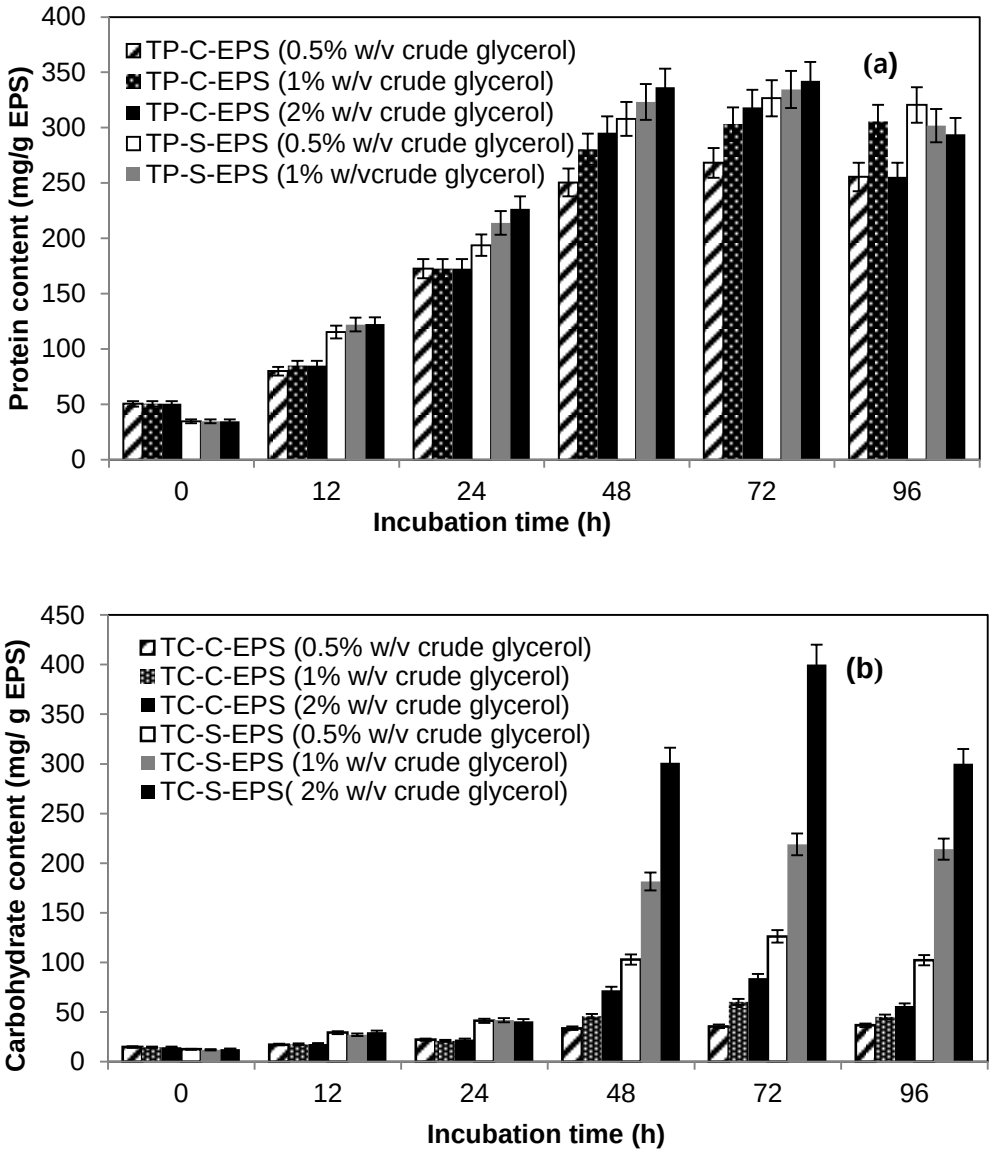

Figure 6-3: (a) Protein content and (b) Carbohydrate content and of S-EPS and C-EPS produced at different fermentation time using sludge fortified with different concentration of crude glycerol as production medium and centrifugation as extraction method

6.5.3 Composition and concentration of EPS extracted by different methods

The variation of protein and carbohydrate content of B-EPS extracted by different methods with fermentation time is presented in Figures 6-4a and 6-4b. No significant variation of the B-EPS concentration was observed using heating (20.8 ± 0.5 g B-EPS/L) or centrifugation method (21.3 ± 0.7 g B-EPS/L) (Table 6-3). However, the BFs concentration of 25.5 ± 0.9 g/L observed in EDTA method was high compared to other extraction methods. The chemical composition (proteins, carbohydrates and nucleic acids, humic acids and lipids) of B-EPS vary with the extraction method (Table 6-3). The carbohydrate and protein content of B-EPS was also higher in case of EDTA extraction, whereas almost similar content was observed in the case of heating or centrifugation methods (Figure 6-4a, 6-4b). EDTA was confirmed as effective in extracting humic substances and nucleic acids (Table 6-3).

Nucleic acid concentration extracted from 72 hours fermentation sample by EDTA method was 3680 ± 102 mg/L (or 144.3 mg nucleic acids per g BFs) for sludge fortified with 2 % (w/v) crude glycerol, whereas 1240 mg/L (or 93 mg nucleic acids per g B-EPS) was observed in the control (without addition of glycerol to sludge) experiment. Deoxyribonucleic Acid (DNA) or nucleic acid content was also lower in case of centrifugation method (2420 ± 144 mg/L or 113.6 mg nucleic acids per g B-EPS) than the EDTA extraction method.

The carbohydrate and protein content of the BFs could be higher because of two reasons; firstly due to the higher efficiency of EDTA to extract capsular EPS tightly attached to the cell and secondly, the EDTA may cause cell lysis secreting intracellular proteins and nucleic acid or some cellular storage polysaccharides such as glycogen. EDTA can cause cell lysis releasing intracellular bio-polymeric substances like nucleic acids, humic substances, polysaccharides and polypeptides. These intracellular polymeric substances contribute to the high final EPS concentration extracted by EDTA method. The carbohydrate and protein content was almost same in case of heating or centrifugation method. These two methods do not cause cell lysis. Centrifugation is often used to separate the soluble (S-EPS) fraction of EPS (Hebbar et al., 1992). It has been stated that the heating method extracted bound EPS or capsular EPS (strongly attached to flocs) as both kinds of EPS (loosely bound and tightly bound) exist in flocs (Comte et al., 2006); however, no such difference in B-EPS extracted by centrifugation or heating method was observed in this study. These results are in agreement with other studies (Frolund et al., 1996; Liu and Fang, 2002; Sheng et al., 2005; Tapia et al., 2009), who revealed that the final EPS concentration and composition (carbohydrate and protein content) is a function of the extraction protocol used.

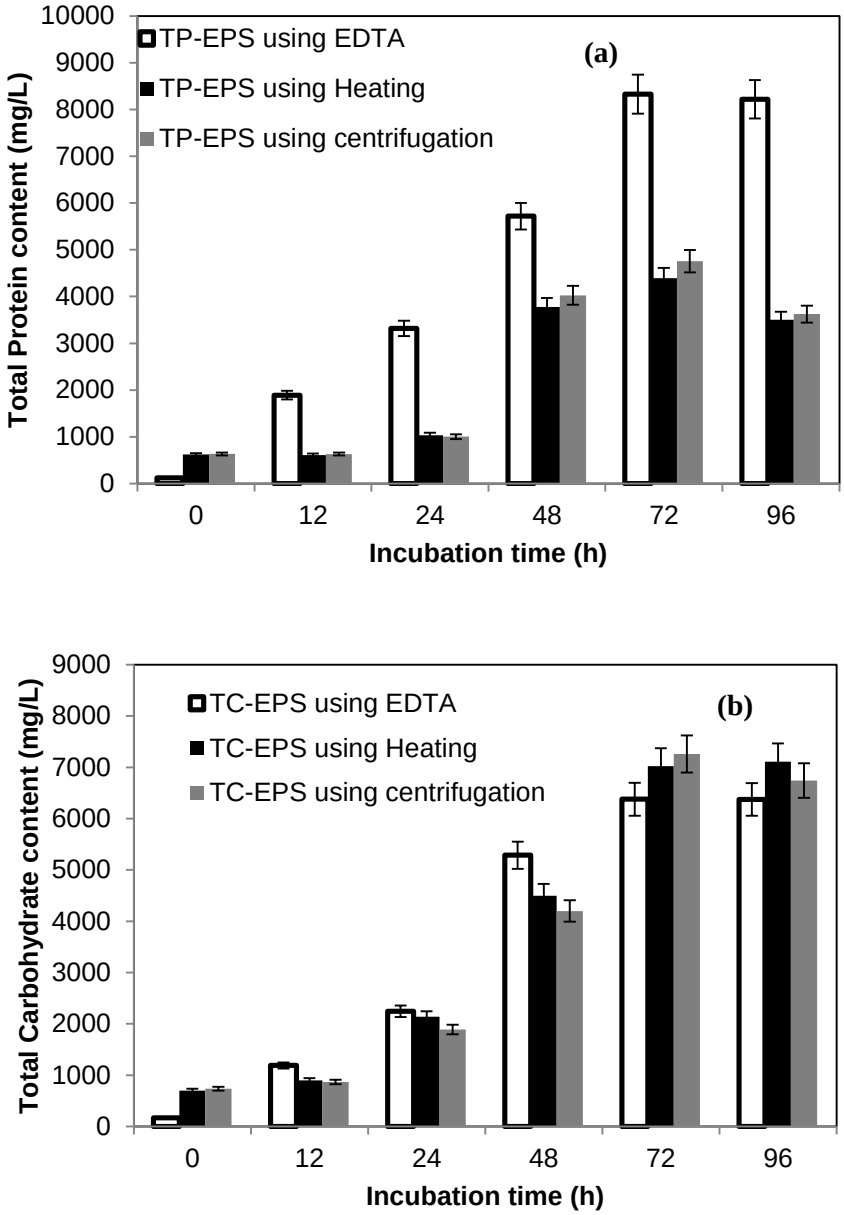

Figure 6-4: (a) The total protein content and (b) total carbohydrate content of B-EPS produced at different fermentation time. The EPS was produced in sludge supplemented with crude glycerol and extracted by different extraction methods.

Table 6-3: Characterization of B-EPS produced in sludge with and without fortification of crude glycerol

Method of EPS extraction	Initial glycerol concentration (g/L)	Total carbohydrate (TC) (mg/L) or (mg/g B-EPS)	Total protein (TP) (mg/L) or (mg/g B-EPS)	Humic substances (HS) (mg/L) or (mg/g B-EPS)	Nucleic acid (NA) (mg/L) or (mg/g B-EPS)	Fermentation time (h)	Total (TC+TP+ HS+N) (mg/L)	EPS concentration (g/L)	Other compounds (mg/L)
Centrifugation	none	1800 (135.3)	2890 (217.3)	3369 (253.3)	1240 (93.2)	48	9300	13.3±03	4000
Centrifugation	19.8	8270 (388.2)	3680 (172.7)	4317 (201.7)	2420 (113.6)	72	18690	21.3±0.5	2600
Heating	19.8	8260 (397.1)	3500 (168.3)	4290 (206.2)	2030 (97.6)	72	18080	20.8±1.2	2800
EDTA (5 g/L)	19.8	8500 (333.3)	4560 (178.8)	4954 (194.3)	3680 (144.3)	72	21694	25.5±0.7	3800

Note: Digits in parenthesis represents the concentration in mg/g B-EPS

6.5.4 Kaolin flocculation activity of EPS extracted by different methods

The variation of kaolin flocculation activity (FA) with EPS dose (volume or mg EPS/g kaolin) is presented in Figure 6-5. B-EPS produced using sludge fortified with 2 % (w/v) crude glycerol and extracted by different methods for the samples collected at 48, 72 and 96 h of fermentation was used to determine FA. In all cases, there is a general trend that FA increased, reached maximum and then decreased with EPS dose irrespective of the type of method used for EPS extraction or EPS collected at different time of fermentation (Figure 6-5). The maximum FA was achieved using the sample of EPS collected at 72 h of fermentation. However, a different dose of EPS was required to achieve maximum FA value for EPS obtained by different extraction methods. Table 6-4 summarizes the maximum FA obtained with a corresponding dose of the B-EPS extracted by different methods. The maximum flocculation activity 93.3 ± 0.5 % of B-EPS (obtained in case of sludge fortified with 2 % crude glycerol) using 2.1 mg B-EPS/g kaolin (mg of EPS added per gram of kaolin suspension in water) was a little lower than the maximum FA of B-EPS (94.2 % using 1.3 mg B-EPS/g kaolin) obtained without glycerol fortification. Centrifugation was used to extract the EPS in both cases, i.e. with or without glycerol addition.

The dosage of B-EPS required to achieve maximum FA value was dependent on the EPS extraction method. The dose of B-EPS or BFs required to achieve highest flocculation activity is approximately 11 times higher for EDTA method than the centrifugation method (Table 6-4). This was due to variation in chemical composition and the structure (functional group of sugar residues) of B-EPS produced with or without supplementation of crude glycerol and the EPS extraction method.

The chemical composition and structure of EPS depends upon the carbon source supplementation in the production medium (Ye et al., 2011). The most abundant monosaccharide was galactose accounting for 70 mole % of the total carbohydrate content of the EPS when purified glycerol was used as a carbon source in the medium for EPS production (Freitas et al., 2009). In case of glucose as a carbon source in the medium, the most abundant monomer of the EPS produced was glucose and small amounts of mannose, rhamnose and glucuronic acid (Monteiro et al., 2012). In the present study, *Cloacibacterium* sp. utilizes the carbon source present in sludge (glucose, proteins, volatile fatty acids and others). After adding glycerol, the bacteria start utilising glycerol as carbon source as well. Therefore, a slight decrease in maximum flocculation

activity (with the addition of glycerol) may be due to sugar residues variation in the composition of EPS produced with the glycerol addition to sludge.

In addition, the variation of flocculation activity of EPS extracted by different methods (Figure 6-5) or different requirement of EPS dose to attain the maximum FA (Table 6-4) was due to a change of composition and molecular weight of the extracted EPS. Past studies investigated the effect of carbon source during EPS production and the method used to extract on the molecular weight of the EPS synthesized (Lee et al., 2007). The molecular weight of EPS obtained by cultivating *Ganoderma applanatum* on glucose or maltose as carbon source was higher than 2000 kDa, whereas, the EPS produced by cultivating the organisms on lactose, sucrose or fructose was lower than 2000 kDa. Also, as described in the previous section, the extraction method may change the composition of the EPS obtained. Therefore, fortification of glycerol in sludge medium to produce EPS and the use of the different extraction methods to extract the EPS influenced the molecular weight and chemical composition of the EPS produced, which consequently affected the EPS bio-flocculation potential.

The flocculation activity of 90.5 ± 0.5 % was observed using 4.2 mg B-EPS/g kaolin extracted by centrifugation. This flocculation activity is higher than the FA (77.0 %) of B-EPS extracted by heating method while adding the same dose of B-EPS. The flocculation activity using the same dose of B-EPS (4.2 mg B-EPS/ g kaolin) extracted by EDTA (obtained by extrapolation) was 77.5%, which is lower than flocculation activity of B-EPS extracted by the centrifugation method, but practically equal to the flocculation activity of B-EPS extracted by heating method (77%). Therefore, it seems that heat treatment can cause some structural and functional changes to the EPS and thus affect their flocculation activity.

In case of EDTA extraction method, the complexation between EDTA molecules and EPS molecules decreases the flocculation functionality of the EPS, although the EDTA method is reported to extract the maximum amount of EPS. Thus, at the same concentration of BFs the FAs were found to be in the order; centrifugation method > heating method ≥ EDTA method.

Table 6-4 : Maximum flocculation activity exhibited by B-EPS produced in sludge fortified with 2 % v/v glycerol and extracted by different methods.

Sample	Kaolin conc. g/L	Concentration of EPS mg EPS/g kaolin	Volume of EPS solution (mL)	Time of fermentation (h)	FA (%)
Water (Control)	5	-	500	-	9.6 ± 0.9
Centrifugation	5	2.1	500	72	93.3 ± 0.5
Heating	5	4.7	500	72	92.0 ± 1.0
EDTA	5	23.1	500	72	95.3 ± 1.2

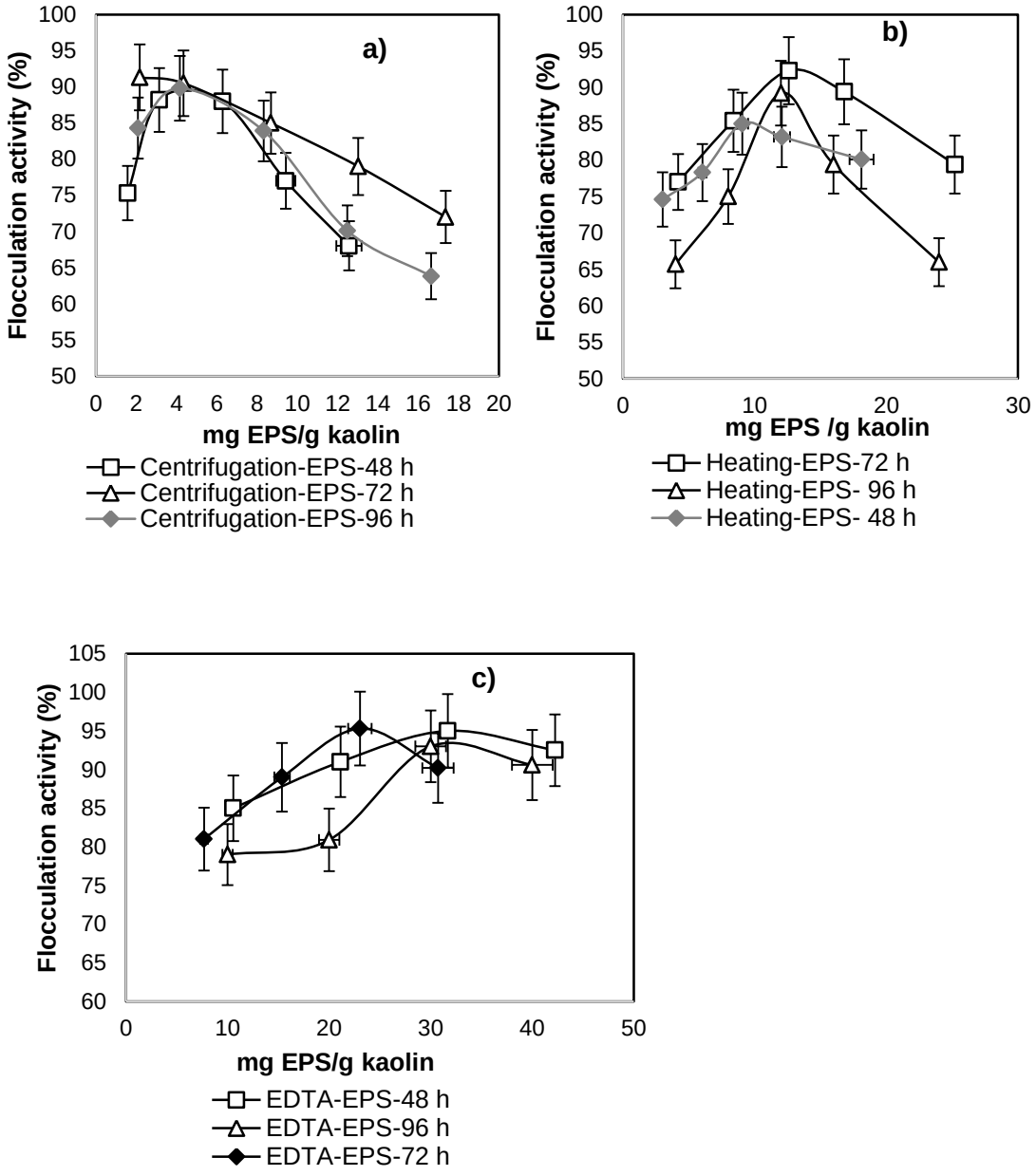

Figure 6-5 : Flocculation activity of B-EPS or BFs extracted by: a) centrifugation, b) heating and c) EDTA method.

