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LA PRODUCTION DE SUBSTANCES POLYMÉRIQUES DÉ EXTRACELLULAIRES PAR CULTURE PURE ET MIXTE UTILISANT LES BOUES D'ÉPURATION COMME MATIÈRES PREMIÈRES ET APPLICATIONS DANS LE TRAITEMENT DES EAUX NATURELLES ET USÉES

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To my family, teachers, friends and well wishers who have lend me a moral support during this travail

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RÉSUMÉ

Les substances polymériques extracellulaires (SPE) sont des biopolymères produits par les micro-organismes, ils sont considérés comme des alternatives durables, non toxiques et moins chères, peuvent remplacer les floculants chimiques grâce à leurs applications dans les différents processus de traitement des eaux usées et de traitement des boues. La production des SPE peur être très efficace en utilisant les boues municipales comme source de carbone et une voie prometteuse pour la gestion durable des déchets. Dans ce contexte, la caractérisation biochimique de 13 SPE produites par des souches bactériennes a été réalisée par le test BIOLOG. Les souches bactériennes ont été cultivées dans des boues stérilisées pour la production des SPE. La floculation et la capacité de déshydratation des SPE produites (bouillon, les SPE solubles et capsulaires brutes) ont été testées en utilisant la solution de kaolin combinée avec du calcium (150 mg de Ca^{2+}/L de suspension de kaolin). Le test BIOLOG a révélé qu'il y avait 9 *Bacillus*, 2 *Serratia* et 2 *Yersinia*. En effet, les concentrations des SPE produites par différentes souches de *Bacillus* étaiest plus élevées que celles de *Serratia* et *Yersinia*. Le Bouillon des EPS a montré une activité de floculation plus de 75% pour *Bacillus* sp.7, *Bacillus* sp.4 et *Bacillus* sp.6, respectivement. Des activités de floculation supérieure à 75% ont été atteintes en utilisant de très faibles concentrations de bouillon des SPE de 1,12 à 2,70 mg SPE /g SS.

La cinétique de fermentation discontinue de treize souches bactériennes (9 *Bacillus*, 2 *Serratia* et 2 *Yersinia*) a été étudiée utilisant des boues stérilisées comme une matière première. De ce fait, la plupart des souches comme *Bacillus* (μmax/h: de 0,11 à 0,27), *Serratia* (μmax/h: 0,23 à 0,27) et *Yersinia* (μmax/h: 0,18 à 0,19) ont la capacité de croître et produire des SPE (1.4 à 2.1 g/L) dans les boues stérilisées. En général, la production des SPE est liée à la croissance bactérienne. En outre, les souches *Bacillus* sp. 7, *Serratia* sp. 2 et *Yersinia* sp. 2 produisent des concentrations des SPE plus élevées (1,9-2,1 g/L) que celles des autres souches bactériennes. Les teneurs en protéines et glucides contenues en SPE demeurent constantes au cours de la fermentation. En effet, le Bouillon SPE présente une forte activité de floculation de kaolin (≥ 75%) dans la plupart des cas, à l'exception de *Bacillus* sp. 1, *Bacillus* sp. 5 et *Bacillus* sp. 9, respectivement. En général, les plus importantes activités de floculation (≥ 75%), ont été atteintes en utilisant 1,31 à 1,70 mg B-SPE/g de kaolin, de 0,45 à 0,97 mg protéine/g de kaolin et de 0,11 à 0,21 mg des glucides/g de kaolin.

Afin d'optimiser les concentrations de solides en suspension de boues et le prétraitement requis pour une meilleure production des SPE, une stérilisation, un traitement alcalin thermique et un traitement thermique et acide ont été appliqués à différents concentrations de solides en suspension de boues (17,0; 22,4; 29,8; 37,3; 44,8 g/L, respectivement). Des boues prétraitées ont été utilisées comme matière première pour la production des SPE par *Serratia* SP.1. Après 72 h de fermentation, La concentration de SPE produite est de 2,3 et 3,4 g/L en cas de traitement thermique et alcalin. Par contre, elle était 1,5 g/L dans les boues traitées thermiquement à pH acide. Des concentrations inférieures des SPE ont été produites à des concentrations relativement élevées de solides en suspension (37,3; 44,8 g/L). Le Bouillon, les SPE capsulaires brutes et slime SPE ont été extraits et utilisés comme agents de conditionnement en combinant 150 mg de Ca^{2+}/L de suspensions de kaolin. L'activité de floculation maximale est de 79,1% et une capacité déshydratation de 52,2% ont été obtenues utilisant le bouillon et les SPE EPS capsulaires brutes, respectivement.

Treize souches bactériennes produisant des EPS (9 *Bacillus*, 2 *Serratia* et 2 *Yersinia*) ont été cultivées (comme culture mixte) dans la boue stérilisée (matières en suspension: 25 g/L). Une Optimisation de la vitesse d'agitation et la cinétique de production de SPE en fermentation discontinue par la culture mixte a été effectuée. La culture mixte produit des concentrations plus élevées de SPE (4,9 g/L) par rapport à celle de la culture pure (2.7-3.7 g/L). La vitesse d'agitation optimale était de 150 tours par minute avec la production de SPE de 4,9 g/L à 72 h. La production de SPE a été liée à la croissance bactérienne. Le Bouillon (B-SPE) a révélé une haute activité de floculation en kaolin (91,2%) à des concentrations très faibles (0,8 mg B-SPE/g kaolin) et elle était comparable à celle du polymère chimique, Magnafloc-155 (90,4% à 0,2 mg/g de kaolin). Le B-SPE a montré une AF de kaolin supérieure lorsqu'il est combiné avec des cations divalents (Ca^{2+} et Mg^{2+}) en comparaison avec les cations trivalents (Fe^{3+} et Al^{3+}). Avec l'ajout de cations trivalents (Fe^{3+} et Al^{3+}), les performances de floculation (turbidité et DCO) de B-SPE dans la rivière (90%), les eaux usées municipales (80,7%) et la brasserie des eaux usées (80-84%) était meilleures que le Ca^{2+} et Mg^{2+}.

La présente étude a montré que les treize souches bactériennes (9 *Bacillus*, 2 *Serratia* et 2 *Yersinia*) ont la capacité de produire des SPE utilisant les boues stérilisées comme matière première. Les souches de *Bacillus* produisent une concentration de SPE plus forte que celle produite par *Serratia* et *Yersinia*. Ainsi, La production de SPE n'a pas été totalement liée à la croissance bactérienne pour toutes les souches étudiées lorsqu' elles sont incubées indépendamment dans la boue stérilisée alors que, elle a été associée à la croissance dans le

cas de la culture mixte. La culture mixte a produit des concentrations de SPE plus élevées en comparaison aux cultures pures. En fait, le consortium de culture mixte peut être utilisé pour la production de flocculants avec un haut rendement. L'activité de floculation était comparable à Magnafloc-155 (polymère chimique) et, par conséquent, il pourrait être utilisé comme floculant dans les applications de contrôle de la pollution environnementale. Les résultats sont très encourageants et justifient la poursuite de la recherche sur l'optimisation de processus dans des fermenteurs de laboratoire avec un contrôle du pH, de la température et la concentration en oxygène dissous, etc.

ABSTRACT

Extracellular polymeric substances (EPS) are biopolymers produced by the microorganisms, they are considered as an eco-friendly, cost effective and sustainable alternatives to substitute the existing chemical flocculants for their applications in various water, wastewater and sludge treatment processes. Highly efficient EPS production using municipal wastewater sludge could be a promising avenue for the sustainable waste management. In this context, the biochemical characterization of 13 extracellular polymeric substances (EPS) producing bacterial strains was carried out by BIOLOG. The bacterial strains were cultured in sterilized sludge for EPS production. Flocculation and dewatering capabilities of produced EPS (broth, crude slime and capsular) were examined using kaolin suspension combined with calcium (150 mg of Ca^{2+}/L of kaolin suspension). BIOLOG revealed that there were 9 *Bacillus*, 2 *Serratia* and 2 *Yersinia* species. Concentration of EPS produced by different *Bacillus* strains was higher than that of *Serratia* and *Yersinia*. Broth EPS revealed flocculation activity more than 75% for *Bacillus* sp.7, *Bacillus* sp.4 and *Bacillus* sp.6, respectively. Flocculation activity higher than 75% was attained using very low concentrations of broth EPS (1.12-2.70 mg EPS/g SS). The kinetics of batch fermentation of thirteen bacterial strains (9 *Bacillus*, 2 *Serratia* and 2 *Yersinia*) was carried out using sterilized sludge as raw material. The most of *Bacillus* (μ_{max}/h: 0.11-0.27), *Serratia* (μ_{max}/h: 0.23-0.27) and *Yersinia* (μ_{max}/h: 0.18-0.19) strains had capability to grow and produce EPS (1.4-2.1 g/L) in sterilized sludge. In general, EPS production was mixed growth associated. *Bacillus* sp. 7, *Serratia* sp. 2 and *Yersinia* sp. 2 produced higher concentration (1.9-2.1 g/L) of EPS than the rest bacterial strains. Protein and carbohydrate contents of EPS remained constant during fermentation. Broth EPS exhibited high kaolin flocculation activity (≥ 75%) in most of the cases except *Bacillus* sp. 1, *Bacillus* sp. 5 and *Bacillus* sp. 9, respectively. In general, high flocculation activities (≥ 75%), were attained using 1.31-1.70 mg B-EPS/g kaolin, 0.45-0.97 mg protein/g kaolin and 0.11-0.21 mg carbohydrates/g kaolin.

To optimize the suspended sludge solids concentration and pre-treatment required for maximum EPS production, the sterilization, alkaline-thermal and acid-thermal treatments were applied to different sludge solids concentrations (17.0; 22.4; 29.8; 37.3; 44.8 g/L, respectively). The pre-treated sludge was used as raw material for *Serratia* sp.1 to produce EPS. After 72 h of fermentation, total EPS of 2.3 and 3.4 g/L were produced in sterilized and alkaline-thermal treated sludge as compared to that of 1.5 g/L in acid-thermal treated sludge. Lower EPS were produced at relatively higher solids concentrations (37.3; 44.8 g/L). Broth, crude forms of

capsular and slime EPS were extracted from fermented broths and used as conditioning agents by combining with 150 mg of Ca^{2+}/L of kaolin suspensions. Maximum flocculation activity of 79.1% and increased dewatering by 52.2% was achieved using broth and crude capsular EPS, respectively.

Thirteen EPS producing bacterial strains (9 *Bacillus*, 2 *Serratia* and 2 *Yersinia* sp.) were cultivated (as mixed culture) in sterilized sludge (suspended solids: 25 g/L). Optimization of the agitation speed and EPS production kinetics in batch fermentation for the mixed culture was carried out. The mixed culture produced higher concentrations of EPS (4.9 g/L) as compared to that of the pure culture (2.7-3.7 g/L). The optimum agitation speed was 150 rpm with the production of 4.9 g/L EPS at 72 h. The EPS production was growth associated. Broth (B-EPS) revealed high kaolin flocculating activity (91.2%) at very low concentrations (0.8 mg B-EPS/g kaolin) and it was comparable to the chemical polymer, Magnafloc-155 (90.4% at 0.2 mg/g kaolin). Broth EPS revealed higher kaolin FA when combined with divalent cations (Ca^{2+} and Mg^{2+}) as compared to the trivalent cations (Fe^{3+} and Al^{3+}). With the addition of trivalent cations (Fe^{3+} and Al^{3+}), flocculation performance (turbidity and COD removal) of B-EPS in river water (90%), municipal wastewater (80.7%) and brewery wastewater (80-84%) was better than the Ca^{2+} and Mg^{2+}.

The study revealed that all the thirteen bacterial strains (9 *Bacillus*, 2 *Serratia* and 2 *Yersinia* species) have capability to produce EPS using sterilized sludge as sole raw material. *Bacillus* strains produced higher concentration of EPS than *Serratia* and *Yersinia.* EPS production was mixed growth associated for all the bacterial strains when incubated independently in the sterilized sludge whereas, it was growth associated in case of the mixed culture. The mixed culture had produced higher concentrations EPS as compared to the pure cultures using sterilized sludge as a raw material. The mixed culture consortium could be used for the production of highly efficient flocculants. The flocculation performance of EPS was comparable to Magnafloc-155 (chemical polymer) and hence, it could be used as a flocculant in environmental pollutions control applications. The results are very encouraging and warrant further research on process optimization in laboratory fermenters under controlled conditions of pH, temperature and dissolved oxygen concentration etc.

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LISTE DES ABRÉVIATIONS

ACT	Acid-thermal treatment
Al^{3+}	Aluminium ions
ALT	Alkaline-thermal treatment
APHA	American Public Health Association
B-EPS	Broth EPS
BIOLOG	BIOLOG bacterial identification system
C/N ratio	Carbon to nitrogen ratio
Ca^{2+}	Calcium ions
Ca-Kaolin-EPS	Calcium (Ca^{2+}), kaolin suspension in water and EPS
C-EPS	Capsular EPS
CER	Cation exchange resin
CFU	Capillary suction time
COD	Chemical oxygen demand
Crude C-EPS	Broth sediments containing C-EPS
CST	Capillary suction time
Da	*Dalton*
DLVO	Derjaguin–Landau–Verwey–Overbeek
DNA	Deoxyribonucleic acids
E-DNA	Extracellular deoxyribonucleic acids
EDTA	Ethylenediamine tetraacetic acid
EPS	Extracellular polymeric substances or microbial polymers or bioflocculants
FA	Flocculation activity
Fe^{3+}	Ferric ions
FF	Filamentous fungi

Flocculation activity	(1-*S/C*) x100 (%), where *C* is control turbidity and *S* is sample turbidity
GN/GP	Gram's negative/gram's positive
HA	Humic acid
INRS-ETE	Institut national de la recherche scientifique, Centre Eau, Terre et Environnement
Kaolin control	Kaolin suspension in deionized water
LB-EPS	Loosely bound EPS
mg EPS/g of kaolin	mg of EPS added per gram of kaolin suspension in water
Mg^{2+}	Magnesium ions
NOM	Natural organic matter
NTU	Nephelometric Turbidity Units
OD	Optical density
P (AM-DMC)	Poly(acrylamide[2-methacryloyloxy)ethyl] trimethylammonium chloride
PAC	Poly aluminium chloride
PAHs	Polycyclic aromatic hydrocarbons
PAM	Polyacrylamide
PAM	Polyacrylamide
PGA	Poly-gamma-glutamate
*q*EPS	Specific EPS production rates
rpm	Revolutions per minute
S-EPS	Slime EPS
sp.	one species
spp.	two or more species
SRF	Specific resistance to filtration
SRT	Solids retention time
SS	Suspended solids

ST	Sterilization
SVI	Sludge volume index
TB-EPS	Tightly Bound EPS
TPHs	Total petroleum hydrocarbons
TS	Total solids
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TSS	Total suspended solids
VS	Volatile solids
VSS	Volatile suspended solids
ζ	Zeta Potential
μmax	Maximum specific growth rate

CHAPITRE 1

SYNTHÈSE

PARTIE 1: SYNTHÉSE BIBLIOGRAPHIQUE

1.1 Les substances polymériques extracellulaires (SPE)

Les substances polymériques extracellulaires (SPE) sont des bio-polymères produits par les micro-organismes. Elles sont considérées comme des substances non toxiques, efficaces, peu coûteuses, et peuvent avantageusement remplacer les floculants chimiques dans divers processus de traitement des eaux usées. Les SPE sont formées par différentes substances chimiques sécrétées par les micro-organismes lors de la lyse cellulaire. La sécrétion des SPE est un élément de caractérisation des micro-organismes dans les milieux naturels. Ces substances peuvent être produites par des cellules procaryotes et eucaryotes (Flemming et Wingender 2001; Wingender et al. 1999). Elles constituent le biofilm visqueux. En général, les SPE dans un biofilm varient de 50% à 90% (Flemming et Wingender 2001). Elles sont définies comme capsulaires (C-SPE), soluble (S-SPE), faiblement liées (LB-SPE) et étroitement liées (TB-SPE) en considérant leur capacité d'association avec les cellules ou la méthode d'extraction utilisée (Tableau 1.1). Les composés organiques produits par des micro-organismes dans les boues activées peuvent être regroupés en trois catégories: i) les composés sécrétés par les micro-organismes en raison de leur interaction avec l'environnement; ii) les composés produits suite au métabolisme de substrat et la troisième consiste à la croissance bactérienne et des composés libérés lors de la lyse cellulaire et/ou la dégradation des micro-organismes ou des composants microbiens.

Les fonctions de base des SPE sont l'agrégation des cellules bactériennes, l'adhésion aux surfaces, la formation de flocs et biofilm et elles représentent, des éléments de communication et de reconnaissance entre cellules (par exemple, l'adhésion cellulaire). De plus, elles sont définies comme des éléments de structure de biofilms, considérés comme une barrière de protection contre les agressions extérieures. Les SPE ont un rôle dans la rétention d'eau pour minimiser la dessiccation de la cellule, l'adsorption de composés organiques exogènes, et l'adsorption d'ions inorganiques. Aussi, ces bio-polymères possèdent des activités enzymatiques et permettent particulièrement l'interaction des polysaccharides avec des enzymes (Tian 2008; Wingender et al. 1999.). Les polysaccharides, les protéines, les substances humiques et les acides nucléiques sont les principaux composants des SPE. Cette matrice polymérique a un caractère d'adsorption, de biodégradabilité et d'hydrophobicité. En effet, Les SPE ont un rôle important dans la formation de biofilm, le transfert de masse par l'intermédiaire d'un biofilm, l'adsorption de différents métaux et des composés

organiques/inorganiques et surtout, elles fournissent un soutien structurel au biofilm (résistant au cisaillement) (Czaczyk 2007; Flemming et Leis 2003; Flemming et al. 2005; Neyens et al. 2004).

Tableau 1.1 Les types des SPE

La base de classification	SPE	Remarques	Références
Nature de l'association de SPE avec les cellules	Slime	Sont les polymères qui sont présents dans le surnageant après centrifugation de la biomasse. La plupart sont sous forme soluble ou détachées des cellules sous forme de colloïdes.	(Barker et Stuckey. 1999; Comte et al. 2006a; Laspidou et Rittmann. 2002; Raszka et al. 2006; Tian. 2008; Wingender et al. 1999)
	Capsulaire	Sont la partie permanente de la membrane cellulaire et sont liées au culot (cellules bactériennes).	
État physico-chimique et la composition des SPE	Solubles	Sont secrétées par les cellules sous forme dissoute dans le milieu. Les principaux composants sont des macromolécules et des colloïdes.	(Barker et Stuckey. 1999; Comte et al. 2006a; Laspidou et Rittmann. 2002; Raszka et al. 2006; Tian. 2008;. Wingender et al. 1999)
	Liées	Les SPE liées aux cellules. Les composants des SPE consolidés sont des gaines, des polymères capsulaires, gel condensée, des polymères faiblement liés et des matières organiques ci-joint.	

1.2 Les principales propriétés des SPE

La matrice de SPE a une grande influence sur les propriétés des agrégats microbiens en raison de leurs caractéristiques particulières telles que l'adsorption, la biodégradation et l'hydrophobicité. Ces propriétés dépendent principalement des composantes des SPE tels que les polysaccharides, les protéines, les acides nucléiques et les substances humiques (Tableau 1.2). Les valeurs présentées dans le Tableau 1.2 représentent la gamme typique des composants des SPE. Des teneurs relativement faibles en protéines et en polysaccharides ont été observées par Neyens et al. (2004). En fait, cette variation pourrait être due à différents facteurs tels que l'échantillonnage, le type de boues d'épuration; les conditions de fonctionnement et les méthodes d'extraction. Cette matrice polymérique est de nature ionique, hydrophile/hydrophobe et liée avec la matrice biologique (de la biomasse) à travers différentes interactions chimiques (Mayer et al. 1999). Les processus microbiens tels que la production de SPE et leur dégradation sont également liés à des enzymes fonctionnellement actives. Par conséquent, la composition chimique de ces bio-polymères peut être corrélée à des propriétés physiques et dynamiques de SPE.

Tableau 1.2 La Composition et les propriétés principales des SPE

Composants des SPE	Contenu typique dans la matrice de SPE	Les principales propriétés de différents composants	Remarques	Références
Les Polysaccharides	40-95%	L'adhésion, l'agrégation de cellules bactériennes, la rétention d'eau, l'adsorption des composés organiques et inorganiques, la liaison des enzymes, une source nutritive, une barrière protectrice pour les cellules	Joue un rôle principal dans la floculation et la bio-sorption. La concentration et les caractéristiques des polysaccharides peuvent décider du sort des propriétés de surface ainsi que la biodégradabilité des SPE.	(Flemming et Wingender, 2010; Tian 2008; Wingender et al, 1999)
Les protéines	1-60%	L'adhésion, l'agrégation de cellules bactériennes, la rétention d'eau, la sorption de composés organiques et inorganiques, la liaison des enzymes, donneur ou accepteur d'électrons, une barrière de protection pour les cellules.	Joue un rôle principal dans la floculation et la bio-sorption. La concentration et les caractéristiques de la protéine décide du sort des propriétés de surface ainsi que la biodégradabilité des SPE.	(Flemming et Wingender 2010; Tian 2008; Wingender et al. 1999)
Les acides nucléiques	1-10%	Adhésion, l'agrégation des cellules bactériennes, source de nutriments, échanger l'information génétique, exportation de composants de la cellule	Les acides nucléiques ont un rôle important dans la floculation et la bio-sorption en fonction de la quantité et des composants disponibles.	(Flemming et Wingender 2010; Tian 2008;.Wingender et al. 1999)
Les Lipides	1-10%	L'exportation de composants cellulaires	rôle majeur et mineur dans la floculation et la bio-sorption.	(Flemming et Wingender 2010; Tian 2008;.Wingender et al. 1999)
Les substances humiques	-	Adhérence, donneur ou accepteur d'électrons	Rôle mineur dans la floculation et bio-sorption. L'impact des substances humiques dépend principalement de la concentration ainsi que la nature des substances humiques.	(Flemming et Wingender 2010; Tian 2008; Wingender et al. 1999)

1.3 La production de SPE

1.3.1 Les Souches bactériennes

Plusieurs études ont rapporté la production de SPE par des cultures pures (Tableau 1.3). Ces recherches ont négligé les interactions importantes entre les microorganismes dans les cultures mixtes. Les avantages de la production de SPE utilisant des cultures mixtes sur des cultures simples ont été cités par quelques études (Ma et al. 2003; Wang et al. 2011; Zhang et al. 2007; Zhu et al. 2008). La croissance coopérative des micro-organismes que l'on trouve dans des cultures mixtes de certaines souches bactériennes productrices des SPE, ou dans un mélange des souches bactériennes productrices et non productrices des SPE (comme dans le cas de boues activées) peut affecter la concentration et les caractéristiques des bio-polymères produits (Mårtensson et al. 2002). En effet, le bouillon de fermentation de la culture mixte est plus visqueux que celui de la culture pure (Wingender et al. 1999). De plus, la présence de certaines souches bactériennes non productrices des SPE ainsi que les bactéries productrices de SPE dans une culture mixte peut améliorer la production de ces substances polymériques. En fait, lorsque deux ou plusieurs souches microbiennes constituent un biofilm, la présence de SPE peut aider à établir ce dernier et favorise sa croissance plus que le biofilm d'espèces similaires. Aussi, les SPE promeuvent non seulement l'adhérence et la croissance des cellules, qui les synthétisent, mais aussi ceux d'autres espèces microbiennes. En effet, la concentration de SPE produite dans une culture mixte est relativement plus élevée que celle produite dans une culture pure (Li et al. 2012; Zhang et al. 2007). Selon Zhang et al. (2007), environ 15 g/L de SPE purifiées pourrait être récupéré par une culture mixte de *Staphylococcus* et *Pseudomonas sp*. Dans le cas de milieu de croissance complexe comme les eaux usées ou les boues d'épuration utilisées comme matière première pour la production de SPE, la relation symbiotique de certaines souches microbiennes pourrait être bénéfique pour la production de SPE par le consortium. Certains microbes peuvent synthétiser les éléments nutritifs, qui sont utilisés par les autres microbes. Par ailleurs, la sécrétion de certaines enzymes par différents microbes peut aider les microorganismes à satisfaire leur besoins nutritifs à partir du milieu complexe. Dans un tel cas, le rendement de SPE peut augmenter avec l'augmentation du substrat utilisé par la culture mixte.

La relation symbiotique entre certaines souches productrices et non productrices des SPE pourrait être utile pour la production des biopolymères et pour une meilleure floculation. Ma et

al. (2003) ont produit une bioflocculant en cultivant une culture mixte de deux souches de *Bacillus* et ont observé que les SPE obtenues présentent une capacité de floculation plus importante en comparaison à celle de la culture pure. Des résultats similaires ont été observés dans d'autres études sur les SPE produites par la culture mixte de *Rhizobium radiobacter* et *Bacillus sphaericus* (Zhu et al. 2008), *Staphylococcus* et *Pseudomonas sp.* (Zhang et al. 2007), *Rhizobium radiobacter* et *Bacillus sphaericus* (Wang et al. 2011). Il est clair que la présence de SPE à composition différente dans certains biofilms mixtes augmente les propriétés adhésives. Cependant, ce qui n'est pas encore connu, est l'effet de l'élimination partielle ou totale de l'un des polymères à partir d'une telle matrice complexe. La culture mixte a révélé une production de bio-polymères de poids moléculaire élevé et une viscosité plus importante que celle de la culture pure (Li et al. 2012). Bien que ce phénomène symbiotique de cultures mixtes a été étudié pour des paires de souches bactériennes, d'autres n'ont montré aucune amélioration. En effet, le comportement des souches bactériennes dans les cultures mixtes dans certains cas peut affecter la production de SPE ainsi que leurs propriétés polymèriques. Dans certains cas, la culture mixte a diminué la concentration de SPE par rapport à celle de la culture pure. Il a été souvent observé que des très faibles concentrations de SPE sont produites par des cultures mixtes dans les milieux naturels, tels que les boues d'épuration en comparaison à celle des souches pures isolées à partir du même milieu (Subramanian et al. 2010). Par conséquent, trouver les combinaisons symbiotiques des différents microbes est l'étape indispensable pour obtenir la meilleure combinaison pour la culture mixte ayant une forte production de SPE et un potentiel de floculation.

1.3.2 La phase de croissance

La production de SPE peut être soit synonyme de croissance, croissance associée ou croissance indépendante (Barker et Stuckey, 1999). Les corrélations entre la croissance cellulaire et la sécrétion de SPE ont été rapportées dans plusieurs études. Les SPE peuvent être utilisées comme source de carbone et d'énergie suite au manque de substrat. Dans certaines conditions, la production de SPE est le résultat de l'excrétion et de la consommation des cellules microbiennes. Cependant, les résultats rapportés dans la littérature sont légèrement contradictoires. Selon Pavoni et al. (1972), la production des substances polymériques se trouve au cours de la phase endogène d'une culture par lot, par contre Hantula et Bamford (1991) ont montré que leur production commence lors de la phase stationnaire de croissance. Dans d'autres études, elle était associée à la croissance cellulaire (Kurane et al.

1991), au milieu de la phase exponentielle (Farrah et Unz 1976), au début, associé et au milieu de la phase exponentielle d'après Kurane et al. (1991), alors qu'elle augmente avec la phase exponentielle et diminue au cours de la phase stationnaire selon Wingender et al. (1999). En fait, la diminution de la concentration de SPE en phase stationnaire ou dans la phase endogène a été liée à leur dégradation par des enzymes produites au cours de la fermentation. Par conséquent, la concentration de SPE a été diminuée pendant la phase exponentielle en fonction du temps, mais elle était constante au cours de la phase stationnaire (Sheng et al. 2006).

1.3.3 Le Substrat

La production de SPE dépend du type de substrat, la source de carbone et d'azote. La majorité des études ont rapporté la production de SPE en milieux synthétiques ou en milieux simples (Tableau 1.3). Il y à certaines sources de carbone et d'azote à être favorisées en comparaison aux autres, par différents micro-organismes producteurs des SPE (Salehizadeh et Shojaosadati, 2001; Sheng et al. 2010; Wingender et al. 1999). Par exemple, le fructose est favorisé sur le saccharose par *Rhodococcus erythropolis* pour la production de biofloculant (Kurane et al. 1991). Les cellules de *Rhodococcus erythropolis* étaient petites et en forme tige lorsque le saccharose est le substrat utilisé comme source de carbone, alors que dans le cas deu fructose, les cellules sont allongées. De plus, la production de SPE par *Chryseobacterium daeguense-W6* était plus élevée en utilisant le maltose, le mannose et le glucose en comparaison à l'acétate de sodium, le galactose, le phtalate, le saccharose, l'éthanol et l'arabinose en tant que source de carbone (Liu et al. 2010a). Aussi, la production de SPE par *Chryseobacterium daeguense-W6* était plus importante en utilisant la tryptone, la levure et l'extrait de bœuf comme source d'azote, par rapport au sulfate d'ammonium, la peptone, le nitrate d'ammonium, l'hydrolysat de caséine, l'urée et le nitrate de sodium. Selon Liu et al. (2010a) le glucose et la tryptone étaient la meilleure combinaison pour la production de SPE par *Chryseobacterium daeguense.* Li et Yang (2007) ont rapporté que la boue activée additionnée du glucose présente une production de SPE plus élevée que celle supplémentée en acétate. Par contre, la boue nourrie d'acétate a montré des meilleurs résultats dans la floculation et la séparation, avec une diminution de matières solides en suspension, un indice de volume des boues inférieur (IVB) et une résistance spécifique à la filtration (RSF), par rapport à celle enrichie en glucose. Cela était dû à une augmentation de SPE solubles (LB-SPE) contenues dans les boues alimentées du glucose, qui a eu un effet négatif sur la floculation, la clarification,

la décantation et déshydratation des boues. La teneur en protéines des SPE augmente et la teneur en acides nucléiques diminue en changeant les eaux usées de produits chimiques, le cuir et les boues d'épuration industrielle de la teinture par les boues municipales et de vin (Sponza 2002). En effet, la carence en azote dans les boues de vin et les eaux usées municipales a été liée à une augmentation de la teneur en protéines dans les SPE. Dans une autre étude, la boue activée alimentée en acétate de sodium et en glucose a une teneur plus élevée en polymères solubles (LB-SPE) que celle alimentée avec de l'amidon (Ye et al. 2011a). Ils ont également observé que la quantité de SPE étroitement liées (TB-SPE), les polysaccharides et la teneur en protéines dans les TB-SPE et la teneur en protéines dans les LB-SPE, sont indépendants de la source de carbone. En effet, l'ajout d'une source de carbone et d'énergie peut contribuer à la mort de certaines bactéries favorisant ainsi la production de SPE. La concentration du substrat (carbone et azote) a un effet sur la production de SPE (Ye et al. 2011a). La dépendance de SPE sur la concentration du substrat (source de carbone et d'azote) est attribuée au fait que la concentration de substrat régit le taux d'utilisation du substrat et par conséquent la vitesse de formation de SPE.

Un accent profond est donné au rapport C/A (carbone/azote) en lien avec la production de SPE. Cela est dû au fait que le rapport C/A a un impact important sur les métabolismes microbiens et donc sur la production de SPE. En fait, le rapport C/A est critique pour la production des bio-polymères autant que le type de source de carbone et d'azote. En dépit de plusieurs rapports sur le rapport C/A par rapport au SPE, il n'y a aucun rapport C/A fixe et favorable dans la littérature. Cela peut être dû à des variations dans le type de sources de carbone et d'azote et des micro-organismes rapportés. Les analyses de Liu et al. (2010a) ont révélé un rapport C/A optimal de 0,5. L'augmentation ou la diminution du rapport C/A de 0,5 conduit à une baisse en activité de floculation. Dans d'autres études réalisées par Ye et al. (2011b), un rapport C/A de 20 présente une meilleure production de SPE. Lorsque le rapport C/A dans les boues diminu (de 100 à 20), la teneur en glucides dans les LB-SPE a diminué, alors que la teneur en protéines dans les SPE solubles (LB-SPE) augmente. D'autre part, lorsque le rapport C/A augmente, la teneur en glucides dans les LB-SPE augmente, et la teneur en protéine contenue dans les LB-SPE diminue. Cependant, la teneur en glucides et en protéines dans les SPE étroitement liées (TB-SPE) n'a pas été influencée par le rapport C/A. En outre, selon Ye et al. (2011b) le rapport C/A de 20 est favorable, alors que le rapport C/A de 100 ou inférieure à 4 pourrait affecter les propriétés des boues.

Les nutriments sont des composants nécessaires à la croissance bactérienne et pour améliorer la production de SPE, qui jouent un rôle dans la décantation des boues. Le rôle de l'azote et du phosphore en tant que nutriments essentiels pour les procédés de traitement biologique des eaux usées est bien connu. La production de SPE peut être contrôlée en optimisant le taux de nutriments. Les oligo-éléments et les vitamines sont requis par des cellules en plus des six macro-éléments (C, O, H, N, S, P, K, Ca, Mg, et Fe). Les oligo-éléments nécessaires incluent le manganèse (Mn), le zinc (Zn), le cobalt (Co), le molybdène (Mo), le nickel (Ni), le cuivre (Cu), le vanadium (V), le bore (B), le fer (Fe), et l'iode (I) et les vitamines nécessaires incluent K, B1, B2, B6, B12, la biotine, la niacine, l'acide pantothénique. Les micronutriments sont souvent nécessaires à une dose de $<$1 mg/L, ce qui signifie que la mise en évidence de conditions de dosage est techniquement difficile. Les taux de nutriments ont un effet significatif sur la production et la composition des bio-polymères. La teneur de la boue en SPE augmente avec l'augmentation de rapport micro-organisme/nourriture (Janga et al. 2007). La production de SPE pourrait être améliorée par une carence en phosphore (Fang et al. 2009). Bura et al. (1998) et Hoa et al. (2003) ont également constaté que la teneur en glucides des SPE extraites de boues activées augmente quand le phosphore est en pénurie.

Durmaz et Sanin (2001) ont trouvé que les SPE extraites de boues activées riches en protéines et à faible teneur en glucide quand le rapport du Carbone/Azote est de 5, par contre si le rapport C/A atteint le 40, la quantité de protéine est diminuée, alors que la quantité des glucides augmente. D'autre part, des études ont rapportés que les boues activées qui présentent un faible rapport carbone/azote tendent à produire une quantité de SPE avec un haut rapport protéines/glucides (Bura et al. 1998; Liu et Fang 2002). Pour la commercialisation, le coût de la production de SPE doit être vérifié. Afin de réduire le coût de production, des études sont nécessaires pour sélectionner des substrats à faible coût et en même temps une optimisation des conditions de fermentation doit être considérée pour améliorer la production des bioflocculants. La boue municipale, les boues de l'industrie du vin, l'eau usée de brasserie et les eaux usées industrielles peuvent être utilisés comme des substrats potentiels pour la production des bio-polymères. En fait, l'utilisation de déchets est considérée comme une voie prometteuse pour la production de SPE réduisant ainsi le coût de production et fournit des solutions aux problèmes de gestion des déchets.

1.3.4 Les Conditions de la croissance

Le pH, la température, la teneur en oxygène sont les facteurs principaux qui influencent la production de SPE. La majorité des études ont rapporté la production des biopolymères en milieux synthétiques ou en milieux simples (Tableau 1.3). La plupart des microorganismes produisent des fortes concentrations des SPE à pH neutre de température entre 25-30°C. Des taux élevés d'oxygène peuvent améliorer la production des biopolymères. En effet, Bayer et al. (1990) ont constaté que la quantité des SPE produites par *Pseudomonas aeruginosa* est fortement dépendante de l'oxygène. Ainsi, le taux d'oxygène le plus élevé (80 vs 40 mm Hg) a amélioré la production de SPE. D'une part, les variations de tensions d'oxygène représentent l'un des mécanismes de déclenchement pour la régulation de la production de SPE. D'autres parts de la recherche ont étudié les effets spécifiques de l'oxygène. La quantité des SPE contenue dans les boues diminuerait dans des conditions anaérobies (Nielsen et al. 1996; Thompson et Leps 1986). Ils ont rapporté que les flocs de boues activées ont tendance à se désintégrer sous limitation d'oxygène ou sous les conditions d'épuisement. Cette désintégration peut être causée par la production limitée ou inhibée de SPE ou aussi par l'hydrolyse ou la dégradation de ces polymères. Shin et al. (2001) ont comparé la production de SPE des boues activées dans trois bioréacteurs en fonction des différentes concentrations en oxygène dissous. Ils ont observé que, à un niveau élevé d'oxygène dissous, les glucides contenus dans les SPE augmentent avec le temps, tandis que la teneur en protéines est constante. À un taux d'oxygène dissous faible, la concentration de SPE a augmenté avec le temps au cours de la fermentation, mais la quantité des glucides et des protéines contenues dans les biopolymères est stable. En revanche, les SPE jouent également un rôle très important dans les bioréacteurs anaérobies citant l'exemple de réacteur à refoulement anaérobie de boues en lit. La distribution des SPE joue un rôle important dans la stabilité de granules (pour maintenir la cohésion du granulé) dans ce réacteur. Applegate et Bryaers (1991) ont signalé que les faibles teneurs en oxygène favorisent une production élevée de SPE. En effet, les faibles concentrations d'oxygène ont ralenti le taux de croissance bactérienne, en leur donnant plus de temps pour se consolider. Au contraire, le déclenchement de la production des biopolymères est également lié à un environnement semi-anaérobie, ce qui favorise la croissance bactérienne et leur production (Gamar-Nourani et al. 1998). En effet, les résultats obtenus par les différentes études sont un peu contradictoires soulignant ainsi l'importance d'étudier l'effet de la disponibilité de l'oxygène sur la production de SPE.

Tableau 1.3 Les caractéristiques chimiques de la production de SPE

Les Micro-organismes	Le Substart	Les conditions-de fermentation enerlenmeyers	La concentration maximale des SPE (g/L)	Les Caractéristiques chimiques					Références
				Les glucides (%, P/P ou Unités molaires)	Protéines (%, P/P)	Autres (%, P/P)	Les Poids moléculaire (Da)	Les groupes fonctionnels	
Rhodococcus erythropolis	-	96-120 h	5,0	-	-	-	-	-	(Takeda et al. 1991)
Alcaligenes cupidus (KT201)	Saccharose	-	1,5	Sucre Total: 72,25 Le galactose, le glucose et le mannose étaient présents dans un rapport approximatif de 6,3: 5,5: 1, respectivement.	-		2×106	Ester acétyle	(Toeda et Kurane 1991)
Klebsiella sp.	Glucose	200 tours par minute, 30°C, 120 h	1,0	Sucre neutre : 69,10 Sucre Total: 72,55, le galactose, du glucose et du mannose étaient présents dans un rapport d'environ 5: 2: 1, respectivement.	-	Acide uronique: 15,81	≥2×106	Méthoxyle	(Dermlim et al. 1999)
Citrobacter	Acétate Propionate	120 tours par minute, 30°C, 72 h	1,5	-	-	-	-	-	(Fujita et al. 2000)
Bacille sp. (AS-101)	Le glucose et de gélose	130 tours par minute, 30°C, 10-15 h	-	Glucides: 83	17	Acide uronique: 11,40, l'acide pyruvique: 6,10, l'acide acétique: 0,40	-	Hydroxyle, amine	(Salehizadeh et al. 2000)
Corynebacterium glutamicum	Saccharose	120 tours par minute, 28 °C, 48 h	2,0	-	-	-	-	-	(He et al. 2002)

Les Micro-organismes	Le Substart	Les conditions-de fermentation enerlenmeyers	La concentration maximale des SPE (g/L)	Les Caractéristiques chimiques					Références
				Les glucides (%, P/P ou Unités molaires)	Protéines (%, P/P)	Autres (%, P/P)	Les Poids moléculaire (Da)	Les groupes fonctionnels	
Bacillus firmus	Glucose	150 tours par minute, 35 °C, 72 h	1,4 (À 33 h)	Sucre Total: 87	Non détecté	Acide uronique: 38,00 L'acide pyruvique: 6,30	2×10^6	Carboxylate, hydroxyle, méthoxyle	(Salehizadeh et Shojaosadati 2002a)
Bacillus mucilaginosus (MBFA9)	Amidon soluble	150 tours par minute, 30 °C, 84 h	-	-	-	Sucre neutre: l'acide uronique 47,40: 19,10 sucre aminé: 2,70	$2.6x10^6$	Carboxyle hydroxyle	(Deng et al. 2003)
*Vagococcus*sp. souche (W31)		120 tours par minute, 25 °C 48 à 72 h	2,3	-	-	-	$2x10^5$	Hydroxyle, carboxyle, méthoxyle	(Gao et al. 2006)
Bacille sp. (MBFF19)	Glucose	200 tours par minute, 30 °C, 48 h	0,8	Sucre neutre: 3,60 sucres aminés: 0,50	16.40	Acide uronique: 37,00	-	Un groupe carboxyle, hydroxyle, méthoxyle	(Zheng et al. 2008)
Bacillus subtilis (E1)	Glycérol et éthanol	150 tours par minute, 28 °C, 48 h	6,3	107 ± 7,5 uM	111 ± 3,74 uM	Acide uronique 27,318 mM; Sucres aminés 41 uM	-	-	(Buthelezi et al. 2010)
Pseudomonas plecoglossicida (A14)	Glycérol et éthanol	150 tours par minute, 28 °C, 48 h	8,3	706 ± 12,2 uM	40 ± 1.631 uM	Acide uronique 26,817 mM; Sucres aminés 5 uM	-	-	(Buthelezi et al. 2010)

Les Micro-organismes	Le Substart	Les conditions-de fermentation enerlenmeyers	La concentration maximale des SPE (g/L)	Les Caractéristiques chimiques					Références
				Les glucides (%, P/P ou Unités molaires)	Protéines (%, P/P)	Autres (%, P/P)	Les Poids moléculaire (Da)	Les groupes fonctionnels	
Pseudomonas pseudoalcaligenes (A17)	Glycérol et éthanol	150 tours par minute, 28 °C, 48 h	15,2	56 ± 4,9 pM	70 ± 1,349 uM	Acide uronique 26,954 mM; Sucres aminés 54 uM	-	-	(Buthelezi et al. 2010)
Klebsiella terrigena (R2)	Glycérol et éthanol	150 tours par minute, 28 °C, 48 h	27,7	356 ± 7,5 uM	4 ± 0,86 uM	Acide uronique de 2 mM; Sucres aminés: 0	-	-	(Buthelezi et al. 2010)
Exiguobacterium acetylicum (D1)	Glycérol et éthanol	150 tours par minute, 28 °C, 48 h	10,2	20 ± 0,9 uM	22 ± 0,221 uM	Acide uronique 26,361 mM; Sucres aminés: 0	-	-	(Buthelezi et al. 2010)
Staphylococcus aureus (A22)	Glycérol et éthanol	150 tours par minute, 28 °C, 48 h	10,8	470 ± 7,5 uM	9 ± 0,098 uM	Acide uronique: 26,178 mM; Sucres aminés: 2 uM	-	-	(Buthelezi et al. 2010)
Proteus mirabilis (TJ-1)	Glucose	160 tours par minute, 30 °C, 48 h	1,3	63,10	30,90	-	$1.2x10^5$	-	(Xia et al. 2008)
Chryseobacterium .daeguense (MBF-W6)	Le glucose, le tryptone	180 tours par minute, 30 °C, 36 h		-13,1	32,4	Acide nucléique: 6,8		Un groupe carboxyle, hydroxyle, méthoxyle	(Liu et al. 2010a)
Bacillus licheniformis	Saccharose	200 tours par minute, 37 °C, 48 h	2,9	89,00	11,00	-	$6.89x10^4$	-	(Xiong et al. 2010)

Les Micro-organismes	Le Substart	Les conditions-de fermentation enerlenmeyers	La concentration maximale des SPE (g/L)	Les Caractéristiques chimiques					Références
				Les glucides (%, P/P ou Unités molaires)	Protéines (%, P/P)	Autres (%, P/P)	Les Poids moléculaire (Da)	Les groupes fonctionnels	
Proteus mirabilis (TJ-1)	Glucose	130 tours par minute, 25 °C, 48 h	1,3	63,10	30,90	-	$1.2x10^5$	-	(Zhang et al. 2010)
Azotobacter indicus (ATCC 9540)	*Madhuca latifolia* l'extrait de fleur	180 tours par minute, 30 °C, 144 h	6,1	97,7	2,3	-	$2x10^6$	O-acétyl, Orcinol, carboxyle, hydroxyle	(Patil et al. 2011)
Halomonas sp. (AAD6)	mélasse de betterave à sucre	180 tours par minute, 37 °C, 10-15 h	-	90,00	0,5	Acides nucléiques: 5,4	-	-	(Sam et al. 2011)
Bacillus megaterium (TF10)	Glucose	30 °C, 24 h	-	76,90	23,00		$1.0\text{-}2.5x10^6$	Hydroxyle Amide Carboxyl Amine primaire	(Yuan et al. 2011)
Paenibacillus elgii (B69)	Le glucose, la peptone	220 tours par minute, 30 °C, 96 h	12,12			Principalement des polysaccharides (glucose, l'acide glucuronique, la xylose, et Le mannose à un rapport de 1: 0,53: 1,15: 0,46)	$3,5\ x10^6$		(Li et al. 2013)
	Le lactose, le peptone de soja	220 tours par minute, 30 °C, 96 h	11,49						
	Saccharose, l'extrait de boeuf	220 tours par minute, 30 °C, 96 h	10,15						
	Amidon soluble	220 tours par minute, 30 ° C, 96 h	9,53						
	Maltose	220 tours par minute, 30 °C, 96 h	9,18						

1.4 Production de SPE en tant que bio-floculant

Les SPE produites par les bactéries dans le bouillon fermenté (par des cultures pures ou mixtes) peuvent être utilisées en tant que biofloculant sous différentes formes possibles (forme brute, forme purifiée ou après traitement). La forme brute des SPE, qui est la forme la plus rentable, représente le bouillon fermenté. Selon la littérature, le bouillon fermenté obtenu par la culture des souches produisant les SPE dans différents milieux (synthétique, boues ou eaux usées), présentent un potentiel de floculation assez élevé (Zhang et al. 2007). L'avantage principal de ce type de biofloculant réside dans le fait qu'aucun traitement supplémentaire n'est nécessaire avant son application. Les SPE brutes sont présentes sous trois différentes formes: sous la forme de bouillon fermenté (contenant des SPE sous formes de capsules et de vases), sous la forme de vase (obtenu dans le surnageant après la centrifugation du bouillon) et sous la forme capsulaire (le résidu restant après la centrifugation du bouillon). Ces trois différentes formes peuvent être utilisées en tant que biofloculant. L'utilisation de la filtration par membrane du bouillon fermenté permet de récupérer à la fois les SPE et les cellules. Ainsi, la formulation du bouillon fermenté récupéré va servir par la suite à la conversion du bouillon liquide à sa forme solide. Suite à la concentration du bouillon, il y aura une réduction de son volume, l'augmentation de la stabilité du produit et l'augmentation son activité relative lors du dosage. La concentration du bouillon contenant ainsi les SPE peut être possible soit par la méthode de pulvérisation ou par la méthode de lyophilisation. En effet, le choix entre les deux méthodes dépend principalement de la stabilité des SPE et de l'aspect économique de chacune. Généralement, la méthode de pulvérisation est plus économique que la lyophilisation mais elle reste sensible vis-à-vis de la dégradation et la dénaturation du produit.

Dans le cas où les SPE sont utilisées sous leur forme soluble, des méthodes physiques et chimiques assez douces peuvent être utilisées afin de produire les SPE par le bouillon. Les cellules sont par la suite séparées par centrifugation et le surnageant sera précipité par des méthodes chimiques appropriées. Zouboulis et al. (2004) ont transformé les SPE produites par *Rhizomonas* sp en poudre. Par la suite, 2 g de cette poudre ont été solubilisé dans un litre d'eau déminéralisée. Ainsi, l'agitation de la solution permet d'obtenir une solution de concentration 2000 mg/L. les SPE obtenues sous cette forme peuvent être utilisées en tant que biofloculant.

1.5 Rôle des EPS dans la floculation et la déshydratation

Généralement, les SPE sont les facteurs les plus importants dans le processus de floculation des eaux usées. Les interactions qui existent entre les SPE et les cellules présentent un effet significatif sur la floculation des microbes. En plus, les SPE sont des facteurs importants dans l'épaississement et la déshydratation des boues dans les systèmes des eaux usées (Houghton et al. 2001). Durant l'épaississement et la déshydratation, deux types d'interactions entre les molécules d'eau et les molécules des SPE peuvent être présentes: les interactions électrostatiques et les liaisons d'hydrogènes. En effet, les SPE la déshydratation des boues dépend principalement des SPE. Selon (Jin et al. 2004; Mikkelsen and Keiding 2002), l'augmentatation de la quantité des SPE permet d'améliorer d'avantage la capacité de déshydrater les boues. En présence d'une quantité élevée en SPE, les boues activées présentent une sensibilité et un degré de dispersion assez faibles ce qui conduit à une meilleur déshydratation (Mikkelsen and Keiding 2002). Selon Houghton et al. (2001), l'effet des SPE sur la déshydratation des boues dépend de la quantité des SPE présentes. En plus, les différents composés des SPE peuvent influencer la capacité des microbes à déshydrater les boues. Par conséquent, la réduction de la fraction protéique (Sponza 2002) ainsi que l'amélioration de la partie carbonique des SPE (Cetin and Erdincler 2004) améliorent la capacité de déshydratation des boues.

1.6 Les facteurs influençant la capacité des SPE à déshydrater différentes suspensions

Différentes études visaient à développer les caractéristiques des SPE synthétisées par différentes microorganismes (Tableau 1,4). La majorité des études ont utilisées des suspensions de kaolin comme étant un standard pour évaluer les caractéristiques de floculation des SPE.

Tableau 1.4 Les caractéristiques de floculation des SPE produites par différents microorganismes

Microorganismes	Structure chimiques des SPE	Concentration optimale de floculant	pH optimal	Activité de floculation (OD à550 nm) ou FA%	Effets du dosage des SPE	Cations utilises avec les SPE	Remarques	Références
Alcaligenes latus (KT201)	Poids moléculaire $2x10^6$ Da	0.002 ml du bouillon après de 72 h	5	9 (1/OD)	-	Effet synergétique positive des cations Al^{3+}, Fe^{3+}, Fe^{2+}, Mg^{2+}, Ca^{2+}, Al^{3+} a plus d'effet	Suspension de 5 g/L de kaoline	(Toeda and Kurane 1991)
Rhodococcus erythropolis	-	10-20 mg/L	-	70-80%	-	1.2 mM de $Al_2(SO_4)_3$	Suspension de 5 g/L de kaoline	(Takeda et al. 1991)
Pseudomonas sp. (A-99)	Protine acide avec une quntté faible de polysaccharides: acide galacturonique, glucose and galactose Groupe carboxylique Poids moléculaire 5×10^3 to 2×10^6 Da	20 mg/L	5-7	12 (1/OD)	-	5 mM de Ca^{2+}, Fe^{2+} et Mg^{2+} (effet positive)	-	(Yokoi et al. 1998)
Citrobacter	Poids moléculaire 232-440 kDa	1-10 mg/L	2-8	90%	-	-	-	(Fujita et al. 2000)
Corynebacterium glutamicum	-	-	-	80%	-	-	-	(He et al. 2002)
Bacillus firmus	-	30 mg/L	3.7		-	-	-	(Salehizadeh and Shojaosadati 2002b)
Bacillus mucilaginosus (MBFA9)	Polysaccharide compose d'acide uronique (19,1%) de sucre neutre (47,4%)et sucre aminé (2,7%)	0.1 ml/L	-	99,6%	-	-	-	(Deng et al. 2003)

Microorganismes	**Structure chimiques des SPE**	**Concentration optimale de floculant**	**pH optimal**	**Activité de floculation** (OD à550 nm) ou FA%	**Effets du dosage des SPE**	**Cations utilises avec les SPE**	**Remarques**	**Références**
Vagococcus sp. strain (W31)	Groupe hydroxylique, carboxyliqe et methoxylique	-	7-10	-	-	-	-	(Gao et al. 2006)
Aeromonas sp. (N11)	**-**	1 mg/L	3-5	92,40%	**-**	**-**	suspension de 5 g/L de kaoline	(Li et al. 2007)
Bacillus mucilaginosus	-	-	-	Élimination de 93,3% De SS	Élemination de COD et BOD de 74,5%, et 42,3%,	-	-	(Lian et al. 2008)
Bacillus mucilaginosus	-	-	-	Élimination de 93,6% De SS	COD, et BOD 70,5%, et 77,4%	-	-	(Lian et al. 2008)
Bacillus mucilaginosus	-	-	-	Élimination de 88,4% De SS	66.2%, COD, et 41,7% BOD	-	-	(Lian et al. 2008)
Proteus mirabilis (TJ-1)	Groups carboxylique, hydroxylique et aminé poids moléculaire $1.2x10^5$ Da	2 ml de bio-floculant		93,13%	-	1% $CaCl_2$	suspension de 4 g/L de kaoline	(Xia et al. 2008)
Bacillus sp. strain (F19)	Sucre neutre (3,6%, w/w), acide uronique (37,0%, w/w), sucre amine (0.5%, w/w) et protéine (16.4%, w/w) Groupe hydroxylique, carboxylique et méthylique	-	-	-	-	Fe^{3+} inhibe la floculation	Kaolin suspension of 5 g/L	(Zheng et al. 2008)

Microorganismes	Structure chimiques des SPE	Concentration optimale de floculant	pH optimal	Activité de floculation (OD à550 nm) ou FA%	Effets du dosage des SPE	Cations utilises avec les SPE	Remarques	Références
Azotobacter indicus (ATCC 9540)	Groups acétyle, carboxyle et hydroxyle Poids moléculaire $2x10^6$ Da	500 mg/L	5-10	72%	Reduction de BOD: 38-80%, COD: 37-79% et SS: 41-68%	No cations addition	Wastewater from Wool industry Starch industry Sugar industry Dairy industry	(Patil et al. 2011)
		10 mg/L	5-10	92%		10 mg/L de $CaCl_2$		
Halomonas sp. (AAD6)	Carbohydrates (90% (w/w)), fable quantité d'acide nucéleique of nucleic acids (4-5% (w/w)) moins que 0,5% (w/w) de protéines	20 mg/L	7,3	93%	-	-	Synthetic and natural sea water	(Sam et al. 2011)
Bacillus megaterium (TF10)	Presence de groups hydroxyls, amine et carboxyle et des amines Poids moléculaire : 1037-2521 kDa	-	-	96%	-	5.6 mM of $CaCl_2$	Kaolin suspension of 4 g/L	(Yuan et al. 2011)
Staphylococcus cohnii sp.	-	0,3 mg/L	7	70.3%	-	2 mL de 1% $CaCl_2$	Kaolin suspension of 5 g/L (100 mL)	(Wong et al. 2012)
Staphylococcus cohnii sp.	-	1,2 mg/L	7	88,9%	-	$AlCl_3$	Kaolin suspension of 5 g/L (100 mL)	(Wong et al. 2012)
Arthrobacter sp. Raats	Glycoproteine, 56% protein et 25% carbohydrate	0,1 mL du surnageant du bouillon	7	84% (par OD)	-	0,1 mLde 1% $CaCl_2$	Suspension de kaoline de 4 g/L (9 mL)	(Mabinya et al. 2012)
Paenibacillus elgii (B69)	Polysaccharides	0,5 mL de 1% EPS solution	-	87%	Wastewater, COD: 68%, Turbidity: 83%, Color: 88%	0,5 mLde 1% $CaCl_2$	Suspension de kaolin de 4 g/L (10 mL)	(Li et al. 2013)
Klebsiella pneumoniae	96,8% de polysaccharide et 2,1% de protéines	10-100 mg/L	7,5			5 mL de 9 mM $CaCl_2$	Suspension de kaoline de 4 g/L (1000 mL)	(Zhao et al. 2013)

1.6.1 Les cations

Les cations jouent un rôle très important dans la floculation. En effet, ces ions neutralisent les particules chargées négativement, stabilisent les surfaces chargées négativement des polymères et agissent en tant qu'agents de liaison entre les particules et les polymères (Higgins and Novak 1997b). Le role majeurs des ions bivalent et trivalents est leur capacité d'améliorer l'adsorption des SPE et des particules en suspensions en diminuant leurs charge négative. Par exemple, l'activité floculante des *Enterobacter* sp diminue en présence d'Al^{3+}, Fe^{3+}, Fe^{2+}, et Ca^{2+} (Yokoi et al. 1997). L'activité floculante du kaolin des SPE produites par *Bacillus* sp était induite en augmentant la concentration de Ca^{2+}, Mg^{2+}, et Fe^{2+}. Un pH optimum situé entre 4 et 5 est nécessaire pour induire la floculation par les ions Ca^{2+} (Yokoi et al. 1997). Pour d'autres ions tels que Mg^{2+}, Fe^{2+}, et Fe^{3+}, le pH optimum est situé entre 6 et 7 (Yokoi et al. 1997). La plus haute activité floculante des SPE était enregistrée pour des concentrations de 2-8 mM de Ca^{2+}. Cependant, une augmentation dans les concentrations de Al^{3+} et Fe^{3+} provoquent une diminution de l'activité du floculant et peut même engendrer une inhibition complète de la floculation (pour des concentrations situées entre 0,2 mM de Al^{3+} et 0,5 mM de Fe^{3+}) (Yokoi et al. 1995; 1998; 1997). Pour les SPE de *Nocardia amarae,* la flocculation est stimulée par l'addition de Na^{+}, Ca^{2+}, Al^{3+}, et Fe^{3+}. Cependant, une addition excessive de Fe^{3+} peut provoquer une inhibition compléte de la floculation. Pour d'autres microorganismes tels que *Rhodococcus erythropolis* (Kurane et al. 1991) et *Alcaligenes cupidus,* l'activité de floculation des SPE est améliorée les cations Ca^{2+} et Al^{3+}. La présence de $CaCl_2$ dans le milieu améliore significativement l'activité de floculation des SPE produites par certains microorganismes tels que *Bacillus*, *Pseudomonas*, *Serratia* et *Yersinia* (Subramanian et al. 2010). En conclusion, l'application des SPE comme biofloculant nécessite la présence des différents ions. Dans le but de mieux comprendre les différents types d'interactions présentes entre les SPE et les ions, des études plus profondes doivent être mise en place.

1.6.2 Les différentes formes des SPE

Suite aux différences physico-chimiques des SPE, plusieurs formes de floculation peuvent être distinguées (Tableau 1.1). Subramanian et al. (2010) ont observé que les SPE solubles produites dans le milieu minéral synthétique présentent une meilleure capacité de floculation et de déshydratation comparativement aux SPE bouillantes et capsulaires. Cette différence peut

être attribuée au fait qu'il existe des différences physiologiques et biochimiques entre les SPE slime, capsulaires et de bouillons produites dans les boues traitées et dans le milieu minérale synthétique. Selon Li and Yang (2007), les SPE facilement détachées de la cellule (LB-SPE) peuvent détoriorer l'attachement des cellules et affaiblir la structure des flocs ce qui conduit à une mauvaise séparation entre les boues et l'eau. Malgré que dans cette étude, la quantité de LB ne dépasse pas les 20% de la quantité totale des SPE, un effet négatif des LB-SPE sur la bio-floculation, sur la clarification des effluents, sur la sédimentation des boues et sur la déshydratation a été observé. Les valeurs de l'efficacité de la séparation de l'eau et des boues défini par les SES (solide en suspension), les VIB et les RTS est reliée à la quantité des LB-SPE dans les boues, mais aucune corrélation ne peut etre établie avec les TB-SPE (Li and Yang 2007). Yu et al. (2009b) ont montré dans leurs études que la fraction des SPE qui présente une forte liaison possède des capacités de floculation des suspensions de kaolin assez élevée comparativement à d'autres types de SPE (slime). D'autre part, Les fractions TB-SPE présentent une capacité élevée de floculation. Ceci est due à la présence importante des macromolécules (330-1200 KDa) et aux cations trivalents (Fe^{3+} et Al^{3+}).

1.6.3 Concentration des SPE

La concentration des SPE joue un rôle très important dans la floculation et la déshydratation des suspensions. Les concentrations optimales des SPE qui donnent le maximum d'activité de floculation sont représentées dans le tableau 1.5. La concentration des SPE est directement liée à la floculation et la déshydratation des boues (Houghton et al. 2001). À des concentrations optimales des SPE, on pbserve une diminution dans le nombre des particules fines présentes dans les boues. Cela améliore considérablement la floculation et la déshydrataion des boues (Gonçalves et al. 2007). Des concentrations de SPE qui dépassent l'optimal, est préjudiciable dans la déshydrataion des boues. Houghton et al. (2001) ont constaté qu'une déshydratation maximale peut etre atteinte pour des concentrations optimales des SPE. Des concentrations optimales d'SPE de l'ordre de 10 mg SPE/g SS, 20 mg SPE/g SS et 35 mg SPE/g SS ont été enregistrés pour les boues digérées, les boues brutes et les boues activées, respectivement. Selon (Shin et al. 2001), les concentrations des SPE qui permettent un maximum de floculation varient selon les différentes souches bactériales. À partir des résultats enregistrés dans le tableau 1.5, on peut constater que les concentrations maximales des SPE varient entre 0.2 mg SPE/g SS to 1000 mg SPE/g SS.

Tableau 1.5 Concentrations optimales des SPE (en présence des ions de calcium) requises pour l'activité floculante de kaoline

Microorganismes producteurs de bio-floculant	Suspension de kaoline (g/L)	Concentration optimales des SPE		Activité de floculation (%)	Références
		mg SPE/g SS	mg/L		
Aeromonas sp. (N11)	5,0	0,2	1	92,4	(Li et al. 2007)
Gyrodinium impudicum	5,0	0,2	1	90,0	(Yim et al. 2007)
Klebsiella mobilis	5,0	0,5	2,5	95,4	(Wang et al. 2007)
Bacillus licheniformis (X14)	4,0	0,5	2	99,2	(Ji et al. 2010)
Bacillus licheniformis	1,0	5,8	5.8	90,0	(Xiong et al. 2010)
Bacillus sp. (AS101)	5,0	6,0	30	92,0	(Salehizadeh et al. 2000)
Bacillus sp. (DYU1)	5,0	8,0	40	97,0	(Wu and Ye 2007)
Azotobacter indicus	5,0	100,0	500	92,0	(Patil et al. 2011)
Bacillus sp. (BS9)	5,0	200,0	1000	78,2	(Subramanian et al. 2010)
Pseudomonas sp. (BS2)	5,0	400,0	2000	78,0	(Subramanian et al. 2010)
Serratia sp. (BS8)	5,0	400,0	2000	78,1	(Subramanian et al. 2010)
Microbacterium sp. (BS15)	5,0	400,0	2000	81,2	(Subramanian et al. 2010)
Pantoea sp. (BS7)	5,0	600,0	3000	71,4	(Subramanian et al. 2010)
Yersinia sp. (BS11)	5,0	600,0	3000	83,7	(Subramanian et al. 2010)
Enterobacter sp. (BS25)	5,0	1000,0	5000	76,1	(Subramanian et al. 2010)

1.6.4 Quantité de carbohydrates et de protéines présents dans les SPE

Ye et al. (2011b) ont constaté qu'il n'existe pas une forte relation entre la concentration totale des SPE et la floculation des boues activées et entre la sédimentation et la déshydratation. D'un autre part, la quantité de protéines produites par LB-SPE est en corrélation avec la floculation des boues activées, la sédimentation et la déshydratation. En effet, la charge à la surface et l'hydrophobilcité des SPE est importantes dans la sédimentation des boues. En plus, la charge de la surface des cellules peut avoir un role dans l'hydrophobilicté des bactéries. Le facteur le plus important qui détérmine la charge à la surface de la cellule est le rapport entre la quantité des carbohydrates et les protéines dans les SPE. Shin et al. (2001) ont montré que la charge à la syface des cellules dépend du rapport entre la quantité de carbohydrate et les protéines dans les SPE. En augmentant ce rapport. La charge négative à la surface des cellules diminue.les cellules ayant une charge de surface assex élevée présentent des taux d'hydrophobicité élevés. Ainsi, les composants hydrophobes sont capables de se lier à des particules ionrganiques

chargé négativement telles que: Ca^{2+}, Mg^{2+}. Suite à l'augmentation de la charge négative, l'agrégation des bactéries est améliorée. Les cations divalents forment des ponts de liaison entre les SPE et les bactéries. La charge négative élevée à la surface des cellules favorise la sédimentation des cellules. Ceci est représenté par une valeur faible de SVI (Shin et al. 2001). Urbain et al. (1993) ont indiqué la sédimentation dépend fortement de la charge négative de surface. Les polysaccharides jouent un role majeur dans la flocculation. Par exemple, Bruus et al. (1992) ont remarqué que les cations divalents intéragissent fortement à l'intérieur des flocs avec des groupements d'alginates chargés négativement tels que les polysaccharides. Les cations multivalents (divalents et trivalents) peuvent combler entre les groupes carboxyles chargés négativement. Ainsi, La quantité de protéines et des polysaccharides dans les SPE sont des facteurs importants dans la floculation, la sédimentation etla déshydratation (Higgins and Novak 1997a). D'autres facteurs tels que le pH de la suspension, la température, la stabilité et la taille du floc, peuvent affecter la floculation des SPE.

1.7 Les applications environmentales des SPE

Récemment, plusieurs études ont été réalisés pour étudier l'effet de l'ajout des cations dans l'eau brute lors de l'application des SPE produites par différentes souches (Buthelezi et al. 2009; Li et al. 2009; Ma et al. 2008; Nontembiso et al. 2011). Des SPE synthétisés par *Bacillus* sp. ont été utilisées dans jar test afin de comparer l'efficacité de l'élimination de la turbidité des kaolin (Ma et al. 2008). Des efficacité de l'ordre de 86% (SPE), 95% ($Al_2(SO4)_3$) et 96% ($Fe_2(SO4)_3$). Ils ont conclut que la combinaison des $Fe_2(SO4)_3$ avec les SPE pour le traitement des eaux brutes était intéressante. Ils n'ont constaté aucun résiduel de fer ou d'aliminuim quand les SPE étaient appliquées dans le tratement des eaux brutes. Li et al. (2009) ont étudié le traitemet des eaux potables par les SPE produites par *Bacillus licheniformis* (combinées avec $CaCl_2$). En effet, les SPE synthétisés ont montré une grande efficacité concernant le traitement des eaux potables. L'élimination maximale de la COD et de la turbidité est de l'ordre de 61,2% et 95,6 %, respectivement. Ils ont constaté aussi que les SPE sont efficaces; a différentes températures (4 et 25^{o}C). Selon Li et al. (2009), les SPE synthétisés par *Bacillus licheniformis* ne présentaient aucun danger lors du traitement des eaux potables. Les SPE produites par différentes souches telles que: *Bacillus subtilis*, *Exiguobacterium acetylicum*, *Klebsiella terrigena*, *Staphylococcus aureus*, *Pseudomonas pseudoalcaligenes* et *Pseudomonas plecoglossicida* sont capables aussi de réduire la turbidité de 84,1 à 93,6% pour 10 mg/L d'SPE (Buthelezi et al. 2009). Ces SPE étaient aussi capables d'éliminer (jusqu'aux 98,3 %) les

bactéries Gram positives (*Staphylococcus aureus* et *Streptococcus faecalis*) et Gram négative (*Escherichia coli* et *Klebsiella oxytoca*) présentes dans les rivières. Buthelezi et al. (2009) ont indiqué que le remplacement de l'alum (coagulant ionrganique) par les SPE peut etre possible. La présence de la matière organique naturelle (MON) dans les eaux est considéré comme un sérieux problème. Les SPE synthétisés par *Pseudomonas aeruginosa* and *Pseudomonas putida* sont capables d'éleminer les MON de l'environnemnt (Wang et al. 2012). Grace à leurs capacité d'adsorption et de biofloculation, les SPE présentent de bons candidats pour le traitement des eaux de rivières contaminées par les MON. En plus, l'aspect non toxique des SPE les rend avantagieux. Malgré leur importance, l'utilisation des SPE n'est pas favorisée. En effet, cela est du au fait que les traitements biologiques des eaux potables peut provoquer une contamination des eaux par les microbes et cela va conduire à l'utilisation d'autres procédés tels que les procédés de filtration, de désinfection etc. Dans ce cas, des études supplémentaires doivent etre faites pour étudier le traitetment des eaux de rivières ou les eaux potables par les SPE.

Dans le domaine de traietemt des eaux, la floculation et la méthode la plus utilisée pour éliminer la matière en suspension et améliorer la qualité de l'eau. Des études récentes ont visé à remplacer les SPE par les polymères chimiques conventionels. Selon Gong et al. (2008), les SPE synthétisées par *Serratia ficaria* peuvent être appliquées pour le traitement des différents eaux uses industrielles. Alors que les SPE produites par *Bacillus mucilaginosus* étaient capables d'éliminer 85% des SS et 68,5% de la DCO de l'eau d'amidon (Deng et al. 2003). Les SPE ont été utilisées aussi pour traiter les eaux usées porcines. Ils ont enregistré qu'une élimination respective de 91 et de 42% de la turbidité et de la DCO sont possibles. En effet, Ces résulats sont meilleur que ceux trouvés par les polymeres conventionnels (chlorure d'aliminum) (Zhang et al. 2012). Selon Lian et al. (2008), les SPE produites par *Bacillus mucilaginosus* peuvent etre utilisées pour traiter les eaux municipales, les eaux de breuvages et les eaux pharmaceutiques. Une élimination de la COD de l'ordre de 74,6, 70,5 et 66,2%, de la DBO de l'ordre de 42,3, 77,4, et 41,7%, et de la SS de l'ordre de 93,3, 93,6, and 88,4% ont été enregistrées pour les eaux usées domestiques, les eaux usées de breuvage et les eaux usées pharmaceutiques, respectivement, en utilisant les SPE produites par *Bacillus mucilaginosus*. Des SPE produites par des cultures de *Staphylococcus* sp. et de *Pseudomonas* sp. ont été utilisés pour traiter efficacement les eaux d'impression et de teinture (élimination de 80% de la COD) (Zhang et al. 2007). Des SPE synthétisés par *Paenibacillus elgii* (B69) sont capables d'éliminer 68% de la DCO and 83% de la turbidité (Li et al. 2013). La majorité des études qui s'intéressent à la production des SPE ont été réalisées à l'échelle de laboratoire. L'utilisation

des SPE à l'échelle pilote permettra certainement de mieux comprendre le mécanisme d'application des SPE. D'autres applications telles que: l'élimination de la couleur, la déshydratation des boues, l'élimination des métaux et des composés toxiques, le traitement des lixiviats de décharge et la remédiation des sols sont possibles en appliquant les SPE.

PARTIE 2: PROBLÉMATIQUE

Les problèmes suivants ont été mis en évidence à partir d'une revue de littérature.

2.1 Traitement des boues d'épuration, réutilisation et problèmes liés à la disposition des boues

Le traitement, la réutilisation et la disposition des boues sont des sujets de grande préoccupation à cause des quantités importantes de boues générées dans les stations de traitement des eaux usées municipales et industrielles. Les principaux problèmes sont les coûts de transport, la présence d'éléments toxiques dans les boues, et la capacité d'enfouissement des boues. Le traitement et la disposition des boues représentent 20-60% du coût total d'exploitation des stations de traitement des eaux usées. Par ailleurs, les boues d'épuration ont un grand potentiel de revalorisation. En effet, les boues sont d'importantes sources de carbone, d'azote, de phosphore et d'autres nutriments. L'utilisation des boues pourrait être un avantage pour la croissance des micro-organismes isolés des boues d'épuration, lesquels sont déjà bien adaptés à elles.

2.2 Problèmes liés à l'utilisation des coagulants et floculants chimiques dans les stations de traitement des eaux usées

Les coagulants et polymères chimiques utilisés dans les stations d'épuration pour le processus de coagulation-floculation sont onéreux, corrosifs et toxiques. Ces produits chimiques induisent également de sérieux problèmes environnementaux et de santé, lorsque les boues générées sont utilisées pour la récupération des sous produits ou pour des usages agricoles.

Lorsque les coagulants ferriques (par exemple, $FeCl_3$) sont utilisés de manière excessive, ils peuvent induire la corrosion et la coloration des équipements et donner lieu à des préoccupations esthétiques désagréables telles que les odeurs, la couleur et le goût métallique. L'utilisation des produits chimiques inorganiques pour le conditionnement des boues augmentent la masse de boues de 15 à 30%, mais également diminue la valeur de combustion des boues à l'incinération. Environ 5 kg de polymères sont utilisés pour conditionner environ 1000 kg de matières sèches dans les processus typiques de déshydratation des boues. Le coût des polymères chimiques utilisés pour les opérations de conditionnement des boues est très élevé. La plupart des produits chimiques induit des préoccupations environnementales et de

santé. Ce qui limite alors leur utilisation à long terme. Par conséquent, l'utilisation de polymères chimiques pour la floculation n'est pas une option viable.

Il y a une demande croissante de polymères naturels afin de remplacer ou réduire l'utilisation des polymères chimiques classiques ou floculants. La biofloculation par l'utilisation des polymères naturels est considérée comme une méthode durable, économique et respectueuse de l'environnement. Les polymères microbiens sont de potentiels polymères naturels. Cependant, divers aspects des floculants microbiens sont encore non élucidés ou peu étudiés, notamment la production, la caractérisation de différentes fractions de polymères extracellulaires (SPE) en lien avec leur capacité à floculer et l'optimisation du processus de floculation utilisant des floculants microbiens.

2.3 Défis dans la production biofloculants microbiens

Les microorganismes responsables de la production des SPE doivent être isolés des boues activées afin d'étudier les caractéristiques de leur matrice sur la floculation, la décantation et la déshydratation des boues. Dans le cas d'une production de SPE au laboratoire, les microorganismes doivent être investigués à la fois dans des cultures pures ou mixtes, en fonction des concentrations de SPE sécrétées et des caractéristiques attendues. Le substrat est un paramètre essentiel critique dans la production de SPE. Le principal facteur limitant pour une production commerciale des SPE est le coût élevé du substrat, notamment le glucose, l'amidon, le saccharose les sources les plus courantes de carbone. Dans les bioprocédés, le coût du substrat représente 20-40% du coût total de production. Par conséquent, un substrat moins coûteux avec des productivités ou (rendement) comparables est essentiel pour réduire les coûts de production d'es SPE. Les milieux synthétiques sont les milieux de cultures les plus utilisés pour la production de SPE. D'un point de vue économique, l'utilisation des milieux de cultures coûteux et tels que les milieux synthétiques n'est pas recommandée. Il est également rapporté que l'utilisation de milieux de culture synthétique réduit à long terme la capacité des microorganismes à produire les SPE. Cela est dû au fait que, bien que la production de SPE soit la propriété de certaines microorganismes, les SPE sont produites principalement dans des conditions de survie des microorganismes, notamment les conditions extrêmes d'assimilation de nutriments et de croissance (faibles teneures en nutriments, pH faible ou élevé, présence de toxines, etc). Le stress environnemental peut forcer ces cellules bactériennes à sécréter des SPE afin de protéger leurs parois cellulaires des toxines et également éviter l'état d'assèchement. Ces conditions ne sont pas présentes dans les milieux synthétiques. En effet

dans les milieux de culture synthétique les concentrations de carbone et d'éléments nutritifs sont sous controle. Par conséquent, il y a une nécessité d'utiliser des milieux de culture moins coûteux et évolutifs. Les boues d'épuration sont un exemple de milieux de culture évolutifs. Les boues d'épuration contiennent naturellement d'importantes quantités de SPE. La présence de SPE dans les boues est souvent à l'origine de certains problèmes au cours des opérations de traitement et de déshydratation des boues. L'utilisation de SPE naturels issus des boues d'épuration peut être une alternative très utile. La production de SPE à partir des boues peut être optimisée par le bon choix du type de prétraitement des boues, la concentration de solides dans les boues, le type de milieu de culture (pur/mixte), ratio carbone/azote, les ions métalliques et les conditions opératoires, etc. Par conséquent, des études systématiques sont nécessaires pour produire et investiguer la production de SPE à partir de boues secondaire.

2.4 Applications potentielles des SPE dans le traitement des eaux naturelles et usées

Les SPE ont trois caractéristiques importantes à savoir, l'adsorption, l'hydrophobicité/hydrophicité et la biodégradabilité. Même s'il est largement admis que les SPE sont un facteur important dans les opérations de floculation et de déshydratation des boues, leur présence dans les boues est considérée à la fois bénéfique et problématique. Dans ce contexte, les concentrations et caractéristiques ou les compositions de SPE sont des facteurs décisifs. En plus de ces facteurs, la présence d'ions métalliques (divalents, trivalents, et monovalents) formant des liaisons les SPE transforment ces derniers rendent en de précieux biopolymères ayant le potentiel d'être utilisé comme des floculants efficaces. Différentes types de SPE, dépendamment de leurs compositions biochimiques spécifiques, présentent des comportements distincts de floculation. La plupart des études publiées à ce jour démontrent que les activités de floculation des polymères microbiens utilisent des milieux de culture (SPE totaux et granulés) ou des fractions. Une étude comparative est nécessaire pour évaluer la capacité de floculation réelle des différents types de SPE. Par ailleurs, la plupart des études rapportées à ce jour sont des études réalisées dans de tubes à essais et utilisant des suspensions de kaolin. Les activités de floculation et les caractéristiques de décantation des polymères microbiens doivent être investiguer à grande échelle pour déterminer leur capacité de floculation comparée à celles des polymères classiques.

PARTIE 3: HYPOTHÈSES, OBJECTIFS, ORIGINALITÉ ET MÉTHODOLOGIE DE LA RECHERCHE

3.1 Hypothèses

1. La capacité de flocculation et déshydratation sont des propriétés techniques très intéressantes des SPE produits dans les boues ou dans les milieux de culture synthétiques. Les fractions solubles et solides des matrices de SPE ont demontré des capacités d'agrégation de solides semblables à celles des floculants commerciales. Par conséquent, les SPE sécrétées par différents microorganismes devraient être produites en laboratoire dans des conditions contrôlées. **Les SPE ayant de bonnes capacités de floculation devraient être étudiés pour des applications potentielles dans les opérations de floculation et déshydratation dans les stations de traitement des eaux usées**. En cas de succès, l'utilisation de produits chimiques (pour la plupart est toxiques, coûteux et difficilement biodégradable) pourrait être partiellement ou complètement réduite en employant des biofloculants respectueux de l'environnement et moins coûteux (Chapter 3).
2. La gestion des boues/réutilisation/recyclage. Les boues d'épuration sont produites en grandes quantités pendant le traitement des eaux usées municipales et industrielles à travers le monde entier. Cela attire une grande attention en raison des problèmes de gestion et de réutilisation/valorisation des boues. **L'utilisation des boues pour la production de produits à valeur ajoutée est donc une stratégie de gestion durable des boues, respectueuse de l'environnement** (Chapitre 3).
3. Les boues d'épuration sont des sources peu couteuses de carbone et de nutriments. Elles peuvent donc potentiellement être utilisées comme substrat pour la production de SPE. L'utilisation des boues comme milieu de culture des bactéries produisant les SPE est avantageux, d'autant que ces bactéries isolées des boues, ont une capacité d'adaptation facile dans la boue. **Le prétraitement des boues est indispensable pour augmenter la disponibilité du carbone et de l'azote et pour améliorer l'état de l'inoculum.** Le type de traitement des boues et la concentration de boues peuvent influer la composition et la structure des SPE. Par conséquent, il est possible que différents prétraitements de boue et concentrations de boues puissent induire des rendements differents de production de SPE ayant des caractéristiques de floculation différentes (Chapitre 4).

4. **Le rendement et les caractéristiques chimiques des SPE produites dépendent principalement des micro-organismes en raison de leurs caractéristiques biochimiques uniques**. Les cultures microbiennes mixtes ou cultures microbiennes pures sont également très critiques dans la production des caractéristiques chimiques souhaitées. Dans le cas des cultures mixtes, les groupes microbiens dominants ont un rôle important. Différents microorganismes produisant des SPE peuvent influencer les teneurs en glucides et protéines des SPE produits et donc affecter la capacité de floculation des SPE (Chapitre 3 et 5).
5. **Le type de SPE (bouillon, capsulaire et slime/limon) influence le comportement de floculation, à cause de la différence au niveau des groupes fonctionnels et des variations des caractéristiques physico-chimiques**. Cependant, il ya une ambiguïté sur le rôle du type de SPE dans la floculation. Dans ce contexte, une étude de floculation (en jar test) à partir de differentes types de SPE est essentielle (Chapitre 4).
6. **Les SPE chargés négativement ont une capacité de fixation des métaux. Cela est due à la présence de différents sites et liaison sur les polymères. La présence de cations divalents ou trivalents est très important pour la floculation à l'aide de SPE**. Par conséquent, l'utilisation des SPE comme floculant demande une bonne combinaison de cations bivalents ou trivalents afin d'initier le processus de floculation (Chapitre 5).

3.2 Les objectifs de recherche

3.2.1 Objectifs général

L'optimisation de la production de SPE à partir des boues secondaires comme substrat pour des milieux de culture pures ou mixtes. L'optimisation du processus de floculation en utilisant des biofloculants sous différentes formes de SPE pour aboutir à une meilleure floculation, décantation et déshydratation.

3.2.2 Ojectifs spécifiques

La détermination ou l'évaluation des points suivants, chacun d'eux est basé sur: a) la capacité de production de SPE à partir des boues d'épuration; et b) L'efficacité de l'activité de floculation et de déshydratation du kaolin:

1. Investigation des souches bactériennes pour leur capacité de production de SPE dans les boues d'épuration et leurs caractéristiques biochimiques (Chapitre 3.1).
2. Étude cinétique de la production de SPE en utilisant des boues d'épuration comme matières premières (Chapitre 3.2).
3. Effet de différentes méthodes de prétraitements des boues (traitement à l'acide, traitement thermique, traitement alcalino-thermique et stérilisation) sur la production de SPE dans les boues d'épuration et capacité de floculation et de déshydratation des SPE (Chapitre 4).
4. Détermination de la capacité de floculation et de déshydratation des différents de SPE (bouillon, capsulaire et slime/limon) produits en utilisant les boues d'épuration comme matières premières (Chapitre 4 et 5.1).
5. Effet des concentrations de solides en suspension sur la production de SPE et les capacités de floculation et déshydratation (Chapitre 4 et 5.1).
6. Utilisation de milieux de culture mixte pour la production de SPE dans les boues d'épuration (Chapitre 5.1).
7. Effet de la vitesse d'agitation sur la production de SPE en culture discontinue (Chapitre 5.2).
8. Effet de différentes combinaisons de cations (Ca^{2+}, Mg^{2+}, Fe^{3+}, Al^{3+}) et de SPE sur les applications (Chapitre 5.2).
9. Applications des SPE dans le traitement de différents types d'eau et d'eaux usées (Chapitre 5).

3.3 Originalité

1. La production de SPE a été réalisée à partir des boues prétraitées, comme seule source de matière première. Il s'agit d'une nouvelle approche pour convertir les boues en produits à valeur ajoutée (Chapitre 3 et 5).
2. La caractérisation biochimique de treize souches bactériennes (isolées de boues d'épuration) pour la production des SPE. La caractérisation sera utile pour plusieurs autres applications de ces souches bactériennes (Chapitre 3).
3. Pour la première fois, les capacités de floculation et de déshydratation de trois différentes types de SPE (boue, capsulaires et slime/limon) (produites en utilisant des boues d'épuration) ont été investigué (Chapitre 4).

4. L'optimisation des méthodes de prétraitements des boues, les concentrations de boues et l'étude cinétique de production de SPE seront utiles pour la mise à l'échelle de la production de SPE (Chapitre 3.2, 4 et 5).
5. L'utilisation de différents cations (Ca^{2+}, Mg^{2+}, Fe^{3+}, Al^{3+}) afin d'améliorer la floculation et de déshydratation (Chapitre 5.2).
6. L'utilisation des SPE produits dans les boues d'épuration comme floculant pour le traitement de différents types d'eaux et d'eaux usées (Chapitre 5).

3.4 Méthodologie

Les boues secondaires des eaux usées (sans polymères chimiques) ont été recueillies de l'unité de biofiltration (média filtrant biolite) de la Communauté Urbaine du Québec (usine municipale de traitement des eaux usées et des boues, CUQ, Québec, Canada). Les boues d'épuration ont été utilisées comme matière première pour la production de SPE en culture discontinue (expériences en fioles) pendant toute l'étude. Dans le chapitre 3, treize souches bactériennes produisant des SPE (9 *Bacillus*, 2 *Serratia* et 2 *Yersinia*) ont été utilisées en tant que cultures pures pour le dépistage (chapitre 3.1) et les expériences d'études cinétiques (chapitre 3.2). Le système BIOLOG a été utilisé pour l'identification et la caractérisation biochimique des souches bactériennes (chapitre 3.1). Dans le chapitre 4, la souche *Serratia* sp.1 a été utilisée pour étudier l'effet de différents prétraitements et différentes concentrations de solides des boues sur la production de SPE et les performances de floculation des SPE produits. Dans les chapitres 5.1 et 5.2, treize souches bactériennes productrices de SPE (9 *Bacillus*, 2 *Serratia* et 2 *Yersinia*) ont été utilisées comme cultures pures et/ou mixtes pour la production de SPE dans les boues stérilisées. Tout au long de cette étude, pour effectuer les analyses quantitatives, qualitatives et comparatives des SPE produites dans les boues, la récolte des SPE a été réalisée selon la méthode de précipitations centrifugation-chauffage-éthanol comme décrit dans le chapitre 3.1. Des séries de tests de jar standards ont été réalisées pour évaluer la performance de floculation de différents floculants. Des suspensions d'argile de kaolin (K2-500, USP, Fisher Scientific, États-Unis) (5 g/L dans de l'eau désionisée) ont été utilisées comme suspensions standards. Les formes brutes de SPE (Bouillon B-SPE, capsulaire C-SPE et Slime S-SPE) ont été utilisées dans les études de floculation. Des mesures de turbidité (NTU) et CST (s) ont été effectuées pour évaluer l'activité de floculation et de déshydratation dans les expériences (chapitre 3, 4, 5). Les potentiels Zeta ont été mesurés pour la compréhension des mécanismes de floculation (chapitre 3, 4) et pour sélectionner les

concentrations de cations (chapitre 5). Dans les chapitres 3, 4 et 5.1, les B-SPE combinées avec le calcium ont été utilisés dans les essais de floculation, alors que, dans la partie 2 du chapitre 5, différents cations tels que le calcium, le magnésium, le fer et l'aluminium ont été utilisés dans les essais de floculation. Dans les chapitres 5.1 et 5.2, un polymère chimique classique (Magnafloc-155) a été utilisé pour comparer les performances de floculation des SPE. Dans le chapitre 5, les SPE ont été testées pour les applications potentielles dans l'eau de la rivière, les eaux usées municipales et les eaux usées de brasserie pour le retrait de la turbidité et l'élimination de la DCO.

PARTIE 4: RÉSULTATS ET DISCUSSION

4.1 Criblage et cinétiques de production des SPE de treize souches microbiennes à partir de boue des eaux usées

4.1.1 Criblage et caractérisation biochimique des bactéries productrices de SPE (Chapitre 3.1, article publié)

Afin de comprendre les capacités de production de SPE des cultures pures à partir de boues des eaux usées, il est essentiel d'acquérir une connaissance de leurs propriétés métaboliques et leur susceptibilité vis-à-vis de certains composés chimiques et antimicrobiens. Dans ce contexte, la technique d'identification biochimique utilisant les plaques BIOLOG représente un outil pratique et efficace pour l'étude et la comparaison des profils physiologiques de différentes souches bactériennes. L'analyse des profils physiologiques permet d'estimer les degrés de similarité entre les souches bactériennes étudiées. Le système BIOLOG permet d'investiguer le potentiel des microorganismes à croitre et se développer sur un nombre donné de sources de carbone. Ainsi, la caractérisation biochimique de 13 souches bactériennes productrices de SPE a été effectuée en utilisant la technologie BIOLOG. Les propriétés métaboliques et biochimiques de chaque souche isolée des boues, leur capacité à produire des SPE en les cultivant sur les boues des eaux usées, ainsi que l'activité floculante des SPE produites ont été étudiées.

Les souches bactériennes ont été cultivées sur des boues des EU stérilisées pour la production de SPE. Les capacités floculantes et déshydratantes des SPE produites (bouillon, capsulaire et limon) ont été examinées en utilisant la technique de suspension au kaolin combiné au calcium (150 mg Ca^{2+}/L de suspension au kaolin). La caractérisation des souches étudiées par le BIOLOG a permis d'identifier 9 souches du genre *Bacillus*, 2 du genre *Serratia* et 2 du genre *Yersinia*. La plupart de ces souches bactériennes ont montré un potentiel important pour d'utilisation d'un large spectre de sources de carbone et d'azote. Une concentration de SPE supérieure à 1 g/L a été produite par la majorité des souches investiguées. La concentration de SPE produite par les différentes souches de *Bacillus* a été supérieure à celle produite par *Serratia* et *Yersinia*. Les SPE sous forme de bouillon ont montré une activité floculante de plus de 75% pour *Bacillus* sp. 7, *Bacillus* sp. 4 et *Bacillus* sp.6. Cette activité a été atteinte en utilisant une très faible concentration de SPE sous forme de bouillon (1,12-2,70 mg SPE/g MES).

4.1.2 Cinétique de production des SPE des treize isolats cultivés sur les boues des eaux usées (Chapitre 3.2, article publié)

La détermination des paramètres cinétiques de croissance et de production de SPE est requise afin d'acquérir la compréhension nécessaire des mécanismes métaboliques impliqués dans la synthèse des SPE. Jusqu'à présent, la littérature ne rapporte aucune étude ayant porté sur la synthèse de SPE à partir des boues des EU. Ainsi, pour être capable d'utiliser ces boues comme substrat pour la production de SPE, une investigation systématique de la croissance des souches bactériennes et la synthèse de SPE en fonction du temps, réalisée à l'échelle du laboratoire, est fortement recommandée. Une telle analyse aboutirait à la production efficace de SPE possédant les propriétés requises. En effet, pour pouvoir appliquer les SPE dans les procédés de contrôle de la pollution environnementale, il est important d'étudier leur mécanisme de biosynthèse durant le procédé de fermentation. De même, la compréhension de la cinétique de croissance et de synthèse des SPE est importante pour assurer une production stable et importante de ces composés à grande échelle. Pour cela, l'étude de l'influence de la croissance cellulaire pour la production de SPE à l'échelle du laboratoire a été effectuée au cours de ce projet. Treize isolats ont été cultivés en mode batch sur les boues des EU stériles afin de déterminer la corrélation entre la croissance cellulaire, la production de SPE et leurs caractéristiques et propriétés de floculation.

Toutes les souches bactériennes cultivées dans les boues des EU ont montré un profil de croissance sigmoïde typique d'une culture en batch. Les profils de croissance de *Bacillus* sp.1 et sp.2, *Serratia* sp.2 et *Yersinia* sp.2 ne montrent pas d'occurrence d'une phase de latence apparente et semblent entamer leurs croissances directement en phase exponentielle. Ceci indique que ces souches sont bien adaptées à la croissance dans les boues stérilisées. D'autre part, les autres souches (*Bacillus* sp.3, *Bacillus* sp.4, *Bacillus* sp.5, *Bacillus* sp.6, *Bacillus* sp.7, *Bacillus* sp.8, *Bacillus* sp.9, *Serratia* sp.1 and *Yersinia* sp.1) ont montré une phase de latence initiale d'une durée de 3-6h avant l'entame de la phase exponentielle de croissance. Cette occurrence semble probablement due à un arrêt de croissance ayant affecté une fraction de la population microbienne fraichement inoculée dans le milieu de culture, avant de s'adapter aux nouvelles conditions de croissance.

La concentration maximale de B-SPE (1,36-2,12 g/L) a été observée entre 60-72 h de fermentation pour la plupart des souches étudiées, à l'exception de *Bacillus* sp.1 et *Yersinia* sp.2. Dans ces deux derniers cas, des concentrations maximales de B-SPE (1,36-1,95 g/L) ont été rapportées à 48 h et 36 h de fermentation, respectivement. *Bacillus* sp.7, *Serratia* sp.2 et

Yersinia sp.2 ont révélé des productivités élevées (0,021, 0,023 et 0,036 g/L/h, respectivement). Dans tous les cas, la concentration des SPE de type limon (S-SPE) a été supérieure (2,5-7,3 fois) à celle des SPE capsulaires (C-SPE). La concentration des protéines a été plus élevée (3,9-4,6 fois et 2,7-3,3 fois pour S-SPE et C-SPE, respectivement) que celle des carbohydrates dans le cas de toutes les cultures. Des variations significatives dans les activités floculantes (AF) (13,8-83,1 %) ont été observées dans le cas de toutes les cultures microbiennes étudiées. En général, les AF les plus élevées (≥ 75%) ont été observées pour les SPE collectées entre 60 - 72 h de fermentation dans le cas de *Serratia* sp.1, sp.2 et *Yersinia* sp.1 ainsi que la plupart des souches de *Bacillus* (sp.2, 3, 4, 7 et 8). Dans le cas de *Bacillus* sp.1, *Bacillus* sp.5, *Bacillus* sp.9 et *Yersinia* sp.2, des AF maximales de 58,8%, 55,2%, 62,4% et 79,5% ont été observées pour les SPE échantillonnées à 48, 60, 72 et 36 h d'incubation, respectivement.

Les SPE synthétisées par *Serratia* sp.2 et *Bacillus* sp.7 ont révélé les AF les plus élevées (83,1 %). Les AF des B-SPE échantillonnées à différents temps d'incubation (et produites par différentes souches) sont distinctes et les variations observées sont attribuées à la concentration des SPE et leurs compositions (fractions protéique et glucidique). Les AF ont augmentées avec l'augmentation de la concentration des protéines et des carbohydrates composant les SPE testées. D'une manière générale, l'atteinte d'une AF élevée (≥ 75%) requiert des concentrations de 1,31-170 mg B-SPE/g kaolin, 0,45-0,96 mg protéine/g kaolin et 0,11-0,21 mg glucides/g kaolin.

4.2 Effet des différents prétraitements (thermo-acide, thermo-alcalin et stérilisation) et de la concentration des matières en suspension de la boue sur la production de SPE (Chapitre 4, article publié)

Parce qu'elles renferment la majeure fraction des nutriments nécessaires à la culture, les matières en suspension des boues (MES) affectent le taux de croissance et la formation de produit des souches bactériennes cultivées. Le prétraitement des boues est essentiel pour la solubilisation des sources de carbone complexes en fragments plus simples, aidant ainsi les microorganismes à les assimiler. Ainsi, dans cette étude, la souche *Serratia* sp.1 a été cultivée dans les boues des EU afin d'optimiser la concentration de MES permettant de maximiser le rendement en SPE. Trois types de prétraitements, la stérilisation (ST), le traitement thermo-acide (ACT) et le traitement thermo-alcalin (ALT), ont été appliqués à différentes concentrations de solides totaux afin d'étudier leur impact sur la production de SPE. L'activité de floculation du

kaolin, ainsi que les capacités de déshydratation des différents types de SPE ont été étudiées en présence de cations divalents (Ca^{2+}).

Les résultats ont clairement montré que les SPE produites par *Serratia* sp.1 varient significativement en fonction du prétraitement appliqué à la boue ainsi qu'à sa teneur en MES. Une production maximale de SPE (B-SPE) de 3,4 g/L, 2,8 g/L et 1,5 g/L a été enregistrée pour les cultures effectuées sur les boues ALT, ST et ACT, respectivement. De plus, pour une même concentration en solides totaux, différentes quantités de B-SPE ont été produites en fonction des prétraitements appliqués. Par exemple, les quantités de B-SPE produites pour les boues ST, ALT et ACT pour une concentration en solides totaux de 17,0 g/L, sont de 2,2 g/L, 3,4 g/L et 1,2 g/L, respectivement. Similairement au profil des B-SPE, les S-SPE produites correspondaient à 2,0 g/L, 2,3 g/L et 1,0 g/L pour les boues ST, ALT et ACT, respectivement. D'une manière générale, les S-SPE pour les boues ST et ALT ont été 1,5-2 fois plus élevées que les C-SPE.

Dans le cas des boues ST, la concentration de B-SPE a augmenté de 2,2 g/L à 2,8 g/L avec une augmentation des solides totaux de 17 g/L à 22,4 g/L. De plus, une augmentation des solides totaux de 29,8 g/L à 44,8 g/L a induit une diminution de la production de B-SPE de 2,8 g/L à 2,5 g/L. Une tendance similaire a été observée pour les B-SPE durant la fermentation des boues ACT. Cependant, dans le cas des boues ALT, la concentration de B-SPE a diminué de 3,4 g/L à 2,0 g/L avec une augmentation des solides totaux de 17,0 g/L à 44,8 g/L. Les résultats confirment aussi que les concentrations des différentes formes de SPE (capsulaire, limon et bouillon) produites dans tous les types de substrats ont été principalement affectées par la teneur en solides totaux dans les boues. La concentration de S-SPE a varié en fonction de la concentration en solides totaux, similairement aux B-SPE. La concentration de C-SPE est restée relativement constante pour une concentration en solides totaux variant de 17 g/L à 44,8 g/L des boues de type ST (environ 0,8 g/L) et ALT (0,8-1,1 g/L), alors que la production de C-SPE a été faible pour les boues ACT (<0,5 g/L).

Les B-SPE possédant l'AF la plus élevée (79,13 %) ont été produites dans des boues de type ST, à une concentration de solides totaux de 17 g/L. Par ailleurs, les B-SPE (0,7-0,9 mg/g kaolin) produites dans le même type de boue, à des concentrations de solides totaux de 22,4, 29,8, 37,3 et 44,8 g/L, ont exhibé des AF respectives de 71,4%, 74,2%, 41,5% et 33,5%. Dans la présente étude, les mêmes concentrations de SPE (utilisées pour la détermination de l'AF) produites en utilisant différentes concentrations en solides totaux de boues dans le milieu ont révélé différentes activités floculantes. Les ratios protéines sur carbohydrates des SPE de type

limon, observés respectivement pour les boues ST, ALT et ACT, ont été de: 2, 1,8 et 1,4, pour une concentration de solides totaux de 17 g/L; de 2,0, 2,1 et 1,2 à 22,4 g/L de solides totaux; de 1,8, 1,6 et 1,1 à 29.8 g/L de solides totaux; de 1,4, 1,2, 1,3 à 33,7 g/L de solides totaux et de 1,3, 1,4, 1,1 à 44,8 g/L de solides totaux. Ces résultats permettent d'établir clairement que la composition des SPE montrait des différences dépendamment que les cultures aient été effectuées dans un milieu de culture synthétique ainsi qu'à différentes concentrations de matières en suspension (présente étude). Ce changement dans le ratio protéines sur carbohydrates représente une des raisons qui ont changé la nature (groupement fonctionnel) des SPE produites. Ainsi, la nature distincte des B-SPE produites à différentes concentrations de solides pourrait aussi expliquer cette variation dans les AF.

Les B-SPE, C-SPE et S-SPE obtenues des boues prétraitées par stérilisation ont montré une activité de floculation maximale de 79,1%, de 72,3% et de 53,0%, respectivement. Cependant, les B-SPE obtenues des boues ST, ALT et ACT ont montré des AF respectives de 79,1%, 60,3% et 44,7%. Des concentrations relativement élevées de C-SPE (2,8 à 3,5 mg C-SPE/g kaolin) ont été requises pour atteindre une AF élevée (>60 %), par comparaison à celles de B-SPE (0,7 à 0,9 mg B-EPS/g kaolin). Les résultats ont clairement montré que les B-SPE possédaient l'activité floculante la plus élevée par rapport aux S-SPE et aux C-SPE obtenues à partir des boues ST. D'une manière générale, la présente étude a montré une très faible quantité de SPE (sous toutes les formes) a été requise pour atteindre des AF maximales, en comparaison à celles rapportées par la littérature. B-SPE, C-SPE et S-SPE, obtenues à partir des mêmes boues prétraitées, ont permis d'augmenter la capacité de déshydratation jusqu'à 44,8%, 52,2% et 31,2%, respectivement. Cependant, les B-SPE obtenues des boues ST, ALT et ACT ont révélé une capacité de déshydratation de 44,8%, 40,6% et 30,9%, respectivement.

4.3 Culture mixte: la production de SPE, la cinétique et l'application des SPE à l'eau et au traitement des eaux usées (Chapitre 5, un article publié et un article soumis)

Treize souches bactériennes produisant des SPE ont été cultivées (en culture mixte) dans la boue stérilisée (solides en suspension: 25 g/L) pour optimiser les conditions d'agitation nécessaires à la production la plus élevée de SPE. Les conséquences de la croissance microbienne de la culture mixte sur la production de SPE au cours de la fermentation en discontinue à l'échelle du laboratoire ont été réalisées. Les SPE récoltées ont été examinées pour leur performance de floculation (élimination de la turbidité et de la déshydratation) dans les

essais de floculation utilisant des suspensions de kaolin avec le Ca^{2+}, Mg^{2+}, Fe^{3+} et Al^{3+}, respectivement. Les SPE ont également été utilisées pour le traitement de l'eau de rivière, les eaux usées municipales et les boues secondaires des eaux usées.

4.3.1 Production de SPE par une culture mixte dans les boues d'épuration (Chapitre 5.2)

La concentration des SPE produites par la culture mixte a considérablement varié avec la vitesse d'agitation. La concentration de B-SPE a augmenté avec l'augmentation de la vitesse d'agitation de 0 à 150 tours par minute. La plus grande concentration de B-SPE (4,950 g/L) ont été observées à 150 tours par minute. La concentration de B-SPE observée à 150 tpm était 1,5 fois plus élevée que la concentration observée sans agitation (1,975 g/L). Des vitesses d'agitation plus élevées (200 tours par minute et 250) ont eu un effet néfaste sur la production de SPE. Les concentrations de B-SPE obtenues à une plus grande vitesse d'agitation étaient de 24-25% moins élevées que les concentrations de B-SPE observées à 150 tours par minute. Les résultats ont indiqué qu'une vitesse de 150 tours par minute est la vitesse d'agitation optimale requise pour la production maximale de SPE. Comme pour les B-SPE, les concentrations les plus élevées de S-SPE (4,125 g/L) ont été observées à 150 tours par minute, soit 2,75 fois plus élevée que la concentration observée sans agitation. Dans le cas des C-SPE, la concentration observée à 150 tpm était 1,73 fois plus élevée que celle observée sous aucune agitation. Les activités de floculation des échantillons de bouillon (FA) ont été recueillies après 72 h d'incubation. La meilleure FA (89,4%) et la meilleure déshydratation (60,5%) ont été observés pour les B-SPE produites à 150 tpm. Les meilleures FA et déshydratation obtenues pour les B-SPE produites à 150 tours par minute sont dues à la concentration plus élevée (4,9 mg/L) de B-SPE présentes dans le dosage par rapport à tous les autres échantillons de B-SPE. La concentration de B-SPE (4,9 g/L) produite par la culture mixte (13 souches bactériennes) cultivée dans la boue stérilisée (à 72 h), est supérieure à celle de la culture pure (2,7-3,7 g/L), dans des conditions de fermentation analogues. La culture mixte produit une concentration de B-SPE 1,3 à 1,8 fois supérieure par rapport à la culture pure. Des concentrations plus élevées de B-SPE produits par des cultures mixtes dans la présente étude pourraient être attribuées à la relation symbiotique entre les différentes souches bactériennes dans le consortium. Dans l'ensemble, la synthèse de SPE en utilisant la culture mixte et des boues stérilisées comme milieu de production a des avantages sur les cultures pures sur le plan de la production de concentrations élevées de SPE. Au cours des 72 h d'incubation à l'aide de la culture mixte, il y avait une légère augmentation des concentrations de protéines et de glucides dans les SPE

jusqu'à 60 h d'incubation. Des contenus en protéines de 0,582 g/g pour les S-SPE et 0,669 g/g pour les C-SPE ont été observés à 72 h. La concentration en protéines est plus élevée que celle des hydrates de carbone dans tous les cas. Des concentrations en glucides de 0,133 g/g pour les S-SPE et de 0,258 g/g pour les C-SPE ont été observées à 60 h. Les concentrations en S-SPE étaient plus élevées par rapport aux C-SPE dans les bouillons. Ainsi, la majeure partie des SPE totaux (S-SPE et C-SPE), protéines (77-82% p/v) et de glucides (67-68% p/v) étaient présents dans les S-SPE. Les C-SPE contiennent 18-23% des protéines et 24-33% des glucides totaux des SPE.

4.3.2 Cinétique de production de SPE par culture mixte en utilisant des boues d'épuration (Chapitre 5.2)

La culture mixte a révélé une courbe de croissance de type sigmoïdale typique de la culture discontinue dans les boues stérilisées. Le profil de croissance des cellules n'a pas montré de phase de latence apparente et a commencé en phase exponentielle. Ceci indique que la culture mixte est bien adaptée à la croissance dans les boues stérilisées. La culture mixte atteint la concentration cellulaire maximum (de 1,76 à 1,78 x 10^{11}) à 60-72 heures d'incubation. Pendant l'incubation, la concentration des cellules a augmenté de deux cycles logarithmiques dans les 36 premières heures, alors qu'il n'y avait qu'une légère augmentation de la concentration cellulaire (de 1,63 à 1,78 x 10^{11}) de 36 h à 72 h. Les taux de croissance spécifiques ont été calculés comme étant la pente du tracé semi-logarithmique du nombre de cellules en fonction du temps de culture au cours de la phase exponentielle. Le taux de croissance spécifique maximal était de 0,35 h^{-1}.

Pendant 96 heures d'incubation, la concentration en B-SPE augmente avec le nombre de cellules totales (CFU/ml). Une concentration maximale de B-SPE de 4,9 g/L a été obtenue à 72 h. Pendant la même période, des concentrations en S-SPE de 4,1 g/L et C-SPE de 0,8 g/L ont été obtenues. La boue brute avait de très faibles concentrations de SPE (moins de 13 mg/g solides en suspensions). Jusqu'à 72 h d'incubation, les concentrations B-SPE et de S-SPE varient proportionnellement au compte des cellules. Une baisse continue de la production de B-SPE a été observée. Le taux de production de B-SPE était maximal, 0,127 à 0,123 g/L/h pour 12-24 h d'incubation, alors qu'il a diminué rapidement à 0,024 g/L/h (à 48 h) et il a diminué progressivement à 0,013 g/L/h pour le restant de l'incubation. Au cours de la phase stationnaire (72 à 96 h), la productivité de B-SPE était soit nulle ou négative, ce qui indique que la dégradation des SPE était supérieure à la production des SPE par les cellules, qui ont tendance

à prendre leurs nutriments nécessaires à partir de ce matériau (matrice SPE) de stockage. Pour les S-SPE, la productivité a augmenté légèrement pendant l'incubation de 12 à 24 h, respectivement, et a diminué par la suite. Dans le cas des C-SPE, la productivité maximale était de 0,026 g/L/h à 12 h. Les résultats démontrent clairement que la production maximale de SPE (toutes les formes) a eu lieu pendant la phase de croissance exponentielle jusqu'à 24 h d'incubation et la production d'EPS avait continué lentement pendant encore 72 h.

Les taux de production spécifiques (q_{SPE}) ont été calculés pour toutes les formes de SPE, respectivement, à la vitesse de variation de la concentration des SPE par million de cellules. Les taux de production spécifiques de SPE (toutes les formes) et les taux de croissance spécifiques des cellules avaient une corrélation linéaire. L'ordonnée à l'origine de l'équation était égal à zéro ou proche de zéro, ce qui indique que la production de SPE est associée à la croissance. Les résultats de cette étude justifient l'évaluation de l'efficacité du processus de production des SPE en tenant compte de la concentration des SPE produits, le coût du milieu de culture et de la durée du procédé.

4.3.3 Effet de l'addition de différents cations sur les performances de la floculation du kaolin par les EPS (Chapitre 5.2)

L'addition de différents cations (150 mg Ca^{2+}/L, 150 mg Mg^{2+}/L, 5 mg Fe^{3+}/L et 5 mg Al^{3+}/L respectivement), suivi par l'addition de SPE ont démontré un profil distinct des FA du kaolin. Les cations divalents (Ca^{2+} et Mg^{2+}) ont révélé une FA plus élevée par rapport aux cations trivalents (Fe^{3+} et Al^{3+}). Un maximum de FA de 81,6%, 79,9%, 54,4% et 68,3% a été observée pour le Ca^{2+}, Mg^{2+}, Fe^{3+} et Al^{3+}, respectivement. Le retrait le plus élevé de la turbidité ou le FA maximum pour les cations Ca^{2+} et Mg^{2+} a été observé de 1,2 à 1,6 mg SPE/g kaolin, alors que la suppression maximale de la turbidité ou la FA maximale pour Fe^{3+} et Al^{3+} a été observée à 1,6 mg SPE/g de kaolin. Le Magnafloc-155, un polymère chimique anionique classique, a été utilisé dans les essais en récipients (jar test) similaires pour les B-SPE, pour comparer les performances de floculation. L'ajout de différents cations avec le Magnafloc-155 a également eu des répercussions importantes sur la FA du kaolin similaire au bouillon de SPE. Comme pour les bouillons de SPE, les cations divalents (Ca^{2+} et Mg^{2+}) ont révélé une FA plus élevée par rapport aux cations trivalents (Fe^{3+} et Al^{3+}) pour le Magnafloc-155. Une FA maximum de 90,4%, 88,5%, 77,9% et 75,4% a été observée pour le Ca^{2+}, Mg^{2+}, Fe^{3+} et Al^{3+}, respectivement. La concentration optimale de Magnafloc-155 était dans la gamme de 0,08 à 0,16 mg/g de kaolin nécessaire pour obtenir la FA maximale atteignable, ce qui est relativement inférieur à celle des

bouillons de SPE (1,2 à 1,6 mg/g de kaolin), quels que soient les cations. Les SPE ou bioflocculants utilisés dans l'étude étaient de la forme la plus simple, c'est-à-dire un bouillon fermenté sans poursuite du processus de purification.

4.3.4 Applications des SPE dans le traitement des eaux de rivière, eaux usées municipales et eaux usées de brasserie

4.3.4.1 Effet de différentes formes de SPE (Chapitre 5.1)

Eau de rivière. Un enlèvement maximum de la turbidité de 93,5, 55,7 et 81,7% a été observé à 2,0, 0,8 et 0,8 mg/L de B-SPE, S-SPE et C-SPE, respectivement. L'élimination de la turbidité de 93,9% a été observée à une concentration de 0,2 mg/L de Magnafloc-155. Une augmentation supplémentaire de la concentration de Magnafloc-155 à 0,5 mg/L a résulté en une augmentation de la turbidité. Cependant, cette augmentation de la turbidité était relativement moindre comparativement à celle des SPE. L'élimination maximale de la DCO (52%) a été observée pour le B-SPE alors qu'il était de 48% pour le Magnafloc-155. La performance des B-SPE est comparable à celle du Magnafloc-155. L'ajout de SPE anionique a conduit au rassemblement des particules neutralisées et des cations pour former des flocs plus gros et plus denses. Une augmentation de la densité des particules SPE-calcium a augmenté la précipitation de ces particules et par le fait même augmenter l'élimination de la turbidité. À la concentration optimale de B-SPE, la turbidité de l'eau de rivière a été réduite à 1,5 NTU. L'application de B-SPE pour le traitement de l'eau de rivière a donné des résultats prometteurs, mais des recherches supplémentaires sont nécessaires pour évaluer son applicabilité sur le terrain.

Eaux usées municipales. Une élimination maximale de la turbidité de 91,7, 74,5 et 86,9% a été observée à 5.0, 4.2 et 0.7 mg/L de B-SPE, S-SPE et C-SPE, respectivement. Une élimination de la DCO de 84,9, 60,6 et 85,8% a été observée dans le cas de B-SPE, S-SPE et C-SPE, respectivement. Des enlèvements de la turbidité et la DCO de 97,8 et de 87,2%, respectivement, ont été observés à une concentration de 0,8 mg/L de Magnafloc-155. La concentration requise en B-SPE pour atteindre plus de 80% d'élimination de la turbidité et de 70% de la DCO était 6,25 fois plus élevée que celle du Magnafloc-155.

Eaux usées de brasserie. Un maximum d'élimination de la turbidité de 81,8, 59,2 et 79,8% a été observé à 12,4, 10,4 et 6,6 mg/L de B-SPE, S-SPE et C-SPE, respectivement. Une élimination de la DCO de 88,4, 67,6 et 87,6% a été observée à 12,4, 10,4 et 5,0 mg/L de B-SPE, S-SPE et

C-SPE, respectivement. L'enlèvement de la turbidité et la DCO de 84,8 et de 89,7% a été observé à une concentration de 5,0 mg/L pour le Magnafloc-155. La performance du Magnafloc-155 dans le traitement des eaux usées de brasserie était comparable à celle des B-SPE et C-SPE respectivement. Le dosage optimal de B-SPE était d'environ 2 fois plus élevé que celui des C-SPE. Les B-SPE ont efficacité de floculation relativement faible (en respectant la concentration des SPE) par rapport aux C-SPE.

4.3.4.2 Effet de l'addition de différents cations sur le traitement de l'eau et des eaux usées à l'aide des SPE (Chapitre 5.2)

La concentration en cations. Pour induire la neutralisation des charges, différents cations devaient être ajoutés aux eaux de rivière, eaux usées municipales et de brasserie. Pour atteindre la performance maximale de floculation des différentes eaux et eaux usées, souvent le potentiel zêta du système est diminué dans la plage de -15 < potentiel Zeta < -10 mV (Jefferson et Parsons, 2005). L'initiation de ce phénomène a été observée 50 mg/L de Ca^{2+}, 50 mg/L de Mg^{2+}, 5 mg/L Fe^{3+} et 5 mg/L Al^{3+} d'eau de rivière, respectivement. Pour les eaux usées municipales, 200 mg/L Ca^{2+}, 200 mg/L Mg^{2+}, 20 mg/L Fe^{3+} et 20 mg/L Al^{3+} d'eaux usées municipales ont été sélectionnés. Pour les eaux usées de brasserie, 250 mg/L Ca^{2+}, 250 mg/L Mg^{2+}, 40 mg/L Fe^{3+} et 40 mg/L Al^{3+} d'eaux usées de brasserie ont été sélectionnés. Par conséquent, les concentrations de cations telles que décrites précédemment au cours de laquelle la formation de flocs étaient visibles ont été choisies pour les tests (jar test) pour étudier l'effet de la concentration des SPE sur la turbidité et la DCO.

Eau de rivière. Il a été constaté que, pour tous les cations, l'enlèvement de la FA a augmenté avec la dose de SPE jusqu'à la dose optimale de B-SPE. Lorsque le dosage de SPE est supérieur à la dose optimale, l'élimination de la turbidité est diminuée. La FA maximale de 93,5%, 93,5%, 94,8% et 93,5% a été observée pour le Ca^{2+}, Mg^{2+}, Fe^{3+} et Al^{3+}, respectivement. La turbidité finale de l'eau de rivière était de 1,5, 1,5, 1,2 et 1,5 NTU, respectivement. Ces réductions de turbidité ont été obtenues à une concentration de cations bivalents de 50 mg/L d'eau de rivière (concentration plus élevée) en comparaison à 5 mg/L d'eau de rivière (d'une concentration plus faible) pour les cations trivalents. Pour des concentrations de cations et des conditions de mélange similaires, les essais de floculation ont été effectués avec le Magnafloc-155, pour comparer la performance des B-SPE pour le traitement de l'eau de rivière. L'ajout de différents cations avec le Magnafloc-155 a également eu des répercussions importantes sur FA similaire à celles des SPE. La FA maximum de 93,9%, 94,3%, 96,1% et 94,3% a été observée

pour le Ca^{2+}, Mg^{2+}, Fe^{3+} et Al^{3+}, respectivement. La concentration optimale de 0,2 mg de Magnafloc-155/L d'eau de rivière était relativement plus faible que les B-SPE (1,0-1,5 mg B-SPE /L kaolin). Une élimination maximale de la DCO de 70,2% et de 67,3% a été observée pour le Fe^{3+} et Al^{3+} (combiné avec 1,5 et 1,0 mg B-SPE /L, respectivement), tandis que, l'élimination DCO maximale de 52,0% et de 51,4% a été observée pour Ca^{2+} et Mg^{2+} (combiné avec 1,0 mg B-SPE /L). Pour le Magnafloc-155 (dose de 0,2 mg/L), l'élimination maximale de la DCO de 48,0%, 53,8%, 53,2% et 46,8% a été observée pour le Ca^{2+}, Mg^{2+}, Fe^{3+} et Al^{3+}, respectivement.

Globalement, les résultats ont montré que les performances de floculation des B-SPE étaient tout à fait semblables et comparables à celles du Magnafloc-155. À un dosage optimal de B-SPE, la turbidité de l'eau rivière a été réduite à 1,2 NTU. Toutefois, selon Santé Canada (2003) l'eau potable traitée doit avoir turbidité inférieure à 0,1 NTU à tout moment. Par conséquent, pour atteindre les limites autorisées de turbidité, la filtration sur sable classique ou des étapes de filtration sur membrane sont nécessaires après la floculation. Le pH initial de l'eau de rivière était de 7,85, ce qui est légèrement alcalin. Après l'addition des B-SPE ou de Magnafloc-155, le pH de l'eau de rivière est resté inchangé. La valeur du pH de l'eau de rivière est diminuée de 7,85 à 7,4 ± 2 par l'addition de cations divalents (Ca^{2+} et Mg^{2+}) au cours des tests en pot. Ceci indique qu'il n'y a pas eu de correction requise de la valeur du pH pour l'eau traitée dans ce cas. Cependant, le pH de l'eau de rivière est passé de 7,85 à 6-6,3 par l'addition de cations trivalents (Fe^{3+} et Al^{3+}). Le pH est passé de légèrement alcalin à acide. La diminution du pH est attribuée à la dissociation des cations par la série de réactions d'hydrolyse et la production d'ions H^{+}. L'amplitude du changement du pH est liée à la concentration de coagulants chimiques. L'application de B-SPE pour le traitement de l'eau de rivière a donné des résultats prometteurs, mais des recherches supplémentaires sont nécessaires pour évaluer son applicabilité sur le terrain.

Eaux usées municipales. L'ajout de cations était nécessaire pour la coagulation ou la neutralisation des charges des solides des eaux usées et c'était une condition préalable à l'ajout de floculants (B-SPE ou Magnafloc-155). Bien que les concentrations de cations ont été maintenues au même, le dosage optimum de B-SPE nécessaire pour atteindre une FA maximum a été déterminée par addition de différentes concentrations de B-SPE dans les eaux usées municipales. L'ajout de différents cations avec les B-SPE à des eaux usées a eu un impact significatif sur la FA. La tendance générale est l'augmentation de la FA avec l'augmentation des B-SPE (à une concentration de cations constante) jusqu'à la dose optimale

de 1,49 mg B-SPE /L eaux usées. L'addition de B-SPE au-delà de la dose optimale n'a pas augmenté la FA. Une FA maximum de 91,7%, 87,6%, 94,3% et 92,2% a été observée pour le Ca^{2+}, Mg^{2+}, Fe^{3+} et Al^{3+}, respectivement. Une suppression maximale de la DCO de 80,4% et de 78,1% a été observée pour le Fe^{3+} et Al^{3+}, alors qu'une élimination maximale de DCO de 74,5% et de 74,5% a été observée pour les ions Ca^{2+} et Mg^{2+} (tableau 1.3). Une élimination maximale de la turbidité et de la DCO a été obtenue à la dose de 1,5 mg B-SPE/L pour les cations bivalents (Ca^{2+} et Mg^{2+}) et 2,0 mg B-SPE/L pour les cations trivalents (Fe^{3+} et Al^{3+}), respectivement. Selon les concentrations de cations et des conditions d'agitation similaires, les essais de floculation ont été effectués à l'aide du Magnafloc-155 pour comparer la performance des B-SPE pour le traitement des eaux usées municipales. L'ajout de différents cations avec le Magnafloc-155 a également eu des répercussions importantes sur la FA des eaux usées municipales similaire au B-SPE. Dans le cas du Magnafloc-155, une FA maximum de 93,9%, 94,3%, 96,1% et 94,3% a été observée pour le Ca^{2+}, Mg^{2+}, Fe^{3+} et Al^{3+}, respectivement. Une suppression maximale de la DCO de 76,8%, 75,5%, 83,8% et 79,7% a été observée pour le Ca^{2+}, Mg^{2+}, Fe^{3+} et Al^{3+}, respectivement. L'élimination maximale de la turbidité et de la DCO a été atteinte à une dose de 0,6 mg Magnafloc-155/L et 0,4 mg Magnafloc-155/L pour les cations divalents (Ca^{2+} et Mg^{2+}) et les cations trivalents (Fe^{3+} et Al^{3+}), respectivement. Les cations peuvent neutraliser les charges négatives à la fois des polysaccharides et des particules en suspension et augmenter l'adsorption des polysaccharides sur des particules en suspensions (Wu et Ye, 2007). Contrairement aux eaux de rivière et lacustre, les eaux usées municipales fournissent un système abondants en matière organique et en anions complexant, ce qui devrait étendre la gamme de concentration dans laquelle les espèces hydrolysantes et coagulantes interagissent avec le contenu de l'eau brute.

Eaux usées de brasserie. L'ajout de différents cations avec les B-SPE à des eaux usées a eu un impact significatif sur la FA. Une FA maximum de 81,8%, 76,4%, 83,9% et 79,5% a été observée pour le Ca^{2+}, Mg^{2+}, Fe^{3+} et Al^{3+}, respectivement. Une suppression maximale de la DCO de 88,4%, 85,7%, 87,4% et 86,2% a été observée pour le Ca^{2+}, Mg^{2+}, Fe^{3+} et Al^{3+}, respectivement. La concentration de B-SPE optimale pour l'élimination maximale de la turbidité et de la DCO était de 12,4 mg B-SPE/L d'eaux usées de brasserie, quel que soit le type de cations ajouté aux eaux usées de brasserie. Les essais de floculation ont été effectués à l'aide du Magnafloc-155 à des concentrations de cations et de mélange similaires, pour comparer la performance des B-SPE pour le traitement des eaux usées de brasserie. L'ajout de différents cations avec le Magnafloc-155 a également eu des répercussions importantes sur la FA des eaux usées de brasserie similaires au B-SPE. Une FA maximum de 84,2%, 81,5%, 85,6% et

86,4% a été observée pour le Ca^{2+}, Mg^{2+}, Fe^{3+} et Al^{3+}, respectivement. Dans le cas d Magnafloc-155, une élimination maximale de la DCO de 89,7%, 88,8%, 89,7% et 89,3% a été observée pour le Ca^{2+}, Mg^{2+}, Fe^{3+} et Al^{3+}, respectivement. Une élimination maximale de la turbidité et de la DCO a été atteinte à une dose de 5,0 mg Magnafloc-155/L pour les cations divalents (Ca^{2+} et Mg^{2+}) et 4,0 mg Magnafloc-155/L pour les cations trivalents (Fe^{3+} et Al^{3+}).

PARTIE 5: CONCLUSIONS ET RECOMMANDATIONS

5.1 Conclusions

1. Toutes les treize souches bactériennes ont la capacité de produire de SPE en utilisant les boues stérilisées comme matière première. Les souches de *Bacillus* produisent une plus forte concentration de SPE que les souches *Serratia* et *Yersinia* (Chapitre 3.1).
2. Les B-SPE (pour toutes les treize souches de bactéries) ont présenté une meilleure activité de floculation (plus de 75%), à de très faibles concentrations de B-SPE (1,12 à 2,70 mg SPE/g SS) (Chapitre 3.1).
3. La production de SPE de l'ensemble des souches bactériennes est associée à la croissance lors d'une incubation de façon individuelle dans la boue stérilisée. Les concentrations de protéines et de glucides par g de SPE étaient restées constantes ou modifiées de manière non significative pendant 72 h d'incubation dans la boue stérilisée, quelle que soit la souche bactérienne (Chapitre 3.2).
4. En général, pour réaliser des fortes activités de floculation (≥ 75%), de 1,31 à 1,70 mg B-SPE/g de kaolin, de 0,45 à 0,97 mg de protéine/g de kaolin et de 0,11 à 0,21 mg glucides/g de kaolin ont été nécessaires (Chapitre 3.2).
5. Des concentrations plus élevées (2,3 g/L et 3,4 g/L) des substances polymères extracellulaires (SPE) ont été produites par *Serratia* sp.1 dans des boues stérilisées et traitées avec un traitement thermique-alcalin par rapport à celle produite (1,5 g/L) dans un traitement thermique-acide (Chapitre 4).
6. Une concentration plus élevée de SPE a été produite dans des solides en suspensions de la boue de 17,0 g/L, 22,4 g/L et 29,8 g/L par rapport à celle produite dans les solides de la boue de 37,3 g/L et 44,8 g/L (Chapitre 4).
7. Les SPE combinées avec le Ca^{2+} agissent comme un agent de conditionnement. Les B-SPE et les formes capsulaires brutes de SPE ont montré une plus grande activité de floculation (79,1%) et une meilleure déshydratation (52,2%) que celles des formes brutes de boues de SPE (S-SPE) (Chapitre 4).
8. La culture mixte a produit des concentrations plus élevées (4,9 g/L) de SPE par rapport aux cultures pures (2,7 à 3,7 g/L) à l'aide de la boue stérilisée comme matière première. Les B-SPE ont révélé une activité de floculation du kaolin élevée (91,2%) à des concentrations très faibles (0,8 mg B-SPE/g kaolin) et elle était

comparable à celle du polymère chimique, Magnafloc-155 (90,4% à 0,2 mg/g de kaolin) (Chapitre 5.1).

9. Les SPE produits étaient capables de maintenir leurs propriétés de floculation dans la plage de température de 4-60°C. Les B-SPE présentaient également une très bonne performance de floculation (% d'élimination de la turbidité) dans l'eau de rivière (93,5%), les eaux usées municipales (91,7%) et des eaux usées de brasserie (81,8%) et il a été comparable au Magnafloc-155 (Chapitre 5.1).
10. Les résultats sont très encourageants et justifient la poursuite des recherches sur l'optimisation des processus dans des fermenteurs de laboratoire à des conditions contrôlées de pH, de la température et de la concentration en oxygène dissous (Chapitre 5.1).
11. Une concentration en SPE de 4,9 g/L (la plus haute) a été produite à 72 h d'incubation dans un incubateur-agitateur à 150 tours par minute (Chapitre 5.2).
12. Avec une culture mixte de 13 isolats, les résultats ont démontré que 150 rpm était l'agitation optimale requise pour la production maximale de SPE et pour celle ayant des capacités de floculation et de déshydratation supérieures. Il a été révélé que l'agitation a eu un impact significatif sur la production de SPE. Ce fait doit être considéré en produisant de SPE dans les grandes cuves de fermentation à grande échelle, car la vitesse d'agitation aura un impact significatif sur la production de SPE (Chapitre 5.2).
13. Les B-SPE ont révélé une FA du kaolin supérieur (80%) en combinaison avec des cations divalents (Ca^{2+} et Mg^{2+}) par rapport aux cations trivalents (Fe^{3+} et Al^{3+}). Les B-SPE présentaient également une très bonne performance de floculation (élimination de la turbidité) dans l'eau de rivière (90%), les eaux usées municipales (80,7%) et les eaux usées de brasserie (80-84%) avec l'ajout de cations trivalents (Fe^{3+} et Al^{3+}). Les performances de floculation du bouillon produit par culture mixte ont été comparables à celles du Magnafloc-155 (polymère chimique) et, par conséquent, il pourrait être utilisé en tant que floculant (Chapitre 5.2).

5.2 Recommandations

1. L'utilisation de la glycérine ou d'autres suppléments de carbone pour améliorer la production de SPE dans les boues d'épuration (Chapitres 3.1 & 4).

2. Le liquide mixte de solides en suspensions ou de la biomasse active de la boue activée peut être utilisé comme inoculum pour la production de SPE (Chapitres 5.1 & 5.2).

3. L'utilisation d'agitation continue ou intermittente ou de cisaillement au cours de la fermentation pour augmenter la production de SPE en culture discontinue (Chapitre 5.2).

4. Récupération du bouillon de SPE fermenté sous forme de poudre (purifié ou brut, par lyophilisation ou séchage à l'air, etc) et de son application en tant que floculant (Chapitres 5.1 & 5.2).

5. Caractérisation chimique des SPE (nature chimique, poids moléculaire, angle de contact, propriétés de surface, etc) (Chapitre 2)

6. Analyse des coûts et de l'énergie de masse de la production de SPE (Chapitre 5).

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CHAPITRE 2

EXTRACELLULAR POLYMERIC SUBSTANCES OF BACTERIA AND THEIR POTENTIAL ENVIRONMENTAL APPLICATIONS

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RÉSUMÉ

Les biopolymères sont considérés comme une alternative potentielle en comparaison à des polymères chimiques classiques en raison de leur facilité de biodégradabilité, de haute efficacité, la non-toxicité et la pollution non-secondaire. Récemment, des substances polymériques extracellulaires (SPE, des biopolymères produits par les micro-organismes) ont été reconnues par de nombreux chercheurs comme un floculant potentiel pour leurs applications dans divers procédés d'eau, d'assainissement et de traitement des boues. Dans ce contexte, l'information de la littérature sur les SPE est largement dispersée et très rare. Ainsi, cette étude marginalise diverses études menées jusqu'à présent sur la nature-de production et de récupération des SPE, leurs propriétés, les applications environnementales et d'ailleurs, examine de manière critique les besoins futurs de la recherche et l'application avancée prospective des SPE. L'un des aspects les plus importants de composition chimique et des détails structurels de différentes fractions de SPE en termes de glucides, de protéines, de l'ADN extracellulaire, de lipides et des agents tensioactifs et les substances humiques sont décrits. Ces caractéristiques chimiques des SPE par rapport à la formation et les propriétés des agrégats microbiens ainsi que la dégradation des SPE dans la matrice (biomasse, des flocs etc.) sont analysés. Les propriétés mécaniques importantes (sur la base de caractéristiques structurelles) tels que l'adsorption, la biodégradabilité, caractère hydrophile/hydrophobe de la matrice de SPE sont également discutés en détail. Différents aspects de processus de production de SPE tels que la croissance bactérienne; l'inoculum et les facteurs affectant la production de SPE ont été présentés. Ainsi, les facteurs importants qui affectent la production de SPE y compris la phase de croissance, les sources de carbone et d'azote et de leur rapport, le rôle d'autres éléments nutritifs (phosphore, oligo-éléments/oligo-éléments et les vitamines), l'impact de pH, de la température, les métaux, les conditions aérobies par rapport à des conditions anaérobies et aussi l'étude sur la culture pure et mixte. La production de SPE à forte concentration avec une productivité élevée est essentielle pour des raisons économiques. Par conséquent, la connaissance de tous les aspects de la production de SPE (ci-dessus) est nécessaire de formuler une base logique et scientifique pour la recherche et les activités industrielles. Une des questions très importantes dans la production/application/biodégradation des SPE C'est ainsi que les SPE sont extraites de la matrice ou de bouillon de culture. En outre, la matrice de SPE disponible sous différentes formes (brute, faiblement liés, étroitement lié, soluble, et capsulaire purifié) peut être utilisée comme bioflocculant. Plusieurs méthodes

chimiques et physiques pour l'extraction de SPE (sous forme brute ou purifiée) à partir de différentes sources ont été analysées et testées. Il y'a amplement d'informations disponibles dans la littérature sur les différentes méthodes d'extraction de SPE. La floculation, la déshydratation et la capacité de biosorption sont les propriétés techniques très intéressantes de la matrice de SPE. Des informations récentes sur les aspects importants de ces propriétés qualitativement ainsi que quantitativement ont été décrites. Des informations récentes sur le mécanisme de floculation médiée par les SPE ont été présentées. Le rôle potentiel de SPE dans la déshydratation des boues et le phénomène de biosorption a été discuté en détail. Différents facteurs qui influencent la capacité de floculation de SPE et la déshydratation de différentes suspensions ont été inclus. Les facteurs pris en compte sont les cations, les différentes formes de SPE, la concentration des SPE, la teneur en protéines et en glucides de SPE, leur poids moléculaire, le pH de la suspension et la température etc. Ces facteurs ont été sélectionnés basés sur leur rôle dans la floculation et le mécanisme de déshydratation ainsi qu'en fonction les plus récentes données de la littérature disponibles sur ces facteurs. Par exemple, ce n'est que récemment qu'il a été démontré qu'il existe une concentration optimale de SPE pour une meilleure floculation et déshydratation de boues Une concentration élevée ou faible de SPE peut conduire à la déstabilisation de flocs. De plus, les rôle des SPE dans les applications environnementales telles que le traitement de l'eau, la floculation et la décantation des eaux usées, la décoloration des eaux usées, la déshydratation des boues, l'enlèvement et la récupération de métaux, l'élimination des composés organiques toxiques, le traitement des lixiviats de décharge, l'assainissement des sols et la remise en état ont été présentés sur la base des informations les plus récentes disponibles. Cependant, les données disponibles sur l'application l'environnementale des SPE sont très limitées. Les enquêtes sont nécessaires pour explorer le potentiel des applications de terrain de SPE. Enfin, les limites de l'écart de connaissances sont décrites et les besoins de recherche ainsi que les perspectives d'avenir sont mis en évidence.

Mots-clés: Bioflocculants; Polymères bactériens; Substances polymères extracellulaires; les eaux usées; boues; Contrôle de la pollution.

ABSTRACT

Biopolymers are considered a potential alternative to conventional chemical polymers because of their ease of biodegradability, high efficiency, non-toxicity and non-secondary pollution. Recently, extracellular polymeric substances (EPS, biopolymers produced by the microorganisms) have been recognised by many researchers as a potential flocculent for their applications in various water, wastewater and sludge treatment processes. In this context, literature information on EPS is widely dispersed and is very scarce. Thus, this review marginalizes various studies conducted so far about EPS nature-production-recovery, properties, environmental applications and moreover, critically examines future research needs and advanced application prospective of the EPS. One of the most important aspect of chemical composition and structural details of different moieties of EPS in terms of carbohydrates, proteins, extracellular DNA, lipid and surfactants and humic substances are described. These chemical characteristics of EPS in relation to formation and properties of microbial aggregates as well as degradation of EPS in the matrix (biomass, flocs etc) are analyzed. The important engineering properties (based on structural characteristics) such as adsorption, biodegradability, hydrophilicity/hydrophobicity of EPS matrix are also discussed in details. Different aspects of EPS production process such as bacterial strain maintenance; inoculum and factors affecting EPS production were presented. The important factors affecting EPS production include growth phase, carbon and nitrogen sources and their ratio, role of other nutrients (phosphorus, micronutrients/trace elements, and vitamins), impact of pH, temperature, metals, aerobic versus anaerobic conditions and pure and mixed culture. The production of EPS in high concentration with high productivity is essential due to economic reasons. Therefore, the knowledge about all the aspects of EPS production (listed above) is highly essential to formulate a logical and scientific basis for the research and industrial activities. One of the very important issues in the production/application/biodegradation of EPS is how the EPS is extracted from the matrix or a culture broth. Moreover, EPS matrix available in different forms (crude, loosely bound, tightly bound, slime, capsular and purified) can be used as a bioflocculant material. Several chemical and physical methods for the extraction of EPS (crude form or purified form) from different sources have been analyzed and reported. There is ample information available in the literature about various EPS extraction methods. Flocculability, dewaterability and biosorption ability are the very attractive engineering properties of the EPS matrix. Recent information on important aspects of these properties qualitatively as well as quantitatively has been described. Recent information on the mechanism of flocculation mediated by EPS is

presented. Potential role of EPS in sludge dewatering and biosorption phenomenon has been discussed in details. Different factors influencing the EPS ability to flocculate and dewaterability of different suspensions have been included. The factors considered for the discussion are cations, different forms of EPS, concentration of EPS, protein and carbohydrate content of EPS, molecular weight of EPS, pH of the suspension, temperature etc. These factors were selected for the study based upon their role in the flocculation and dewatering mechanism as well the most recent available literature findings on these factors. For example, only recently it has been demonstrated that there is an optimum EPS concentration for sludge flocculation/dewatering. High or low concentration of EPS can lead to destabilization of flocs. Role of EPS in environmental applications such as water treatment, wastewater flocculation and settling, color removal from wastewater, sludge dewatering, metal removal and recovery, removal of toxic organic compounds, landfill leachate treatment, soil remediation and reclamation has been presented based on the most recent available information. However, data available on environmental application of EPS are very limited. Investigations are required for exploring the potential of field applications of EPS. Finally, the limitations in the knowledge gap are outlined and the research needs as well as future perspectives are highlighted.

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

1 Introduction

Microbial flocculants (EPS, extracellular polymeric substances) are considered an eco-friendly, cost effective and sustainable alternatives to substitute the existing chemical flocculants. Microbial EPS are biopolymers (Wingender et al., 1999). EPS matrix is formed by different biochemicals secreted by microbes, release of cellular material/products by cell lysis or organic matter in the medium. EPS secretion is a general attribute of microorganisms in natural environments and occurs in prokaryotic as well as in eukaryotic microorganisms (Flemming and Wingender, 2001; Wingender et al., 1999). EPS consists of quite viscous biofilm matrix. In general, EPS in a biofilm varies from 50% to 90% of the total organic matter (Flemming and Wingender, 2001). EPS are defined as capsular (C-EPS), slime (S-EPS), loosely bound (LB-EPS) and tightly bound (TB-EPS) on the basis of the nature of their association with the cells or the method used to extract/separate EPS from the cells (Table 1.1). The organic compounds produced by activated sludge microorganisms can be grouped into three categories: the first category compounds are secreted by microorganisms due to their interaction with the environment, the second category includes the compounds produced as a result of substrate metabolism and the third involves bacterial growth and compounds released during the lysis and/or degradation of microorganisms or microbial components.

Basic functions of EPS are aggregations of bacterial cells, adherence to surfaces, formation of flocs and biofilms, cell-cell recognition (e.g., cell adhesion), structural elements of biofilms, protective barrier for cells, and water retention to minimise desiccation of the cell, sorption of exogenous organic compounds, and sorption of inorganic ions, enzymatic activities and interaction of polysaccharides with enzymes (Tian, 2008; Wingender et al., 1999). Carbohydrates, proteins, humic substances and nucleic acids are the main EPS components. Because of special EPS components, EPS matrix shows adsorption abilities, biodegradability and hydrophilicity/hydrophobicity. EPS have an important role in biofilm formation, mass transfer via biofilm, adsorption of different metals and organic/inorganic compounds by biofilm and most importantly it provides structural support to the biofilm (resistant to shear) (Czaczyk, 2007; Flemming and Leis, 2003; Flemming et al., 2005; Neyens et al., 2004).

The occurrence or production of EPS in different systems (e.g., membrane bioreactors (MBR)) is unwanted and a serious issue. Fouling is a serious problem in membrane processes. The fouling problem makes MBR operation and control very difficult. This is mainly due to the prevalence of EPS in the system (Drews et al., 2006). EPS interactions with the membrane

surface are not well established till date. There are numerous theories or considerations reported in the literature, which are often contradictory and very confusing. In general MBRs are operated at a constant-flux, however, due to fouling trans-membrane pressure increases. The increase in trans-membrane pressure occurs in three phases and fouling occurs because of pore clogging, floc adhesion and cake layer formation, changes of cake layer and/or osmotic pressure effect. As per the literature, in general, EPS concentrations and chemical characteristics are the two important factors that determine the extent and severity of the fouling condition (Drews et al., 2006; Lin et al., 2014). Based upon the present understanding, there are mainly three strategies to mitigate membrane fouling such as EPS production control, modifications of EPS characteristics and EPS removal from the system (Lin et al., 2014). Fouling mitigation strategies require extensive research on advanced analysis of different components of EPS and chemical and functional characterization of the EPS produced by different microorganisms to increase fundamental understanding of EPS's role in fouling mechanism. With the advancement of knowledge about EPS-fouling nexus, simplified and economical strategies for the fouling control are expected to come out in near future.

Recently, the use of bacterial EPS for environmental applications has been the focus of many researchers. On the other hand, the information on the environmental applications of EPS are scattered and there is no consolidated report so far, which highlights the various aspects of EPS and their correlation to the potential environmental applications. This review provides current state-of-the-art knowledge with regard to bacterial EPS and their environmental applications. Special emphasis has been laid on the EPS nature-production-recovery, interrelation among EPS characteristics and properties, and potential application of EPS in environmental pollution control. Throughout the review, the term EPS has been used synonymously to address microbial polymers, extracellular polysaccharides substances, bacterial polymers, biofilms matrix and/or bioflocculants.

Table 2.1 Types of EPS

Classification basis	EPS	Remarks	References
Nature of EPS association with cells	Slime	Slimes are the polymers present in the supernatant after centrifugation of the biomass. Slimes are mostly in soluble form or unattached to the cells in the form of colloids.	(Barker and Stuckey, 1999; Comte et al., 2006a; Laspidou and Rittmann, 2002; Raszka et al., 2006; Tian, 2008; Wingender et al., 1999)
	Capsular	Capsular EPS are the permanent part of the cell membrane and are bound to pellets (bacterial cells).	
Physical-chemical states and composition of EPS	Soluble	Soluble EPS are the secretion from the cells in dissolved form in the surrounding environment. The main components are macromolecules, colloids and slimes.	(Barker and Stuckey, 1999; Comte et al., 2006a; Laspidou and Rittmann, 2002; Raszka et al., 2006; Tian, 2008; Wingender et al., 1999)
	Bound	The bound EPS attach to the cells. The components of bound EPS are sheaths, capsular polymers, condensed gel, loosely bound polymers and attached organic material.	

2 Chemical characteristics of EPS

The chemical nature of EPS is diverse and varies in terms of carbohydrates, protein, nucleic acid, lipids, and humic substances (non-secreted fraction) concentration and their form. The dominant components of EPS are protein and carbohydrates (75-90%). The EPS also contain carbohydrate and protein derivatives such as lipopolysaccharides, glycoproteins and lipoproteins (Marvasi et al., 2010; Sheng et al., 2010; Simões et al., 2010). The details of EPS matrix components are described in the following paragraphs.

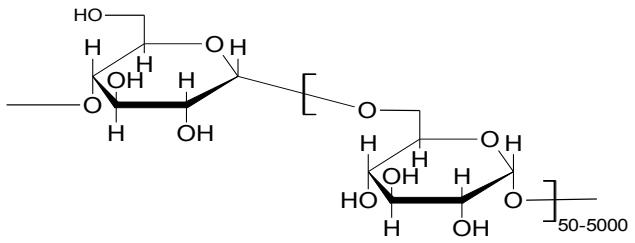
2.1 Carbohydrates (exopolysaccharides)

The carbohydrates are the major component of extracellular EPS. The microbial exogenous secretion contains neutral carbohydrates (mainly hexose, rarely pentose) and uronic acids (such as glucuronic, galacturonic and mannuronic acid). The presence of uronic acids or common substitutes like acetate ester, pyruvate ketals, formats, succinates and inorganic (phosphate and sulphate) decides the nature (neutral, anionic or cationic) of EPS macromolecules. EPS exopolysaccharides are either homopolysaccharides or heteropolysaccharides. The homopolysaccharides are generally neutral and composed of only one monosaccharide type such as D-glucose or L-fructose (Czaczyk, 2007; Pal and Paul, 2008; Sutherland, 2001; Vu et al., 2009). The homopolysaccharides are divided into three distinct groups. The first group

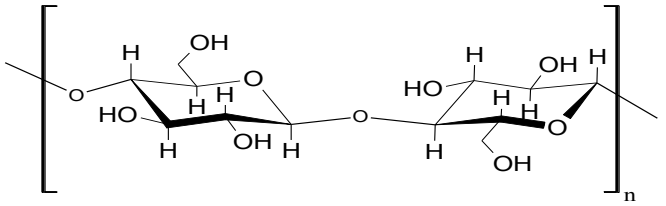
consists of α-D-glucans produced by *Leuconostoc mesenteroides*. These macromolecules contain mainly α-(1,6)-linked D-glucosyl units. The degree of branching involves α-(1,3)-linkage, rarely α-(1,2)-linkage and α-(1,4)-linkage. Second group consists of β-D-glucan synthesized by the bacteria such as *Pediococcus* sp. and *Streptococcus* sp. The molecules are composed of β-(1,3)-linked D-glucosyl units involving with branching β-(1,2)-linkage. Third group consists of fructans produced by *Streptococcus salivarius* contains β-(2,6)-linked fructosyl units (Czaczyk, 2007). The examples of homopolysaccharides produced by microorganisms are Dextran, Curdlan, and Cellulose. Dextran is produced by *Leuconostoc* spp. and *Streptococcus* spp. The basic unit of dextran is glucose that contains several consecutive α-(1,6)-links in the chain. The α-D-glucan have side chains through α-(1,-6)-linkages, which mostly join from α-(1,3)-linkage and sporadically from α-(1,4)-linkage or α-(1,2)-linkage (Figure 2.1a), Curdlan is a linear polysaccharide made up of β-(1,3)-linked glucose residues produced by *Alcaligenes faecalis* and *Agrobacterium* sp. Cellulose is also a well-known biopolymer formed by repetitive units of D-glucose with β-(1,4)-linkage (Figure 2.1b). Cellulose is mainly produced by Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and Gram-positive bacteria (Rehm, 2010; Vu et al., 2009).

Heteropolysaccharides (alginate, xanthan gum, gellan gum, welan gum, colanic acid, K30-antigen and hyaluronic acid) are produced by lactic acid bacteria and created by repeated units of monosaccharides (such as D-glucose, D-galactose, L-fructose, L-rhamnose, D-glucuronic acid, L-guluronic acid and D-mannuronic acid). Physical properties of microbial heteroplysaccharides are determined by the nature of bonding between monosaccharides units and the branching of the chains. Most of the heteropolysaccharides also processes substituents of pyruvates, succinates and formates (Czaczyk, 2007). One of the most studied heteroexpolysaccharides (alginate) involved in the EPS formation of the opportunistic pathogen species such as *Pseudomonas aeruginosa* and *Azotobacter* (Flemming and Wingender, 2010). Alginate is not essential for *Pseudomonas aeruginosa* in biofilm formation, but its presence has a notable effect on biofilm architecture. Alginate is a linear β-(1,4)-linked non-repeating heteropolymer of mannuronic acid and guluronic acid (Figure 2.1c). Xanthan gum, which is produced by strains of *Xanthomonas campestris* is a branched anionic heteropolymer. The main chain of xanthan gum is composed of glucose units while the side chain is trisaccharides, formed by α-D-mannose with acetyl group, β-D-glucuronic acid and a terminal β-D-mannose unit linked to a pyruvate group (Figure 2.1d). *Sphingomonas* spp. produces gellan. Gellan is a linear heteropolymer composed of a tetrasaccharides repeating units of two molecules of D-glucose, D-glucuronic acid and L-rhamose (Figure 2.1e). The Hyaluronic acid produced by

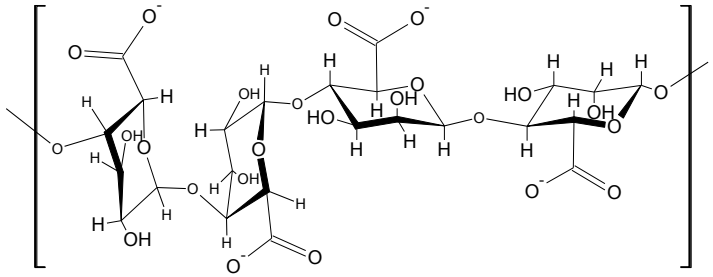
Streptococcus spp. and *Pasteurella multocida* is a β-(1,4)-linked repeating heteropolymer composed of disaccharide units of glucuronic acid and N-acetylglucosamine (Figure 2.1f) (Rehm, 2010; Vu et al., 2009).



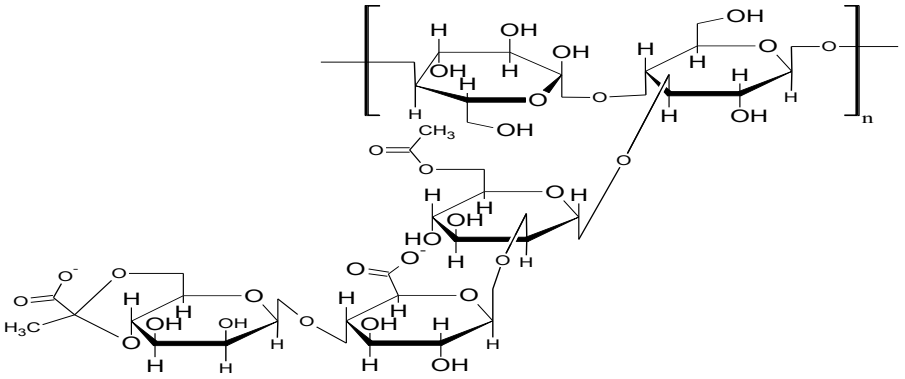
a) Dextran



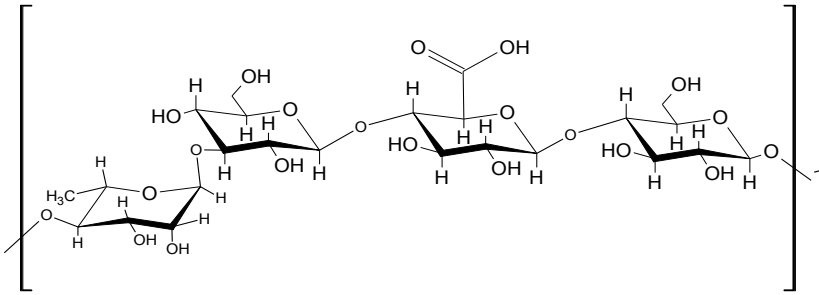
b) Cellulose



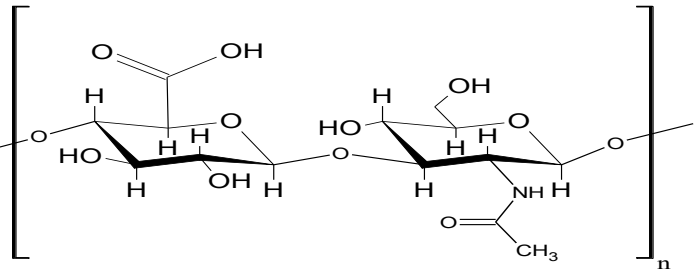
c) Alginate



d) Xanthan



e) Gellan



f) Hyaluronic acid

Figure 2.1 Chemical structure of some exopolysaccharides: a) Dextron, b) Cellulose, c) Alginate, d) Xanthan, e) Gellan, f) Hyaluronic acid

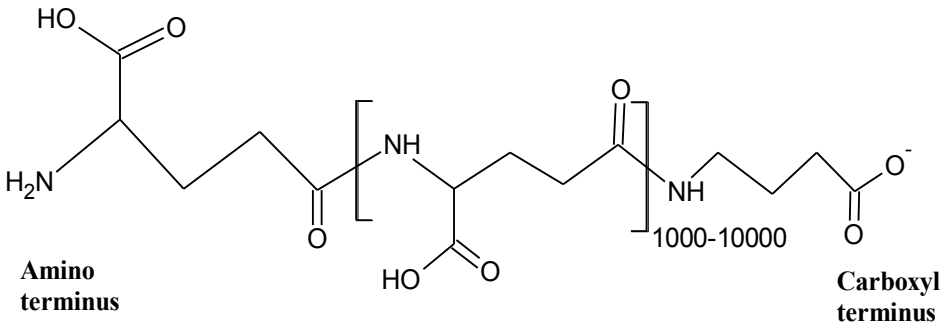
2.2 Protein

EPS contains a substantial amount of proteins as enzymes and structured proteins. Several enzymes have been detected in EPS matrix, and many of these are involved in the degradation of EPS. These extracellular enzymes act on the substrates like water soluble/insoluble polymers as well as organic particles trapped in EPS. The water soluble substrates include some polysaccharides, proteins and nucleic acids, whereas water insoluble compounds include cellulose, chitin and lipids. During starvation, enzymes secreted by the microorganisms can potentially degrade the EPS components. These enzymes can target the EPS of the same bacteria or of other species (Flemming and Wingender, 2010). *Bacillus subtilis* produces peptide antibiotics, which exhibit rigid structure and resistant to hydrolysis by proteases (Marvasi et al., 2010).

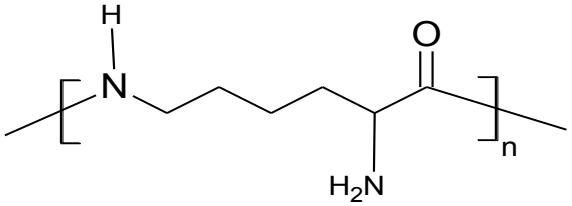
The structural proteins or non-enzymatic proteins in the EPS matrix, such as the cell surface-associated and extracellular carbohydrates binding proteins (called as lectins), are involved in the formation of polysaccharides matrix network and constitute a link between bacterial surface and extracellular surface (Flemming and Wingender, 2010). The examples include lectin-like protein in the matrix of activated sludge flocs, which helps in bacterial aggregations and form flocs (Park and Novak, 2009). Polyamides are the non-ribosomally synthesized peptides biopolymer. There are two well-studied cases, which were found ubiquitously among bacterial species, poly-gamma-glutamate (PGA) (Figure 2.2a) and £-poly-L-lysine (Figure 2.2b) (Lee et al., 1999). The PGA is an anionic homo-polyamide can either exist in capsular or slime form. The *Bacillus anthracis* PGA is an example of capsular EPS, whereas *Bacillus subtilis* is known to produce soluble PGA. The use of the polyamide as cross linked PGA is the established bioflocculant (Rehm, 2010; Shih and Van, 2001).