6.5.5 EPS efficiency to remove heavy metals from primary treated wastewater

The removal of heavy metals present in primary treated wastewater at different contact time is presented in Figure 6-6. Two different concentrations of B-EPS (35 mg/L and 50 mg/L) extracted by centrifugation were employed. The EPS extracted by EDTA method was not used in this study because of the ability of EDTA to sequester metal ions can affect to find the exact capacity of metals to remove metals. EDTA usually binds to a metal cation through two amines and four carboxylates groups. Therefore, it will interfere with the capacity of EPS to remove heavy metals.

Using 35 mg/L of EPS dose, 85% Nickel (Ni) was removed in 120 min, whereas 68, 72 % removal of Aluminium (Al) and Iron (Fe) was observed in 60 and 30 min, respectively. Removal efficiencies of Zinc (Zn) and Copper (Cu) were 65% and 40% in 120 min and 720 min, respectively. In spite of the high electronegativity of Cu (1.9) comparing with Zn (1.6), Cu adsorption was slow. This can be explained by the competitive metal binding process to EPS. In the presence of many metals, the highest affinity towards Zn or Fe probably reduced the amount of Cu binding efficiency of EPS (Sara et al., 2011).

The contact time and the concentration of B-EPS affected the removal efficiency of heavy metals (Figures 6-6a and b). The removal efficiency of Ni and Al increased from 40 to 80 % and 60 to 70 %, respectively, as the contact time increased from 30 to 180 min. The maximum removal was attained within the first 180 min by using 35 mg/L of B-EPS. The rate of metal adsorption (or removal) was higher at the beginning due to the availability of a large number of the adsorbent sites. As these sites are exhausted, the uptake rate is controlled by the rate of mass transfer of the metal ions at the solid-liquid interface (media-adsorbent interface). These results are in agreement with those obtained by Dorris et al. (2000) who found an increase in removal efficiency of cadmium, copper, nickel and zinc with contact time. In present study, it is apparent that the metal removal efficiency increases as the concentration of EPS increased in case of Ni and Zn. The removal efficiency of nickel and zinc increases from 80 to 85% and 56 to 65%, respectively when the EPS concentration was increased from 35 to 50 mg/L. However, the removal capacity of Al, Cu and Fe decreases with increase in EPS concentration (Figure 6-6). As the EPS concentration increases the number of binding sites available for the metal ions also increases. After certain EPS concentration, the adsorption capacity was steady or decreasing with EPS concentration due to screening effect

between EPS molecules. The screening effect produced a hindrance to EPS active sites by an increase of EPS concentration. Higher concentration of adsorbent (EPS) could agglomerate the adsorbent (EPS) particles decreasing the surface to volume ratio (S/V) hence decreasing the adsorption capacity (Addagalla et al., 2009) (Figure 6-6).

An adsorption isotherm is commonly used to correlate the adsorption capacity of an adsorbent (EPS) and the concentration of metal ions. Many studies have investigated the efficiency of EPS or low cost adsorbent for heavy metal removal from wastewater (Chien et al., 2013; Rani et al., 2010, Shaheen et al., 2013; Sikder et al., 2014).

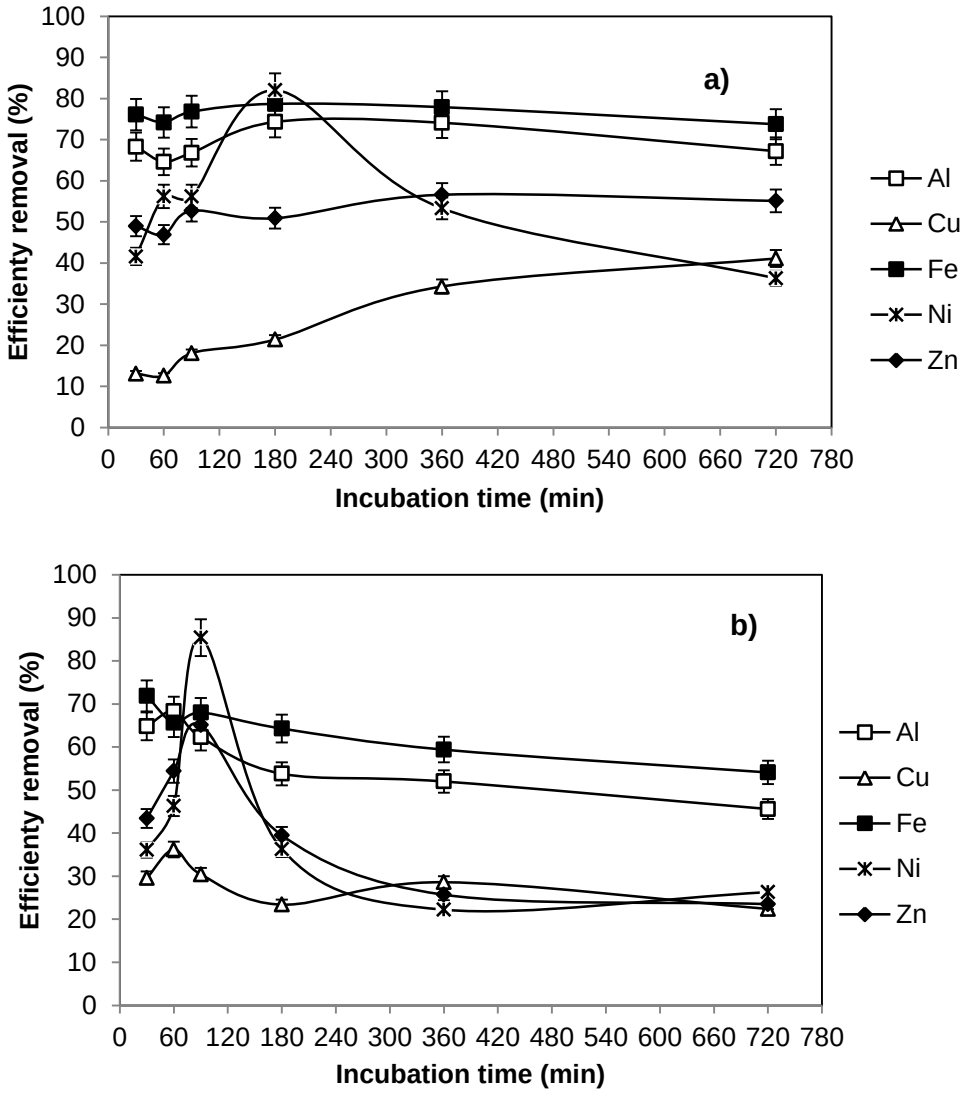

Figure 6-6: Heavy metal removal efficiency of B-EPS: (a) at 35 mg/L concentration. B-EPS was extracted by centrifugation; (b) at 50 mg/L concentration. B-EPS was extracted by centrifugation.

Table 6-5 presents a comparison of heavy metal adsorption capacity of EPS obtained from *Cloacibacterium* sp. with other reported bio-adsorbents.

Comparatively the EPS obtained from *Cloacibacterium* sp was more efficient to remove Ni (85%) and Zn (65%) compared to those produced by pseudomonas (Ni 28% and Zn 20%) (Chien et al., 2013) and egg shell as adsorbent in dyeing wastewater (62% in case of Zn) (Shaheen et al., 2013) (Table 6-5).

The copper removal efficiency was less effective in the present study as compared to other studies reported in literature. The removal efficiency for copper was only 36 % as compared to 40, 69, 73, 79% using *Sinorhizobium meliloti*, *Bacillus*, egg shell and chitosan respectively as adsorbent.

Conclusively, the EPS produced by *Cloacibacterium* could to be an alternative low cost adsorbent replacing the chemical polymers used in secondary sludge. The EPS from sludge produced in this study demonstrated a very good capacity to remove Nickel in comparison to secondary treatment and a similar efficiency of iron and aluminium elimination were obtained. However the secondary treatment revealed a better capacity to eliminate Cu and Zn (Table 6-5).

Table 6-5 : Comparison of heavy metal sorption capacity of EPS of *Cloacibacterium* sp with other reported biosorbents.

Metal	Adsorbent	Initial concentration (mg/L)	Final concentration (mg/L)	Removal capacity (%)	References
Ni	EPS of *Cloacibacterium sp*	48.0	7.0	85	This work
	EPS of *Pseudomonas EJ01*	125	85	28	Chien et al., (2013)
	Primary treatment (using chemical polymers, CUQ)	48.0	18.0		This work
	Secondary treatment	18.0	8.1	83	
Fe	EPS of *Cloacibacterium sp*	14.2	4.0	71	This work
	Primary treatment (using chemical polymers, CUQ)	14.2	3.8		This work
	Secondary treatment	3.8	3.0	78	
Zn	EPS of *Cloacibacterium sp*	17.4	5.5	65	This work
	Primary treatment (using chemical polymers,CUQ)	17.4	5.4		This work
	Secondary treatment	5.4	2.7	84	
	EPS of *Pseudomonas* EJ01	11.0	8.8	20	Chien et al., (2013)
	Egg Shell	18.0	6.84	62	Shaheen et al., (2013)

Al	EPS of *Clocibacterium sp*	26.9	7.2	73	This work
	Primary treatment (using chemical polymers, CUQ)	26.9	9.1		This work
	Secondary treatment	9.1	7.0	74	
Cu	EPS of *Clocibacterium sp*	26.0	16.5	36	This work
	Primary treatment (using chemical polymers,CUQ)	26.0	3.9		This work
	Secondary treatment	3.9	2.8	63	
	Bassillus sp	2.88	1.6	44	Rani et al., (2010)
	Egg Shell	553	150	73	Shaheen et al., (2013)
	Chitosan	-	-	79	Sikder et al., (2014)
	EPS of Sinorhizobium meliloti	-	-	40	Hou et al., 2013

The two most common adsorption isotherms are:

Freundlich isotherm $Qe = k\ Ce\ 1/n$ ---------- (1)

Langmuir isotherm $Qe = Q_m bCe / (1 + bCe)$ -------- (2)

Where Qe is the adsorption capacity (mg of metal adsorbed/g-adsorbent or EPS) and Ce is the equilibrium concentration of the adsorbed species (metal ion).

Both isotherms have two parameters that are specific to the adsorbent and adsorbed species. The Langmuir parameter Q_m represents the maximum adsorption capacity of the adsorbent (EPS) (Sugihhartati et al., 2013).

Table 6-6 summarizes the best-fitted Freundlich parameters, k and n, and the best-fitted Langmuir parameters, Qm and b, for the adsorption of five metal species by EPS.

Results show that the Freundlich isotherm correlated satisfactorily with the adsorption data of Al, Fe and Zn (R^2 ranging 0.98-0.99), but poorly for Cu and Ni (R^2 ranging 0.84-0.87). On the other hand, the Langmuir isotherm correlated satisfactorily with those of Al, Fe and Ni (R^2 ranging 0.96–0.99), but poorly for Cu (R^2 of 0.79) and Zn (R^2 of 0.47). These results are different from those obtained by Liu and Fang, (2002) using EPS extracted from activated sludge. The Freundlich isotherm correlated with the adsorption data of Ni and Cu (R^2 ranging 0.89–0.97), whereas the Langmuir isotherm correlated with Zn (R^2 of 0.96).

The complex and diverse EPS properties make it usually difficult to understand the adsorption behaviors of EPS or microbial biomass. According to the results obtained, the adsorption capacity shows that the adsorbent (EPS) has a higher affinity towards nickel, aluminum and iron than copper and zinc (Fig 6-6 and Table 6-6). This is most likely due to differences in EPS structure and chemical composition. As described previously high protein and polysaccharide content of EPS could have an important role for metals removal. To investigate into the structural changes of EPS after binding with metals, FTIR spectroscopy has been commonly used (Li et al., 2014).

The previous study (Ruan et al., 2013) conducted Fourier Transform Infrared Spectroscopy (FTIR) analysis and revealed that the main groups located on proteins moiety of the EPS were C=O, C–N, –OH, and amide groups, which could react and remove heavy metals by electrostatic interaction. Furthermore, the C–O–C group on polysaccharides, –OH assigned to alcohols or carboxyl, C=O belonging to carboxyl or phenolic alcohols, and the sulfur- and phosphorus-containing groups were also involved in the complexation reaction.

Table 6-6: Freundlich and Langmiur isotherm constants.

Metal	B-EPS	Freundlich isotherm			Langmuir isotherm		
		K_F (mg/g EPS)	n	R^2	Q_m (mg/g EPS)	b (L/mg)	R^2
Al	35	$1.1\ 10^{-3}$	-2.31	0.99	2.0	-11.3	0.99
Al	50	$7.1\ 10^{-4}$	-0.20	0.98	1.0	-9.2	0.98
Cu	35	$5.3\ 10^{-10}$	-0.26	0.86	0.07	-23.2	0.86
Cu	50	$1.0\ 10^{-10}$	-0.24	0.84	0.06	-22.7	0.79
Fe	35	$1.4\ 10^{-3}$	-1.26	0.95	3.0	-3.7	0.96
Fe	50	$11.5\ 10^{-3}$	-1.51	0.98	2.0	-4.3	0.98
Ni	35	$2.7\ 10^{-8}$	-1.14	0.85	0.01	-1012.3	0.97
Ni	50	$5.4\ 10^{-7}$	-2.56	0.87	0.007	-2672.6	0.99
Zn	35	$1.7\ 10^{-6}$	-0.81	0.96	0.09	-28.0	0.47
Zn	50	$1.0\ 10^{-8}$	-0.35	0.98	0.04	-24.7	0.56

6.6 Conclusion

This work describes the production of biopolymer by *Cloacibacterium normanence* grown in activated sludge supplemented with 2%(v/v) of crude glycerol. The highest concentration of EPS was obtained using EDTA compared to centrifugation or heating method. However, 11 times higher amount of EDTA extracted EPS was required to approximately give similar flocculation activity as given by the EPS extracted by centrifugation method.

High metal removal efficiency was observed using 35 mg/L of B-EPS extracted by centrifugation method. The flocculating capacity and metal removal ability make the EPS produced by *Cloacibacterium* sp. a good alternative as bioflocculant and low cost adsorbent.

6.7 Acknowledgements

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CHAPITRE 7

Fourier transform infrared spectroscopy and liquid chromatography-mass spectrometry study of extracellular polymer substances produced on secondary sludge fortified with crude glycerol.

Ce chapitre est constitué d'un article publié :

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7 Fourier transform infrared spectroscopy And Liquid chromatography-mass spectrometry study of extracellular polymer substances produced on secondary sludge fortified with crude glycerol

7.1 Abstract

This study was conducted to characterize the extracellular polymeric substances (EPS) produced by growing *Cloacibacterium normanense* in wastewater sludge alone and fortified crude glycerol. The EPS highest concentration of 17.5 g/L was produced using 25 g/L sludge suspended solids supplemented with 25 g/L of crude glycerol. Galactose and Glucose was the main compound of EPS produced with or without crude glycerol, respectively. EPS FTIR spectra revealed a variation in the different functional groups such as amines, carboxyl, and hydroxyl groups for EPS produced. Different functional groups were observed in the EPS produced with or without glycerol. The EPS exhibited kaolin flocculation activity up to 95% and was stable at high temperature. The high viscosity bioflocculant properties of EPS make it suitable for potential industrial applications.

7.2 Keywords

Cloacibacterium normanense, EPS, Activated sludge, crude glycerol, FTIR, Rheology, Bioflocculation

7.3 Introduction

Microbial polysaccharides have been demonstrated with a vast range of functional properties and applications including food products, pharmaceuticals, bioemulsifiers (Xie et al., 2013), bioflocculants (Sathiyanarayanan et al., 2013), chemical products (Wang et al., 2008) and the biosorption of heavy metals (Mohamad et al., 2012). The chemical compositions of EPSs produced by different bacterial strains are very diverse. The main constituents of EPS are sugars such as galactose, glucose and rhamnose. Acetate and puryvate are characteristic substituents. Whilst the structures are very complex, consist of branched repeating units and different linkages that exist between the monosaccharides (Klock et al., 2007).

Depending on their specific composition, EPSs have different cellular functions, including accumulation of nutrients, diffusion barrier for toxins and heavy metals, cell motility, attachment to surfaces, protection against desiccation. Thus, several researchers have discussed recent advancements in the understanding of the structure–function relationships, i.e., to relate EPSs structure with their properties (bioflocculant, bioemulsfiers) in order to improve EPSs synthesis and applications (Klock et al., 2007; Pepi et al., 2005). Therefore, there is a need to develop an understanding of structure–function relationships to relate EPS structure, chemical composition and molecular weight with bioflocculation properties, especially for the EPS produced in wastewater sludge by new isolated bacterial strain.

In this work, a novel microbial biopolymer produced by *Cloacibacterium normanense* is described. Along with the fermentation process for the extracellular polysaccharides (EPSs) production from sludge supplemented with crude glycerol, a preliminary polymer characterization in terms of its chemical composition and structure is presented. Glycosyl composition analysis was performed by HPLC assigning the different polysaccharide monomers. EPS was also characterized by quantification of the content of proteins and carbohydrates, its structure by Fourier transform infrared spectroscopy (FT-IR), and its morphology and surface attachment by scanning electron microscopy (SEM).

7.4 Methods

7.4.1 EPS production

Cloacibacterium normanense was grown on 25 g/L suspended solids (SS) of activated sludge collected from bio-filtration unit of Communauté Urbaine du Québec (Municipal wastewater treatment plant, CUQ, Québec, Canada). For inoculum preparation, *Cloacibacterium normanense (*NK6, accession number KF675204) was inoculated in Tryptic soy broth (TSB) and incubated at 30° C, 180 rpm for 24 h. The pre-culture was added (5% v/v) to each experimental flask containing 150 mL sterilized sludge (25 g/L SS, pH 7). Crude glycerol 25 g/L was added at 24h to obtain the desired C/N concentration ratio 25. The C/N ratio was calculated taking into account the initial concentration of nitrogen in sludge and carbon content of glycerol. The flasks were then incubated in a shaker at 180 rpm and 30°C for 96 h. The control experiments were also performed without adding crude glycerol. During the experiments, samples of the broth were collected every 24 h to determine EPS concentration, glycerol concentration and cell population.

The crude glycerol solution also contains other components (soap and methanol), which can act as carbon source. Therefore, consumption of soap and methanol was determined during the fermentation (Hu et al. 2012).

7.4.2 EPS extraction and dry weight

The centrifugation method was used to extract S-EPS (slime EPS) and C-EPS (capsular EPS). Broth EPS (B-EPS) consists of both S-EPS and C-EPS, or the fermented broth is named as B-EPS. The fermented broth was centrifuged at 9000 g, 4 °C for 20 min to obtain supernatant (containing S-EPS). The biomass pellet was re-suspended in deionized water equal to the initial volume and then heated at 60°C for 20 min followed by centrifugation at 9000 g, 4 °C for 20 min to extract capsular EPS (Bala Subramanian et al., 2010).