Moreover, glycoproteins which are well studied in eukaryotes and their presence have also been reported in prokaryotes. Glycoproteins are generated when sugar moiety is covalently attached to protein, which perform various cellular functions such as maintain structural integrity and stability to signaling and inter-cellular communication (Hug and Feldman, 2011). Two main types of glycosylation are found in prokaryotes: N-glycosylation and O-glycosylation (Hitchen and Dell, 2006). Glycoprotein distribution in bacterial cells is very diverse and classified as surface-layer, membrane-associated, flagelli/pilli-associated, cellular and secreted glycoproteins (Upreti et al., 2003). The secreted glycoproteins have been reported in both gram-positive (e.g., *Bacillus amyloliquefaciens, Bacillus macerans*, *Cellulomonas fimi* and *Clostridium*

acetobutylicum) and gram-negative bacteria (e.g., *Flavobacterium meningosepticum, Mycobacterium tuberculosis* and *Mycobacterium bovis*) (Upreti et al., 2003).



a) Poly-gamma-glutamate



b) Poly-L-lysine

Figure 2.2 Chemical structure of peptide EPS a) Poly-gamma-glutamate, b) Poly-L-lysine

2.3 Extracellular DNA

The EPS of various origins (e.g., wastewater sludge, soils, marine sediments) have been found to contain extracellular DNA (E-DNA, i.e., extracellular deoxyribonucleic acids), and these contents occur particularly in large amounts in wastewater EPS, although the amount produced can vary even between closely related species. E-DNA is a major structural component in the EPS matrix of *Staphylococcus aureus*, whereas it is only a minor component in the EPS formed by *Staphylococcus epidermidis* (Flemming and Wingender, 2010). EPS produced by *Bacillus cereus* requires E-DNA as part of the EPS matrix (Marvasi et al., 2010). The presence of E-DNA in the EPS matrix of *Streptococcus mutans* is associated with the export of a large quantity of competence-signaling peptides to the medium that helps in horizontal gene transfer (Czaczyk, 2007).

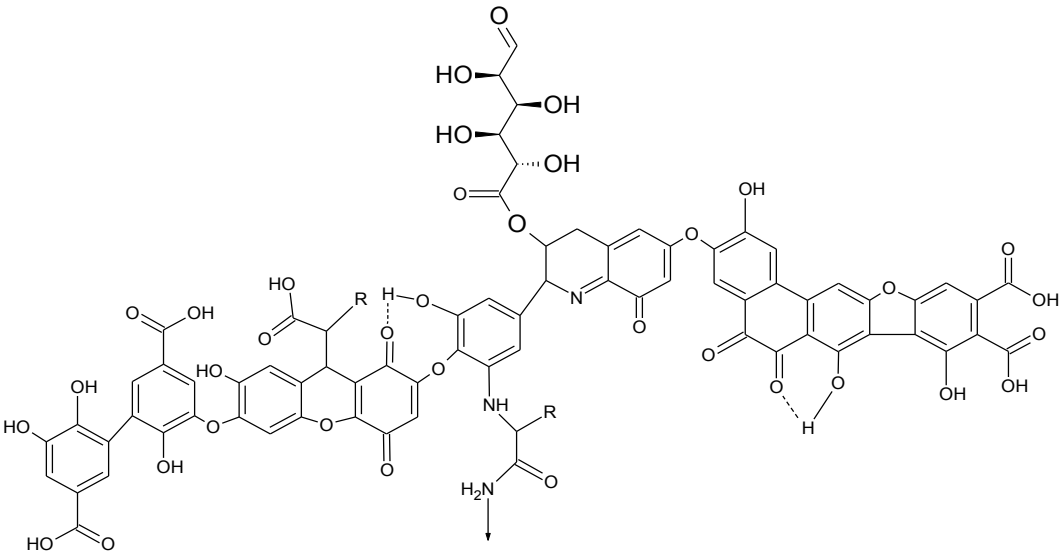
2.4 Lipids and Surfactant

The hydrophilic nature of EPS is mainly due to hydrated polysaccharides; protein and DNA molecules, but some EPS also possess the hydrophobic properties (for example, a *Rhodococcus* spp.). The hydrophobic characteristics of EPS were attributed to polysaccharides-linked methyl and acetyl groups. The EPS matrix also contains lipids and lipids derivatives. Lipopolysaccharides participates in adhesion (e.g., adhesion of *Thiobacillus ferrooxidans* on to pyrite surfaces) (Flemming and Wingender, 2010). There are different biosurfactants (surface active EPS) such as surfactin, viscosin and emulsion. These biosurfactants are capable of dispersing hydrophobic substances in the medium. Biosurfactants can have antibacterial and antifungal properties and are important for bacterial attachment and detachment from oil droplets. The example of biosurfactants, rhamnolipid (a glycolipid) found in the EPS matrix of *Pseudomonas aeruginosa* (Flemming and Wingender, 2010; Soberón-Chávez et al., 2005). The lipopeptide, surfactin produced by *Bacillus subtilis.* The main function of surfactin is to help in colonization and during competition of resources (Marvasi et al., 2010).

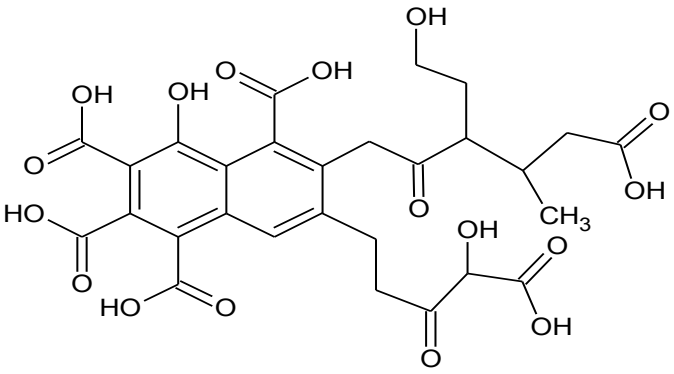
2.5 Humic substances

Humic substances are the integral part of the EPS, although the humic substances are not secreted by microbes. Humic substances get adsorbed by EPS matrix from the natural

environment (e.g., wastewater sludge, soils). The presence of humic substances in EPS matrix is important because it can influence the properties of the EPS such as biodegradability, adsorption ability (Wingender et al., 1999). Humic substances are naturally occurring complex ligand widely distributed in nature. In natural aquatic system, humic substances share around 30-80% of total dissolved organic matter (Moura et al., 2007; Pena-Mèndez et al., 2005). Humic substances make up the bulk of organic matter because they represent most of the organic materials of soils, peat, lignites, brown coals, sewage, natural waters and their sediments. Humic substances can be divided into three components: fulvic acids, humic acids and humin. Humic acids are the major part of the humic substances. Fulvic and humic acids represent the alkali-soluble humus fragments while humin represent the insoluble residue (Pena-Mèndez et al., 2005). The humic substances system is formed through the association of several components during the humification process, such as amino acids, lignin, pectin or carbohydrates; through inter molecular forces (donor-acceptor, ionic, hydrophilic and hydrophobic). The humic substances can be formed in several ways and lignin plays an important role in these processes (Burdon, 2001; Davies and Ghabbour, 2001). According to Burdon (2001) humic organic matters consist of the mixture of plant and partially/fully decomposed microbial constituents (such as carbohydrates, protein, lipids and lignins, tannins melanins, etc.). The chemical nature of the humic substances is very complex and proposed structure of humic acid according to Stevenson (1982) has been shown in Figure 2.3a. The structure reveals that it is a very complex structure with multiple phenolic and carboxylic groups. The model structure of fulvic acid also shows a complex structure (Figure 2.3b) with multiple carboxyl and phenolic functional groups (Buffle et al., 1977).



a) Humic acid



b) Fulvic acid

Figure 2.3 Chemical structures of humic substances a) Humic acid, b) Fulvic acid

3 Important properties of EPS

The EPS matrix has great influence on the properties of microbial aggregates because of their special characteristics such as adsorption, biodegradation and hydrophobicity/hydrophilicity. These special EPS properties mainly depend upon the components of EPS such as exopolysaccharides, proteins, nucleic acids, humic substances (Table 2.2). The values presented in Table 2.2 are the typical range of the EPS components. Relatively low values of protein and polysaccharides were observed by Neyens et al. (2004). This variation could be due to various factors such as sampling, type of wastewater sludge; operating conditions and extraction methods. EPS matrix is ionic, hydrophilic/hydrophobic in nature and its link up with the biological matrix through different chemical interactions (Mayer et al., 1999). Important microbial processes such as EPS production and degradation are also related to the functionally active enzymes. Therefore, chemical composition of EPS can be correlated to the physical and dynamic properties of EPS. The important engineering properties of EPS matrix are discussed in the following sections.

Table 2.2 Composition and important properties of EPS

Components of the EPS	Typical content in the EPS matrix	Important properties of different components	Remarks	References
Polysaccharides	40-95%	Adhesion, aggregation of bacterial cells, retention of water, adsorption of organic and inorganic compounds, binding of enzymes, nutrient source, and protective barrier to cells.	Play main role in flocculation and biosorption. The concentration and characteristics of the polysaccharides decide the fate of surface properties as well as biodegradability of the EPS.	(Flemming and Wingender, 2010; Tian, 2008; Wingender et al., 1999)
Proteins	1-60%	Adhesion, aggregation of bacterial cells, retention of water, sorption of organic and inorganic compounds, binding of enzymes, electron donor or acceptor, and protective barrier to cells.	Play main role in flocculation and biosorption. The concentration and characteristics of the protein decide the fate of surface properties as well as biodegradability of the EPS.	(Flemming and Wingender, 2010; Tian, 2008; Wingender et al., 1999)
Nucleic acids	1-10%	Adhesion, aggregation of bacterial cells, nutrient source, exchange of genetic information, export of cell components, and exchange of genetic information.	Nucleic acids have an important role in flocculation and biosorption depending upon the quantity and available components.	(Flemming and Wingender, 2010; Tian, 2008; Wingender et al., 1999)
Lipids	1-10%	Export of cell components	Major to minor role in flocculation and biosorption.	(Flemming and Wingender, 2010; Tian, 2008; Wingender et al., 1999)
Humic substances	-	Adhesion, Electron donor or acceptor	Minor role in the flocculation and biosorption. The impact of humic substances mainly depends upon its concentration as well as the nature of humic substances.	(Flemming and Wingender, 2010; Tian, 2008; Wingender et al., 1999)

3.1 Adsorption

The EPS have many adsorption sites for metals and organic matters, such as aromatics, aliphatic in proteins, and hydrophobic regions in carbohydrates (Flemming and Leis, 2003). This reveals the potential roles of EPS in heavy metal sorption to bacterial cells and transporting in environments (Guiné et al., 2006; Hu et al., 2006). Because of the presence of many functional anionic groups in EPS, such as carboxyl, phosphoryl, sulfhydryl, phenolic and hydroxyl groups, they offer cation exchange potential and hence can complex with heavy metals (Ha et al., 2010; Joshi and Juwarkar, 2009; Liu and Fang, 2002). The bacterial EPS typically contains 20-50% of uronic acids in polysaccharides. Based on the estimated numbers of the available carboxyl and hydroxyl groups, the EPS are regarded to have a very high binding capacity (Guibaud et al., 2003). Proteins, carbohydrates and nucleic acids in EPS all have the abilities to complex with heavy metals (Priester et al., 2006; Zhang et al., 2006). The binding capability and strength of the bonds between EPS and heavy metals were known to be high, and the adsorption obeyed the Langmuir or Freundlich equations (Bhaskar and Bhosle, 2006; Moon et al., 2006; Zhang et al., 2006). Furthermore, the soluble EPS might have a greater adsorptive ability for heavy metals than the bound EPS from the wastewater sludge (Comte et al., 2006a).

Several types of metal ions present in surrounding medium are bound to EPS. The binding between EPS and divalent cations, such as Ca^{2+} and Mg^{2+}, is one of the main intermolecular interactions in maintaining the microbial aggregate structure (Mayer et al., 1999). During the adsorption of heavy metals onto activated sludge, Ca^{2+} and Mg^{2+} were found to be released into the solution simultaneously, indicating that the ion exchange mechanism was involved (Yuncu et al., 2006). Theoretical predictions carried out based on estimated numbers of available carboxyl and hydroxyl groups in the acidic polysaccharides; suggest a very high metal binding capacity. EPS reportedly had a high binding capacity (0.13 mMol/mg of EPS) for lead (Pb^{2+}), irrespective of the EPS concentration present in the marsh sediments (Flemming et al., 2000). EPS can absorb very high (22 ng/mg of EPS) quantity of copper (Flemming et al., 2000). The stability constants for Ni, Cu, Pb, Cd and Zn complexes with EPS range from 10^5 to 10^9 (Flemming et al., 2005). The stability constants of these complexes with EPS strongly depend upon pH. This is due the competition between H^+ and metal ions. EPS have adsorbed metal ions up to 25% the EPS concentration. Similar to the EPS, adsorption of dissolved copper from aqueous media have been carried out using alginate (Flemming et al., 2005). EPS can also adsorb organic pollutants, such as phenanthrene (Liu et al., 2001), benzene (Späth et al.,

1998), humic acids (Esparza-Soto and Westerhoff, 2003), and dye (Sheng et al., 2008). It might be attributed to some hydrophobic regions in EPS (Flemming and Leis, 2003). Spath et al. (1998) reported that more than 60% of benzene, toluene and m-xylene were absorbed by EPS, and only a small fraction of these pollutants was absorbed by the cells. The EPS are negatively charged and bind with the positively charged organic pollutants through electrostatic interaction (Esparza-Soto and Westerhoff, 2003). Moreover, proteins have a high binding strength and capability than humic substances. The soluble EPS (or loosely bound EPS) have a higher fraction of proteins than the bound EPS, and thus they may have higher binding capacity than the bound EPS (Pan et al., 2010).

3.2 Biodegradability

EPS can also be used by bacteria as source of carbon and energy. Usually, the main components of the EPS are carbohydrates and proteins, and in biological wastewater treatment system the enzymes for the degradation of these polymers are abundant. The bacteria in activated sludge can use the EPS that are excreted by other microorganisms for their metabolic activity (Zhang and Bishop, 2003). However, Laspidou and Rittmann (2002) argued that certain parts of EPS cannot be degraded by microorganisms. Wang et al. (2005) also revealed that part of EPS from aerobic granular sludge were biodegradable and elaborated that the EPS, which were present in the outer layer of aerobic granular sludge could not be biodegraded, although those located in the inner layer were biodegradable. Park and Novak (2007) reported that the EPS extracted from the sludge using various methods had different biodegradability. For instance, the EPS extracted using the cation exchange resin (CER) method were aerobically biodegradable, while the EPS extracted using the sulfide method were anaerobically biodegradable. The smaller molecular substances that are produced as a result of EPS degradation could be used as carbon and energy source for cell growth under the nutrients limiting conditions. EPS degradation can also result in the deflocculation of sludge flocs. The non-degradable portion of EPS may flow with the effluent from reactors or a treatment system and deteriorate the quality of the effluent.

3.3 Hydrophilicity/Hydrophobicity

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 (Tian, 2008). The EPS in microbial aggregates has many charged groups (e.g., carboxyl, phosphoric, sulfhydryl, phenolic and hydroxyl groups) and polar groups (e.g., aromatics, aliphatics in proteins, and hydrophobic regions in carbohydrates) (Flemming and Leis, 2003). The formation of hydrophobic areas in EPS would be beneficial for organic pollutant adsorption (Späth et al., 1998). The presence of hydrophilic and hydrophobic groups in EPS molecules indicates that EPS are amphoteric. The relative ratio of these two groups is related to the composition of EPS. Jorand et al. (1998) used XAD resin to separate the hydrophilic and hydrophobic EPS fractions and found that approximately 7% were hydrophobic and mainly comprised proteins, whereas the hydrophilic fraction mainly consisted of carbohydrates. The analysis of monosaccharide and amino acid contents in EPS after hydrolysis revealed that approximately 25% of the amino acids were negatively charged and approximately 24% were hydrophobic (Dignac et al., 1998). The properties of EPS significantly influence the hydrophobicity of microbial aggregates and their formation in the treatment systems or bioreactors. It also demonstrates the importance of the EPS as the sorption sites for organic pollutants (Flemming and Leis, 2003).

4 Production of EPS for bioflocculants recovery

4.1 Bacterial strain maintenance

Some of the EPS producing bacterial strains are likely to lose their EPS producing capabilities during laboratory storage on synthetic medium (Wingender et al., 1999). Therefore, such microbial strains must be maintained in such a way that they do not lose their desirable characteristics. Bacterial strains can be maintained either by regular sub-culturing, or by lyophilised (freeze-dried), or by freezing under nitrogen, or deep freezing at -80°C. The purity of the cultures should be maintained with known viability to prepare the inoculum when needed.

4.2 Inoculum

The medium used to prepare the inoculum should be designed for rapid cells growth without EPS production during the inoculum preparation step. Inoculum production may also involve many transfers, which increase the risk of contamination. In general, to avoid any contamination during inoculation, the inoculum steps are kept as minimum as possible, which is also more

economical. Contamination during inoculation or production step can significantly increase the costs of production. To reduce the risk of contamination during sampling it is usual to take a sample from the residue left in each vessel after its contents have been transferred to the next stage reactor. The growth kinetics of the culture is very important to check on the contamination issues. In this regard, the kinetic parameters such as cell growth rate and oxygen uptake rate are useful to determine the inoculums quality (Tyagi et al., 2009).

4.3 Factors affecting EPS production

The EPS secretion from the cells in their growth environment is influenced by the factors that govern bacterial metabolism. These factors include: genotype, physical, chemical and biological factors. Specific factors including carbon to nitrogen ratio (C/N ratio), pH, and temperature of the culture medium; incubation conditions (agitation or mixing speed), i.e. the growth conditions of the culture.

4.3.1 Growth phase

EPS production is either growth-synonymous, growth-associated, growth independent (Barker and Stuckey, 1999). The correlations between cell growth and secretion of EPS have been reported in many studies. EPS can be degraded by bacteria as a source of carbon and energy, when there is substrate shortage condition. In certain conditions the production of EPS is the result of the excretion and consumption from the microbial cells. However, the results reported in literature are somewhat contradictory. EPS production occurs during the endogenous phase of a batch culture (Pavoni et al., 1972), at the beginning of the stationary growth phase (Hantula and Bamford, 1991), parallel to cell growth (Kurane et al., 1991), from the middle of the exponential growth phase (Farrah and Unz, 1976), simultaneously during the early and middle of the exponential growth phases (Kurane et al., 1991), increases with exponential phase and decreases during stationary phase (Wingender et al., 1999). The EPS concentration decline during stationary or in endogenous growth phase was linked with the production of an enzyme that degrades EPS. The EPS concentration had decreased with cultivation time during the exponential growth phase, but it was constant during the stationary phase (Sheng et al., 2006).

4.3.2 Carbon and nitrogen sources

The EPS production depends upon the type of substrate, i.e., carbon and nitrogen source. There are a number of carbon and nitrogen sources reported to be favoured over others by different EPS producing microorganisms (Salehizadeh and Shojaosadati, 2001; Sheng et al., 2010; Wingender et al., 1999). Fructose was favorable over sucrose by *Rhodococcus erythropolis* for the flocculant production (Kurane et al., 1991). The cells of *Rhodococcus erythropolis* were short and rod-shaped with sucrose as a carbon source, whereas the cells elongated when fructose was used. The production of EPS by *Chryseobacterium daeguense*-W6 was higher for maltose, mannose and glucose compared to the sodium acetate, galactose, phthalate, sucrose, ethanol and arabinose as a carbon source (Liu et al., 2010a). The production of EPS by *Chryseobacterium daeguense*-W6 was higher for nitrogen source tryptone, yeast and beef extract as compared to ammonium sulfate, peptone, ammonium nitrate, casein hydrolysate, urea and sodium nitrate. According to Liu et al. (2010a) glucose and tryptone were the best combination for EPS production by *Chryseobacterium daeguense*. Li and Yang (2007) reported that the activated sludge fed with glucose exhibited higher EPS production than that fed with acetate. However, the acetate-fed sludge performed better in flocculation and separation, with lower effluent suspended solids, sludge volume index (SVI) and specific resistance to filtration (SRF) values as compared to the sludge that was fed on glucose. This was due to an increase in higher loosely bound EPS (LB-EPS) content in the glucose fed sludge, which had a negative effect on sludge flocculation, clarification, sludge settling and dewatering. The protein content of the EPS increased and the nucleic acids content decreased by changing the wastewater from chemical, leather and dye industrial wastewater sludge to wine and municipal wastewater sludge (Sponza 2002). Nitrogen deficiency of the wine and municipal wastewater sludge was related to increased protein content in the EPS. In another study, the activated sludge fed with sodium acetate and glucose had higher LB-EPS content than that fed with starch (Ye et al., 2011a). They also observed that the amount of tightly bound EPS (TB-EPS), polysaccharide and protein content in TB-EPS and protein content in LB-EPS, were independent of the carbon source. Sudden addition of a carbon and energy source to the bacteria starved for carbon and energy may accelerate the death of some bacteria and EPS may be produced as a result of this process. The concentration of substrate (carbon and nitrogen) has a greater influence on EPS production (Ye et al., 2011a). The EPS dependence on the concentration of a substrate (carbon and nitrogen source) is attributed to the

fact that the concentration of substrate governs the substrate utilization rate and accordingly the EPS formation rate.

A profound emphasis is given to C/N (carbon/nitrogen) ratio in relation with EPS production. This is due to the fact that the C/N ratio had a great effect on microbial metabolisms and hence on the EPS production. In fact, C/N ratio is critical for EPS production than the type of carbon and nitrogen source. In spite of several reports on C/N ratio relation to EPS, there is no fixed favorable C/N ratio in the literature. This might be due to variations in the type of carbon-nitrogen sources and microorganisms reported. The analysis of Liu et al. (2010a) revealed an optimum C/N ratio of 0.5. Increase or decrease in the C/N ratio from 0.5 resulted in a reduced flocculation activity. In another study by Ye et al. (2011b), a C/N ratio of 20 was favorable for EPS production. When the C/N ratio in the sludge decreased (from 100 to 20), the carbohydrate content in LB-EPS decreased, whereas the protein content in LB-EPS increased. On the other hand, when the C/N ratio increased, the carbohydrate in LB-EPS increased, and the content of protein in LB-EPS decreased. However, the carbohydrate and protein contents in TB-EPS were not influenced by the C/N ratio. Moreover, according to Ye et al. (2011b) optimum C/N ratio of 20 is favourable, whereas C/N ratio about 100 or below 4 could adversely affect the sludge properties. Durmaz and Sanin (2001) found EPS in activated sludge to be rich in proteins, but low in carbohydrates at a carbon to nitrogen ratio of 5, but as the carbon to nitrogen ratio increased to 40, the amount of protein decreased sharply, whereas the amount of carbohydrates increased. Other researchers have found that activated sludge growing on wastewater with a low carbon to nitrogen ratio tends to produce EPS with a high protein/carbohydrate ratio (Bura et al., 1998; Liu and Fang, 2002). For commercialization, the cost of EPS production should be checked. In order to reduce the cost of production, research is needed to select low-cost substrates and at the same time fermentation conditions should be optimised to enhance bioflocculant production. Municipal, wine, food, brewery and canning industrial wastewater sludge could be the potential substrates.

4.3.3 Role of other nutrients (other than C and N)

Nutrients are necessary components for growth of bacteria as well as to stimulate the production of EPS, which play a part in sludge settling. The role of nitrogen and phosphorus as essential nutrients for biological wastewater treatment processes is well known. EPS production can be controlled by optimising the nutrient ratio. Trace elements and vitamins are required by cells in addition to the six macronutrients (C, O, H, N, S, P, K, Ca, Mg, and Fe). The trace

elements required include manganese (Mn), zinc (Zn), cobalt (Co), molybdenum (Mo), nickel (Ni), copper (Cu), vanadium (V), boron (B), iron (Fe), and iodine (I) and the vitamins required include K, B1, B2, B6, B12, biotin, niacin, and pantothenic acid. Micronutrients are often required at a dose of $<$1 mg/L, which means that the demonstration of dosage requirements is technically difficult.

Nutrient levels have a significant effect on EPS production and composition. The EPS content of the sludge increased with an increase in food to microorganism ratio (Janga et al., 2007). EPS production could be promoted by phosphorus deficiency (Fang et al., 2009). Bura et al. (1998) and Hoa et al. (2003) also found that the carbohydrate content in EPS extracted from activated sludge increases when phosphorus is in short supply.

4.3.4 Impact of pH

The pH of the culture medium significantly influences the EPS production. However, the effect of pH on the production of EPS varies with different microorganisms, operational conditions and medium composition (Shu and Lung, 2004). The pH effects are often investigated using the same microorganism in flask experiments with different initial pH values. In general, the optimal medium pH for EPS production varies from 5.0 to 7.0. Several microorganisms have the capability to produce EPS at pH 7 in different media (Gandhi et al., 1997). Most of the EPS producing microorganisms require a constant pH for maximum production of EPS. Some of the microorganisms produce more EPS in acidic pH 5.5-6.5 (Lee et al., 1999). The extreme pH of the medium (pH 2.0-3.0 or pH higher than 10) affect the biosynthesis of EPS (Czaczyk, 2007). The morphological changes of the cells depend mainly upon pH. Lee et al. (1999) reported that the EPS production was reduced due to the morphological changes of *Aureobasidium* cells occurred at the medium pH 2. They found that the optimal pH profile of the medium for the EPS production was pH 5.5 to 6.5. The pH also affects the molecular weight distribution of EPS. A relatively high molecular weight EPS with a lower yield was obtained at low pH (pH 3) values while a relatively low molecular weight EPS with a high yield was obtained at higher pH values (Shu and Lung, 2004). In some microorganism's regulation of biosynthetic pathway of EPS production may be dependent upon the pH. Table 2.3 shows the EPS production by different microorganisms. Most of the listed microorganisms had produced maximum EPS near neutral pH.

4.3.5 Temperature

The temperature is one of the most important parameters influencing EPS production (Wingender et al., 1999). Most of the microorganisms reported to produce higher concentrations of EPS in the temperature range of 25-30^{o}C (Table 2.3) in batch culture under aerobic conditions. In contrast, to these reports, the EPS production (in batch culture, lab study) has been also observed at temperature range from -2^{o}C to 42^{o}C (Nichols et al., 2005; Wingender et al., 1999). The literature reports suggest that the EPS production could vary for different microbes at a similar growth temperature. The optimum growth temperature for the maximum EPS production mainly depends upon the bacterial strain as well as the temperature of the natural environment of the bacteria (Nichols et al., 2005). It is also possible that optimum temperature for growth and optimum for EPS production may be different depending upon the microbial strain. The difference between optimum temperature for growth and EPS production could also result of increased enzymatic activity in the synthesis of the EPS precursors. A decrease in temperature causes a decrease in growth rate and cell wall polymer synthesis, making more precursors available for EPS synthesis (Sutherland, 2001). Therefore, it is important to find out the optimum temperature for the EPS production as well as optimum for growth considering the type of bacteria and the temperature of natural environments from where the bacteria originally belong.

4.3.6 Metal ions

As a major part of the EPS (soluble and/or loosely bound EPS) is bound with cells mainly through ion bridging with multivalent metals, the metal concentration may also influence the EPS composition. Higgins and Novak (1997c) found that the protein content in the sludge EPS increased at higher Ca^{2+} or Mg^{2+} concentrations, and that higher Na^{+} concentration led to a lower protein content. With an increasing concentration of Fe fed to waste activated sludge, the EPS characteristics and components could also be altered (Lu et al., 2005). In the presence of toxic substances such as heavy metals, microbial cells in activated sludge and biofilms produced more EPS to protect themselves from the harsh environment (Priester et al., 2006). Furthermore, under toxic conditions (increased heavy metals concentration) the increase of the protein content in EPS far exceeded than that of the other components. As the concentration of toxic substances (heavy metals) went over a threshold, its effect on the promotion of EPS production became less significant (Sheng and Yu, 2006). However, some toxic substances,

e.g., bismuth dimercaptopropanol, can also inhibit the production of EPS. At a level just below the minimum inhibitory concentration (bismuth dimercaptopropanol), the production of EPS of *Brevundimonas diminuta* could be significantly reduced (Badireddy et al., 2008).

4.3.7 Aerobic versus anaerobic conditions and EPS production

Favourable growth conditions, i.e., oxygen content of the system are one of the key influencing factors in the EPS production. High levels of oxygen could increase EPS production. Bayer et al. (1990) found that the EPS produced by *Pseudomonas aeruginosa* was highly oxygen-dependent. Higher oxygen level (80 Vs 40 mm Hg) enhanced the EPS production. Variations in oxygen tensions represent one of the trigger mechanisms for the up-regulation of EPS production. On the other hand, other researchers have investigated less specific effects of oxygen. The EPS content of sludge would decrease under anaerobic conditions (Nielsen et al., 1996; Thompson and Leps, 1986). It is reported that activated sludge flocs tend to disintegrate under oxygen limitation or depletion conditions. Such disintegration might be caused by the suppression of EPS production or the hydrolysis of EPS as well. Shin et al. (2001) compared the EPS production of activated sludge in three bioreactors operated at different dissolved oxygen concentration and found that at a high dissolved oxygen level the carbohydrates content in EPS increased with time, whereas the protein content remained unchanged. At a low dissolved oxygen level, the EPS concentration increased with fermentation time but the carbohydrates and proteins content of EPS remained the same. In contrast, EPS also plays a very important role in anaerobic bioreactors such as an upflow anaerobic sludge blanket (UASB) reactor. EPS distribution has an important function in granule stability (to maintain granule cohesion) in UASB reactor. Applegate and Bryaers (1991) reported that oxygen-limited conditions exhibited high EPS production. Low oxygen concentrations slowed bacterial growth rate, giving them more time to consolidate. On the contrary, triggering of EPS production is also linked to a semi-anaerobic environment, which favours the growth and EPS production (Gamar-Nourani et al., 1998). The conflicting conclusions of these researchers highlight the importance of studying the effect of oxygen availability on EPS production.

4.3.8 Pure and mixed cultures

Most of the researchers have reported the production of EPS by pure cultures (Table 2.3). These reports have overlooked the important interactions between microbes in mixed cultures.

The advantages of EPS production using mixed cultures over single cultures have been sited few researchers (Ma et al., 2003; Wang et al., 2011; Zhang et al., 2007; Zhu et al., 2008). The co-operative growth of the microorganisms that occur in mixed cultures of certain EPS producing bacterial strains or mixed cultures of an EPS producing and a non-EPS producing bacterial strains (as in case of activated sludge process) can influence the concentration and characteristics of the produced EPS (Mårtensson et al., 2002). The viscosity of the fermented broth of the mixed culture was reportedly much higher than that of the pure culture (Wingender et al., 1999). The presence of certain non-EPS producing bacterial species along with EPS producing species in a mixed culture can stimulate the production of EPS. When two or more microbial strains form a biofilm, the presence of EPS may not only assist in establishing the biofilm, but also promote greater biofilm growth than the comparable single species biofilms. The EPS promote not only the adhesion and growth of the cells, which synthesise it, but also those of other microbial species. Relatively higher concentration of EPS is produced in a mixed culture of the EPS producing bacterial strains than that of the pure culture (Li et al., 2012; Zhang et al., 2007). According to Zhang et al. (2007), around 15 g/L of purified EPS could be recovered by a mixed culture of *Staphylococcus* sp. and *Pseudomonas* sp.. In case of complex growth medium such as wastewater or wastewater sludge when used as a raw material for EPS production, symbiotic relationship of certain microbial strains might be beneficial for the EPS production by the consortium. Certain microbes can synthesize the nutrients, which are utilized the other microbes. Moreover, secretion of certain enzymes by different microbes may help microbes to fulfill their nutrient requirement from a complex medium. In such a case, the yield of the EPS may increase due to increase in the substrate utilization by the mixed culture.

Symbiotic relationship between certain EPS and non-EPS producing bacterial strains might be useful for producing EPS of higher flocculation efficiency. Ma et al. (2003) produced a bioflocculant by growing mixed culture of two *Bacillus* strains and observed that the EPS obtained exhibited higher flocculation ability as compared to the pure culture of these strains. Similar results were observed in some other studies carried out for EPS produced by mixed culture of *Rhizobium radiobacter* and *Bacillus sphaericus* (Zhu et al., 2008), *Staphylococcus* sp. and *Pseudomonas* sp. (Zhang et al., 2007), *Rhizobium radiobacter* and *Bacillus sphaericus* (Wang et al., 2011). It is clear that the presence of EPS of different composition in some mixed biofilms increases the adhesive properties. However, what is not yet known is the effect of partial or complete removal of one of the polymers from such a complex matrix. Mixed culture revealed higher viscosity and higher molecular weight EPS production than that of the pure culture (Li et al., 2012). While this symbiotic phenomenon of mixed cultures has been observed

for some pairs of bacterial strains, others show no enhancement. This behaviour of the bacterial strains in mixed cultures under certain cases may also have detrimental effects on the EPS production as well as its polymeric properties. In some cases, the mixed culture decreased the EPS concentration as compared to that of pure culture. It has been often seen that very low concentration of EPS is produced by mixed cultures in natural environments such as wastewater sludge as compared to that by the pure strains isolated from the same environment (Subramanian et al., 2010). Thus, finding out the symbiotic combinations of different microbes is the essential step for obtaining the best combination for the mixed culture having high EPS production and flocculating potential.

Table 2.3 Production and chemical characteristics of EPS

Microorganism	Production medium	Fermentation conditions (shake flask)	Maximum EPS produced (g/L)	Chemical characteristics					References
				Carbohydrates (%, w/w or Molar units)	Proteins (%, w/w)	Others (%, w/w)	Molecular weight in Da	Functional groups	
Rhodococcus erythropolis	-	96-120 h	5.0	-	-	-	-	-	(Takeda et al., 1991)
Alcaligenes cupidus (KT201)	Sucrose	-	1.5	Total sugar : 72.25 Galactose: glucose: mannose - 6.3:5.5:1	-		2×10^6	Acetyl ester	(Toeda and Kurane, 1991)
Klebsiella sp.	Glucose	200 rpm, 30°C, 120 h	1.0	Neutral sugar :69.10 Total sugar : 72.55 Galactose: glucose: mannose - 5:2:1	-	Uronic acid: 15.81	$\geq 2\times10^6$	Methoxyl	(Dermlim et al., 1999)
Citrobacter	Acetate-Propionate	120 rpm, 30°C, 72 h	1.5	-	-	-	-	-	(Fujita et al., 2000)
Bacillus sp. (AS-101)	Glucose and agar	130 rpm, 30°C, 10-15 h	-	Carbohydrates : 83	17	Uronic acid: 11.40, Pyruvic acid: 6.10, Acetic acid: 0.40	-	Hydroxyl, Amine	(Salehizadeh et al., 2000)
Corynebacterium glutamicum	Sucrose	120 rpm, 28°C, 48 h	2.0	-	-	-	-	-	(He et al., 2002)
Bacillus firmus	Glucose	150 rpm, 35°C 72 h	1.4 (at 33 h)	Total sugar : 87	Not detected	Uronic acid: 38.00 Pyruvic acid: 6.30	2×10^6	Carboxylate, Hydroxyl, Methoxyl	(Salehizadeh and Shojaosadati, 2002a)
Bacillus mucilaginosus (MBFA9)	Soluble starch	150 rpm, 30°C 84 h	-	-	-	Neutral sugar: 47.40; Uronic acid: 19.10; Amino sugar: 2.70	$2.6\text{x}10^6$	Carboxyl Hydroxyl	(Deng et al., 2003)
Vagococcus sp. strain (W31)		120 rpm, 25°C 48 to72 h	2.3	-	-	-	$2\text{x}10^5$	Hydroxyl, Carboxyl, Methoxyl	(Gao et al., 2006)
Bacillus sp. (MBFF19)	Glucose	200 rpm, 30°C 48 h	0.8	Neutral sugar : 3.60 Amino sugars : 0.50	16.40	Uronic acid : 37.00	-	Carboxyl, Hydroxyl, Methoxyl	(Zheng et al., 2008)

Microorganism	Production medium	Fermentation conditions (shake flask)	Maximum EPS produced (g/L)	Chemical characteristics					References
				Carbohydrates (%, w/w or Molar units)	Proteins (%, w/w)	Others (%, w/w)	Molecular weight in Da	Functional groups	
Bacillus subtilis (E1)	Glycerol and Ethanol	150 rpm, 28°C, 48 h	6.3	107±7.5 µM	111±3.74 µM	Uronic acid 27.318 mM; Amino sugars 41 µM	-	-	(Buthelezi et al., 2010)
Pseudomonas plecoglossicida	Glycerol and Ethanol	150 rpm, 28°C, 48 h	8.3	706±12.2 µM	40±1.631 µM	Uronic acid 26.817 mM; Amino sugars 5±0.141 µM	-	-	(Buthelezi et al., 2010)
Pseudomonas pseudoalcaligenes (A17)	Glycerol and Ethanol	150 rpm, 28°C, 48 h	15.2	56±4.9 µM	70±1.349 µM	Uronic acid 26.954 mM; Amino sugars 54 µM	-	-	(Buthelezi et al., 2010)
Klebsiella terrigena (R2)	Glycerol and Ethanol	150 rpm, 28°C, 48 h	27.7	356±7.5 µM	4±0.86 µM	Uronic acid 2 mM; Amino sugars 0	-	-	(Buthelezi et al., 2010)
Exiguobacterium acetylicum (D1)	Glycerol and Ethanol	150 rpm, 28°C, 48 h	10.2	20±0.9 µM	22±0.221 µM	Uronic acid 26.361 mM; Amino sugars 0	-	-	(Buthelezi et al., 2010)
Staphylococcus aureus (A22)	Glycerol and Ethanol	150 rpm, 28°C, 48 h	10.8	470±7.5 µM	9±0.098 µM	Uronic acid: 26.178 mM; Amino sugars: 2 µM	-	-	(Buthelezi et al., 2010)
Proteus mirabilis (TJ-1)	Glucose	160 rpm, 30°C, 48 h	1.3	63.10	30.90	-	$1.2x10^5$	-	(Xia et al., 2008)
Chryseobacterium.daeguense (MBF-W6)	Glucose, tryptone	180 rpm, 30°C, 36 h		-13.1	32.4	Nucleic acid: 6.8		Carboxyl, Hydroxyl, Methoxyl	(Liu et al., 2010a)
Bacillus licheniformis	Sucrose	200 rpm, 37°C, 48 h	2.9	89.00	11.00	-	$6.89x10^4$	-	(Xiong et al., 2010)
Proteus mirabilis (TJ-1)	Glucose	130 rpm, 25°C, 48 h	1.3	63.10	30.90	-	$1.2x10^5$	-	(Zhang et al., 2010)

Microorganism	Production medium	Fermentation conditions (shake flask)	Maximum EPS produced (g/L)	Chemical characteristics					References
				Carbohydrates (%, w/w or Molar units)	Proteins (%, w/w)	Others (%, w/w)	Molecular weight in Da	Functional groups	
Azotobacter indicus (ATCC 9540)	*Madhuca latifolia* flower extract	180 rpm, 30°C, 144 h	6.1	97.7	2.3	-	$2x10^6$	O-Acetyl, Orcinol, Carboxyl, Hydroxyl	(Patil et al., 2011)
Halomonas sp. (AAD6)	Sugar beet molasses	180 rpm, 37°C, 10-15 h	-	90.00	0.5	Nucleic acids: 4-5	-	-	(Sam et al., 2011)
Bacillus megaterium (TF10)	Glucose	30 °C, 24 h	-	76.90	23.00	-	$1.0\text{-}2.5x10^6$	Hydroxyl Amide Carboxyl Primary Amine	(Yuan et al., 2011)
Paenibacillus elgii (B69)	Glucose, peptone	220 rpm, 30°C, 96 h	12.12			Mainly polysaccharides Glucose: glucuronic acid: xylose: mannose at a ratio of 1:0.53:1.15:0.46	$3.5\ x10^6$		(Li et al., 2013)
	Lactose, soy peptone	220 rpm, 30°C, 96 h	11.49						
	Sucrose, Beef extract	220 rpm, 30°C, 96 h	10.15						
	Soluble starch	220 rpm, 30°C, 96 h	9.53						
	Maltose	220 rpm, 30°C, 96 h	9.18						

5 Recovery of EPS

5.1 EPS Extraction

There are several methods for EPS extraction from different bacterial cultures grown in different medium and different types of wastewater sludge (activated/granular and or aerobic/anaerobic) (Table 2.4). The EPS extraction methods include various physical (centrifugation, cation exchange resin (CER), heating and sonication), chemical methods (alkaline-NaOH, acid-H_2SO_4, trichloroacetic acid, boiling benzene, crown ether, ethylenediamine tetraacetic acid (EDTA), ethanol, enzymes, glutaraldehyde, sulfide, and NaCl treatments) and a combination of both physical and chemical methods (alkaline + heating, ion exchange + stirring, formaldehyde + heating, CER + sonication). The selection and evaluation of the different physical and chemical extraction methods have been discussed in brief by different authors (Comte et al., 2006b; Sheng et al., 2010; Wingender et al., 1999). To study the compositions and functions of the LB-EPS and TB-EPS in a sludge sample, the two fractions of the bound EPS may be extracted separately. As the LB-EPS bound with cell loosely, a mild method (e.g., high-rate shear, heating at low temperatures, or high speed centrifugation) should be chosen to avoid the inclusion of the TB-EPS. Subsequently, a harsh methods (e.g., heating at high temperatures, sonication or chemical extraction methods) should be applied for the TB-EPS extraction. Li and Yang (2007) modified a heating extraction method, which included a mild step and a harsh step for extracting the LB-EPS and TB-EPS from activated sludge subsequently. The cell lysis was not significant after such extraction process. The high speed centrifugation and ultra-sonication were also used to extract the LB-EPS and TB-EPS from sludge (Ramesh et al., 2007).

Water solubility of the components extracted is the important factor in all the extraction methods, whereas highly hydrophobic compounds cannot be extracted. Quantification of EPS is mainly dependent upon the extraction method (Wingender et al., 1999). Based on the literature, it is evident that there is no universal extraction method available so far to determine accurately the quantitative extraction of the EPS from the different microbial suspensions or aggregates. Not a single extraction method exists, which can demonstrate high extraction efficiency without unwanted cell lysis and disruption of different components or macromolecules of EPS. Comparative analysis of the published results is extremely difficult because of the methodological limitations.

The yield of EPS greatly depends upon the extraction method (Wingender et al., 1999). Chemical methods have a higher extraction yield of EPS as compared with the physical methods. However, the chemical methods also have problems such as contamination of the extracted sample from added chemicals (e.g., EDTA) and some chemicals reacts with EPS and induce changes in the EPS composition. Therefore, selection of appropriate EPS extraction methods is necessary.

EPS extraction yield for physical methods is comparatively lower than that of chemical methods and combined chemical-physical methods. On the other hand, physical methods extract comparatively more EPS molecules with higher molecular weight than the chemical methods (Comte et al., 2007). Generally, EPS extracted by the chemical methods get contaminated by the extracting reagents and this sometimes prevents the EPS composition from being determined (Comte et al., 2006b). The complexion properties of the isolated EPS may be altered during the extraction procedure as well as extraction reagent. For example, new binding sites can be formed during the destruction of the native gel matrix (Wuertz et al., 2001). The efficiency of the extraction methods depend upon several factors such as cell lysis, extraction yield, the specificity of the extraction, and the carry-over of the chemicals from the extraction solution towards EPS extract (D'Abzac et al., 2010). Moreover, the nucleic acid content and the protein/polysaccharide ratio also need to be considered to ensure that an abnormal cell lysis does not occur during the extraction procedure.

5.2 EPS recovery as a bioflocculant material

The EPS produced by the bacterial species in the fermented broth (by pure or mixed cultures) can be used as the bioflocculants in different forms (crude form, purified form or after treatment). The crude form of EPS is the fermented broth, which can be used as cost effective bioflocculant material. In the literature there are several reports stating the high flocculation potential of the fermented broth obtained by cultivating the EPS producing bacterial strains in different medium (synthetic, sludge or wastewater) (More et al., 2012a; Zhang et al., 2007). The main advantage of this type of bioflocculant is that there is no need for further treatment to prepare bioflocculant for its application. Crude EPS in three different forms , namely, fermented broth (containing slime and capsular EPS), slime (obtained in the supernatant after centrifuging the broth) and capsular (the residue left after centrifuging the broth) can be used as bioflocculant materials (More et al., 2012a; More et al., 2012b). More et al. (2012a) used pretreated wastewater sludge as a raw material for the production of EPS and the EPS were

recovered in the form of fermented broth, slime and capsular EPS. Fermented broth can be concentrated by membrane filtration to recover the EPS along with cells. Formulation of the fermented broth can be used to convert liquid broth into solid form. The concentration of broth reduces the volume of the broth, increases the product stability and the relative activity of the dosage. The broth containing EPS can be concentrated either by spray drying or through lyophilisation. The selection of spray drying or lyophilisation depends upon the stability of EPS and the economy. In general, the spray drying process is more economical than the lyophilisation process, but it is sensitive towards the product degradation/denaturation. In case of utilization of EPS in soluble form, the mild physical or chemical treatment can be used to release soluble EPS from the broth. The cells can be separated by centrifugation and supernatant can be precipitated through chemical precipitation (More et al., 2012a). Zouboulis et al. (2004) reported that the EPS produced by *Rhizomonas* sp. had first transformed into powder, and then 2 g of the fine powder were dissolved in 1 L of deionized water. The stirring was maintained during dissolving the solids, in order to obtain the final solution of 2000 mg/L. The EPS obtained in this form could also be used as a bioflocculant material.

Table 2.4 Different EPS extraction methods

Methods		Mechanism	Remarks
Physical	Centrifugation	EPS separates from cell surface and dissolve to solution under the centrifugal force.	Used for separation of soluble EPS fraction from cell biomass. Comparatively less cell lysis. Bound EPS cannot be extracted significantly.
	Heating	The molecular movement is enhanced that accelerates the EPS dissolution.	Significant lysis and cells disruption.
	Sonication	The EPS part of the biofilm matrix under the impulsive pressure that is created by the sonication.	Effectively disintegrate sludge flocs and release enzymes. Mild and effective. Most commonly used method for sludge EPS.
	Sonication/centrifugation	The EPS dissolve into solution under the impulsive pressure created by the sonication and centrifugal force.	Effectively disintegrate sludge flocs and release enzymes. Mild and effective. Widely used method.
Chemical	Acidic treatment	Improves the repulsive force and disrupts the interaction between EPS and cells, causing the EPS to fall away from the cell surface.	Effectively disintegrate sludge flocs. Sever cell lysis and cell disruption.
	Alkaline treatment	Alkaline treatment with the addition of NaOH causes the groups, such as the carboxylic groups, to be ionized, resulting in a strong repulsion between the EPS and the cells, and thus makes the EPS dissolve in water.	Effective to extract Al-bound EPS. Sever cell lysis and the disruption of macromolecules.
	CER	CER removes the divalent cations, thus causing the EPS to fall apart.	High efficiency and low cell lysis. Highly selective for the Ca and Mg bound EPS.
	Crown ether	Crown ether is used to combine divalent metals and disrupt the binding interaction between EPS and cells.	Effectively disintegrate sludge flocs.
	EDTA	Divalent cations are very important for the cross-linking of charged compounds in the EPS matrix, and thus the removal of these cations using EDTA causes the EPS matrix to fall apart.	High extraction efficiency and cause a low degree of cell lysis. A low degree of cell lysis, Residual EDTA can contaminate the EPS extraction and induce errors in protein determination in the Lowry method.
	Enzymatic extraction	The carbohydrate and protein-hydrolyzing enzymes were used to disrupt the structure of sludge and dissolve the EPS.	Mild and effective. Low extraction efficiency.
	Ethanol extraction	Denatures the EPS and reduces the binding force between EPS and cells.	Mild and effective. Most commonly used method.
	Glutaraldehyde	As glutaraldehyde has the ability to fix cells and denatures EPS, it can also be used to extract EPS.	Mild and effective. Low extraction efficiency.
	HCHO/NaOH	The addition of HCHO reduces the cell lysis that is caused by the addition of NaOH.	Formaldehyde could fix the cell, and thus prevent cell lysis, by reacting with functional groups of proteins and nucleic acids. The dose of HCHO can change the EPS characteristics and influence the determination of carbohydrates.
	NaCl	Cation exchange is promoted by using a high concentration of NaCl.	Mild, effective, and cheaper method.
	NH_4OH/EDTA	This method combines the pH adjustment and ion exchange methods to improve the extraction efficiency. Using a strong alkali such as NH_4OH reduces cell lysis.	Effectively disintegrate sludge flocs
	Sulfide	The addition of sulfide to sludge could remove Fe by formation of FeS, resulting in the disintegration of the sludge floc structure.	Highly selective for Fe-bound EPS.

References: (Comte et al., 2007; Sesay et al., 2006; Sheng et al., 2010; Wingender et al., 1999)

6 Role of EPS in flocculation, dewatering and biosorption

6.1 Flocculation

In general, EPS are the important factors in the flocculation process of the wastewater systems. The interactions between EPS and cells have a significant effect on microbial flocculation ability. Bioflocculation process has been explained by the number of theories. For example, the microbial cell flocculation has been described using the classical Derjaguin-Landau-Verwey-Overbeek (DLVO) or extended DLVO theories (Bos et al., 1999; Liu et al., 2007). In the DLVO theory the total energy of adhesion is the result of van der Waals attractive forces and the repulsive interactions due to the interpenetration of the electrical double layers (Rijnaarts et al., 1999). Van der Waals forces, polar interactions, electrical double layer interaction and Brownian movement forces are taken into consideration of the extended DLVO theory. If the cell kinetics could overcome the total energy barrier in the DLVO curves, the cell could aggregate and flocculation would occur. The DLVO theory provides an effective way to evaluate the contribution of EPS to the sludge flocculation (Liu et al., 2010b).

In contrast to the well-known mechanisms of flocculation in colloidal systems, the mechanisms of flocculation are not entirely clear in biological systems. Flocculation in biological systems is generally explained by the bridging mechanism. Particles and cells get aggregated by biopolymer flocculants through bridging and charge neutralization mechanism. During bridging, the biopolymers bring different particles closer by encouraging aggregation. In this case, the biopolymer can absorb to other particles to form flocs. This mechanism explains flocculation by neutral or like-charged EPS (Hantula and Bamford, 1991). Charge neutralization occurs when the particle surface charge is reduced by oppositely charged cations and/or bioflocculants. The distance between particles decreases and attractive forces become more effective than the repulsive forces between the particles (Hantula and Bamford, 1991). The effectiveness of the bridging mechanism depends on the molecular weight of the EPS, the charge on the polymer and the particle, the ionic strength of suspension, and the nature of mixing. The flocculation of yeast cells with a flocculant produced by *Aspergillus sojae* has been explained in terms of the bridging between discrete cells and linearly extended biopolymer chains, leading to the formation of a three-dimensional matrix that is capable of settling under quiescent conditions (Nakamura et al., 1976). There are two flocculation mechanisms induced by cations: double layer compression and ion bridging through EPS. A high ionic concentration would promote the

bacterial flocculation and this phenomenon was attributed to the compressing double electric layer (Liu et al., 2007). The ion bridging interactions between multivalent cations and EPS also play the important roles in microbial flocculation (Nguyen et al., 2007; Sobeck and Higgins, 2002). Multivalent cations (e.g., Ca^{2+} and Mg^{2+}) tend to bridge with EPS, and thus improve the flocculation of microbial aggregates (Higgins and Novak, 1997a; Liu et al., 2007).

The EPS are involved in the structure of microbial aggregates and the interactions between cells. In addition, the main intermolecular interactions between cells, including polymer entanglement (Mikkelsen and Nielsen, 2001), ion bridging through EPS, and electrostatic interaction (Klausen et al., 2004), as well as van der Waal's force and hydrogen bonds (Mayer et al., 1999), contribute to the stability of microbial aggregates. EPS are expected to govern, to a certain extent, the stability of microbial aggregates. A higher EPS content in the sludge would result in greater sludge stability (Mikkelsen and Nielsen, 2001).

6.2 Dewatering

The EPS are very important factors in the wastewater sludge thickening and dewatering processes (Houghton et al., 2001). During thickening and dewatering, two types of binding mechanisms between water molecules and EPS are generally involved: electrostatic interactions and hydrogen bonds. The former (electrostatic interactions) are active between the permanent dipoles of water and the functional groups of EPS. The latter (hydrogen bonds) are active between EPS hydroxyl groups and water molecules (Neyens et al., 2004). The EPS have both the beneficial and detrimental effects on the sludge dewaterability. An increase in EPS generally leads to a poorer sludge dewatering ability, possibly because; the steric force generated by EPS prevents contact between cells. In addition, the macromolecules in EPS cause the retention of a lot of water in the sludge flocs and increase the amount of interstitial water in such flocs. EPS can also form a stable gel that prevents water seepage from the pores of flocs, which deteriorates the dewatering ability of the sludge. After removing EPS, the sludge dewatering ability would be improved (Chen et al., 2001; Neyen et al., 2004). The EPS content in sludge can be reduced by different sludge treatments such as ultrasonication, peroxidation, thermal hydrolysis (Neyen et al., 2004; Dewil et al., 2006; Appels et al., 2008).

Some studies have shown that the sludge dewatering ability improves as the EPS content increases (Jin et al., 2004; Mikkelsen and Keiding, 2002). With a higher EPS content, activated sludge had a lower shear sensitivity and lower degree of dispersion, leading to a good

dewatering ability (Mikkelsen and Keiding, 2002). Houghton et al. (2001) proposed that the effect of EPS on the dewatering ability of sludge depended on the concentration of EPS in sludge. Using sludge samples from eight wastewater treatments, they found that the dewatering ability of activated sludge initially increased with the EPS content, but then decreased once the EPS content exceeded a certain threshold. This suggests that a lower EPS content is more beneficial for sludge flocculation and dewatering, as this causes the cells to bind more tightly. As the EPS content further increased and exceeded a certain threshold, the water that was retained by the EPS significantly increased, which resulted in a lower sludge dewatering ability (Houghton and Stephenson, 2002). Further, the various components in EPS have different effects on the dewatering ability of microbial aggregates. The proteins had a high water-holding capacity. Thus, a reduced protein fraction in sludge EPS improved the sludge dewatering ability (Sponza, 2002). Increasing the carbohydrate fraction in EPS would enhance the sludge dewatering (Cetin and Erdincler, 2004).

6.3 Biosorption

There are several studies, which reported that the EPS are the important factors in biosorption of heavy metals (Comte et al., 2006a; Wingender et al., 1999). The biosorption capacity of EPS matrix is attributed to the proteins, polysaccharides and lipid contents. The EPS contains different functional groups (such as amino, carboxyl, hydroxyl, phosphate etc.), which induce strong attractive forces between the cations and biomass (Solís et al., 2012). Total binding sites present in the EPS matrix define the EPS biosorption capacity. In general, metal biosorption by EPS involves physical-chemical interactions between the metal and the functional groups of the EPS matrix. Biosorption involves several mechanisms, including physical adsorption, ion exchange, complexation, and precipitation (Wingender et al., 1999). The effectiveness of biosorption by EPS depends on the pH, temperature, effective contact area between EPS and adsorbate, time of contact, ionic strength, and concentration of the adsorbate, adsorbate structure and the type of microorganism (Comte et al., 2006a; Solís et al., 2012). According to Lopez et al. (2000), metal biosorption increases with the increase in pH (1-10). This is because, ion exchange is more effective when fewer protons are available to compete with the metals for negatively charged metal binding sites of EPS matrix (Wingender et al., 1999). The medium pH affects the ionization state of the functional groups (such as carboxylate, phosphate and amino groups) of EPS. The carboxylate and phosphate groups carry negative charges that allow the EPS components to be potent scavengers of cations (Comte et al., 2006a). According to Loaec

et al. (1997), the biosorption properties of bacterial exopolysaccharides (*Alteromonas macleodii* subsp. *fijiensis*), they suggested that the formation of a metal-ligand coordination bond is based on the theory of hard and soft acids (*i.e.* electron acceptor) and bases (*i.e.* electron donor).The main electron donor atoms of exopolymers are the nitrogen compounds (present as amino-sugars, oxygen as hydroxyl and carboxyl and sulphate ester). According to Gadd, (1992), Pb has a strong preference for ligands such as O and N, while Cd ions prefer soft ligands such as sulphide. In contrast to these theories, it is also pointed out that the binding of cations with polyacids cannot be reduced to pure ion exchange (Comte et al., 2006a). This interaction proved to be specific of the counter ion and it was not only of an electrostatic nature. It has also suggested that the global electric field, surrounding the partially neutralized polyacid molecules, has a role to play in the resulting metal-polymer complex stability.

6.4 Factors influencing the EPS ability to flocculate and dewaterability of different suspensions

The several studies have reported the flocculation characteristics of the EPS synthesized by different microorganisms (Table 2.5). Most of the studies used kaolin suspensions as a standard to evaluate the flocculation characteristics of the EPS. The factors affecting the flocculation characteristics of the EPS are summarized and discussed in the following sections.

Table 2.5 Flocculation characteristics of the EPS synthesized by different microorganisms

Microorganisms	Chemical characteristics of EPS	Optimum flocculant concentration[a]	Optimized pH[b]	Flocculation activity (as OD at 550 nm) or FA%[c]	Other effects of the EPS dosage	Cations used with the EPS during flocculation	Remarks	References
Alcaligenes latus (KT201)	Molecular weight $2x10^6$ Da	0.002 ml culture broth after 72 h of fermentation	5	9 (1/OD)	-	Positive synergic effect of bivalent/trivalent cations Al^{3+}, Fe^{3+}, Fe^{2+}, Mg^{2+}, Ca^{2+} Al^{3+} being the most effective cation	Kaolin suspension of 5 g/L	(Toeda and Kurane, 1991)
Rhodococcus erythropolis	-	10-20 mg/L	-	70-80%	-	1.2 mM of $Al_2(SO_4)_3$	Kaolin suspension of 5 g/L	(Takeda et al., 1991)
Pseudomonas sp. (A-99)	An acidic protein and contained a small amount of an acidic polysaccharide consisting of galacturonic acid, glucose and galactose Carboxylixc group Molecular weight 5 x 10^3 to 2 x 10^6 Da	20 mg/L	5-7	12 (1/OD)	-	5 mM of Ca^{2+}, Fe^{2+} and Mg^{2+}, respectively had positive effect on flocculation	-	(Yokoi et al., 1998)
Citrobacter	Molecular weight 232-440 kDa	1-10 mg/L	2-8	90%	-	-	-	(Fujita et al., 2000)
Corynebacterium glutamicum	-	-	-	80%	-	-	-	(He et al., 2002)
Bacillus firmus	-	30 mg/L	3.7		-	-	-	(Salehizadeh and Shojaosadati, 2002b)
Bacillus mucilaginosus (MBFA9)	Polysaccharide composed mainly of uronic acid (19.1%) neutral sugar (47.4%) and amino sugar (2.7%)	0.1 ml/L	-	99.6%	-	-	-	(Deng et al., 2003)

Microorganisms	**Chemical characteristics of EPS**	**Optimum flocculant concentration**[a]	**Optimized pH**[b]	**Flocculation activity** (as OD at 550 nm) or FA%[c]	**Other effects of the EPS dosage**	**Cations used with the EPS during flocculation**	**Remarks**	**References**
Vagococcus sp. strain (W31)	Hydroxyl, carboxyl and methoxyl group	-	7-10	-	-	-	-	(Gao et al., 2006)
Aeromonas sp. (N11)	-	1 mg/L	3-5	92.40%	-	-	Kaolin suspension of 5 g/L	(Li et al., 2007)
Bacillus mucilaginosus	-	-	-	SS removal 93.3%	74.5%, COD and, 42.3% BOD removal	-	-	(Lian et al., 2008)
Bacillus mucilaginosus	-	-	-	SS removal 93.6%	70.5%, COD, and 77.4% BOD	-	-	(Lian et al., 2008)
Bacillus mucilaginosus	-	-	-	SS removal, 88.4%	66.2%, COD, and 41.7%, BOD	-	-	(Lian et al., 2008)
Proteus mirabilis (TJ-1)	Carboxyl, hydroxyl and amino groups Molecular weight $1.2x10^5$ Da	2 ml of bioflocculant		93.13%	-	1% $CaCl_2$	Kaolin suspension (4.0 g/L)	(Xia et al., 2008)
Bacillus sp. strain (F19)	Neutral sugar (3.6%, w/w) uronic acid (37.0%, w/w), amino sugars (0.5%, w/w) and protein (16.4%, w/w Hydroxyl, Carboxyl, Methoxyl Groups	-	-	-	-	Fe^{3+} inhibited flocculation	Kaolin suspension of 5 g/L	(Zheng et al., 2008)
Azotobacter indicus (ATCC 9540)	Acetyl, carboxyl, and hydroxyl groups and Molecular weight $2x10^6$ Da	500 mg/L	5-10	72%	Reduction in BOD: 38-80%, COD: 37-79% and SS: 41-68%	No cations addition	Wastewater from Wool, Starch, Sugar industry and Dairy industry	(Patil et al., 2011)
Azotobacter indicus (ATCC 9540)	Acetyl, carboxyl, and hydroxyl and Molecular weight $2x10^6$ Da	10 mg/L	5-10	92%		10 mg/L of $CaCl_2$	Wool, Starch, Sugar, Dairy industry	(Patil et al., 2011)

Microorganisms	**Chemical characteristics of EPS**	**Optimum flocculant concentration**[a]	**Optimized pH**[b]	**Flocculation activity** (as OD at 550 nm) or FA%[c]	**Other effects of the EPS dosage**	**Cations used with the EPS during flocculation**	**Remarks**	**References**
Halomonas sp. (AAD6)	Carbohydrates (90% (w/w)), minor amounts of nucleic acids (4-5% (w/ w)) and less than 0.5% (w/w) protein	20 mg/L	7.3	93%	-	-	Synthetic and natural sea water	(Sam et al., 2011)
Bacillus megaterium (TF10)	Presence of hydroxyl, amide, carboxyl and primary amine groups Molecular weight : 1037-2521 kDa	-	-	96%	-	5.6 mM of $CaCl_2$	Kaolin suspension of 4 g/L	(Yuan et al., 2011)
Staphylococcus cohnii sp.	-	0.3 mg/L	7	70.3%	-	2 mL of 1% $CaCl_2$	Kaolin suspension of 5 g/L (100 mL)	(Wong et al., 2012)
Staphylococcus cohnii sp.	-	1.2 mg/L	7	88.9%	-	$AlCl_3$	Kaolin suspension of 5 g/L (100 mL)	(Wong et al., 2012)
Arthrobacter sp. Raats	Glycoprotein, 56% protein and 25% carbohydrate	0.1 mL of supernatant of fermented broth	7	84% (by OD)	-	0.1 mL of 1% $CaCl_2$	Kaolin suspension of 4 g/L (9 mL)	(Mabinya et al., 2012)
Paenibacillus elgii (B69)	Mainly polysaccharides	0.5 mL of 1% EPS solution	-	87%	Wastewater, COD:68%, Turbidity:83%, Color: 88%	0.5 mL of 1% $CaCl_2$	Kaolin suspension of 4 g/L (10 mL)	(Li et al., 2013)
Klebsiella pneumoniae	Mainly polysaccharides 96.8% and protein 2.1%	10-100 mg/L	7.5			5 mL of 9 mM $CaCl_2$	Kaolin suspension of 4 g/L (1000 mL)	(Zhao et al., 2013)

6.4.1 Cations

Cations play important role in flocculation activity. Cations neutralize negatively charged particles as well as stabilize the negative surface charge of polymers and acts as binding agent in forming bridges between particles and polymers (Higgins and Novak, 1997b). The role of bivalent and trivalent cations is to increase the initial adsorption of EPS and suspended particles by decreasing the negative charge on both the EPS and the particle (More et al., 2012a). As an example, the flocculating activity of *Enterobacter* sp. increases in the presence of Al^{3+}, Fe^{3+}, Fe^{2+}, and Ca^{2+} (Yokoi et al., 1997). The kaolin flocculating activity of EPS produced by *Bacillus* sp. was induced by increasing the concentrations of Ca^{2+}, Mg^{2+}, and Fe^{2+}. The optimum pH for stimulating the flocculation by Ca^{2+} was in the range of pH 4-5 (Yokoi et al., 1997). For Mg^{2+}, Fe^{2+}, and Fe^{3+}, the optimum pH was 6-7 (Yokoi et al., 1997). The flocculating activity of EPS was the highest in the Ca^{2+} concentration range of 2-8 mM. In contrast, an increase in Al^{3+} or Fe^{3+} concentrations resulted in a sharp decrease in the flocculating activity and no flocculation was observed at concentrations of 0.2 and 0.5 mM for these ions, respectively (Yokoi et al., 1995; Yokoi et al., 1998; Yokoi et al., 1997). For the EPS of *Nocardia amarae*, the flocculation activity was stimulated by addition of Na^{+}, Ca^{2+}, Al^{3+}, and Fe^{3+}. However, an excessive addition of Fe^{3+} inhibited flocculation. The flocculating activity of EPS produced by *Rhodococcus erythropolis* (Kurane et al., 1991) and *Alcaligenes cupidus* polysaccharide (Toeda and Kurane, 1991) was enhanced by increasing the concentrations of Ca^{2+} and Al^{3+}. The presence of $CaCl_2$ significantly increased the flocculating activity of the EPS produced by several species such as *Bacillus*, *Pseudomonas*, *Serratia* and *Yersinia* (More et al., 2012b; Subramanian et al., 2010). Interactions of the EPS with different cations are very crucial for the potential applications of EPS as a bioflocculant material. Optimization studies must be conducted to understand these interactions and to figure out the best combination of EPS and cations.

6.4.2 Different forms of EPS

As there is the difference in physicochemical nature of these different EPS forms, flocculation through these different forms of EPS are distinct (Table 2.1). More et al. (2012a) reported that the slime, capsular and broth EPS produced in the treated sludge by different bacterial strains revealed distinct flocculation and dewatering capability. Broth and capsular EPS produced in sludge exhibited better flocculation and dewatering capability than that of slime EPS (More et

al., 2012a). On the other hand, Subramanian et al. (2010) reported that the slime EPS produced in the synthetic mineral medium had better flocculation and dewatering potential than that of broth and capsular EPS respectively. The variation in the flocculation and dewatering ability were attributed to physiological and biochemical differences of slime, capsular and broth EPS produced in treated sludge and synthetic mineral medium. Li and Yang (2007) found that excessive EPS in the form of loosely bound EPS (LB-EPS) may deteriorate cell attachment and weaken the floc structure, which results in poor sludge water separation. Although in this study, the LB content was less than 20% of the total EPS. The presence of LB-EPS had a clearly negative effect on bioflocculation, effluent clarification, sludge settleability and dewaterability. The sludge-water separation performance as specified by the ESS (effluent suspended solids), SVI and SRT values was closely related to the amount of LB-EPS in the sludge, but no correlation could be established with the TB-EPS content (Li and Yang, 2007). Yu et al. (2009b), showed that the tightly bound EPS fractions possessed a high flocculating rate to kaolin suspensions than other EPS fractions (slime and loosely bound EPS). High flocculability of TB-EPS fraction was attributed to high contents of macromolecules (330-1200 kDa) and trivalent cations (Fe^{3+} and Al^{3+}) as compared to that of other EPS fractions.

6.4.3 Concentration of EPS

Concentration of the EPS plays an important role in the flocculation and dewatering of the suspensions. The optimum concentrations of the EPS at maximum flocculation activity are presented in Table 2.5. EPS concentration is directly related to sludge dewatering and sludge flocculation (Houghton et al., 2001). At optimum EPS concentration, there was the decrease in number of small particles present in the sludge, which enhanced the sludge flocculation and dewatering (Gonçalves et al., 2007). Excess EPS concentration (than the optimum) is detrimental to sludge dewatering. This is mainly due to high water retention by excess EPS matrix decreases the dewaterability of the sludge. Houghton et al. (2001) also reported that maximum dewaterability could be achieved at optimum concentrations of EPS. The optimum concentration of EPS for digested sludge, raw sludge and activated sludge were 10 mg EPS/g SS, 20 mg EPS/g SS and activated sludge 35 mg EPS/g SS, respectively (Houghton et al., 2001). More et al. (2012a) found that a very low concentration of EPS was required to attain high kaolin flocculation activity (>75%) and the lowest CST, whereas the dose of EPS produced in the synthetic media by the same bacterial strains required 250-2500 mg EPS/g SS to attain a similar flocculation activity (Subramanian et al., 2010). EPS produced in the sterilized sludge

were effective in the kaolin flocculation as compared with the EPS produced in the synthetic media. This was probably due to the method and conditions (such as mixing condition, pH, temperature and sequence of addition of EPS and cations to the kaolin suspension) used in the flocculation tests of the two studies (More et al., 2012a; Subramanian et al., 2010). EPS concentration for maximum flocculation activity varies with EPS obtained from different bacterial strains (Shin et al., 2001). As shown in the Table 2.6, the reported optimum EPS concentration varies from 0.2 mg EPS/g SS to 1000 mg EPS/g SS.

6.4.4 Protein and carbohydrate content of EPS

Ye et al. (2011b) observed that there was no strong relationship between total EPS concentration and activated sludge flocculation, settling and dewatering. On the other hand, the protein content in LB-EPS correlated with activated sludge flocculation, settling and dewatering. Surface charge and hydrophobicity of the EPS are important in sludge settling. Cell surface charge may also play a role in the hydrophobicity of the bacteria. The important factor determining the charge of the cell surface is the ratio of carbohydrates to protein in the EPS. Shin et al. (2001) revealed that the surface charge of the cell was positively related with carbohydrates to protein ratio in the EPS. Negative surface charge decreased with increase in the carbohydrate to protein ratio in the EPS. Cells having higher negative surface charge show higher hydrophobic and these hydrophobic components form bonds with positively charged inorganic particles such as Ca^{2+}, Mg^{2+}. Bacterial aggregation is more at higher negative surface charge. Between the EPS constituents and bacterial cells, divalent cations may act as bridging agents. The strong negative charge on the cell surface was favorable for settling, which was represented by the low SVI values (Shin et al., 2001). Urbain et al. (1993) reported that strength of negative surface charge is correlated with sludge settling. Polysaccharides play the major role in flocculation. For example, Bruus et al. (1992) suggested that the divalent cations interact with negatively charged groups of alginate like polysaccharides within bioflocs. Multivalent cations (divalent, trivalent) may bridge among negatively charged carboxyl groups (of Uronic acids) (Subramanian et al., 2009). Both proteins and polysaccharides contents of EPS are important factors, which participate in flocculation, settling and dewatering process (Higgins and Novak, 1997a).

6.4.5 Molecular weight of EPS

The molecular weights of the EPS reported in different studies have been presented in Table 2.3. The molecular weight range of EPS varies from 10^5 to $2.5x10^6$ Da (Yokoi et al., 1995). The EPS produced by *Flavobacterium* sp. is a protein with a molecular weight of $1.4x10^5$ Da (Tong et al., 1999). The molecular weight of the EPS produced by *Aspergillus sojae* has been estimated at $>2x10^5$ Da by size exclusion chromatography (Nakamura et al., 1976). The molecular weights of EPS were estimated to be $2.75x10^5$ Da (Yamayuchi et al., 1996). The smallest molecular weight reported for EPS is approximately $3.7x10^4$ Da and this flocculant is produced by *Hemimysis anomala* (Saito et al., 1990). The molecular weight of anionic polysaccharide of *Oscillatoria* sp. was reported to be more than $2x10^5$ Da (Bender et al., 1994). The efficiency of the bridging mechanism in flocculation is related to the size of the EPS. Flocculation with high molecular weight EPS involves more adsorption points, stronger bridging, and higher flocculating activity than does flocculation with a low-molecular-weight EPS.

6.4.6 pH of the suspension

The pH of the suspension is one of the most important factors influencing flocculating activity (Yokoi et al., 1995; Yokoi et al., 2002). The EPS were reported to be active at acidic conditions (pH 3-6) (Yim et al., 2007), while the EPS from *Nannocystis* sp. was active in alkaline conditions ranging from pH 12-14 in the presence of 30 mg/L $CaCl_2 \cdot 2H_2O$ (Zhang et al., 2002). The flocculant from *Streptomyces griseus* was active in acidic conditions ranging from pH 2-6, with the maximum activity observed at pH 4 (Shimofuruya et al., 1995). The maximum activity of the flocculant from *Enterobacter* sp. was observed at pH 3 and the activity decreased with increasing pH (Yokoi et al., 1997). The EPS produced by *Bacillus licheniformis* showed strong flocculating activity at a wide range of pH values from 2-12 (Ji et al., 2010).

The flocculating activity of EPS from *Bacillus* sp. was high in acidic pH (pH 3-5) (Yokoi et al., 1995). The maximum flocculation activity of EPS from *Enterobacter* sp. was observed at pH 3 and the activity decreased with increasing pH (Yokoi et al., 1997). The EPS of *Streptomyces griseus* was active in acidic conditions (pH 2-6) and the maximum activity was observed at pH 4 (Shimofuruya et al., 1995). The EPS produced by *Rhodococcus erythropolis* is reported to be active at neutral pH (Kurane et al., 1991). At pH higher than 7, there was increase in flocculating activity of EPS synthesized by *Aspergillus sojae* (Nakamura et al., 1976). The EPS synthesized by *Aspergillus* sp. had maximum flocculating activity at pH 3-8 (Nam et al., 1996). The variations

in the optimum pH of the suspensions for EPS depend on the bacterial species (EPS composition), nature of the suspensions (e.g., surface charge), type (mono or multivalent) and concentrations of additive cations used. Finding out the optimum pH range is basic step during the flocculation via EPS.

6.4.7 Temperature

The EPS with protein or peptide backbone in the structure are generally thermally labile, but those made of carbohydrates are heat-stable (Takegi and Kadowaki, 1985). The EPS derived from *Rhodococcus erythropolis* (Kurane et al., 1991), *Paecilomyces* sp. (Takegi and Kadowaki, 1985), *Aspergillus sojae* (Nakamura et al., 1976), and *Bacillus* sp. (Suh et al., 1997) are heat-stable and retain more than 50% of the initial flocculating activity after 15 min of boiling in water. The EPS produced by *Aspergillus* sp. was stable at 100 ^{o}C for more than 1 h (Nam et al., 1996). The flocculants of *Bacillus* sp. (Yokoi et al., 1995), *Nocardia amarae* (Takeda et al., 1992), and *Streptomyces griseus* (Shimofuruya et al., 1995) were reported as being heat-labile. Optimal temperature range of 20-40^{o}C for flocculating activity of pectin (heteropolysaccharide), with the highest activity at 30^{o}C was also observed (Yokoi et al., 2002). On the other hand, the flocculating activity of the EPS from *Enterobacter aerogenes* had an optimal temperature range of 35 to 65 ^{o}C and the highest activity at 45 ^{o}C (Lu et al., 2005). EPS have also showed strong flocculating activity over a broad range of temperature (4 to 95 ^{o}C), and it maintained excellent flocculating activity at lower temperatures, exhibiting its great application potential in treating low temperature (below room temperature) drinking water compared with commonly used chemically flocculants, polyaluminium chloride (PAC) and polyacrylamide (PAM) (Li et al., 2009).

6.4.8 Other factors

Floc strength/stability and floc size are important operational parameters in solid/liquid separation techniques for the efficient removal of aggregated particles (Jarvis et al., 2005). Flocs must be resistant to the shear stresses to hold together the aggregated particles. Larger the aggregated particles, higher are the removal efficiencies. Floc strength is dependent on the inter-particle bonds between the components of the aggregate (bonds strength and number of bonds) (Bruus et al., 1992; Jarvis et al., 2005). Flocs breakage occurs when the shear stress become more than the bond strength. EPS act as glue for binding the different floc constituents

together by different interactions (Keiding and Nielsen, 1997), entanglement of EPS molecules and hydrophobic interactions (Urbain et al., 1993). Since the stability of these aggregates follows the general rules for colloidal chemistry, changes in physical-chemical factors such as ionic strength and ionic composition, pH, and detergent addition can influence the inter particle forces between the floc constituents and hence floc strength.

EPS of high molecular weight can form stronger flocs (Ni and Yu, 2011). The presence of large number of bonds among aggregated components increases the floc compaction and strength (Jarvis et al., 2005; Jin et al., 2003). The floc size and shape are critical parameters of floc strength (Bruus et al., 1992; Jarvis et al., 2005). Poor floc stability leads to an increase in the turbidity of the effluent (Bruus et al., 1992). As the EPS participate in maintaining the structure and strength of microbial aggregates, the quantity and properties of EPS components are therefore, expected to be crucial to the stability of biomass flocs.