For measuring dry weight, one volume of supernatant obtained after centrifugation (crude S-EPS) was mixed with two volumes of chilled ethanol (95% v/v) and precipitated at -20°C overnight. After precipitation, the sample was centrifuged at 6000 g for 15 min to obtain the precipitated pellets. The pellet was dried at 60°C until constant weight.

The EPS concentration was estimated by the following formula: $[\mathrm{EPS}](\mathrm{g/L}) = \frac{W2-\mathrm{W1}}{V}$

Where, w_1: Initial dry weight of the empty aluminium dish without a sample (g)

w_2: Dry weight of the aluminum dish with dried sample (g)

v: volume of the sample (L)

The total EPS (B-EPS) contained in the broth was calculated as sum of S-EPS and C-EPS. All the measurements were carried out in triplicates and the average was presented

7.4.3 Analytical methods

Glycerol concentration in the cell-free supernatant, methanol and soap content was determined according to Hu et al. (2010). The growth was measured based on the dry weight per volume of the culture. The cell dry weight (CDW) or biomass was determined by centrifugation (8000g, 4◦C, 15 min) and after the C-EPS extraction, followed by overnight drying the sample to a constant weight in an oven at 60◦C. The cell concentration of all the samples (diluted with saline solution), was measured as CFU employing standard agar-plate technique. Total nitrogen and organic carbon in the samples collected at various times of fermentation were measured by the CHNS analyzer (Shimadzu VCPH).

7.4.4 EPS characterization

7.4.4.1 Chemical composition of the EPS

Glycosyl composition analysis was performed by combining Liquid Chromatography/Mass Spectrometry (LC/MS) with Hypersil Gold column (100*2.1 mm ID), using 85% Water, 0.1 % formic acid (Phase A)/ 15% acetonitrile (CAN), 0.1 % formic acid (Phase B), as eluent, at a flow rate of 0.4 mL/min and a temperature of 30 °C (Freitas et al. 2009). EPS sample 20 µl was used for the identification and quantification of acyl group and monosaccharides present in the purified EPS (the ethanol precipitated EPS). The monosaccharides were identified by their retention times in comparison to standards.

7.4.4.2 FT-IR spectroscopy

Precipitated EPS were collected by centrifugation at 4000 g, 30 min at 4° C and dried at 60° C. The purified and dry S-EPS (0.1 – 0.2 mg) and 100 mg of potassium bromide (KBr) were mixed and pressed in a die (at five tons and one minute) to form a pellet. Afterwards, the pellet was immediately put into the sample holder and FT-IR spectra were recorded. The transmission FT-IR spectra were obtained using a Perkin Elmer 2000 FT-IR spectrometer. FT-IR scanning was conducted in ambient conditions. The resolution was set to 4 cm^{-1} and the operating range was 400 to 4000 cm^{-1} (Madejová, 2003).

7.4.5 EPS properties

7.4.5.1 Rheology

To investigate the stability of the EPS at different temperatures, the crude EPS solution was incubated for 10 min at 80 °C to 200 °C in an incubator. The viscosity of the above EPS solution (20 mL) was measured using a ULA S 34 spindle (Digital Viscometer, DV- II+ Pro, Brookfield), at 60 rpm and room temperature.

7.4.5.2 Enzymatic digestions test

Proteinase K was used as the proteolytic enzyme and 80 units of the enzyme were added to 200 mL sample. Proteinase K is a non specific enzyme that hydrolyses proteins at a number of cleavage sites. Cellulase (β glucosidase) was used as an extracellular polysaccharide degrading enzyme and 150 active units were added to 200 mL sample of B-EPS. Cellulase hydrolyses the polysaccharides present in EPS (Xie et al., 2010). Enzymes were added to each 200-mL sample of the fermented broth collected at 48h and the enzymatic digestion was conducted for 36h at 30° C. The samples, each 20 mL, were collected at every 12h to measure ζ-potential, viscosity and turbidity index. All the measurements were carried out in duplicates and the average result was presented.

7.4.5.3 Scanning electron microscopy (SEM)

Samples of enzymatic digestion test were collected 6 hr after enzyme addition. Broth sample of 10 µl was taken on the glass slide and the cells were fixed with 3% glutaraldehyde. After fixing, the cells were washed with ethanol solution of different concentration (30–100% v/v) and air dried between each wash to completely remove the water adhered to the cells. After final washing, cells were subjected to overnight drying. The dried samples were mounted on conventional

12.7 mm or 25.4 mm diameter aluminum stubs using double sided adhesive carbon discs and coated with gold film to a thickness of 10–20 nm using a sputter coater (SPI™ sputter coater module) to examine their morphology under scanning electron microscopy (Model: Carl Zeiss EVO®50 smart SEM) (Ajila and PrasadaRao, 2008).

7.4.5.4 Zeta potential

The charge of B-EPS after enzymatic digestion was determined by adding 50-1000 µL of individual EPS sample to 100 mL of deoinzed water. Characterization of charge (zeta potential) was implemented using Zetaphoremeter (Zetaphoremeter IV, Zetacompact Z8000, CAD Instrumentation, France) with the application of the Smoluckowski equation. Surface charge of the wastewater sludge was also measured. The zeta potential values were obtained from an average of around 10 measurements, the average values are presented with its half-width confidence interval at 95% confidence level.

7.4.5.5 Measurement of Turbidity index

Kaolin clay was used as a test material to measure the flocculation activity or the turbidity index of EPS samples digested by enzymes. Flocculation activity or turbidity index of EPS was carried out through the jar test method (Bala Subramanian et al., 2010). Kaolin with concentration of 5g/L was suspended in distilled water, 150 mg/L of Ca^{2+} was added to the kaolin suspension and pH was adjusted to 7.5 after addition of $Ca^{2+.}$ The samples collected at different times of (6 h, 12 h, 24 h and 36 h) after adding enzymes, were added in different volumes (corresponding to desired concentrations of EPS, which was calculated through dry weight and the volume of EPS solution required) to kaolin suspension and rapidly mixed at 100 rpm for an initial 5 min then slowly mixed for an additional 30 min at 70 rpm. After 35 min of mixing, samples were transferred to a 500 mL cylinder and allowed to settle for 1 h. The supernatants of the settled samples were collected to measure the turbidity using turbidimeter (Micro 100 turbidimeter, Scientific Inc.). The flocculation activity (FA) was determined using the formula [100*(B-A)/B]; where ‘A’ is the turbidity of the sample (treated with EPS; Slime, Capsular or Broth-EPS) and ‘B’ is the turbidity of the control (in which equal volume of EPS solution was replaced with distilled water).

7.4.6 Statistical analysis

All analysis reported in this manuscript was performed in triplicate, and the results are presented as the mean values. The results were analysed by analysis of variance (ANOVA), using Excel's Analysis ToolPak**.**

7.5 Results and discussion

7.5.1 EPS production

The variation of various EPS concentration and C/N ratio (with and without adding crude glycerol) during the fermentation was summarised in Table 7-1. The C/N ratio was calculated as the concentration ratio (i.e. concentration of organic carbon/concentration of total nitrogen).

The crude glycerol was added at 24 h fermentation as an extra carbon source to aid EPS production by *Cloacibacterium normanense*, which increased the C/N ratio to 25 compared to 11 in the control (Table 7-1). The maximum EPS production (about 17.5 g/L) was observed at 72 h of fermentation with glycerol fortification (C/N 22, Table 7-1, Figure 7-1), whereas EPS concentration of 13.3 g/L was observed at 48 h (C/N reached 14) fermentation time without glycerol fortification. Without glycerol, the C/N ratio initially increased (0 to 48 h) and was due to higher consumption of nitrogen than carbon, both required for growth (Figure 7-1). Further, decrease in the C/N ratio after 48 h could be due to higher consumption of carbon required for maintenance of cells, because EPS did not increase during this time. In this sense, C/N 22 is considered to be the most favourable for EPS production by *Cloaciacterium* sp. However, Miqueleto et al. (2010) have reported that C/N 10 was recommended for high EPS production in an anaerobic sequencing batch biofilm reactor. In the study of Liu et al. (2011), the C/N ratio was 12 for maximum EPS concentration (8.90 g/L) produced by *Zunongwangia profunda* SM-A87. Nevertheless, for most EPS-synthesizing microorganisms, the highest polymer productivities are usually achieved at high C/N ratio that is strain dependent.

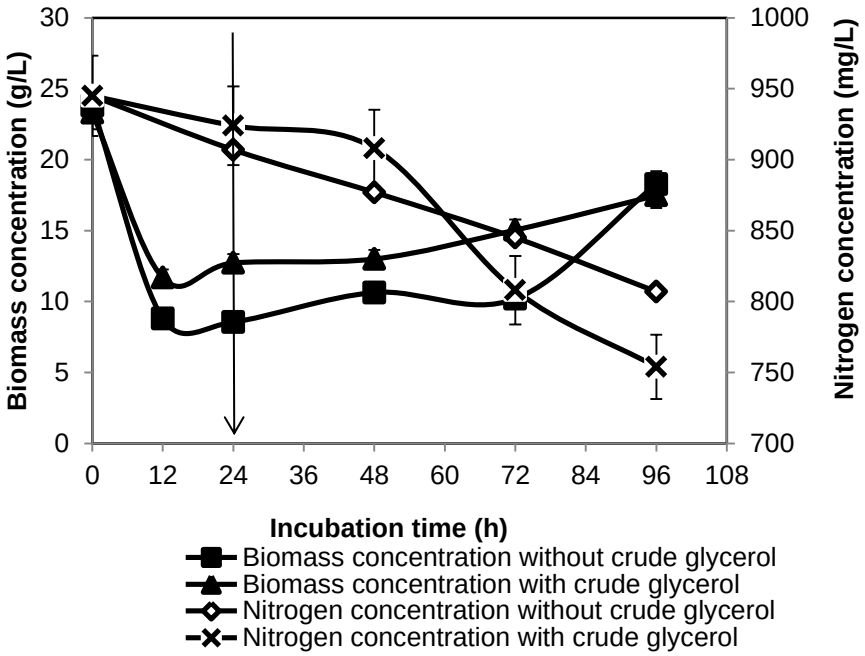

Figure 7-1: Variation of biomass and nitrogen concentration consumption during fermentation with and without addition of crude glycerol as carbon source. (Arrow represents the addition of glycerol at this point).

With glycerol addition, EPS concentration steadily increased until 72 h, whereas C/N ratio first increased to 25 (at 24 h due to the addition of extra glycerol) and then decreased to 22 at 72 h. Most of the added glycerol was consumed at 48 h (residual glycerol 2.0 g/L, Figure 7-2); however, EPS still substantially increased from 48 to 72 h. Therefore, the microbe used carbon from sludge decreasing the C/N ratio (even in spite of nitrogen decrease, Table 7-1).

Table 7-1 : Production of biomass and EPS produced with and without crude glycerol fortification during fermentation.

Experiments	Incubation time (h)	0	24	48	72	96
with crude glycerol addition	**C/N ratio**	3	25	21	22	16
	S-EPS (g/L)	1.4 ± 0.3	4.6± 0.3	13.9 ± 0.4	15.6 ± 0.5	4.3± 0.3
	B-EPS (g/L)	1.8 ± 0.3	5.2 ± 0.2	14.9± 0.2	17.5± 0.4	15.4± 0.6
	EPS/biomass ratio (g/g)	0.08 ± 0.01	0.4 ± 0.2	1.1 ± 0.3	1.1 ± 0.1	0.9 ± 0.4
Experiments	**C/N ratio**	3	11	14	12	11
without crude glycerol addition	**S-EPS (g/L)**	1.5 ± 0.3	3.9 ± 0.3	12.9 ± 0.5	12.2 ± 0.3	10.4± 0.2
	B-EPS (g/L)	1.9± 0.3	4.7± 0.3	13.3± 0.2	12.5± 0.1	10.8± 0.4
	EPS/biomass ratio (g/g)	0.08 ± 0.3	0.55 ± 0.2	1.25 ± 0.3	1.27± 0.1	0.60 ± 0.4

The CFU (colony forming units) count increased during the first 48 hours of fermentation (with the addition of glycerol) and nitrogen used was 37 mg/L, whereas between 48 and 72 h, the increase in CFU was relatively same but nitrogen consumed was almost 100 mg/L (Figure 7-2, Table 7-1). The excess consumption of nitrogen (48-72 h) was routed toward formation of other unknown metabolites (Lee et al., 2004). In this context, Duenas et al. (2003) reported that EPS production from *Pediococcus damnosus* was mainly enhanced by an increase in glucose concentration, but not by an increase in nitrogen concentration. In addition, the crude glycerol is source of soap and methanol, which can be used as substrate for the EPS production. Figure 7-2 shows the decrease of crude glycerol content during the fermentation concomitant with the growth of biomass on semi log scale (X0 is the initial CFU and Xt is CFU at time 't').

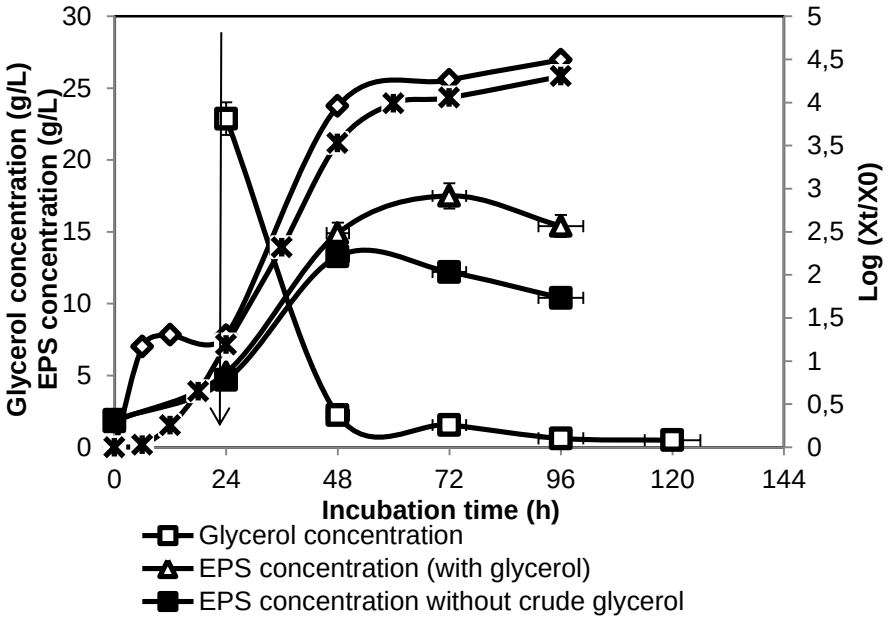

Figure 7-2: Variation of glycerol, cell and B-EPS concentration during fermentation. Xt and X0 are CFU at time t and t=0, respectively. (Arrow represents the addition of glycerol at this point)

Figure 7-3 illustrates the decrease of methanol and soap concentration during the fermentation process indicating that soap and methanol are degraded by the strain for growth and to generate EPS. Kumar et al. (2007) have also reported that non sugar sources like methanol could contribute to produce microbial EPSs.

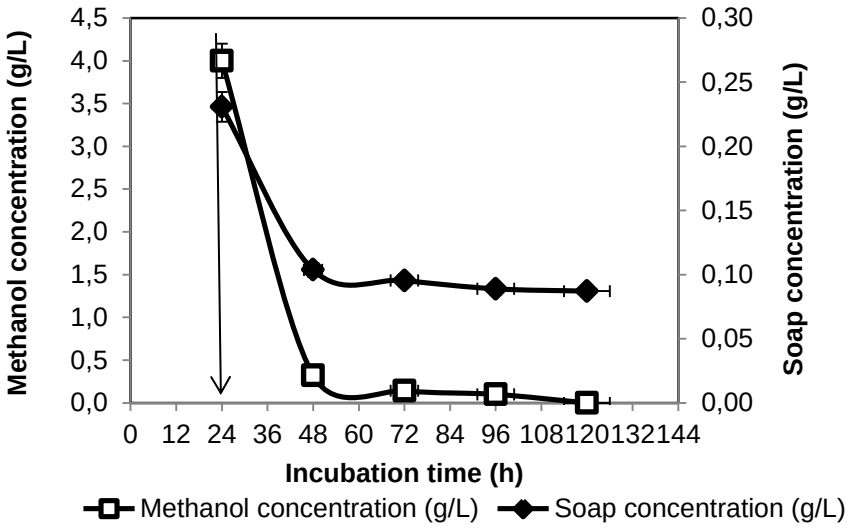

Figure 7-3 : Soap and methanol concentration profile during fermentation. (Arrow represents the addition of glycerol at this point).

The carbon source was studied for the EPS and biomass production of *Cloacibacterium normanense*, determining the relative significance of two variables (crude glycerol added and sludge media without crude glycerol addition), using ANOVA Excel's Analysis ToolPak. The ANOVA analysis for EPS production and biomass is shown in Table 7-2. The value of regression coefficient R^2 was 0.9890 for biomass and 0.9723 for EPS production. The p-values of the models were 0.0002 and 1.3632E-05, in case of crude glycerol fortification, 4.27E-06 and 3.4637E-05 without crude glycerol for biomass and EPS production, respectively, indicating that the models were significant. Usually, a model term is considered to be significant when its value of ‘‘p-value’’ is less than 0.05 (Zhou et al., 2010)

Table 7-2: Identification of significant variables for EPS production and Biomass of *Cloacibacterium normanense* using ANOVA of Excel.

Variables	Biomass[a]			EPS production[b]		
	coefficient estimate	F value	p value	coefficient estimate	F value	p value
Sludge + crude glycerol	0.93	0.07	0.0002[c]	1.01	0.013	1.3632E-05[c]
Sludge without crude glycerol	0.99	0.01	4.2769E-06[c]	1.0087	0.016	3.4637E-05[c]

[a] R^2=0.9890;
[b] R^2=0.9723;
[c] Model terms are significant.

7.5.2 EPS physico-chemical characterization

The B-EPS obtained in the experiments were analysed for their sugar and acyl group composition (Figure 7-4). Five main constituent sugar residues were identified by the glycosyl composition analysis, namely, glucose, galactose, lactose, sucrose and xylose. Galactose was the most abundant monosaccharide, accounting for 67 mol% of the total carbohydrate content of the B-EPS. Glucose represented 13 mol%, but lactose, sucrose and xylose was present in only minor amounts i.e. 3, 8 and 9 mol%, respectively.

EPS synthesized without the addition of crude glycerol was distinct from the EPS produced with glycerol addition. The EPS without glycerol addition was characterized by only one type of monosaccharide, i.e. eighty mol% of glucose. The appearance of galactose and lactose monosaccharide after adding crude glycerol was demonstrated. This can be explained by the fact that the strain *Cloacibacterium normanense* converts glucose to galactose or/and glucose to lactose in the presence of glycerol. We can, therefore, anticipate that the addition of glycerol has influenced the level of activation of the enzymes necessary for the different pathways and involved in the synthesis and assembling of the sugar nucleotides, thus resulting in the production of polymers with such a diverse sugar composition. Similar observations have been recorded by Yolunda et al. (2005). Polysaccharides containing galactose are produced by several bacteria, such as *Pseudomona*s, *Lactobacillus* and *Streptococcus* (Freitas et al., 2009). The data obtained in our study are agreed with the findings reported by Freitas et al., (2009). They demonstrated high galactose content of the EPS produced by *P. oleovorans* grown on pure glycerol.

The present results have also shown that the EPS contained (with or without glycerol addition) non-saccharide components, namely, acyl groups. Two different acyl groups were identified in small quantity. Pyruvate (0.014 wt%) appeared after addition of glycerol; however, succinate (0.057wt.%) was present in the absence of glycerol (Figure 7-4D). These components are frequently present in microbial EPS and notably influence polymer's properties, namely, solubility and rheology (Rinaudo, 2004). According to Freitas et al. (2009), three acyl groups were identified using media containing pure glycerol, i.e. pyruvate (3.35 wt%), succinate (1.04 wt%) and acetate (0.38 wt%).

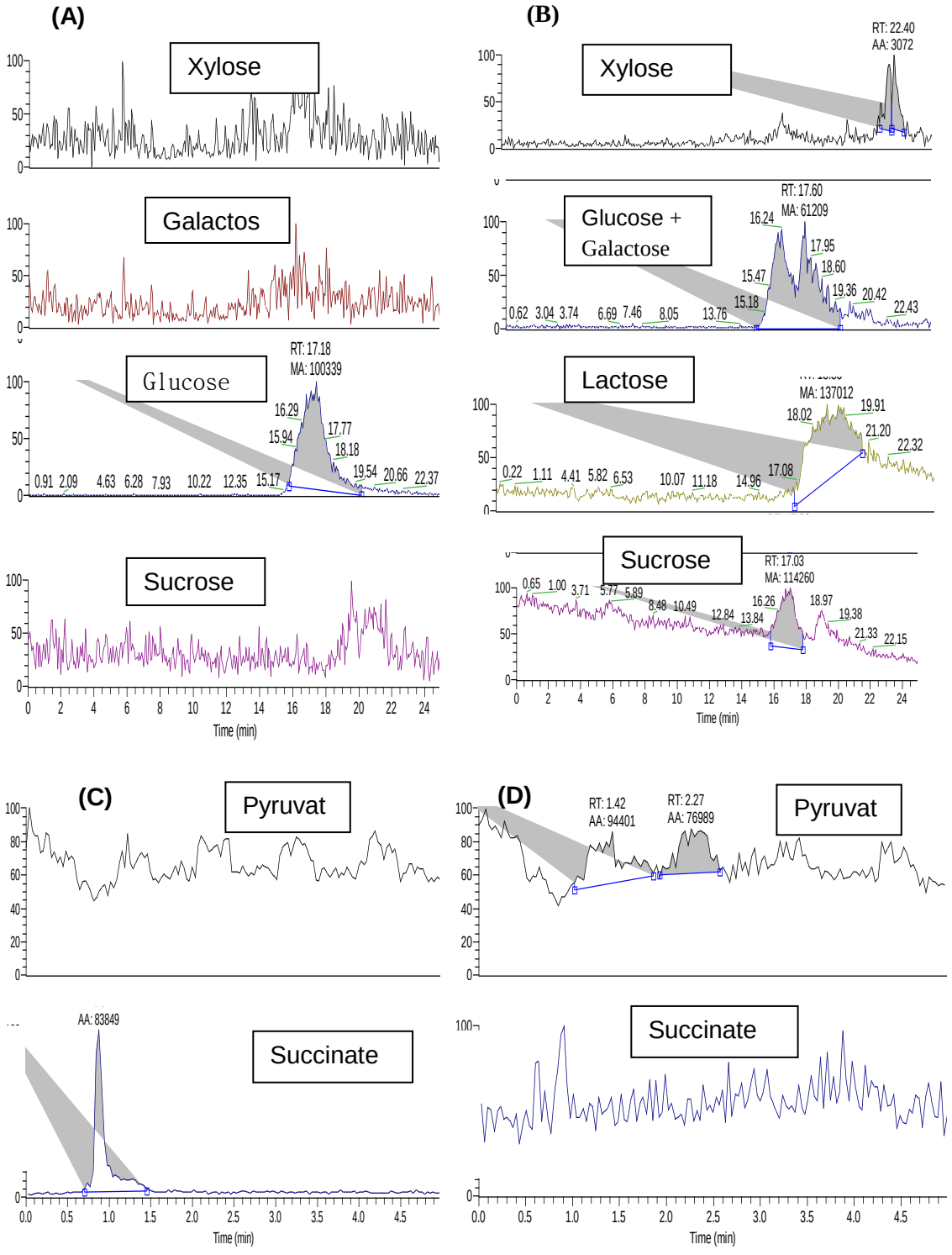

Figure 7-4: LC/MS analysis of the EPS produced by *Cloacibacterium normanense* : (A) Monosaccharide analysis without crude glycerol, (B) Monosaccharide analysis with crude glycerol, (C) Acyl group analysis without crude glycerol, (D) Acyl group analysis with crude glycerol.

7.5.3 FT-IR spectroscopy

The FTIR analysis of EPS (produced with or without crude glycerol) was presented in Figure 7-5a and b. The FT-IR spectrum of the exopolysaccharide is in agreement with the chemical analysis (LC/MS) described above. The main functional groups are hydroxyl, carboxyl, acyl and amino, which corresponds mostly to the presence of carbohydrates and proteins in the EPS.

In the two cases (with and without crude glycerol addition), it revealed a broad stretched peak at 3455 cm^{-1} (range 3600–3200 cm^{-1}), corresponding to the hydroxyl group. A weak absorption at 2925 cm^{-1} (range 300-2500 cm^{-1}) was assigned to an asymmetrical C H stretching vibration of the aliphatic CH_2 group, which represents the presence of organic substances like sugars and proteins. The amide II band at 1729 cm^{-1} originates from N-H bonding and C-N stretching vibrations in -CO-NH of proteins. Presence of an asymmetric stretching peak or vibration at 1643 cm^{-1} may correspond to the ring stretching of galactose as the LC/MS analysis indicated in the last section. Another peak at 1404 or 1455 cm^{-1} could be attributed to the symmetric stretching of the COO^- group. The absorption peaks (1271 or 1233 cm^{-1}) ranging from (1500–1200 cm^{-1}) were designated to C-O-C and C=O, which indicates the occurrence of carbohydrates. A peak at 1058 cm^{-1} (1000–1125 cm^{-1} range) may be attributed to O-acetyl ester linkage bond of uronic acid. Absorption peak approximately in the range of 781–522 cm^{-1} corresponded to stretching of alkyl-halides. These findings are in agreement with the studies of (Kavita et al., 2014; Freitas et al., 2009; Nwodo & Okoh, 2012).

However, the EPS produced with sludge supplemented with crude glycerol differs from the EPS produced without crude glycerol by having additional peaks at different regions. IR spectra of EPS produced in the case of crude glycerol addition (Figure 7-5a) show particular bands, which do not appear for EPS produced in the absence of crude glycerol (Figure 7-5b). Three bands around 2855cm^{-1} (symmetric stretching vibration of CH_2, C=O stretching), 1678 cm^{-1} (deformation vibration of N-H or Amide I and C–N stretching) and 1578 cm^{-1} (deformation vibration of N-H or Amide II) were appeared when crude glycerol was added to the production medium. These characteristic bands can be attributed to protein and polysaccharide functional groups. By the presence of these peaks, FTIR of EPS (with crude glycerol) demonstrates relatively higher quantity of hydroxyl (-OH), amide (-CO-NH), carboxyl (-COO-), and primary amine ($-NH_2$) groups compared with those observed without crude glycerol. The abundance of these groups in EPS produced with supplementation of crude glycerol in the medium may contribute to the quiet difference in flocculation activity (93.4%) compared to flocculation activity (90.2%) of EPS produced without crude glycerol fortification. Further, the excess presence of carboxyl, hydroxyl,

and amine groups are very important for bioflocculation, providing surface charges, which serve as the binding sites for suspended particles causing aggregation or floc formation as also discussed in the study of Li et al. (2008) and Kavita et al. (2014). The difference in the occurrence, position and frequency of these groups could affect the flocculation activity and that could be the reason of high flocculation activity of the EPS obtained in this study (with or without fortification of crude glycerol) compared to those reported by other researchers.

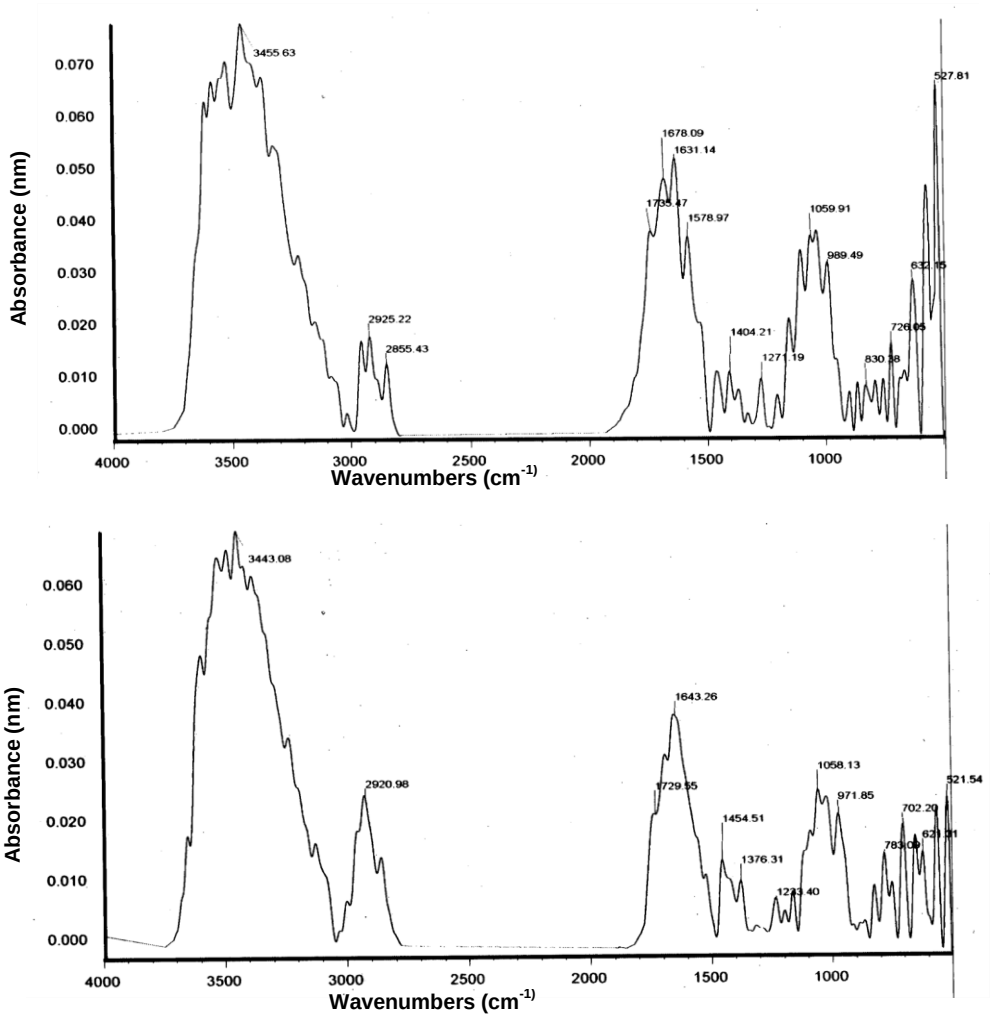

Figure 7-5 : IR spectra of the S-EPS produced in sludge by pure culture of *Cloacibacterium normanense* grown: a) with crude glycerol and b) without crude glycerol addition.

The flocculation activity of EPS synthesised by *Pseudomonas* SM9913 was 49.3% (Li et al., 2008) and 40% of the EPS produced by *Oceanobacillus iheyensis* (Kavita et al., 2014). These groups (C-O-C; O-H; C=O…etc.) were either absent or present in insufficient quantity in EPSs as described in Table 3. Table 3 recaps the functional groups corresponding to bands observed in the IR spectra of B-EPS produced by the strain with or without glycerol fortification of sludge as well as those available in the literature.

First, we should explain the interaction between the cations and the EPS functional groups and their effect on floc formation and other parameters. Several researchers (Sobeck and Higgins, 2002) have performed experiments that support the DLVO theory, as London force, for the mechanism of bioflocculation. London forces are present between all chemical groups. The source of this type of interaction is the spontaneous formation of transient dipoles due to fluctuations in the electron distribution within the molecule. This temporary dipole polarise the molecule and then creating dipolar attraction forces. It represents the main cohesive force between hydrocarbon chains.

In the presence of stretching vibration of C=O band, the carboxyl ion (−COO−) prefers to accept the H+ of the solution and the −COOH group can be formed, which destroys the ionic bonds formed between Ca^{2+} (cation used in flocculation) and the bioflocculant (EPS). Consequently, leads to the disintegration of the flocs. These results were confirmed by (Li et al., 2008). They proposed that a large number of carboxyl groups of EPS can also serve as binding sites for divalent cations (Ca^{2+}). When bridged by cations, the negatively charged EPS combines the flocs together. This enables EPS to serve a key role in the flocculation of activated sludge. The 1678 cm^{-1} band appeared in the IR spectrum of EPS produced in the presence of crude glycerol, whereas it was absent in the IR spectrum of EPS without glycerol (Figure 7-5a and b). This band represents the carboxyl group, which could bind to cations and form flocs by bridging mechanism. In relation to the carboxyl group, the EPS produced by *Pseudoalteromonas* sp. SM991 (Li et al., 2008) exhibited low FA of 37.7% compared to 93.4% FA of EPS produced in this study with crude glycerol supplementation. The low activity can be related to a relatively smaller strength (15.4%) of the band at 1678 cm^{-1} in EPS produced by Li et al. than 40% strength in EPS obtained in the present study (with crude glycerol).

The bands (1678, 1630-1660, and 1550-1580) are characteristic of C–N stretching and deformation vibration of N-H (Amide II). Hydrogen bonds may contribute to the active reaction between water molecules and amide group (N-H) as present frequently in proteins of EPS (with crude glycerol). The researchers also suggested that higher content of protein moiety in EPS, as

indicated by the presence of peptide bond in the IR spectrum of EPS obtained with supplementation of crude glycerol (corresponding to wave numbers 1678, 1630-1660, and 1550-1580 in Table 7-3 and Figure 7-5a), could bring more negatively charged amino groups, thus strengthening electrostatic interaction between cations. This plays an important role in flocculation. Further, the large band of Amide II (wave number 1550-1580, Table 7-3) is present in the EPS (produced with glycerol) and absent in the EPS produced without glycerol. This implies a high concentration of protein moiety in EPS with glycerol, which leads to a high flocculation activity. Furthermore, the EPS obtained in the present study revealed better flocculation comparing to those produced by *Klebsiella pneumoniae* strain NY1 (85.3%) or the consortium of *Cobetia* sp. and *Bacillus* sp. MAYA (90.2%) due to the absence of the amide band (in the latter), where the bioflocculant structure was a polysaccharide (Nie et al., 2011; Ugbenyen et al., 2014) (Table 7-3).

The symmetric vibration of CH_2 vibration was observed in the EPS obtained in the present study (in glycerol case corresponding to wavenumber 2850-2865, Table 7-3). This type of bond was absent in the other IR spectrum of EPS obtained by other studies (Yuan et al., 2011; Li et al., 2008; Nie et al., 2011; Ugbenyen et al., 2014) as indicated in Table 7-3. This bond could offer the covalent C=C bond, which is more effective in playing an important role in the aggregation of flocs compared to any other type of main interactions (such as hydrogen bond, London force…) (Yuan et al., 2011)

Table 7-3 : Main functional group observed from IR spectra of broth EPS (B-EPS) with and without crude glycerol fortification.

Wave number (cm^{-1})	**Absorbance (nm)**							**Vibration type**	**Functional type**
	EPS with glycerol (present study)	**EPS without glycerol** (present study)	**EPS by *Pseudoaltermonas* SM9913** (Li et al., 2008)	**EPS by *Oceanobacillis iheyensis* BK6** (Kavita et al., 2014)	**EPS by *Klebsiella pneumoniae*** (Nie et al., 2011)	***EPS by Cobetia* sp. and *Bacillus* sp.** (Ugbenyen et al., 2014)	**EPS by *Bacillus megaterium* TF10** (Yuan et al., 2011)		
3200–3420	0.05	0.065	0.3	0.4	0.5	0.6	0.3	Stretching vibration of OH	OH into polymeric compounds
2930-2935	0.025	0.02	0.3	0.3	0.4	0.5	0.2	Asymmetric stretching vibration of CH_2	Proteins (peptidic bond)
2850-2865	0.015	-		-	-	-	-	Symmetric stretching vibration of CH_2	Proteins (peptidic bond)
1678	0.05	-		-	-	-	-	Stretching vibration of C=O and C–N (Amide I)	Proteins (peptidic bond)
1630-1660	0.06	0.04	0.8	0.3	0.4	0.3	0.3	Stretching vibration of C=O and C–N (Amide I)	Proteins (peptidic bond)
1550–1580	0.04	-	-	-	-	-	0.02	Stretching vibration of C–	Proteins (peptidic

							N and deformation vibration of N–H (Amide II)	bond)
1450–1460	-	0.01	-	-	-	0.6	Deformation vibration of CH2	phenols
1400–1410		-	0.7	0.3	0.2	-	0.2	Stretching vibration of C=O / Deformation vibration of OH	Carboxylates
1235-1245	0.015	0.01	-	-	0.3	-	0.09	Deformation vibration of CH_2/ Stretching vibration OH	Alcohols and phenols
1130-1160	0.04	0.04	-	0.3	-	-	-	Stretching vibration C-O-C	phenols
1060–1100	0.03	0.03	0.01	-	0.4	0.6	0.4	Stretching vibration of OH	polysaccharides
<1000	0.06	0.06	-	0.2	-	0.2	0.02	Several bands visible	Phosphorus or sulphur functional group

7.5.4 EPS properties

7.5.4.1 Effect of Temperature on EPS viscosity and FA

The high temperature resistance of the polymers or EPS is important for two reasons: Firstly, the EPS will be formulated as powder using atomiser, which is prepared by spray drying at high temperature (more than 80° C). Secondly, there are many other applications of EPS where high temperature resisting polymers are used (Patil et al., 2011). In medical devices for sterilization of polymers (as alginate) in encapsulation of living cells that release biologically active substances to treat conditions such as diabetes and brain tumors, as gelling agent in food industries (when the polymer is not soluble in water they use heat) (the temperature range 60-80%), and films for food preservation and such as coatings in textile industries (Alginate –chitosan is the polymer used in this application) (Carneiro-da-Cunha et al., 2010).

In the present study, the stability of S-EPS produced by *Cloacibacterium normanense* (NK6) was investigated by exposing the EPS for 10 minutes at different temperatures (from 80 to 200° C) and then measuring their viscosity. The S-EPS of the sample collected at 72h of fermentation with glycerol addition to sludge and possessing highest EPS concentration was used. Figure 7-6 presents the impact of temperature on the flocculation activity and viscosity of the EPS, which revealed that the S-EPS from *Cloacibacterium normanense* strain exhibited a good stability at high temperature. The viscosity starts to decrease slowly from 80° C until 150° and then decreased rapidly at 200° C. The flocculation activity decreased in a similar way as the viscosity (Figure 7-6). The decrease in viscosity could be attributed to the polymer degradation due to the cleavage of glycosidic bonds within the polysaccharide structure (Freitas et al., 2009)

Past studies have discussed the degradation of polymers in aqueous and organic solutions, which was accelerated by strong acids, certain oxidizing agents, ultraviolet light and temperature (Salehizadeh et al., 2001). They observed various reaction mechanisms and revealed that redox reactions involving free radicals were probably the cause of polymer degradation and concomitant viscosity losses, which could affect their ability to flocculate.