Table 2.6 Optimum EPS concentration (with calcium ions) required for kaolin flocculation activity

Bioflocculant producing microorganism	Kaolin suspension (g/L)	Optimum EPS concentration		Flocculation activity (%)	References
		mg EPS/g SS	mg/L		
Aeromonas sp. (N11)	5.0	0.2	1	92.4	(Li et al., 2007)
Gyrodinium impudicum (KG03)	5.0	0.2	1	90.0	(Yim et al., 2007)
Klebsiella mobilis	5.0	0.5	2.5	95.4	(Wang et al., 2007)
Bacillus licheniformis (X14)	4.0	0.5	2	99.2	(Ji et al., 2010)
Bacillus licheniformis	1.0	5.8	5.8	90.0	(Xiong et al., 2010)
Bacillus sp. (AS101)	5.0	6.0	30	92.0	(Salehizadeh et al., 2000)
Bacillus sp. (DYU1)	5.0	8.0	40	97.0	(Wu and Ye, 2007)
Azotobacter indicus	5.0	100.0	500	92.0	(Patil et al., 2011)
Bacillus sp. (BS9)	5.0	200.0	1000	78.2	(Subramanian et al., 2010)
Pseudomonas sp. (BS2)	5.0	400.0	2000	78.0	(Subramanian et al., 2010)
Serratia sp. (BS8)	5.0	400.0	2000	78.1	(Subramanian et al., 2010)
Microbacterium sp. (BS15)	5.0	400.0	2000	81.2	(Subramanian et al., 2010)
Pantoea sp. (BS7)	5.0	600.0	3000	71.4	(Subramanian et al., 2010)
Yersinia sp. (BS11)	5.0	600.0	3000	83.7	(Subramanian et al., 2010)
Enterobacter sp. (BS25)	5.0	1000.0	5000	76.1	(Subramanian et al., 2010)

Note: Percent difference in optical density (OD) at 550 nm for the sample and control respectively was the flocculating activity.

7 Environmental applications of EPS

7.1 Water treatment

Recently several studies have been reported on the application of EPS produced by different bacterial strains to the raw water treatment with or without cations addition (Buthelezi et al., 2009; Li et al., 2009; Ma et al., 2008; Nontembiso et al., 2011). EPS synthesized by *Bacillus* sp. was used in the jar tests to compare the kaolin turbidity removal (Ma et al., 2008) with the following efficiency, EPS (86%), $Al_2(SO_4)_3$ (95%) and $Fe_2(SO_4)_3$ (96%). They concluded that the combination of EPS and $Fe_2(SO_4)_3$ was promising for the raw water treatment. There was no residual Ferric or Aluminium ion accumulation when EPS were applied for the treatment of raw water. Li et al. (2009) investigated EPS synthesized by *Bacillus licheniformis* (combined with $CaCl_2$) for drinking water treatment. The EPS synthesized showed good flocculating performance and industrial potential for the treatment of drinking water. The maximum COD and turbidity removal from the water were 61.2% and 95.6%, respectively. They also observed that the EPS were effective at different temperature (4 ^oC and 25 ^oC) for the water treatment. According to Li et al. (2009), the EPS synthesized by *Bacillus licheniformis* were safe for their application in the drinking water treatment. EPS produced by several bacterial strains such as *Bacillus subtilis*, *Exiguobacterium acetylicum*, *Klebsiella terrigena*, *Staphylococcus aureus*, *Pseudomonas pseudoalcaligenes* and *Pseudomonas plecoglossicida* were also able to remove river water turbidity from 84.1 to 93.6% at 10 mg/L of EPS dosage (Buthelezi et al., 2009). These EPS were also able to remove (up to 98.3%) both Gram positive (*Staphylococcus aureus* and *Streptococcus faecalis*) and Gram-negative (*Escherichia coli* and *Klebsiella oxytoca*) bacteria from the river water samples. Buthelezi et al. (2009) indicated that the application of bacterial EPS could be a potential alternative to alum (inorganic coagulants) in the river water treatment. Natural organic matters (NOM) present in the river water are of great health concern. EPS synthesized by *Pseudomonas aeruginosa* and *Pseudomonas putida* were able to remove NOM from the aqueous environment (Wang et al., 2012). The EPS are potential flocculants to use in river water treatment for the NOM removal due to their biosorption and bioflocculation capabilities. Moreover, EPS are considered advantageous for coagulation treatment for water purification due to their non-toxic and biodegradable nature. Although, several studies have advocated the application of EPS to treat river or drinking water, however, the treatment has the restricted acceptance. This is because the biological treatment of drinking water is not a

favourable option due to the fear of water contamination by microbes, which leads to biological treatment demands for extra unit operations such as filtration, disinfection, etc for the safety reasons. Further studies are required on the safe applications of EPS in the treatment of river or drinking water.

7.2 Wastewater flocculation and settling

In wastewater treatment, flocculation is the most commonly used method for removing suspended solids and to increase the effluent quality. Recent studies have projected EPS as an alternative to the conventional chemical polymers in wastewater treatment. Gong et al. (2008) reported that the EPS synthesized by *Serratia ficaria* were potentially applicable to the different industrial (brewery, soy sauce brewing, meat processing and pulp and paper) wastewater treatments. Whereas the EPS synthesized by *Bacillus mucilaginosus* were able to remove more than 85% of SS and 68.5% of COD from starch wastewater (Deng et al., 2003). The EPS were also used to treat swine wastewater with turbidity and COD removal efficiency of 91% and 42%, respectively, which was better than the conventional polymer (polyaluminium chloride, PAC) alone (Zhang et al., 2012). Lian et al. (2008) reported that the EPS synthesized by *Bacillus mucilaginosus* can be used to treat municipal, brewage and pharmaceutical wastewater. The COD removal of 74.6, 70.5 and 66.2%, and BOD removal of 42.3, 77.4, and 41.7%, and SS removal of 93.3, 93.6, and 88.4% were achieved for domestic, brewage, and pharmaceutical wastewater, respectively using EPS synthesized by *Bacillus mucilaginosus*. A mixed culture consortium also was reported to produce highly efficient EPS. The EPS produced by a mixed culture of *Staphylococcus* sp. and *Pseudomonas* sp. were successfully applied for the treatment of indigotin printing and dyeing wastewater (80% COD removal) (Zhang et al., 2007). Polysaccharides based EPS synthesized by *Paenibacillus elgii* (B69) revealed the wastewater COD and turbidity removal of 68% and 83%, respectively (Li et al., 2013). Most of the studies about the EPS applications in wastewater treatment were carried out in the laboratory scale. The pilot scale studies of wastewater treatment by EPS could bring more insights to the practical applications of the EPS.

7.3 Colour removal from wastewater

Dyes are responsible for the changes in colour, increase in organic load and toxicity of textile industrial wastewater (Solís et al., 2012). Release of untreated textile industrial wastewater into

the natural water bodies has harmful effects on the aquatic life (Solís et al., 2012; Zhang et al., 2009). Recently, biosorption has been recognized a potential technology for the treatment of textile industrial wastewaters (Solís et al., 2012). The biosorption capacity of the bacteria is attributed to the EPS (mainly heteropolysaccharides and lipids) of the cell wall, which contains different functional groups (e.g., amino, carboxyl, hydroxyl, phosphate etc), causing an attractive force between the dye and the cell wall (Buthelezi, 2008; Solís et al., 2012; Wang et al., 2007; Zhang et al., 2009). There are several studies, which reported potential applicability of EPS the biosorbents material (Buthelezi, 2008; Inbaraj et al., 2008; Liu et al., 2009; Zhang et al., 2009; Zhang et al., 2007).

The effective dye removal using EPS produced by several bacterial species (such as *Bacillus*, *Exiguobacterium*, *Klebsiella*, *Pseudomonas* and *Staphylococcus*) have been reported by Buthelezi et al. (2008). Decolourization efficiency was 20-99.9% using EPS as biosorbents (Buthelezi, 2008). EPS had high efficiency in decolorizing dye solutions of basic fuchsine (93%), whereas Chromium (VI) removing efficiency was 28% (initial Chromium (VI) concentration of 280 mg/L) (Buthelezi, 2008). The effectiveness of the biosorbents or decolourization efficiency depends on EPS concentration, pH, temperature, ionic strength, and time of contact, dye concentration, dye structure and microorganism (Solís et al., 2012). EPS synthesized by *Bacillus* species were investigated by Inbaraj et al. (2008) for basic brown 1 dye removal at different temperature and pH. They observed that the dye adsorption rate was increased with increasing solution temperature, whereas adsorption capacity was decreased with decrease in temperature. The maximum dye adsorption by EPS was observed at pH 5 (Inbaraj et al., 2008). The EPS produced in dairy wastewater by *Klebsiella mobilis* were efficient in flocculating some disperse dyes in aqueous solutions with a removal efficiency of 91% for disperse Violet HFRL (Wang et al., 2007). The EPS synthesized by *Proteus mirabilis* were effective in hazardous dye (basic blue 54) removal from aqueous solution in batch systems (Zhang et al., 2009). The maximum dye (basic blue 54) uptake rate of 2.005 g/g of EPS was observed. According to Zhang et al. (2009), the dye adsorption occurs due to the presence of a large number of binding sites available in EPS matrix of high molecular weight along with stronger van der Waals forces, which binds with dye molecules. As a result, EPS matrix was completely embedded in adsorbed dye molecules. Bacteria in the consortium possess higher colour removal efficiency as compared to the single bacterium (Solís et al., 2012). Zhang et al. (2007) observed that the EPS synthesized by a consortium of *Staphylococcus* sp. and *Pseudomonas* sp. had capability of treating printing and dye wastewater. Liu et al. (2009) have reported that the EPS extracted from the backwashed sludge revealed high (82.9 and 77.8%) decolourization efficiency in

methylene blue and fast blue aqueous solutions. Overall, the EPS revealed the potential to become a good biosorbent for removal and recovery of dye from different solutions and wastewaters. Several researchers are focused on developing strategies to utilize EPS for biosorption. The knowledge about the biosorption using EPS is expected to increase in near future as it is an attractive cost effective treatment option.

7.4 Sludge dewatering

Large quantities of chemical polymers are used in mechanical sludge dewatering operations, which makes the sludge dewatering an expensive process in a wastewater treatment plant (More et al., 2010). Sludge also contains EPS and has been identified as one of the important parameters for the efficiency of sludge dewatering. The concentration and characteristics of EPS are the important parameters, which affect the sludge dewatering (Houghton et al., 2001; Houghton and Stephenson, 2002; More et al., 2010). Only recently, EPS have been studied for their applications as bioflocculant materials in sludge dewatering. The EPS synthesized by *Klebsiella* sp. showed a similar performance for sludge dewatering as compared with the chemical flocculants such as Alum, PAC and PAM at their optimal dosage (Yang et al., 2012). Final dry sludge solids of 17.5% (w/w) and specific resistance to filtration (SRF of the sludge) of 3.36 x 10^{12} m/kg were obtained after preconditioning the sludge with the EPS. Yang et al. (2012) also observed that the combined use of EPS and alum reduced SRF from 10.87 x 10^{12} m/kg to 1.72 x 10^{12} m/kg, and dry solids increased from 13.1 to 21.3%. Zhang et al. (2010) reported that 0.17% (w/w) EPS (synthesized by *Proteus mirabilis*) along with 1.3% (w/w) $CaCl_2$ enhanced sludge dewaterability as compared to that of conventional chemical polymers. Moreover, EPS showed better sludge conditioning performance at neutral pH. Although very few reports are available, there is a lack of detailed investigations on the applicability of the EPS in sludge dewatering. Effects of sludge type and concentration, cations type and their concentration are required to systematically evaluate the real potential of EPS in sludge dewatering operations.

7.5 Metal removal or recovery

Heavy metal removal from wastewater is usually achieved by activated sludge. This is achieved mainly through biosorption of heavy metals by activated sludge EPS (during flocculation and settling process) (Guibaud et al., 2003). The EPS produced by several bacterial species (such

as *Bacillus, Halomonas, Herbaspirillum, Pseudomonas* and *Paenibacillus*) were recognised as potential flocculating agents in the treatment of industrial wastewater effluents (Lin and Harichund, 2012). In this study, a significant (more than 50%) removal of Pb^{+2}, Zn^{+2} and Hg^{+2} were observed at an optimum EPS concentration (1 to 10 mg/L, irrespective of the bacterial strains). The EPS used in this study, removed Cd^{+2} effectively only at higher EPS concentrations (10000 mg/L) but not at a lower concentration. The Cd^{2+} removing capacity of EPS was enhanced significantly (up to 95%) by an increase in temperature (up to 45^{o}C). The EPS producing *Pseudomonas* sp. (strain EJ01) was also observed to tolerate cadmium ions (2 mM and above) present in the growth medium and it was directly related to the increase (50%) in the EPS concentration (Chien et al., 2013). The EPS synthesized by microorganisms in soils get adsorbed on mineral surfaces and they affect the mineral's ability to immobilize heavy metals. Mikutta et al. (2012) studied the sorption kinetics of Pb^{2+}, Cu^{2+} and Zn^{2+} to the composite of EPS (from *Bacillus subtilis*), Ca-saturated bentonite and ferrihydrite. Bentonites had adsorbed higher EPS-C (18.5 mg/g) than ferrihydrite (7.9 mg/g). During sorption, the EPS had chemically altered with bentonite and the uptake of low-molecular weight components and EPS-N was preferred over others. The rate of Pb^{2+}, Cu^{2+}, and Zn^{2+} sorption for bentonite was increased by mineral-bound EPS. On the other hand, EPS aggregated ferrihydrite and changed the structure of mineral-EPS associations. Ferrihydrite selectively retained high-molecular weight and P-rich components of EPS and it had a negative effect on the metal uptake for ferrihydrite. The number of binding sites and complexion capacities of EPS are correlated with proteins, polysaccharides and humic substances content because of the presence of functional groups such as carboxylates, hydroxyls and amines (Guibaud et al., 2003; Lesmana et al., 2009). Metal ions also can get accumulated in the bacterial cell cytoplasm or get adsorbed on to the bacterial cell wall (Brown and Lester, 1979).

7.6 Removal of toxic organic compounds

The EPS have ability to remove toxic organic compounds from different wastewater, sludge and soils. Polycyclic aromatic hydrocarbons (PAHs) degradation using microorganisms is considered the most feasible bioremediation technique (Zhang et al., 2011). The EPS producing bacteria are reportedly applied for the removal of PAHs from contaminated soils (Jia et al., 2011; Liu et al., 2001; Zhang et al., 2011). In situ inoculation of PAHs degrading bacteria having EPS producing capability can efficiently clean up soils and sediments polluted with PAHs (Zhang et al., 2011). EPS play an important role in PAHs biodegradation (Jia et al., 2011). The

interaction between EPS and PAHs is considered the spontaneous and exothermic and the bindings of PAHs to EPS are mainly dominated by the hydrophobic interactions. EPS are able to display variety of surface activities that allow solubility of hydrophobic substrates. *Phenanthrene (*PHE) biodegradation proceeds mainly at the silicone oil-water interfaces. The bacteria overcome PHE mass transfer limitation by producing EPS, which facilitates mass transfer of PHE dissolved in silicon oil. The EPS enhances PHE mass transfer in the bulk water, thus increasing the bioavailability of PHE in water. Liu et al. (2001) reported that the EPS producing bacteria enhanced the extent of release of soil-bound phenanthrene. Jia et al. (2011) observed that *Zoogloea* sp. and *Aspergillus niger* had degraded more than 30% of pyrene after 35 days. For both of these microbes, the pyrene degradation was increased with increase in EPS concentration. Pyrene degradation could be enhanced by increasing the specific surface area of the EPS in contact with pyrene. So far, the studies reported on the applications of EPS in toxic organic compound removal mostly are at laboratory scale.

7.7 Landfill leachate treatment

Coagulation-flocculation process can be used for organic content removal from landfill leachate and also for treating stabilized and old landfill leachates, which is simpler and less expensive process compared to a membrane process or use of adsorbents (Renou et al., 2008). Instead of conventional chemical coagulants and flocculants, bacterial EPS can be used in leachate treatment. Zouboulis et al. (2004) found that the application of EPS from *Rhizomonas* sp. was quite efficient (when compared with alum or PAC) in the removal of humic acids from synthetic solutions as well as in the reduction of COD (45%) content from landfill leachate. An optimum EPS dosage of 20 mg/L at pH 7-7.5 was required to attain more than 85% of humic acid removal. The advantage of the EPS in this case was that there was no need of pH adjustment to have optimum treatment results. Very low dosage of EPS (50 mg/L) produced similar COD removal as that of alum (500 mg/L). Use of EPS in the leachate treatment is also advantageous either to eliminate or to decrease the concentration of aluminum or iron in the liquid phase of the treated leachate.

EPS producing bacterial species can also be grown in leachate for in situ leachate treatment. Young landfill leachate (age less than five years) is generally rich in organic matter; it can be used as a suitable raw material for the EPS producing bacterial strains (Fusconi et al., 2006; Zouboulis et al., 2004). Leachate infiltration in to land and aquifers are the main issues with landfill sites. Reduction in leachate production, efficient collection and treatment prior to

discharge are the main areas of landfill engineering, which need to be improved (Renou et al., 2008). In that context, EPS are of great ecological importance because EPS producing bacteria are capable of hindering the flow of water, reducing the volume of the pores available for fluid transport and lowering the hydraulic conductivity. The EPS producing bacterial strains are especially applicable in this case because of their bacterial growth and survival strategies in different environments. By this way EPS producing microbes can be used as a biological barrier to prevent contamination of groundwater. Bacterial EPS matrix and biomass form a biobarrier, which clogs the aquifer and physically separate adjacent areas of an underground region. Thus, the biobarriers prevent the further infiltration of leachate contamination in to land and aquifer. Aquifer bioclogging can be achieved by on-site microbes and spiking the nutrients in the target site (Ross et al., 2001), or by spiking microbes and nutrient in the target site (Dennis and Turner, 1998). Given the potential of the EPS producing bacteria for biotechnological exploitation (both in bioremediation of environmental pollutants and in the synthesis of chemicals), the EPS-producing species are required to be further studied. EPS producing bacterial strains can be isolated to study their possible application in bioremediation of the contaminated area. In this context, it is advantageous to isolate EPS producing bacterial species from the landfill leachate for their application in landfill leachate treatment. There is still a large room for exploring the possible microbial applications in bioremediation in surface and subsurface environments. In particular, a fundamental understanding is needed on the exact role played by EPS in the clogging process, of the factors that influence their clogging efficiency, and of whether their morphology has an effect on their ability to clog soils or aquifer materials.

7.8 Soil remediation and reclamation

Terrestrial and aquatic environments are contaminated with various kinds of pollutants. Among these, total petroleum hydrocarbons (TPHs), polycyclic aromatic hydrocarbons (PAHs) and pesticides from anthropogenic sources pose a risk to human health. Release of hydrocarbons into the environment contaminates the soil and water. Chemical precipitation, electrochemical deposition, evaporation, membrane processes, ion exchange and activated carbon adsorption are the main technologies to remove metals from these environments. Recently, there is growing interest in developing techniques to remove or reduce levels of hydrocarbons and heavy metals from contaminated soil by means of specially selected microorganisms (Peter, 2011). Bioremediation has been recently considered a potential soil remediation technology especially in Polar Regions with hydrocarbon-contamination (Aislabie et al., 2006). There are

many studies focused on the development of biofilm reactors for remediation of xenobiotic compounds (Singh et al., 2006). Biofilms consist of microorganisms in communities supported by EPS matrix. Biofilms have higher microbial biomass, which can degrade recalcitrant compounds and biofilms also have the capability to immobilize such compounds via EPS matrix (Singh et al., 2006). Enhanced gene transfer among biofilm microbial communities and their chemo taxis makes the biofilms suitable for bioremediation (Singh et al., 2006). In a recent study, biofilms have shown very high (97%) diesel oil degradation capability (Chandran and Das, 2011). The successful application of bioremediation depends on the degradation capacity of microbes and in situ environmental conditions (Aislabie et al., 2006). Bioremediation efficiency can be improved by genetic engineering. The microbial properties of interest to genetic engineering are chemotactic ability, the mixed culture biofilms and optimization of physical-chemical conditions (Singh et al., 2006). There is the need for extensive research of biofilm communities and gene transfer within biofilms, which would facilitate the development of better techniques for the bioremediation of contaminated soils.

The EPS matrix can potentially be used to reduce transport of solids in runoff water, to reduce heavy metals transport, to stabilize soil and to reduce dust generation (Gerbersdorf et al., 2008). EPS derived from different pure or mixed cultures can be produced in aerobic bioreactors. The EPS could be separated from the growth media and extracted to produce a non-reactive (non-cross-linking) material. This non-reactive material can be transported as a low-density, dry solid and applied to the soil by either mixing the dry EPS with water at the site of use, producing a viscous liquid, or gel that is directly applied to the soil surface. The EPS can be applied to the soil by mixing the dry EPS into the soil, then applying water. EPS can form a gel within the soil matrix and EPS binds with soil molecules. The EPS play the important role in surface adhesion, cell aggregation to form a biofilm matrix, formation of protective shield, water absorption, and nutrient accumulation. Secretion of EPS by bacteria is recognized as a cohesive force in promoting surface erosion resistance in sediments (Droppo, 2009). Cyanobacteria have been applied for promoting soil adhesion in arid regions (Prasanna et al., 2008). Soil modification using the natural products of indigenous soil bacteria has introduced the concept of bio-geo-civil engineering (Ivanov and Chu, 2008). The EPS from *Rhizobium tropici* can improve the soil strength and helps in erosion control and slope stability. This is especially highly suitable in situations where conventional methods (geotextiles and vegetative cover) are not suitable.

Table 2.7 Potential environmental pollution control applications of the EPS

Applications	Role of EPS matrix	Remarks
Water treatment	Bioflocculant, biosorbent	EPS can be used for removal of suspended solids, natural organic matter. EPS can be used as a flocculant in combination with multivalent cations (Ca^{2+}, Mg^{2+}, Al^{3+}, Fe^{3+}). There are safety issues about its wide application.
Wastewater flocculation and settling	Bioflocculant, biosorbent	Application in the removal of suspended solids, organic matter removal, improving effluent quality. EPS can reduce the dependency upon chemical polymers bioflocculant combined with conventional chemical polymers (PAC, PAM). EPS application to wastewater treatment is very attractive.
Colour removal from the wastewater	Bioabsorbent, bioflocculant	EPS can remove of dye from wastewater and hence the colour. Dye accumulated by can be degraded by the microorganism. The application of EPS in dye removal is quite promising.
Sludge dewatering	Thickener, bioflocculant	EPS application in sludge thickening and dewatering, presence of multivalent cations (Ca^{2+}, Mg^{2+}, Al^{3+}, Fe^{3+}) along with EPS is must. The application of EPS on higher concentrations of sludge solids is not yet reported.
Metal removal from waste effluents	Bioabsorbent, bioleaching, bioflocculant	Heavy and toxic metals removal or recovery from wastewater, wastewater sludge or industrial wastes. EPS application to metal removal or recovery from waste effluents is very attractive and promising.
Removal of toxic organic compounds	Bioabsorbent, biodegradation, bioflocculant	Application of EPS for the removal toxic organic compounds from wastewater, wastewater sludge and soils could be a promising strategy in future.
Landfill leachate treatment	Bioleaching, biodegradation	Bioleaching of the heavy metals and toxicants from the landfill leachate is becoming popular among the researchers. EPS can play important role in landfill leachate treatment.
Soil remediation and reclamation	Biosorbent, biodegradation, bioaugmentation	EPS can potentially applicable in soil adhesion, reducing the soil erosion and reduction in transportation of metals from soils to water bodies.

8 Concluding remarks

The quantity of available data on environmental applications of EPS is rather limited compared to the data that can be found on conventional polymers. Therefore, there is scarcity of knowledge about relevant EPS properties, different aspects of EPS production and recovery and potential of EPS in pollution control applications. Moreover, so far, the work on the application of EPS in water, wastewater and wastewater sludge flocculation, dewatering and treatment is still in the research phase. Extensive investigation is required before its possible implementation to the field processes. The flocculation studies reported so far were carried out at the lab scale (test tube) experiments. Moreover, the studies have reported optical density a main parameter to determine flocculation, but very few reports are available on flocculation performed in standard jar tests. The standard jar tests are the prerequisite of the field application. Test tube studies results do not simulate actual potential of EPS as compared to the conventional chemical polymers at field operating conditions. Flocculation studies should be investigated as compared with the proper controls. Most of the studies have ignored this very basic and important aspect. All the limitations to shake flasks studies necessitate the scale up studies. The floc strength of the EPS is also an important factor, which is yet to be explored according to the environmental applications of EPS. The literature witnesses that the EPS production media or raw material used and the microbial strain employed also have an undisputed role in the chemical composition of the produced EPS, which will affect/alter the flocculation mechanism. Further, the flocculation studies must consider the effect of these components in their final prediction about the applicability of their bioflocculant. Also, EPS produced by different microorganisms in natural systems (wastewater, wastewater sludge etc.) or in the control systems (in laboratory, using different raw materials) possess special (or varying) engineering properties. Therefore, it warrants optimizing the process of production to have desired EPS characteristics. The cost of the EPS production is an important factor when considering its field application. Most of the studies have reported the production of EPS in the synthetic medium. Use of synthetic medium may not be cost effective option. Therefore, low cost medium (raw material) must be investigated for the production EPS. There are many such options, such as crude glycerol, municipal wastewater sludge, and different industrial wastewater sludge such as food industrial wastes. A very limited research has been conducted on these options and therefore requires systematic exploration for the production of EPS and its application as flocculent.

Extraction of EPS has been also a critical issue in the EPS study. Literature reports the EPS characterization based upon various extraction methods, physical, chemical or combination of both. The yield of the EPS and the effect of the different extraction methods need to be taken into consideration while investigating EPS literature. It is also not very well clear if extraction or purification or both or none is required for application of EPS as coagulants/flocculants in various sectors. Presently, there is very scarce information on this aspect. Further, the literature is also scanty on structural changes of different form of EPS during extraction with different methods (chemical or physical). The contamination of EPS during chemical extraction process is also an important issue, which can have serious consequences on the purification process of the extracted EPS. These structural changes along with contamination could certainly affect the flocculation ability of the EPS and need systematic investigation. Many studies have successfully demonstrated the treatment of wastewater and color removal employing EPS synthesized by individual or a consortium of various microorganisms, however, the literature is scanty on large scale studies.

Very limited information exists on the flocculation efficiency of different types of EPS (crude, purified, LB, TB etc.) for real practical application. Most of the studies have been conducted using kaolin as the medium to flocculate. This warrants investigating which form of EPS produced by different microbial strains on different media (semi-synthetic or complex or waste material) will be efficient for flocculation of targeted system (water, wastewater, sludge, color removal, metal removal, toxic organic removal, landfill leachate etc.). Moreover, symbiotic relationship between different strains to produce desired properties EPS also needs to be studied. Research is also warranted on safety concern of use of EPS to flocculate the river and drinking water. On the other hand, there is also a need to further explore role of EPS in soil remediation and clogging of aquifers.

Owing to an increasing environmental awareness and the limitation of fossil resources, it is anticipated that renewable biopolymers will replace a substantial fraction of the market for synthetic polymers. There will also probably be a growing demand for the production of bacterial EPS having material properties that are specifically tailored for environmental applications such as water, wastewater, wastewater sludge, leachate treatments, etc. The competitive advantage of the environmentally friendly and highly biodegradable and, in many cases, biocompatible polymers will become increasingly attractive mainly for the environmental industry. Bacteria remain ideal organisms for the production of EPS with properties of interest. Microbial polymers produced in different forms of EPS have been reportedly used as flocculants. So far in literature,

very limited studies had been reported solely to examine the potential of EPS in various environmental applications. Therefore, extensive research is required to be carried out ~~on~~ in the areas such as the chemical characterization of EPS produced using different substrates, different microorganisms, synthesis of biopolymers, study of mechanism of flocculation through EPS and finding out the ways to modify EPS properties to increase flocculation efficiency. Bacterial polymers could be used for the flocculation of secondary wastewater. The concentrated wastewater or the flocculated material contains unutilised organic matter, which could be used as a substrate for bioconversion. For example, it could be the best raw material for the production of lipids, biodiesel or biopolymers production. The use of biopolymers for settling the secondary wastewater solids can eliminate the biological reactor required for the treatment of the secondary wastewater. Moreover, the concentrated secondary wastewater solids are rich in organic matter, which is better raw material than the secondary wastewater sludge. The secondary wastewater sludge contains organic matter mostly in the form of biomass and secondary metabolites accumulated after the biological treatment of the secondary wastewater sludge. Because of the higher concentrations of unutilised carbon and nitrogen, the concentrated sediments of secondary wastewater would yield higher production of biodiesel, lipids or other such products.

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List of Abbreviations

APHA	American Public Health Association
B-EPS	Broth EPS
C-EPS	Capsular EPS
CER	Cation exchange resin
C/N ratio	Carbon to nitrogen ratio
COD	Chemical oxygen demand
CST	Capillary suction time
Da	*Dalton*
DLVO	Derjaguin–Landau–Verwey–Overbeek
DNA	Deoxyribonucleic acids
E-DNA	Extracellular deoxyribonucleic acids
EDTA	Ethylenediamine tetraacetic acid
EPS	Extracellular polymeric substances or microbial polymers or bioflocculants
FA	Flocculation activity
Flocculation activity	(1-*S/C*) x100 (%), where *C* is control turbidity and *S* is sample turbidity
HA	Humic acid
LB-EPS	Loosely bound EPS
OD	Optical density
mg EPS/g of kaolin	mg of EPS added per gram of kaolin suspension in water
NOM	Natural organic matter
NTU	Nephelometric Turbidity Units
PAC	Poly aluminium chloride
PAHs	Polycyclic aromatic hydrocarbons
PAM	Polyacrylamide

PGA	Poly-gamma-glutamate
S-EPS	Slime EPS
SS	Suspended solids
SRF	Specific resistance to filtration
SRT	Solids retention time
SVI	Sludge volume index
TB-EPS	Tightly Bound EPS
TPHs	Total petroleum hydrocarbons
ζ	Zeta Potential

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CHAPITRE 3

CRIBLAGE ET CINÉTIQUES DE PRODUCTION DES SPE DE TREIZE SOUCHES MICROBIENNES À PARTIR DE BOUE DES EAUX USÉES

PARTIE 1

BIOCHEMICAL DIVERSITY OF THE BACTERIAL STRAINS AND THEIR BIOPOLYMER PRODUCING CAPABILITIES IN WASTEWATER SLUDGE

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RÉSUMÉ

La caractérisation biochimique de 13 souches polymériques extracellulaires (SPE) a été réalisée par BIOLOG. Les souches bactériennes ont été cultivées dans les boues stérilisées pour la production de SPE. La floculation et la capacité de déshydratation de SPE produites (bouillon, les SPE solubles et capsulaires brutes) ont été examinées par la solution de kaolin combinée avec le calcium (150 mg de Ca^{2+}/L de suspension de kaolin). BIOLOG a révélé qu'il y avait 9 *Bacillus*, 2 *Serratia* et 2 *Yersinia*. La plupart de ces souches bactériennes ont la capacité d'utiliser un large spectre de sources de carbone et d'azote. Une concentration de plus de 1 g du SPE/L a été produite par la plupart des souches de bactéries. La concentration des SPE produite par différentes souches de *Bacillus* était plus élevée que celle produite par *Serratia* et *Yersinia*. Les B-SPE ont une activité de floculation de plus de 75% dans le cas de *Bacillus* sp.7, *Bacillus* sp.4 et *Bacillus* sp.6, respectivement. L'activité de floculation supérieure à 75% a été obtenue en utilisant de faibles concentrations de B-SPE (de 1,12 à 2,70 mg SPE/g SS).

Mots clés: biofloculation; biopolymères; Substances polymères extracellulaires; boues; floculation; déshydratation

ABSTRACT

The biochemical characterization of 13 extracellular polymeric substances (EPS) producing bacterial strains was carried out by BIOLOG. The bacterial strains were cultured in sterilized sludge for EPS production. Flocculation and dewatering capabilities of produced EPS (broth, crude slime and capsular) were examined using kaolin suspension combined with calcium (150 mg of Ca^{2+}/L of kaolin suspension). BIOLOG revealed that there were 9 *Bacillus*, 2 *Serratia* and 2 *Yersinia* species. Most of these bacterial strains had capability to utilize wide spectrum of carbon and nitrogen sources. EPS concentration of more than 1 g/L was produced by most of the bacterial strains. Concentration of EPS produced by different *Bacillus* strains was higher than that of *Serratia* and *Yersinia*. Broth EPS revealed flocculation activity more than 75% for *Bacillus* sp.7, *Bacillus* sp.4 and *Bacillus* sp.6, respectively. Flocculation activity higher than 75% was attained using very low concentrations of broth EPS (1.12-2.70 mg EPS/g SS).

Key words: Bioflocculation; Biopolymers; Extracellular polymeric substances; Sludge; Flocculation; Dewatering

1 Introduction

Chemical flocculants commonly used in wastewater sludge treatment are inorganic such as alum, ferric chloride, lime, and polyaluminum chloride (PAC), and organic synthetic polymers such as polyacrylamide (PAM) and polyethylene amine (Salehizadeh and Shojaosadati 2001, 2002). There are environmental and health concerns about neurotoxicity and less degradability of organic synthetic polymer flocculants and also, carcinogenic nature of their degraded monomers such as acrylamide (Subramanian et al., 2010; Zhang et al., 2010). Therefore, environmentally safe, biodegradable and sustainable bioflocculants such as chitosan, sodium alginate, and microbial flocculants (e.g. extracellular polymeric substances (EPS), filamentous fungi (FF)) have been studied for their potential applications in wastewater sludge flocculation to replace chemical polymers completely or partially (Salehizadeh and Shojaosadati, 2001; More et al., 2010; Subramanian et al., 2010; Zhang et al., 2010).

EPS are the biopolymers secreted by microorganisms outside or on the cell wall, which are the structural and functional integrity of microbial aggregates such as biofilms, flocs and sludge (Wingender et al., 1999). Polysaccharides and proteins are the major components of EPS, whereas lipids, nucleic acids and other biopolymers as minor components (Higgins and Novak, 1997a, 1997b; Wingender et al., 1999). Viscous matrixes of EPS play an important role in the sludge flocculation because of their adsorption capability, biodegradability, hydrophobicity/hydrophilicity and the presence of charged functional groups (Urbain et al., 1993; Higgins and Novak, 1997a). EPS occur in the sludge naturally but sludge seldom settles as its own and hence it requires addition of flocculating agents (Flemming and Wingender, 2001; Houghton et al., 2001; Tian, 2008). The concentration and type of EPS present in the sludge are considered as the important parameters for sludge settling and dewatering (Houghton et al., 2001). Recent studies have reported that microbial flocculants/polymers i.e. EPS produced from various microorganisms were applied as a flocculant material which could be used alone, or in combination with multivalent cations, or in combination with conventional polymers to improve the sludge settling and dewatering characteristics (Subramanian et al., 2010; Zhang et al., 2010). Microbial flocculant, TJ-F1 has been reported to improve the sludge dewaterability when used alone as well as in combination with conventional poly (acrylamide((2-Methacryloyloxy)ethyl) trimethylammonium chloride) (P(AM-DMC)) and $CaCl_2$ (Zhang et al., 2010). The previous study conducted in our lab (Subramanian et al., 2010) revealed that bacterial strains isolated from wastewater sludge were capable of producing viscous EPS in synthetic mineral medium having high flocculating ability.

The flocculating activity and production cost of bioflocculants are the two major limiting factors with regard to their applications. The economical and efficient production of the biopolymers can be achieved by cheaper production medium such as wastewater sludge. Wastewater sludge is cheap and readily available source of carbon, nitrogen and other nutrients. Microorganisms can utilize carbon, nitrogen, phosphorus and micronutrients available in the municipal and industrial wastewater sludge (Drouin et al., 2008). As per our knowledge, there is no study reported so far where wastewater sludge has been used as a raw material for EPS production by microorganisms. 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 producing capabilities of pure microbial cultures in wastewater sludge, it is essential to have knowledge of their metabolic, biochemical properties, and susceptibility to certain chemicals and antimicrobial agents. In this context, BIOLOG plate technique is a rapid and convenient tool for studying and comparing physiological profiles of the bacterial strains. Analysis of physiological profiles allows estimation of similarity between bacterial strains. BIOLOG systems investigate the metabolic potential of the bacterial strains which are capable of metabolically active and growing in the certain conditions (Konopka et al., 1998). Therefore, the objective of this work was to study metabolic and biochemical properties of each individual strains isolated from sludge, their EPS producing capabilities using sludge as raw material and to determine flocculation activity of the EPS.

2 Materials and Methods

2.1 Identification and biochemical characterization

Thirteen EPS producing bacterial strains isolated from the wastewater sludge and identified by 16S rDNA sequence were used in this study (Subramanian et al., 2010). BIOLOG system (BIOLOG Inc., Hayward, USA) was used for the identification and the biochemical characterization of bacterial strains. Identified strains were grown on the tryptic soy agar plates and stored at 4 ^{o}C and sub-cultured fortnightly.

BIOLOG system was used to investigate biochemical diversity or metabolic potential of the bacterial strains which is based on their ability to oxidize different carbon and nitrogen sources

(Konopka et al., 1998). All the steps were performed according to the manufacturer's instructions. BIOLOG plates were inoculated with 100 µL of the bacterial suspensions and incubated at 25 ^{o}C for 24 h, respectively to allow the utilization of carbon and nitrogen sources. Utilization reactions are indicated when a purple colour forms in the wells and no reactions remain colourless. Growth of the bacterial strains in different pH, carbon sources, amino acids/proteins, carboxylic acids, esters and fatty acids and presence of antibiotic, reducing power, Grams staining characteristics were studied (Becker et al., 2009). BIOLOG system enables to understand what stimulates or inhibits growth of the strains.

2.2 Sludge as a growth medium

Secondary wastewater sludge (without chemical polymers) was collected from biofiltration unit (biolite filter media) of Communauté Urbaine du Québec (Municipal wastewater sludge treatment plant, CUQ, Québec, Canada). The sludge was first settled by gravity for 1 h and the concentrated sludge was collected by discarding the supernatant. Characteristics of the sludge, such as pH, total suspended solids (TSS), and volatile suspended solids (VSS), total carbon, total nitrogen, and total phosphorous were determined using Standard Methods (APHA, 2005). The sludge used was at pH 6.5, TSS of 10 g/L and VSS of 7.4 g/L. Viscosity was measured by viscometer (DV-II+PRO, Brookfield Engineering Laboratories, Inc., Stoughton, MA, USA), at constant higher shear rate of 30 s^{-1} and room temperature. Zeta potential (ζ) was measured using Zetaphoremeter (Zetaphoremeter IV, Zetacompact Z8000, CAD Instrumentation, France). The sludge was stored at 4 ^{o}C for further use.

2.3 EPS production and harvesting

Sludge sterilization was done by autoclaving at 121 ^{o}C for 15 min. After cooling to ambient temperature, pH of sterilized sludge was adjusted to 7. All the bacterial strains were grown in sterilized sludge by incubating at 250 rpm, 25 ^{o}C for 24 h and used as inoculum. Standard plate count method was used to study growth of the bacterial strain by colony forming unit (CFU) on the TSA plates and was reported as CFU/mL. Inoculum (3×10^{6} CFU) was used to inoculate in 100 mL of sterilized sludge in 500 mL Erlenmeyer flasks and then incubated at 250 rpm, 25 ^{o}C for 72 h, respectively. EPS produced were measured at the end of the cultivation. After incubation (72 h), broth samples were centrifuged at 6000 x g for 15 min at 4^{o}C to obtain supernatant (containing slime EPS; termed as crude S-EPS) and pellets (containing capsular

EPS (C-EPS) with bacterial cells along with residual sludge material and termed as crude C-EPS) (Subramanian et al., 2010; Wingender et al., 1999).

S-EPS in the supernatant was precipitated with 2.2 volume of absolute chilled ethanol by incubating the mixture at -20 ^{o}C for 1 h. The precipitates (containing pure S-EPS) were collected by centrifugation at 6000g for 15 min at 4 ^{o}C. Precipitates of S-EPS were dried at room temperature in a laminar hood for 6 h and dry weight of the precipitates was measured and denoted as S-EPS (APHA, 2005). To determine C-EPS, the pellet (crude C-EPS) was re-suspended in deionized water. The re-suspended pellet was first heated at 60^{o}C in water bath for 30 min to release C-EPS followed by centrifugation at 6000 x g for 15 min at 4^{o}C (Wingender et al., 1999; Li and Yang, 2007). The supernatant (containing C-EPS) was used to precipitate C-EPS using same procedure as for S-EPS. Dry weight of the precipitates was measured and denoted as C-EPS. Sum of dry weights of S-EPS and C-EPS (measured above) was denoted as B-EPS.

The protein content in the S-EPS was determined using bovine serum albumin as a standard (Lowry et al., 1951). The carbohydrate content of the S-EPS was determined by the phenol-sulphuric acid method using glucose as the standard solution (DuBois et al., 1956). Viscosity and zeta potential measurements were carried out for the sterilized sludge before and after 72 h of incubation.

2.4 Flocculation activity and dewatering

Flocculation activities of the EPS were based on a decrease in turbidity of the standard kaolin suspension after jar tests (Yokoi et al., 1997). Flocculation activity was calculated according to the equation: Flocculation activity = (1-*S/C*) x100 (%), where *C* is control turbidity and *S* is sample turbidity. Crude EPS (S-EPS and C-EPS) were used in the jar tests. The jar tests were performed using kaolin suspension prepared in deionized water (5 g/L). Divalent cations solution i.e. Ca^{2+} of 150 mg/L ($CaCl_2$ dissolved in deionized water) (Zhang et al., 2010) was added to the kaolin suspension of 500 mL by rapid mixing (175 rpm for 3 min) and then pH was adjusted to 7.5 by NaOH (1M). During rapid mixing, 4 mL of each EPS obtained in different forms from the broth was added and rapid mixing continued for 3 min followed by slow mixing at 75 rpm was allowed for 30 min. After slow mixing, the samples were allowed to settle in 500 mL graduated cylinders to determine their turbidity (Hantula and Bamford, 1991) and capillary suction time (CST). Characteristics such as pH and turbidity of the samples were determined using standard methods (APHA, 2005). CST was the measure of dewaterability (Scholz, 2005)

and determined by the CST instrument (Trition electronics, model 304 M CST, Dunmow, Essex) using a 10-mm diameter reservoir. Lower CST value means better dewaterability or filterability.

3 Results and Discussion

3.1 Biochemical diversity

3.1.1 Identification

BIOLOG provided genus identification of the 13 bacterial strains tested at 24 h. Of the 13 identifications reported, nine were belonging to *Bacillus* species and others were two *Serratia* and two *Yersinia*. These identification results were concurrent with the 16S rDNA sequences (100% similarity) of all the 13 bacterial strains as shown in Table 3.1.1.

3.1.2 Physiological and biochemical properties

Nine bacterial strains (*Bacillus*) were Gram-positive whereas others (two *Serratia* and two *Yersinia*) were Gram-negative. Most of the bacterial strains revealed growth at pH 5, 6 and 7, except *Bacillus* sp.1 and *Bacillus* sp.9, which could not grow at pH 5. Growth of different bacterial strains at various NaCl concentrations was observed. Most of the *Bacillus* spp. 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 as also reported in the literature (Upadhyay et al., 2011; Zahran, 1997; Zhang et al., 2011). The osmotic pressure created by salinity may stimulate these bacterial strains to produce EPS. EPS provides a self-protective mechanism for these bacterial strains by providing a buffering zone against saline conditions (Upadhyay et al., 2011; Zahran, 1997). *Serratia* strains were able to grow in 1% and 4% NaCl, whereas *Yersnia* spp. could grow only at 1% NaCl. *Serratia* spp. and *Yersinia* spp. were less tolerant to the osmotic shock or environmental stress of high salt concentration.

All thirteen strains grew in 1% w/v sodium lactate. This indicates all strains are tolerant to the antimicrobial effects of sodium lactate or lactic acid. These results are concurrent with the reports that EPS producing bacterial strains exhibits tolerance towards the organic acids such as lactic acid (Kubota et al., 2009).

There was no growth of *Bacillus* spp. observed in the presence of tetrazolium compared to *Serratia* spp. and *Yersinia* spp. that exhibited growth. Growth in presence of tetrazolium indicates the reducing power of the microorganisms i.e. the overall electron transport chain activity or a presence of a variety of biological redox pathways in microbial energy metabolism ecosystem (Maier et al., 2009). Bacterial cell components and redox enzymes facilitate the reduction of the tetrazolium salt extracellularly. Extracellular redox activity is associated with the production of EPS which may help microbial systems to deal with organic materials which are difficult to utilize (Wuertz et al., 1998).

3.1.3 Carbon and nitrogen utilization profile

Biochemical characteristics of all the isolates showed different patterns of carbon and nitrogen source utilization under similar conditions of incubation (Table 3.1.2). Observed profile of carbon and nitrogen sources metabolized in BIOLOG plates reflects the catabolic potential and nutrient requirements of a certain microbial strain.

It was observed that, after 24 h of incubation, most of the carbon (such as lactose, fructose, glycerol, tween 40 and acetic acid) and nitrogen (different amino acids/proteins) sources were utilized by *Bacillus*, *Serratia* and *Yersinia* spp., except *Bacillus* sp.1 and *Bacillus* sp.9. The results of bacterial response to different carbon and nitrogen sources have been listed in Table 3.1.2 and Table 3.1.3, respectively.

Bacillus sp.1 utilized few carbon sources such as L-lactic acid, L-malic acid, acetoacetic acid, acetic acid and formic acid, and nitrogen sources such as L-aspartic acid, L-glutamic acid and L-histidine. *Bacillus* sp.9 utilized only L-fucose and L-glutamic acid. According to Garland et al.(1999), sometimes BIOLOG systems are insensitive to the certain bacterial structure due to their metabolic redundancy. Species composition could change without a shift in the profile of the positive responses, which might be the case of *Bacillus* sp.1 and *Bacillus* sp.9. The 95 substrates in the BIOLOG plate are comprised of simple, common substrates selected on their ability to discriminate among bacterial isolates. In this study, *Bacillus* sp.1 and *Bacillus* sp.9 which utilized very few carbon sources probably required recalcitrant or unusual substrates which might be of particular importance for these strains such as cellulose in terrestrial ecosystems and halogenated phenolics in wastewater treatment (Konopka et al., 1998). These two strains were grown in our lab using tryptic soy agar (TSA) media in which dextrose or D-glucose was the energy source, it contained enzymatic digests of casein and soybean meal

which provides amino acids and other nitrogenous substances. Further, these strains had shown growth along with EPS production in sterilized sludge (Table 3.1.1). This could be due to the fact that the wastewater sludge contains a variety of organic matters including monomers, oligomers and polymers (different molecular weights) in the form of particulate or dissolved fractions (Burgess and Pletschke, 2008). Major chemical fractions in municipal wastewater are proteins, sugars, volatile fatty acids, lipids and others such as humic acid, DNA-RNA, tannic acid, fibers which could be useful as raw material by the bacterial strains (Huang et al., 2010). Among all thirteen strains, only *Bacillus* sp.6 was found to utilize N-acetyl neuraminic acid. N-acetyl-D-galactosamine was utilized by *Bacillus* sp.5 and *Bacillus* sp.8, respectively. The derivatives of these amino sugars are generally found in bacteria and distributed in glycoproteins.

3.1.4 Antibiotic resistance

Antibiotic resistance of the strains to fusidic acid, troleandomycin, rifamycin SV, minocycline, lincomycin, vancomycin, nalidixic acid, aztreonam were also examined in the BIOLOG tests. It was observed that after 24 h, *Serratia* spp. and *Yersinia* spp. were resistant to most of the antibiotics, except fusidic acid and minocycline. *Serratia* spp. was resistant to aztreonam, but *Yersinia* spp. was suppressed. Except for aztreonam, there was no growth of most of the *Bacillus* strains in other antibiotics tested. *Bacillus* sp.1, 5, 6, 8 and 9 exhibited growth in presence of aztreonam. These results revealed that *Serratia* spp. and *Yersinia* spp. were resistant to most of the antibiotics tested. Moreover, *Serratia* spp. and *Yersinia* spp. demonstrated growth in presence of D-glucose-6-PO_4 and D-fructose-6-PO_4, whereas *Bacillus* spp. did not exhibit growth on these substrates. Certain EPS producing bacterial strains exhibit antibiotic resistance either due to the physical restriction to diffusion of the antimicrobial agents provided by their extracellular matrix, or slow growth rate of the bacterial cells inside the biofilms covered by extracellular polymeric matrix or some physiologic changes brought about by interaction of the organisms with a surface (Donlan, 2000).

3.2 EPS production and harvesting

All the bacterial strains were individually cultivated in sterilized sludge under similar conditions to ascertain their capacity of EPS production. Quantity of total (slime and capsular) EPS harvested from different strains was in the range of 0.7 to 1.7 g/L after 72 h of fermentation. The

control sample of sterilized raw sludge (without inoculation) had very low concentration of EPS (less than 31 mg/g SS). Therefore, the high EPS concentration (Figure 3.1.1) observed in the fermented broth was due to EPS secretion by individual bacterial strains during 72 h of fermentation. EPS production was higher in most of the *Bacillus* spp. than in the *Serratia* spp. and *Yersinia* spp. Total EPS of about 1 g/L or more was produced by most of the strains, except *Bacillus* sp.1 and *Yersinia* sp.2. The highest production of total EPS in sterilized sludge was 1.689 g/L in case of *Bacillus* sp.3 followed by 1.6 g/L in case of *Bacillus* sp.8 and 1.5 g/L in case of *Serratia* sp.1.

The control sample viscosity i.e. initial (before fermentation) viscosity of the sterilized sludge was 4.66 mPas. The viscosity of fermented broth (at the end of fermentation) for all individual strains was substantially increased (Table 3.1.1). An increase in viscosity of 18.25 to 21.94% was observed in cases where total EPS concentration produced by individual strains was more than 1 g/L. Fermented broths of *Bacillus* sp.1 and *Yersinia* sp.2 with viscosity increase of 2.92% and 6.05% revealed EPS concentrations of 0.7 g/L and 0.8 g/L, respectively. However, in case of *Yersinia* sp.1, comparatively lower viscosity increase (8.81%) of the culture broth was observed despite of total EPS concentration of 1.1 g/L.

All the EPS producing bacterial strains were isolated from the sludge of same wastewater treatment plant as used for production of EPS and also these strains were cultured in same sterilized sludge The total carbon, total nitrogen, total phosphorous, soluble protein and carbohydrate contents of the sludge sample used for EPS production for all bacterial strains was 401 g/Kg, 52.1 g/Kg, 1 g/Kg, 23.6 g/Kg and 2.3 g/Kg of dry sludge weight, respectively. However, EPS producing ability of individual strain was different (Tables 3.1.1). This is due to the fact that the individual bacterial strains possess different metabolic pathways to utilize different available carbon sources present in the sludge (as also revealed from the BIOLOG study). The production of EPS in sterilized sludge was comparatively less than that in synthetic media as reported in our earlier study (Subramanian et al., 2010). Slime EPS production in synthetic media by *Bacillus* sp.5, *Serratia* sp.1 and *Yersinia* sp.1, was 2.4 g/L, 3.0 g/L and 2.5 g/L, respectively, whereas slime produced EPS in sterilized sludge varied from 0.4 to 1.0 g/L. This could be attributed to the limited availability of carbon and nitrogen sources in the sterilized sludge as compared to the synthetic medium. BIOLOG study revealed a low spectrum of carbon utilization in case of *Bacillus* sp.1. The limited availability of carbon and nitrogen sources from the sludge along with low spectrum of carbon utilization resulted in the lowest total EPS (slime + capsular) production capacity (0.7 g/L) (Table 3.1.1) of this strain. However, in case of *Bacillus*

sp.9, which also revealed low spectrum of carbon utilization, the total EPS produced was 1.2 g/L (which was comparatively higher than *Bacillus* sp. 1) (Table 3.1.1). It clearly demonstrated that *Bacillus* sp.9 had specific metabolic pathways which helped to utilize less number of nutrients and carbon sources available in the sludge but still synthesized high concentration of EPS.

3.3 Flocculation activity and dewaterability

The jar tests without addition of calcium ions showed very low flocculation activity (16.3%). In this case, turbidity of the kaolin suspension reduced from 135 NTU to 113 NTU by the addition of calcium ions. Significant changes in the flocculation activities were observed when 150 of Ca^{2+} mg/L was added to kaolin suspensions followed by addition of 4 mL of different forms of EPS (B-EPS, C-EPS and S-EPS, Figure 3.1.1). The concentration of each EPS (4 mL) added was presented as mg of EPS per g of kaolin solids. Zeta potential of kaolin suspension (5 g/L) without addition of Ca^{2+} and EPS was -30.94±1.33 mV. Negative charge of kaolin suspension was reduced from -30.94±1.33 to -11.31±0.50 mV by addition of Ca^{2+}. All the EPS had negative zeta potential. Zeta potential of the B-EPS, C-EPS and S-EPS were -28.97±0.34 mV, -30.24±0.44 mV and -27.70±0.94 mV, respectively. It is reported that the presence of uronic acids (such as D-glucuronic, D-galacturonic, and mannuronic acids) or ketal-linked pyruvates in EPS confers the anionic property (Wingender et al., 1999). This property is important because it allows association of divalent cations such as calcium, which has been shown to cross-link with the polymer strands and provide greater binding force. Subsequent additions of EPS after mixing calcium ions with kaolin suspensions had very small change in the charge. Therefore, charge neutralization of the kaolin particles was achieved by addition of Ca^{2+}. EPS addition in presence of cations stimulated the flocculation by neutralization and destabilization of residual negative charges of carboxyl groups of uronic acid in an acidic polysaccharide, forming bridges which bind kaolin particles to each other (Higgins and Novak, 1997a; Yokoi et al., 1997; Zhang et al., 2010).

Higher flocculation activity was observed for EPS produced by most of the *Bacillus* spp. compared to *Serratia* and *Yersinia*. Eight out of nine *Bacillus* spp. revealed comparatively higher flocculation activity (>50%), except *Bacillus* sp.1. EPS from *Serratia* sp.2 and *Yersinia* sp.2 revealed comparatively lower flocculation activity (<50%). Among all three EPS forms (B-EPS, C-EPS and S-EPS), B-EPS revealed the highest flocculation activity of 81.73%, 77.78% and 76.59% in case of *Bacillus* sp.7, *Bacillus* sp.4 and *Bacillus* sp.6, respectively. Flocculation

activity of 68.80% was observed in case of C-EPS of *Bacillus* sp.2 and flocculation activity of 68.11% was observed in case of S-EPS of *Bacillus* sp.8.

Increase in dewaterability i.e. decrease in the CST was observed using 150 mg/L of Ca^{2+} followed by 4 mL of different forms of EPS (Figure 3.1.2). The control sample (kaolin suspension without any EPS or Ca^{2+} addition) CST was 38.7 s. The samples with the addition of Ca^{2+} (without EPS) to kaolin suspension had CST of 29.4 s. CST was reduced by 24% with only $CaCl_2$ addition (without any EPS) to the kaolin suspension. In general, B-EPS and crude C-EPS proved to be more efficient in increasing dewaterability than crude S-EPS. Dewaterability was improved by 36.43% in case of B-EPS from *Bacillus* sp.7 which was the highest among all. Crude C-EPS from most of the *Bacillus* spp. were able to improve dewaterability of about 30%, except in case of *Bacillus* sp.1. Enhancement in the dewaterability observed in the kaolin suspension was due to the reduction in the charge density by supplied Ca^{2+}, leading to inter-particle bridging between kaolin particles and subsequent adsorption and interparticle bridging induced by supplied different forms of EPS as also noted by other studies (Bruus et al., 1992; Higgins and Novak, 1997b). Enhanced flocculation influences particles size distribution of the suspension, binding small particles together and hence improving the dewatering characteristics (Houghton et al., 2001).

Very low concentration of B-EPS (1.12-2.70 mg EPS/g SS, here SS represents target kaolin suspension concentration of 5 g/L) for kaolin flocculation was required to attain high flocculation activity (>75%), whereas EPS produced in the synthetic media by same bacterial strains required 250-2500 mg EPS/g SS to attain similar flocculation activity (Subramanian et al., 2010). Houghton et al. (2001) also reported that for each type of sludge examined, the optimum concentration of EPS (digested sludge 10 mg EPS/g SS, raw sludge (primary or mixed sludge with unknown ratio of primary and secondary sludge) 20 mg EPS/g SS and activated sludge 35 mg EPS/g SS, here SS represents target sludge solids concentrations), could exhibit maximum dewaterability, respectively. Further work on optimal EPS for maximum flocculation activity is warranted. This study revealed that EPS produced in the sterilized sludge were effective in the flocculation as compared with the EPS produced in the synthetic media, although, method and conditions used in the flocculation tests such as mixing condition, pH, temperature and sequence of addition of EPS and cations to the kaolin suspension were different (Subramanian et al., 2010).

The nature of EPS was important in the bioflocculation. Quantities as well as nature of the EPS produced by the bacteria in the synthetic medium were different to the EPS produced in sludge,

because bacterial capacity to express EPS varies with growth medium (Wingender et al., 1999). Carbohydrate/Proteins ratios of EPS produced in synthetic medium were 3.5, 1.46, and 1.88 for *Bacillus* sp.5, *Serratia* sp.1 and *Yersinia* sp.1, respectively; whereas these ratios for EPS produced in sterilized sludge were 0.58, 2.93 and 3.37, respectively. Different studies have suggested that there was dominant role of either carbohydrates or proteins content present in the EPS on sludge flocculation. Carbohydrate could play an important role in flocculation through bridging mechanism between negatively charged groups from carbohydrates and divalent cations (Bruus et al., 1992), Whereas proteins could play a dominant role in flocculation (Higgins and Novak, 1997a), through hydrophobic interactions and polyvalent cation bridging which increases the floc binding strength and hence by enhancing the stability of the biopolymer network. However, the present study could not establish clear relation between carbohydrate to protein ratio (Carbohydrate/Proteins) ratio and flocculation activity (Table 3.1.1).

4 Conclusions

BIOLOG revealed that most of the *Bacillus*, *Serratia* and *Yersinia* strains had capability to utilize wide spectrum of carbon and nitrogen sources. All the tested bacterial strains have capability to produce EPS using sterilized sludge as raw material. *Bacillus* strains produced higher concentration of EPS than *Serratia* and *Yersinia.* EPS of more than 1 g/L was produced by most of the bacterial strains. Broth EPS exhibited flocculation activity more than 75% in case of *Bacillus* sp.7, *Bacillus* sp.4 and *Bacillus* sp.6, respectively. Flocculation activity higher than 75% was attained using very low concentrations of broth EPS (1.12-2.70 mg EPS/g SS).

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Abbreviations

B-EPS	Broth EPS
BIOLOG	BIOLOG bacterial identification system
Ca-Kaolin-EPS	Calcium (Ca^{2+}), kaolin suspension in water and EPS
C-EPS	Capsular EPS
CFU	Colony forming unit
CST	Capillary suction time
EPS	Extracellular polymeric substances
GN/GP	Gram's negative/Gram's positive
INRS-ETE	Institut national de la recherche scientifique, Centre Eau, Terre et Environnement
Kaolin control	Kaolin suspension in deionized water
NTU	Nephelometric Turbidity Units
P (AM-DMC)	poly(acrylamide[2-Methacryloyloxy)ethyl]trimethylammonium chloride
PAC	Polyaluminium chloride
PAM	Polyacrylamide
rpm	Revolutions per minute
S-EPS	Slime EPS
sp.	One species
spp.	Two or more species
TS	Total solids
TSA	Tryptic soy agar
TSS	Total suspended solids
VS	Volatile solids
VSS	Volatile suspended solids

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Table 3.1.1 Identification of the bacterial strains and their EPS concentrations produced using sterilized sludge as raw material

Isolate	Identification by 16S rDNA sequence (Subramanian et al. 2010)	BIOLOG Genus ID	*Viscosity of broth (72 h fermentation) (mPas)	**Increase in broth viscosity (%)	S-EPS (g/L)	C-EPS (g/L)	B-EPS (g/L)	Carbohydrate to Protein to ratio in the EPS
Bacillus sp.1	*Bacillus* strain BS3 (Accession NO: EU031753)	*Bacillus*	4.96±0.049	2.92	0.440	0.264	0.704	2.18
Bacillus sp.2	*Bacillus* strain BS4 (Accession NO: EU031754)	*Bacillus*	5.86±0.085	20.48	0.840	0.437	1.277	0.91
Bacillus sp.3	*Bacillus* strain BS5 (Accession NO: EU031755)	*Bacillus*	5.97±0.067	21.94	1.032	0.657	1.689	1.16
Bacillus sp.4	*Bacillus* strain BS6 (Accession NO: EU031756)	*Bacillus*	5.86±0.031	20.48	0.862	0.427	1.289	0.97
Bacillus sp.5	*Bacillus* strain BS9 (Accession NO: EU031759)	*Bacillus*	5.71±0.094	18.39	0.784	0.457	1.241	0.58
Bacillus sp.6	*Bacillus* strain BS10 (Accession NO: EU031760)	*Bacillus*	5.92±0.085	21.28	0.942	0.516	1.458	0.98
Bacillus sp.7	*Bacillus* strain BS14 (Accession NO: EU031764)	*Bacillus*	5.80±0.076	19.66	0.926	0.636	1.562	0.89
Bacillus sp.8	*Bacillus* strain BS17, (Accession NO: EU031767)	*Bacillus*	5.86±0.055	20.48	1.038	0.612	1.650	0.95
Bacillus sp.9	*Bacillus* strain. BS19 (Accession NO: EU031769)	*Bacillus*	5.70±0.139	18.25	0.970	0.268	1.238	2.00
Serratia sp.1	*Serratia* strain BS8 (Accession NO: EU031758)	*Serratia*	5.70±0.007	18.25	0.942	0.575	1.517	2.93
Serratia sp.2	*Serratia* strain BS18 (Accession NO: EU031768)	*Serratia*	5.67±0.420	17.81	0.648	0.329	0.977	3.37
Yersinia sp.1	*Yersinia* strain BS11 (Accession NO: EU031761)	*Yersinia*	5.11±0.058	8.81	0.750	0.427	1.177	7.76
Yersinia sp.2	*Yersinia* strain BS13 (Accession NO: EU031763)	*Yersinia*	4.96±0.049	6.05	0.490	0.304	0.794	3.00

Note: *The control viscosity i.e., viscosity of the sterilized sludge before inoculation was 4.66 mPas.

**The initial viable cell count 3×10^6 CFU.

Table 3.1.2 Compounds which serve as sole carbon sources for EPS producing bacterial strains

Carbon sources	*Bacillus* sp.1	*Bacillus* sp.2	*Bacillus* sp.3	*Bacillus* sp.4	*Bacillus* sp.5	*Bacillus* sp.6	*Bacillus* sp.7	*Bacillus* sp.8	*Bacillus* sp.9	*Serratia* sp.1	*Serratia* sp.2	*Yersinia* sp.1	*Yersinia* sp.2
Dextrin	-	+	+	+	+	+	+	+	-	+	+	+	+
D-Maltose	-	+	+	+	+	+	+	+	-	+	+	+	+
D-Trehalose	-	+	+	+	+	+	+	+	-	+	+	+	+
D-Cellobiose	-	+	+	+	+	+	+	+	-	+	+	+	+
Gentiobiose	-	+	+	+	+	+	+	+	-	+	+	+	+
Sucrose	-	+	+	+	+	+	+	+	-	-	-	+	+
D-Turanose	-	+	+	+	+	+	+	+	-	-	+	-	-
Stachyose	-	-	+	-	+	+	+	+	-	+	+	-	-
D-Raffinose	-	+	+	+	+	+	+	+	-	+	+	-	-
α-D-Lactose	-	-	+	-	+	+	+	+	-	+	+	-	-
D-Melinoise	-	+	+	+	+	+	+	+	-	+	+	+	+
β-Methyl-D-Glucoside	-	+	+	+	+	+	+	+	-	+	+	+	+
D-Salicin	-	+	+	+	+	+	+	+	-	+	+	+	+
N-Acetyl-D-Glucosamine	-	+	+	+	+	+	+	+	-	+	+	+	+
N-Acetyl- β-D-Mannosamine	-	-	+	+	+	+	+	+	-	+	+	-	+
N-Acetyl-D-Galactoseamine	-	-	-	-	+	-	-	+	-	+	+	+	+
N-Acetyl Neuraminic Acid	-	-	-	-	-	+	-	-	-	-	-	-	-
α-D-Glucose	-	+	+	+	+	+	+	+	-	+	+	+	+
D-Mannose	-	+	+	+	+	+	+	+	-	+	+	+	+
D-Fructose	-	+	+	+	+	+	+	+	-	+	+	+	+
D-Galactose	-	-	+	+	+	+	-	+	-	+	+	+	+
3-Methyl Glucose	-	-	+	-	-	+	-	+	-	+	+	-	-
D-Fucose	-	-	+	+	+	-	+	+	-	+	+	-	-
L-Fucose	-	-	+	+	+	-	+	+	+	+	+	+	+
L-Rhamnose	-	+	+	+	+	-	+	+	-	-	-	-	+
Inosine	-	-	+	+	+	-	+	+	-	+	+	+	+
Pectin	-	+	+	+	+	+	+	+	-	+	+	+	+
D-Galacturonic Acid	-	+	+	+	-	-	+	+	-	+	+	+	+
L-Galactonic Acid Lactone	-	-	-	-	-	-	-	-	-	-	+	-	-
D-Gluconic Acid	-	+	+	+	+	+	+	+	-	+	+	+	+

Carbon sources	*Bacillus* sp.1	*Bacillus* sp.2	*Bacillus* sp.3	*Bacillus* sp.4	*Bacillus* sp.5	*Bacillus* sp.6	*Bacillus* sp.7	*Bacillus* sp.8	*Bacillus* sp.9	*Serratia* sp.1	*Serratia* sp.2	*Yersinia* sp.1	*Yersinia* sp.2
Glucuronic Acid	-	+	+	+	-	-	+	+	-	+	+	+	+
Glucuronamide	-	-	-	-	-	-	-	+	-	-	+	-	-
Mucic Acid	-	+	+	+	-	-	+	+	-	-	-	-	+
Quinic Acid	-	-	-	-	+	-	-	-	-	-	-	-	-
D-Saccharic Acid	-	+	+	+	-	-	+	+	-	+	+	-	-
P-Hydroxy-Phenylacetic Acid	-	-	-	-	-	-	-	-	-	+	+	-	-
Methyl Pyuvate	-	+	+	+	+	+	+	+	-	-	+	-	+
D-Lactic Acid Methyl Ester	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Lactic Acid	+	+	+	+	+	+	+	+	-	+	+	-	-
Citric Acid	-	+	+	+	+	-	+	+	-	+	+	-	+
α-Keto-Glutaric Acid	-	-	-	-	-	-	-	-	-	+	+	-	-
D-Malic Acid	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Malic Acid	+	+	+	+	+	+	+	+	-	+	+	+	+
Bromo-Succinic Acid	-	-	+	+	+	+	+	+	-	+	+	+	+
Tween 40	-	+	+	+	+	+	+	+	-	+	+	-	-
γ-Amino-Butyric Acid	-	+	+	-	+	-	+	+	-	-	-	-	-
α-Hydroxy Butyric Acid	-	-	-	-	-	-	-	+	-	-	-	-	-
β-Hydroxy-D,L-Butyric Acid	-	-	-	-	-	-	-	-	-	-	-	+	-
α-Keto-Butyric Acid	-	-	-	-	-	-	-	-	-	-	-	-	-
Acetoacetic Acid	+	+	+	+	+	+	+	+	-	-	-	-	-
Propionic Acid	-	-	-	-	-	-	-	+	-	-	-	-	-
Acetic Acid	+	+	+	+	+	-	+	+	-	+	+	+	+
Formic Acid	+	+	+	+	-	-	+	-	-	+	+	+	-

Note: + growth; - no growth

Table 3.1.3 Compounds which serve as sole nitrogen sources for EPS producing bacterial strains

Nitrogen sources	*Bacillus* sp.1	*Bacillus* sp.2	*Bacillus* sp.3	*Bacillus* sp.4	*Bacillus* sp.5	*Bacillus* sp.6	*Bacillus* sp.7	*Bacillus* sp.8	*Bacillus* sp.9	*Serratia* sp.1	*Serratia* sp.2	*Yersinia* sp.1	*Yersinia* sp.2
Gelatin	-	+	+	+	+	+	+	+	-	-	-	-	-
Glycyl-L-Proline	-	-	-	+	-	-	-	+	-	+	+	+	+
L-Alanine	-	+	+	+	+	+	+	+	-	+	+	+	+
L-Arginine	-	+	+	+	+	+	+	+	-	-	-	-	-
L-Aspartic Acid	+	+	+	+	+	+	+	+	-	+	+	+	+
L-Glutamic Acid	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Histidine	+	+	+	+	+	+	+	-	-	+	+	+	+
L-Pyroglutammic Acid	-	+	+	+	-	-	-	+	-	-	-	-	-
L-Serine	-	+	+	+	+	-	+	+	-	+	+	+	+
D-Aspartic Acid	-	-	-	-	+	-	-	+	-	-	-	-	-
D-Serine	-	-	-	-	-	-	-	-	-	+	+	+	+

Note: + growth; **-** no growth

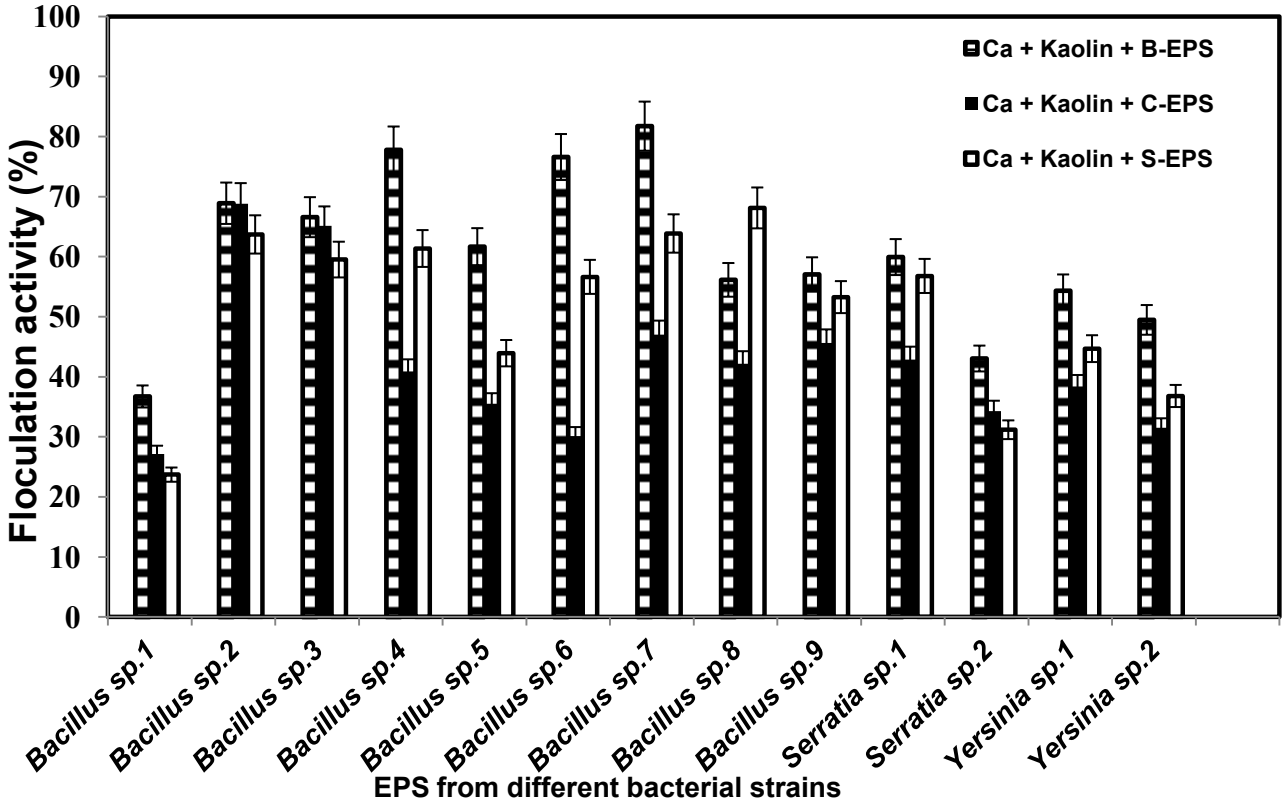

Figure 3.1.1 Variations in the kaolin flocculation activity due to addition of Ca^{2+} followed by different forms of the EPS produced in the sludge by different bacterial strains

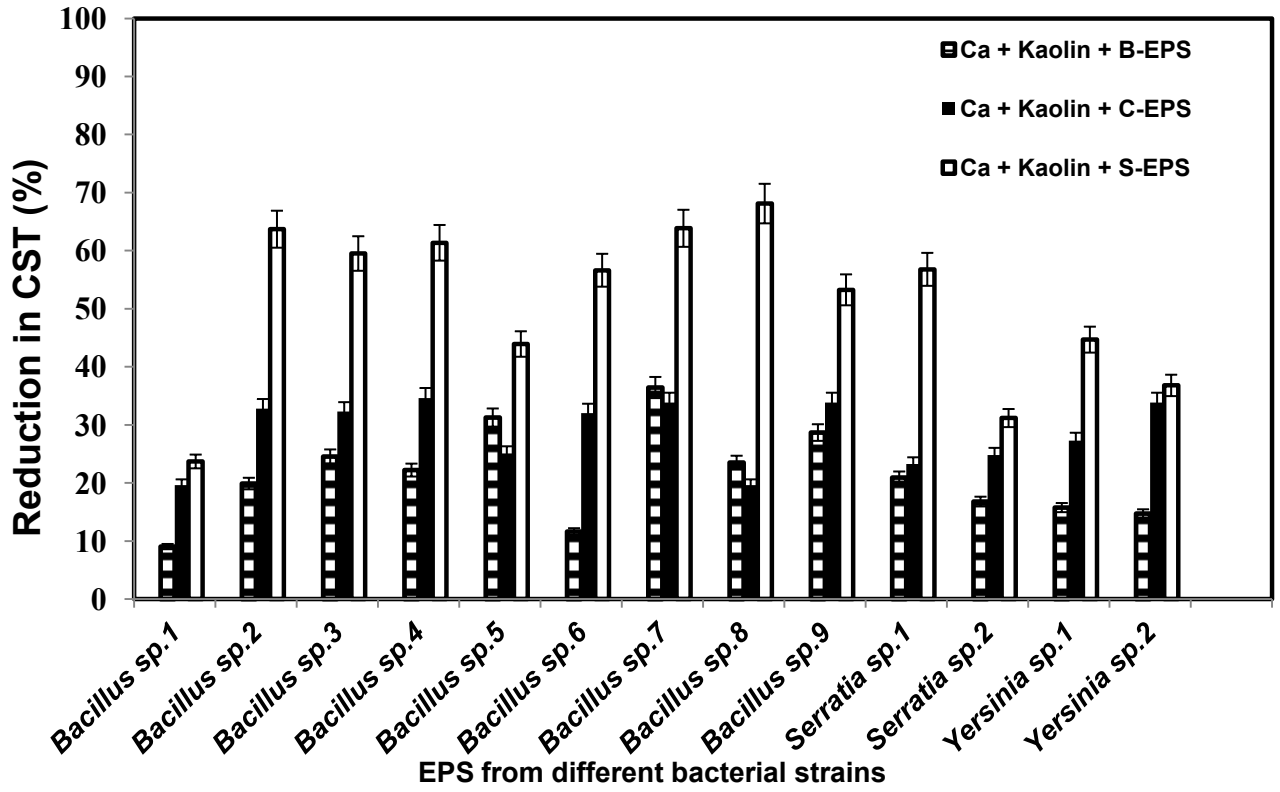

Figure 3.1.2 Reduction in the CST values of the kaolin suspension due to addition of Ca^{2+} followed by different forms of the EPS produced in the sludge using different bacterial strains

PARTIE 2

EXTRACELLULAR POLYMERIC SUBSTANCES (EPS) PRODUCTION KINETICS OF THIRTEEN SLUDGE ISOLATES USING WASTEWATER SLUDGE AS RAW MATERIAL AND ITS FLOCCULATION POTENTIAL

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RÉSUMÉ

La cinétique de fermentation discontinue de treize souches bactériennes productrices des substances polymériques extracellulaires (SPE) (9 *Bacillus*, 2 *Serratia* et 2 *Yersinia*) a été effectuée en utilisant des boues stérilisées comme matière première. Il a été montré que la plupart des *Bacillus* (μmax: 0,11 à 0,27 h^{-1}): et *Yersinia* (μmax: 18 à 0,19 h^{-1}), les souches de *Serratia* (0,23-0,27 h^{-1} μmax) ont la capacité de se développer et de produire des SPE (de 1,36 à 2,12 g/L) dans les boues stérilisées. En général, la production de SPE était partiellement liée à la croissance bactérienne pour les différentes souches cultivées de façon indépendante. *Bacillus* sp. 7, *Serratia* sp. 2 et 2 *Yersinia* sp. produisent une concentration plus élevée (1,95 à 2,12 g/L) de SPE que les autres souches bactériennes. Les teneurs en protéines et en glucides contenues dans les SPE restent constantes pendant la fermentation. Les B-SPE présentaient une forte activité de floculation du kaolin (≥ 75%) dans la plupart des cas, sauf pour *Bacillus* sp. 1, *Bacillus* sp. 5 et *Bacillus* sp. 9, respectivement. En général, les meilleures activités de floculation (≥ 75%), ont été atteintes en utilisant 1,31 à 1,70 mg B-SPE/g de kaolin, de 0,45 à 0,97 mg de protéine/g et 0,11 à 0,21 mg de glucides/g de kaolin. L'étude suggère, q'une exploration systématique est nécessaire pour optimiser le processus de production des SPE. En effet, Les SPE produites dans les boues peuvent potentiellement être utilisées pour l'eau et pour le traitement des eaux usées.