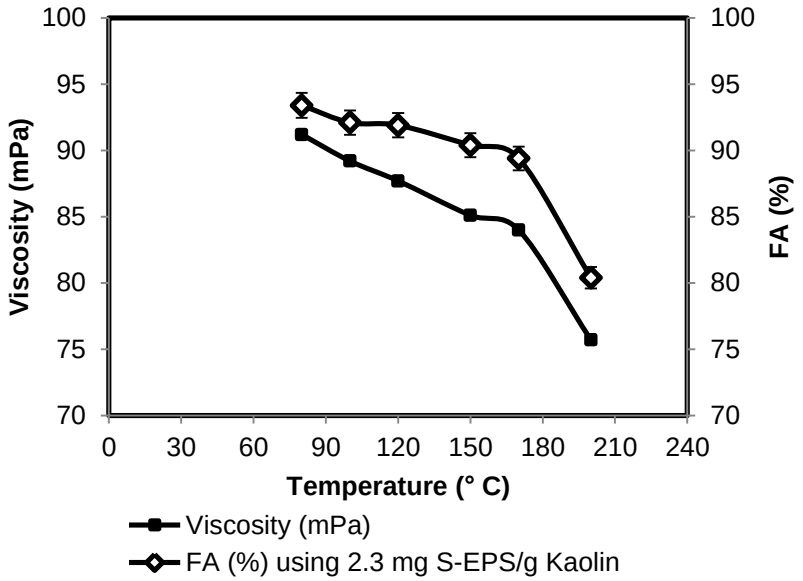

Figure 7-6 : Variation of viscosity and floculation activity of S-EPS at different temperatures.

7.5.4.2 7.5.4.2 Enzymatic study of S-EPS

Protein and carbohydrate are the main components of EPS. Many previous reports proposed that protein was more important than carbohydrate to floc formation and demonstrated that activated sludge deflocculated after incubation with a proteolytic enzyme (Liao et al., 2001; Wilen et al., 2003). In order to better understand the role of physicochemical properties, as well as the role of protein and carbohydrate in bioflocculation, enzymes were used in this study to degrade biopolymers of S-EPS produced by *Cloacibacterium normanense*. Viscosity, surface charge and turbidity index were measured at different incubation time. Proteinase K was used to degrade extracellular proteins moiety and cellulase was used to degrade extracellular polysaccharide moiety of the EPS. As shown in Figure 7-7, the viscosity decreased after the addition of both enzymes. Cellulase addition caused the viscosity to drop to around 50% of the original value after 24 h.

Proteinase K addition caused a viscosity drop to about 60% of the original value within three hrs. This clearly demonstrated that protein in S-EPS is more important to viscosity drop than carbohydrate. With the degradation of the carbohydrate and protein in S-EPS, the cell surface charge decreased (Figure 7-7b). The ζ-potential values decreased from –41 mV to around –57.3 mV and –47.1 mV after adding cellulose and Proteinase K, respectively. Increased negative surface charges would cause deterioration in flocculation (Liu and Fang, 2002). Six

hours after the Proteinase K addition (Figure 7-7c), turbidity index was measured by adding EPS to kaolin solution (2.3 mg S-EPS/g Kaolin). During the first six hrs, the turbidity decreased and then increased rapidly reaching 19.8 NTU after 36 hours. There was no sign of turbidity recovery with an increase in incubation time. Cellulase addition gave almost the same trend. Turbidity decreased, followed by a relatively slow increase (compared with proteinase K results) with incubation time. These findings suggested that hydrolysis of carbohydrate and protein moieties in S-EPS decreased the viscosity and increased the surface charge, which caused deflocculation. Thus, these results established that both protein and carbohydrate moiety of the EPS play an important role in floc formation.

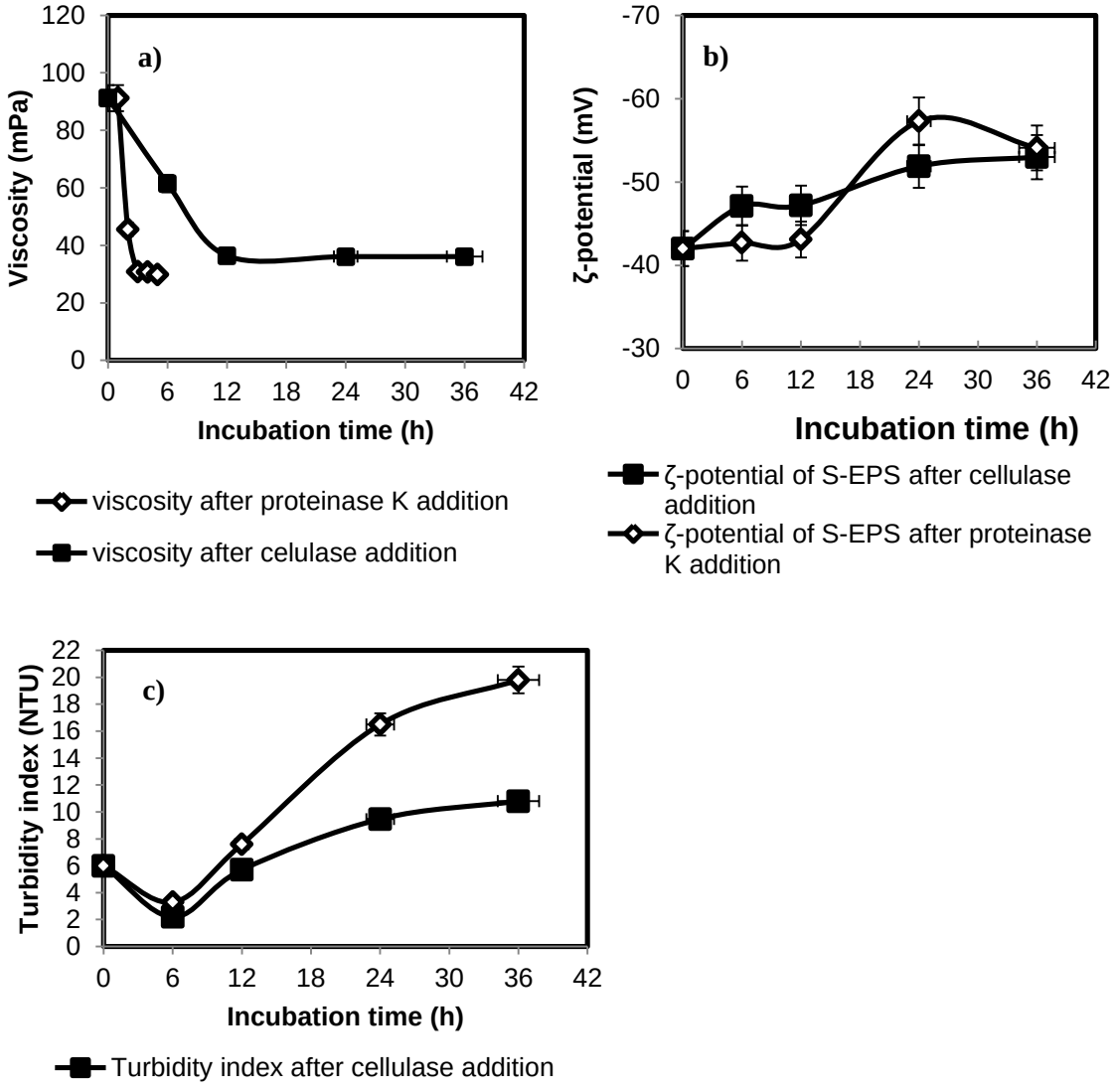

Figure 7-7 : Change of (a) Viscosity, (b) ζ-potential and (c) Turbidity index of B-EPS after addition of cellulase or Proteinase K to the EPS solution.

Figure 7-8 demonstrates SEM photos presenting the state of bacterial aggregate before and after enzyme addition. Bacterial flocs before enzymes digestion were more visible than those treated with enzymes for six hours (Figure 7-8a–c). This suggested that the enzymes destroyed the EPS structure and the cells were dispersed.

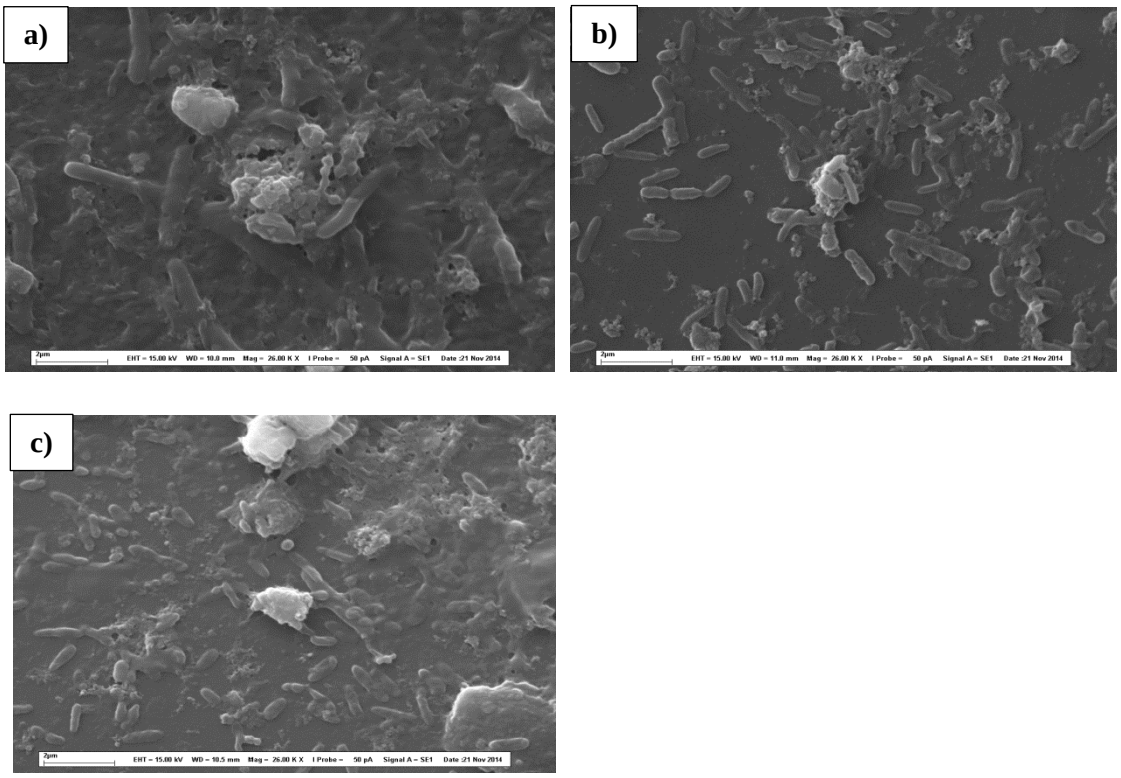

Figure 7-8: Scanning electron micrograph of *Cloacibacterim normanense:* (a) Control or without enzyme treatment; (b) 6 hrs after treated with Cellulase; (c) 6 hrs after treated with Proteinase K.

7.6 Conclusion

This research investigated the carbon and nitrogen content influence on EPS production, the chemical characterisation of EPS produced and their degree of stability as bioflocculant. The following conclusions could be drawn from the foregoing research:

- 17.5 g/L EPS was produced by *Cloacibacterium normannese*.
- The EPS obtained had higher galactose contents (67 mol%) and lower content of glucose (13 mol%), xylose (9 mol%), sucrose (8 mol%) and lactose (3 mol%).
- Distinct functional groups were demonstrated using IR spectra.
- The degree of EPS stability decreased under high temperature (150° C).
- Deflocculation was induced due to digestion of protein and carbohydrate moieties of the EPS by proteinase K and cellulase enzymes, respectively.
- Detailed structural analysis using SEM photos revealed that both protein and carbohydrate moieties of EPS are important factors to impact surface properties and bioflocculation ability of the EPS.

7.7 Acknolwledgements

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CHAPITRE 8

Simultaneous Extracellular Polymeric Substances (EPS_s) and Lipid Production by *Cloacibacterium normanense* via Fermentation of sterilized Activated Sludge fortified with crude glycerol

Ce chapitre est constitué d'un article suivant :

Klai Nouha, Ram Saurab Kumar,Tyagi RD, (2016). Simultaneous Extracellular Polymeric Substance (EPS) and Lipid Production by Cloacibacterium normanense via Fermentation of sterilized Activated Sludge fortified with crude glycerol (Soumis Biotechnology and bioengineering)

8 Concomitant Extracellular Polymeric Substance and lipid Production by *Cloacibacterium normanense* via fermentation of sterilized activated Sludge fortified with crude glycerol.

8.1 Abstract

Abstract

Exopolymeric substances (EPS) are produced by numerous microorganisms as their defense mechanism in hostile conditions. Recently EPS have been reported to be used as a potential bioflocculant for settling and dewatering of solid waste generated in wastewater treatment plants. In our previous study (Klai et al., 2015) we reported the production of EPS by newly isolated strain *Cloacibacterium normanense NK6.* In this study, we report the simultaneous EPS production and lipid accumulation by C. normanense. The EPS production and lipid accumulation by the bacterium were enhanced by varying the carbon-nitrogen (C/N) ratio in sterilized activated sludge media supplemented with crude glycerol as additional carbon source. Sterilized activated sludge was inoculated with 5% (v/v) of *Cloacibacterium normanense*. At low C/N ratio, lipid content was found to be low, but EPS concentration was significantly high while at high C/N ratio, an increase in lipid % (g/g cell dry weight) and a decrease in EPS concentration was observed. The best results were obtained using C/N 25. *Cloacibacterium normanense* accumulated a lipid content of 27.6% (w/w) and 22 g/L of EPS in 72 h. Therefore, *Cloacibacterium sp.* appears to be a suitable candidate for fermentation processes involving renewable resources.

8.2 Keywords

Wastewater solid sludge, crude glycerol, *Cloacibacterium normanense*, EPS, microbial lipid, Techno-economic evaluation.

8.3 Introduction

Extracellular polymeric substances (EPS) is a complex mixture of high molecular weight biopolymers like polysaccharide, protein, nucleic acids, and humic substances. They can protect the cells from the external environment and provide energy and carbon source when the substrate is in short supply (Wingender et al., 1999). It has been reported that EPS produced by bacteria can play a significant role in controlling the flocculation and floc properties, including settling and dewatering (More et al., 2012, Klai et al., 2015). EPS composition is very complex, and its components and content vary with many factors such as a microorganism, cultivation time, carbon source, and growth state, etc. (Sheng and Yu, 2006). For many microorganisms, EPS synthesis is stimulated by the abundance of carbon availability and limitation of other nutrients, such as nitrogen, oxygen or phosphorus (Kumar et al., 2007). High C: N ratios (10-20) are commonly required for attaining high yield of EPS synthesis (Rosalam et al., 2006) although high C: N ratio is reportedly unfavorable for biomass growth. Hence, a compromise in the C: N ratios is required to promote cell growth whilst favoring EPS synthesis. Nevertheless, for most EPS-synthesizing microorganisms, the highest polymer productivities are usually achieved for low nitrogen concentrations (Farhadi et al., 2012) that are strain dependent.

Accordantly, lipid accumulation is receiving considerable attention because of their potential as a source of feedstock for biofuel production. Numerous studies have successfully transferred fourth generation microbial oil to biodiesel (Meng et al. 2009; Gao et al. 2014). Lipids serve as storage materials in some lipid accumulating yeasts and bacteria. According to many studies (Zhang et al., 2014), under nitrogen limiting and carbon-excess conditions, organisms tend to store lipids. Therefore, the limitation of nitrogen source could be the key for simultaneous production of EPS and lipid.

Lipid and EPS are produced respectively inside and outside the cell by microorganisms. They are able to serve as energy and carbon source when microorganisms are under starvation conditions. Thus, it is essential to understand the relationship between the intracellular and extracellular product by the same microorganism.

Cloacibacterium normanense NK6 has been reported to produce high EPS concentration, using activated sludge and crude glycerol as the sole carbon source (Nouha et al., 2016). The crude glycerol feeding was used for EPS synthesis. High and reproducible EPS productivity (0.28 g/L.h) were achieved with initial glycerol concentrations of 20 g/L. The EPS produced is composed of galactose (67 mol %) and was poor in other sugars like glucose (13 mol %),

xylose (9 mol %), sucrose (8 mol %) and lactose (3 mol %). Pyruvyl, succinyl, and acetyl substituent groups accounted for 0.4–3.3 wt% of the EPS dry weight.

The impact of nitrogen concentration on the balance between growth and polymer synthesis by *Cloacibacterium normanense* was not previously assessed nor their influence on the polymer's production or flocculation properties. EPS can allow microorganisms to live at a high-cell density in stable mixed population communities and significantly influence their topographical properties, which are of considerable importance in governing bacterial flocculation and adhesion (Sheng and Yu, 2006). Thus, elucidation of the relationship between EPS, Lipid, and biomass growth would benefit for the lipid accumulation by *C. normanense*. Moreover, information about the lipid production by *C. normanense* is never studied. Thus, considering EPS production along with lipid formation is essential for understanding the technical and economic value of the whole process. Therefore, in this study, the simultaneous production of EPS and lipid by this strain was investigated at optimal C/N ratio. The results obtained in this study will be helpful in understanding the biosynthesis of the extracellular and intracellular product by *Cloacibacterium normanense*.

8.4 Material and methods

8.4.1 Preparation of Cloacibacterium normanense culture and

For inoculum preparation, *Cloacibacterium* sp. (NK6, accession number KF675202) was inoculated into an Erlenmeyer flask (150 mL) containing 50 mL TSB(Tropic soy broth) media and it was incubated for 48 h at 30 °C, 180 rpm to logarithmic phase. Further, 5 ml of the TSB pre-grown broth was transferred to 100 mL of sterilized sludge (25 g/L suspended solids or SS) and incubated for 24 h.

8.4.2 Culture conditions for flask fermentation

For fermentation, 5% (v/v) pre-culture was added to the flask (500 mL) containing 150 mL sterilized sludge. The pH was adjusted to 7. Crude glycerol and peptone were used as carbon and nitrogen source respectively for EPS production and Lipid accumulation. Different initial C/N ratios (18, 25, 30, 50 and 100) were adjusted by changing the nitrogen quantity to determine the optimum C/N (carbon/nitrogen) ratio for high EPS production and lipid accumulation yield. The initial concentration of 40 g/L of crude glycerol was used as supplemented carbon source in sterilized sludge. This concentration was optimal for high lipid accumulation yield (Zhang et al., 2014). The crude glycerol utilized contains other compounds such as soap, methanol and free fatty acid (FFA) (Table8-1). These components can act as a carbon source for growth and product formation. The composition of crude glycerol was determined according to Hu et al. (2012). The peptone content was determined by calculating

the total carbon as given inTable 8-2. The nitrogen requirements were calculated as per following equations:

Total Carbon (TC) = C-Glycerol + C-Soap + C-Methanol all in grams of C

Total Nitrogen required (TN) = TC/ (C: N ratio required)

Peptone Required (Pep) = TN/ N content in peptone

The nitrogen content of peptone is assumed to be 10% (w/w). The carbon and nitrogen content from available from sludge is assumed negligible as compared to the supplemented carbon source.

Table 8-1: Crude glycerol (C.G.) stock composition, production reactor's initial composition and Fermented broth composition after 72 hours.

Component	Stock C.G. (% w/w)	0^{th} hour (g/L)	72 hours (g/L)
Glycerol	13.4	40.0	14.45
Methanol	4.6	13.6	0.35
soap	23.5	70	41.2
Peptone	NA	28.6	0
Biomass	NA	ND	29.28
Density	0.845		ND
pH	6.8		8.7
Ash	1.5		ND

Table 8-2: Flask fermentation conditions to maintain C/N ratio

	C/N 18			C/N 25			C/N 30			C/N 50			C/N 100		
	Soap	**Glyc**	**MeOH**	**Soap**	**Glyc**	**MeOH**	**Soap**	**Glyc**	**MeOH**	**Soap**	**Glyc**	**MeOH**	**Soap**	**Glyc**	**MeOH**
Concentration of C.G. components in 44.77 mL (g/L)	10.5	6.0	2.0	10.5	6.0	2.0	10.5	6.0	2.0	10.5	6.0	2.0	10.5	6.0	2.0
Gram of Carbon	7.8	2.3	0.8	7.8	2.3	0.8	7.8	2.3	0.8	7.8	2.3	0.8	7.8	2.3	0.8
Total grams of Carbon		10.9			10.9		10.9				10.9			10.9	
Peptone N content (g/150 mL)	6.04			4.3			3.6			2.2			1.1		
Initial concentration of C.G. component compounds (g/L)	70.0	40.0	13.6	70.0	40.0	13.6	70.0	40.0	13.6	70.0	40.0	13.6	70.0	40.0	13.6

8.4.3 Estimation of bacterial growth and analytical methods

During the fermentation, samples were withdrawn at each 12 h interval to measure the cell concentration and biomass concentration. Cell concentration was measured as CFU (colony format unit) per mL employing the standard serial dilution technique on agar plate. 25 mL of culture was centrifuged at 6000 g at 4°C for 20 min for biomass determination. After C-EPS extraction, as described by Nouha *et al*. (2016), the residual biomass pellet was dried at 105°C to get the dry weight of the biomass. Glycerol concentration, methanol and soap content in the cell-free supernatant were determined according to Hu et al. (2010). Total carbon and nitrogen in the samples were measured by the CHNS analyzer (Shimadzu VCPH).

8.4.4 EPS and lipid extraction

EPS was extracted by centrifugation method. Samples were centrifuged at 9000 g, 4 °C for 20 min to obtain supernatant (containing slime EPS, termed as S-EPS). The pellets were re-suspended to the initial volume and then heated at 60 °C for 20 min to obtain capsular EPS (C-EPS) (Nouha et al., 2015). Broth EPS (B-EPS) contains both S-EPS and C-EPS. The supernatant obtained after centrifugation (crude S-EPS) was mixed with two volumes of chilled ethanol (95% v/v (volume/volume)) and incubated at -20°C to get the pellets S-EPS. After precipitation, the pellets were separated by centrifugation. The pellet was further dried at 60°C to determine S-EPS dry weight.

Lipid was extracted using standard method (Folch et al., 1957, Vicente et al., 2009). The washed pellet (2.6 ± 0.1g) was mixed with 15 mL solvents of chloroform-methanol (2:1 v/v) and 5 mL zirconium beads (diameter 0.7mm) were added to the mix. The cells were lysed by a bead beater for 12 h (BioSpec Products, Bartlesville, OK, USA). The mixture was separated into three different layers by 10 min centrifugation. The residual biomass was present in the bottom layer; intermediate phase was lipid dissolved in chloroform, and top phase was methanol and water. The chloroform containing lipid was taken out and transferred into a pre-weighed glass tube (W1). The rest of the solution (containing cell debris, methanol, and water) was again supplemented with a 15mL chloroform-methanol (1:1 v/v) solvent and kept for agitation. After 12 hours of agitation, the solution was filtered using Whatman filter paper. The filtrate was mixed with a previously extracted solution (chloroform solution containing lipid), and the mix was allowed to settle for phase separation. The bottom phase containing lipid in chloroform (the other phase was water and methanol) was collected and subjected to overhead nitrogen sparging until total chloroform was evaporated completely. The remaining samples were further dried at 60°C in a hot air oven until the sample reached a constant

weight (W2). The weight, thus recorded, represents the amount of lipid (or lipid-like cellular components, which are soluble in chloroform) in that sample. The dried extract is trans-esterified and analyzed by GC (gas chromatography) in the form of FAMEs (Fatty acid methyl esters) to quantify the actual lipid. The chloroform extract (crude lipid) content of the biomass was calculated as Crude Lipid content= (W2-W1)/dry biomass weight × 100%.

Where W1 expresses the pre-weighed glass tube, and W2 denotes the oven dried microbial crude lipid in a pre-weighed glass tube.

8.4.5 EPS flocculation activity

The sludge flocculation activity of EPS was determined by modified jar test method (Klai et al., 2015). Municipal wastewater (5 g/L) sludge sample was used for the test. 600 mg/L of Alum ($Al_2 (SO_4)_3$) was added to the sludge sample. In this study, instead of Ca^{2+}, alum was used as a coagulant to compare the results with the actual treatment condition in wastewater treatment plant. Further, Ca^{2+} as a coagulant is not sufficiently active with municipal sludge as concluded by our preliminary studies (data not shown). Further, same alum concentration (600 mg/L) is used to simulate the exact treatment plant scenario (Communauté Urbaine du Québec, (CUQ) Quebec). The pH of sludge was changed to 7 after it has been optimized. S-EPS produced by *Cloacibacterium normanense* was selected because of high flocculation activity observed in kaolin solution. The supernatant of the fermented broth without ethanol precipitation was used as S-EPS. The samples were mixed at 120 rpm for first 5 min after addition of 600 mg/L of alum (which enables the neutralization) followed by addition of EPS (different concentration) with mixing at 50 rpm for 25 min allowing floc formation (Li and Yang, 2007; Nouha et al., 2015).

The well-mixed sludge samples (1L, 5g/L SS) were transferred into beakers and 600 mg/L of $Al_2 (SO_4)_3$) was added along with a different dosage of S-EPS. Another two beakers served as positive control (addition of Zetag in place of S-EPS) and negative control (without the addition of Alum, EPS or Zetag). Zetag is the cationic chemical polymer, used by municipal wastewater treatment plant (CUQ, Québec). It was utilized to compare its flocculation efficiency with that of EPS. After addition of S-EPS, each mixed sludge sample was then transferred into 1L graduated measuring cylinder for turbidity measurement (Nouha et al., 2015). After 30 minutes, the supernatant of each sample was then collected to measure the turbidity using turbidimeter (Micro 100 turbidimeter, Scientific Inc.).

8.4.6 Lipide transesterification

For transesterification, lipid was dissolved in hexane (25 mL/ gram lipid), then mixed with methanol (in 6:1 (mol/mol) ratio or 0.4 mL methanol per gram lipid) containing 1% w/w (1 g NaOH/ 100g oil) sodium hydroxide. Afterwards the mixture was heated to 55 °C for 12 h. After the reaction, NaCl solution was added (50 mL of 5% w/v NaCl solution per gram lipid) and the solution was allowed to stand for 15 min. FAMEs were extracted with hexane (top) phase. The bottom phase was again mixed with hexane (25 mL per gram lipid, to remove non-recovered FAMEs) and FAMEs were then mixed with the fraction separated earlier. The FAMEs in hexane was washed with sodium bicarbonate solution (10 mL of 2% w/v solution per gram lipid), and the top hexane layer was then dried at 60 °C in an oven (Halim et al., 2011). The FAMEs were dissolved in hexane (0.01 g lipid/10 mL hexane) and analyzed using a Gas Chromatography linked with FID (Flame ionization detector) (GC-FID) (Perkin Elmer, Clarus 500). 1µl of trans-esterified sample was injected with an automated sample injector, and the sample analysis was performed with Agilent Chem Station module software from Agilent technologies. The calibration curve was prepared using a standard mix of 37 FAMEs (47885-U, 37 Component FAME Mix; Supelco, Bellefonte, PA, USA). 1, 3- Dichlorobenzene was used as an internal standard with a concentration of 50 ppm.

8.4.7 Kinetic parameters calculation

The maximum specific growth rate (µmax, h−1) was determined using equation 1:

$$\ln\left(\frac{x}{x0}\right) = \mu max \times t \quad (1)$$

Where, X0(g/L) is the initial cell concentration. The yields of biomass (Yx/s, gCDW/gGlyc) and EPS (Yp/s, gEPS/gGlyc; gLipid /gGlyc) on glycerol (Glyc.) as substrate were determined using Eqs. (2) and (3),

$$\Delta X = \text{final biomss (Xf)} - \text{Initial Biomass (Xi)}$$

$$\Delta S = \text{final Substrate(Sf)} - \text{Initial Substrate (Si)}$$

$$\Delta P = \text{final Product (Pf)} - \text{Initial Product (Pi)}$$

$$Yx/s = \frac{\Delta x}{\Delta s} \quad (2)$$

$$Yp/s = \frac{\Delta p}{\Delta s} \quad (3)$$

Where Δx (g/L) and Δp (g/L) are the biomass and EPS produced, respectively, and Δs (g/L) is the substrate up taken during the same cultivation time. The EPS volumetric productivity (rp, g/L h) was determined as follows: $r_p = \frac{\Delta p}{\Delta t}$ (4)

Where p corresponds to concentration of the product EPS (g/L) or lipid (g/L) at time t (h). The EPS specific productivity (qp, gEPS/gCDW.h) was determined using Equation 5.

$q_p = \frac{rp}{\Delta x}$ (5)

8.5 Results and discussion

8.5.1 Effect of C/N ratio on EPS and lipid production in sterilized sludge

In present study, five set of experiments were tested. The initial glycerol concentration used was fixed at 40 g/L with variation of nitrogen concentration (peptone) to adjust the desired C/N ratio (18, 25, 30, 50 and 100). As presented in Table 8-1 (crude glycerol composition) the methanol concentration is very high which could affect the *Cloacibacterium* sp. growth. Taking this in consideration the fact that *Cloacibacterium normanense* could tolerate 6% (v/v), the concentration of methanol contains in the broth was maintained at 6% (v/v) or 4.6% (w/v).

Table 8-3 presents the culture condition such as the carbon source and nitrogen source used in flask fermentation to maintain the desired C/N ratio. In the five flask fermentation experiments, C/N ratio was set by changing only nitrogen concentration while the total carbon concentration was fixed to 10.9 g/L in the media.

EPS production seems to be affected by variable C/N ratios (Table-8-3**Erreur ! Source du renvoi introuvable.**, Figure 8-1). It decreases when the C/N ratio increases. For C/N 18, the EPS concentration was 22.5 g/L comparing to 4.9 g/L in case of C/N ratio 100. In contrast, lipid accumulation steadily increased at higher C/N ratio until C/N ratio reached 100; concomitant with biomass concentration **Figure 8-**(Figure 8-1). The highest lipid concentration was measured at C/N ratio of 100 with 14.1 g/L.

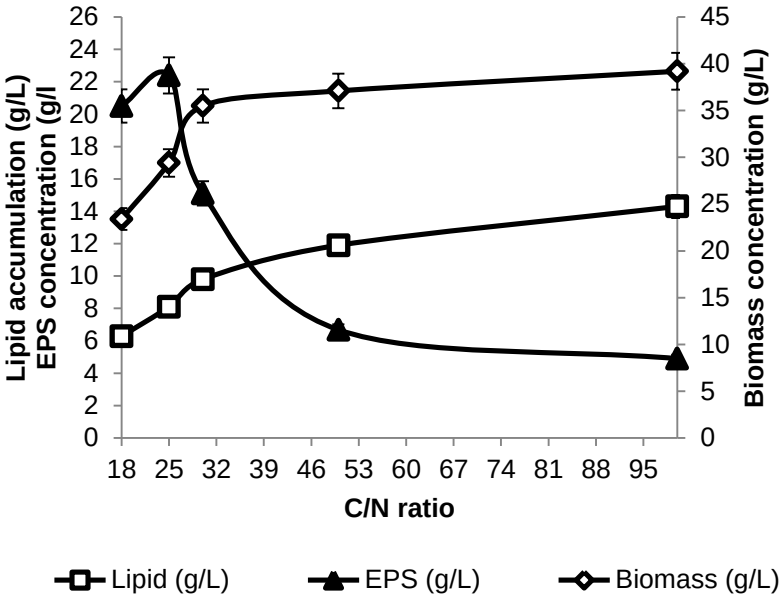

Figure 8-1: Effect of different C/N ratios on biomass, lipid accumulation and exopolysaccharide production in shake flask cultures of *C. normanense.*

Table 8-3: Kinetic parameters obtained in flask fermentation experiments using fixed glycerol concentration and different nitrogen concentrations.

C/N ratio	Initial concentration (g/L)		Cultivation time (h)	CDW (g/L)	μmax h^{-1}	EPS (g/L)	Lipide (g/L)	$Y_{X/S}$ (g CDW/g Gly)	Product content per unit biomass		r_p (g/Lh)		q_p (g/CDW h)	
	Glycérol	Peptone							g EPS/ g CDW)	g Lipid/ g CDW)	rp_E	rp_L	EPS	Lipide
18	40	40.3	72	23.4	0.076	22.5	5.8	0.6	0.96	0.26	0.31	0.07	0.013	0.0040
25	40	28.6	72	29.4	0.070	22.4	7.8	0.7	0.76	0.27	0.31	0.11	0.010	0.0038
30	40	23.9	72	35.5	0.064	15.1	9.8	0.8	0.42	0.28	0.21	0.14	0.006	0.0037
50	40	14.5	72	37.1	0.046	6.7	11.9	0.9	0.18	0.32	0.09	0.16	0.002	0.0044
100	40	7.25	72	39.2	0.035	4.9	14.1	1.0	0.12	0.36	0.07	0.20	0.001	0.0050

The results obtained show that increasing the initial nitrogen concentration (decrease in C/N ratio) led to higher EPS synthesis (the highest EPS productivity obtained at peptone concentrations of 40.3 g peptone/L for C/N ratio 18 at 72h,(Figure 8-2 A,B).

The nitrogen source is used mainly for cell growth and enzyme production for catabolic and anabolic pathways. Several authors (González-García et al., 2015; Liu et al., 2011) reported that EPS producing bacteria need a specific C/N ratio to promote EPS synthesis. In balanced optimum nitrogen availability the nitrogen is efficiently used to produce required cell machinery (enzymes) and simultaneously process the excess carbon for extracellular polymers. The EPS concentration was mainly affected by decrease in peptone concentration in the event of limiting nitrogen source condition during which the strain starts to store lipid (Gao et al., 2013; X. Zhang et al., 2014) (Figure 8-2).

The reduction of EPS polymer production at high C/N ratios might have caused by the low nitrogen concentration used for cultivation. This behavior can be accounted by the fact that while increasing the C/N ratio the availability of nitrogen is decreased, which disrupts the normal anabolism of the cell thereby the tendency of the cell is to accumulate the available carbon in the form of intracellular products (lipid) rather than to metabolize it in normal ways. The extracellular polysaccharides are primary functional products of the microbe to serve as protection matrix of the microbes and they are produced in normal balanced growth situation with little excess of carbon. In high C/N ratio the scarcity of nitrogen disables this normal functionality of the cell. On the other hand with C/N ratio when growth metabolism is disrupted the carbon availability in the cell increases for lipid accumulation and microbes tends to preserve it in the form of polymeric substances like lipid.

The yields and productivities for EPS are listed in Figure 8-2A-B, when the C/N ratio was between 18 and 30 the yield of EPS ($Y_{P/S}$, gEPS/gGlyc) increases with the fermentation time however the productivity of EPS rp_E (g/L.h) decreases drastically (Figure 8-2 A, B). According to C/N ratio, the productivity of EPS is higher (0.28 g/L.h) at lower C/N 18 comparing to 0.07 g/L.h at C/N 100 at 72 h. These results were in agreement with (Torres et al., 2014). Torres et al. reported that when the nitrogen feed concentration was increased from 4.5 g/L to 45 g/L the productivity and yield of EPS increased from 2.04 (g-EPS/L.d) and 0.17(g-EPS/g-Glycerol) to 5.6(g-EPS/L.d) and 0.23(g-EPS/g-Glycerol), respectively.

As apparent in Figure 8-2 C-D, the yield of lipid on glycerol (Yp/s) increases with fermentation time while contrarily the productivity (rp_L) decreases. According to C/N ratio, the productivity of lipid is higher at C/N 100 (0.2 g/L.h) than at C/N 18 (0.08 g/L.h) at 72 h. These results were better than that obtained by (Angerbauer, Siebenhofer, Mittelbach, & Guebitz, 2008). They

observed lipid productivity of 0.0045g/L.h at C/N 100 while growing *Lipomyces starkeyi* in sewage sludge. The results obtained in this study are also in accordance with the results obtained by cultivating *Trichosporon oleaginous* in sludge fortified with glycerol and peptone and obtained a lipid productivity of 0.11 g/L.h in our laboratory (unpublished data, Table 8-4).