Mots clés: les substances polymères extracellulaires; Le taux de croissance; la cinétique; production; Les boues d'épuration.

ABSTRACT

The kinetics of batch fermentation of thirteen extracellular polymeric substances (EPS) producing bacterial strains (9 *Bacillus*, 2 *Serratia* and 2 *Yersinia*) were carried out using sterilized sludge as a raw material. The most of *Bacillus* (μ_{max}: 0.11-0.27 h^{-1}), *Serratia* (μ_{max}: 0.23-0.27 h^{-1}) and *Yersinia* (μ_{max}: 0.18-0.19 h^{-1}) strains had capability to grow and produce EPS (1.36-2.12 g/L) in sterilized sludge. In general, EPS production was mixed growth associated for all the bacterial strains cultivated independently. *Bacillus* sp. 7, *Serratia* sp. 2 and *Yersinia* sp. 2 produced higher concentration (1.95-2.12 g/L) of EPS than the other remaining bacterial strains. Protein and carbohydrate contents of EPS remained constant during fermentation. Broth EPS exhibited high kaolin flocculation activity (≥ 75%) in most of the cases except *Bacillus* sp. 1, *Bacillus* sp. 5 and *Bacillus* sp. 9, respectively. In general, high flocculation activities (≥ 75%), were attained using 1.31-1.70 mg B-EPS/g kaolin, 0.45-0.97 mg protein/g kaolin and 0.11-0.21 mg carbohydrates/g kaolin. The study suggests, further systematic exploration is required for optimizing the process of EPS production. EPS produced in the sludge can potentially be used for different water and wastewater treatment.

Key words: Extracellular polymeric substances; Growth rate; Kinetics; Production; Wastewater sludge.

1 Introduction

Biopolymers produced by microorganisms represent the novel approach for the development of environmental friendly and sustainable alternatives to the conventional chemical polymers [1, 2]. Biopolymers production in the form of extracellular polymeric substances (EPS) is a general property of several bacterial species in natural environments. Recently, there is a surge in the research in producing EPS by various bacterial strains using a variety of production media [3-7]. Relatively lower flocculation efficiency and high costs of production are the major restrictions on development of EPS as flocculant. The production of EPS reported to date is mostly performed on synthetic media or costly carbon sources such as glucose or sucrose [8, 9]. A variety of non-expensive substrates or raw materials were used in previous investigations such as brewery wastewater sludge [3], dairy industry wastewater [10] and glycerol waste [7]. In the recent studies it was found that wastewater sludge could be used as raw material for EPS production using several bacterial strains isolated from wastewater sludge [11, 12]. The wastewater sludge is a potentially economical culture medium as it is a rich source of carbon, nitrogen, phosphorus and other nutrients. Moreover, the use of wastewater sludge will be advantageous for sludge isolated microorganisms cultivation, which is already well adapted to it. However, in the earlier study time dependent EPS production during fermentation was not investigated [11, 12], which is a decisive factor in economic aspects of the production process.

On the other hand, there are different views on correlation of EPS production with microbial growth. According to these views, EPS production occurs either during the endogenous phase of a batch culture, or at the beginning of the stationary growth phase, or parallel to cell growth, or from the middle of the exponential growth phase, or simultaneously during the early and middle of the exponential growth phases, or increases with exponential phase and decreases during stationary phase [13]. EPS production is considered as growth-synonymous, growth-associated or growth-independent [14]. Most of these studies related to EPS production and cell growth have been carried out using either simple substrates or synthetic media [7, 15]. Knowledge of kinetic parameters of EPS synthesis in wastewater sludge is needed to enhance EPS production and to obtain a better understanding of the mechanisms involved in the EPS synthesis [16]. However, till now there is no report on kinetics of EPS synthesis by bacterial strains using wastewater sludge as a raw material. Therefore, to utilize wastewater sludge as a raw material for EPS production, systematic lab scale investigation on time dependent increase in the microbial population and EPS concentration is warranted. The analysis of this kind might

lead to an efficient production of EPS with novel, desired properties. Indeed, it is important for the application of EPS in environmental pollution control activities to investigate the process of EPS biosynthesis during the fermentation process. The understanding of kinetic parameters is also important for the stable production of high amounts of EPS at large scale.

Therefore, an investigation of the consequences of microbial growth on EPS production during lab scale batch fermentation was carried out in this study. Thirteen sludge isolates were cultivated in sterilized sludge (as raw material) to determine the correlation between cells growth rate, EPS concentration, characteristics and flocculation activity during batch fermentation.

2 Materials and methods

2.1 Microorganism

Thirteen EPS producing bacterial strains (9 *Bacillus*, 2 *Serratia* and 2 *Yersinia*) isolated from the wastewater sludge were used in this study [12]. All the strains were cultivated individually on tryptic soy agar plates (TSA) and stored at 4^{o}C and sub-cultured fortnightly.

2.2 Wastewater sludge as a growth medium

Secondary wastewater sludge (without chemical polymers) was collected from Municipal wastewater treatment plant (Québec city, Canada). Sludge was first settled by gravity for 1 h and concentrated sludge was collected by discarding the supernatant. Characteristics of sludge, such as pH, total solids (TS), suspended solids (SS) and volatile suspended solids (VSS) were determined using Standard Methods [17]. The wastewater sludge characteristics used in the experiments are shown in Table 3.2.1. The sludge was stored at 4^{o}C for further use.

2.3 Sludge pre-treatment

The raw sludge samples were sterilized by autoclaving (steam sterilization) at 121^{o}C for 15 min. EPS production at pH 7.0 was the highest therefore, after sterilization, the sludge samples were cooled to room temperature and adjusted to pH 7.0 using 1 M NaOH [6]. The pre-treated sludge samples (without any nutrient supplementation) were used as raw material for cultivation and EPS production.

2.4 EPS production

The pre-culture was prepared in two steps as per the previous study [11]. In the first step, each microbial strains was aseptically (and individually) transferred from TSA plates to 500 mL Erlenmeyer containing 100 mL of tryptic soy broth (TSB) and then incubated at 30^{o}C under an agitation rate of 200 rpm for 24 h. In the second step, the TSB grown culture was inoculated (3% v/v) in the sterilized sludge (100 mL) and incubated at 30^{o}C and 200 rpm for 24 h in an orbital shaking incubator. This is an important step in inoculums production as it allows the strains to adapt to their new complex substrate. This pre-culture was inoculated (3% v/v) in the sterilized sludge medium (150 mL) contained in a 500 mL Erlenmeyer flask and incubated at 30^{o}C and 200 rpm, for 72 h. To study the time course of EPS production, broth samples (2 mL) were withdrawn at each 6 h for total cells counts, then EPS from broths were harvested from the samples at 12 h interval throughout the fermentation process. For each strain, a total dedicated six number of fermentation flasks were inoculated. Each flask broth was harvested at 12 h intervals in order to ensure an appropriate amount of sample for EPS harvesting and analyses. Moreover, this methodology allows checking the reproducibility of the fermentation process.

2.5 EPS harvesting

To harvest EPS, broth samples (50 mL) were centrifuged at 6000g for 15 min at 4^{o}C to obtain supernatant (crude slime EPS or crude S-EPS) and pellets (crude capsular EPS or crude C-EPS) with bacterial cells along with residual sludge material [11]. To extract the S-EPS, the crude S-EPS was precipitated by adding 1:2.2 volumes of absolute chilled ethanol followed by storing the mixture at -20^{o}C overnight. The precipitates were collected by centrifugation at 6000g for 15 min at 4^{o}C. S-EPS precipitates were heated at 105^{o}C for 24 h and dry weights obtained were represented as S-EPS concentration [17]. For protein and carbohydrate analysis, precipitates of S-EPS (before heat drying) were re-suspended in deionised water (to initial broth volume). To determine C-EPS concentration, the crude C-EPS was re-suspended in deionized water (to initial broth volume). The re-suspended crude C-EPS were heated at 60^{o}C in water bath (with shaking 30 rpm) for 30 min to release C-EPS then centrifugated at 6000g, 4^{o}C for 15 min [11]. The supernatant (containing C-EPS) was used to precipitate C-EPS with absolute chilled ethanol. Dry weight content of protein and carbohydrates of C-EPS was measured using the same procedure as for S-EPS. Sum of dry weights of S-EPS and C-EPS (measured above) was denoted as broth EPS (B-EPS).

2.6 Kinetic parameters

The specific growth rates were calculated as the slope of semilog plot of the cell number versus cultivation time during exponential phase. In batch culture assays, the maximum specific growth rate (μ_{max}) was experimentally determined in the exponential growth phase as per equation: $\mu_{max} = [\ln(X_0/X_1)]/(t_1-t_0)$, where, X_0 and X_1 are the numbers of CFU/mL and t_0 and t_1 are the times along the exponential phase, respectively. Volumetric productivities were graphically estimated by mean of differentiation of EPS produced (g/L) per unit time (1/h). Specific EPS production rates (q_{EPS}; g EPS/million cells/h) were determined as per equation: $q_{EPS} = 1/N(dEPS/dt)$, where *N* is the microbial population as a multiple of million cells, *dEPS/dt* are the volumetric productivities.

2.7 Kaolin flocculation tests

Jar tests (PB-700 Standard Jar Testers, Phipps & Bird, DARCO international inc) were carried out to investigate flocculation capabilities of EPS produced by the thirteen bacterial strains at different incubation time (12, 24, 36, 48, 60 and 72 h), respectively. Kaolin clay (K2-500, USP, Fisher scientific, US) suspensions (5 g/L in deionized water) were used. During jar tests, first rapid mixing of suspensions was carried out at 175 rpm for 3 min to allow homogeneous dispersion of solids and to foster solids interactions. During the rapid mixing (at 175 rpm), 150 mg Ca^{2+}/L kaolin solution was added followed by addition of EPS. Addition of calcium ions was necessary to for the kaolin surface charge neutralization and to initiate coagulation [11]. EPS as broth (crude B-EPS, 2 mL) were employed in the jar tests unless and otherwise stated. Calcium stock solution was prepared by dissolving equivalent quantity of anhydrous $CaCl_2$ (solid pellets) in deionised water to make final concentration of 25 g of Ca^{2+}/L). After rapid mixing, pH of the suspension was adjusted to 7.5 and slow mixing at 75 rpm was allowed for 30 min to ensure flocculation. The pH of the suspensions was adjusted to 7.5 because at slightly alkaline condition, highest kaolin flocculation activity using calcium was observed. After mixing, the samples were transferred to measuring cylinders of 500 mL for 30 min settling. After settling, supernatant and sediments of the samples were used to measure flocculation activity (FA) and capillary-suction-time (CST), respectively. All the tests were carried out in triplicates and the means were presented (with standard error less than 5% of the mean).

2.8 Analysis

Characteristics of sludge, such as pH, total solids (TS), suspended solids (SS), volatile solids (VSS), chemical oxygen demand (COD), and turbidity (NTU) were determined using Standard Methods [17]. Viscosity (mPas) and zeta potentials (ζ) were measured using Viscometer (DV DV-II PRO + (Brookfield Engineering Laboratories, Inc., Stoughton, MA, USA)), elemental analysis (CHNS-932 and TOC analyser) and Zetaphoremeter (Zetaphoremeter IV, Zetacompact Z8000, CAD Instrumentation, France), respectively. Measurement of cells concentration as colony forming units (CFU) was carried out by standard agar-plate technique. The protein content of the extracted S-EPS was determined using bovine serum albumin as a standard [18]; the carbohydrate content of the extracted C-EPS was determined by the phenol-sulfuric acid method using glucose as the standard solution [19]. To determine flocculation activity or FA, turbidity of the samples supernatant was measured using Micro 100 Turbidity meter, Hach Company. FA measure was based on the relative decrease in turbidity of suspension after settling [11]. FA was calculated according to the equation: FA = (1-*S/C*) x100 (%), where *C* is control turbidity and *S* is sample turbidity. Kaolin suspension without EPS was the control. For quantifying the effects of EPS on dewaterability, Capillary-suction-time (CST) of the settled kaolin sediments were measured. CST was determined by the CST instrument (Triton electronics, model 304 M CST, Dunmow, Essex), using a 10-mm diameter reservoir. Lower CST value as compared to the control CST indicated better dewaterability [11]. The sediments from jar test samples without EPS addition was the control. The increase in the dewaterability was calculated by equation: increase in dewaterability (%) = {(control CST) – (sample CST)} / (control CST) x100.

3 Results and discussion

3.1 The bacterial growth

Batch fermentations of all the thirteen bacterial strains, individually cultivated in sterilized sludge, were carried out for 72 h. The total cells concentrations measured at different incubation time are presented in Figure 3.2.1. All the bacterial strains revealed typical sigmoid batch culture growth curve in sterilized sludge. Cells growth profiles of *Bacillus* sp. 1, *Bacillus* sp. 2, *Serratia* sp. 2 and *Yersinia* sp. 2 did not show any occurrence of apparent lag phase and started directly their growth in exponential phase. This indicated that these cultures were well adapted

to the growth in sterilized sludge. On the other hand, remaining bacterial strains (*Bacillus* sp. 3, *Bacillus* sp. 4, *Bacillus* sp. 5, *Bacillus* sp. 6, *Bacillus* sp. 7, *Bacillus* sp. 8, *Bacillus* sp. 9, *Serratia* sp. 1 and *Yersinia* sp. 1) showed initial lag phases of 3-6 h before starting exponential growth. This phenomenon was most likely due to a growth cessation affecting a fraction of the freshly inoculated cells until the culture was completely adapted to the new fermentation environment, and consequently increased their specific growth rate. In case of *Bacillus* sp. 3, *Bacillus* sp. 7 and *Serratia* sp. 1 (Figures 3.2.1c, 1g, 1j), the occurrence of a true lag phase was observed during the first 3-6 h of cultivation. During this phase, cell growth was completely stopped and resulted in a decrease of the total cell count. In this study, despite the fact that inoculum was prepared in sterilized sludge under similar conditions, the studied bacterial strains had different response to the substrates available in the culture medium, which resulted in the initial lag of some cultures. The appearance of a latency phase was most likely due to the effect of changes in nutrients, physical environment or a combination of both. Therefore, the cells required time to adapt to their new culture medium by inducing the appropriate metabolic pathways to degrade the available carbon and energy sources [20]. The initial lag phase could be avoided by optimizing the age of inoculum or by enriching the inoculum preparation medium.

Most of the bacterial strains attained maximum cells concentration (8.32×10^7 - 2.90×10^9 CFU/mL) at 60-72 h of fermentation. However, *Bacillus* sp. 1 and *Yersinia* sp. 2 (Figures 3.2.1a, 1m) attained the maximum cells concentration (3.73×10^7 and 2.30×10^9 CFU/mL) at 48 and 36 h, respectively. During fermentation, the cells concentrations were increased by two to three log cycles in case of *Bacillus* sp. 2, *Bacillus* sp. 3, *Bacillus* sp. 4, *Bacillus* sp. 6, *Bacillus* sp. 7, *Bacillus* sp. 8, *Serratia* sp. 1, *Serratia* sp. 2, *Yersinia* sp. 1 and *Yersinia* sp. 2 (Figures 3.2.1b-1d, 1f-1h and 1j-1m). Whereas in case of *Bacillus* sp. 1, *Bacillus* sp. 5 and *Bacillus* sp. 9, the increase in cells concentrations were less than 2 log cycles. In general, the pattern of cells growth is directly related to the metabolic characteristics of the bacterial strains. In the earlier study [12], the biochemical profile based on BIOLOG system had demonstrated that all the bacterial strains used in this study had different patterns of carbon and nitrogen source utilization under similar incubation conditions. Therefore, this explains the different growth patterns in sterilized sludge observed for the studied strains under similar incubation conditions. Also, due to a high level of complexity of the substrate composition, distinct metabolic responses for each bacterial strain could be observed during batch cultivation. Relatively lower cell concentrations observed in case of *Bacillus* sp. 1 and *Bacillus* sp. 9 were probably due to their limited metabolic competence i.e., able to utilize only few nutrients available in the

sterilized sludge [12]. The depletion of their exclusive assimilable compounds and their inability to shift to the other available substrates induced a precocious growth cessation.

The experimental data for bacterial growth were analysed to determine the maximum specific growth rates of the studied strains cultivated in sterilized sludge (Table 3.2.2). The analysis of kinetic parameter estimations showed that the bacterial strains revealed distinct specific growth rates ranging from 0.11 to 0.27 h^{-1}. Among all the strains, *Serratia* sp. 1, *Bacillus* sp. 3 and *Bacillus* sp. 7 revealed relatively higher specific growth rates of 0.27, 0.27 and 0.25 h^{-1}, respectively. In contrast, *Bacillus* sp. 1 revealed relatively lower specific growth rate (0.11 h^{-1}). The most of *Bacillus* strains had growth rates lower than the *Serratia* and *Yersinia* strains. As discussed earlier, the metabolic characteristics of these strains might be responsible for the lower growth rates. Knowledge of the bacterial specific growth rates in batch culture environment is essential for operating and evaluating the state of the process and also to simulate the growth of the bacterial population that converts sterilized sludge to EPS and bacterial biomass.

The differences in cell growth patterns and specific growth rates observed among the studied bacterial strains, and moreover among the same species, would be directly related to the metabolic capacity of each microbial strain to assimilate several nutrients in the culture medium. In fact, bacterial strains such as *Bacillus* sp. 1 and 9 showed very low maximal cell concentrations and specific growth rates as compared to *Bacillus* sp. 7 and *Serratia* sp. 1. These latter had metabolic capacities of wide spectra of C and N-sources utilization, whereas *Bacillus* sp. 1 and 9 had preference to assimilate a very few compounds as per their biochemical profiles performed by BIOLOG system [12]. It is believed that this polyvalence in C and N-sources assimilation capacities give place to the occurrence of a diauxic growth pattern that would characterize the strains where a high cell concentration was reached. Indeed, wastewater sludge is a complex mixture of different organic matters and therefore, there is possibility of metabolic shift due to the depletion of certain preferred carbon/nitrogen sources or other nutrients. Hydrolytic enzyme activities are also of higher interest in the case of sterilized sludge medium. Indeed, wider panoply of the hydrolytic enzymes for a strain, will lead to its higher ability to uptake complex insoluble matters (such as proteins, polysaccharides or fibres) would increase.

3.2 EPS production

The concentrations of EPS synthesized by different bacterial strains in sterilized sludge at different incubation time are presented in Figure 3.2.1. Maximum B-EPS concentration (1.363-2.125 g/L) was observed at 60-72 h incubation for most of the bacterial strains except *Bacillus* sp. 1 and *Yersinia* sp. 2. In case of *Bacillus* sp. 1 and *Yersinia* sp. 2, maximum EPS concentration (1.363 and 1.950 g/L) was observed at 48 and 36 h of incubation, respectively. EPS productivities of the bacterial strains are presented in Table 3.2.2. *Bacillus* sp. 7, *Serratia* sp. 2 and *Yersinia* sp. 2 revealed higher productivities (0.021, 0.023 and 0.036 g/L/h, respectively). EPS productivities (0.008-0.036 g/L/h) observed in this study are within the range of productivity values of xanthan production (0.040-0.440 g/L/h) [21] and also comparable to the productivity values reported for bacterial alginate (0.014 g/L/h)[22]. However, the EPS productivity values are relatively lower than those reported for xanthan production (0.130-0.510 g/L/h) [23] and gellan gum production (0.160-0.480 g/L/h) [14].

A decrease in EPS (all forms) concentration was observed after attaining maximum EPS concentrations at respective incubation times for most of the bacterial strains (Figure 3.2.1). According to Figure 3.2.1, the EPS production started during exponential growth phase of the cells and continued up to stationary phase. The bacterial strains used in this study have a wide range of EPS productivities even though they were cultivated under similar conditions. Batch mode fermentations are characterized by a continuous change in the physicochemical composition of the medium as well as in the physiological state of the growing cells. Over time, the culture medium is depleted from essential nutrients required to ensure adequate microbial growth, especially C and N-sources. Consequently, cells turn to their reserve materials, stored among others in the form of EPS, to hydrolyse them and use as C and N-sources required for their growth and maintenance. This metabolic shift depends strongly on the metabolic properties and the enzymatic baggage (capability of certain microbial strain to express certain enzymes) of each bacterial species [12, 24]. Therefore, longer incubation times might result in a decrease in EPS content due to a bacterial utilization of produced EPS to overcome nutrient starvation.

In all the cases, slime EPS concentration (S-EPS) was higher (2.5-7.3 times) than the capsular EPS (C-EPS). The S-EPS concentrations had followed the trend of B-EPS at different incubation time (Figure 3.2.1). *Bacillus* sp. 7 had produced highest S-EPS concentration (1.662 g/L) as compared with rest of the bacterial strains (1.104-1.572 g/L). *Yersinia* sp. 2, *Bacillus* sp. 7 and *Serratia* sp. 2 had produced higher C-EPS (0.525, 0.580 and 0.600 g/L) as compared with

rest of the bacterial strains (0.215-0.354 g/L). Most of the *Bacillus* strains had produced lower C-EPS concentrations as compared to *Serratia* and *Yersinia* strains.

Sterilized sludge used as a substrate for these cultivations is a complex medium that contains several compounds serving as potential nutrients (C and N-sources, oligoelements, growth factors etc.) to support bacterial growth. Therefore, it was difficult to ascertain complete kinetic characterization because of the high degree of substrate complexity and the difficulty to quantify the potential nutrients contained in it. The estimated kinetic parameters of EPS production are presented in Table 3.2.2. Maximum specific EPS production rates were determined as per million cells. In all the cases, specific EPS production rate and specific growth rate of the cells had linear correlation (Table 3.2.2). The y-intercepts of these correlations indicated mixed-growth associated EPS production scheme [20]. During stationary growth phase, EPS productivities were either zero or negative. At this point, it seems that EPS degradation dominated the EPS production as cells tend to uptake their required nutrients from this storage material. Moreover, non-growing cells also had been reported to contribute in EPS matrix which might be expressed in positive y-intercept observed in case of *Bacillus* sp. 1 and *Bacillus* sp. 3. Similar linear correlations between EPS production rates and biomass growth rates had been also reported [16] for the aerobic granular sludge. However, the kinetic parameters reported [16] were based upon the substrates and EPS measured in terms of COD (chemical oxygen demand) unlike the present batch scale fermentation. Therefore, kinetic constants obtained in present study could not be compared to the literature [16]. The correlation between specific EPS production and growth rate was relatively poor for some bacterial strains (Table 3.2.2). The poor correlation between specific EPS production and specific growth rate (for *Bacillus* sp. 1, 2, 6, 8, *Yersinia* sp. 1, 2) indicate that factors other than the live cells were contributing more to the EPS production. Other factors that could influence the EPS concentration are the presence of EPS producing and non EPS producing cells, the production and degradation of EPS which occurs simultaneously at varying rates during the incubation.

During fermentation, the suspended solids increased in case of all the bacterial strains (data not shown). Maximum increase in the suspended solids coincided with that of maximum cells concentration and seemed therefore correlated to it. This indicated that the increase in the suspended solids was mainly due to the growth of the bacterial strains in sterilized sludge. The bacterial strains had used nutrients available in the sterilized sludge for growth, cell maintenance and production of EPS. It is difficult to determine the exact substrate concentration present in the sterilized sludge during fermentation which could be available for bacteria. The

wastewater sludge is a complex matter composed of easily degradable, degradable, difficult to degrade and non-degradable compounds in either soluble or non-soluble forms. Therefore, the rate at which the cells utilize sludge materials (components) as a substrate is unknown or very difficult to measure simply by chemical oxygen demand (COD) or total organic compounds (TOC) degradation measurements, specifically in the present study. This is because of the sludge (soluble as well as suspended) is being used as a raw material for the EPS production (which also accumulates sludge solids or biomass) therefore; there is conversion of organic matter in one form to another form (which remains with the sludge solids and cannot be separated). The COD or TOC measurements of specific sample (incubated sludge) will simply provide the information about the exact quantity that is present in it but it does not correctly represent the amount that was used by the cells for the growth and EPS production. In the literature, kinetic studies involving mixed substrates in batch cultures are mostly restricted to the effects reported on the specific growth rate [25]. For each batch test, the increase in the suspended solids was determined as the maximum increase in the biomass concentration. In batch culture experiments, either the consumption of the growth-controlling substrate or the increase in biomass concentration can be examined as a function of time. Traditional kinetics theories are based on the assumption that a single compound is controlling the rate of microbial cells. However, in this study, the bacterial strains had the potential to utilize different carbon substrates either simultaneously or sequentially [12], depending on the solicited metabolic route and the metabolic control mechanism for each potential nutrient source.

3.3 EPS characteristics

The viscosity of the broths had increased 1.4-3.0 times during the 72 h cultivation as shown in Table 3.2.3. The most significant increase of broths viscosity was observed during 60-72 h of incubation (Table 3.2.3). The viscosity increase is attributed to increase of EPS concentration in the broth. This was due to the establishment of novel or stronger interactions which are likely to occur, either among EPS molecules, or between polymer chains and the other components of the fermented broth, contributing for the viscosity built-up [7]. The EPS matrix is generally viscous in nature and has the glue like properties which makes the broth more viscous [8].

There were significant variations in the protein and carbohydrate concentrations present in EPS synthesized by different bacterial strains at any given incubation time (Figure 3.2.2). However, it was observed that irrespective of the bacterial strain, the concentration of protein and carbohydrate present in EPS had remained constant or changed insignificantly at different

incubation time during 72 h of the fermentation. In case of *Bacillus* sp. 4 and *Bacillus* sp. 7, relatively higher protein concentrations (in S-EPS) 0.649 and 0.697 g/L were observed at 60 and 72 h of incubation, respectively (Table 3.2.4). Among all the bacterial strains, *Bacillus* sp. 1 and *Bacillus* sp. 9, revealed relatively lower protein concentrations (in S-EPS) of 0.330 and 0.276 g/L, respectively. In case of *Bacillus* sp. 4 and *Bacillus* sp. 7, relatively higher carbohydrate concentrations (in C-EPS) of 0.156 and 0.162 g/L were observed at 60 and 72 h of incubation, respectively (Table 3.2.4). S-EPS had 1.6 to 5.1 times higher protein concentrations than the C-EPS. It is widely reported that centrifuged pellet contains 80.7% (w/v) of total protein concentration present in sludge [26]. However, in present study, C-EPS were extracted from pellets by excluding cells. Therefore, large portion of proteins could have remained in excluded cells.

EPS produced by pure cultures (using same bacterial strains as this study) in synthetic medium had 1.9-3.4 times higher carbohydrates content as compared with the proteins [6]. However, in present study, Proteins concentrations was higher (3.9-4.6 and 2.7-3.3 times in S-EPS and C-EPS, respectively) than the carbohydrates in all cases (Table 3.2.4). Similarly, presence of higher protein concentrations in EPS was reported by several other workers [1, 26-28]. The concentration of protein and carbohydrates present from the EPS also depend on the type and concentrations of carbon and nitrogen sources used in growth medium [29]. Carbon to nitrogen ratio of raw sludge used in the present study was very low (8.8) and could also be the reason for higher protein content than carbohydrates in the EPS. The Ca^{2+} concentration in raw sludge was higher (4.5 times) than Na^{+} (Table 3.2.1). The higher divalent cations (calcium or magnesium) are reported to enhance lectin binding activity influencing adhesion between the substrate and microbial population and consequently enhance the biofilm formation (structural stability) and which encourages the change in metabolic dynamics by inducing EPS synthesis [30]. In general, the composition of EPS is heterogeneous and varies based on many factors such as medium composition, growth phase, extraction method and different process operation parameters (temperature, pH, agitation speed, cultivation time etc.) [9].

3.4 Flocculation activity and dewaterability

Kaolin flocculation activities (FAs) were determined for the B-EPS (for 2 mL broth volume) sampled at each incubation time and the results of highest FAs for different bacterial strains were presented in Table 3.2.5. Significant variations in the FAs (13.8-83.1%) were observed for all the cases. In general, higher FA (≥ 75%) was observed for EPS sampled at 60-72 h of

incubation time in case of *Serratia* sp. 1, *Serratia* sp. 2 and *Yersinia* sp. 1 and most of the *Bacillus* strains (*Bacillus* sp. 2, 3, 4, 7 and 8). In case of *Bacillus* sp. 1, *Bacillus* sp. 5, *Bacillus* sp. 9 and *Yersinia* sp. 2, maximum FA of 58.8, 55.2, 62.4, and 79.5% were observed for EPS sampled at 48, 60, 72 and 36 h of incubation, respectively. EPS synthesized by *Serratia* sp. 2 and *Bacillus* sp. 7 revealed the highest FA of 83.1%. FAs of B-EPS sampled at different incubation times (and produced by different bacterial cultures) were distinct mainly because of the variations in B-EPS concentration (added to kaolin suspensions) present in the 2 mL broth tested (Table 3.2.5). Optimum dosage of EPS is required for achieving maximum flocculation activity (or minimum turbidity). The addition of EPS (dose) lower or higher than the optimum value can lead to decrease in flocculation activity (or decrease in the turbidity) [11]. The variations in FAs were also attributed to the EPS components i.e., protein and carbohydrates (Table 3.2.5). FAs increased with increase in protein and carbohydrate concentrations (present in the EPS tested). In general, to achieve high FA (≥ 75%), 1.31-1.70 mg B-EPS/g kaolin, 0.45-0.96 mg protein/g kaolin and 0.11-0.21 mg carbohydrates/g kaolin were required. A relatively poor performance of B-EPS synthesized by *Bacillus* sp. 1, *Bacillus* sp. 5 and *Bacillus* sp. 9 strains was mainly due to the presence of lower EPS concentration (1.0 mg B-EPS/g kaolin) in the dose as compared to that the rest bacterial strains. *Bacillus* sp. 1 and *Bacillus* sp. 9 strains also had relatively lower protein and carbohydrate concentrations (in the added EPS dose) as compared to the rest strains. Similar to the trend in FA, significant variation in dewaterability was observed in all cases (Table 3.2.5). The increase in dewaterability was caused by the addition of B-EPS to the kaolin suspensions. Among all the bacterial strains, increases in dewaterabilities of 54.3% and 50.7% were observed for B-EPS synthesized by *Serratia* sp. 2 and *Bacillus* sp. 7, respectively. EPS synthesized by *Bacillus* sp. 1 and *Bacillus* sp. 9 revealed relatively lower (27.9 and 32.3%) dewaterability. Moreover, B-EPS synthesized by *Bacillus* sp. 1, *Bacillus* sp. 5 and *Bacillus* sp. 9 had also revealed relatively lower (27.9 and 32.3%) dewaterability. Dewaterabilities of B-EPS were also varied with different incubation times as presented in Table 3.2.5. Similar to FAs, dewaterabilities of B-EPS produced by *Bacillus* sp. 7 at different incubation time were varied mainly due to B-EPS, protein and carbohydrates concentrations.

Zeta potential of kaolin suspension (5.0 g/L) was -29.52±3.15 mV. When calcium was added to kaolin suspension, the zeta potential was reduced from -29.52±3.15 mV to 13.50±1.46 mV. All the broth EPS exhibited negative zeta potential (Table 3.2.3). The negative surface charge of the EPS allows association of calcium to cross-link with the EPS/kaolin and provide greater binding force and promote aggregation phenomenon. The results of the study highlighted the fact that FA and dewaterability are highly dependent on the concentration of EPS (B-EPS), EPS

protein and carbohydrate [11, 26]. It was observed [12] that higher FAs (≥ 75%) were attained using 1.31-2.70 mg EPS/g kaolin produced by the same strains cultivated in sterilized sludge (10.0 g/L suspended solids and 72 h incubation time) under similar conditions. Similarly, high kaolin FAs (≥ 90%) were obtained using bioflocculants produced by different bacterial strains at bioflocculants concentration of 0.2-8.0 mg EPS/g kaolin [2, 5, 10, 31-34]. In contrast, EPS produced in the synthetic media by the same bacterial strains required 250.0-2500.0 mg EPS/g kaolin to attain similar flocculation activity [6]. This might suggest that EPS produced in the sterilized sludge (as in present study) showed better flocculation capacities as compared with the EPS produced in the synthetic media. However, the method and conditions used in the flocculation tests such as mixing, pH, kaolin suspension volume, mixing and settling time, temperature and the sequence of EPS and calcium addition to the kaolin suspension were different in present study with the one of [6].

4 Conclusions

EPS production was mixed growth associated for all the bacterial strains when incubated independently in the sterilized sludge. Protein and carbohydrate concentrations per g of EPS had remained constant or changed insignificantly during 72 h of incubation in sterilized sludge, irrespective of the bacterial strain. In general, to achieve high flocculation activities (≥ 75%), 1.31-1.70 mg B-EPS/g kaolin, 0.45-0.97 mg protein/g kaolin and 0.11-0.21 mg carbohydrates/g kaolin were required. Based upon this screening study, further studies such as bench scale fermentations and EPS production optimization are warranted to analyse the feasibility of the process at an industrial scale.

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Abbreviations

APHA	American Public Health Association
B-EPS	Broth EPS
C-EPS	Capsular EPS
Crude C-EPS	Broth sediments containing C-EPS
CST	Capillary suction time
EPS	Extracellular polymeric substances
FA	Flocculation activity
NTU	Nephelometric Turbidity Units
S-EPS	Slime EPS
SS	Suspended solids
TSA	Tryptic soy broth
TSB	Tryptic soy broth
TS	Total solids
TSS	Total suspended solids
VS	Volatile solids
VSS	Volatile suspended solids
ζ	Zeta Potential
μ_{max}	Maximum specific growth rate
q_{EPS}	Specific EPS production rates

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Table 3.2.1 Wastewater sludge characteristics used in the experiments

Parameters	Concentration
Total solids (TS)	13.45 g/L
Total volatile solids (TVS)	8.85 g/L
Total suspended solids (TSS)	10.82 g/L
Total volatile suspended solids (TVSS)	6.28 g/L
VS/TS	0.66
VSS/TSS	0.58
pH	6.43
Total carbon	427523.1 mg/kg
Total nitrogen	48635.6 mg/kg
Total phosphorus	1037.2 mg/kg
Al	6713.2 mg/kg
Ca	17445.0 mg/kg
Cd	5.8 mg/kg
Cr	72.5 mg/kg
Cu	332.4 mg/kg
Ni	31.6 mg/kg
Fe	11859.5 mg/kg
K	3061.1 mg/kg
Mn	94.0 mg/kg
Na	3898.4 mg/kg
Pb	46.5 mg/kg
S	3806.1 mg/kg
Zn	489.8 mg/kg

Table 3.2.2 Kinetic parameters of EPS production by different cultures during fermentation (for 72 h) in sterilized sludge

Culture	Maximum cells, EPS concentration, productivity at the incubation time				Specific growth rate of cells μ_{max} (h^{-1})	[a]Maximum specific production rate (g EPS/million cells/h)	Relation between specific EPS production (q_{EPS}) and specific growth rate of cells (μ) [c][y = a (x) + b]
	CFU/mL	B-EPS (g/L)	Productivity (g/L/h)	[b]Time (h)			
Bacillus sp. 1	3.73E+07	1.363	0.016	48	0.11	0.80	y = 6.8756x + 0.1677; R^2 = 0.55
Bacillus sp. 2	2.45E+08	1.652	0.016	60	0.15	5.27	y = 37.457x - 1.9907; R^2 = 0.36
Bacillus sp. 3	1.73E+09	1.646	0.013	72	0.27	0.26	y = 1.2202x + 0.0288; R^2 = 0.92
Bacillus sp. 4	1.48E+09	1.787	0.014	72	0.14	0.44	y = 4.0957x - 0.0837; R^2 = 0.88
Bacillus sp. 5	8.32E+07	1.496	0.012	72	0.14	2.50	y = 17.062x - 0.2764; R^2 = 0.85
Bacillus sp. 6	1.65E+09	1.595	0.014	72	0.13	4.58	y = 19.041x - 0.4277; R^2 = 0.27
Bacillus sp. 7	2.50E+09	2.025	0.021	60	0.25	0.35	y = 2.7771x - 0.0876; R^2 = 0.92
Bacillus sp. 8	2.90E+09	1.716	0.016	60	0.19	3.45	y = 18.302x - 0.9474; R^2 = 0.60
Bacillus sp. 9	3.43E+08	1.265	0.008	72	0.13	0.15	y = 1.2587x - 0.0225; R^2 = 0.78
Serratia sp. 1	2.50E+09	1.813	0.017	60	0.27	0.22	y = 1.724x - 0.0637; R^2 = 0.97
Serratia sp. 2	2.20E+09	2.125	0.023	60	0.23	0.26	y = 1.8764x - 0.0366; R^2 = 0.82
Yersinia sp. 1	2.30E+09	1.650	0.010	72	0.18	7.67	y = 42.88x - 1.5866; R^2 = 0.40
Yersinia sp. 2	2.30E+09	1.950	0.036	36	0.19	0.49	y = 3.4836x - 0.0751; R^2 = 0.68

Note: [a]Specific rates were determined per 10^6 CFU/mL. [b]Time of maximum EPS production. [c]In the linear relation, y: specific EPS production (q_{EPS}); **x:** specific growth rate (μ); **a:** slope or yield of EPS per 10^6 CFU/mL; **b:** x or y-intercept. Data are the means of three independent experiments. Standard error is less than 5%.

Table 3.2.3 Rheological and surface charge properties of the fermented broth of different bacterial strains

Culture	Fermented broth		[c]Time (h)
	[a]Viscosity (mPas)	[b]Zeta potential (mV)	
Bacillus sp. 1	3.11	-42.87	48
Bacillus sp. 2	4.72	-33.72	60
Bacillus sp. 3	5.24	-35.31	72
Bacillus sp. 4	5.62	-33.09	72
Bacillus sp. 5	4.36	-38.44	72
Bacillus sp. 6	5.62	-35.85	72
Bacillus sp. 7	5.94	-32.46	60
Bacillus sp. 8	4.72	-36.84	60
Bacillus sp. 9	3.64	-42.18	72
Serratia sp. 1	5.53	-34.16	60
Serratia sp. 2	6.70	-36.44	60
Yersinia sp. 1	4.55	-37.98	72
Yersinia sp. 2	5.62	-36.32	36

Note: [a]The viscosity of wastewater sludge before and after sterilization was 3.11 and 2.24 mPas, respectively. [b]Zeta potential of the fermented broth. [c]Time of maximum EPS production. Data are the means of three independent experiments. Standard error is less than 5%.

Table 3.2.4 Slime EPS (S-EPS) and capsular (C-EPS) concentrations, protein and carbohydrates contents of S-EPS and C-EPS observed for the fermented broths of different bacterial cultures sampled at optimum incubation time.

Culture	[a]Time (h)	[b]S-EPS (g/L)			[d]C-EPS (g/L)		
		[b]Dry weight	[c]Protein	[c]Carbohydrates	[d]Dry weight	[e]Protein	[e]Carbohydrates
Bacillus sp. 1	48	1.104	0.330	0.077	0.260	0.163	0.049
Bacillus sp. 2	60	1.390	0.493	0.125	0.262	0.165	0.054
Bacillus sp. 3	72	1.292	0.437	0.101	0.354	0.223	0.067
Bacillus sp. 4	72	1.572	0.697	0.162	0.215	0.135	0.045
Bacillus sp. 5	72	1.232	0.403	0.095	0.264	0.166	0.056
Bacillus sp. 6	72	1.309	0.447	0.103	0.286	0.180	0.059
Bacillus sp. 7	60	1.662	0.649	0.156	0.580	0.365	0.114
Bacillus sp. 8	60	1.412	0.506	0.121	0.304	0.192	0.059
Bacillus sp. 9	72	1.009	0.276	0.065	0.256	0.161	0.059
Serratia sp. 1	60	1.457	0.531	0.125	0.356	0.224	0.070
Serratia sp. 2	60	1.525	0.570	0.125	0.600	0.378	0.114
Yersinia sp. 1	72	1.375	0.485	0.105	0.275	0.173	0.054
Yersinia sp. 2	36	1.425	0.513	0.121	0.525	0.331	0.103

Note: [a]Time of maximum EPS production; [b]Slime EPS concentration at the time of maximum EPS production; [c]S-EPS of sterilized sludge contained 0.262 g/L protein and 0.031 g/L of carbohydrates; [d]Capsular EPS concentration at the time of maximum EPS production; [e]C-EPS of sterilized sludge contained 0.058 mg/L protein and 0.015 mg/L of carbohydrates; Data are the means of three independent experiments. Standard error is less than 5%.

Table 3.2.5 Flocculation activity (FA) and dewaterability (ΔCST) observed for broth (B-EPS, 2 mL) added to kaolin suspensions (5 g/L) produced by different bacterial strains in sterilized sludge at different incubation times.

Time (h)	*Bacillus* sp. 1					*Bacillus* sp. 2					*Bacillus* sp. 3				
	mg dose/g kaolin			[a]FA (%)	[b]ΔCST (%)	mg EPS/g kaolin			[a]FA (%)	[b]ΔCST (%)	mg EPS/g kaolin			[a]FA (%)	[b]ΔCST (%)
	EPS	Protein	Carbohydrates			EPS	Protein	Carbohydrates			EPS	Protein	Carbohydrates		
12	0.58	0.22	0.05	13.8	16.9	0.84	0.345	0.09	44.8	22.8	0.85	0.35	0.08	57.1	18.7
24	0.77	0.28	0.07	45.8	26.7	1.06	0.428	0.12	55.2	24.9	0.96	0.39	0.09	59.2	21.4
36	0.92	0.34	0.08	53.0	27.3	1.13	0.453	0.12	61.0	30.9	1.14	0.46	0.11	70.1	26.7
48	1.09	0.39	0.10	58.8	27.9	1.25	0.499	0.13	65.3	34.4	1.21	0.49	0.12	72.5	34.4
60	1.06	0.38	0.10	58.1	24.0	1.32	0.526	0.14	75.8	37.7	1.30	0.52	0.13	73.8	36.2
72	1.04	0.38	0.09	55.2	24.6	1.31	0.521	0.14	75.1	36.8	1.32	0.53	0.13	75.8	40.1

Time (h)	*Bacillus* sp. 4					*Bacillus* sp. 5					*Bacillus* sp. 6				
	mg dose/g kaolin			[a]FA (%)	[b]ΔCST (%)	mg dose/g kaolin			[a]FA (%)	[b]ΔCST (%)	mg dose/g kaolin			[a]FA (%)	[b]ΔCST (%)
	EPS	Protein	Carbohydrates			EPS	Protein	Carbohydrates			EPS	Protein	Carbohydrates		
12	0.93	0.442	0.104	64.5	22.0	0.75	0.294	0.072	39.5	22.0	0.88	0.342	0.086	65.6	26.4
24	1.11	0.525	0.126	66.8	24.0	0.84	0.327	0.082	45.2	24.0	0.91	0.356	0.088	66.8	27.3
36	1.20	0.565	0.138	69.9	27.9	0.97	0.372	0.095	48.6	27.9	0.98	0.378	0.101	70.1	29.7
48	1.35	0.630	0.154	71.9	29.4	1.13	0.435	0.109	50.9	29.4	1.07	0.410	0.106	72.2	27.9
60	1.34	0.624	0.158	74.5	32.9	1.17	0.450	0.118	54.3	32.9	1.21	0.457	0.117	76.4	33.8
72	1.43	0.666	0.166	78.6	46.0	1.20	0.455	0.121	55.2	46.0	1.28	0.502	0.130	78.3	39.5

Time (h)	*Bacillus* sp. 7					*Bacillus* sp. 8					*Bacillus* sp. 9				
	mg dose/g kaolin			[a]FA (%)	[b]ΔCST (%)	mg dose/g kaolin			[a]FA (%)	[b]ΔCST (%)	mg dose/g kaolin			[a]FA (%)	[b]ΔCST (%)
	EPS	Protein	Carbohydrates			EPS	Protein	Carbohydrates			EPS	Protein	Carbohydrates		
12	1.08	0.670	0.140	71.7	24.9	0.83	0.363	0.082	50.1	26.7	0.65	0.178	0.076	18.3	22.0
24	1.32	0.810	0.171	74.0	29.4	0.98	0.421	0.094	59.6	28.8	0.72	0.198	0.082	30.8	25.2
36	1.38	0.846	0.183	77.3	39.8	1.01	0.435	0.104	69.4	32.3	0.82	0.217	0.096	34.2	27.0
48	1.58	0.966	0.210	79.7	46.0	1.15	0.490	0.126	74.0	33.8	0.88	0.232	0.105	36.5	29.4
60	1.62	0.811	0.216	83.1	50.7	1.37	0.558	0.144	79.4	45.4	0.84	0.270	0.100	54.2	30.9
72	1.54	0.939	0.205	82.4	48.1	1.36	0.572	0.142	79.4	43.6	1.01	0.272	0.121	62.4	32.3

Time (h)	*Serratia* sp. 1					*Serratia* sp. 2					*Yersinia* sp. 1				
	mg dose/g kaolin			[a]FA (%)	[b]ΔCST (%)	mg dose/g kaolin			[a]FA (%)	[b]ΔCST (%)	mg dose/g kaolin			[a]FA (%)	[b]ΔCST (%)
	EPS	Protein	Carbohydrates			EPS	Protein	Carbohydrates			EPS	Protein	Carbohydrates		
12	1.10	0.507	0.116	62.7	24.9	1.08	0.627	0.118	73.1	34.4	1.24	0.490	0.118	69.6	33.8
24	1.27	0.582	0.133	66.8	27.0	1.24	0.714	0.142	74.0	36.8	1.26	0.501	0.122	72.5	32.9
36	1.32	0.601	0.147	70.1	29.4	1.46	0.726	0.163	77.3	38.9	1.28	0.510	0.122	73.0	32.3
48	1.45	0.658	0.154	76.1	40.1	1.58	0.747	0.182	81.7	43.0	1.30	0.513	0.125	75.4	40.1
60	1.45	0.604	0.156	80.9	43.1	1.70	0.758	0.191	83.1	54.3	1.30	0.518	0.125	76.0	41.8
72	1.42	0.644	0.158	80.1	39.8	1.54	0.762	0.170	82.2	45.1	1.32	0.526	0.127	76.1	42.7

Time (h)	*Yersinia* sp. 2				
	mg dose/g kaolin			[a]FA (%)	[b]ΔCST (%)
	EPS	Protein	Carbohydrates		
12	0.98	0.433	0.110	59.0	24.9
24	1.26	0.546	0.140	66.5	27.0
36	1.56	0.675	0.179	79.5	41.2
48	1.52	0.660	0.170	79.0	40.1
60	1.38	0.594	0.154	65.4	37.4
72	1.38	0.588	0.133	61.5	38.6

Note: [a]FA-flocculation activity; the control turbidity i.e., turbidity without EPS was 139 NTU. [b]ΔCST - increase in dewaterability; the control turbidity i.e., turbidity without EPS was 33.7 s. Data are the means of three independent experiments. Standard error is less than 5%.

Figure (a) *Bacillus* sp. 1

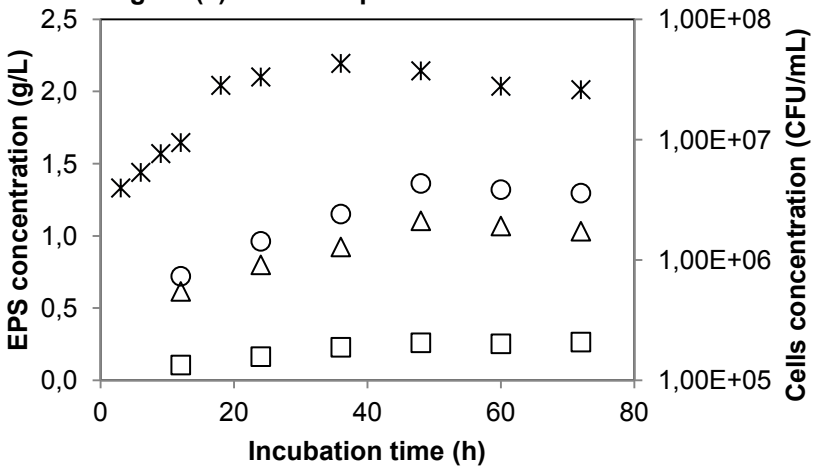


Figure (b) *Bacillus* sp. 2

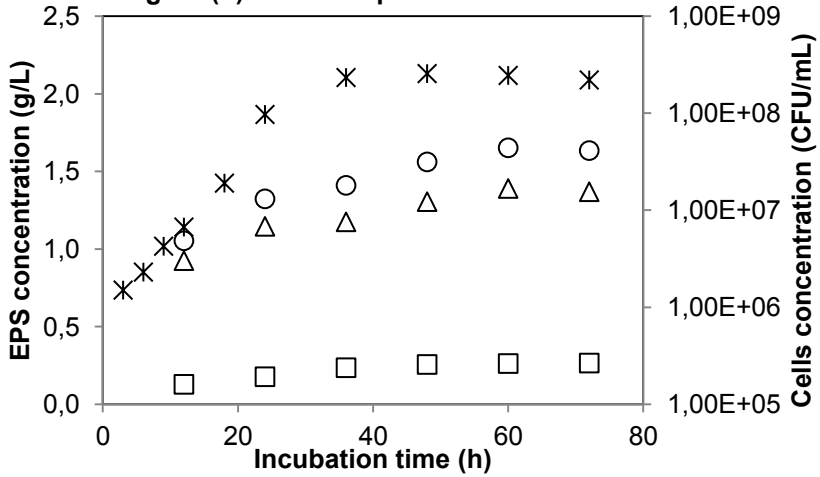


Figure (c) *Bacillus* sp. 3

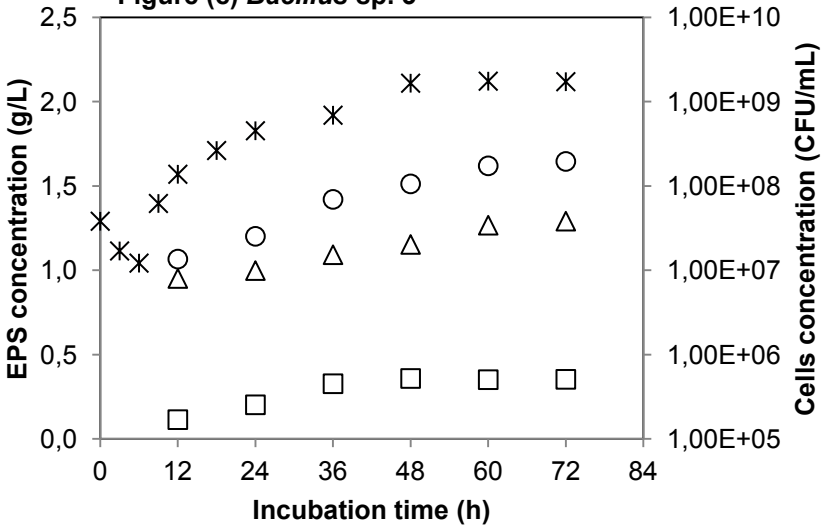


Figure (d) *Bacillus* sp. 4

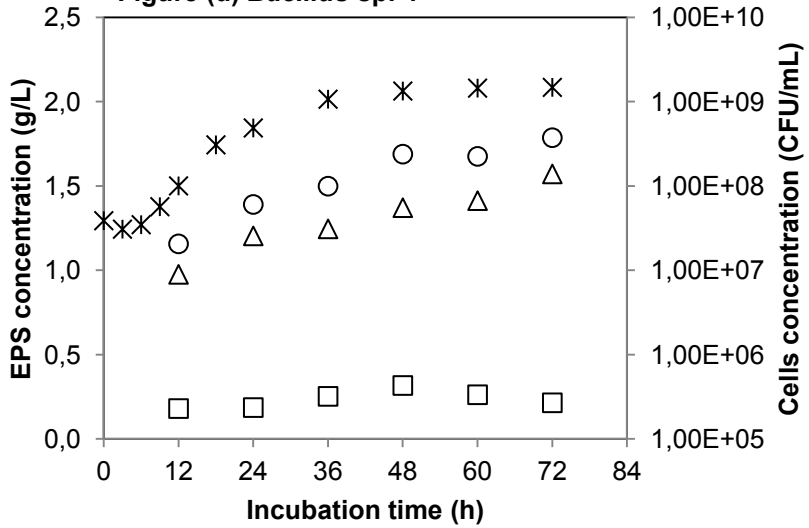


Figure (e) *Bacillus* sp. 5

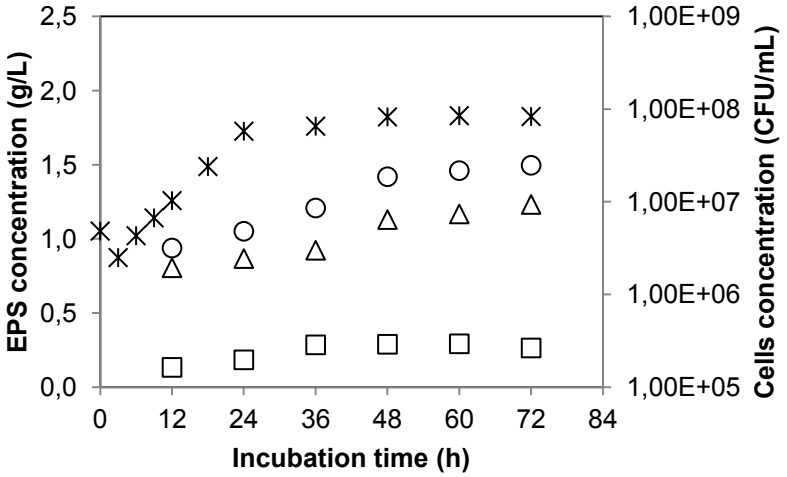


Figure (f) *Bacillus* sp. 6

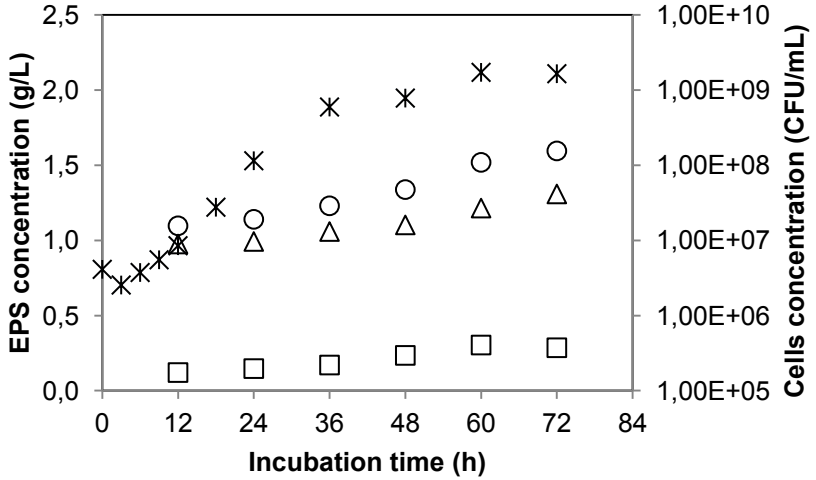


Figure (g) *Bacillus* sp. 7

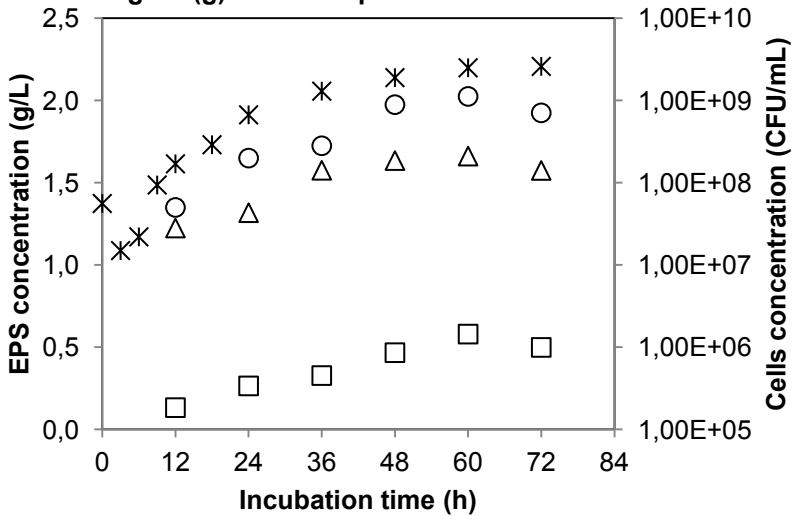


Figure (h) *Bacillus* sp. 8

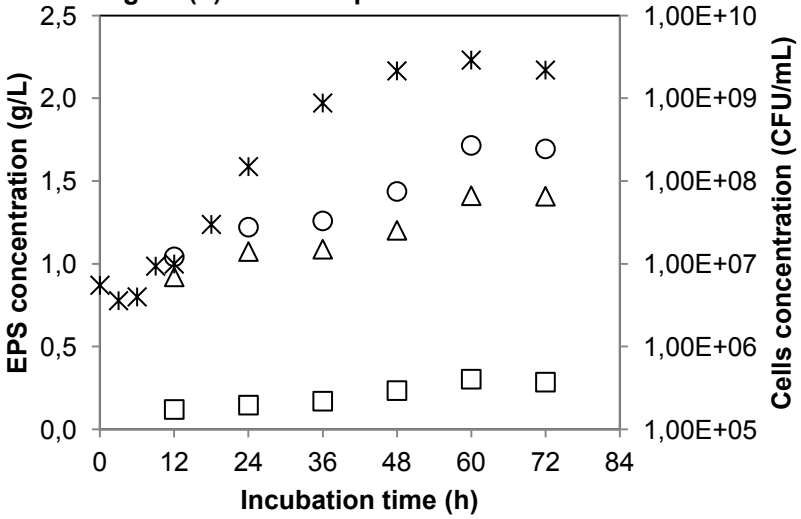


Figure (i) *Bacillus* sp. 9

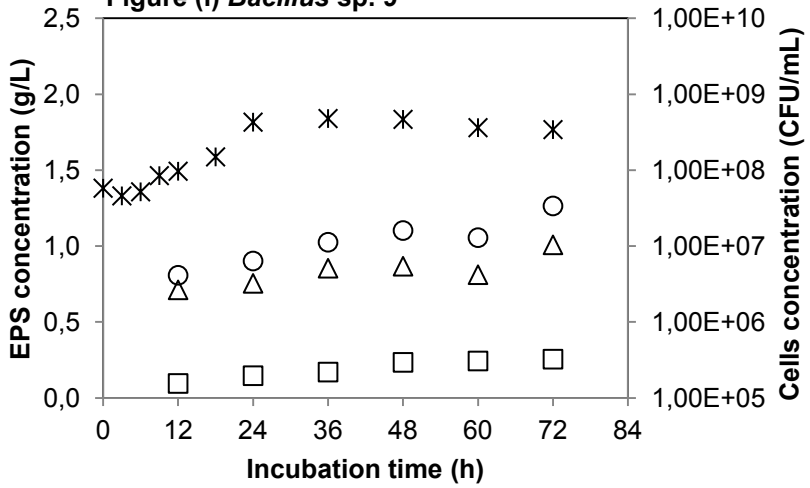


Figure (j) *Serratia* sp. 1

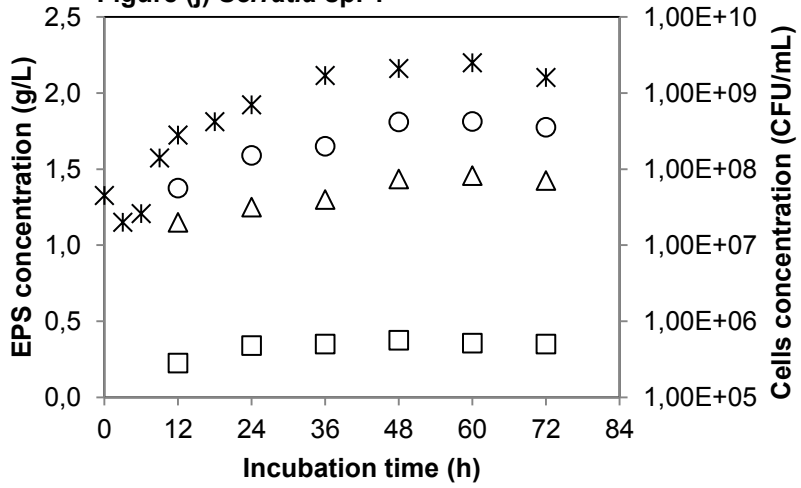


Figure (k) *Serratia* sp. 2

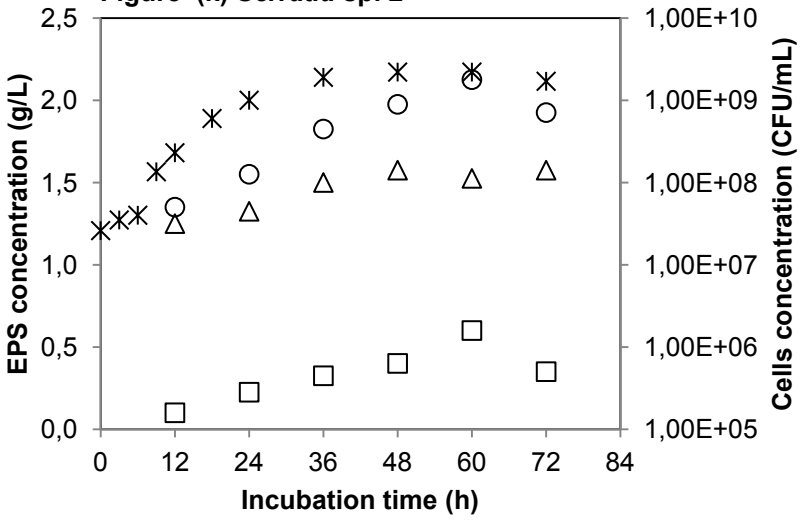
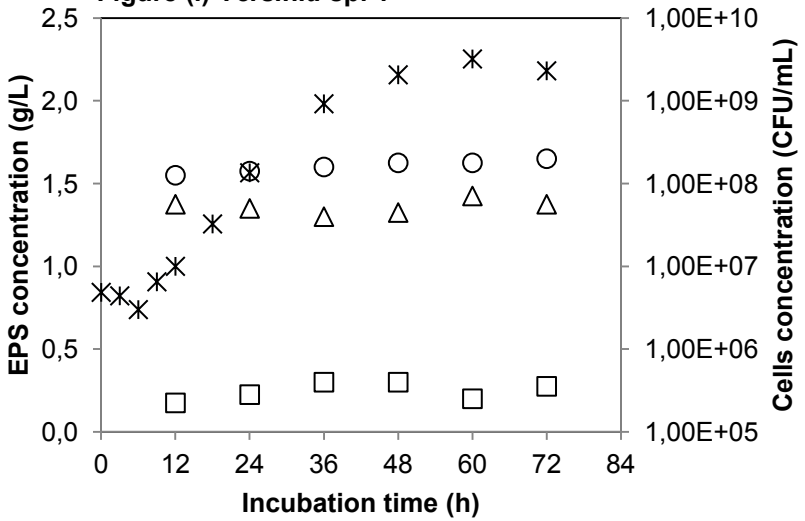


Figure (l) *Yersinia* sp. 1



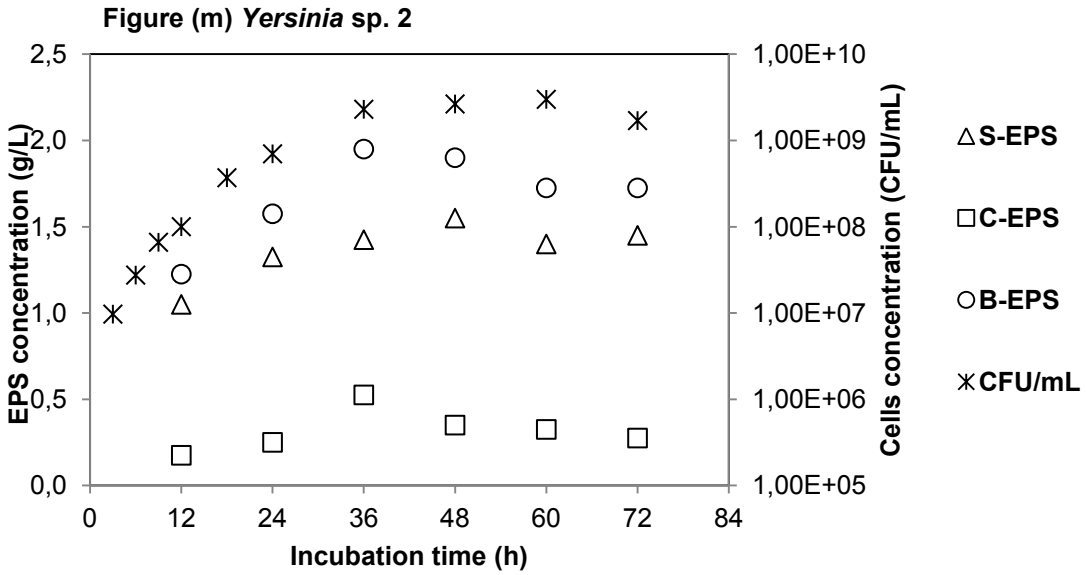
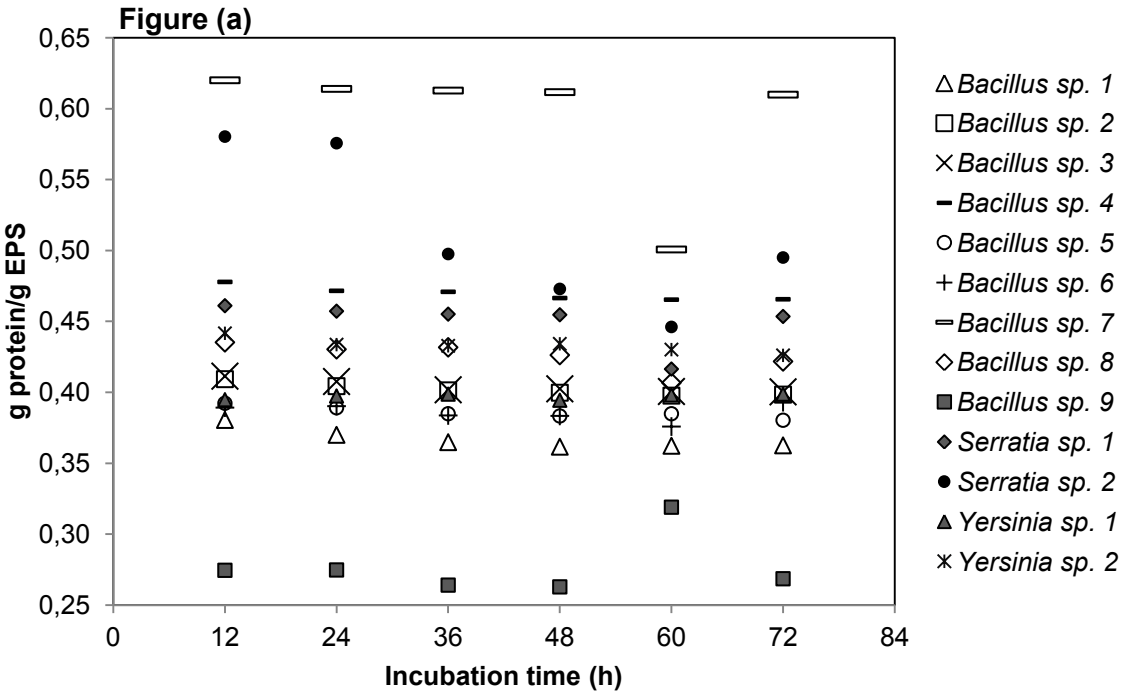


Figure 3.2.1 Time course of concentrations of cells (CFU/mL), broth (B-EPS), slime (S-EPS) and capsular (C-EPS) EPS produced by different bacterial strains such as Figure (a) *Bacillus* sp. 1, Figure (b) *Bacillus* sp. 2, Figure (c) *Bacillus* sp. 3, Figure (d) *Bacillus* sp. 4, Figure (e) *Bacillus* sp. 5, Figure (f) *Bacillus* sp. 6, Figure (g) *Bacillus* sp. 7, Figure (h) *Bacillus* sp. 8, Figure (i) *Bacillus* sp. 9, Figure (j) *Serratia* sp. 1, Figure (k) *Serratia* sp. 2, Figure (l) *Yersinia* sp. 1 and Figure (m) *Yersinia* sp. 2, respectively.



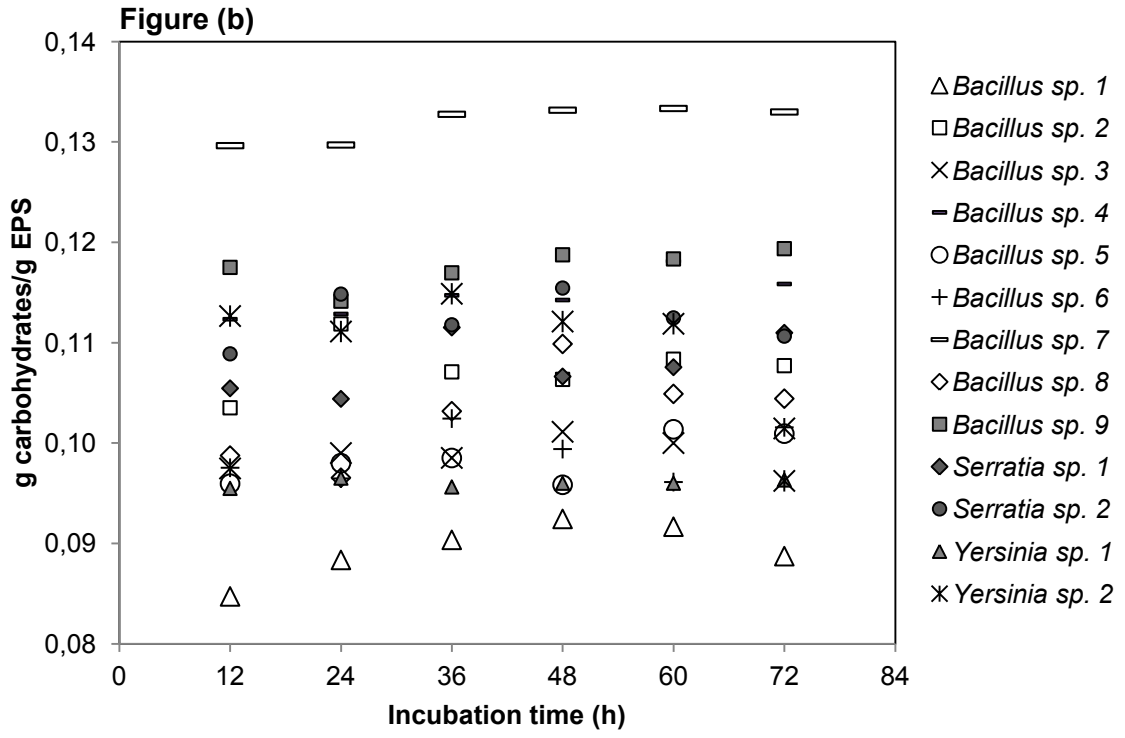


Figure 3.2.2 Protein Figure (a) and carbohydrate Figure (b) content present in the broth EPS synthesized by different bacterial strains in sterilized sludge at different incubation times

CHAPITRE 4

BACTERIAL POLYMER PRODUCTION USING PRE-TREATED SLUDGE AS RAW MATERIAL AND ITS FLOCCULATION AND DEWATERING POTENTIAL

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RÉSUMÉ

La stérilisation, le traitement alcalin-thermique et le traitement acide-thermique ont été appliqués à différentes concentrations des solides en suspension des boues (17.0, 22.4, 29.8, 37.3, 44.8 g/L, respectivement). Ainsi, les boues prétraitées ont été utilisées comme matière première pour la production de SPE par la souche bactérienne *Serratia* sp.1. Après 72 h de fermentation, des SPE totaux de 2,3 et 3,4 g/L ont été produites dans les boues stérilisées et dans les boues prétraitées par traitement alcalin-thermique. Elle était faible plus (1.5 g/L) dans les boues traitées thermiquement et avec un pH acide. Les plus faibles quantités de SPE ont été produites à des concentrations relativement élevées de solides en suspension (37,3; 44,8 g/L). Le bouillon, les formes capsulaires et solubles (slime) brutes des SPE ont été extraites à partir de bouillons fermentés et utilisés comme agents de conditionnement par la combinaison avec 150 mg de Ca^{2+}/L de suspensions de kaolin. L'activité de floculation maximale de 79,1% et une meilleure déshydratation de 52,2% ont été atteintes utilisant les B-SPE et les C-SPE, respectivement. Les résultats ont démontré que les SPE ayant une capacité de floculation élevée pourraient être produites en utilisant les boues d'épuration comme matière première.

Mots clés: La floculation; Déshydratation; Substances polymères extracellulaires; Prétraitement; Les boues d'épuration

ABSTRACT

Sterilization, alkaline-thermal and acid-thermal treatments were applied to different sludge solids concentrations (17.0; 22.4; 29.8; 37.3; 44.8 g/L, respectively) and the pre-treated sludge was used as raw material for *Serratia* sp.1 to produce extracellular polymeric substances (EPS). After 72 h of fermentation, total EPS of 2.3 and 3.4 g/L were produced in sterilized and alkaline-thermal treated sludge as compared to that of 1.5 g/L in acid-thermal treated sludge. Lower EPS were produced at relatively higher solids concentrations (37.3; 44.8 g/L). Broth, crude forms of capsular and slime EPS were extracted from fermented broths and used as conditioning agents by combining with 150 mg of Ca^{2+}/L of kaolin suspensions. Maximum flocculation activity of 79.1% and increased dewatering by 52.2% was achieved using broth and crude capsular EPS, respectively. The results demonstrated that EPS having high flocculating capability could be produced using wastewater sludge as sole raw material.

Key words: Flocculation; Dewatering; Extracellular polymeric substances; Pre-treatment; Wastewater sludge

1 Introduction

Inorganic flocculants and organic synthetic polymers widely used for coagulation-flocculation processes in wastewater treatment plants are reported to be expensive, corrosive, toxic and non-readily degradable. Some of these flocculants and/or their degraded monomers (e.g., monomers of polyacrylamide derivatives) carry serious health and environmental concerns especially if chemically treated sludge is recovered and used or disposed off to the agricultural land (Salehizadeh and Shojaosadati, 2001; Tyagi et al., 2009). Thus, recently flocculation potential of economical, environment friendly and sustainable biopolymers (e.g., chitosan, sodium alginate and microbial flocculants) is being studied by many researchers. Microbial flocculants (extracellular polymeric substances-EPS) are the least explored such option which is reported to carry the potential of substituting conventional flocculants partly or completely (Gong et al., 2008; More et al., 2010; Subramanian et al., 2010).

EPS are biopolymers, located at or outside the cell surface. They are the products of active secretion, cell surface material shedding, cell lysis, and sorption from the environment (Wingender et al., 1999). EPS are produced by both prokaryotes and eukaryotes in a wide variety of environments (e.g., soils, sludge). Due to special properties (adsorption capability, hydrophobicity/hydrophilicity and degradability), naturally occurring EPS in wastewater treatment plants have important role in the sludge flocculation, settling and dewaterability. Broadly, EPS are classified as capsular and slime based on nature of their association with microbial cells (Tian, 2008; Wingender et al., 1999). Each fraction of EPS along with their association with certain cations (e.g. Ca^{2+}, Mg^{2+}) behaves differently when subjected to shear in flocculation process (Higgins and Novak, 1997a). Recently, attempts have been made by some researchers to explore the potential of EPS as microbial flocculant in sludge conditioning operations (Gong et al., 2008; Subramanian et al., 2010). In earlier study, *Serratia* sp.1 was found to produce EPS in synthetic mineral media and also it had shown high kaolin flocculation activity (Subramanian et al., 2010). The production of EPS reported till date mostly is in synthetic media. Wastewater sludge is potentially economical media as it is a rich source of carbon, nitrogen, phosphorus and other nutrients. The use of sludge will be advantageous for growth of the microorganisms isolated from the wastewater sludge which are already well adapted to it. Suspended sludge solids concentrations affects the growth rate and product formation by the bacterial strain cultured due to the fact that nutrients required for bacterial growth are mostly embedded into it (Drouin et al., 2008; Vidyarthi et al., 2002). Pre-treatment of

the sludge is essential for solubilising complex carbon sources to simpler ones which helps in accelerating growth and product formation by bacteria. Therefore, in this study, EPS producing *Serratia* sp.1 was cultured in sludge to determine the optimum suspended solids concentration for the maximum EPS yield. Three types of sludge pre-treatments at different solids concentration, sterilization (ST), alkaline-thermal (ALT) and acid-thermal (ACT) for their impact on EPS production were investigated. Kaolin flocculation activities and dewatering capacity of different EPS forms (broth, crude capsular and slime or B-EPS, C-EPS and S-EPS, respectively) were studied in presence of divalent cations (Ca^{2+}).

2 Materials and methods

2.1 Microorganism

EPS producing bacterial strain *Serratia* sp.1 (EU031758), isolated from the wastewater sludge (Subramanian et al., 2010) was cultivated on tryptic soy agar plates and stored at 4 ^{o}C and sub-cultured fortnightly.

2.2 Wastewater sludge

Wastewater sludge (without addition of chemical polymers) was collected from biofiltration unit at Ville de Québec (Québec city, Canada). The sludge was first settled by gravity for 1 h and concentrated sludge was collected by discarding supernatant. Characteristics of sludge, such as pH, total solids (TS), suspended solids (SS), volatile solids (VS), volatile suspended solids (VSS), turbidity (NTU) were determined using Standard Methods (APHA, 2005). Viscosity (mPas) and zeta potentials (ζ) were measured using Viscometer (DV DV-II PRO + (Brookfield Engineering Laboratories, Inc., Stoughton, MA, USA)) and Zetaphoremeter (Zetaphoremeter IV, Zetacompact Z8000, CAD Instrumentation, France), respectively. The sludge was stored at 4 ^{o}C for further use.

2.3 Sludge treatments

The SS of raw sludge (before various treatments) was adjusted to 17.0; 22.4; 29.8, 37.3 and 44.8 g/L. The pH and VSS/TSS of the raw sludge was 6.16 and of 62.5%, respectively. The raw sludge samples were treated by sterilization treatments (ST or steam sterilization), alkaline-

thermal (ALT) and acid-thermal (ACT) treatments, respectively. Sterilization was carried out by autoclaving (steam sterilization) at 121 ^{o}C for 15 min. In ALT treatment, first, pH of the sludge was raised to 10 by using 1 M NaOH and autoclaved at 121 ^{o}C for 15 min, then the sludge was cooled down to room temperature and adjusted to pH 7.0 using 1 M HCl. In ACT treatment, first, pH was reduced to 2 using 1 M HCl and autoclaved at 121 ^{o}C for 15 min, then the sludge was cooled down to room temperature and adjusted to pH 7.0 using 1 M NaOH.

2.4 EPS production and harvesting

The tryptic soy broth (TSB) grown *Serratia* sp.1 culture was first inoculated in the sterilized sludge (100 mL, SS: 17.0 g/L) in 500 mL Erlenmeyer, and the sample was incubated at 25^{o}C and 250 rpm for 24 h. After 24 h, 3 % v/v of the inoculum (prepared in sterilized sludge) was used to inoculate different treated sludge samples (100 mL of ST, ALT and ACT sludge samples of different SS: 17.0; 22.4; 29.8, 37.3 and 44.8 g/L, respectively) in 500 mL Erlenmeyer. These inoculated sludge samples were incubated at 25 ^{o}C and 250 rpm, for 72 h. After incubation (72 h), broth samples were centrifuged at 6000g for 15 min at 4^{o}C to obtain supernatant (containing slime EPS and termed as crude S-EPS) and pellets (containing capsular EPS (C-EPS) with bacterial cells along with residual sludge material and termed as crude C-EPS) (Subramanian et al., 2010; Wingender et al., 1999).

To determine the S-EPS, the supernatant (crude S-EPS) was precipitated with 2.2 volumes of absolute chilled ethanol by incubating the mixture at -20 ^{o}C for 1 h. The precipitates (containing pure S-EPS) were collected by centrifugation at 6000g for 15 min at 4 ^{o}C. Precipitates of S-EPS were dried at room temperature in laminar hood for 6 h and its dry weight was measured and denoted as S-EPS (APHA, 2005). To determine C-EPS, the pellet (crude C-EPS) was resuspended in deionized water. The resuspended pellet was first heated at 60^{o}C in water bath for 30 min to release C-EPS followed by centrifugation at 6000 x g, 4^{o}C for 15 min (Li and Yang 2007). The supernatant (containing C-EPS) was used to precipitate C-EPS using same procedure as for S-EPS. Dry weight of the precipitates was measured and denoted as C-EPS. Sum of dry weights of S-EPS and C-EPS (measured above) was denoted as B-EPS. All the EPS measurements were carried out in triplicates and the average values were presented (with standard error less than 5% of the mean).

Viscosity and zeta potentials of the different forms of EPS were measured as described earlier. The protein content in the supernatants (containing slime) was determined using bovine serum

albumin as a standard (Lowry et al., 1951); the carbohydrate content in the supernatants was determined by the phenol-sulfuric acid method using glucose as the standard solution (DuBois et al., 1956).

2.5 Evaluation of flocculation activity (FA) and dewaterability

Kaolin clay (K2-500, USP, Fisher scientific, US) was used in jar tests as a model suspension (5 g/L) prepared in deionized water (500 mL) to examine flocculation and dewatering capabilities of the produced EPS. Equivalent volume of broth, supernatant (crude S-EPS) and resuspended pellets (crude C-EPS) corresponding to the measured amount of B-EPS, S-EPS and C-EPS, respectively, was employed in the jar tests. Effects of different EPS concentrations (B-EPS, C-EPS and S-EPS) along with Ca^{2+} (obtained from solution of $CaCl_2$ prepared in the deionized water) were examined.

In jar tests, first rapid mixing of kaolin suspensions was carried out at 175 rpm for 3 min to allow homogeneous dispersion of solids and to encourage solids interactions. During the rapid mixing, 150 mg of Ca^{2+}/L of kaolin were added followed by different dosage of EPS (B-EPS, C-EPS and S-EPS). After rapid mixing, pH of the suspensions of calcium, kaolin and EPS were adjusted to 7.5 and slow mixing at 75 rpm was allowed for 30 min to ensure flocculation. Then all the jar test samples were transferred to measuring cylinders of 500 mL for 30 min settling. After 30 min of settling, supernatants and sediments of the samples were used to measure flocculation activity and dewatering, respectively. All the tests were performed in triplicates and average values were presented (with standard error less than 5% of the mean).

To determine flocculation activity, turbidity of the supernatants was measured using Micro 100 Turbidity meter, Hach Company. Flocculation activity measure was based on the relative decrease in turbidity of suspension after settling (Yokoi et al., 1995). Flocculation activity was calculated according to the equation: Flocculation activity = (1-*S/C*) x100 (%), where *C* is control turbidity and *S* is sample turbidity. Kaolin suspension with Ca^{2+} addition (without EPS) was the control.

For quantifying the effects of EPS on dewaterability, Capillary-suction-time (CST) of the settled sediments were measured. CST was determined by the CST instrument (Triton electronics, model 304 M CST, Dunmow, Essex), using a 10-mm diameter reservoir (Electronics, 1998; Scholz, 2005). Lower CST value as compared to the control CST indicated better dewaterability. The sediments from jar test samples with added Ca^{2+} (without EPS) was the control. The

increase in the dewaterability was calculated by equation: increase in dewaterability (%) = {(control CST) – (sample CST)} / (control CST) x100.

3 Results and discussion

3.1 EPS production

In general, *Serratia* sp.1 showed high growth rate (the highest cell count after 36 h was in the range of 10^9 colony forming unit (CFU)/mL) in treated sludge. *Serratia* sp.1 has wide range of carbon utilization ability (More et al. 2011, unpublished manuscript), which eventually helped it to survive and utilize available carbon and nutrients sources present in the treated sludge for its growth and EPS secretion. Concentrations of EPS harvested (at 72 h of fermentation) from different samples of fermented broths are as shown in Fig. 4.1.The control sample of sterilized sludge (without culturing) had very low concentration of EPS (less than 31.0 mg/g SS). High EPS concentrations (Fig. 4.1) observed in the fermented broths were due to EPS secretion by *Serratia* sp.1 in treated sludge during 72 h of fermentation. Results clearly showed that EPS secreted by *Serratia* sp.1 significantly varied with different sludge treatments and sludge solids concentrations.

3.2 Effect of different sludge treatments

Although the sludge from the same sample was used for the EPS production using same bacterial strain, application of different sludge treatments produced distinct EPS concentrations. Maximum EPS (B-EPS) of 3.4 g/L, 2.8 g/L and 1.5 g/L were harvested from fermented broths of ALT, ST and ACT sludge, respectively (Fig. 4.1). Moreover, at the same sludge solids concentration with different pre-treatment, different quantities of B-EPS were harvested. For example, quantity B-EPS harvested from fermented broths of ST, ALT and ACT sludge at SS of 17.0 g/L was 2.2 g/L, 3.4 g/L and 1.2 g/L, respectively. Similar to the trend of B-EPS, S-EPS of 2.0 g/L, 2.3 g/L, and 1.0 g/L was produced in ST ALT, and ACT sludge, respectively. In general, S-EPS in ST and ALT sludge was (1.5 to 2 times) higher compared to C-EPS.

The variations in EPS harvested from different fermented broths were attributed to the specific chemical/physical changes induced by different treatments in the pre-treated sludge. The studies previously conducted in our lab (Verma et al., 2007) have confirmed the facts that the soluble carbon and nitrogen concentrations increased after sludge pre-treatments (irrespective

of sludge treatment method). Increase in soluble carbon and nitrogen concentration was higher in case of alkaline-thermal and sterilization as compared to acid-thermal treatment (irrespective of sludge solids concentration) (Verma et al., 2007). In general, sludge treatments (thermal and or chemical) disintegrated the organic fractions (including natural sludge EPS matrix, extracellular proteins, polysaccharides, and enzymes) and released volatile fatty acids in to the sludge medium. At alkaline conditions, distribution patterns of these released substances in the treated sludge reported to be different than at acidic conditions. Capacity of acids (HCl) to induce cell lysis (cell hydrolysis) is lower than alkaline reagents (NaOH) and hence, sludge solubilisation significantly increased with alkaline treatment and to a lesser extent by acid treatment (Chen et al., 2007). Moreover, sterilization of sludge increased soluble COD (chemical oxygen demand) (Verma et al., 2007) and volatile fatty acids and other low molecular weight soluble carbon compounds to an extent which was higher than acid treatments. The alkaline conditions increased the dissociation of acidic groups from sludge biomass and hence, enhanced the solubility of sludge proteins and carbohydrates. Alkaline treatment solubilises mostly proteins of the sludge whereas acid treatment solubilises mostly carbohydrate portion of the sludge (Aravinthan et al., 2001). The solubilisation of larger cellular proteins and other nitrogenous organic material in the ALT and ST sludge lead to decrease in carbohydrates to protein ratio as compared to ACT sludge. The carbon sources and nature of nutrients available in the media change with the type of treatment and therefore can change microbial EPS secretion pattern. The EPS content is reported to increase with food to microorganism ratio (Jorand et al., 1994). Therefore, in the present study, specific carbon and nutrients content released in the ALT and ST sludge medium favored EPS secretion by *Serratia* sp.1 as compared to that of ACT sludge.

3.3 Effect of suspended solids (SS) concentrations

The quantity of EPS harvested from the fermented broths varied with the initial SS concentration and pre-treatment method used. In case of ST sludge, concentration of B-EPS increased from 2.2 g/L to 2.8 g/L with increase in SS concentrations from 17.0 g/L to 22.4 g/L. Further, increase in SS concentration from 29.8 g/L to 44.8 g/L decreased the B-EPS concentration from 2.8 g/L to 2.5 g/L. Similar trend of B-EPS concentration was observed during fermentation of ACT sludge (Fig. 4.1). However, in case of ALT sludge, concentration of B-EPS decreased from 3.4 g/L to 2.0 g/L with the increase in SS concentrations from 17.0 g/L to 44.8 g/L. The results also confirmed that the concentration of different EPS forms (B-EPS, C-EPS, S-EPS) produced in all

the treated sludge were mainly affected by sludge solids concentrations. The concentration of S-EPS varied with SS concentration similar to B-EPS. Concentration of C-EPS was almost constant for SS from 17.0 g/L to 44.8 g/L, of ST (around 0.8 g/L) and ALT (0.8-1.1 g/L) sludge whereas, concentration of C-EPS was comparatively lower for ACT (less than 0.5 g/L) sludge.

In spite of the fact that higher SS (37.3 g/L and 44.8 g/L) represent higher carbon and nutrient contents, lower concentrations of EPS were produced. The amount of particulate carbon and other organic complexes solubilised into available (readily biodegradable) substrate might not be the same at different SS concentrations (Verma et al., 2007). This probably resulted in the variation of readily biodegradable carbon and nitrogen contents of the treated sludge. The non availability (or less availability) of degradable carbon and other nutrients in the sludge at higher solids concentrations would have affected the bacterial growth and hence the concentration of EPS produced. In addition, the mass transfer limitation and an inadequate supply of oxygen to microorganisms because of poor mixing at high SS concentrations also attributed to lower concentration of EPS at the end of fermentation (Maria, 2004). High solids concentration increased viscosity of the broths having non-Newtonian, pseudo plastic flow behavior. High viscosity exerts a negative impact on the mass transfer properties of the broth, especially the gas-liquid mass transfer rate (Maria, 2004).

3.4 Flocculation activity of different EPS

Addition of different forms of EPS to kaolin suspensions in presence of calcium ions (Ca^{2+}) enhanced kaolin flocculation activity (FA). In general, flocculation activity increased with quantity of EPS added to kaolin suspension, reached a maximum and then decreased with further addition of EPS (Fig. 4.2). EPS formed flocs in combination with calcium ions present in kaolin suspension. This phenomenon lead to increased settling of flocs formed (larger in size and dense) and hence increased the flocculation activity.

In all jar tests, there was an optimum concentration of EPS at which the highest flocculation activity was observed (Fig. 4.2). However, B-EPS produced at different solids concentration in ST sludge revealed different optimum concentration (0.6 to 0.8 mg of B-EPS/g of kaolin) for maximum flocculation activity (Fig. 4.2). In case of B-EPS produced in maximum flocculation activity of 79.13% was obtained with B-EPS produced in ST sludge at SS 17.00 g/L. Further, B-EPS (0.7-0.9 mg of EPS/g of kaolin) produced in ST sludge at SS concentration of 22.4, 29.8, 37.3and 44.8 g/L, exhibited maximum flocculation activities of 71.4%, 74.2%, 41.5% and 33.5%,

respectively. In present study, same concentration of EPS (used to determine flocculation activity) produced using different sludge suspended solids concentrations (of the same sludge) in the medium revealed different flocculation activity. For example for the B-EPS concentration of about 0.7 g/g SS (kaolin) had flocculation activity of about 79.1% (EPS produced at SS-17.0 g/L) and 41.5% (EPS produced at SS-37.3 g/L). Further, in our earlier study (Subramanian et al. 2010), B-EPS produced by same bacterial strain (as used in present study) in synthetic medium revealed that 100 to 1000 mg of EPS/g of SS was required for attaining kaolin flocculation activity higher than 60%, which is different (much higher) than that required in present study (of sludge). In present study, carbohydrates to protein ratios (Wingender et al., 1999) of the slime EPS observed respectively for ST, ALT and ACT sludge were 2, 1.8, and 1.4 at 17.0 g SS/L; 2.0, 2.1, 1.2 at 22.4 g SS/L; 1.8, 1.6, 1.1 at 29.8 g SS/L; 1.4, 1.2, 1.3 at 33.7 g SS/L and 1.3, 1.4, 1.1 at 44.8 g SS/L. The carbohydrate to protein ratio of slime EPS was 1.8 when produced in synthetic medium (Subramanian et al., 2010). These results clearly established that the EPS composition was different when produced in synthetic medium (Subramanian et al., 2010) as well as at different sludge suspended solids concentrations (present study). This change in carbohydrate/protein ratio was one of the reasons that changed the nature (functional groups) of the EPS produced. Thus, the distinct nature (protein and carbohydrate contents) of B-EPS produced at different SS concentrations also might be the reason for variations in flocculation activities.

C-EPS and S-EPS exhibited similar trends as that of B-EPS. Decrease in flocculation activity with excess EPS addition was due to destabilization of flocs, caused by increased surface charge on the kaolin particles. On the other hand, excess EPS also increased colloidal particles, which increased the suspension turbidity and decreased flocculation activity. Concentration of colloidal particles affects amount of polymer dosage required. Flocculation decreases if the concentration of colloidal particles (in the size range of 1-100 μm) increases (Higgins and Novak, 1997b).

Apart from the general trends of flocculation activity versus EPS concentration, different types of EPS produced in the sludge pre-treated by different methods and EPS produced in the same sludge but at different sludge solids concentration revealed distinct patterns of flocculation activity. In general, B-EPS and C-EPS exhibited higher flocculation activity as compared to S-EPS. EPS (all forms) obtained from ST and ALT sludge exhibited higher flocculation activity as compared to that of ACT sludge (Table 4.1). B-EPS, C-EPS and S-EPS obtained from ST sludge revealed maximum flocculation activity of 79.1%, 72.3% and 53.0%, respectively.

Whereas, B-EPS obtained from (different treated sludge) ST, ALT and ACT sludge revealed maximum flocculation activity of 79.1%, 60.3% and 44.7%, respectively. The results of maximum flocculation activities obtained for different forms of EPS at corresponding optimum concentration of EPS are presented in Table 4.1.

Relatively higher concentration of C-EPS (2.8 to 3.5 mg of C-EPS/g of kaolin) was required to attain higher flocculation activity (more than 60%), as compared to that of B-EPS (0.7 to 0.9 mg of B-EPS/g of kaolin). The results clearly showed that B-EPS had higher flocculating capability than C-EPS and S-EPS from ST sludge. Overall, in present study, very low quantity of EPS (all forms) was required for achieving maximum flocculation activity as compared to the reported in literature (Subramanian et al., 2010). These results indicated that EPS produced in the sludge had high binding capacity as compared to EPS produced in the synthetic medium. The flocculation behaviour of the EPS depends upon its adsorbability, hydrophobicity and metal binding ability (Wingender et al., 1999).

The variations in flocculation activity of different forms of EPS produced by *Serratia* sp.1 were attributed to the presence of different functional groups on the EPS. EPS are generally negatively charged due to functional groups that it carries such as carboxyl groups in EPS. It is reported that bacterial EPS from the activated sludge contains carboxyl, hydroxyl, amide and amino groups and which preferably participates in flocculation (Higgins and Novak, 1997b). As a consequence, multivalent cations play a significant role in binding negatively charged biopolymer to enhance bioflocculation. EPS participates in the flocculation mainly through available carboxyl and hydroxyl groups which induces very high binding capacity of EPS. EPS which generally reported to have high molecular weight 100 kDa (Kumar et al., 2004) carries large number of binding sites with high flocculation capability.

The nature of EPS produced with different sludge treatment methods had significant impact on the flocculation properties. Alkaline treatment solubilises mostly proteins in the sludge whereas acid treatment solubilises mostly carbohydrate portion of the sludge (Aravinthan et al. 1998). Similarly, sterilization of sludge increases protein content than carbohydrate content of the sludge. Composition of the produced EPS mainly depends upon nutrients available in the sludge. Growth medium contains high protein content leads to the production of EPS with high protein content and which possesses different flocculation properties. The solubilisation of larger cellular proteins and other nitrogenous organic material in the ALT and ST sludge lead to decrease in carbohydrate to protein ratio as compared to ACT sludge (Chen et al., 2007) and higher flocculation activity. The results clearly indicated that the EPS produced at high SS

concentration was functionally different than the EPS produced at lower SS concentration which also reflected in different flocculation behaviour. These results are in agreement to the existing reports (Keiding and Nielsen, 1997) that EPS from different origins have different and unique characteristics.

Various studies concluded that physical, chemical and biological parameters play a major role in bioflocculation. The biological parameters include type of microorganisms and their EPS. The important factors in the bioflocculation using EPS which can affect the floc characteristics are mainly: EPS concentration, charge and hydrophobicity, and cations requirements (Houghton et al., 2001; Tian, 2008). Apart from these factors, as discussed above, the characteristics of EPS (content of proteins, polysaccharides, and other components) were also reported to play a major role in bioflocculation (Wingender et al., 1999). Thus, different patterns of flocculation activity were observed using different forms of EPS and also using EPS produced in sludge treated with different methods and at different SS concentrations. The characteristics of EPS (composition) need to be studied thoroughly to justify the variations in flocculation behaviour of EPS.

3.5 Impact of EPS on Dewaterability

CST tests revealed that EPS (combined with Ca^{2+}) dosage lowered CST of kaolin suspension. In general, similar to flocculation activity trend (discussed earlier), dewaterability of kaolin sediments increased with EPS concentration up to certain EPS dosage (optimum quantity of EPS) followed by a decrease of dewaterability with further addition of EPS (Fig. 4.3). Initial increase in dewaterability attributed to the formation of stabilized flocs. EPS has floc stabilizing effects through polymer entanglement. These interactions are either purely physical (adsorption or through van der waals forces) or could be due to the gel-networks formed by the bridging of EPS through divalent cations. At higher dosage of EPS than optimum increases the colloidal matter (with the addition of excess EPS), increase the viscosity and produces smaller size flocs which lead to decrease in the sludge dewaterability. Cumulative effect of all of these parameters was that the filter surface (used in the CST test) was blocked by these smaller sized flocs as well as the colloidal matter which increased CST. This phenomenon occurred at the excess EPS addition in all the jar test samples.

Different forms of EPS revealed distinct dewaterability patterns (Fig. 4.3, Table 4.1). In general, B-EPS and C-EPS had higher dewatering capacity as compared to S-EPS whereas overall, EPS (all forms) harvested from ST and ALT sludge exhibited higher dewaterability as compared

to that of ACT sludge (Table 4.1). B-EPS, C-EPS and S-EPS, obtained from same treated sludge (ST sludge, irrespective of solids concentrations) were able to increase dewaterability up to 44.8%, 52.2% and 31.2%, respectively. Whereas, B-EPS obtained from ST, ALT and ACT sludge (irrespective of solids concentrations) revealed maximum dewaterability of 44.8%, 40.6% and 30.9%, respectively. All other results of maximum dewaterability obtained by dosage of EPS from different treated sludge and solids concentrations are presented in Table 4.1.

The EPS produced by *Serratia* sp.1 mainly contained proteins and polysaccharides (Subramanian et al., 2010). It is reported that protein and polysaccharides i.e., main EPS components have an excellent affinity and ample binding sites with strong Van der Waals forces due to their high molecular weight (Xia et al., 2008). A crystal-linear structure of the EPS has the capability to bridge counter charged particles and out layer organic materials to form compact flocs. Strong adsorption ability between functional groups (carboxyl, hydroxyl and amino groups) present on the EPS and cations in the suspension made the resulting flocs more compact than the original ones, which further improved the dewaterability by expelling the water in the exterior and the interior the flocs (Salehizadeh and Shojaosadati, 2001). Moreover, hydrophobic properties of EPS are of specific importance. Settling and dewatering of the sludge having optimum concentration of EPS improves due to higher internal hydrophobicity (e.g., presence of hydrophobic amino acids in EPS protein) of the EPS (Higgins and Novak, 1997b; Urbain et al., 1993).

Crude C-EPS (pellet suspended in deionized water) used in the present study contained the sediment matrix of tightly bound EPS, cellular biomass and other organic and inorganic matters from the broth. Tightly bound EPS have been reported to have positive impact on the dewaterability due to their active participation in the formation of stabilized flocs. Whereas, crude S-EPS (supernatant of fermented broth) consists mainly of soluble or slime EPS, certain portion of loosely bound EPS and large amount of colloidal matter in the supernatant from the broth. Loosely bound EPS in the sludge reported to have significant and adverse effect on the sludge settleability and compressibility (Li and Yang, 2007; Yang and Li, 2009). The weak flocs formed by S-EPS which is in soluble form was responsible for its least effectiveness in dewaterability. Optimum dosage of EPS can make all the flocculated particles to become compact flocs. On the other hand, an excessive EPS concentration may prevent the small flocs to grow big because of the charge similarity. Most of the water will then remain trapped in the interior of the small flocs, making it difficult to dewater.

3.6 Zeta potentials (ζ) and flocculation mechanism

The zeta potentials (ζ) of different kaolin suspensions with or without calcium and EPS additions were all negatively charged (Fig. 4.4). Zeta potentials of kaolin suspensions (5 g/L) without addition of Ca^{2+} and EPS were -30.9±1.3 mV. Addition of EPS alone (without Ca^{2+}) to kaolin suspension had negative effect on flocculation activity (data not shown). This was due to the fact that both the EPS (all forms) and kaolin suspension were negatively charged. The surface charge of kaolin particles must be reduced to induce particle agglomeration. To reduce the electrical repulsion of identically charged layers around kaolin particles a polymer must carry an opposite charge to that of the kaolin surface. Zeta potential appears to be at or near zero in the conditioning with cationic polymers (Abu-Orf et al., 2001). Negative charge of kaolin suspensions was reduced from - 30.9±1.3 to -11.3±0.5 mV by addition of 150 mg of Ca^{2+}/L of kaolin suspension. Subsequent additions of EPS after mixing calcium ions with kaolin suspensions had very small change in the charge. Therefore, charge neutralization of the kaolin particles was achieved only by addition of Ca^{2+}. However, calcium addition followed by EPS revealed high flocculation activity and enhanced dewaterability. These results suggested that the specific interactions of EPS and calcium with kaolin particles can be supported by adsorption and bridging mechanism. Charge neutralization of the suspended negatively charged suspended solids and bridging of the particles can be accomplished by Ca^{2+} (Neyens et al., 2004; Xia et al., 2008).

Flocculation of the kaolin particles was completed by charge neutralisation and bridging mechanism in two steps. First step was the coagulation, in which individual calcium ions (positively charged) draw closer kaolin particles (negatively charged); through columbic attraction and calcium-kaolin complexes were formed. Calcium reduced the thickness of the diffuse double layer of adjacent kaolin particles, thus reducing the inter particle distance between clay particles (Mekhamer and Assaad, 1999). This phenomenon was evidenced by the reduction in zeta potentials of kaolin suspensions after calcium addition. These interactions were encouraged by rapid mixing in jar tests. The pH had important role in the process. To obtain full dispersion of the particles, it was necessary to raise the suspension pH to about 7.5. The suspended particles can be easily aggregated around neutral pH (7.5) (Chang et al., 1997).

Second step was the flocculation, in which polymers from added EPS matrix acts like a bridging agent of two or more calcium-kaolin complexes and reduces interparticle distances through the hydrogen bond mechanism (Mekhamer and Assaad, 1999). Bridging occurred after the

neutralized calcium-kaolin complexes get adsorbed on to EPS. Moreover, EPS adsorbed to vacant sites remained on the surface of kaolin or calcium particles. Many particles could be adsorbed onto a long molecular chain, and the particles adsorbed on the chain could be adsorbed simultaneously by other polymer chains, leading to the formation of three-dimensional flocs with a better settling capacity. Slow mixing allows the aggregates to combine two or more calcium-kaolin complexes to form larger floc particles, and settle down and precipitate as evidenced in the jar tests.

Addition of biopolymer (EPS) strengthened the floc structure, which reduced floc breakage and increased dewaterability. Improved dewaterability in the study indicated that the addition of EPS was improving the confined floc structure and strength (Wu et al., 2003). The oppositely charged particles reduced the particle surface charge density such that the attractive forces become effective, however, the results show that the flocculation activity and dewaterability were increased by EPS addition i.e., mainly through adsorption and bridging mechanism and least by charge neutralization. Flocs composed of coagulant and particles can be one to two orders of magnitude stronger than charge neutralized flocs (Jarvis et al., 2005). The results of the flocculation tests clearly showed that flocs formed through bridging of the EPS and calcium-kaolin complexes were reported stronger than the flocs formed through charge neutralization.

4 Conclusions

Higher concentration (2.3 g/L and 3.4 g/L) of extracellular polymeric substances (EPS) were produced by *Serratia* sp.1 in sterilized and alkaline-thermal treated sludge as compared to that produced (1.5 g/L) in acid-thermal treated sludge. Higher concentrations of EPS were produced in sludge solids of 17.0 g/L, 22.4 g/L and 29.8 g/L compared to that produced in the sludge solids of 37.3 g/L and 44.8 g/L. EPS combined with Ca^{2+} acted as conditioning agent. Broth and crude forms of capsular EPS had shown higher flocculation activity (79.1%) and better dewaterability (52.2%) respectively, than those of crude forms of slime EPS.

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Abbreviations

APHA	American Public Health Association
ALT	Alkaline-thermal treatment
ACT	Acid-thermal treatment
B-EPS	Broth EPS
C-EPS	Capsular EPS
Ca^{2+}	Calcium ions
COD	Chemical oxygen demand
CST	Capillary suction time
EPS	Extracellular polymeric substances
FA	Flocculation activity
Flocculation activity	(1-*S/C*) x100 (%), where *C* is control turbidity and *S* is sample turbidity
mg EPS/g of kaolin	mg of EPS added per gram of kaolin suspension in water
NTU	Nephelometric Turbidity Units
S-EPS	Slime EPS
ST	Sterilization
Serratia sp.1	*Serratia* sp. accession number EU031758
SS	Suspended solids
TSB	Tryptic soy broth
TS	Total solids
TSS	Total suspended solids
VS	Volatile solids
VSS	Volatile suspended solids
ζ	Zeta Potential

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Table 4.1 Maximum flocculation activity and maximum dewatering efficiency at optimum concentration for different types of EPS produced in different treated (ST, ALT and ACT) sludge at different SS concentrations (SS: 17.0, 22.4, 29.8, 37.3 and 44.8 g/L)

EPS from treated sludge	B-EPS				C-EPS				S-EPS			
	mg EPS/g of kaolin	FA_{max} (%)	mg EPS/g of kaolin	CST (%)	mg EPS/g of kaolin	FA_{max} (%)	mg EPS/g of kaolin	CST (%)	mg EPS/g of kaolin	FA_{max} (%)	mg EPS/g of kaolin	CST (%)
17.0-ST	0.7	79.1	0.7	34.7	3.5	59.2	5.2	52.1	3.7	31.6	2.5	03.7
22.4-ST	0.7	71.4	0.7	32.4	3.5	64.9	3.5	40.3	3.2	53.0	3.2	31.2
29.8-ST	0.9	74.2	0.9	44.8	2.8	72.3	2.1	32.7	1.5	48.1	1.5	23.6
37.3-ST	0.7	41.5	0.6	25.1	2.1	47.2	3.5	33.0	1.2	40.0	2.4	17.6
44.8-ST	0.8	33.5	0.8	17.0	1.4	51.0	1.4	31.8	0.6	40.0	1.9	13.0
17.0-ALT	0.9	46.8	0.3	14.7	4.9	51.4	4.9	31.5	2.3	07.7	4.6	14.3
22.4-ALT	0.9	60.3	0.9	40.6	3.2	66.2	2.6	43.0	3.1	49.4	43.9	21.8
29.8-ALT	1.0	48.2	0.9	32.1	2.6	65.2	3.3	34.5	3.1	48.1	40.3	34.5
37.3-ALT	0.7	38.0	0.7	23.0	1.3	45.6	2.5	31.8	1.8	11.8	11.8	25.8
44.8-ALT	0.3	37.8	0.3	19.1	1.3	31.4	2.0	20.9	-	-	0.9	20.9
17.0-ACT	1.4	44.7	0.1	22.5	1.7	50.2	0.4	12.5	0.2	8.7	0.5	21.3
22.4-ACT	0.3	44.1	0.3	27.0	1.2	43.9	1.2	23.6	0.8	43.9	1.2	21.8
29.8-ACT	0.4	42.2	0.4	31.0	2.8	40.7	2.8	31.5	1.1	40.7	1.1	25.4
37.3-ACT	0.4	23.4	0.4	23.6	1.2	31.9	2.7	22.4	0.6	31.9	0.9	22.4
44.8-ACT	0.1	22.8	0.3	19.7	0.8	26.4	1.1	17.6	1.0	26.4	1.05	17.0

Note:

FA: Flocculation activity

mg EPS/ g of kaolin: Optimised concentration of the EPS required per gram of kaolin suspension

17.0-ST; 22.4-ST; 29.8-ST; 37.3-ST ; 44.8-ST: 17.0-ALT; 22.4-ALT; 29.8-ALT; 37.3-ALT; 44.8-ALT; 17.0-ACT; 22.4-ACT; 29.8-ACT; 37.3-ACT and 44.8-ACT: Production of EPS in different treated (ST, ALT and ACT) sludge with different suspended solids concentrations (SS: 17.0; 22.4; 29.8; 37.3 and 44.8 g/L), respectively.

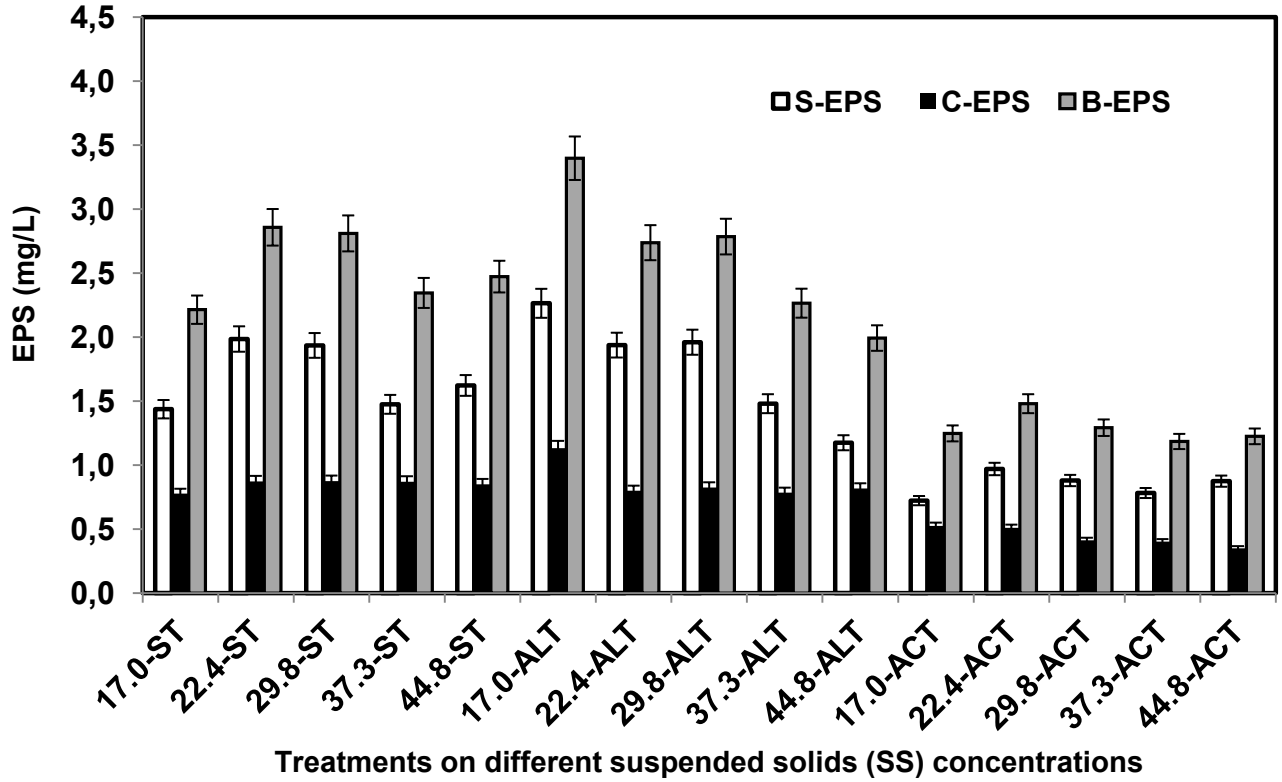

Figure 4.1 Production of EPS in treated (ST, ALT and ACT) sludge with different suspended solids concentration (SS: 17.0, 22.4, 29.8, 37.3 and 44.8 g/L)

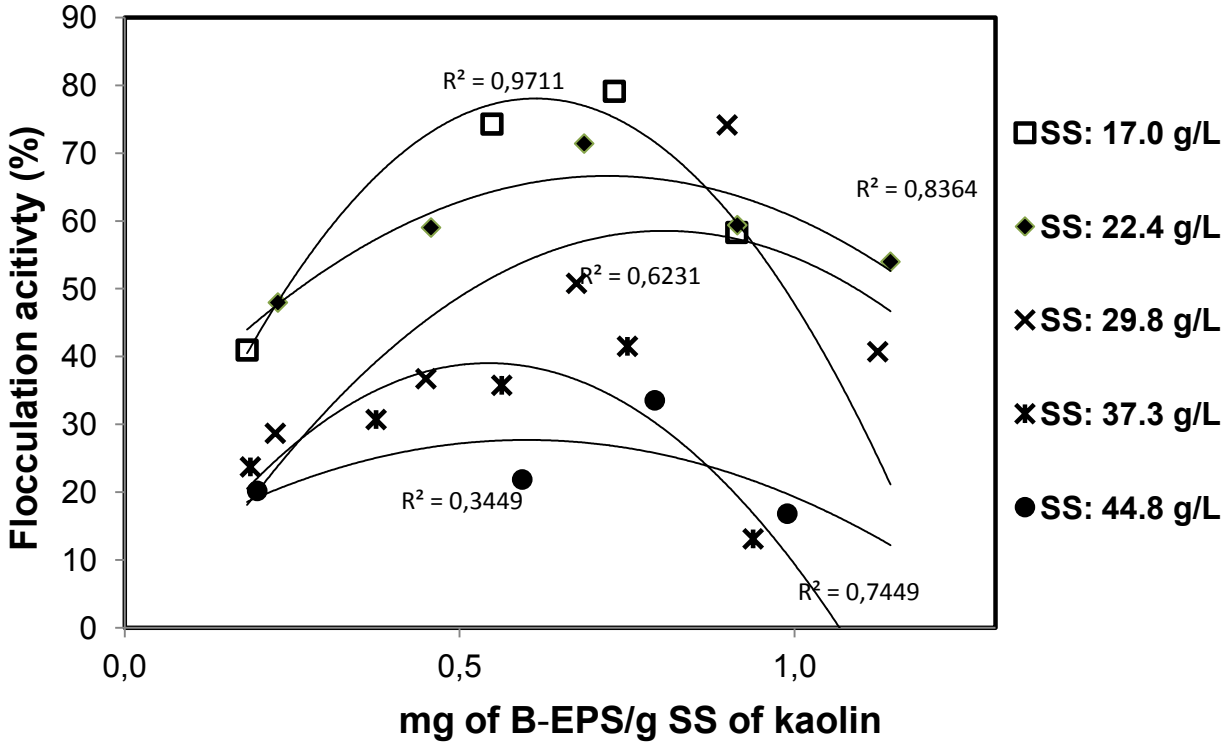

Figure 4.2 Variation in kaolin flocculation activity with different concentration of B-EPS (B-EPS produced in ST sludge at SS concentrations of 17.0, 22.4, 29.8, 37.3 and 44.8 g/L)

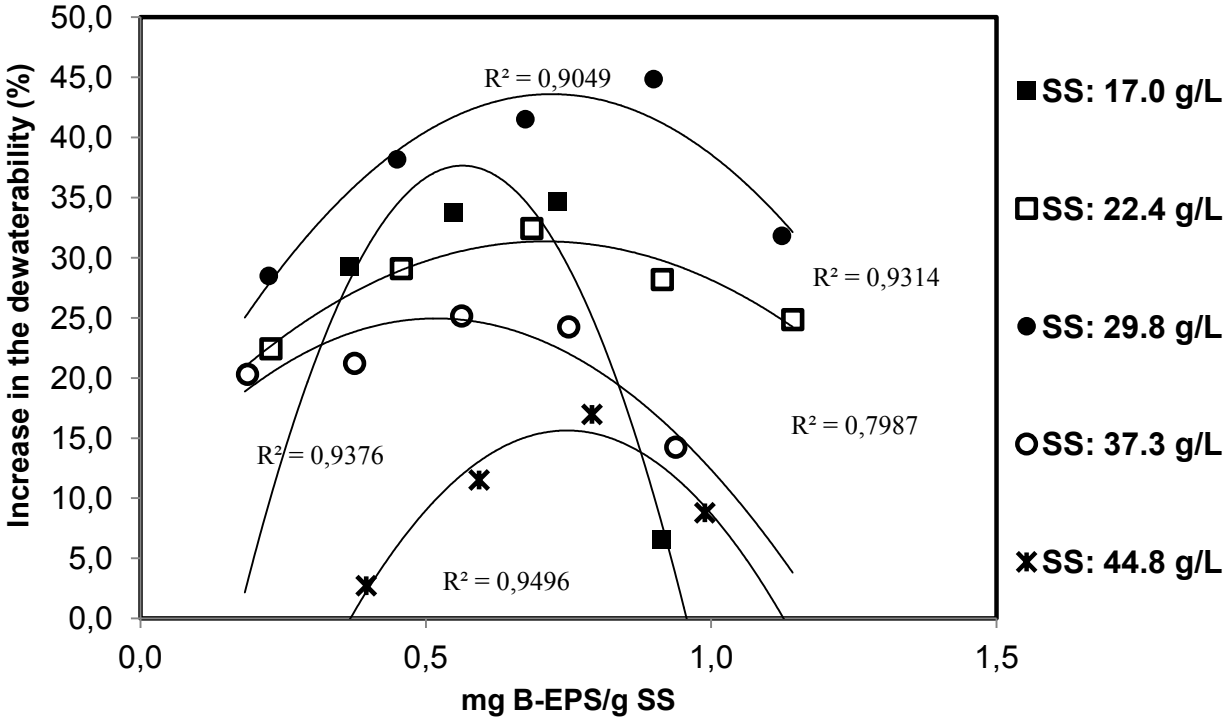

Figure 4.3 Variation of dewaterability (%) of the kaolin suspension with different concentration of B-EPS (B-EPS produced in ST sludge at SS concentrations of 17.0, 22.4, 29.8, 37.3 and 44.8 g/L)

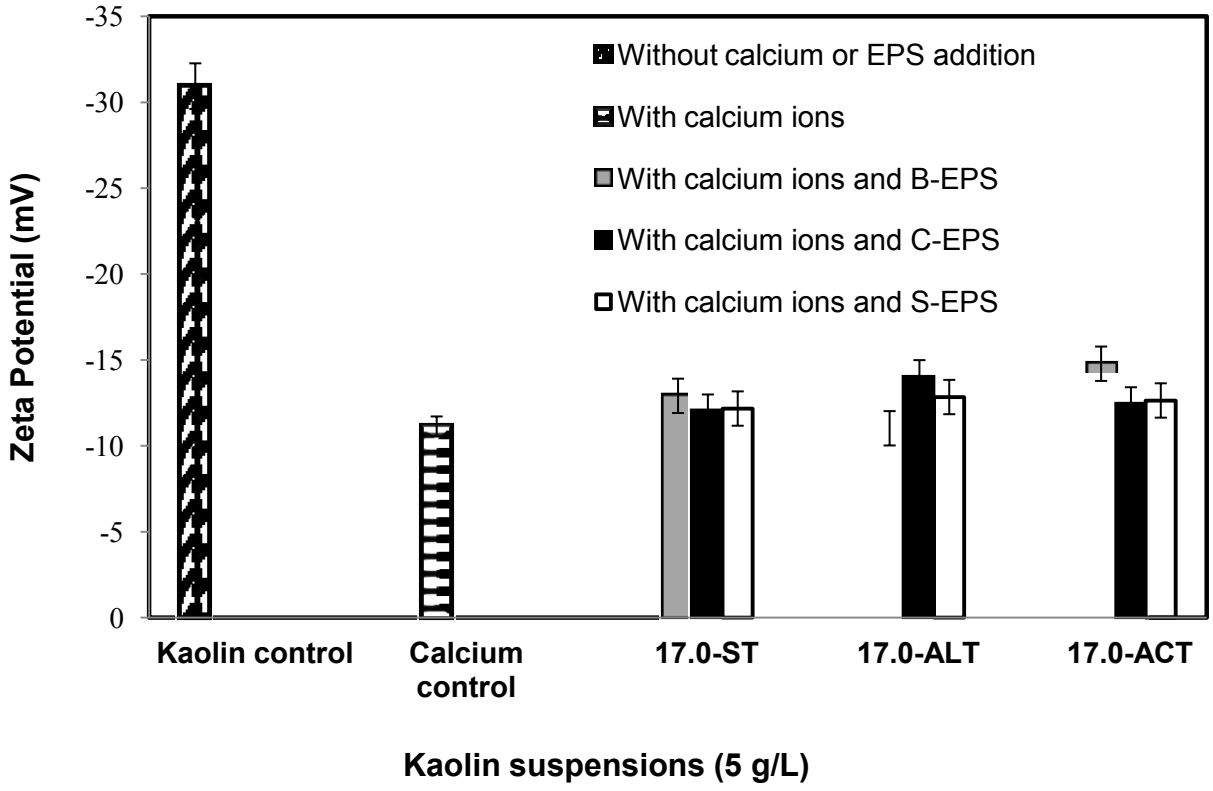

Figure 4.4 Zeta potential of the kaolin suspension (kaolin control and calcium control) and kaolin suspension (combined with calcium) with the addition of different EPS types (B-EPS, C-EPS and S-EPS) produced with different treatments (ST, ALT and ACT) at SS concentration of 17.0 g/L. (Note: **Zeta potential of the B-EPS, C-EPS and S-EPS was -28.9±0.3 mV, -30.2±0.4 mV and -27.7±0.9 mV, respectively)**

CHAPITRE 5

CULTURE MIXTE: LA PRODUCTION DE SPE, LA CINÉTIQUE ET L'APPLICATION AU TRAITEMENT DES EAUX NATURELLES ET USÉES

PARTIE 1

BIOPOLYMERS PRODUCTION BY MIXED CULTURE AND THEIR APPLICATIONS IN WATER AND WASTEWATER TREATMENT

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RÉSUMÉ

Treize souches bactériennes productrices des substances polymériques extracellulaires (SPE) ont été cultivées (comme culture pure/mixte) dans la boue stérilisée (matières en suspension: 25 g/L). La culture mixte a produit des concentrations plus élevées de SPE (4,9 g/L) par rapport à celle de la culture pure (2.7-3.7 g/L), respectivement. Les SPE extraites ont été examinées pour leur activité de floculation (élimination de la turbidité et la déshydratation) en utilisant la solution de kaolin avec des ions Ca^{2+}. Le bouillon (B-SPE) à revélé une activité de floculation de kaolin élevée (91,2%) à des concentrations très faibles (0,8 mg B-SPE/g kaolin) et elle était comparable à celle du polymère chimique, Magnafloc-155 (90,4% à 0,2 mg/g de kaolin). Le B-SPE a présenté également une très bonne activité de floculation (élimination de la turbidité %) dans l'eau de la rivière (93,5%), dans les eaux usées municipales (91,7%) et des eaux usées de brasseries (81,8%). L'étude a révélé que le consortium de culture mixte pourrait être utilisé pour la production des floculants efficaces

MOTS-CLÉS: Biopolymères; Bioflocculants; SPE; Floculation; Culture mixte; Boues des eaux usées; Traitement des eaux usées; Traitement de l'eau.

ABSTRACT

Thirteen extracellular polymeric substances (EPS) producing bacterial strains were cultivated (as pure/mixed culture) in the sterilized sludge (suspended solids: 25 g/L). The mixed culture produced higher concentrations of EPS (4.9 g/L) as compared to that of the pure culture (2.7-3.7 g/L), respectively. The harvested EPS were examined for their flocculation performance (turbidity removal and dewatering) in the jar tests using kaolin suspensions with Ca^{2+}. Broth (B-EPS) revealed high kaolin flocculating activity (91.2%) at very low concentrations (0.8 mg B-EPS/g kaolin) and it was comparable to the chemical polymer, Magnafloc-155 (90.4% at 0.2 mg/g kaolin). B-EPS also exhibited very good flocculation performance (turbidity removal %) in river water (93.5%), municipal wastewater (91.7%) and brewery wastewater (81.8%). The study revealed that the mixed culture consortium could be used for the production of highly efficient flocculants.

KEYWORDS: Biopolymers; Bioflocculants; EPS; Flocculation; Mixed culture; Wastewater sludge; Wastewater treatment; Water treatment.

1 Introduction

Recently there is growing interest in developing biopolymers that are eco-friendly, economical, and sustainable (More et al., 2010). Among different microbial polymers, bacterial extracellular polymeric substances (EPS) are the least explored (Patil et al., 2011). The EPS are biopolymers produced due to active secretion by cells, cell lysis and/or cell surface material shedding. The EPS matrix also contains the organic or inorganic matters that are adsorbed from the environment (Wingender et al., 1999). The EPS possess special properties (adhesion, sorption capability), and therefore, EPS play important role in the wastewater and sludge settling and dewatering. Recently, the potential of EPS as a flocculating material has been explored (Luvuyo et al., 2013, Patil et al., 2011, Wang et al., 2011, Zhang et al., 2007a). There is scarcity of comprehensive studies about the most suitable and highly flocculating bacterial EPS in comparison to the chemical coagulants. The studies on the application of EPS in water and wastewater treatments are also very limited (Li et al., 2013, Li et al., 2009, Gong et al., 2008). Low flocculating capability compared to the chemical polymers and higher production costs due to expensive culture media are the main preventive factors for commercial application of the EPS. However, EPS are biodegradable and therefore the solids produced by the use of EPS in flocculation applications can be safely disposed of in the environment (Subramanian et al., 2010). Thus, the search for microorganisms with better EPS producing abilities, high flocculating activity of the EPS and reduced production costs continues to be the principal goal of the researchers in this field.

The majority of the EPS studies reported so far have been carried out using pure cultures. There are few EPS studies using mixed culture consortium (Luvuyo et al., 2013, Wang et al., 2011, Zhang et al., 2007a). Symbiotic relationships between the mixed culture microorganisms expectedly increase the substrate utilization and the EPS yield. The mixed cultures are also important because such microbial relationships (e.g., biofilms) are commonly present in the natural systems. Most of the EPS synthesis studies have used either synthetic or conventional medium. Cost effective culture medium with higher concentrations of EPS with high flocculation activity are urgently required to apply commercially. In order to develop a cost effective process, wastewater sludge could be used as a raw material to cultivate the culture. Wastewater sludge is a good source of carbon, nitrogen, phosphorus and other nutrients (More et al., 2012a). Moreover, usage of flocculants derived from bacterial cultures grown in the wastewater sludge represents an important development in sustainable environmental technology since it focuses on the utilization of wastes for the treatment of the wastes.

In the present study, mixed culture was cultivated in the sterilized sludge to produce EPS. The EPS production, chemical characterization and their kaolin flocculation potential have been investigated. The EPS production by mixed culture and their flocculation performance was compared to the pure culture. The flocculation performance of EPS was also compared to the conventional chemical polymer to ascertain their field applicability. Applications of EPS for the treatment of river water, municipal and brewery wastewater were also studied.

2 Materials and methods

2.1 Wastewater sludge

Secondary wastewater sludge (without chemical polymers) was collected from Municipal water resource recovery facility at Québec city, Canada. The sludge was first sedimented for 1 h and concentrated sludge was collected by discarding supernatant. The wastewater sludge characteristics are presented in Table 5.1.1. The sludge was stored at 4^{o}C for further use.

2.2 Microorganisms

Thirteen EPS producing bacterial strains (nine of *Bacillus*, two of *Serratia* and two of *Yersinia*) were used (More et al., 2012b, Subramanian et al., 2010). The bacterial strains used in the study were EU031753 (*Bacillus* sp. 1), EU031754 (*Bacillus* sp. 2), EU031755 (*Bacillus* sp. 3), EU031756 (*Bacillus* sp. 4), EU031759 (*Bacillus* sp. 5), EU031760 (*Bacillus* sp. 6), EU031764 (*Bacillus* sp. 7), EU031767 (*Bacillus* sp. 8), EU031769 (*Bacillus* sp. 9), EU031758 (*Serratia* sp. 1), EU031768 (*Serratia* sp. 2), EU031761 (*Yersinia* sp. 1) and EU031763 (*Yersinia* sp. 2). These bacterial strains were previously isolated from the secondary wastewater sludge (Municipal water resource recovery facility, Québec city, Canada). These bacterial strains were selected in the study based upon their ability to grow and produce EPS in the sludge (More et al., 2012a). These bacterial strains had quite distinct behavior of producing EPS when cultivated in the sludge. The EPS producing capabilities of these strains also were different even among the strains of the same species. Produced EPS also had quite distinct flocculation characteristics for all the strains irrespective of the species. Metabolic properties of these strains were responsible for these variations (More et al., 2012a). This clearly indicated that the single/pure strains have metabolic limitations in utilizing the carbon, nitrogen and other nutrients from the sludge. To catch on these leftover nutrients and to increase the EPS production in

sludge, the mixed culture was considered as the best way. All the bacterial strains were grown over tryptic soy agar (TSA) plates and stored at 4^{o}C and sub-cultured fortnightly.

2.3 Sludge pretreatment

The raw sludge samples of 25 g/L were sterilized by autoclaving (steam sterilization) at 121^{o}C for 15 min (More et al., 2012a). Thermal pretreatment of the sludge was the necessary step for the EPS production. Pretreatment increases the biodegradability of the sludge by disintegrating the organic matter and organisms that are originally present in the raw sludge. After sterilization, the sludge samples were cooled to room temperature and adjusted to pH value of 7.0 using 1 M NaOH. The pretreated sludge samples were used as raw material for culturing and EPS production.

2.4 EPS Production

The EPS production by mixed and pure cultures was carried out in the sterilized sludge. The pre-cultures of individual bacterial strains were prepared in two steps, respectively. In the first step, pure cultures were aseptically transferred from TSA plates to 500 mL Erlenmeyer flasks containing 100 mL of tryptic soy broth (TSB) and independently incubated at 30^{o}C and 150 rpm for 12 h. In the second step, the TSB grown cultures were reinoculated (3% v/v) in the sterilized sludge (100 mL) and then independently incubated for 12 h in an orbital shaking incubator. To prepare the preculture of the mixed bacterial consortium, preculture (prepared in sterilized sludge) of each bacterial strain (1% v/v) (mixed together) were first inoculated in the sterilized sludge (100 mL) followed by incubation for 12 h. This pre-culture of the consortium or mixed culture, was transferred (3% v/v) to the sterilized sludge (150 mL) to produce EPS and then incubated for 96 h. The initial cells concentration of the inoculum was 6.70 x 10^{9} CFU. To study the time course of EPS production, broth samples (2 mL) were taken out at each 6 h for total cells counts. EPS from broths were harvested at each 12 h of incubation. To compare EPS synthesis by mixed culture with the pure culture, the same inoculum amount was used for the cultivation, respectively. The preculture (of pure culture) prepared in second step was inoculated (3% v/v, an equivalent amount to the mixed culture inoculum) individually in the sterilized sludge (150 mL). The initial concentrations of inoculums were 5.71, 2.95, 5.01, 5.09, 6.32, 5.40, 7.33, 7.24, 7.60, 5.90, 3.41, 6.35 and 2.62 x 10^{9} CFU for *Bacillus* sp. 1-9, *Serratia* sp. 1-2 and *Yersinia* sp. 1-2, respectively. The inoculated samples were then incubated for 72 h. The

fermented broths at 72 h incubation were used for EPS harvesting, flocculation tests and further analysis.

2.5 EPS Harvesting

To do quantitative, qualitative and comparative analysis of the EPS produced in sterilized sludge, the EPS harvesting was carried out according to More et al. (2012a,b). To harvest EPS, broth samples were centrifuged at 6000g for 15 min at 4^{o}C to obtain supernatant (crude slime EPS or crude S-EPS) and pellets (crude capsular EPS or crude C-EPS) with bacterial cells along with the residual sludge material (More et al., 2012a,b). To determine the S-EPS, the crude S-EPS was precipitated by adding 1:2.2 volumes of absolute chilled ethanol followed by storing the mixture at -20^{o}C overnight. The precipitates were collected by centrifugation at 6000g for 15 min at 4^{o}C. To measure dry mass of S-EPS, precipitates of S-EPS were first dried at room temperature in laminar hood for 6 h. Air dried samples were then heated at 105^{o}C for 24 h and dry mass were measured as S-EPS (APHA, 2005). For protein and carbohydrate analysis, precipitates of S-EPS (before heat drying) were resuspended in deionised water (to initial broth volume). To determine C-EPS, the crude C-EPS was resuspended in deionized water (to initial broth volume). The resuspended crude C-EPS were heated at 60^{o}C in water bath (with shaking 30 rpm) for 30 min to release C-EPS followed by centrifugation at 6000g, 4^{o}C for 15 min (More et al., 2012a,b). The supernatant (containing C-EPS) was used to precipitate C-EPS. The dry mass content of protein and carbohydrates of C-EPS was measured using the same procedure as for S-EPS. Sum of dry mass of S-EPS and C-EPS (measured above) was denoted as B-EPS.

2.6 Kaolin Flocculation Tests

A series of standard jar tests were performed to evaluate the flocculation performance of different flocculants. Kaolin clay (K2-500, USP, Fisher scientific, US) suspensions (5 g/L in deionized water) were used as a standard suspension. Previously optimized jar test conditions were used to evaluate and compare the flocculation performance of different flocculants (More et al. 2012a, b). During jar tests, first rapid mixing of suspensions was carried out at 175 rpm for 3 min to allow homogeneous dispersion of solids and to foster interactions of the solids. During the rapid mixing, 150 mg Ca^{2+}/L kaolin solution was added followed by addition of EPS. Calcium ions participate in charge neutralization of kaolin particles and the added EPS participate in

bridging of neutralized kaolin particles in presence of calcium (More et al., 2012a). Different types of EPS such as the broth (crude B-EPS, 1 mL), the supernatant (crude S-EPS, 1 mL) and the re-suspended pellets (in deionised water to initial broth volume, called crude C-EPS, 5 mL) were employed in the jar tests unless and otherwise stated. The dosages of different type of EPS were selected based on the dosage of each type of EPS that gave maximum flocculation performance in the series of previous jar tests carried out using different forms of EPS. In these tests, in general, fermented broth contains approximately five times less concentration of C-EPS than the S-EPS. Calcium stock solution was prepared by dissolving equivalent quantity of anhydrous $CaCl_2$ (solid pellets) in deionised water to make a final concentration of 25 g of Ca^{2+}/L). After rapid mixing, pH value of the suspensions was adjusted to 7.5 and slow mixing at 75 rpm was allowed for 30 min to ensure flocculation. The pH value of the suspensions (mixture of kaolin-calcium) was adjusted to 7.5 because at slightly alkaline condition, highest flocculation was observed. After mixing samples were transferred to measuring cylinders of 500 mL for 30 min settling. After settling, the supernatant and the sediments of the samples were used to measure flocculation activity (FA) and capillary suction time (CST), respectively. All the tests were carried out in triplicates and the average values were presented (with standard error less than 5% of the mean).

To determine flocculation activity or FA, turbidity of the samples supernatant was measured using Micro 100 Turbidity meter, Hach Company. FA measure was based on the relative decrease in turbidity of suspension after settling (More et al., 2012a, b). FA was calculated according to the equation 5.1.1 described below.

$$FA = (1-S/C) \times 100\ (\%) \qquad (5.1.1)$$

Where

C = Control turbidity (NTU), kaolin suspension without addition of calcium and EPS was the control, unless otherwise stated.

S = Sample turbidity (NTU)

Kaolin suspension without EPS was the control. For quantifying the effects of EPS on dewaterability, Capillary-suction-time (CST) of the settled kaolin sediments were measured. CST was determined by the CST instrument (Triton electronics, model 304 M CST, Dunmow, Essex), using a 10-mm diameter reservoir. Lower CST value as compared to the control CST indicated better dewaterability (More et al., 2012a). The sediments from jar test samples without

EPS addition was the control. The increase in the dewaterability was calculated according to equation 5.1.2.

$$\text{Increase in dewaterability (\%)} = (1-A/B) \times 100\ (\%) \qquad (5.1.2)$$

Where

B = CST of the control (s), kaolin suspension without addition of calcium and EPS was the control, unless otherwise stated.

A = CST of the sample (s)

Flocculation activity of EPS Produced at Different Incubation Time. Jar tests were carried out similarly as discussed earlier to investigate flocculation capabilities of EPS produced at different incubation time (12, 24, 36, 48, 60, 72 and 96 h) by mixed culture, respectively.

Comparison of Flocculation Performance of EPS Produced by Mixed Culture to that by the Pure Culture. To compare the performance of mixed culture EPS with pure culture EPS, jar tests were performed similarly as described earlier using broth (72 h incubation) produced by mixed and pure cultures, respectively. One mL of broth from each culture (mixed and pure) were employed in the jar tests unless and otherwise stated.

Comparison of Flocculation Performance of EPS Produced by Mixed Culture to the Conventional Chemical Polymer. The flocculation performance of EPS was investigated against a conventional chemical polymer, Magnafloc-155 (Anionic Granular Grade Polymer, CIBA Specially Chemicals, Virginia, USA). Magnafloc-155 is presently used for secondary wastewater flocculation and sludge dewatering operations at Municipal water resource recovery facility, Quebec City. The sludge samples were collected from the same facility for the present study. Magnafloc-155 (white powder) was dissolved in deionised water to obtain (1 g/L) stock solution. For comparison, different forms (broth: B-EPS, crude slime: S-EPS and crude capsular: C-EPS) of EPS were used. The EPS used in this study was harvested from the broth sample (at 72 h) that revealed a highest EPS concentration and the highest flocculation activity in previous step, respectively. The series of independent jar tests were conducted similarly as described earlier using different forms EPS and Magnafloc-155, respectively. In these tests, optimum concentration of each flocculant, required for obtaining highest flocculation activity was determined.

Thermal stability of the EPS Produced by Mixed Culture. During storage of the produced EPS (in broth), the biochemical properties could change because of its biological origin and

environmental temperature. Therefore, the effects of temperature on the FA of B-EPS were examined by heating/cooling the crude EPS at different temperatures (-20, -4, 4, 23±1, 40, 60, 80 and 121°C) for 30 min. For heating at 40, 60 and 80°C, fermented broths were placed in a water bath for 30 min, respectively. For heating at 121 °C, EPS were autoclaved (steam sterilization) at 121°C for 30 min. After the respective treatments, EPS samples were examined for their kaolin FA at room temperature by performing jar tests as discussed earlier. In jar tests, B-EPS (2 mg B-EPS/g kaolin) from each of the above treated samples were used.

2.7 Applications of EPS produced by mixed culture to the treatment of river water, municipal and Brewery wastewater

The river water was collected from Saint Charles river (Québec city, Canada) and its characteristics are presented in Table 5.1.2. The wastewater (without chemical polymers) was collected from municipal water resource recovery facility (Québec city, Canada). Brewery wastewater was collected from La Barberie (Québec city, Canada). The municipal and brewery wastewater characteristics are presented in Table 5.1.2. Preliminary jar tests were carried out to select the calcium concentration. In these preliminary jar tests, different calcium concentrations (50, 100, 150, 200, 250 and 300 mg/L) were added to river water, municipal and brewery wastewater, respectively. Calcium stock solution was prepared by dissolving equivalent quantity of anhydrous $CaCl_2$ (solid pellets) in deionised water to make a final concentration of (25 g of Ca^{2+}/L). The supernatant turbidity and chemical oxygen demand (COD) were measured to evaluate the effect of calcium. Zeta potential of the suspensions was also measured at different calcium concentration.

To investigate the potential application of EPS as a flocculant material, the EPS produced (all forms) obtained at the incubation time having highest FA and EPS concentration was used in jar tests. Jar tests were carried out as described earlier with calcium (selected from preliminary tests) and EPS (all forms). Different dosages of the broth (B-EPS), the supernatant (crude S-EPS) and the re-suspended pellets (crude C-EPS), were employed in all jar tests to investigate the effect of EPS concentration. To compare the performance of EPS (all forms), similar jar tests were carried out using different concentrations of Magnafloc-155 along with calcium ions.

2.8 Analysis

Characteristics of sludge, such as pH, total solids (TS), suspended solids (SS), volatile solids (VS), volatile suspended solids (VSS), chemical oxygen demand (COD), turbidity (NTU) and other physical-chemical properties were determined using Standard Methods (APHA, 2005). Viscosity (mPas) and zeta potentials (ζ) were measured using Viscometer (DV DV-II PRO + (Brookfield Engineering Laboratories, Inc., Stoughton, MA, USA)) and Zetaphoremeter (Zetaphoremeter IV, Zetacompact Z8000, CAD Instrumentation, France), respectively. The concentrations of metals in the sludge were analyzed by ICP-AES (Varian Vista-AX CCO, Palo Alto, Ca) after partial digestion of dried, powdered sludge. About 0.5 g of dried sludge samples were digested using analytical grade nitric acid (50% w/w, 20 mL) and hydrogen peroxide (30% w/w, 9 mL). Quality control was also performed with certified samples (multi-element standard, catalogue number 900-Q30-002 and 900-Q30-101) to ensure conformity of the measurement values. Measurement of cells concentration as colony forming units (CFU) was carried out by standard agar-plate technique. The protein content of the extracted S-EPS and C-EPS was determined using bovine serum albumin as a standard (Lowry et al., 1951); the carbohydrate content of the extracted S-EPS and C-EPS was determined by the phenol-sulfuric acid method using glucose as the standard solution (DuBois et al., 1956). The sum of protein content of S-EPS and C-EPS were denoted as the protein content of B-EPS. Similarly, the sum of carbohydrate content of the S-EPS and C-EPS were denoted as the carbohydrate content of B-EPS.

3 Results and discussion

3.1 EPS Production by mixed Culture

The time course of growth and EPS production by mixed culture (of 13 bacterial strains) in sterilized sludge is shown in Fig. 5.1.1. The mixed culture revealed typical sigmoid batch culture growth curve in sterilized sludge. Cells growth profile did not show any occurrence of apparent lag phase and started directly their growth curve in exponential phase. This indicated that the mixed culture was well adapted to the growth in sterilized sludge. Mixed culture attained maximum cells concentration (1.76-1.78 x 10^{11}) was observed at 60-72 h of incubation. During incubation, the cells concentration was increased by two log cycles in first 36 h, whereas there was small increase (1.63 to 1.78 x 10^{11}) in the cells concentration for incubation from 36 h to 72

h. Wastewater sludge is a very complex mixture of organic and inorganic matter and therefore, it is extremely difficult to determine the exact substrate utilization profile. However, dry weights of the extracted EPS (the broths samples collected at different incubation time) represented the amount of EPS produced by the mixed culture in sterilized sludge. During 96 h of incubation, the B-EPS concentration increased with total cells (CFU/mL). The EPS production rate was maximum (0.127-0.123 g/L/h) at 12-24 h of incubation, whereas it decreased with further incubation. The EPS production rate was 0.058, 0.024, 0.021 and 0.013 g/L/h at 36, 48, 60, 72 h of incubation, respectively. A maximum B-EPS concentration of 4.9 g/L was obtained at 72 h. During the same period, S-EPS of 4.1 g/L and C-EPS of 0.8 g/L were obtained. The raw sludge had very low concentrations of EPS (less than 13 mg/g SS). Up to 72 h of incubation, B-EPS and S-EPS concentration varied proportional to cell count. During this period, EPS production was growth associated. These observations were in accordance with the unified theory for EPS production proposed by Laspidou and Rittmann (2002). As per this theory, the formation of EPS was growth associated and was produced in direct proportion to substrate utilization. A small decrease in S-EPS concentration from 4.1 to 3.8 g/L was observed in late stationary phase (72-96 h). This is concurrent with the observations of Ni and Yu (2010). They observed that the total EPS concentration did not change significantly even after the depletion of substrate. Small decrease in S-EPS concentration could be attributed to carbon limitations and enzymatic degradation of EPS. During substrate depletion condition, EPS might undergo the decay process and results in an EPS loss.

The initial broth pH value decreased from 7.0 to 6.16 during the first 24 h (Fig. 5.1.2). The pH value increased again to 6.82 (at 24 to 60 h). The drop in pH value suggests that organic acids were produced by mixed culture, which was consumed later resulting in pH value increase. The metabolic activity of the mixed culture influenced the pH value of sludge (Scheidle et al., 2011). It is widely known that medium pH changes (increase or decrease) during fermentation. The sludge pH value was not controlled during incubation. However, the pH value of the medium stabilized at 6.82 after 48 h of culture. A continuous increase in the viscosity (from 12.3 to 19.3 mPas) of during fermentation was observed up to 72 h (Fig. 5.1.2). The viscosity was decreased from 19.3 to 18.8 mPas (at 72 to 96 h). The viscosity of sterilized sludge (without inoculation) was 12.3±0.07 mPas. The maximum increase in the broth viscosity was 56.9%. When mixed culture, comprising of same bacterial strains, was grown in TSB, EPS produced was visibly viscous as shown in Fig. 5.1.3. The fermented broth was a highly sticky matrix. The presence of more than one type of exopolysaccharides in mixed cultures leads to increased adhesive properties (Wingender et al., 1999). Thus, the increase in viscosity of fermented broth was

mainly attributed to EPS concentration. As EPS concentration had increased with incubation time, the viscosity of the broth is also proportional to the incubation time. The viscosity of the broth was proportional mainly to the EPS concentration and relatively less due to growth. These observations were already reported in previous work by More et al. (2012).

3.2 Comparison of EPS production by mixed culture to the pure culture

The concentration of B-EPS (4.9 g/L) produced by the mixed culture (of 13 bacterial strains) grown in sterilized sludge (at 72 h), was higher than that the pure culture (2.7-3.7 g/L) (Table 5.1.3), under similar fermentation conditions. The mixed culture produced 1.3-1.8 time higher B-EPS concentrations as compared to the pure culture. The increase in broth viscosity with B-EPS concentration was observed in all the cultures (Table 5.1.3). In case of pure cultures, the increase in viscosity was in the range of 8.9-32.5%, respectively. Subramanian et al. (2010) reported that mixed culture produced relatively lower EPS concentrations (2.4 g/L) than the pure cultures (3.0 g/L) while grown in synthetic mineral medium. However, the mixed culture (0.1% v/v secondary sludge consortium as inoculum) used by Subramanian et al. (2010) consists of all types of microorganisms (non-EPS as well as EPS producing microbial species). The bacterial strains (sludge isolates) used in the present study have distinct biochemical characteristics and different capacity to produce EPS in sterilized sludge (More et al., 2012b). Higher concentrations of B-EPS produced by mixed cultures in the present study could be attributed to the symbiotic relationship between different bacterial strains in the consortium. Similar results were obtained by Kurane and Mastuyama (1994), where they observed that mixed culture of four EPS producing bacterial strains (*Oerskovia*, *Acinetobacter*, *Agrobacterium* and *Enterobacter*) had higher yield of EPS as compared to the pure cultures in the same synthetic medium. Zhang et al. (2007a) also found that the mixed culture consortium of *Staphylococcus* sp. and *Pseudomonas* sp. were able to produce higher concentrations of bioflocculants than the individual strains while cultivated in brewery wastewater. According to Wingender et al. (1999), during EPS production by mixed cultures, the exopolysaccharides secreted by some bacterial strains influences other strains to promote EPS synthesis. The degradable organic compounds present in wastewater sludge are of different forms. The sludge contains, easily biodegradable, biodegradable and difficult to biodegrade materials. Our previous study (More et al. 2012) had revealed that all the thirteen strains had distinct behaviour of EPS production. The present study also showed that the EPS producing capabilities were different even among the strains of the same species. Produced EPS also had quite distinct flocculation characteristics for all the

strains irrespective of the species. Metabolic properties of these strains were responsible for these variations. This clearly indicated that the single/pure strains have metabolic limitations in utilising the carbon, nitrogen and other nutrients from the sludge. These bacterial strains may follow different metabolic pathways due to utilization of different forms of available carbon, nitrogen and other nutrients present in the sludge. To catch on these leftover nutrients and to increase the EPS production in sludge, the mixed culture was considered as the best way. The results of the present study using mixed culture have positively supported this hypothesis. The mixed culture might assimilate these nutrients easily and hence produce higher concentration of EPS than the individual strains. Therefore, the symbiotic relationship might have helped these strains in consortium to grow and produce higher concentrations of EPS. The zeta potential (-28.98±1.09 mV) of the fermented broth of mixed culture was relatively less than the pure cultures (-35.18±4.18 mV) (Table 5.1.3). According to Zhang et al. (2007b), the cell surface hydrophobicity was positively correlated to an increase in EPS protein content. The protein contents of EPS produced by mixed culture growing in sludge were higher than the carbohydrate contents (Table 5.1.4). There was a strong correlation between the proteins to carbohydrate ratio and the surface charge values of sludge. The increase in protein content (of EPS) might decrease the surface negative charge of bacterial cells and reduce the electrostatic repulsions between the cells and further favoring EPS production. The positively charged amino groups in proteins can neutralize the negative charge from carboxyl and phosphate groups and therefore can reduce the surface negative charge of the bacterial cells. Lower electrostatic repulsion between the cells encourages the growth of the microbial aggregates by accommodating immobilized cells and keeping them in close proximity. This allows increase in the cell to cell communication, nutrient transport and formation of synergistic micro-consortia that leads to the increase in the growth of the biofilms or EPS production (Wingender et al., 1999). Overall, the synthesis of EPS using mixed culture and sterilized sludge as production medium has advantages over the pure cultures in terms of producing higher concentrations of EPS.

3.3 Chemical characteristics of the EPS produced by mixed culture

There was a slight increase in protein and carbohydrate concentration in EPS up to 60 h of incubation (Table 5.1.4). Protein of 0.582 g/g S-EPS and 0.669 g/g C-EPS was observed at 72 h. Protein concentration was higher than the carbohydrates in all cases. Carbohydrate of 0.133 g/g C-EPS and 0.258 g/g C-EPS was observed at 60 h. S-EPS concentrations in broths were

higher as compared to the C-EPS (Fig. 5.1.1). Thus, the major portion of total EPS (S-EPS and C-EPS) protein (77-82%, w/v) and carbohydrates (67-68% w/v) were present in S-EPS. C-EPS contains 18-23% protein and 24-33% carbohydrates of the total EPS. It is widely reported that centrifuged pellet contains 80.7% (w/v) of total protein concentration present in sludge (Shao et al., 2009). In present study, C-EPS were extracted from pellets by excluding cells. Therefore, large portion of proteins could have remained in excluded cells.

EPS produced by pure cultures (using same bacterial strains as this study) in synthetic medium had 1.9-3.4 times higher carbohydrate concentrations as compared with the proteins (Subramanian et al., 2010). However, in present study, protein concentrations in EPS were higher than the carbohydrates (Table 5.1.4). This was mainly because of higher initial protein content in the sterilized sludge, which was the raw material used in the EPS production. Similar observations were reported by several other studies (McSwain et al., 2005, Zhang et al., 2007b). The concentration of protein and carbohydrates present in the EPS also depend on the type and concentrations of carbon and nitrogen sources used in growth medium (Hoa et al., 2003, Wang et al., 2013). Carbon to nitrogen ratio of raw sludge used in the present study was very low (8.8) and could also be the reason for higher protein content than carbohydrates in the EPS. The composition (carbohydrates and protein) of EPS is sensitive to the operational and environmental conditions. If certain culture condition is induced, then the nature of the EPS changes accordingly (Hoa et al., 2003). C/N ratio affect the microbial physiology, thereby affect the composition of EPS. At the low C/N ratio, the amount of nitrogen available in the sludge is high as compared to carbon. Microorganisms in the sludge flocs utilize this nitrogen in the synthesis of proteins and nucleic acids. However, major part of the carbon is utilized by microorganisms in the biomass synthesis, rather than EPS production. Thus, it leads to lower carbohydrate content in EPS than the protein (Ye et al., 2011). The Ca^{2+} concentration in raw sludge (used in the present study) found to be higher than the Na^{+}, which could be responsible for high protein concentration in the EPS (Higgins and Novak, 1997). The divalent cations such as Ca^{2+} bind extracellular protein within biological flocs through bridging negatively charged sites on biopolymers present on microbial surfaces and other biopolymer network. Calcium bind lectins within the floc matrix and lectins with their multiple binding sites may act to bind polysaccharides within the biopolymer network. The displacement of calcium ions by sodium ions reduces the binding of biopolymer within the floc, leading to a decrease in the bound polymer concentration (Higgins and Novak, 1997). In general, the composition of EPS is heterogeneous and varies based on many factors such as medium composition, growth phase,

extraction method and different process operation parameters (temperature, pH, agitation speed, cultivation time etc.) (Wingender et al., 1999).

3.4 Kaolin flocculation activity of different forms of eps produced by mixed culture (of 13 bacterial strains) using sterilized sludge

Different forms of EPS in the sample drawn at 72 h of incubation were first tested for their flocculation activity (FA) with addition of calcium ions to kaolin suspension. It was observed that the B-EPS had highest FA of 75.7% followed by 59.2% and 48.6% of FA for C-EPS and S-EPS, respectively. Addition of B-EPS had produced flocs of visibly big size (Fig. 5.1.4 and 5.1.5). The kaolin solution with B-EPS addition was very clear (good separation of kaolin particles from water) as compared to that without B-EPS addition (Fig. 5.1.4). Therefore, B-EPS was chosen for further flocculation studies. Addition of one mL of B-EPS (obtained at different incubation time) to kaolin suspensions in the presence of calcium ions (Ca^{2+}) enhanced FA. FA of B-EPS was increased from 33.1% to 75.7% with increase in incubation time from 12 h to 72 h (Fig. 5.1.6). Thereafter, a decrease in the FA was observed. A similar trend was observed for the dewatering characteristics. The increase in dewaterability by 48.1% (highest) was observed for B-EPS at 72 h. Increase in the FA obtained up to 72 h was directly attributed to the increase in B-EPS concentration (Fig. 5.1.1). Moreover, the chemical characteristics (mainly the composition of protein and carbohydrates) of the EPS obtained at different incubation times were different, as discussed earlier. FA variation was attributed to both the EPS concentration as well as to its chemical characteristics. This is concurrent with More et al. (2012a), where for similar EPS concentration, there was variation in FA and it was attributed to variations in EPS concentration and chemical characteristics. Zhang et al. (2007a) also reported that EPS produced by the mixed culture of *Staphylococcus* sp. and *Pseudomonas* sp. revealed high (96.8%) kaolin FA when combined with calcium, which was higher than the individual culture.