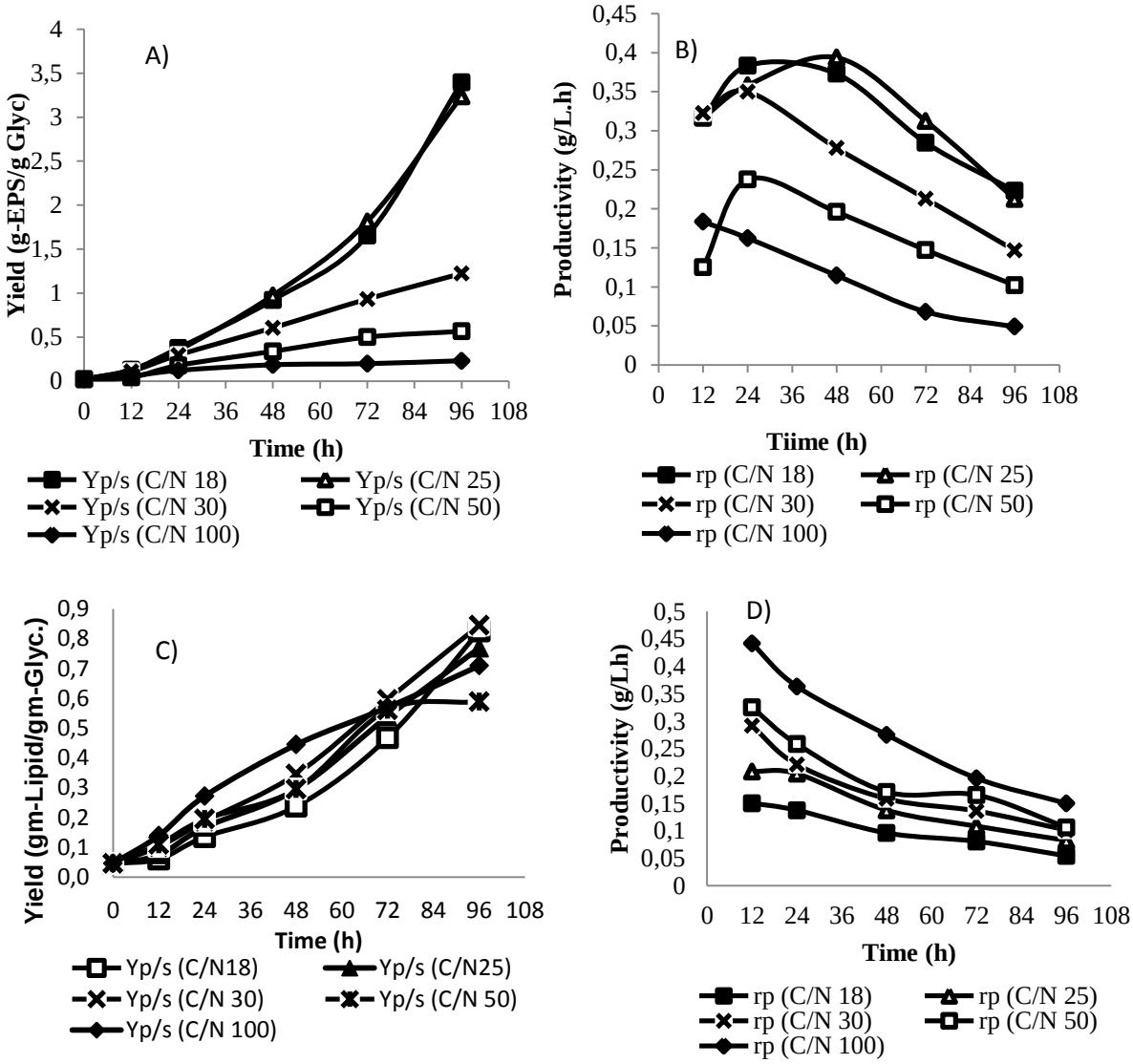

Figure 8-2: A) Yield of EPS per unit gm of glycerol consumed during the fermentation B) EPS formation rate (g/L.h) during the fermentation C) Yield of lipid per unit gm of glycerol consumed during the fermentation D)Lipid formation rate (g/L.h) during the fermentation

As shown in Figure 8-3(a, b and c), There is insignificant lipid accumulation during first 24 hours of fermentation by *Cloacibacterium normanense* in case of C/N 18, 25 and 30, respectively. A decrease of biomass concentration was observed in the first 24 hours of fermentation, this can be accounted by the fact that the insoluble fraction of sludge is metabolized by the bacteria to produce EPS, which is extracellular thus the production of EPS is not reflected in the SS of the samples taken during first 24 hours. Simultaneously, a large amount of glycerol was consumed during the first 24 hours, indicating that the carbon source was probably being directed towards the synthesis of EPS, thus making the carbon substrate unavailable for significant lipid accumulation rendering low lipid yield on substrate (Figure 8-C). Typically, biomass production and lipid accumulation by *Cloacibacterium* sp. occurs mostly under nitrogen limiting conditions in case of C/N 50 and 100. At higher C/N ratio, the biomass concentration increased at the beginning of fermentation indicating the lipid accumulation by this strain (Figure 8-3d and e).

(Angerbauer et al., 2008) and (Mulder, Deinema, Van Veen, & Zevenhuizen, 1962) observed that under nitrogen limiting conditions and the presence of an excess carbon-source, organisms starts to store lipids. Therefore, a high carbon to nitrogen (C/N)-ratio, around 100, is a basic requirement for the accumulation of lipids. However, some nitrogen is also furnished by sludge, which is not possible to exactly determine.

Table 8-4: Comparison of critical parameters between *T. oleagnious* and *C. normanense*

Parameters	**Nouha et al. 2016**	**Xiaolei et al. 2015**
Organisms	*Cloacibacterium normanense*	*Trichosporon oleaginous*
Carbon	Sludge (municipal), Crude glycerol	Sludge (municipal), Crude glycerol
Nitrogen	Peptone	Peptone
C/N	25	30
Yield biomass	0.7 g-CDW/g-glycerol	NA
Yield Lipid	0.305 g-Lipid/g-glycerol	0.26 g-Lipid/g-glycerol
Yield EPS	0.87 g-Lipid/g-glycerol	NA
Lipid Productivity	0.108 g/L/h	0.113 g/L/h
EPS Productivity	0.31 g/L/h	NA
Lipide content	0.27 g-Lipid/g-CDW	0.4 g-Lipid/g-CDW
Time of fermentation	72 hours	84 hours
Max lipid concentration	7.8 g/L	16.4 g/L (4.43 g/L from sludge)
Max EPS concentration	22.4 g/L (extracellular)	NA
Glycerol consumed	40g/L to 14.45 g/L	40 g/L to 1.26 g/L

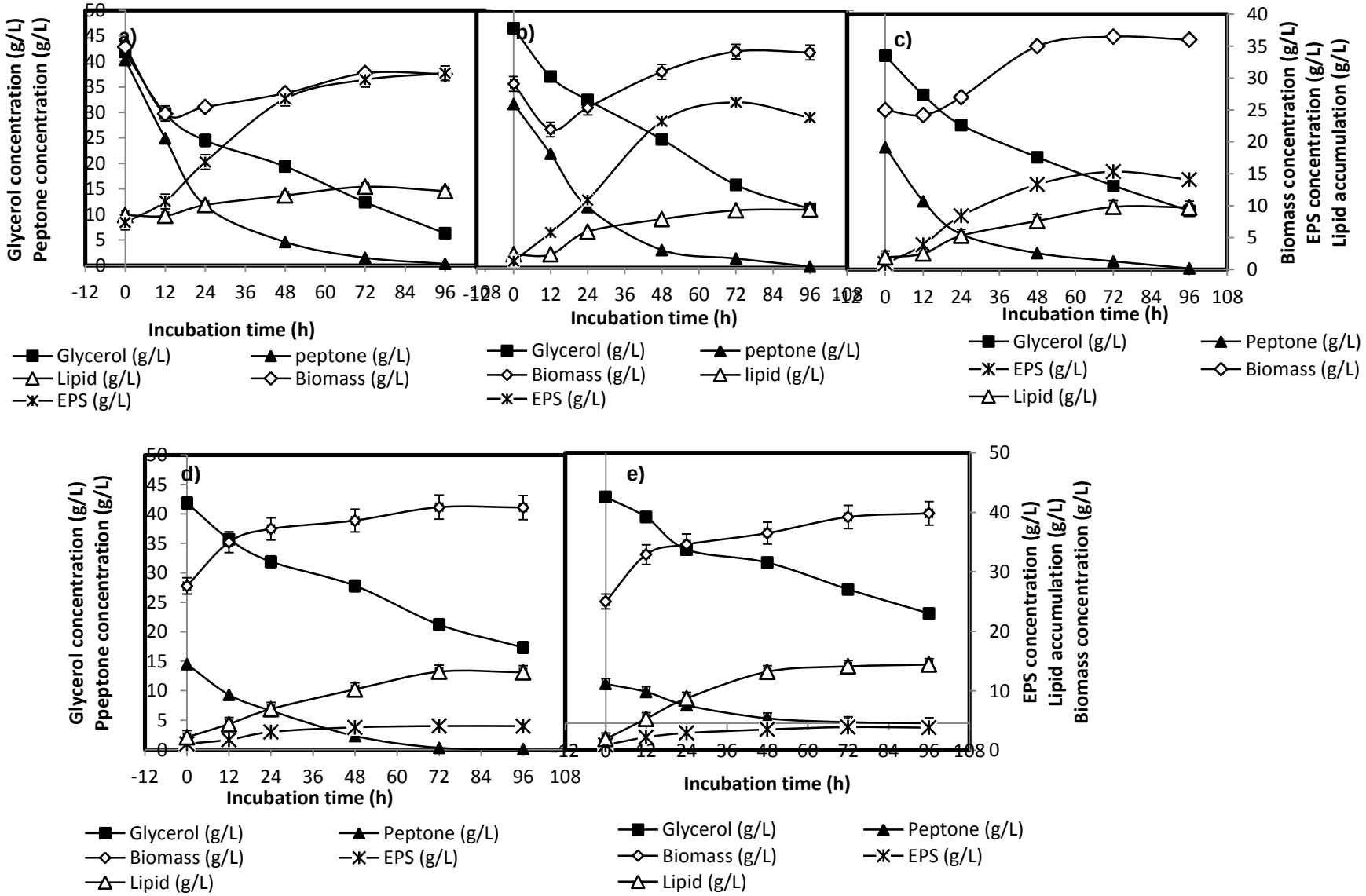

Figure 8-3: Cultivation profile of *C. normanense* with initial glycerol and different nitrogen concentrations a) Run 1 C/N 18 b) Run 2 C/N 25 c) Run 3 C/N 30 d) Run 4 C/N 50 e) Run 5 C/N 100.

Cloacibacterium normanense used glycerol, methanol and soap as carbon source for EPS production and lipid storage. Figure 8-5 a, b and c indicates that the glycerol, methanol and soap were consumed during the fermentation. Increasing the C/N ratio, the rate of consumption of glycerol decreases however, the rate of soap consumption increases particularly at C/N 50 and 100, which could explain the low utilization of glycerol by *Cloacibacterium normanense*. The soap is metabolized favorably for lipid accumulation (at C/N ≥ 30) as it goes through direct assimilation by the microbe for forming lipid molecules (Figure 8-4). The soap was significantly consumed by the strain. As presented in **Erreur ! Source du renvoi introuvable.**C, in case of C/N ratios 18, 25 and 30 the soap concentration decreases only after 24 hours. In contrast *Cloacibacterium* sp. starts to consume soap at the beginning of fermentation (0h) in case of C/N 50 and 100, which led to lipid production. Soap formation in trans-esterification reaction depends on the catalyst used in the reaction. When alkalis such as NaOH or KOH are used, the oleic acid present in lipid fraction is converted to sodium or potassium oleate during FAMEs generation. Soap is reported to enhance the lipid accumulation by microbes. A significant consumption of sodium oleate (representative soap) was observed by biomass for lipid production as reported by (Xu et al., 2012). Increasing the concentration of sodium oleate from 0.5 g/L to 2 g/L increased the lipid content of biomass from 34% (w/w) to 59% (w/w).

These results conclude that the optimum C/N ratio for accumulation of lipid by *Cloacibacterium normanense* was C/N ratio 100, which leads to a lipid content 36.5% (w/w) equivalent to concentration of 13.4 g/L while, C/N 18 was the optimum for EPS production by this strain.

The accumulation of lipids by *Lipomyces starkeyi* was evaluated in media containing sewage sludge, in which sludge pre-treatment with ultrasound resulted in lipid accumulation values greater than 1 g/L with lipid production 35.6 % (w/w) (Angerbauer et al. 2008). In fact the lipid percentage obtained in present study (36.5 % (w/w)) is almost the same as obtained by Angerbauer et al. study; however the productivity of lipid (0.11 g/L/h) at 72 h of fermentation was better than obtained (0.02 g/l/h) at 220h by Angerbauer et al. (2008). Further, in present study two co-products (EPS and lipid) are simultaneously generated, which is not the case in previous studies. In addition, not all bacteria accumulate large quantities of fatty acids. Bacterial strains *Nocardioides sp., Sphingomonas sp., Oceanicaulis alexandrii* sp. and *Micrococcus* sp. isolated from marine living cells, contain a fatty acid (FA) content from 0.3 to 4% (w/w) dry weight (Zabeti, Daud, & Aroua, 2010).

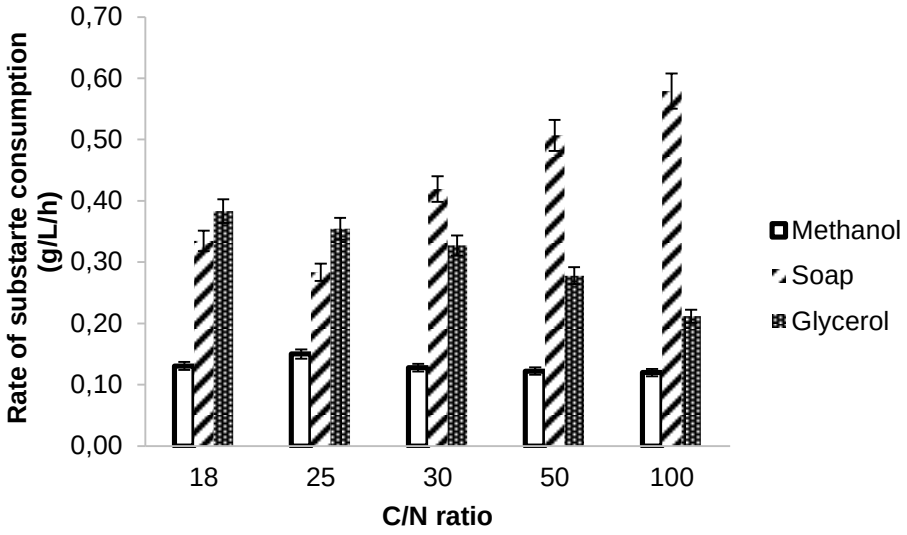

Figure 8-4: Rate of substrate consumption for glycerol, methanol and soap after 72 hours of fermentation for various C/N ratio.

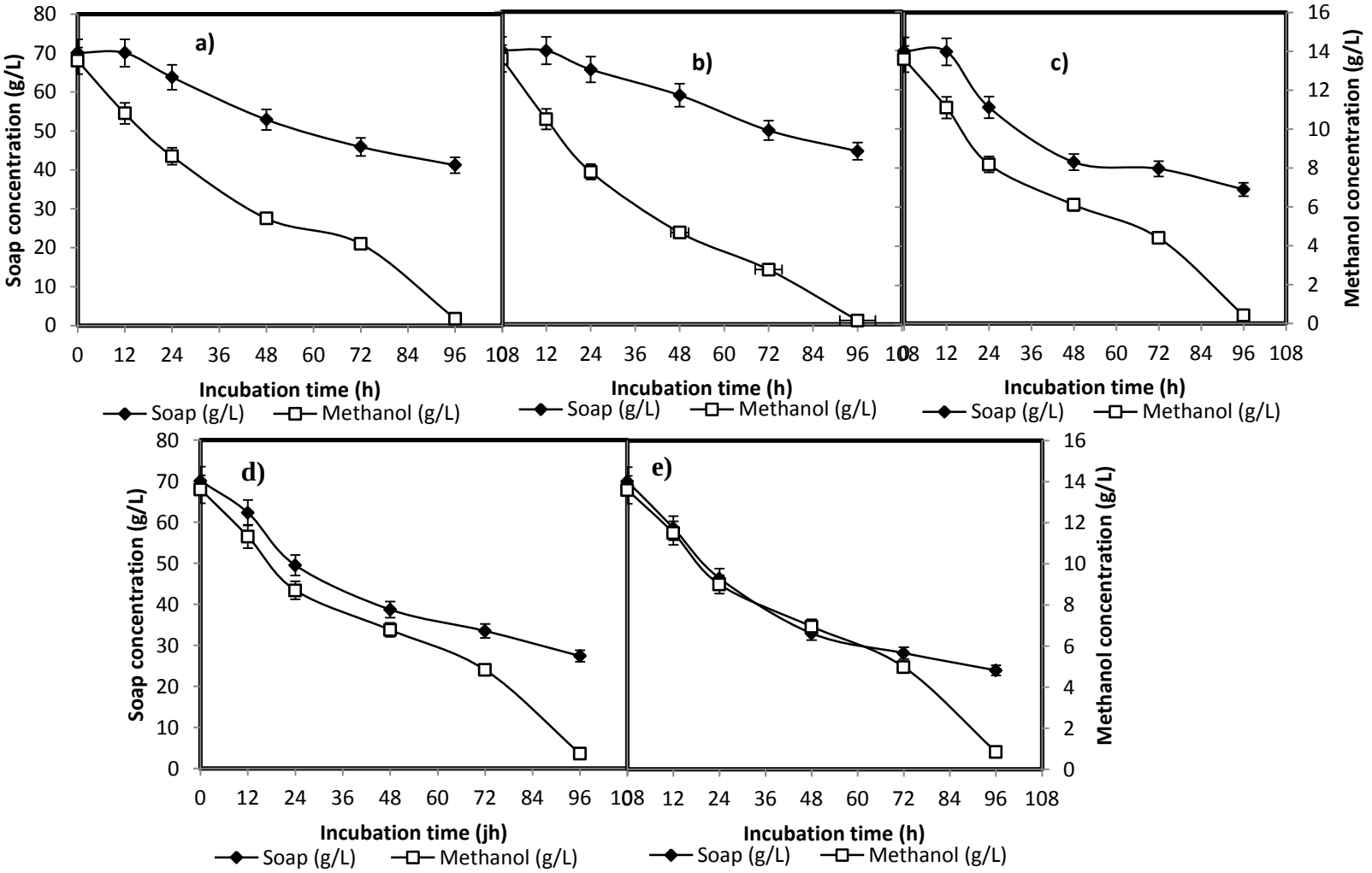

Figure 8-5: Methanol and soap consumption during the fermentation a) Run1 C/N 18 b)Run 2 C/N 25 c) Run 3 C/N 30 d) Run 4 C/N 50 e) Run 5 C/N 100.

8.5.2 Properties of EPS produced by Cloacibacterium normanense as bioflocculant

The results of sludge flocculation activity (FA) using S-EPS are presented in Figure 8-6A. The turbidity decreased with addition of 13 mg S-EPS/g sludge. For the conditions tested, the exopolysaccharide shows a higher flocculating capacity (72 ± 1.3 %) compared to 66 ±1.5 % using 10 mg Zetag /g sludge. Increasing the S-EPS concentration beyond 13 mg S-EPS/gm sludge had no significant improvement in supernatant turbidity; therefore, 13 mg S-EPS/g sludge was selected as optimum concentration. **Erreur ! Source du renvoi introuvable.**B presents the beakers used in the laboratory test for the sludge flocculation experiments. It is apparent from the figure that the turbidity of S-EPS is comparable to that of Zetag. Further, it is evident from the picture that the clarity of the supernatant for 13 mg S-EPS/ g SS is similar to that of 18 mg S-EPS/ g SS.

The efficiency of EPS produced by *Cloacibacterium normanense* as bioflocculant for secondary sludge settling was studied by (Nouha, 2016). The high flocculation activity of EPS was due to structure–function relationships. The EPS structure and chemical composition rich on proteins and carbohydrates could explain the highest flocculation activity obtained by EPS producing *Cloacibacterium normanense*.