3.5 Comparison of flocculation performance of EPS produced by mixed culture to the pure culture

Significant variations in the FA (40.1-76.4%) was observed when calcium (150 mg/L) was added to kaolin suspension followed by addition of one mL of B-EPS synthesized by pure or mixed cultures (Table 5.1.3). Zeta potential of kaolin suspension (5 g/L) was -29.52±3.15 mV. When calcium was added to kaolin suspension, the zeta potential was changed from -29.52±3.15 mV to 13.50±1.46 mV. All the EPS (pure/mixed culture) exhibited negative zeta potential (Table

5.1.3). The negative surface charge of the EPS allows association of calcium to cross-link with the EPS/kaolin and provide greater binding force and promote aggregation phenomenon.

Higher FA was observed for EPS synthesized by mixed culture as compared to that of pure cultures. Among all the EPS tested, EPS synthesized by mixed culture, *Bacillus* sp.7, *Serratia* sp.1 revealed the highest FA of 76.4, 72.5 and 71.7%, respectively. Similar to the trend in FA, significant variation in dewaterability was observed when calcium (150 mg/L) was added to kaolin suspension followed by addition of 1 mL of B-EPS synthesized by pure or mixed cultures. B-EPS synthesized by mixed culture proved to be the most efficient to increase dewaterability as compared to that of pure cultures. Increase in dewaterability of 48.1% was observed for B-EPS synthesized by mixed culture whereas, whereas increase in dewaterability for B-EPS synthesized by pure cultures was between 16.9 to 36.8%. In case of mixed culture, 1 mL of broth dosage represented relatively higher quantity of EPS (2 mg B-EPS/g kaolin) as compared with that of pure cultures (1.0-1.5 mg B-EPS/g kaolin). Therefore, a better performance of B-EPS synthesized by mixed culture was mainly due to the presence of higher EPS concentration (rather than difference in characteristics of the produced EPS) as compared to that of pure cultures. The FA and dewaterability are highly dependent on the concentration of EPS (More et al., 2012a). Therefore, better performance of EPS synthesized by mixed culture as compared to that of pure cultures was also attributed to its better adhesive properties and relatively lowers surface charge.

3.6 Comparison of flocculation performance of eps produced by mixed culture (of 13 bacterial strains) to the conventional chemical polymer

Kaolin flocculation activity (FA) of different concentrations of EPS (all forms) (sampled at 72 h incubation) are presented in the Fig. 5.1.7. In general, FA increased with EPS (all forms) concentration, reached maximum and then decreased with further increase in EPS concentration. In case of Magnafloc-155, there was relatively less decrease in the FA with the addition of excess flocculant dosage. On the other hand, in case of EPS (all forms), there was significant decrease in the FA with the excess EPS concentration. This was due to increase in the colloidal matter with the excess EPS concentration, which decreased the FA. Because of this difference in decrease in the FA, there is difference between the FA profile of Magnafloc-155 and EPS. Thus, the similar graphical expression could not fit perfectly (R^2 = 0.78) in case of Magnafloc-155. This was repeated normal behavior during the jar tests. B-EPS revealed 91.2% (highest) FA at 0.8 mg B-EPS/g kaolin. FA of 88.5% and 76.8% were observed for C-EPS and

S-EPS, respectively. The crude S-EPS (broth supernatant) contain higher number of colloidal (non settleable) particles, which are originally present in the crude S-EPS. Colloidal particle increases the turbidity and decreases the FA. Thus, S-EPS revealed lowest FA value. According to Higgins and Novak (1997), flocculation decreases with increase in the colloidal particles concentration (in the range of 1-100 µm).

Maximum FA of 90.4% was observed at 0.2 mg Magnafloc-155/g kaolin. The results indicated that the B-EPS performance was comparable to the Magnafloc-155. However, relatively lower concentration was required to attain maximum FA for Magnafloc-155 as compared to the B-EPS. B-EPS represents the mixture of S-EPS, C-EPS and organic-inorganic sludge solids. The presence of non-flocculating materials in crude B-EPS could interfere with FA. FA of the B-EPS could be increased by producing higher concentrations of EPS in broth. The B-EPS concentration during cultivation could be increased by either optimizing production conditions (carbon, nitrogen, trace metals or environmental conditions) or by removing colloids from broth (by centrifugation) or by purification of B-EPS or by formulation (making dry powder of the EPS).

3.7 Thermal stability of the EPS produced by mixed culture (of 13 bacterial strains)

To investigate the effect of heating/cooling on its flocculation properties of EPS, broths were subjected to heating/cooling treatment. FA of 75.7, 76.0, 74.2 and 73.3% was observed for B-EPS with treatment at 4, 23, 40 and 60°C, respectively. This indicated that heating the broth (after EPS production) up to 60°C for 30 min did not appear to significantly affect the FA value, suggesting it to be stable up to 60°C. FA of 68.3% and 65.4% was observed after broth heated at 80 and 121°C for 30 min, respectively. For broth kept at -4°C and -20°C for 30 min followed by heating to room temperature, the FA of 68.5% and 63.4% was observed, respectively. Thus, the EPS appears to sustain its FA for temperature range 4 to 60°C. Yokoi *et al.* (1995) reported that FA was lost completely when the bioflocculants produced by *Bacillus* were heated at 100°C for 40 min. The thermal stability (up to 60°C) of the B-EPS produced by our bacterial consortium might be a result of the structure of polysaccharides, which is the backbone of EPS. The large quantities of carboxyl and hydroxyl functional groups in the polysaccharide could interact with each other to create a considerable amount of hydrogen bonds within the molecule, necessary for thermal stability of the bioflocculants. The FA of crude B-EPS decreased from 60 to 121°C due to the influence of high temperature on protein constituents (of B-EPS).

3.8 Applications of EPS produced by mixed culture (of 13 bacterial strains) to the treatment of river water, municipal and Brewery wastewater

Addition of EPS (all forms) without any cations to the river water, municipal wastewater and brewery wastewater had very little and/or negative effect on the turbidity (i.e., no change and/or increase in turbidity). The EPS (all forms), the river water, the municipal and brewery wastewater had negative zeta potentials (Fig. 5.1.8). To induce charge neutralization, calcium ions were added to the river water, municipal and brewery wastewater. A decrease in zeta potential of the suspensions with increase in the concentration of calcium ions was observed. Visible observation revealed that the small sized visible flocs were started to form when zeta potentials of river water, municipal wastewater and brewery wastewater were at -13.1, -12.6 and -14.5 mV, respectively. This phenomenon was observed at the calcium concentration of 50, 200 and 250 mg Ca^{2+}/L of river water, municipal wastewater and brewery wastewater. To attain the maximum flocculation performance of different water and wastewater, often zeta potential of the system is decreased in the range of -15 <zeta potential<-10 mV (Jefferson and Parsons, 2005). The water and wastewater are susceptible to small changes in the input conditions such as addition of flocculants or shear stress within this range of zeta potentials. In present study, addition of calcium ions leads to a reduction in zeta potential value and an increase in the turbidity removal. Therefore, the calcium concentrations at which the flocs formation was visible were chosen for jar tests to investigate the effect of EPS concentration on turbidity and COD removal.

River water treatment. Maximum turbidity removal of 93.5, 55.7 and 81.7% was observed at 2.0, 0.8 and 0.8 mg/L of B-EPS, S-EPS and C-EPS, respectively (Table 5.1.5). For excess EPS addition than the optimum, the river water became very turbid, especially in case of S-EPS. Flocs formed by S-EPS were visibly weaker (easily breakable by mixing) than the B-EPS and C-EPS. The turbidity removal of 93.9% was observed at Magnafloc-155 concentration of 0.2 mg/L. Further increase in Magnafloc-155 concentration up to 0.5 mg/L, the turbidity was increased. However, this turbidity increase was relatively less as compared to the EPS. Maximum COD removal of 52% was observed for B-EPS whereas; it was 48% for Magnafloc-155. The performance of B-EPS was comparable with Magnafloc-155. Li et al. (2009) reported that EPS produced by pure culture (*Bacillus licheniformis*) in synthetic medium revealed high turbidity (95.6%) and COD (61.2%) removal from river water than the chemical polymers (PAC and PAM). Their reported optimum bioflocculants concentration was 2.5 times higher than the present study. Similarly Gong et al. (2008) also reported that the bioflocculants produced by

Serratia ficaria revealed turbidity (84.2%) and COD removal (87.1%) turbidity from river wastewater. Their reported optimum bioflocculants concentration was 5 times higher than the present study. This clearly indicates the B-EPS had very high flocculation efficiency. The addition of (anionic) EPS had lead to the bridging of neutralized particles and cations to form large and dense flocs (Table 5.1.3). Increase in the density of EPS-calcium-particles increased the settlement of these particles and hence increase in the turbidity removal (More et al., 2012a). At optimum dosage of B-EPS, river water turbidity was reduced to 1.5 NTU. However, as per Health Canada (2003) treated drinking water must have turbidity less than 0.1 NTU at all times. Therefore, to achieve allowed turbidity limits, conventional sand filtration or membrane filtration steps are necessary after flocculation. The pH value of the river water was decreased from 7.85 to 7.4±2 during jar tests. This indicates that there was no pH value correction required for the treated water. The application of B-EPS to river water treatment has promising results, however further research is required to evaluate its field applicability.

Municipal wastewater. Maximum turbidity removal of 91.7, 74.5 and 86.9% was observed at 5.0, 4.2 and 0.7 mg/L of B-EPS, S-EPS and C-EPS, respectively (Table 5.1.5). The COD removal of 84.9, 60.6 and 85.8% was observed in case of B-EPS, S-EPS and C-EPS, respectively. The turbidity and COD removal of 97.8 and 87.2% was observed at Magnafloc-155 concentration of 0.8 mg/L, respectively. B-EPS concentration required for achieving more than 80% of turbidity and 70% of COD removal was 6.25 times higher than the Magnafloc-155. Li et al. (2013) reported that polysaccharide-based bioflocculants produced in sucrose medium by *Paenibacillus elgii* revealed municipal wastewater turbidity and COD removal of 83 and 68%, respectively at bioflocculants concentrations of 768.9 mg/L.

Brewery wastewater. Maximum turbidity removal of 81.8, 59.2 and 79.8% was observed at 12.4, 10.4 and 6.6 mg/L of B-EPS, S-EPS and C-EPS, respectively (Table 5.1.5). COD removal of 88.4, 67.6 and 87.6% was observed at 12.4, 10.4 and 5.0 mg/L of B-EPS, S-EPS and C-EPS, respectively. The turbidity and COD removal of 84.8 and 89.7% was observed at Magnafloc-155 concentration of 5.0 mg/L. The performance of Magnafloc-155 in brewery wastewater treatment was comparable to that of the B-EPS and C-EPS respectively. The optimum dosage of B-EPS was around 2 times higher than the C-EPS. B-EPS had relatively lower flocculation efficiency (with respect to the EPS concentration) as compared to the C-EPS. Gong et al. (2008) reported that the bioflocculants (S-EPS) produced by *Serratia ficaria* revealed 80.7% COD removal and 91.8% turbidity removal from brewery wastewater. The optimum bioflocculants concentration reported by Gong et al. (2008) was 4 mg/L, which was relatively lower than the present study. It

should be noted that, Gong et al. (2008) had used different carbon (lactose, glucose and ethanol) and nitrogen (beef extract, urea, peptone, $(NH_4)_2SO_4$ and yeast extract) sources for the production of bioflocculants. On the other hand, in the present study only wastewater sludge was used as a carbon and nitrogen source for the bioflocculants production.

4 Conclusions

The mixed culture had produced higher concentrations (4.9 g/L) of EPS as compared to the pure cultures (2.7-3.7 g/L) using sterilized sludge as a raw material. Broth (B-EPS) revealed high kaolin flocculating activity (91.2%) at very low concentrations (0.8 mg B-EPS/g kaolin) and it was comparable to the chemical polymer, Magnafloc-155 (90.4% at 0.2 mg/g kaolin). EPS produced were able to maintain their flocculating properties in the temperature range of 4-60 ^{o}C. B-EPS also exhibited very good flocculation performance (turbidity removal %) in river water (93.5%), municipal (91.7%) and brewery wastewater (81.8%) and it was comparable to the Magnafloc-155. The results are very encouraging and warrant further research on process optimization in laboratory fermenters under controlled conditions of pH, temperature and dissolved oxygen concentration.

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Table 5.1.1 Wastewater sludge characteristics used in the experiments

Parameters	Concentration
Total solids (TS)	29 g/L
Total volatile solids (TVS)	18 g/L
Total suspended solids (TSS)	25 g/L
Total volatile suspended solids (TVSS)	16 g/L
VS/TS	0.62
VSS/TSS	0.64
pH	6.5
Total carbon	4271000 mg/kg
Total nitrogen	48650 mg/kg
Total phosphorus	1040 mg/kg
Al	6720 mg/kg
Ca	17500 mg/kg
Cd	5.8 mg/kg
Cr	73 mg/kg
Cu	333 mg/kg
NI	32 mg/kg
Fe	11900 mg/kg
K	3100 mg/kg
Mn	94 mg/kg
Na	3900 mg/kg
Pb	47 mg/kg
S	3800 mg/kg
Zn	490 mg/kg

Note: All the data are the means of triplicates. Standard error is less than 5%.

Table 5.1.2 The river water, municipal and brewery wastewater characteristics

Sample	Characteristics				
	pH	Total solids (mg/L)	Turbidity (NTU)	COD (mg/L)	Zeta potential (mV)
River water	7.85±0.1	340±5	23±0.2	34.6±2	19.5±1
Municipal wastewater	6.7±0.2	184±3	58±0.5	37.3±5	31.6±2
Brewery wastewater	4.5±0.2	2362±10	4063±8	1985±8	48±2

Table 5.1.3 EPS concentrations, rheology and jar tests results of B-EPS (with addition of Ca^{2+}) produced by different (pure/mixed) cultures in sterilized sludge

Culture		S-EPS (g/L)	C-EPS (g/L)	B-EPS (g/L)	Broth viscosity (72 h fermentation) (mPas)[a]	Increase in broth viscosity (%)	Zeta potentials (mV)	Flocculation activity (%)[b]	Increase in dewaterability (%)[b]
Name	**Accession Number**								
Bacillus sp-1	EU031753	2.036	0.682	2.718	13.4	08.9	-45.55	40.1	16.9
Bacillus sp-2	EU031754	2.744	0.809	3.553	15.7	27.6	-38.20	54.4	26.7
Bacillus sp-3	EU031755	2.815	0.801	3.616	16.0	30.1	-37.57	69.4	33.2
Bacillus sp-4	EU031756	2.850	0.805	3.655	16.2	31.7	-35.18	62.8	33.4
Bacillus sp-5	EU031759	2.365	0.832	3.197	14.2	15.4	-40.76	47.8	22.9
Bacillus sp-6	EU031760	2.650	0.812	3.462	15.7	27.6	-38.11	65.5	27.0
Bacillus sp-7	EU031764	2.872	0.852	3.724	16.3	32.5	-34.49	71.7	32.0
Bacillus sp-8	EU031767	2.603	0.734	3.337	14.7	19.5	-38.36	59.6	18.9
Bacillus sp-9	EU031769	2.101	0.670	2.771	13.6	10.6	-45.02	41.4	17.2
Serratia sp-1	EU031758	2.852	0.781	3.633	15.8	28.5	-36.40	72.5	36.8
Serratia sp-2	EU031768	2.570	0.737	3.307	14.5	17.9	-38.66	61.5	30.6
Yersinia sp-1	EU031761	2.592	0.764	3.356	14.6	18.7	-40.17	60.9	34.7
Yersinia sp-2	EU031763	2.813	0.788	3.601	15.9	29.3	-38.72	65.4	33.8
Mixed culture (of 13 bacterial strains)	-	4.125	0.850	4.975	19.3	56.9	-28.98	76.4	48.1

Note: [a]The viscosity of the sterilized sludge before inoculation was 12.3 mPas. [b]Kaolin flocculation activity and dewaterability were measured using B-EPS (1 mL) sampled at 72 h fermentation. All the data presented are the means of triplicates and standard error is less than 5%.

Table 5.1.4 The protein and carbohydrate contents of S-EPS and C-EPS produced by mixed culture (of 13 bacterial strains) at different fermentation time

Fermentation time (h)	S-EPS[a]			C-EPS[b]		
	Protein g/g S-EPS	Carbohydrates g/g S-EPS	Protein/carbohydrate ratio	Protein g/g C-EPS	Carbohydrates g/g C-EPS	Protein/carbohydrate ratio
12	0.558	0.106	5.3	0.638	0.190	3.4
24	0.560	0.120	4.7	0.645	0.193	3.3
36	0.565	0.126	4.5	0.651	0.195	3.3
48	0.572	0.125	4.6	0.660	0.233	2.8
60	0.581	0.133	4.4	0.665	0.258	2.6
72	0.582	0.132	4.4	0.669	0.254	2.6
84	0.581	0.129	4.5	0.673	0.251	2.7
96	0.574	0.123	4.7	0.679	0.246	2.8

Note: [a]S-EPS of sterilized sludge contained 0.567 g protein/g S-EPS and 0.099 carbohydrates g/g S-EPS. [b]C-EPS of sterilized sludge contained 0.680 g protein/g C-EPS and carbohydrates 0.180 g/g C EPS. All the data presented are the means of triplicates and standard error is less than 5%.

Table 5.1.5 Turbidity and COD removal from river water, municipal and brewery wastewater using different flocculants (with concentration)

Flocculant	Flocculant concentration (mg/L)	River water[a]		Flocculant concentration (mg/L)	Municipal wastewater[b]		Flocculant concentration (mg/L)	Brewery wastewater[c]	
		Turbidity removal (%)	COD removal (%)		Turbidity removal (%)	COD removal (%)		Turbidity removal (%)	COD removal (%)
B-EPS	0.5	67.8	36.4	1.0	58.4	50.2	2.5	53.2	50.3
	1.0	86.1	42.7	2.0	65.0	55.6	5.0	57.4	62.8
	2.0	93.5	52.0	3.0	80.2	65.3	7.5	67.9	65.2
	3.0	32.2	15.2	4.0	87.6	73.8	10.0	75.7	72.5
	4.0	n/a	n/a	5.0	91.7	74.5	12.4	81.8	88.4
	5.0	n/a	n/a	6.0	77.8	69.7	14.9	79.5	74.2
S-EPS	0.4	40.9	23.8	0.8	57.1	44.5	2.1	44.2	47.2
	0.8	55.7	28.6	1.7	65.0	48.0	4.2	46.0	55.3
	1.7	n/a	n/a	2.5	66.4	52.7	6.2	52.8	60.0
	2.5	n/a	n/a	3.3	70.3	54.1	8.3	57.7	64.3
	3.3	n/a	n/a	4.2	74.5	60.6	10.4	59.2	67.6
	4.2	n/a	n/a	5.0	58.8	58.3	12.5	51.3	62.5
C-EPS	0.1	57.4	28.3	0.2	40.0	46.8	0.8	55.5	58.0
	0.2	81.7	49.1	0.3	82.2	51.0	1.7	61.1	65.2
	0.3	56.1	32.8	0.5	85.9	60.7	3.3	65.2	69.5
	0.5	n/a	n/a	0.7	86.9	75.4	5.0	79.5	87.6
	0.7	n/a	n/a	0.8	76.0	68.5	6.6	79.8	74.2
	0.8	n/a	n/a	1.0	60.5	52.1	8.3	76.3	70.6
Magnafloc-155	0.1	80.0	30.1	0.2	55.0	50.2	1.0	54.7	40.9
	0.2	93.9	48.0	0.4	75.3	59.8	2.0	64.5	57.1
	0.4	88.7	43.6	0.6	80.2	69.0	3.0	69.6	65.3
	0.6	84.8	37.3	0.8	97.8	76.8	4.0	70.1	70.6
	0.8	67.0	30.1	1.0	97.8	74.3	5.0	84.8	89.7
	1.2	13.9	n/a	1.2	92.2	67.2	6.0	83.2	73.3

Note: [a]Initial turbidity and COD of river water was 23.0 NTU and 34.6 mg/L, respectively. [b]Initial turbidity and COD of municipal wastewater was 58.0 NTU and 193 mg/L, respectively. [c]Initial turbidity and COD of brewery wastewater was 4063 NTU and 1985 mg/L, respectively. n/a – The sample had negative turbidity removal (%) and COD removal (%). All the data are the means of three independent experiments. Standard error is less than 5%.

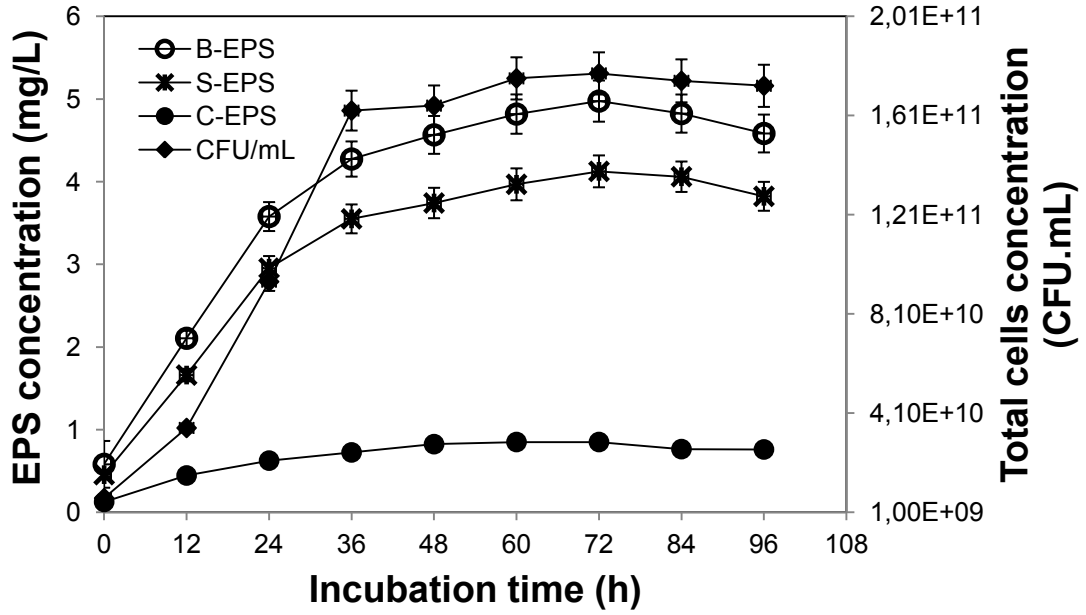

Figure 5.1.1 Variation of EPS concentrations (B-EPS, S-EPS, and C-EPS) and total cells concentrations (CFU/mL) during mixed culture growth (of 13 bacterial strains) in sterilized sludge

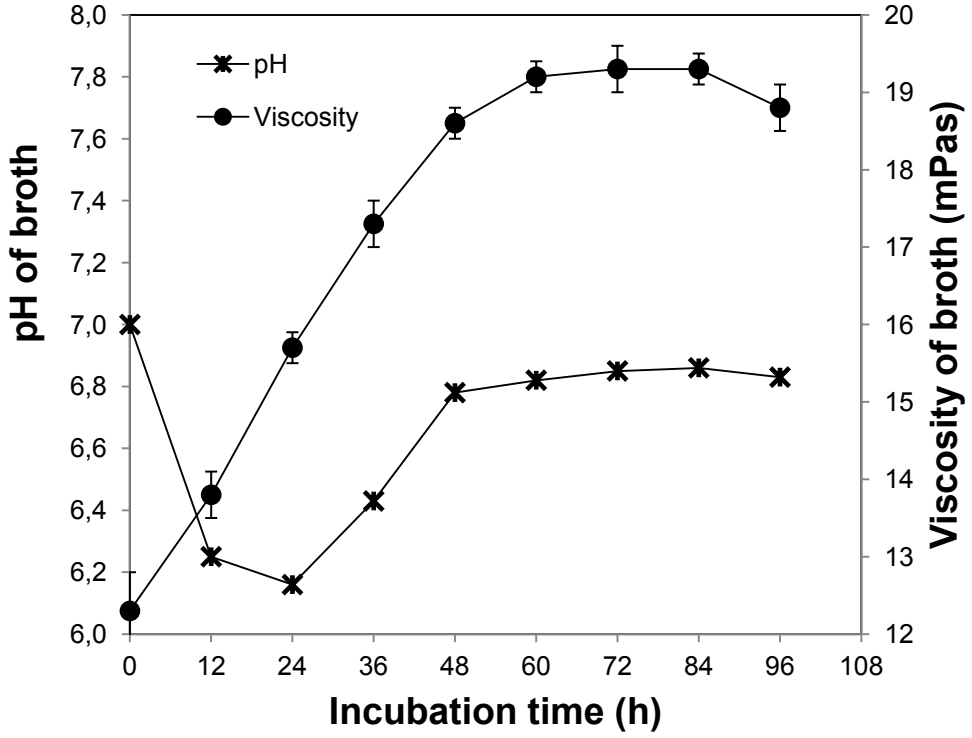

Figure 5.1.2 The pH and viscosity of the broth measured at different incubation time during growth of mixed culture in sterilized sludge

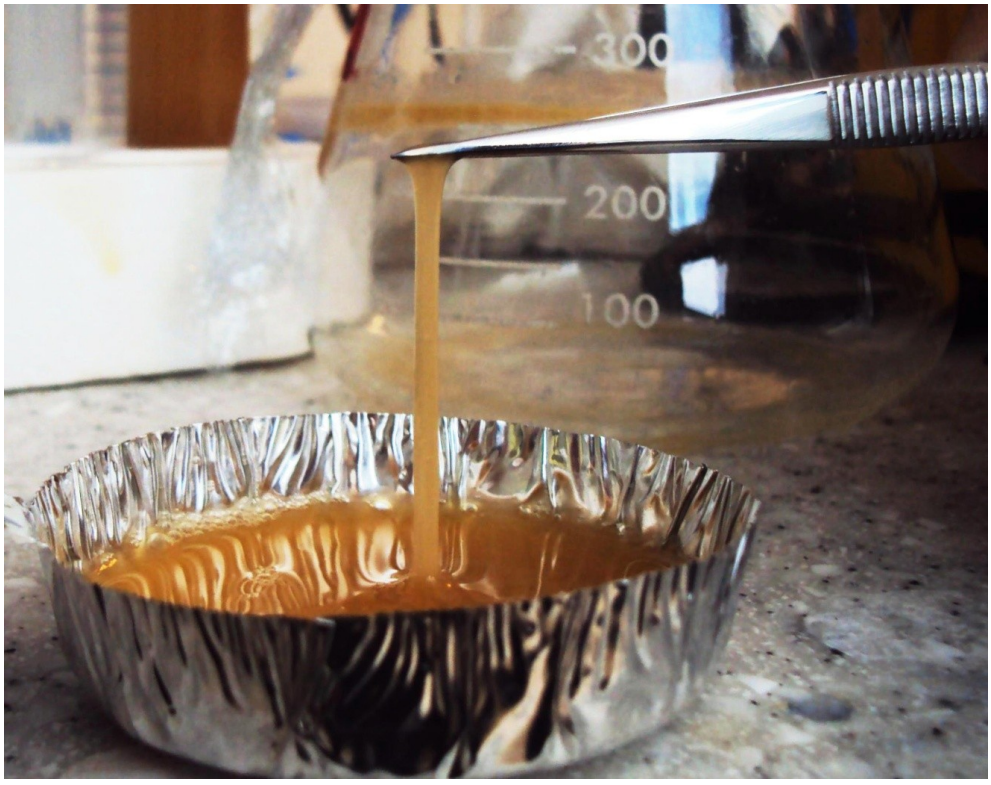

Figure 5.1.3 Viscous EPS produced by mixed culture in synthetic medium

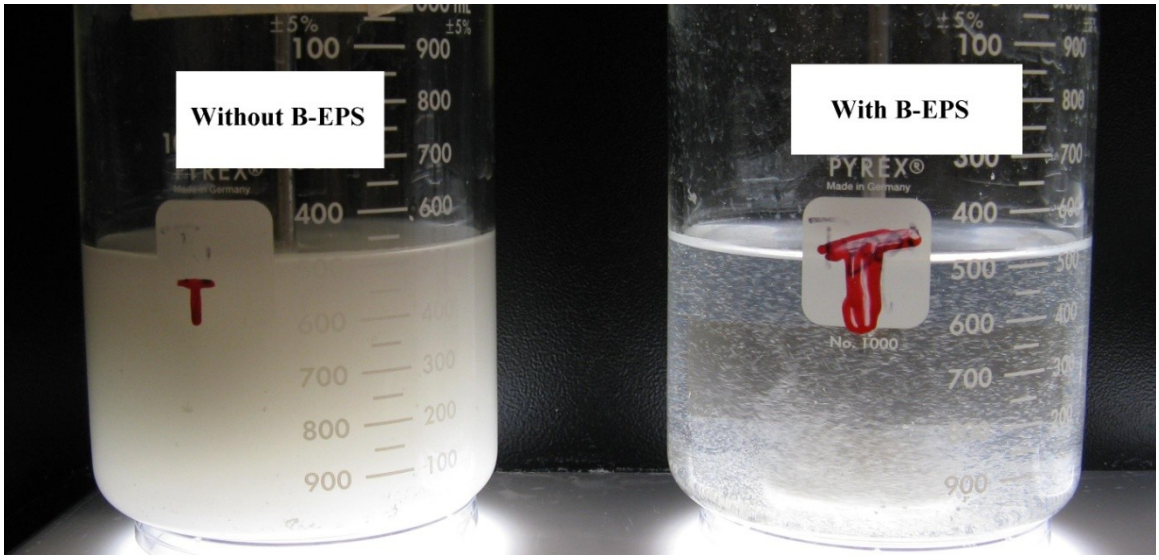

Figure 5.1.4 During Jar tests - kaolin suspension with and without addition of B-EPS (produced by mixed culture in sterilized sludge) combined with calcium

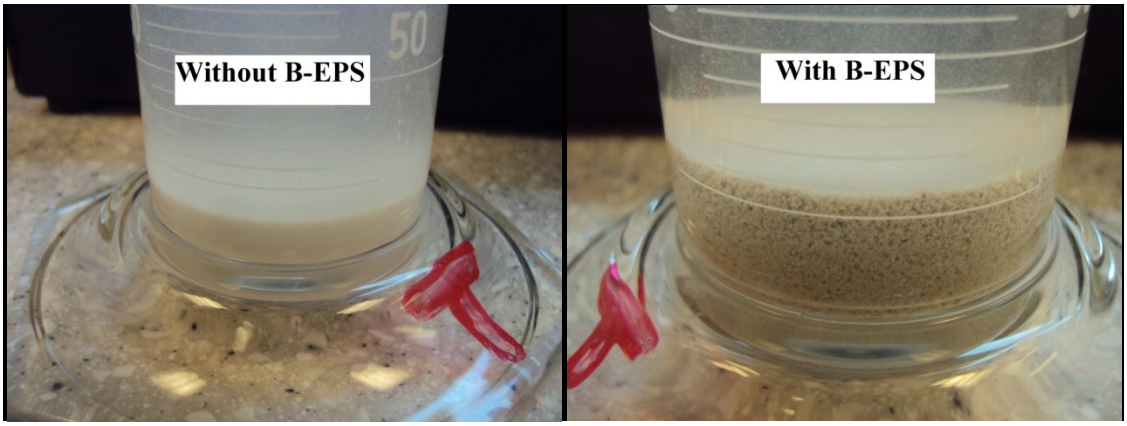

Figure 5.1.5 After jar tests - kaolin flocs with and without addition of B-EPS (produced by mixed culture in sterilized sludge) combined with calcium

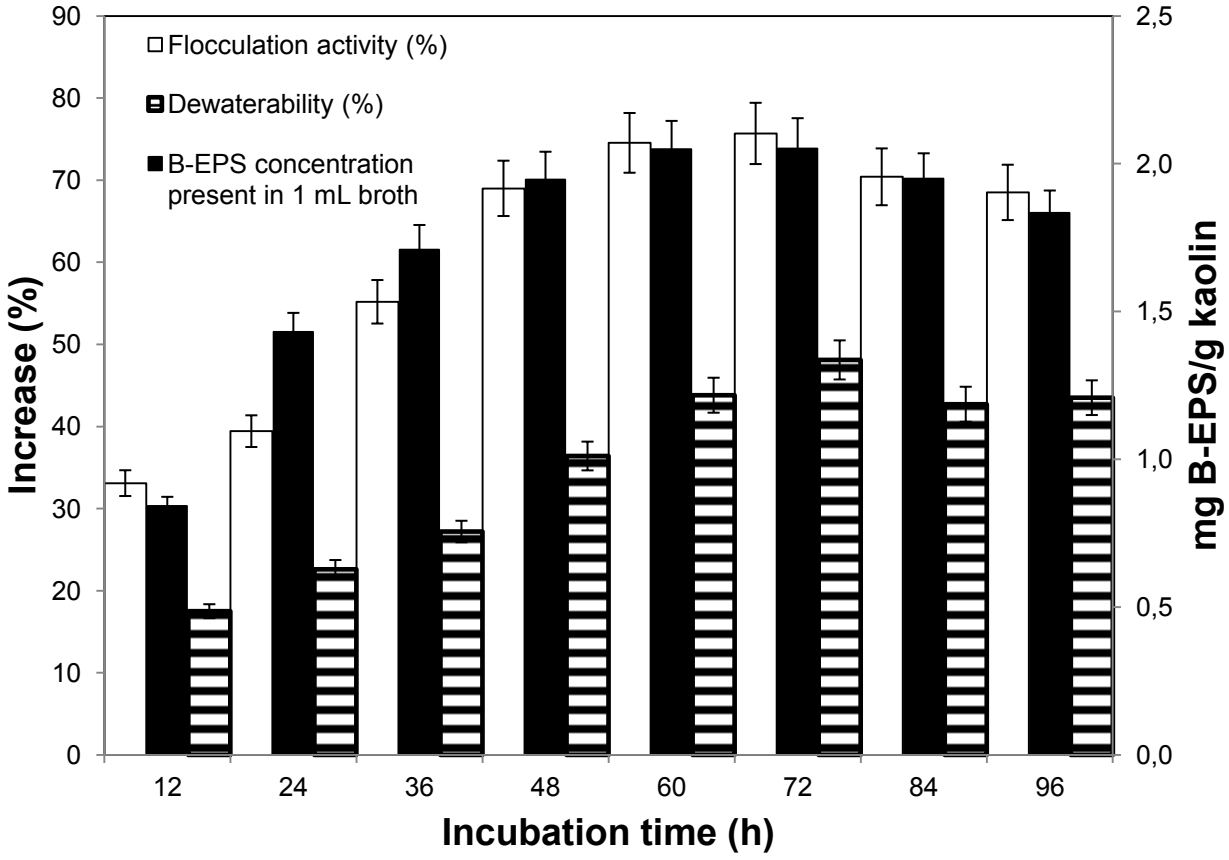

Figure 5.1.6 Variation in kaolin flocculation activity and dewaterability using B-EPS (1 mL broth) sampled at different incubation time during growth of mixed culture in sterilized sludge

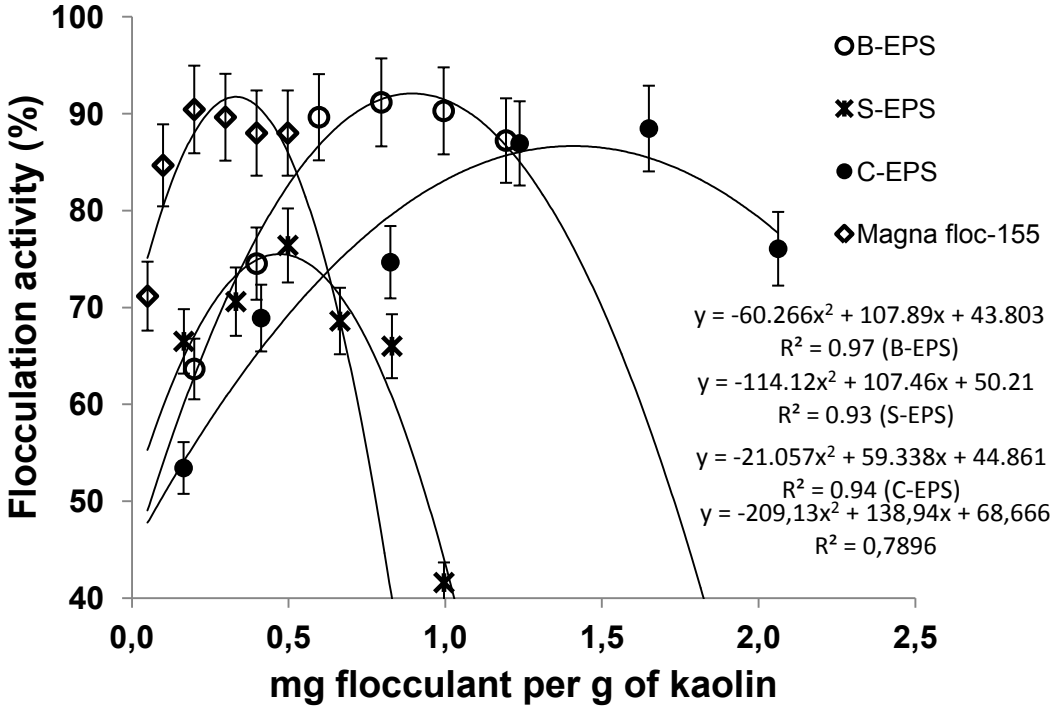

Figure 5.1.7 Effect of various flocculants (B-EPS, S-EPS, C-EPS and Magnafloc-155) concentration on kaolin flocculation activity

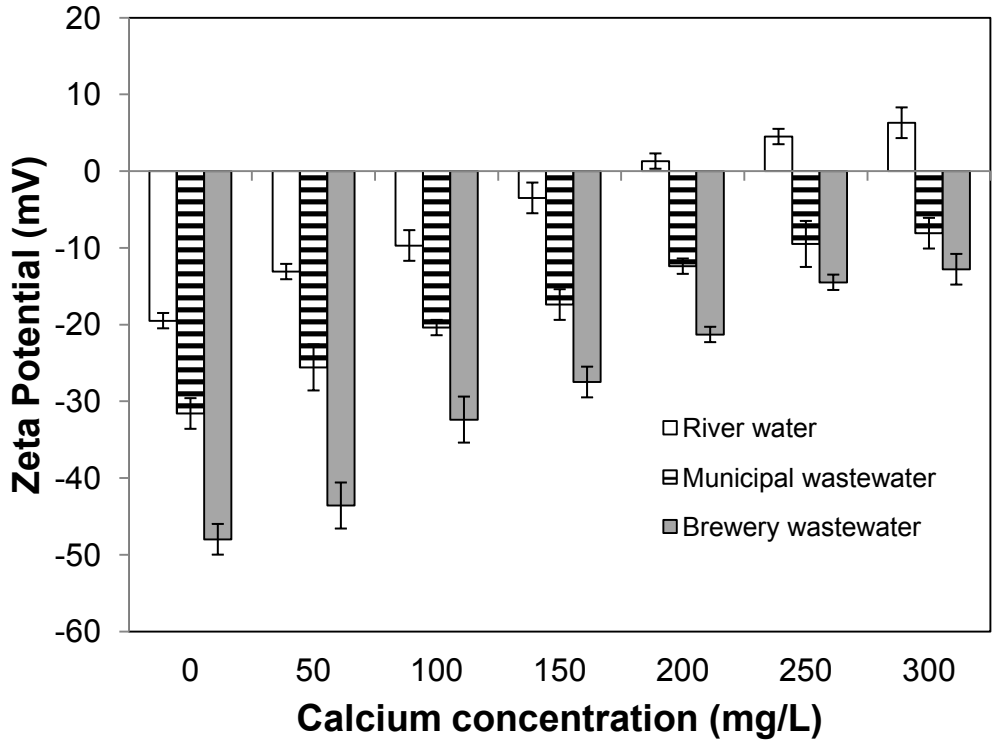

Figure 5.1.8 Zeta potential (mV) of river water, municipal and brewery wastewater at different calcium concentration.

PARTIE 2

BIOPOLYMERS PRODUCTION BY MIXED CULTURE AND EFFECT OF CATIONS ON THEIR FLOCCULATION PERFORMANCE

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RÉSUMÉ

Treize souches bactériennes productrices de substances polymériques extracellulaires (SPE) ont été cultivées (comme culture mixte) dans la boue stérilisée (matières en suspension: 25 g/L) à fin d'optimiser l'agitation et d'effectuer la cinétique de la production des SPE dans une fermentation discontinue. La vitesse d'agitation optimale était de 150 tours par minute avec la production de 4,9 g/L de SPE à 72 h. En effet, la production de SPE est liée à la croissance bactérienne. Les SPE ont montré une meilleure activité de floculation du kaolin (80%) lorsqu'il est combiné avec de cations divalents (Ca^{2+} et Mg^{2+}) en comparaison avec les cations trivalents (Fe^{3+} et Al^{3+}) (moins de 70%). Avec l'ajout d'cations trivalents (Fe^{3+} et Al^{3+}), l'activité de floculation (turbidité et la DCO) des B-SPE dans l'eau de la rivière (90%), les eaux usées municipales (80,7%), les eaux usées de brasseries (80-84%) était meilleure que celle avec le Ca^{2+} et Mg^{2+}. La floculation des SPE était comparable à celle du Magnafloc-155 (polymère chimique) et, par conséquent, elles pourraient être utilisées en tant que floculants.

MOTS-CLÉS: biopolymères; cations; EPS; floculation; Culture mixte; Boues des eaux usées; Traitement des eaux usées; Traitement de l'eau.

ABSTRACT

Thirteen extracellular polymeric substances (EPS) producing bacterial strains were cultivated (as mixed culture) in the sterilized sludge (suspended solids: 25 g/L) and the batch fermentation was carried out. Mixed culture revealed high specific growth rate 0.35 h^{-1}. The EPS production rate was higher up to 24 h which gradually decreased with further incubation. The kinetic estimates demonstrated growth associated EPS production. Broth EPS revealed higher flocculation activity when combined with different cations (Ca^{2+}, Mg^{2+}, Fe^{3+}, Al^{3+}) in river water (≥90%), municipal (≥90%) and brewery wastewater (≥80%), respectively. Low dose (5-40 mg/L) of trivalent cations was required to achieve higher flocculation as compared to the divalent cations (50-250 mg/L). Flocculation performance of EPS was comparable to Magnafloc-155 (chemical polymer) and hence, it could be used as a flocculant.

KEYWORDS: Biopolymers; Cations; EPS; Flocculation; Mixed culture; Wastewater sludge; Wastewater treatment; Water treatment.

1 Introduction

Coagulation and flocculation are widely applied process for various water and wastewater treatments. The choice of flocculants is very important factor in the flocculation process. To date, inorganic polymers and synthetic polymers are the most widely used flocculants because of their high efficiency and performance (Li et al., 2009). However, many of the most commonly used chemical flocculants are reported to be expensive, corrosive, toxic and non-readily degradable (McLachlan, 1995; More et al., 2014a). Recently, there is growing interest in the research on natural polymeric flocculants including starch, chitosan, cellulose, konjac glucomannan, alginate and extracellular polymeric substances (EPS) (Yang et al., 2011; More et al., 2010; More et al., 2014a). The natural polymeric flocculants are considered economical, environment friendly and sustainable. Among these natural polymeric substances, the potential of EPS or microbial flocculants in environmental pollution control applications is yet to be established.

EPS are biopolymers which consist of different biochemicals secreted by microbes, cell lysis products and organic matter adsorbed from the surrounding environment/medium (Wingender et al., 1999; More et al., 2014a). EPS consists of quite viscous biofilm matrix. Because of special EPS components (Carbohydrates, proteins, humic substances and nucleic acids), EPS matrix shows adsorption abilities, biodegradability and hydrophilicity/hydrophobicity (Tian, 2008; More et al., 2014a). Recently, the use of bacterial EPS for environmental application has been the focus of many researchers. However, very limited information exists on the flocculation efficiency of the bacterial EPS for real practical application. Moreover, so far, the work on the application of EPS in water, wastewater and wastewater sludge flocculation, dewatering and treatment is still in the research phase (Li et al., 2013; Luvuyo et al., 2013; Patil et al., 2011; Wang et al., 2011; Zhang et al., 2007). Extensive investigation is required before its possible implementation to the field processes.

Low flocculating capability and higher costs of the production medium, compared to that of conventional chemical polymers, are the main limiting factors in commercial applications of the EPS (More et al., 2014a). Thus, the search for microorganisms with high EPS producing abilities and reduced production costs continues among researchers in this field. Finding out the cheaper culture medium with higher yield of EPS having higher flocculation activity (FA) are the immediate needs of the bioflocculants research. In order to develop cost effective processes for EPS production several different strategies are being applied. One of them is the use low cost

raw materials, such as wastewater sludge for EPS production. Wastewater sludge is potentially economical media as it is a rich source of carbon, nitrogen, phosphorus and other nutrients. The use of sludge will be advantageous for growth of the microorganisms isolated from the wastewater sludge which are already well adapted (More et al. 2012a, b; More et al., 2014a). Moreover, use of bioflocculants derived from bacterial cultures grown in the wastewater sludge represents an important development in sustainable environmental technology.

In aerobic bioprocesses, the EPS production could be enhanced by optimizing agitation (Radchenkova et al., 2014). The agitation is required for the culture to allow mass transfer to occur between the cells and the medium. The agitation also ensures the homogeneous distribution of organic matter to cells for growth and EPS production. Knowledge of kinetic parameters of EPS synthesis in wastewater sludge is needed to enhance EPS production and to obtain a better understanding of the mechanisms involved in the EPS synthesis (Ni et al., 2010). However, there is hardly any report on kinetics of EPS synthesis by bacterial strains using wastewater sludge as a raw material. Therefore, to utilize wastewater sludge as a raw material for EPS production, systematic lab scale investigation on time dependent increase in the microbial population and EPS concentration is warranted. The analysis of this kind might lead to an efficient production of EPS with novel, desired properties. Indeed, it is important for the application of EPS in environmental pollution control activities to investigate the process of EPS biosynthesis during the fermentation process. The understanding of kinetic parameters is also important for the stable production of high amounts of EPS at large scale. Flocculation study demonstrated ions present in the sludge were not enough to initiate flocculation mechanism (More et al., 2012a, b). Therefore, addition of cations was needed for the flocculation to occur using EPS as biopolymer. Cations play important role in the flocculation process. Cations neutralize the negative surface charge of the polymers and acts as binding agent in forming bridges between particles and polymers (Higgins and Novk 1997). Interactions of the EPS with different cations are very crucial for the potential applications of EPS as a bioflocculant material. Optimization studies must be conducted to understand these interactions and to figure out the best combination of EPS and cations.

Therefore, in the present study, thirteen EPS producing bacterial strains were cultivated (as mixed culture) in the sterilized sludge to optimize agitation conditions required for higher EPS production. The consequences of microbial growth in mixed culture on EPS production during lab scale batch fermentation was carried also out in this study. The harvested EPS were examined for their flocculation performance (turbidity removal and dewatering) in the jar tests

using kaolin suspensions with Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}, respectively. The EPS were also used for the treatment of river water, municipal wastewater and secondary wastewater sludge.

2 Materials and methods

2.1 Wastewater sludge

Secondary wastewater sludge (without addition of chemical polymers) was collected from biofiltration unit at Municipal water resource recovery facility at Québec city, Canada. The sludge was first sedimented for 1 h and concentrated sludge was collected by discarding the supernatant. The wastewater sludge characteristics are presented in Table 5.2.1. The sludge was stored at 4^{o}C for further use.

2.2 Microorganisms

Thirteen EPS producing bacterial strains isolated from secondary municipal sludge (Municipal water resource recovery facility, Québec city, Canada) were used (More et al., 2012). The bacterial strains used in the study were EU031753 (*Bacillus* sp. 1), EU031754 (*Bacillus* sp. 2), EU031755 (*Bacillus* sp. 3), EU031756 (*Bacillus* sp. 4), EU031759 (*Bacillus* sp. 5), EU031760 (*Bacillus* sp. 6), EU031764 (*Bacillus* sp. 7), EU031767 (*Bacillus* sp. 8), EU031769 (*Bacillus* sp. 9), EU031758 (*Serratia* sp. 1), EU031768 (*Serratia* sp. 2), EU031761 (*Yersinia* sp. 1) and EU031763 (*Yersinia* sp. 2). These bacterial strains were selected in the study based upon their distinct metabolic properties and distinct capability of EPS production in the sludge and the EPS produced by these strains also had different flocculation characteristics (More et al., 2012a). The single/pure strains have metabolic limitations in utilizing the carbon, nitrogen and other nutrients from the sterilized sludge. To catch on these leftover nutrients and to increase the EPS production in sludge, the mixed culture was considered as the best way (More et al., 2014b). All the bacterial strains were grown over tryptic soy agar (TSA) plates and stored at 4^{o}C and sub-cultured fortnightly.

2.3 Sludge pretreatment

The suspended solids of raw sludge (SS): 25 g/L were sterilized by autoclaving (steam sterilization) at 121^{o}C for 15 min (More et al., 2012a). In previous work (More et al., 2012a), the

maximum EPS concentration of 2.8 g/L was obtained at 22.4-29.8 g/L of sludge solids using *Serratia* sp. 1. Thermal pretreatment of the sludge was the necessary step for the EPS production and it was selected based upon the findings of previous study (More et al., 2012a). Pretreatment increases the biodegradability of the sludge by disintegrating the organic matter and organisms that are originally present in the raw sludge. After sterilization, the sludge samples were cooled to room temperature and adjusted to pH value of 7.0 using 1 M NaOH. These strains had demonstrated higher EPS production at neutral pH (Subramanian et al., 2010). The pretreated sludge samples were used as raw material for culturing and EPS production.

2.4 EPS production

The EPS production by mixed and pure cultures was carried out in the sterilized sludge. The precultures of individual bacterial strains were prepared in two steps, respectively. In the first step, pure cultures were aseptically transferred from TSA plates to Erlenmeyer flasks (containing 100 mL of tryptic soy broth (TSB)) and independently incubated at 30^{o}C and 150 rpm for 12 h. In the second step, the TSB grown individual cultures were reinoculated (3% v/v) in the sterilized sludge (100 mL) and then independently incubated for 12 h in an orbital shaking incubator. To prepare the preculture of the mixed bacterial consortium, preculture (prepared in sterilized sludge) of each bacterial strain (1% v/v) (mixed together) were inoculated in the sterilized sludge (100 mL) followed by incubation for 12 h. Precultures were prepared at 12 h of incubation because shorter lag phases were obtained during batch fermentations using 12 h inoculum as compared to the late-stationary growth phase inoculum. The initial concentrations of inoculums (prior to mixing) were 5.71, 2.95, 5.01, 5.09, 6.32, 5.40, 7.33, 7.24, 7.60, 5.90, 3.41, 6.35 and 2.62 x 10^{9} CFU for *Bacillus* sp. 1-9, *Serratia* sp. 1-2 and *Yersinia* sp. 1-2, respectively.

Effect of Agitation Speed. Agitation speed is one of the important factors affecting the production of EPS. To determine the effect of agitation speed on the EPS production by mixed culture, preculture of the mixed culture was transferred (3% v/v) to the six flasks (500 mL) of the sterilized sludge (150 mL) and the samples were incubated at agitation speed of 0, 50, 100, 150, 200 and 250 rpm, respectively. The initial cells concentration of the inoculum was 6.70 x 10^{9} CFU. All the samples were incubated at 30^{o}C for 72 h. After incubation, the samples were then used for the measurement of EPS concentration and kaolin FA and dewatering.

Kinetics of the EPS production. The preculture of the mixed culture, was transferred (3% v/v) to the sterilized sludge (150 mL) to produce EPS as described earlier in More et al. (2014b). Then the samples were incubated at 30^{o}C and 150 rpm for 96 h. The initial cells concentration of the inoculum was 6.70 x 10^{9} CFU. To study the time course of EPS production, broth samples (2 mL) were drawn at each 6 h for total cells counts. EPS from broths were harvested at each 12 h of incubation.

The specific growth rates were calculated as the slope of semilog plot of the cell number versus cultivation time during exponential phase. In batch culture assays, the maximum specific growth rate (μ_{max}) was experimentally determined in the exponential growth phase as per equation: $\mu_{max} = [\ln(X_0/X_1)]/(t_1-t_0)$, where, X_0 and X_1 are the numbers of CFU/mL and t_0 and t_1 are the times along the exponential phase, respectively. Volumetric productivities (g/L/h) were graphically estimated by mean of differentiation of EPS produced (g/L) with respect to time (1/h). Specific EPS production rates (q_{EPS}; g EPS/million cells/h) were determined as per equation: $q_{EPS} = 1/N(dEPS/dt)$, where *N* is the microbial population as a multiple of million cells, *dEPS/dt* are the volumetric productivities.

2.5 EPS harvesting

To do quantitative, qualitative and comparative analysis of the EPS produced in sterilized sludge, the EPS harvesting was carried out according to More et al. (2014b). To harvest EPS, broth samples were centrifuged at 6000g for 15 min at 4^{o}C to obtain supernatant (crude slime EPS or crude S-EPS) and pellets (crude capsular EPS or crude C-EPS) with bacterial cells along with the residual sludge material (More et al., 2014b). To determine the S-EPS, the crude S-EPS was precipitated by adding 2.2 volumes of absolute chilled ethanol followed by storing the mixture at -20^{o}C overnight. The precipitates were collected by centrifugation at 6000g for 15 min at 4^{o}C. To measure dry mass of S-EPS, precipitates of S-EPS were first dried at room temperature in laminar hood for 6 h to remove volatile ethanol from the sample. Air dried samples contained significant moisture content. The dry weights of the samples were measured as S-EPS (APHA, 2005). Air dried samples were then heated at 105^{o}C for 24 h and dry mass were measured as S-EPS (APHA, 2005). For protein and carbohydrate analysis, precipitates of S-EPS (before heat drying) were resuspended in deionized water (to initial broth volume). To determine C-EPS, the crude C-EPS was resuspended in deionized water (to initial broth volume). The resuspended crude C-EPS were heated at 60^{o}C in water bath (with shaking 30 rpm) for 30 min to release C-EPS followed by centrifugation at 6000g, 4^{o}C for 15 min (Li and

Yang, 2007; More et al., 2012a,b). The supernatant (containing C-EPS) was used to precipitate C-EPS and the dry mass C-EPS was measured using the same procedure as for S-EPS. Sum of dry mass of S-EPS and C-EPS (measured above) was denoted as B-EPS. Crude EPS (broth or supernatant or sediments obtained without drying process as described earlier) were used as bioflocculants for the flocculation tests. The crude EPS were stored at 4^{o}C for two weeks during the experiments. The aging time of EPS was approximately one month when samples stored at 4^{o}C. Crude EPS forms were used in the study to reduce the costs of bioflocculant production.

2.6 Effect of different cations on kaolin flocculation activity

Jar Tests. Kaolin clay (K2-500, USP, Fisher scientific, US) suspensions (5 g/L in deionized water) were used as a standard suspension. The pH of the kaolin suspension was adjusted to 7.5 unless otherwise stated. The flocculation performance of different cations and flocculants were evaluated according to More et al. (2014b). Previously optimized jar test conditions were used to evaluate and compare the flocculation performance of different cations and flocculants (More et al. 2014b). Divalent cations such as Calcium and Magnesium and trivalent cations such as Ferric and Aluminum were used in the study. Calcium (25 Ca^{2+}g/L), Magnesium (25 Mg^{2+}g/L), Ferric (5 Fe^{3+}g/L) and Aluminum (5 Al^{3+}g/L) stock solutions were prepared by dissolving equivalent quantity of anhydrous $CaCl_2$, $MgSO_4$, $FeCl_3$ and $Al_2(SO_4)_3$ in deionised water.

First rapid mixing of each suspension (500 mL in 1 L glass beaker) was carried out at 175 rpm for 3 min to allow homogeneous dispersion of solids and to enhance solids interactions. During the rapid mixing, cations (Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}) of different concentrations were added (individually) followed either by flocculants addition or no flocculants unless otherwise stated specifically. Cations participate in charge neutralization of kaolin particles and the added flocculants participate in bridging of neutralized kaolin particles in presence of cations (More et al., 2012a). After rapid mixing, pH value of the suspensions was adjusted to 7.5 and slow mixing at 75 rpm was allowed for 30 min to ensure flocculation. The pH value of the suspensions (mixture of kaolin-cation) was adjusted to 7.5 because at slightly alkaline condition, highest flocculation was observed. After mixing samples were transferred to measuring cylinders of 500 mL for 30 min settling. After settling, the supernatant and the sediments of the samples were used to measure flocculation activity (FA) and capillary suction time (CST), respectively. All the tests were carried out in triplicates and the average values were presented (with standard error less than 5% of the mean).

To determine FA, turbidity of the samples supernatant was measured using Micro 100 Turbidity meter, Hach Company. FA measure was based on the relative decrease in turbidity of suspension after settling (Yokoi et al., 1995; More et al., 2012a, b). FA was calculated according to the equation 5.2.1 described below.

$$FA = (1-S/C) \times 100\ (\%) \qquad (5.2.1)$$

Where

C = Control turbidity (NTU), kaolin suspension without addition of calcium and EPS was the control, unless otherwise stated.

S = Sample turbidity (NTU)

Kaolin suspension without flocculants was the control. For quantifying the effects of flocculants on dewaterability, Capillary-suction-time (CST) of the settled kaolin sediments were measured. CST was determined by the CST instrument (Triton electronics, model 304 M CST, Dunmow, Essex), using a 10-mm diameter reservoir. Lower CST value as compared to the control CST indicated better dewaterability (More et al., 2012a). The sediments from jar test samples without EPS addition was the control. The increase in the dewaterability was calculated according to equation 5.2.2.

$$\text{Increase in dewaterability } (\%) = (1-A/B) \times 100\ (\%) \qquad (5.2.2)$$

Where

B = CST of the control (s), kaolin suspension without addition of calcium and EPS was the control, unless otherwise stated.

A = CST of the sample (s)

Comparison of Effect of Different Cations Addition on Kaolin Flocculation Performance of B-EPS. Series of preliminary jar tests carried out as described earlier to select the cation (Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}) concentration. First, different concentrations of Calcium (50, 100, 150, 200, 250, 300 mg/L of kaolin suspension), Magnesium (50, 100, 150, 200, 250 and 300 mg/L of kaolin suspension), Ferric (5, 10, 20, 30, 40 and 50 mg/L of kaolin suspension) and Aluminum (5, 10, 20, 30, 40 and 50 mg/L of kaolin suspension) were added (independently) to the kaolin suspensions and jar testes were carried out as described earlier without any flocculant addition. The kaolin FAs of the samples were measured to evaluate the effect of cations. Zeta potential of the suspensions was also measured at different cation concentrations. To determine the effect of different cations on kaolin flocculation performance of B-EPS, series of jar tests were carried

out as described earlier with the selected cation concentrations. For comparison, B-EPS used in this study was obtained from the fermented broth sample (at 72 h) that revealed the highest EPS concentration and the highest FA. In these tests, different broths volumes (0.1, 0.2, 0.4, 0.6, 0.8, 1.0 mL) were used and optimum concentration of B-EPS, required for obtaining highest FA in each case was determined.

Comparison of Kaolin Flocculation Performance of EPS to that of Magnafloc-155. The flocculation performance of B-EPS (along with the addition of different cations as described earlier) was investigated against a conventional chemical polymer, Magnafloc-155 (Anionic Granular Grade Polymer, CIBA Specially Chemicals, Virginia, USA). Magnafloc-155 is presently used for secondary wastewater flocculation and sludge dewatering operations at Municipal water resource recovery facility, Quebec City. The sludge samples were collected from the same facility for the present study. Magnafloc-155 (white powder) was dissolved in deionised water to obtain (1 g/L) stock solution. The aging time of the Magnafloc-155 was 2-3 days. The series of jar tests were conducted by replacing B-EPS with the Magnafloc-155 and by keeping all other conditions (mixing, cation concentration etc) similar to those described earlier for B-EPS. In these tests, optimum concentration of Magnafloc-155, required for obtaining highest FA in each case was determined.

2.7 Applications of EPS produced by mixed culture to the treatment of river water, municipal and Brewery wastewater

The river water was collected from Saint Charles river (Québec city, Canada) and its characteristics are presented in Table 5.2.2. The wastewater (without chemical polymers) was collected from municipal water resource recovery facility (Québec city, Canada). Brewery wastewater was collected from La Barberie (Québec city, Canada). The municipal and brewery wastewater characteristics are presented in Table 5.2.2. Preliminary jar tests similar to described earlier were carried out to select the cations (Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}) concentration. In these preliminary jar tests, different cation concentrations were added to river water, municipal and brewery wastewater, respectively. The supernatant turbidity and chemical oxygen demand (COD) were measured to evaluate the effect of cations. Zeta potential of the suspensions was also measured at different cation concentration.

To investigate the potential application of B-EPS as a flocculant material, the B-EPS obtained at the incubation time having highest FA and B-EPS concentration was used in jar tests. Jar tests were carried out as described earlier using different cations (selected from preliminary tests)

and B-EPS. Different dosages of the broth (B-EPS) were employed in all jar tests to investigate the effect of B-EPS concentration when combined with different cations. To compare the performance of B-EPS, similar jar tests were carried out using different concentrations of Magnafloc-155 along with cations.

2.8 Analysis

Characteristics of sludge, such as pH, total solids (TS), suspended solids (SS), volatile solids (VS), volatile suspended solids (VSS), total chemical oxygen demand (COD), turbidity (NTU) and other physical-chemical properties were determined using Standard Methods (APHA, 2005). Viscosity (mPas) and zeta potentials (ζ) were measured using Viscometer (DV DV-II PRO + (Brookfield Engineering Laboratories, Inc., Stoughton, MA, USA)) and Zetaphoremeter (Zetaphoremeter IV, Zetacompact Z8000, CAD Instrumentation, France), respectively. The concentrations of metals in the sludge were analyzed by ICP-AES (Varian Vista-AX CCO, Palo Alto, Ca) after partial digestion of dried, powdered sludge. About 0.5 g of dried sludge samples were digested using analytical grade nitric acid (50% w/w, 20 mL) and hydrogen peroxide (30% w/w, 9 mL). Quality control was also performed with certified samples (multi-element standard, catalogue number 900-Q30-002 and 900-Q30-101) to ensure conformity of the measurement values. Measurement of cells concentration as colony forming units (CFU) was carried out by standard agar-plate technique. The protein content of the extracted S-EPS and C-EPS was determined using bovine serum albumin as a standard (Lowry et al., 1951); the carbohydrate content of the extracted S-EPS and C-EPS was determined by the phenol-sulfuric acid method using glucose as the standard solution (DuBois et al., 1956). The sum of protein content of S-EPS and C-EPS were denoted as the protein content of B-EPS. Similarly, the sum of carbohydrate content of the S-EPS and C-EPS were denoted as the carbohydrate content of B-EPS.

3 Results and discussion

3.1 Effect of agitation on EPS production and its flocculation characteristics

The concentration of EPS produced by the mixed culture was considerably varied with the agitation speed (Figure 5.2.1). B-EPS concentration had increased with the increase in the agitation speed from 0 to 150 rpm. The control sample of sterilized raw sludge (without

inoculation) had very low concentration of EPS (less than 23.2 mg/g SS). Therefore, the high concentration (Figure 5.2.1) observed in the fermented broths was due to EPS secretion by individual bacterial strains during 72 h of fermentation. The highest B-EPS (4.950 g/L) concentration was observed at 150 rpm. B-EPS concentration observed at 150 rpm was 2.5 times higher than the B-EPS concentration observed with no agitation (1.975 g/L). Higher agitation speed (200 and 250 rpm) had detrimental effect on the EPS production. The B-EPS concentrations at higher agitation speed were 24-25% lower than the B-EPS concentrations observed at 150 rpm. The results indicated that 150 rpm was the optimum agitation speed required for the maximum EPS production. Similar to the B-EPS trend, highest S-EPS concentration (4.125 g/L) was observed at 150 rpm which was 2.75 times higher than the S-EPS concentration observed at no agitation condition. Whereas, C-EPS concentration observed at 150 rpm was 1.73 times higher than that observed at no agitation condition.

Homogeneous distribution of organic matter present in the sludge and mass transfer rates (oxygen and nutrients) to cells for growth and EPS production could have been better at 150 rpm than at lower agitation (0, 50, 100 rpm). In these conditions (agitation lower than 150 rpm), oxygen was also likely to be rate-limiting substrate for EPS production. Radchenkova et al. (2014) also reported that the EPS production by *A*. *Pallidus* was increased by increasing agitation in a 5-1 single impeller jacketed glass reactor with 2.4 L culture medium with maltose as carbon source. According to Radchenkova et al. (2014), agitation was the important factor influencing the availability of nutrients and dissolved oxygen and controlling the rate of metabolite release from the cells. Bandaiphet and Prasertsan (2006) had also observed that the EPS production by *Enterobacter cloacae* WD7 at bench scale was decreased by 44% when the agitation was increased from 200 rpm to 800 rpm. The mechanical stresses on cells growth and physiology associated with vigorous agitation could affect the product formation.

FAs of the broth samples collected at 72 h of incubation are presented in Figure 5.2.2. The control sample of sterilized raw sludge (without inoculation) had no positive effect on FA because of its very low concentration of EPS. Therefore, FA observed for the fermented broths (Figure 5.2.2) were due to higher EPS content present in these samples. In this kaolin jar tests; calcium (150 mg/L kaolin) was used along with the broth (1 mL). The highest FA (89.4%) and dewaterability (60.5%) was observed for the B-EPS produced at 150 rpm. The highest FA and dewaterability obtained for the B-EPS produced at 150 rpm was due to the higher concentration (4.950 mg) of B-EPS present in the dosage as compared to all other B-EPS samples. These results demonstrated that 150 rpm was the optimum agitation required for maximum EPS

production and for the production of EPS having higher flocculation and dewatering capability. This study revealed that agitation has net impact on EPS production. This fact should be considered while producing EPS in large scale fermenters, because the agitation speed will have significant impact on the EPS production.

3.2 Kinetics of EPS production by the mixed culture in sterilized sludge

The time course of growth and EPS production by mixed culture (of 13 bacterial strains) in sterilized sludge is shown in Figure 5.2.3. The mixed culture revealed typical sigmoidal batch culture growth curve in sterilized sludge. Cells growth profile did not show any occurrence of apparent lag phase and started growing in exponential phase. This indicated that the mixed culture was well adapted to the growth in sterilized sludge. Mixed culture attained maximum cells concentration (1.76-1.78 x 10^{11}) at 60-72 h of incubation. During incubation, the cells concentration was increased by two log cycles in first 36 h, whereas there was small increase (1.63 to 1.78 x 10^{11}) in the cells concentration from 36 h to 72 h. The specific growth rates were calculated as the slope of semilog plot of the cell number versus cultivation time during exponential phase. Maximum specific growth rate was 0.35 h^{-1}.

During 96 h of incubation, the B-EPS concentration increased with total cells (CFU/mL) (Figure 5.2.3a). A maximum B-EPS concentration of 4.9 g/L was obtained at 72 h. During the same period, S-EPS of 4.1 g/L and C-EPS of 0.8 g/L were obtained. The raw sludge had very low concentrations of EPS (less than 23.2 mg/g SS). Up to 72 h of incubation, B-EPS and S-EPS concentration varied proportional to cell count. EPS production rates or productivity (for all forms of EPS) observed during the fermentation are shown in Figure 5.2.3b. For B-EPS, continuous decrease in the productivity was observed. The B-EPS production rate was maximum i.e., 0.127-0.123 g/L/h at 12-24 h of incubation, whereas it had decreased rapidly to 0.024 g/L/h (at 48 h) and it had decreased gradually with further incubation to 0.013 g/L/h (Figure 5.2.3b). During the stationary phase (72 to 96 h), B-EPS productivities were either zero or negative, that indicated that EPS degradation had dominated the EPS production as cells tend to take their required nutrients from this (EPS matrix) storage material. For S-EPS, productivity had increased slightly during incubation from 12 to 24 h, respectively and had decreased thereafter. This was because up to 24 h, the S-EPS production dominated the degradation. For C-EPS, maximum productivity was 0.026 g/L/h at 12 h. The results clearly demonstrated that the maximum EPS (All forms) production had occurred during exponential growth phase up to 24 h of incubation and EPS production had continued slowly for further 72 h.

Specific EPS production rates (q_{EPS}) were calculated for all forms of EPS, respectively as the rate of change of EPS concentration per million cells. Specific EPS (all forms) production rate and specific growth rate of the cells had linear correlation as shown in (Figure 5.2.3c). The y-intercept of the equations was zero or close to zero, indicating the growth associated EPS production. EPS (all forms) production occurred during growth and stopped when the cells stopped growing (stationary phase, 72 to 96 h). These observations were in accordance with the unified theory for EPS production proposed by Laspidou and Rittmann (2002). As per this theory, the formation of EPS (in activated sludge aerobic fermentation) was growth associated. Growth-associated EPS synthesis has been reported for glucan (Kambourova et al., 2009), xanthan (Kalogiannis et al., 2003) and scleroglucan (Survase et al., 2007) production. The results of present study warrant the evaluation of the effectiveness of EPS production process by taking into consideration produced EPS concentration, the cost of the medium and the duration of the process.

3.3 Effect of different cations on kaolin flocculation activity

B-EPS used in this study was obtained from the fermented broth sample (at 72 h) that revealed the highest EPS concentration and the highest FA (Figures 5.2.1 and 5.2.2). From the preliminary jar tests, it was observed that, B-EPS addition (without cations) to kaolin suspension had negative effect on FA. This is because the zeta potentials of B-EPS (-28.98 mV) as well as kaolin suspensions (-30.9 mV) were negative. The repulsion between the negatively charged particles did not allow the aggregation of the suspended particles. To reduce the electrical repulsion of identically charged layers around kaolin particles, addition of oppositely charged cations was required. However, addition of monovalent cations (NaCl) along with B-EPS to kaolin suspension also did not reveal positive effect on FA (data not shown). Therefore, series of jar tests were carried out for the determination of optimal cation concentration using different concentrations of Ca^{2+}, Mg^{2+}, Al^{3+} and Fe^{3+}. To attain the maximum flocculation performance of different water and wastewater, often zeta potential of the system is decreased in the range of -15 <zeta potential<-10 mV (Jefferson and Parsons, 2005). From these experiments, the cation concentration of 150 mg Ca^{2+}/L, 150 Mg^{2+}/L, 5 mg Fe^{3+}/L and 5 mg Al^{3+}/L were selected for further jar tests on the basis of their minimum concentration required to bring zeta potentials of the suspensions -15 <zeta potential<-10 mV. Zeta potential of kaolin suspension (5.0 g/L) was -29.52±3.15 mV. When selected cation concentrations (150 mg Ca^{2+}/L, 150 Mg^{2+}/L, 5 mg Fe^{3+}/L and 5 mg Al^{3+}/L) were added to kaolin suspension, the zeta potentials were reduced from -

29.52±3.15 mV to 13.50±1.46 mV. Addition of excess concentration (more than 5 mg Fe^{3+}/L, 5 mg Al^{3+}/g SS) had negative impact on the kaolin FA. At these cation dosages, the floc formation was clearly visible to naked eye. Selected cation concentrations were then combined with different B-EPS concentrations to find out the optimum B-EPS concentrations. The results of these jar tests are shown in Figure 5.2.4. The addition of different cations (150 mg Ca^{2+}/L, 150 Mg^{2+}/L, 5 mg Fe^{3+}/L and 5 mg Al^{3+}/L respectively) followed by the addition of broth EPS demonstrated distinct kaolin FA profile. Divalent cations (Ca^{2+} and Mg^{2+}) had revealed higher FA as compared to the trivalent cations (Fe^{3+} and Al^{3+}). The maximum FA of 81.6%, 79.9%, 54.4%, and 68.3% was observed for Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}, respectively. The highest turbidity removal or FA for (Ca^{2+} and Mg^{2+}) was observed at 1.2 to 1.6 mg EPS/g kaolin, whereas, the highest turbidity removal or FA for (Fe^{3+} and Al^{3+}) was observed at 1.6 mg EPS/g kaolin. To achieve maximum FA, relatively higher concentrations of divalent cations were required as compared to the trivalent cations. However, maximum FA attained using trivalent cations was quite lower as compared to the divalent cations. Enhancement in the FA of B-EPS by addition of different cations is due to neutralizing and stabilizing the residual negative surface charge of B-EPS and then forming bridges between kaolin particles and B-EPS (Salehizadeh and Shojaosadati, 2001). The EPS and kaolin clay could form solid complexes mediated by the cations (Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}) through neutralization and bridging mechanism and the formation of these solid complexes lead to increase in the settling of the kaolin particles. However, most of the reported literature showed that the trivalent cations (Fe^{3+}, Al^{3+} etc.) had negative impact on the kaolin FA (Yokoi et al. 1995; Gong et al., 2008; Zheng et al., 2008; Feng and Xu, 2008; Yim et al., 2007). On the other hand, in the present study, it was observed that at relatively lower dose of trivalent cations (5 mg/L) also had positive impact on the kaolin FA. The dose required for divalent cations was 150 mg/L. This clearly indicates that the literature reports have used very high dose (excess than the optimum dose) of trivalent ions (Fe^{3+} or Al^{3+}) than the required dose. Gong et al. (2008) had used same divalent (Ca^{2+} and Mg^{2+}) and trivalent (Fe^{3+} and Al^{3+}) cation concentration for the kaolin flocculation tests and concluded that trivalent cations had negative impact on kaolin FA. Li et al., (2008) had also observed that divalent cations (Ca^{2+} and Mg^{2+}) had higher kaolin FA for EPS as compared to the trivalent cations (Fe^{3+} and Al^{3+}). Wu and Ye (2007) also had observed that FA of bioflocculants was enhanced by addition of Fe^{3+}. At neutral pH values, the trivalent ions have more significant adverse effect on the kaolin flocculation than the divalent cations (Peng and Di, 1993). The hydrolyzed metal ions adsorb on the particle surfaces and reduce the double layer charge to near zero. In this condition, these metal ions have positive impact on the flocculation. However, an excess of

hydrolyzed metal ions might cause the reversal of double layer charge and lead to a stable suspension with positive double layer charge. In such cases, it has negative impact on the flocculation.

Magnafloc-155, a conventional chemical polymer (anionic) was used in the jar tests similar to the B-EPS for comparing the flocculation performance. The addition of different cations along with Magnafloc-155 also had significant impact on kaolin FA similar to the broth EPS. Similar to the broth EPS, in case of Magnafloc-155 also, the divalent cations (Ca^{2+} and Mg^{2+}) had revealed higher FA as compared to the trivalent cations (Fe^{3+} and Al^{3+}). The maximum FA of 90.4%, 88.5%, 77.9% and 75.4% was observed for Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}, respectively. Relatively lower concentration of Magnafloc-155 (0.08-0.16 mg/g kaolin) was required for obtaining maximum attainable FA as compared to the broth EPS (1.2-1.6 mg/g kaolin), irrespective of the cations. The EPS or bioflocculant used in the study was the simplest form i.e., fermented broth without any further process of purification. The broth EPS contains higher number of colloidal (non settleable) particles, which are originally present in the sludge or produced during fermentation. Colloidal particle increases the turbidity and decreases the FA. According to Higgins and Novak (1997), flocculation decreases with increase in the colloidal particles concentration (in the range of 1-100 μm). Moreover, B-EPS also contains large number of inert particles which did not participate in the coagulation-flocculation process. Their presence in large quantities in the broth might also have affected the coagulation-flocculation process and thus, B-EPS needed relatively higher dosage and attained relatively lower FA as compared to the Magnafloc-155.