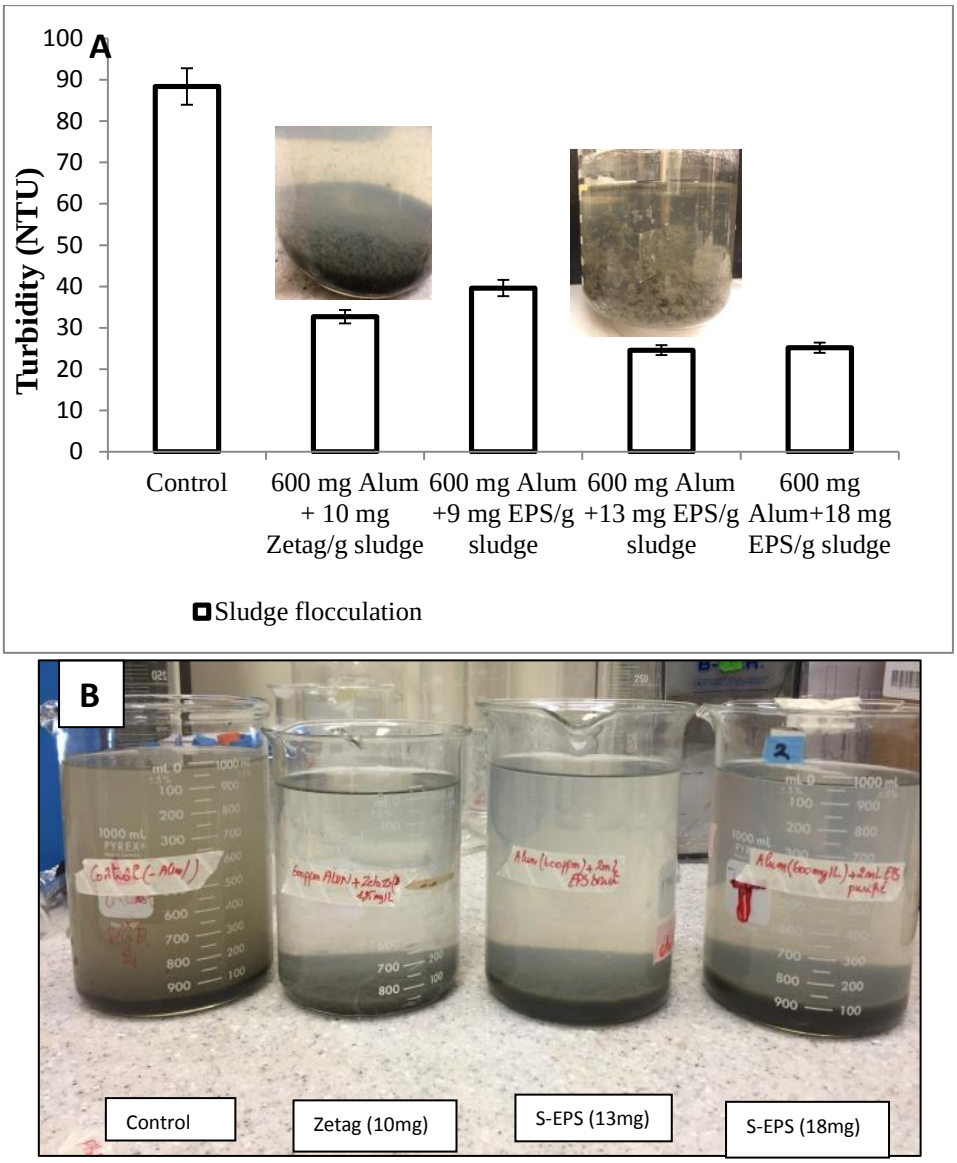

Figure 8-6: (A)Comparative study of sludge flocculation using Zetag and EPS produced by *Cloacibacterium normanense* (B) Jar test pictures of secondary municipal sludge flocculation by control (-), Zetag and S-EPS.

8.5.3 Profiles of the accumulated lipids

The efficiency of lipid conversion to FAMEs is 36.5 % (w/w) of total lipids in case of C/N 100 at 72 h (Table 8-5). Lipid mainly contains C16, C18 and C20 fatty acid methyl ester, which is an indication that the lipids produced is suitable for the production of a biodiesel with excellent burning characteristics (Giroud et al., 2013). This can be accounted by the fact the esterification reaction was catalyzed by base (NaOH) and the lipid obtained in this study contains high content of free fatty acids (FFAs, 11% w/v data not shown) and principally all the FFA was saponified to soap which may yield low transesterification. The FFA can be contributed by the residual crude glycerol or from thermal hydrolysis of sludge which was used as carbon source. Further the lipid extraction was conducted by chloroform, thus the crude chloroform extract may include cellular bodies which are soluble in chloroform like FFA, triacylglycerides, PHA etc.

Table 8-5 presents the lipid composition of *Cloacibacterium normanense* (NK6) for C/N 25 and 100 at 48 and 72 h of fermentation. We choose these two ratios due to the optimum concentration of lipid accumulated (C/N 100) and the significant results of both lipid and EPS concentration in case of C/N 25.

The composition of lipid considerably depends on the C/N ratio of the medium. There are several studies which showed that the lipid profile changed with fermentation time (Papanikolaou & Aggelis, 2002). Elaidic (C18:1n9t) was found to be the major component in all cases. *Cloacibaterium normanense* isolate produced the following unsaturated fatty acids in high quantity: Linoleic (C18:2n6c) and Palmitic (C15:0) (Table 8-5), Palmitoleate (C16:1n7), Linoleic acid (C18:2n6c), stearic (C 18:0) and Eicosenoate (C20:1n9) in minor quantities. Elaidic acid fraction increased from 38.6% (C/N 25) at 48th hour to 42.45 % (C/N 25) at 72nd hour. Similarly for C/N 100, the Elaidic acid fraction increases from 50.5% w/w (C/N 100) at 48th h of fermentation to 53.49% w/w (C/N100) at 72nd hour. In both cases (C/N 25 and 100), the Elaidic acid increases with the fermentation time (Table 8-5). For other fatty acids (stearic, palmitic linoleic and palmitoleate), the FFA fractions remained constant with fermentation time.

Patil et al., (2011) investigated the effect of the C/N ratio on lipid production and on fatty acid composition of lipids in *L. starkeyi* cultivated under different operating conditions. In his study lipid content in cells increased from 19 to 30%. As the C/N ratio increased from 20 to 61, the fatty acid composition of the lipid were also increasing for C16:0 (38.7 to 44.8%), C18:1 (40.7 to 50.2%) and small amounts of C16:1 (5.9 and 14.5%) were also detected. (H. Hu & Gao, 2006) found that the fatty acid composition was influenced by the concentration of the nitrogen. The nitrogen content present in the medium significantly altered the saturated and

unsaturated fatty acid compositions. Similar observations were reported by Huang et al. (2010).

Table 8-5 : Lipid profile of *Cloacibacterium normanense* grown in activated sludge in different culture conditions

C/N ratio/fermentation time	The efficiency of lipid conversion to FAMEs (% w/w)	Fatty acids	Relative amount of total fatty acid (% w/w)
25/ 48 h	24.8	Palmitic (C16:0)	14.85
		Palmitoleate (C16:1n7	1.27
		Stearic (C18:0)	4.19
		Elaidic (C18:1n9t)	38.60
		Linoleic (C18:2n6c)	36.69
		Linolenate (C18:3n3)	4.40
25/ 72 h	27.6	Palmitic (C16:0)	15.87
		Palmitoleate (C16:1n7	2.07
		Stearic (C18:0)	4.59
		Elaidic (C18:1n9t)	42.45
		Linoleic (C18:2n6c)	35.02
100/ 48h	23.9	Palmitic (C16:0)	18.44
		Palmitoleate (C16:1n7	2.90
		Stearic (C18:0)	5.65
		Elaidic (C18:1n9t)	50.57
		Linoleic (C18:2n6c)	21.09
		Eicosenoate (C20:1n9)	0.65
100/ 72 h	36.5	Palmitic (C16:0)	18.53
		Palmitoleate (C16:1n7	2.90
		Stearic (C18:0)	5.49
		Elaidic (C18:1n9t)	53.49
		Linoleic (C18:2n6c)	19.59

8.6 Conclusion

The C/N ratio is a crucial parameter for the EPS production and the accumulation of lipids by *Cloacibacterium normanense*. The study elucidates the importance of C/N ratio; the highest amounts of lipids and EPS concentration were obtained in activated sludge using C/N 25. Further a comparison of S-EPS produced by the microbe was compared with commercial flocculants, and it was found that S-EPS has comparable flocculating efficiency in lab experiments. The fermentation produces simultaneously two products, microbial oil (7.8 g/L) and EPS (22.4 g/L) using C/N ratio 25. The lipids obtained can be used for biodiesel production while EPS can be used to replace chemical flocculants after pilot scale testing.

8.7 Acknolwledgements

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8.8 References

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Annexes

Annexe 1: Données complémentaires au Chapitre 3

Gamme Étalon de carbohydrates, de protéines

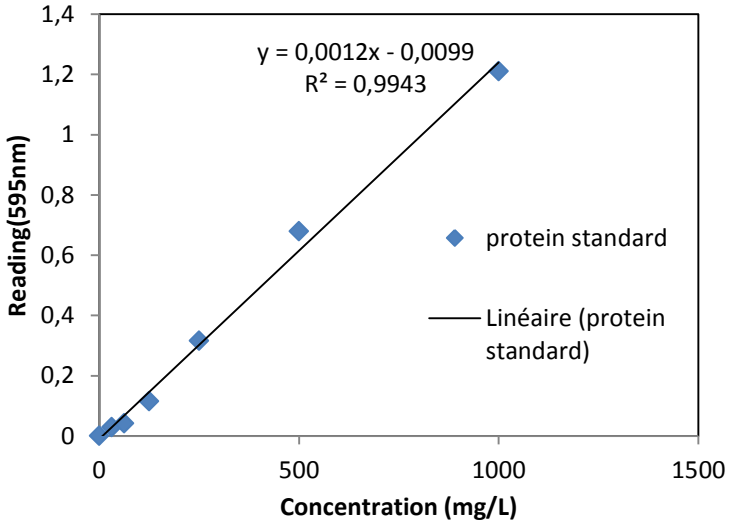

Figure A : Courbe d'étalonnage du dosage des protéines par la méthode Bradford

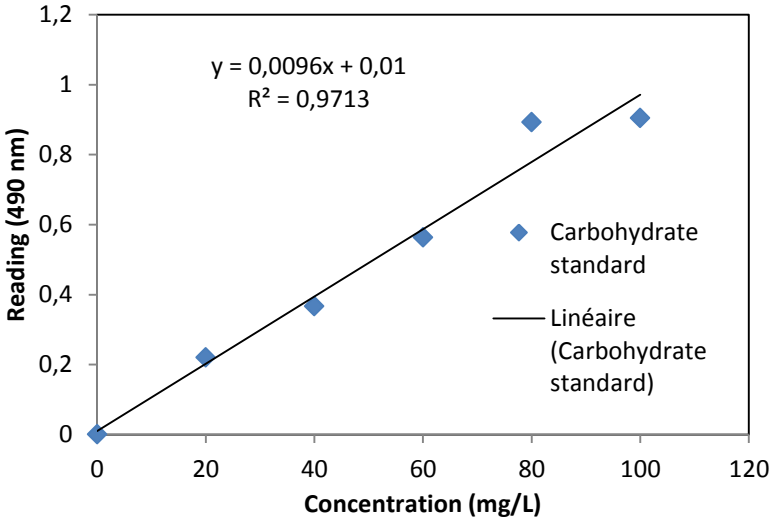

Figure B : Courbe d'étalonnage du dosage des hydrates de carbone par la méthode de Debois

Annexe 2: Données complémentaires au Chapitre 4

Optimisation de paramètres opérationnels

(Matière en suspension, pH, Température, Taille d'inoculum)

Tableau A : Optimisation de la matière en suspension

Temps	CFU/mL (15 g/L)	S-EPS	C-EPS	Total EPS
0	1.55E+06	1.5	0.2	1.7±0.2
12	9.10E+06	2.1	0.35	2.45±0.5
24	1.12E+07	4.4	0.25	4.65±0.5
36	1.52E+09	6.5	0.35	6.85±0.3
48	7.90E+10	10.8	0.3	11.1±0.5
72	7.51E+10	11.5	0.2	11.7±0.5
Temps	**CFU/mL (20 g/L)**	**S-EPS**	**C-EPS**	**Total EPS**
0	9.30E+06	1.58	0.2	1.78±0.7
12	1.32E+07	4.6	0.2	4.8±0.2
24	3.32E+08	6.1	0.25	6.35±0.5
36	4.95E+11	6.6	0.3	6.9±0.5
48	1.84E+12	12.1	0.27	12.37±0.8
72	1.92E+12	10.6	0.2	10.8±0.4
Temps	**CFU/mL (25 g/L)**	**S-EPS**	**C-EPS**	**Total EPS**
0	8.60E+06	1.7	0.2	1.9±0.2
12	1.28E+07	4.6	0.2	4.8±0.3
24	7.80E+08	6.2	0.25	6.4±0.5
36	8.82E+11	7.8	0.28	8.08±0.5
48	3.52E+12	13	0.32	13.32±1.5
72	2.73E+12	11.4	0.2	11.6±0.3
Temps	**CFU/mL (30 g/L)**	**S-EPS**	**C-EPS**	**Total EPS**

0	8.00E+06	1.9	0.2	2.1±0.2
12	3.90E+07	4.5	0.2	4.7±0.3
24	7.20E+08	6.5	0.25	6.75±0.5
36	9.10E+10	7.2	0.35	7.55±0.5
48	2.88E+12	12.8	0.32	11.92±0.9
72	1.69E+12	12.1	0.2	12.3±0.5

Tableau B : Optimisation de la taille d'inoculum

Temps	**CFU/mL (3%)**	**EPS (3%)**	**CFU/mL (1%)**	**EPS (1%)**	**CFU/mL (5%)**	**EPS (5%)**	**CFU/ml (10%)**	**EPS (10%)**
0	8.60E+04	1.9±0.2	1.30E+03	1.95±0.2	8.40E+04	1.85±0.2	1.50E+03	1.9±0.2
6	3.20E+05		6.40E+03		2.80E+05		6.40E+05	
12	4.54E+05	3.8±0.5	7.50E+04	2.6±0.4	4.64E+05	3.6±0.5	3.50E+05	4.3±0.2
18	3.82E+06		9.30E+04		3.85E+06		9.83E+06	
24	2.33E+08	7.4±0.5	1.90E+05	6.8±0.4	2.10E+08	7.8±0.3	4.54E+07	8.4±0.5
30	4.61E+08		3.60E+06		4.61E+08		2.56E+07	
36	5.80E+08		9.40E+06		5.70E+08		4.40E+07	
48	7.51E+08	13.3±0.4	3.70E+07	11.1±0.5	7.11E+08	10.8±0.5	5.70E+07	9.3±0.5
60	7.54E+08		4.30E+07		7.24E+08		3.02E+07	
72	7.80E+08	11.4±0.5	4.50E+07	11.2±0.3	6.10E+08	10.4±0.5	3.50E+07	9.7±0.3
96	6.63E+08		5.10E+07		5.63E+08		3.10E+07	

Tableau B : Optimisation de la taille d'inoculum

Agitation (rpm)	150	180	200	220	250
EPS (g/L)	12.2 ± 1.2	13.3 ± 0.5	13 ± 0.5	12.4 ± 0.3	11.8 ± 0.4
DO 600 nm	1.76	1.76	1.71	1.69	1.66

Tableau C : Optimisation du pH

pH	4	5	6	7	8	9
EPS (48h)	8.4	9.9	12.2	13.3	11.9	9.1
DO 600 nm	1.41	1.47	1.66	1.72	1.6	1.45

Tableau D : Optimisation du Température

Température (°C)	16	30	40
EPS (g/L) (48 h)	10.2	13.3	8.1
DO 600 nm	1.47	1.75	1.37

Tableau E : Optimisation de la taille d'inoculum

Temps	Inoculum (5%)	EPS (g/L)	Inoculum 3%	EPS (g/L)	Inoculum 10 %	EPS (g/L)
0	8.60E+05	1.9±0.2	2.60E+05	1.85±0.3	4.50E+05	1.7±0.5
6	1.20E+06		4.20E+05		3.82E+05	
12	1.54E+06	3.7±0.5	9.54E+05	3.4±0.5	6.54E+05	4.6±0.9
18	3.82E+06		1.32E+06		1.02E+06	
24	2.33E+07	4.8±0.4	7.33E+07	4.2±0.5	5.33E+07	5.8±0.7
30	4.30E+07		1.11E+07		9.11E+06	
36	6.80E+08		6.80E+07		5.60E+07	
48	2.91E+09	15.7±1.5	5.10E+08	14.1±0.5	2.99E+08	10.4±0.5
72	2.80E+09	21.7±1.3	4.80E+08	19.7±0.9	4.30E+08	14.8±1.1
96	1.73E+09	20.8±0.5	9.70E+08	16.5±1	8.20E+08	15.9±1

Annexe 4: Données complémentaires au Chapitre 5

Dosage des acides nucléiques

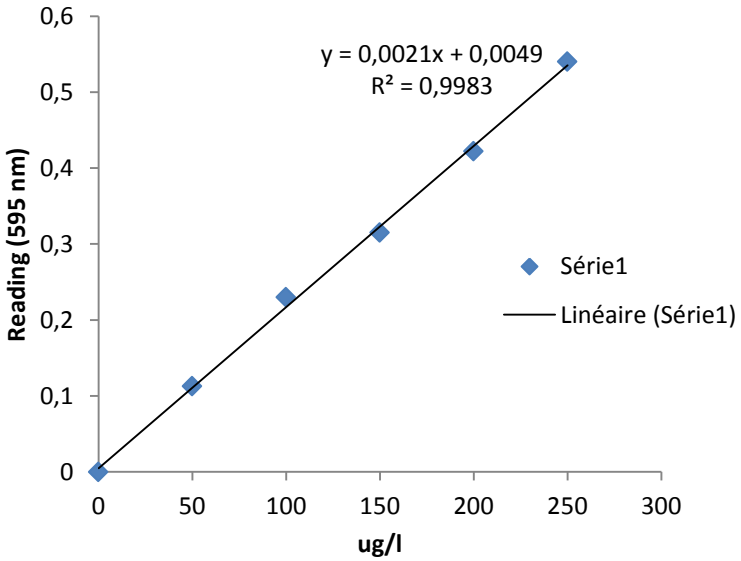

Figure C : Courbe d'étalonnage du dosage des acides nucléiques par la méthode de Burton

Annexe 4: Données complémentaires au Chapitre 6

Effet de la source de carbone sur la production de SPEs

Tableau E : Effet de la source de carbone

2%	EPS (24h)	EPS (24)	EPS(48)	EPS(72)
Glucose	6.48±0.5	16.2±0.6	23.8±0.5	22.2±0.5
Glycérol	6.48±0.5	15.7±0.5	21.7±0.5	20.8±0.5

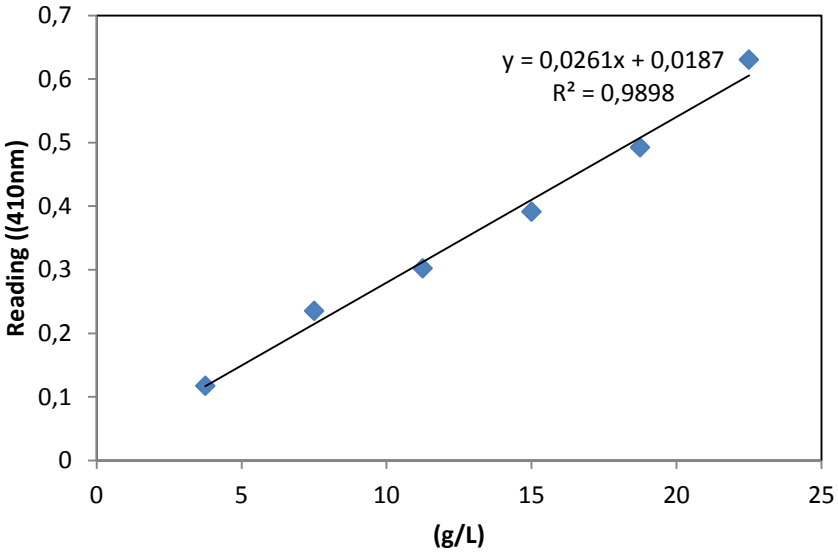

Figure C: Courbe d'étalonnage du dosage de glycérol par la méthode de Bondioli