3.4 Treatment of river water, municipal and Brewery wastewater

Selection of cations concentration. Addition of B-EPS alone (without any cation addition) to the river water, municipal wastewater and brewery wastewater had no and/or negative effect on the turbidity (i.e., no change and/or increase in turbidity). To induce charge neutralization, different cations were required to be added to the river water, municipal and brewery wastewater. It is prerequisite of the coagulation process. A decrease in surface negative charge of the suspensions with increase in the concentration of cation concentrations was observed (Figure 5.2.5). The small sized visible flocs were started to form when zeta potentials of river water, municipal wastewater and brewery wastewater were reaching to -15 mV, respectively. To attain the maximum flocculation performance of different water and wastewater, often zeta potential of the system is decreased in the range of -15 <zeta potential<-10 mV (Jefferson and Parsons,

2005). The water and wastewater are susceptible to small changes in the input conditions such as addition of flocculants or shear stress within this range of zeta potentials. This phenomenon was observed to initiate at the 50 mg Ca^{2+}/L, 50 mg Mg^{2+}/L, 5 mg Fe^{3+}/L and 5 mg Al^{3+}/L of river water, respectively. Therefore these cation concentrations were selected for further experiments. Similarly the selection of cation concentrations for municipal wastewater and brewery wastewater were also carried out (Figure 5.2.5). For municipal wastewater, 200 mg Ca^{2+}/L, 200 mg Mg^{2+}/L, 20 mg Fe^{3+}/L and 20 mg Al^{3+}/L of municipal wastewater were selected (Figure 5.2.5). For brewery wastewater, 250 mg Ca^{2+}/L, 250 mg Mg^{2+}/L, 40 mg Fe^{3+}/L and 40 mg Al^{3+}/L of municipal wastewater were selected (Figure 5.2.5). Therefore, the cation concentrations as described earlier at which the flocs formation was visible were chosen for jar tests to investigate the effect of EPS concentration on turbidity and COD removal.

River water treatment. Addition of cations to river water was necessary for coagulation or charge neutralization. The results suggest that the FA of the river water was influenced by different cations as well as the concentration of B-EPS (Figure 5.2.6a). It was found that, for all the cations, turbidity removal was increased with increasing EPS dosage up to optimum B-EPS dose. When the EPS dosage was in excess than the optimum dosage, the turbidity removal was decreased. The maximum FA of 93.5%, 93.5%, 94.8%, and 93.5% was observed for Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}, respectively. Final turbidity of river water was 1.5, 1.5, 1.2 and 1.5 NTU, respectively. These turbidity reductions were attained at divalent cations concentration of 50 mg/L of river water (higher concentration) as compared to the trivalent cations of 5 mg/L of river water (lower concentration). Under the similar cation concentrations and mixing conditions, the jar tests were performed using Magnafloc-155, to compare the performance of B-EPS for river water treatment. The addition of different cations along with Magnafloc-155 also had significant impact on FA similar to the broth EPS (Figure 5.2.6b). The maximum FA of 93.9%, 94.3%, 96.1% and 94.3% was observed for Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}, respectively. Optimum concentrations of 0.2 mg Magnafloc-155 /L of river water was relatively lower than the broth EPS (1.0-1.5 mg B-EPS/L kaolin). Maximum COD removal of 70.2% and 67.3% was observed for the Fe^{3+} and Al^{3+} (combined with B-EPS of 1.5 and 1.0 mg/L, respectively) whereas, maximum COD removal of 52.0% and 51.4% was observed for $Ca^{2+}$and Mg^{2+} (combined with B-EPS of 1.0 mg/L). In case of Magnafloc-155 (dose of 0.2 mg/L), maximum COD removal of 48.0%, 53.8%, 53.2% and 46.8% was observed for Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}, respectively.

Overall, the results had revealed that the flocculation performance of B-EPS was quite similar and comparable to the Magnafloc-155. At an optimum dosage of B-EPS, river water turbidity

was reduced to 1.2 NTU. However, as per Health Canada (2003) treated drinking water must have turbidity less than 0.1 NTU at all times. Therefore, to achieve allowed turbidity limits, conventional sand filtration or membrane filtration steps are necessary after flocculation. Initial pH of river water was 7.85, slightly alkaline. After the addition of broth B-EPS or Magnafloc-155, the pH of the river water remained unchanged. The pH value of the river water was decreased from 7.85 to 7.4±2 by the addition of divalent cations ($Ca^{2+}$and Mg^{2+}) during jar tests. This indicates that there was no pH value correction required for the treated water in this case. However, the pH of river water dropped from 7.85 to 6-6.3 upon the addition of trivalent cations (Fe^{3+} and Al^{3+}). The pH shifted from slightly alkaline to acidic. The reduction in pH is attributed to dissociation of cations via series of hydrolysis reactions and production of H^{+} ions. The magnitude of the pH shift is related to the chemical coagulant concentrations. The application of B-EPS to river water treatment has promising results, however further research is required to evaluate its field applicability.

Municipal wastewater treatment. The addition of cations was necessary for the coagulation or charge neutralization of the wastewater solids and it was prerequisite for the addition of flocculants (B-EPS or Magnafloc-155). While the cation concentrations were maintained the same, the optimum B-EPS dosage required to achieve maximum FA was determined by adding different concentrations of B-EPS to municipal wastewater. The addition of different cations along with broth EPS to the wastewater had significant impact on FA as shown in Figure 5.2.7a. The general trend was the increase in the FA with increase in broth EPS (at constant cation concentration) up to the optimum dosage of 1.49 mg B-EPS/L wastewater. Excess addition of B-EPS beyond the optimum dosage did not increase the FA. The maximum FA of 91.7%, 87.6%, 94.3%, and 92.2% was observed for Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}, respectively. Maximum COD removal of 80.4% and 78.1% was observed for the Fe^{3+} and Al^{3+} whereas, maximum COD removal of 74.5% and 74.5% was observed for Ca^{2+} and Mg^{2+} (Table 5.2.3). Maximum turbidity removal and maximum COD removal was achieved at a dose of 2.0 mg B-EPS/L for divalent ($Ca^{2+}$and Mg^{2+}) and 1.5 mg B-EPS/L for trivalent cations (Fe^{3+} and Al^{3+}), respectively.

Under the similar cation concentrations and mixing conditions, the jar tests were performed using Magnafloc-155, to compare the performance of B-EPS for municipal wastewater treatment. The addition of different cations along with Magnafloc-155 also had significant impact on municipal wastewater FA similar to the broth EPS (Figure 5.2.7b). In case of Magnafloc-155, the maximum FA of 93.9%, 94.3%, 96.1% and 94.3% was observed for Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}, respectively. The maximum COD removal of 76.8%, 75.5%, 83.8% and 79.7% was

observed for Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}, respectively. Maximum turbidity removal and maximum COD removal was achieved at a dose of 0.6 mg Magnafloc-155/L and 0.4 mg Magnafloc-155/L for divalent ($Ca^{2+}$and Mg^{2+}) and trivalent cations (Fe^{3+} and Al^{3+}), respectively. Cations can neutralize negative charges of both polysaccharide and suspended particles and increase adsorption of polysaccharide onto suspended particles (Wu and Ye, 2007). Unlike river or lake waters, municipal wastewater provides a system with abundant organic matter and complexing anions that should extend the concentration range in which hydrolyzing coagulant species interact with the raw water content. Lian et al. (2008) reported that the bioflocculant produced by *Bacillus mucilaginosus* was able to remove 93% of suspended solids and 75% of COD from municipal wastewater. Li et al. (2013) reported that polysaccharide-based bioflocculants produced in sucrose medium by *Paenibacillus elgii* revealed municipal wastewater turbidity and COD removal of 83 and 68%, respectively at bioflocculant concentrations of 768.9 mg/L.

Brewery wastewater treatment. The addition of cations was necessary for the coagulation or charge neutralization of the brewery wastewater solids and it was prerequisite for the addition of flocculants (B-EPS or Magnafloc-155). While the cation concentrations were maintained the same, the optimum B-EPS dosage required to achieve maximum FA was determined by adding different concentrations of B-EPS to brewery wastewater. The addition of different cations along with broth EPS to the wastewater had significant impact on FA as shown in Figure 5.2.8a. The general trend was the increase in the FA with increase in broth EPS at constant cation concentration up to the optimum dosage. Excess addition of B-EPS beyond the optimum dosage did not increase the FA. The maximum FA of 81.8%, 76.4%, 83.9%, and 79.5% was observed for Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}, respectively. Maximum COD removal of 88.4%, 85.7%, 87.4% and 86.2% was observed for the Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}, respectively (Table 5.2.3). Optimum B-EPS concentration for maximum turbidity removal and COD removal was 12.4 mg B-EPS/L brewery wastewater, irrespective of the type of cations added to brewery wastewater. Under the similar cation concentrations and mixing conditions, the jar tests were performed using Magnafloc-155, to compare the performance of B-EPS for brewery wastewater treatment. The addition of different cations along with Magnafloc-155 also had significant impact on brewery wastewater FA similar to the broth EPS (Figure 5.2.8b). The maximum FA of 84.2%, 81.5%, 85.6% and 86.4% was observed for Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}, respectively. In case of Magnafloc-155, maximum COD removal of 89.7%, 88.8%, 89.7% and 89.3% was observed for Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}, respectively (Table 5.2.3). Maximum turbidity removal and maximum COD removal was achieved at a dose of 5.0 mg Magnafloc-155/L for divalent ($Ca^{2+}$and Mg^{2+}) and 4.0 mg Magnafloc-155/L for trivalent cations (Fe^{3+} and Al^{3+}), respectively. Lian et al. (2008)

reported that the bioflocculant produced by *Bacillus mucilaginosus* was able to remove 94% of suspended solids and 70% of COD from brewery wastewater. Gong et al. (2008) reported that the bioflocculants (S-EPS) produced by *Serratia ficaria* revealed 80.7% COD removal and 91.8% turbidity removal from brewery wastewater. The optimum bioflocculant concentration reported by Gong et al. (2008) was 4 mg/L, which was relatively lower than the present study. It should be noted that Gong et al. (2008) used different carbon (lactose, glucose and ethanol) and nitrogen (beef extract, urea, peptone, $(NH_4)_2SO_4$ and yeast extract) sources for the production of bioflocculants. On the other hand, in the present study only wastewater sludge was used as a carbon and nitrogen source for bioflocculant production.

4 Conclusions

The optimum agitation speed required for the maximum EPS production (4.9 g/L EPS at 72 h) in batch culture by mixed culture cultivated in sterilized sludge was 150 rpm. The kinetic estimates revealed that the EPS production was growth associated. EPS (in broth form) revealed higher kaolin FA (80%) when combined with divalent cations (Ca^{2+} and Mg^{2+}) as compared to the trivalent cations (Fe^{3+} and Al^{3+}) (less than 70%). With the addition of trivalent cations (Fe^{3+} and Al^{3+}), EPS exhibited very good flocculation performance (turbidity and COD removal) in river water, municipal wastewater and brewery wastewater as compared to the divalent cations (Ca^{2+} and Mg^{2+}). The flocculation performance of broth produced by mixed culture was comparable to Magnafloc-155 (chemical polymer) and hence, it could be used as a flocculant with the addition of trivalent cations (Fe^{3+} and Al^{3+}) for different water and wastewater treatment.

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Table 5.2.1 Wastewater sludge characteristics used in the experiments

Parameters	Concentration
Total solids (TS)	29 g/L
Total volatile solids (TVS)	18 g/L
Total suspended solids (TSS)	25 g/L
Total volatile suspended solids (TVSS)	16 g/L
VS/TS	0.62
VSS/TSS	0.64
pH	6.5
Total carbon	4271000 mg/kg
Total nitrogen	48650 mg/kg
Total phosphorus	1040 mg/kg
Al	6720 mg/kg
Ca	17500 mg/kg
Cd	5.8 mg/kg
Cr	73 mg/kg
Cu	333 mg/kg
NI	32 mg/kg
Fe	11900 mg/kg
K	3100 mg/kg
Mn	94 mg/kg
Na	3900 mg/kg
Pb	47 mg/kg
S	3800 mg/kg
Zn	490 mg/kg

Note: All the data are the means of triplicates. Standard error is less than 5%.

Table 5.2.2 The characteristics of river water, municipal and brewery wastewater

Sample	Characteristics				
	pH	Total solids (mg/L)	Turbidity (NTU)	COD (mg/L)	Zeta potential (mV)
River water	7.85±0.1	340±5	23±0.2	34.6±2	-19.5±1
Municipal wastewater	6.7±0.2	184±3	58±0.5	193±5	-31.6±2
Brewery wastewater	4.5±0.2	2362±10	4063±8	1985±8	-48±2

Note: All the data are the means of triplicates. Standard error is less than 5%.

Table 5.2.3 COD removal from river water, municipal and brewery wastewater using different cations and flocculants (at optimum dosage)

Different waters	Cation dose prior to the flocculants addition		COD removal by different flocculants			
	Cation used	Dose (mg/L)	B-EPS dose (mg/L)	Maximum COD removal efficiency	Magnafloc-155 dose (mg/L)	Maximum COD removal efficiency
River water[a]	Ca^{2+}	50	1.0	52.0±2.61	0.2	48.0±2.22
	Mg^{2+}	50	1.0	51.4±2.52	0.2	53.8±2.40
	Fe^{3+}	5	1.5	70.2±3.15	0.2	53.2±2.31
	Al^{3+}	5	1.0	67.3±3.33	0.2	46.8±2.22
Municipal wastewater[b]	Ca^{2+}	200	2.0	74.5±3.73	0.6	76.8±3.13
	Mg^{2+}	200	2.0	74.6±3.43	0.6	75.5±2.74
	Fe^{3+}	20	1.5	80.4±3.62	0.4	83.8±2.29
	Al^{3+}	20	1.5	78.1±3.05	0.4	79.7±3.31
Brewery wastewater[c]	Ca^{2+}	250	12.4	88.4±4.24	5.0	89.7±3.31
	Mg^{2+}	250	12.4	85.7±3.91	5.0	88.8±3.03
	Fe^{3+}	40	12.4	87.4±3.55	4.0	89.7±3.12
	Al^{3+}	40	12.4	86.2±3.82	4.0	89.3±3.10

Note: [a]Initial turbidity and COD of river water was 23.0 NTU and 34.6 mg/L, respectively.
[b]Initial turbidity and COD of municipal wastewater was 58.0 NTU and 193 mg/L, respectively.
[c]Initial turbidity and COD of brewery wastewater was 4063 NTU and 1985 mg/L, respectively.
n/a – The sample had negative turbidity removal (%) and COD removal (%).
All the data are the means of three independent experiments. Standard error is less than 5%.

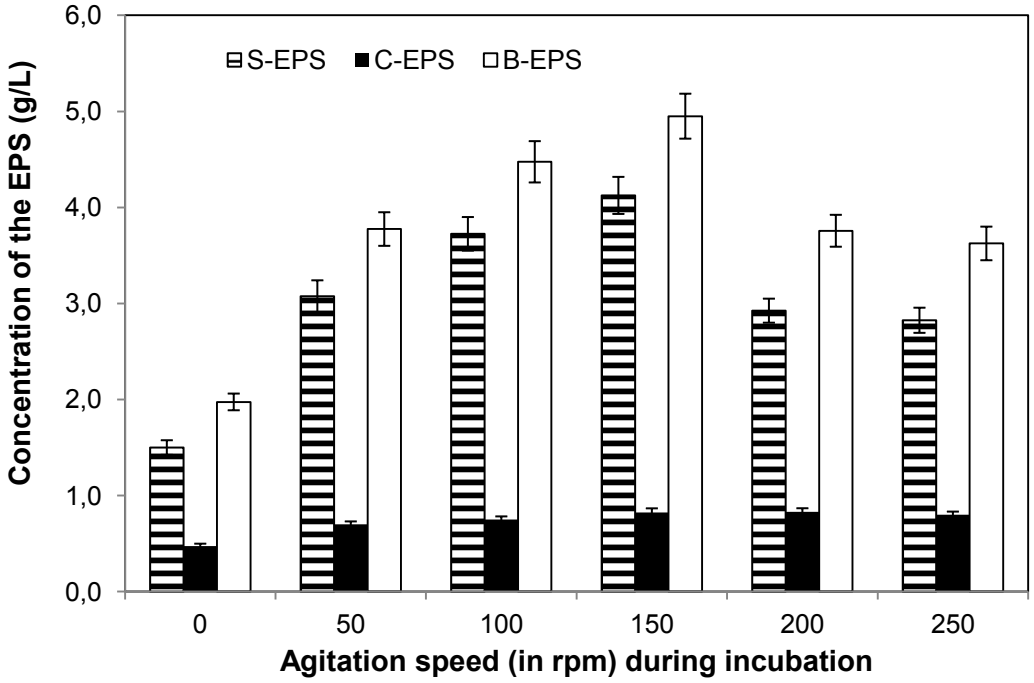


Figure 5.2.1 Effect of agitation speed on different forms of EPS (S-EPS, C-EPS and B-EPS) produced by the mixed culture at 72 h of incubation

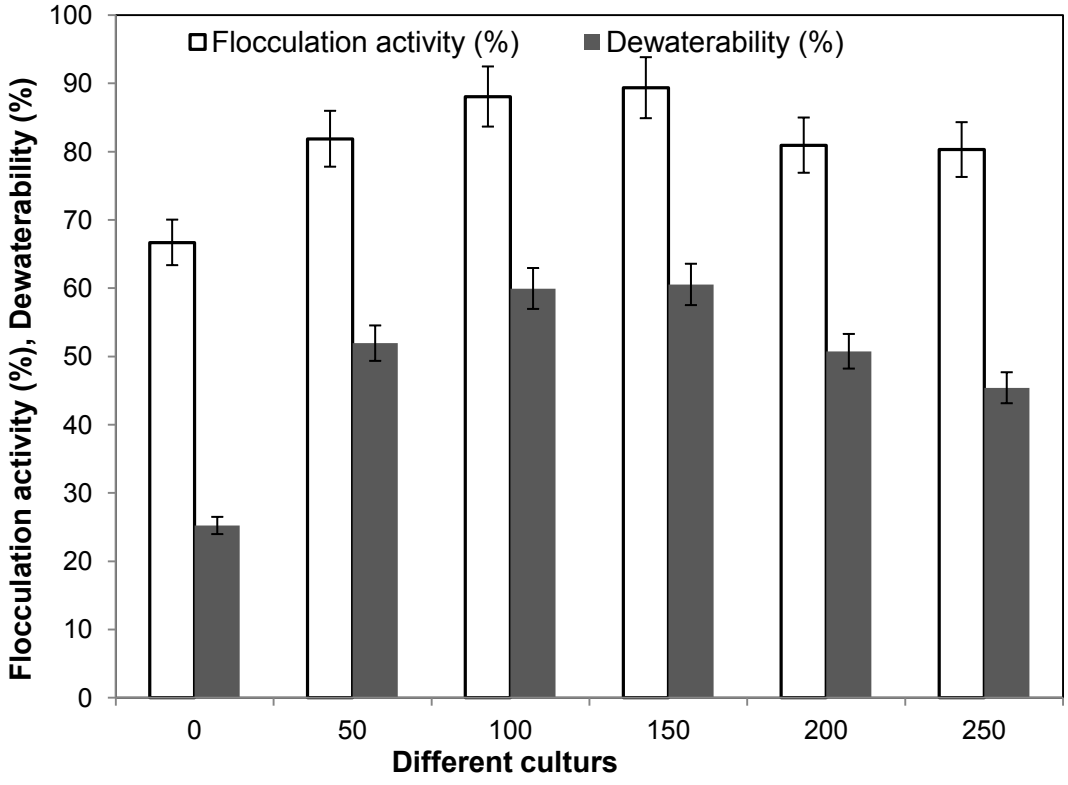


Figure 5.2.2 Kaolin flocculation activity and dewaterability of B-EPS (1 mL) produced by the mixed culture at 72 h of incubation

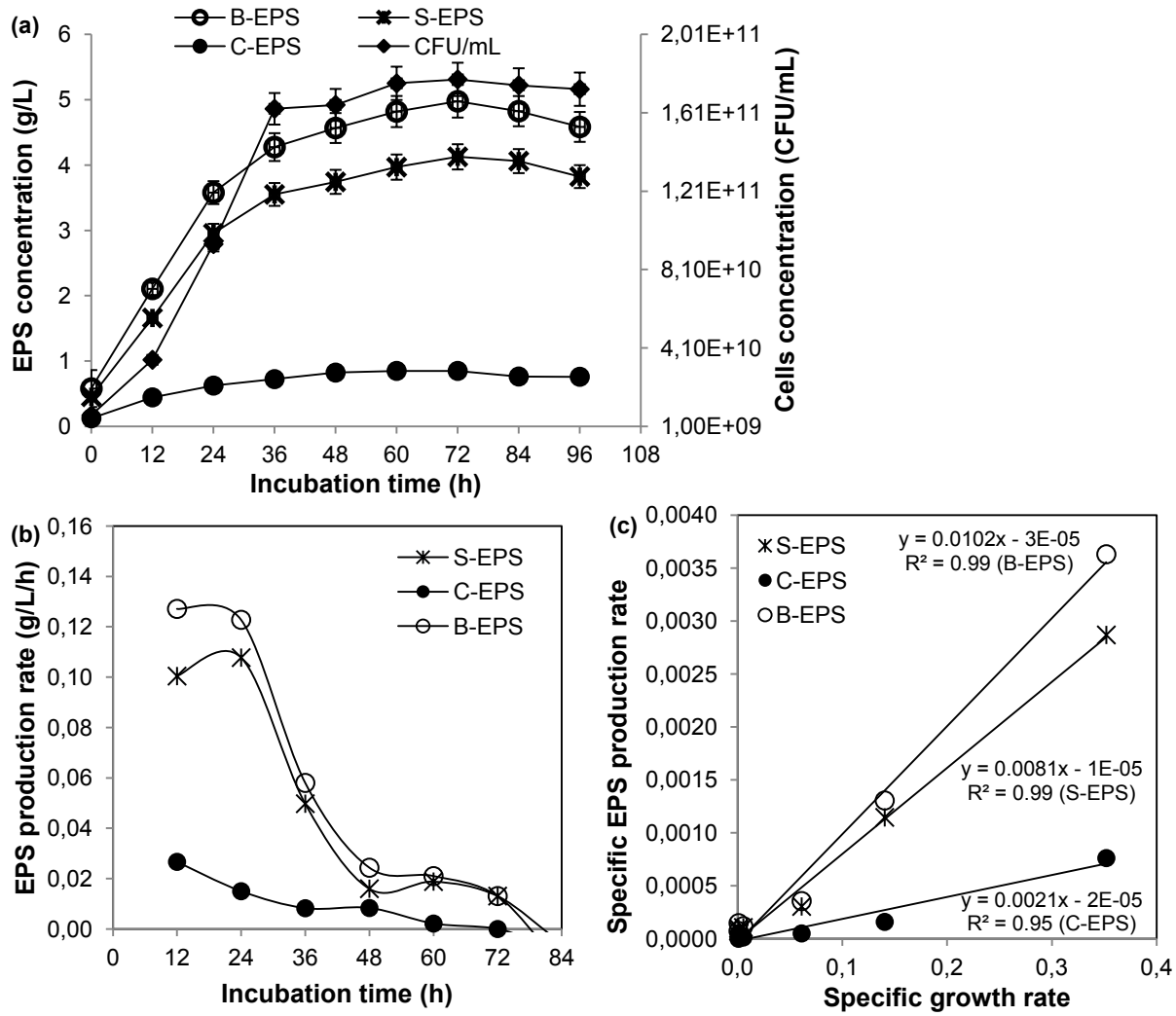


Figure 5.2.3 a) Variation of EPS concentrations (B-EPS, S-EPS, and C-EPS) and total cells concentrations (CFU/mL) during mixed culture growth (of 13 bacterial strains) in sterilized sludge; b) EPS productivity (g/L/h) of the mixed culture in sterilized sludge; c) Relation between specific EPS production (q_{EPS}: g EPS/million cells/h) and specific growth rate of cells (μ), Note: Specific rates were determined per 10^6 CFU/mL. Data are the means of three independent experiments. Standard error is less than 5%.

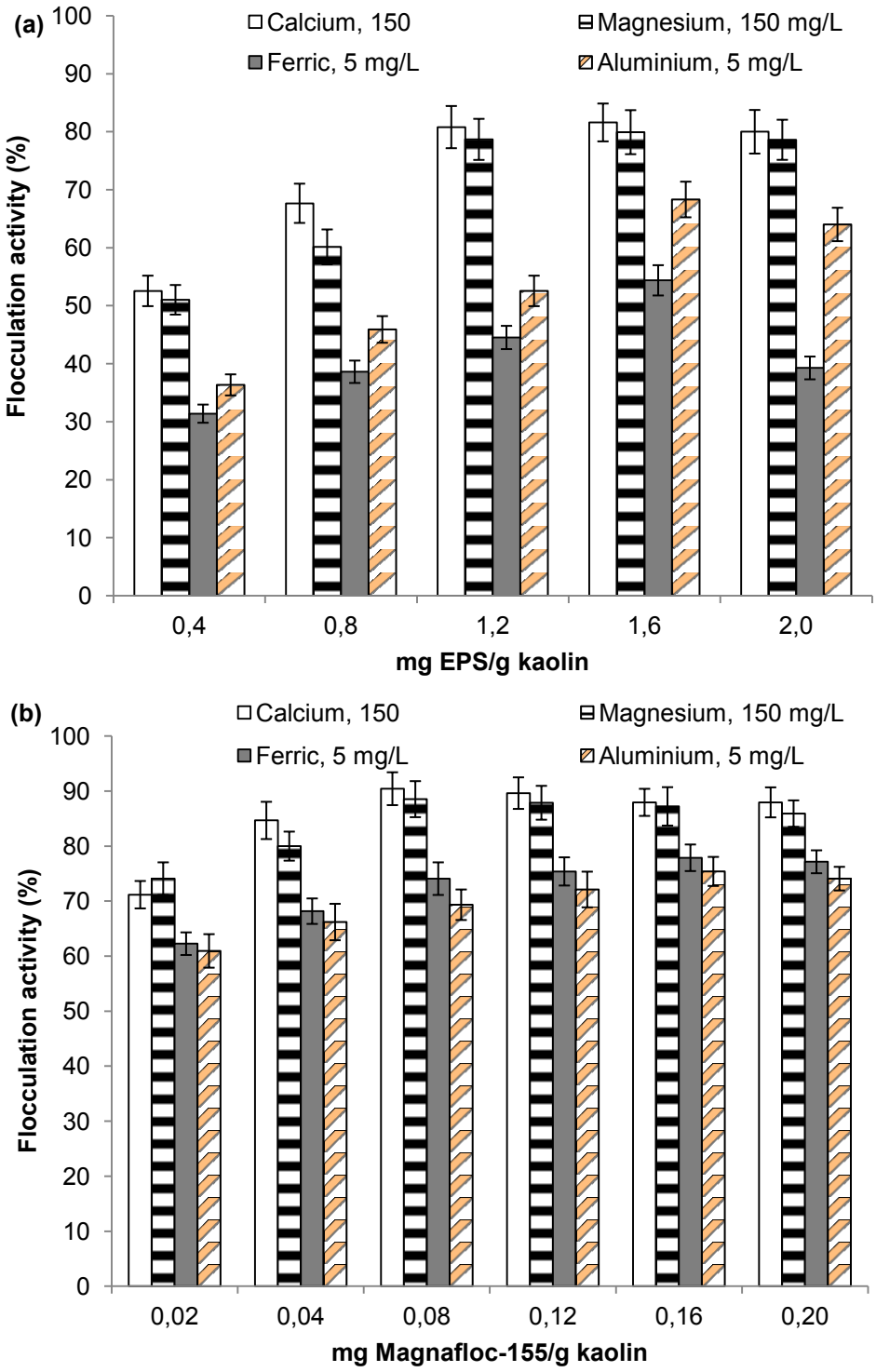


Figure 5.2.4 a) Effect of Broth EPS concentration (produced by mixed culture in sterilized sludge, 25 g/L) and different cations on kaolin flocculation activity; b) Effect of Magnafloc-155 and different cations on kaolin flocculation activity

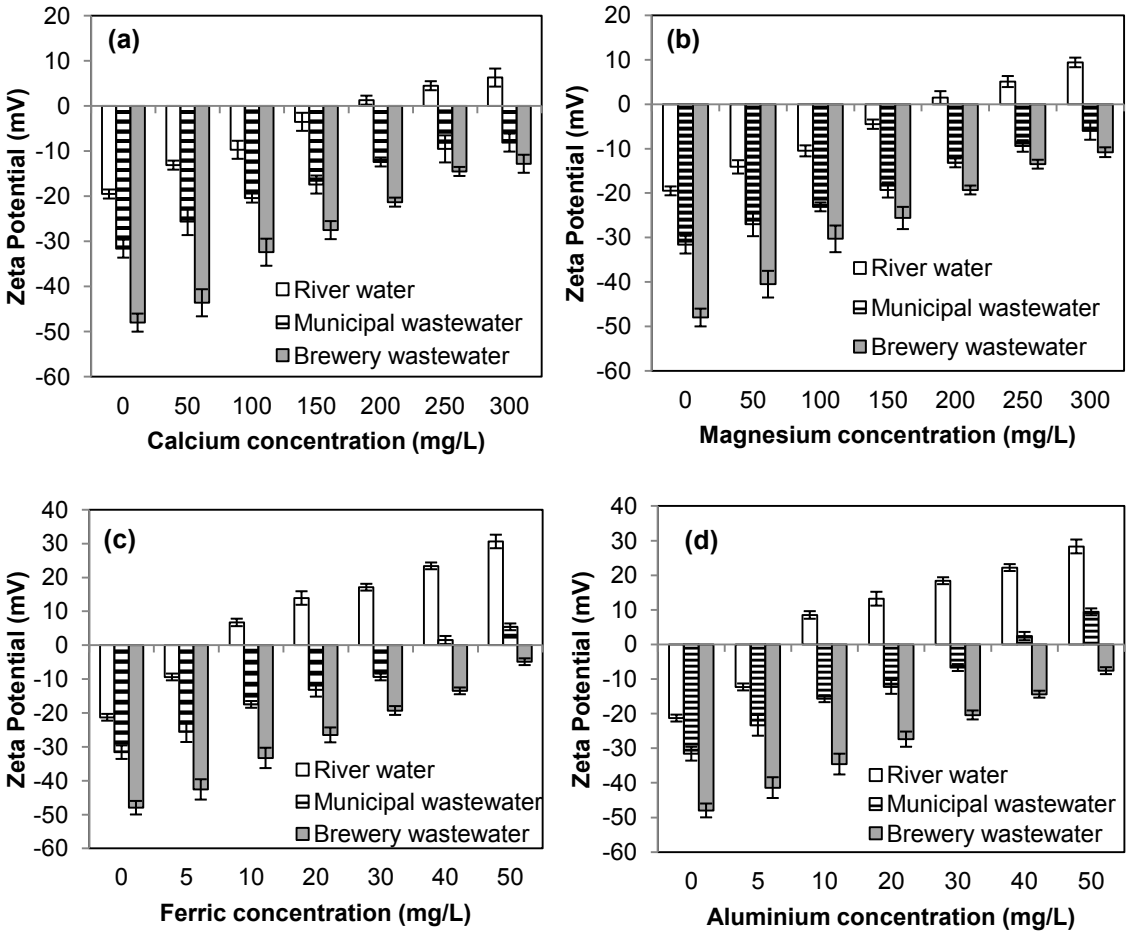


Figure 5.2.5 Zeta potential (mV) of river water, municipal and brewery wastewater at different cation concentrations respectively; a) Calcium (Ca^{2+}), b) magnesium (Mg^{2+}), c) Ferric (Fe^{3+}) and d) Aluminum (Al^{3+})

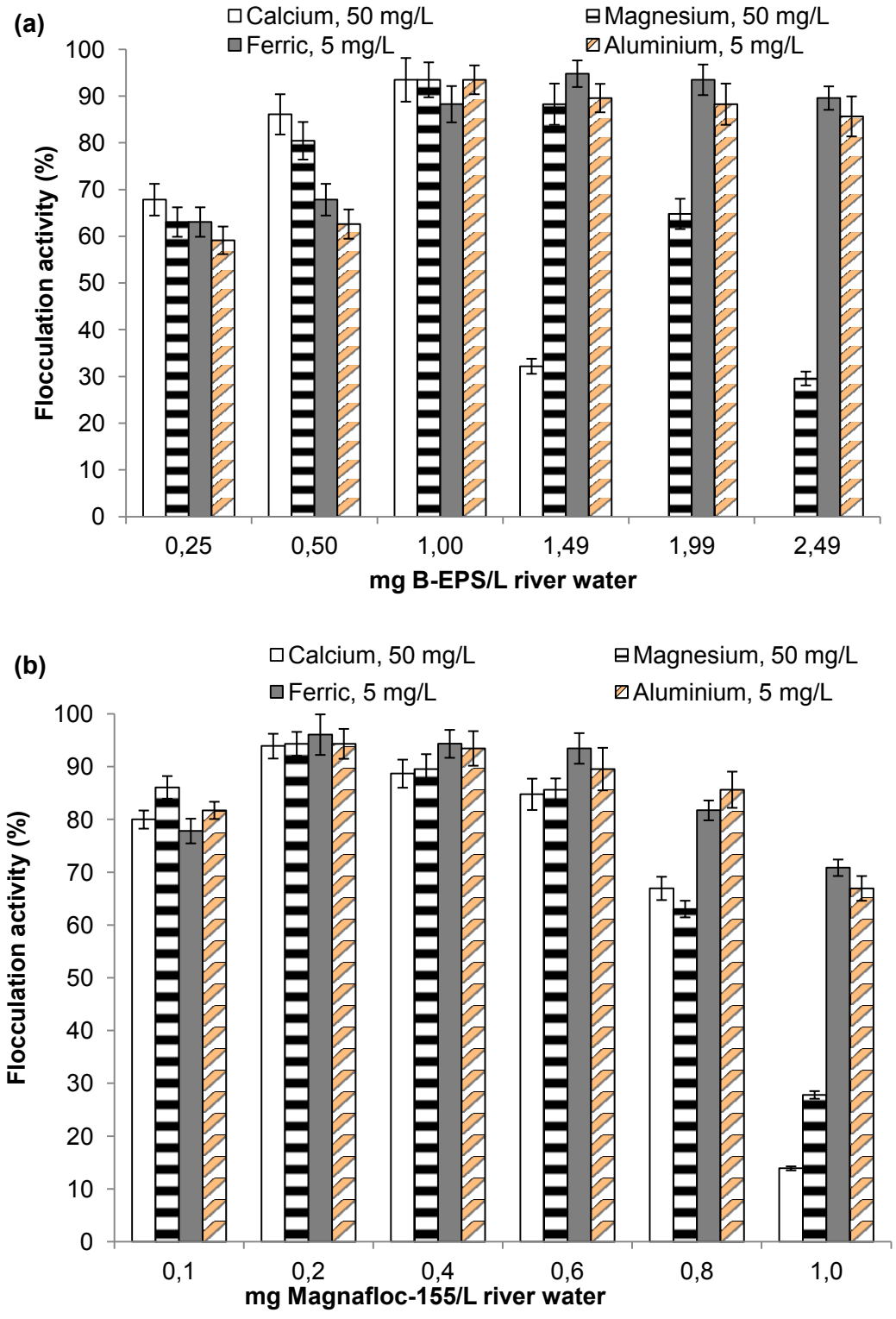


Figure 5.2.6 a) Effect of Broth EPS concentration (produced by mixed culture in sterilized sludge, 25 g/L) and different cations on river water flocculation activity; b) Effect of Magnafloc-155 and different cations on river water flocculation activity.

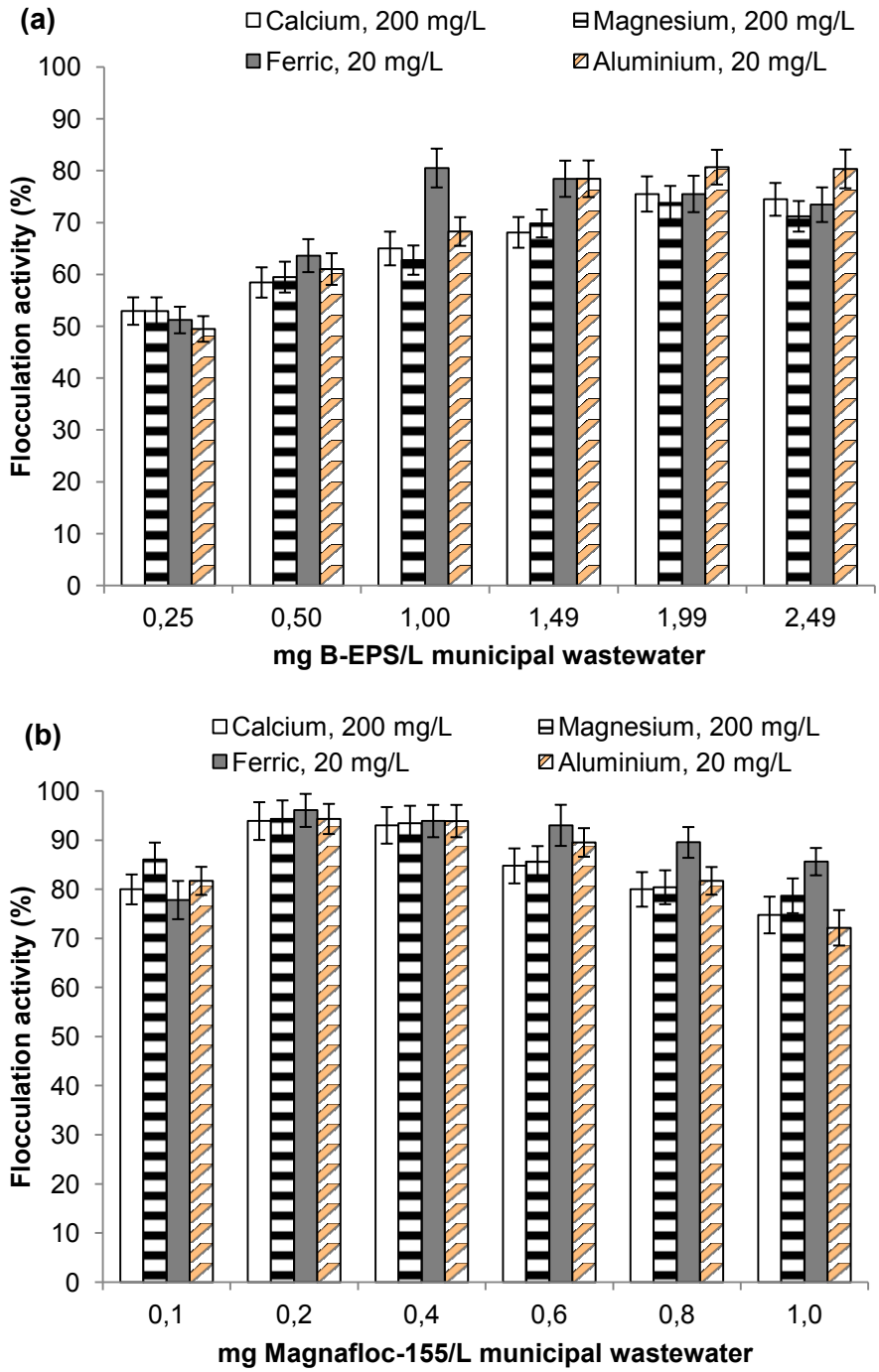


Figure 5.2.7 a) Effect of Broth EPS concentration (produced by mixed culture in sterilized sludge, 25 g/L) and different cations on municipal wastewater flocculation activity; b) Effect of Magnafloc-155 and different cations on municipal wastewater flocculation activity

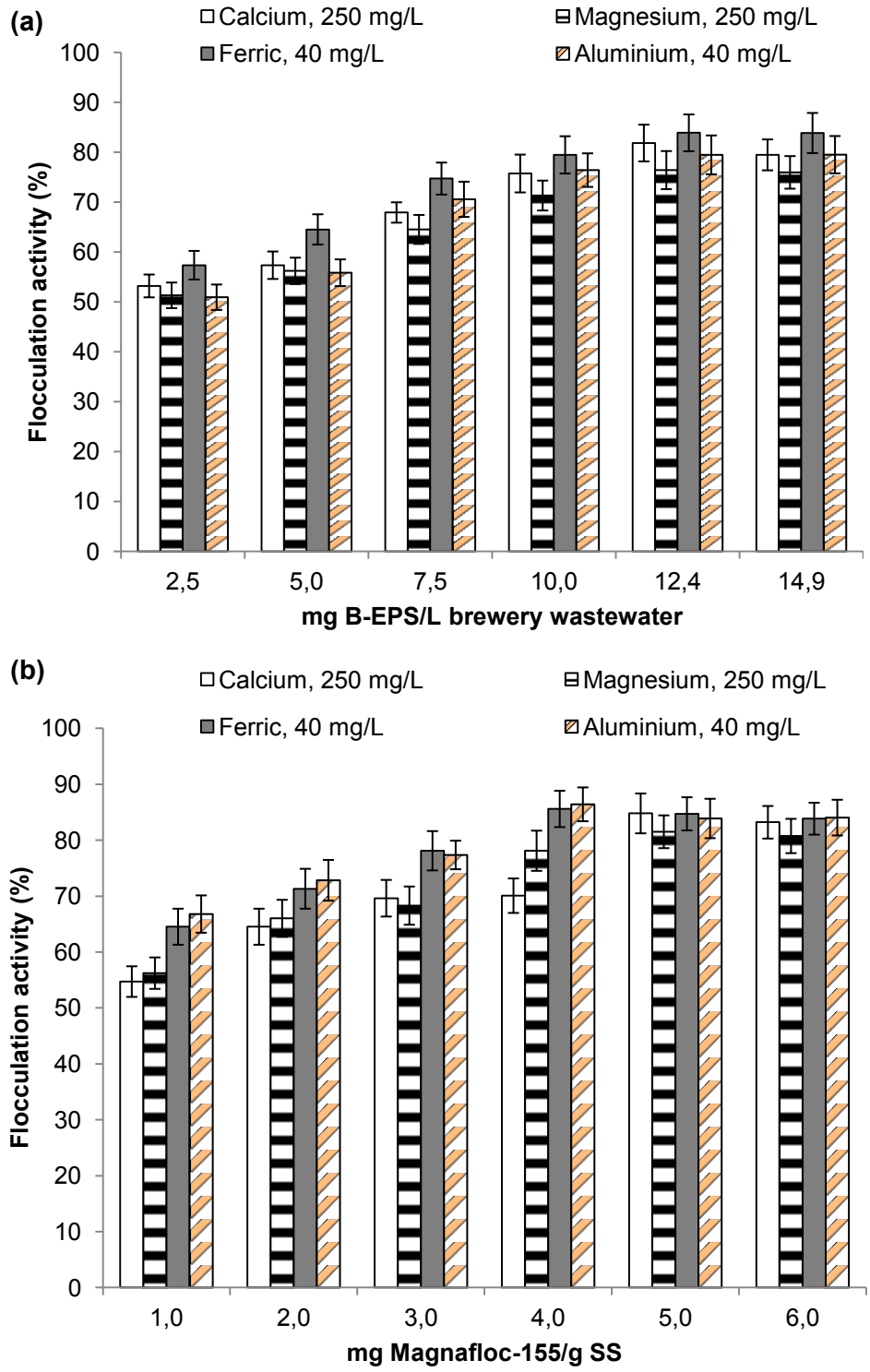


Figure 5.2.8 a) Effect of Broth EPS concentration (produced by mixed culture in sterilized sludge, 25 g/L) and different cations on brewery wastewater flocculation activity; b) Effect of Magnafloc-155 concentration and different cations on brewery wastewater flocculation activity

ANNEXES

Annexe I

Données

Biochemical diversity of the bacterial strains and their biopolymer producing capabilities in wastewater sludge

Table A1.1: Growth of isolated bacterial strains in different pH

pH	*Bacillus* sp.1	*Bacillus* sp.2	*Bacillus* sp.3	*Bacillus* sp.4	*Bacillus* sp.5	*Bacillus* sp.6	*Bacillus* sp.7	*Bacillus* sp.8	*Bacillus* sp.9	*Serratia* sp.1	*Serratia* sp.2	*Yersinia* sp.1	*Yersinia* sp.2
7	+	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+
5	-	+	+	+	+	+	+	+	-	+	+	+	+
Gram's	positive	positive	positive	positive	positive	positive	positive	positive	positive	negative	negative	negative	negative

Note: + growth; **-** no growth

Table A1.2: Growth of isolated bacterial strains in different NaCl (%)

NaCl	*Bacillus* sp.1	*Bacillus* sp.2	*Bacillus* sp.3	*Bacillus* sp.4	*Bacillus* sp.5	*Bacillus* sp.6	*Bacillus* sp.7	*Bacillus* sp.8	*Bacillus* sp.9	*Serratia* sp.1	*Serratia* sp.2	*Yersinia* sp.1	*Yersinia* sp.2
1%	+	+	+	+	+	+	+	+	+	+	+	+	+
4%	+	+	+	+	+	+	+	+	+	+	+	-	-
8%	-	+	+	+	+	+	+	+	+	-	-	-	-

Note: + growth; **-** no growth

Table A1.3: Growth of isolated bacterial strains in Lactic acid

Lactic acid	*Bacillus* sp.1	*Bacillus* sp.2	*Bacillus* sp.3	*Bacillus* sp.4	*Bacillus* sp.5	*Bacillus* sp.6	*Bacillus* sp.7	*Bacillus* sp.8	*Bacillus* sp.9	*Serratia* sp.1	*Serratia* sp.2	*Yersinia* sp.1	*Yersinia* sp.2
1% Sodium Lactate	+	+	+	+	+	+	+	+	+	+	+	+	+

Note: + growth; **-** no growth

Table A1.4: Growth of isolated strains in presence of tetrazolium

Reducing power	*Bacillus* sp.1	*Bacillus* sp.2	*Bacillus* sp.3	*Bacillus* sp.4	*Bacillus* sp.5	*Bacillus* sp.6	*Bacillus* sp.7	*Bacillus* sp.8	*Bacillus* sp.9	*Serratia* sp.1	*Serratia* sp.2	*Yersinia* sp.1	*Yersinia* sp.2
Tetrazolium Violate	-	-	-	-	-	-	-	-	-	+	+	+	+
Tetrazolium Blue	-	-	-	-	-	-	-	-	-	+	+	+	+

Note: + growth; **-** no growth

Table A1.5: Growth of isolated bacterial strains in different carbon sources

Sugars	*Bacillus* sp.1	*Bacillus* sp.2	*Bacillus* sp.3	*Bacillus* sp.4	*Bacillus* sp.5	*Bacillus* sp.6	*Bacillus* sp.7	*Bacillus* sp.8	*Bacillus* sp.9	*Serratia* sp.1	*Serratia* sp.2	*Yersinia* sp.1	*Yersinia* sp.2
Dextrin	-	+	+	+	+	+	+	+	-	+	+	+	+
D-Maltose	-	+	+	+	+	+	+	+	-	+	+	+	+
D-Trehalose	-	+	+	+	+	+	+	+	-	+	+	+	+
D-Cellobiose	-	+	+	+	+	+	+	+	-	+	+	+	+
Gentiobiose	-	+	+	+	+	+	+	+	-	+	+	+	+
Sucrose	-	+	+	+	+	+	+	+	-	-	-	+	+
D-Turanose	-	+	+	+	+	+	+	+	-	-	+	-	-
Stachyose	-	-	+	-	+	+	+	+	-	+	+	-	-
D-Raffinose	-	+	+	+	+	+	+	+	-	+	+	-	-
α-D-Lactose	-	-	+	-	+	+	+	+	-	+	+	-	-
D-Melinoise	-	+	+	+	+	+	+	+	-	+	+	+	+
β-Methyl-D-Glucoside	-	+	+	+	+	+	+	+	-	+	+	+	+
D-Salicin	-	+	+	+	+	+	+	+	-	+	+	+	+
N-Acetyl-D-Glucosamine	-	+	+	+	+	+	+	+	-	+	+	+	+
N-Acetyl- β-D-Mannosamine	-	-	+	+	+	+	+	+	-	+	+	-	+
N-Acetyl-D-Galactoseamine	-	-	-	-	+	-	-	+	-	+	+	+	+
N-Acetyl Neuraminic Acid	-	-	-	-	-	+	-	-	-	-	-	-	-
α-D-Glucose	-	+	+	+	+	+	+	+	-	+	+	+	+
D-Mannose	-	+	+	+	+	+	+	+	-	+	+	+	+
D-Fructose	-	+	+	+	+	+	+	+	-	+	+	+	+
D-Galactose	-	-	+	+	+	+	-	+	-	+	+	+	+
3-Methyl Glucose	-	-	+	-	-	+	-	+	-	+	+	-	-
D-Fucose	-	-	+	+	+	-	+	+	-	+	+	-	-
L-Fucose	-	-	+	+	+	-	+	+	+	+	+	+	+
L-Rhamnose	-	+	+	+	+	-	+	+	-	-	-	-	+
Inosine	-	-	+	+	+	-	+	+	-	+	+	+	+

Note: + growth; **-** no growth

Table A1.6: Growth of isolated bacterial strains in different Hexose-PO4's

Hexose-PO4's	*Bacillus* sp.1	*Bacillus* sp.2	*Bacillus* sp.3	*Bacillus* sp.4	*Bacillus* sp.5	*Bacillus* sp.6	*Bacillus* sp.7	*Bacillus* sp.8	*Bacillus* sp.9	*Serratia* sp.1	*Serratia* sp.2	*Yersinia* sp.1	*Yersinia* sp.2
D-Glucose-6-PO4	-	-	+	-	-	-	-	-	-	+	+	+	+
D-Fructose-6-PO4	-	-	-	-	-	-	+	+	-	+	+	+	+

Note: + growth; **-** no growth

Table A1.7: Growth of isolated bacterial strains in different amino acids/proteins

Amino acids/Proteins	*Bacillus* sp.1	*Bacillus* sp.2	*Bacillus* sp.3	*Bacillus* sp.4	*Bacillus* sp.5	*Bacillus* sp.6	*Bacillus* sp.7	*Bacillus* sp.8	*Bacillus* sp.9	*Serratia* sp.1	*Serratia* sp.2	*Yersinia* sp.1	*Yersinia* sp.2
Gelatin	-	+	+	+	+	+	+	+	-	-	-	-	-
Glycyl-L-Proline	-	-	-	+	-	-	-	+	-	+	+	+	+
L-Alanine	-	+	+	+	+	+	+	+	-	+	+	+	+
L-Arginine	-	+	+	+	+	+	+	+	-	-	-	-	-
L-Aspartic Acid	+	+	+	+	+	+	+	+	-	+	+	+	+
L-Glutamic Acid	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Histidine	+	+	+	+	+	+	+	-	-	+	+	+	+
L-Pyroglutammic Acid	-	+	+	+	-	-	-	+	-	-	-	-	-
L-Serine	-	+	+	+	+	-	+	+	-	+	+	+	+
D-Aspartic Acid	-	-	-	-	+	-	-	+	-	-	-	-	-
D-Serine	-	-	+	-	-	-	-	-	-	+	+	+	+

Note: + growth; **-** no growth

Table A1.8: Growth of isolated bacterial strains in different Hexose acids

Hexose acids	*Bacillus* sp.1	*Bacillus* sp.2	*Bacillus* sp.3	*Bacillus* sp.4	*Bacillus* sp.5	*Bacillus* sp.6	*Bacillus* sp.7	*Bacillus* sp.8	*Bacillus* sp.9	*Serratia* sp.1	*Serratia* sp.2	*Yersinia* sp.1	*Yersinia* sp.2
Pectin	-	+	+	+	+	+	+	+	-	+	+	+	+
D-Galacturonic Acid	-	+	+	+	-	-	+	+	-	+	+	+	+
L-Galactonic Acid Lactone	-	-	-	-	-	-	-	-	-	-	+	-	-
D-Gluconic Acid	-	+	+	+	+	+	+	+	-	+	+	+	+
Glucuronic Acid	-	+	+	+	-	-	+	+	-	+	+	+	+
Glucuronamide	-	-	-	-	-	-	-	+	-	-	+	-	-
Mucic Acid	-	+	+	+	-	-	+	+	-	-	-	-	+
Quinic Acid	-	-	-	-	+	-	-	-	-	-	-	-	-
D-Saccharic Acid	-	+	+	+	-	-	+	+	-	+	+	-	-

Note: + growth; **-** no growth

Table A1.9: Growth of isolated bacterial strains in different Carbolic acids, esters and fatty acids

Carboxylic acids, esters, and fatty acids	*Bacillus* sp.1	*Bacillus* sp.2	*Bacillus* sp.3	*Bacillus* sp.4	*Bacillus* sp.5	*Bacillus* sp.6	*Bacillus* sp.7	*Bacillus* sp.8	*Bacillus* sp.9	*Serratia* sp.1	*Serratia* sp.2	*Yersinia* sp.1	*Yersinia* sp.2
P-Hydroxy-Phenylacetic Acid	-	-	-	-	-	-	-	-	-	+	+	-	-
Methyl Pyuvate	-	+	+	+	+	+	+	+	-	-	+	-	+
D-Lactic Acid Methyl Ester	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Lactic Acid	+	+	+	+	+	+	+	+	-	+	+	-	-
Citric Acid	-	+	+	+	+	-	+	+	-	+	+	-	+
α-Keto-Glutaric Acid	-	-	-	-	-	-	-	-	-	+	+	-	-
D-Malic Acid	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Malic Acid	+	+	+	+	+	+	+	+	-	+	+	+	+
Bromo-Succinic Acid	-	-	+	+	+	+	+	+	-	+	+	+	+
Tween 40	-	+	+	+	+	+	+	+	-	+	+	-	-
γ-Amino-Butyric Acid	-	+	+	-	+	-	+	+	-	-	-	-	-
α-Hydroxy Butyric Acid	-	-	-	-	-	-	-	+	-	-	-	-	-
β-Hydroxy-D, L-Butyric Acid	-	-	-	-	-	-	-	-	-	-	-	+	-
α-Keto-Butyric Acid	-	-	-	-	-	-	-	-	-	-	-	-	-
Acetoacetic Acid	+	+	+	+	+	+	+	+	-	-	-	-	-
Propionic Acid	-	-	-	-	-	-	-	+	-	-	-	-	-
Acetic Acid	+	+	+	+	+	-	+	+	-	+	+	+	+
Forminc Acid	+	+	+	+	-	-	+	-	-	+	+	+	-

Note: + growth; **-** no growth

Table A1.10: Growth of isolated bacterial strains in antibiotics

Antibiotics	*Bacillus* sp.1	*Bacillus* sp.2	*Bacillus* sp.3	*Bacillus* sp.4	*Bacillus* sp.5	*Bacillus* sp.6	*Bacillus* sp.7	*Bacillus* sp.8	*Bacillus* sp.9	*Serratia* sp.1	*Serratia* sp.2	*Yersinia* sp.1	*Yersinia* sp.2
Fusidic Acid	-	-	-	-	-	-	-	-	-	-	-	-	-
Troleandomycin	-	-	-	-	-	-	-	-	-	+	+	+	+
Rifamycin SV	-	-	-	-	-	-	-	-	-	+	+	+	+
Minocycline	-	-	-	-	-	-	-	-	-	-	-	-	-
Lincomycin	-	-	-	-	-	-	-	-	-	+	+	+	+
Vancomycin	-	-	-	-	-	-	-	-	-	+	+	+	+
Nalidixic Acid	-	-	-	-	-	-	-	-	-	+	+	+	+
Aztreonam	+	-	-	-	+	+	-	+	+	+	+	-	-

Note: + growth; **-** no growth

Table A1-11: Growth of isolated bacterial strains in sugar alcohols

Antibiotics	*Bacillus* sp.1	*Bacillus* sp.2	*Bacillus* sp.3	*Bacillus* sp.4	*Bacillus* sp.5	*Bacillus* sp.6	*Bacillus* sp.7	*Bacillus* sp.8	*Bacillus* sp.9	*Serratia* sp.1	*Serratia* sp.2	*Yersinia* sp.1	*Yersinia* sp.2
D-Sorbitol	-	+	+	+	+	+	+	+	-	+	+	+	+
D-Mannitol	-	+	+	+	+	+	+	+	-	+	+	+	+
D-Arabitol	-	-	-	-	-	+	-	+	-	+	+	+	-
Myo-Inositol	-	+	+	+	+	+	+	+	-	+	+	+	+
Glycerol	-	+	+	+	+	+	+	+	-	+	+	+	+

Note: + growth; **-** no growth

Annexe II

Données

Extracellular polymeric substances (EPS) production kinetics of thirteen sludge isolates using wastewater sludge as raw material and its flocculation potential

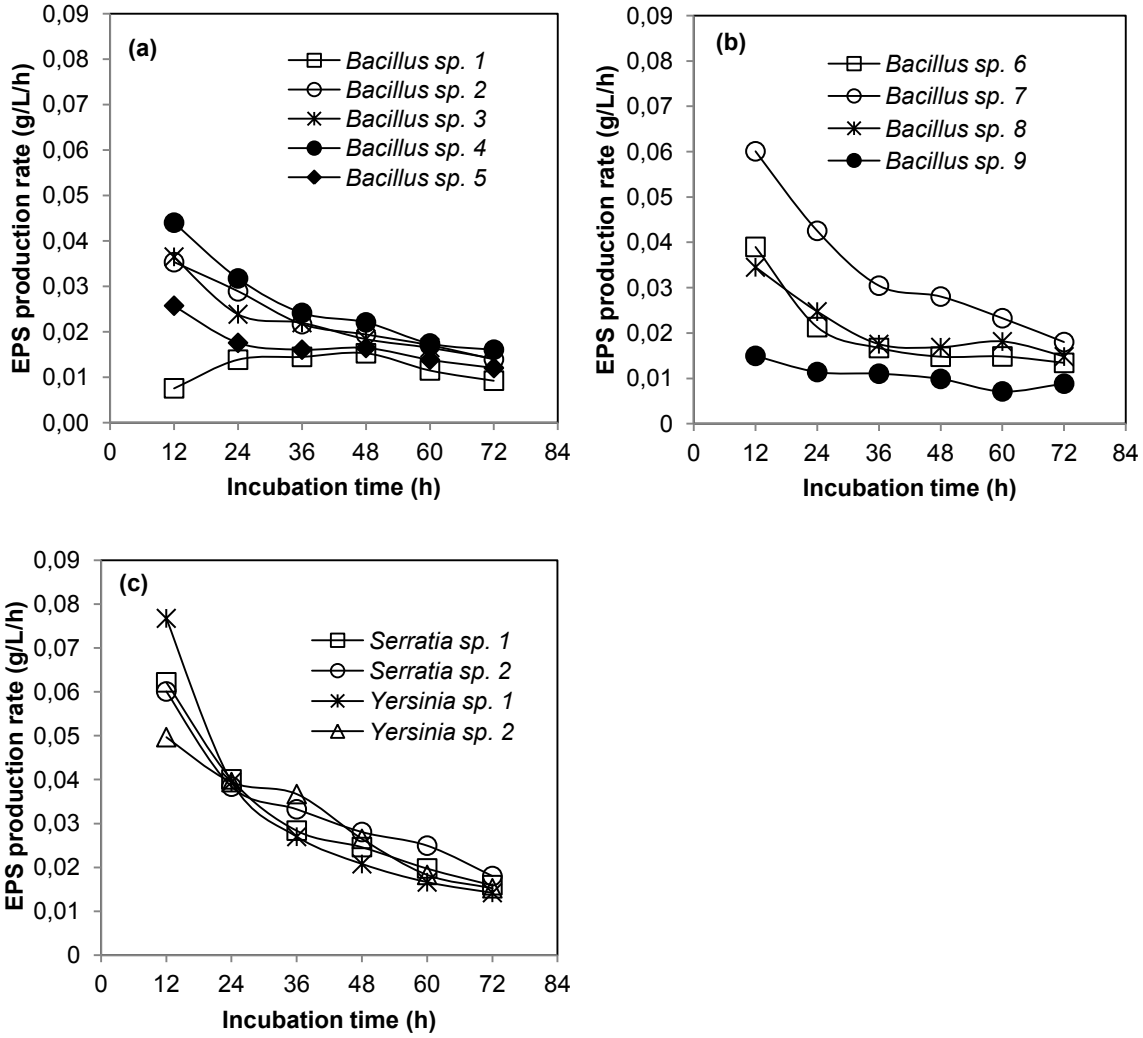
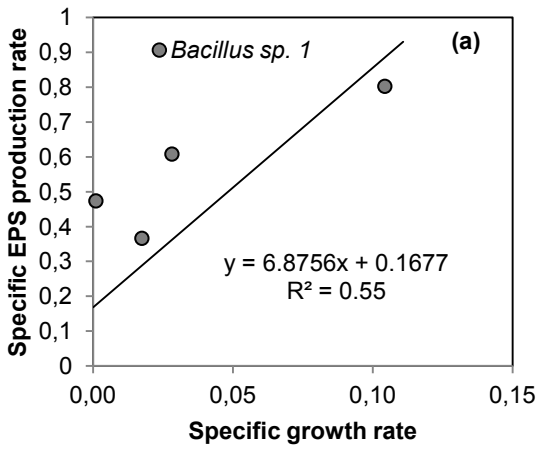
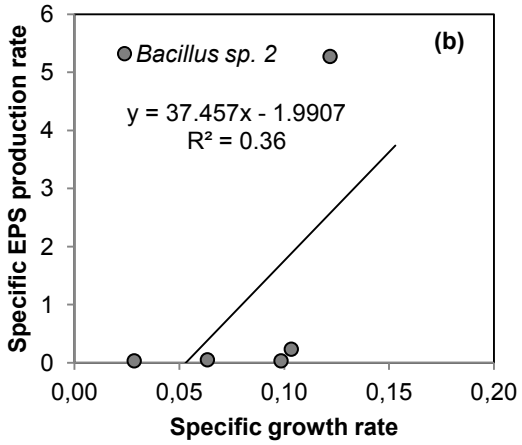
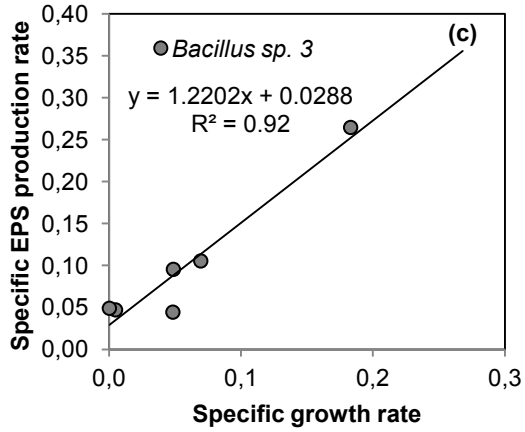
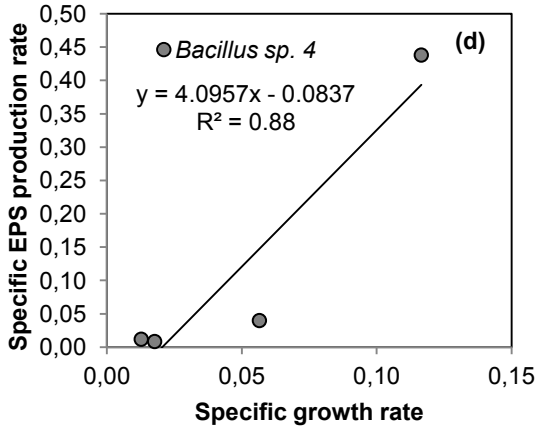


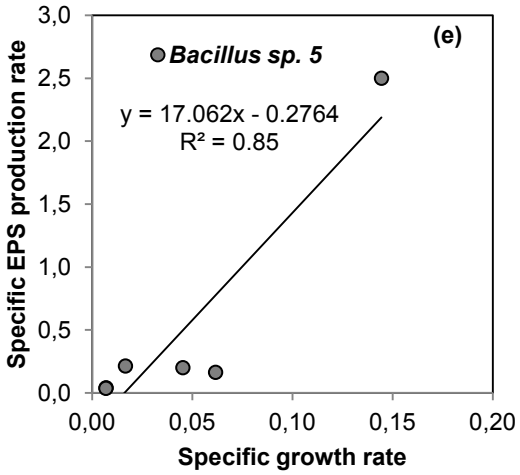
Figure A2.1: Variations in the EPS production rates of bacterial strains cultivated in sterilized sludge at different incubation time; (a) for *Bacillus* sp. 1, *Bacillus* sp. 2, *Bacillus* sp. 3, *Bacillus* sp. 4 and *Bacillus* sp. 5, (b) for *Bacillus* sp. 6, *Bacillus* sp. 7, *Bacillus* sp. 8 and *Bacillus* sp. 9; (c) for *Serratia* sp. 1, *Serratia* sp. 2, *Yersinia* sp. 1, *Yersinia* sp. 2, respectively.

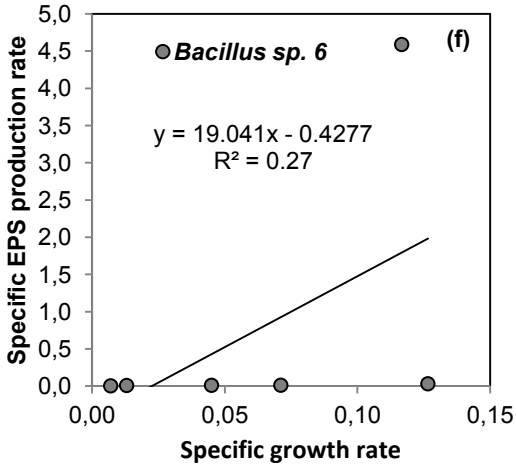

Bacillus sp. 1
(a)
y = 6.8756x + 0.1677
R² = 0.55
Specific EPS production rate
Specific growth rate

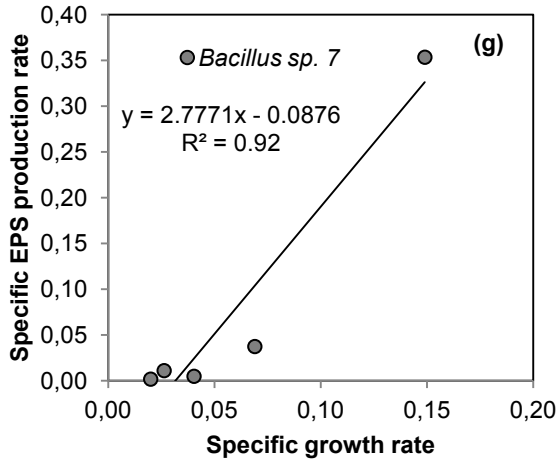

Bacillus sp. 2
(b)
y = 37.457x - 1.9907
R² = 0.36
Specific EPS production rate
Specific growth rate

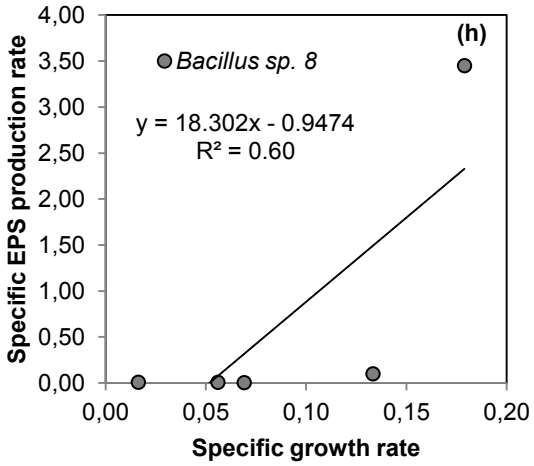

Bacillus sp. 3
(c)
y = 1.2202x + 0.0288
R² = 0.92
Specific EPS production rate
Specific growth rate

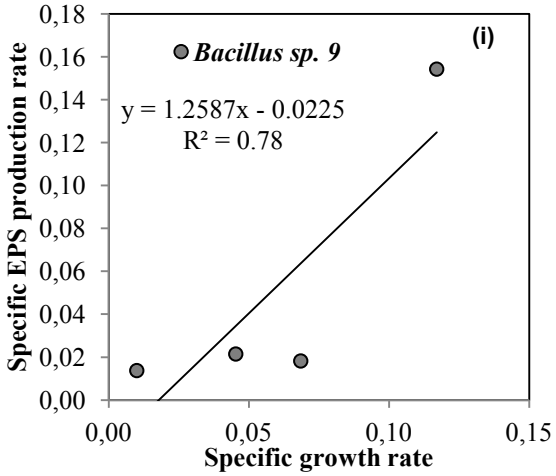

Bacillus sp. 4
(d)
y = 4.0957x - 0.0837
R² = 0.88
Specific EPS production rate
Specific growth rate

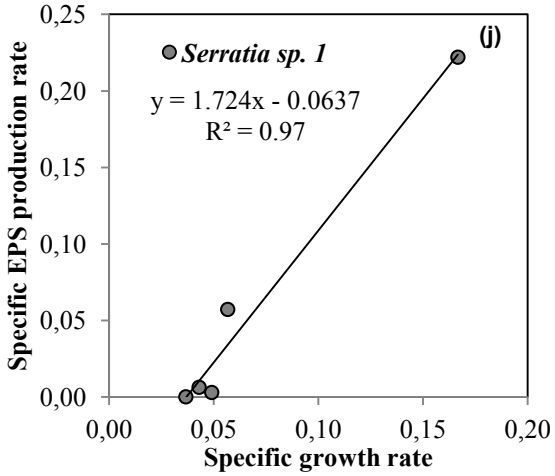

Bacillus sp. 5
(e)
y = 17.062x - 0.2764
R² = 0.85
Specific EPS production rate
Specific growth rate

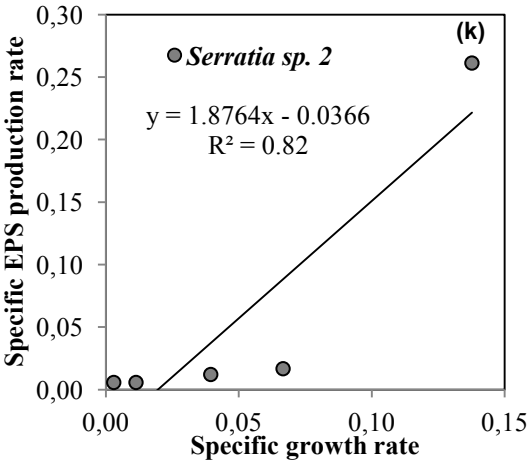

Bacillus sp. 6
(f)
y = 19.041x - 0.4277
R² = 0.27
Specific EPS production rate
Specific growth rate

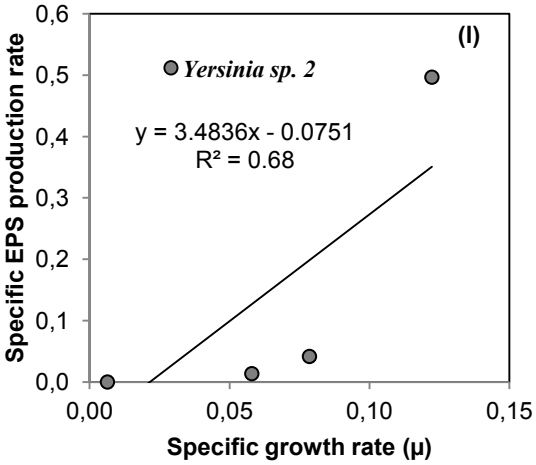

(g)
Bacillus sp. 7
y = 2.7771x - 0.0876
R² = 0.92
Specific EPS production rate
Specific growth rate
0,00
0,05
0,10
0,15
0,20
0,25
0,30
0,35
0,40


(h)
Bacillus sp. 8
y = 18.302x - 0.9474
R² = 0.60
Specific EPS production rate
Specific growth rate
0,00
0,50
1,00
1,50
2,00
2,50
3,00
3,50
4,00


(i)
Bacillus sp. 9
y = 1.2587x - 0.0225
R² = 0.78
Specific EPS production rate
Specific growth rate
0,00
0,02
0,04
0,06
0,08
0,10
0,12
0,14
0,16
0,18


(j)
Serratia sp. 1
y = 1.724x - 0.0637
R² = 0.97
Specific EPS production rate
Specific growth rate
0,00
0,05
0,10
0,15
0,20
0,25


(k)
Serratia sp. 2
y = 1.8764x - 0.0366
R² = 0.82
Specific EPS production rate
Specific growth rate
0,00
0,05
0,10
0,15
0,20
0,25
0,30


(l)
Yersinia sp. 2
y = 3.4836x - 0.0751
R² = 0.68
Specific EPS production rate
Specific growth rate (μ)
0,0
0,1
0,2
0,3
0,4
0,5
0,6

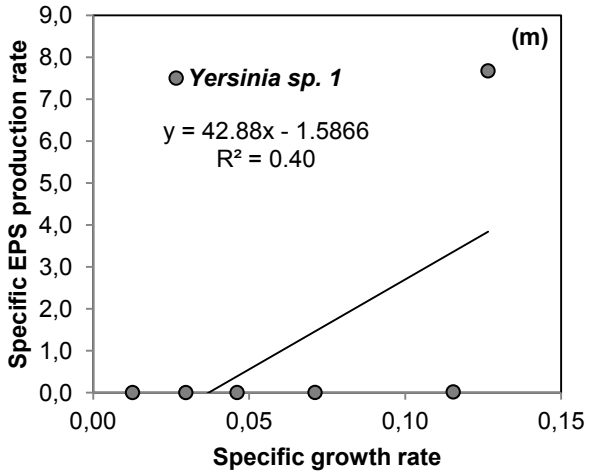


Figure A2.2: Relation between specific EPS production (*q*EPS) and specific growth rate of cells (*μ*); (a) *Bacillus* sp. 1, (b) *Bacillus* sp. 2, (c) *Bacillus* sp. 3, (d) *Bacillus* sp. 4, (e) *Bacillus* sp. 5, (f) *Bacillus* sp. 6, (g) *Bacillus* sp. 7, (h) *Bacillus* sp. 8, (i) *Bacillus* sp. 9, (j) *Serratia* sp. 1, (k) *Serratia* sp. 2, (l) *Yersinia* sp. 1 and (m) *Yersinia* sp. 2, respectively.

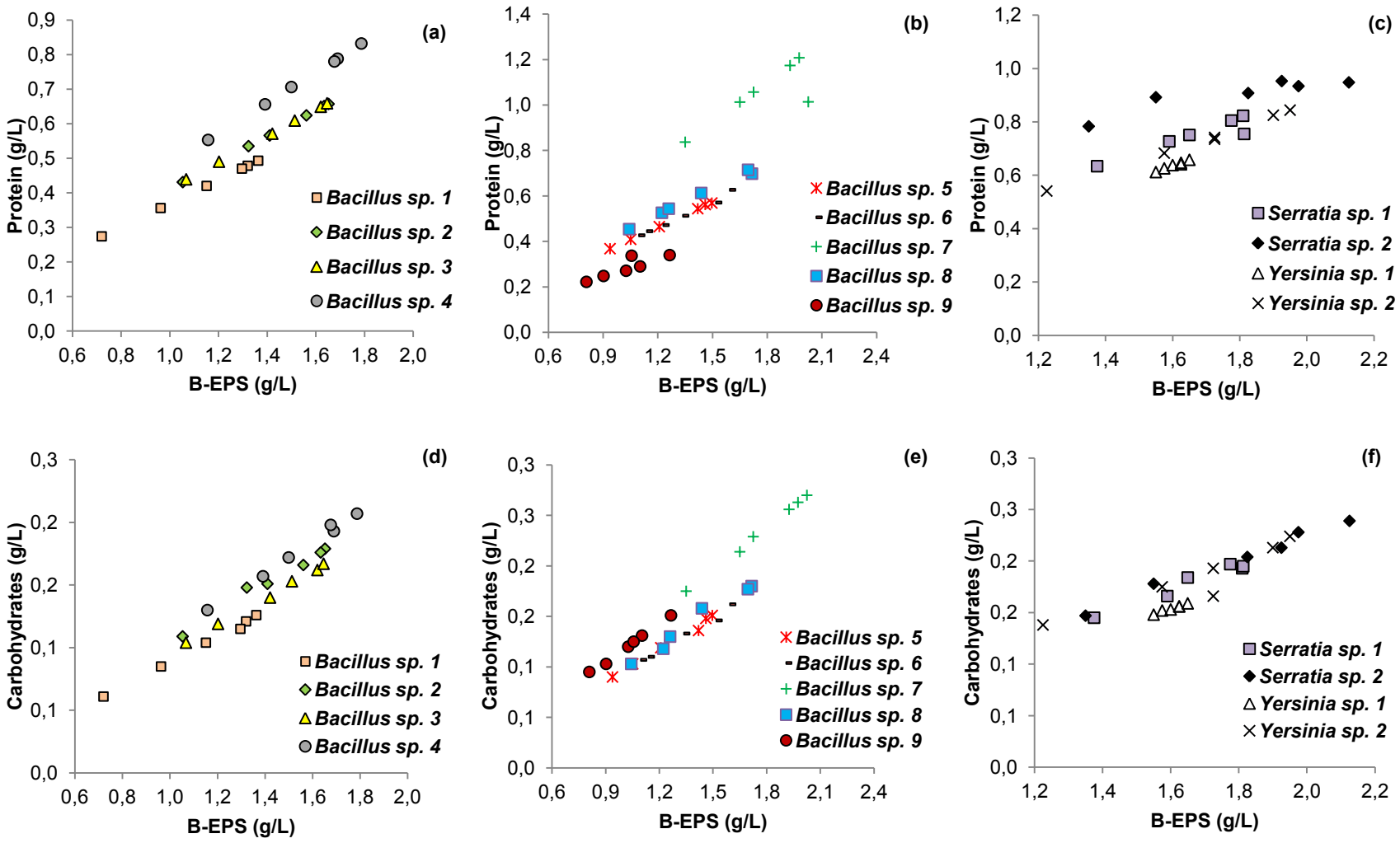


Figure A2.3: Proteins (a, b and c) and Carbohydrates (d, e and f) concentrations observed at different B-EPS concentrations for different bacterial strains.

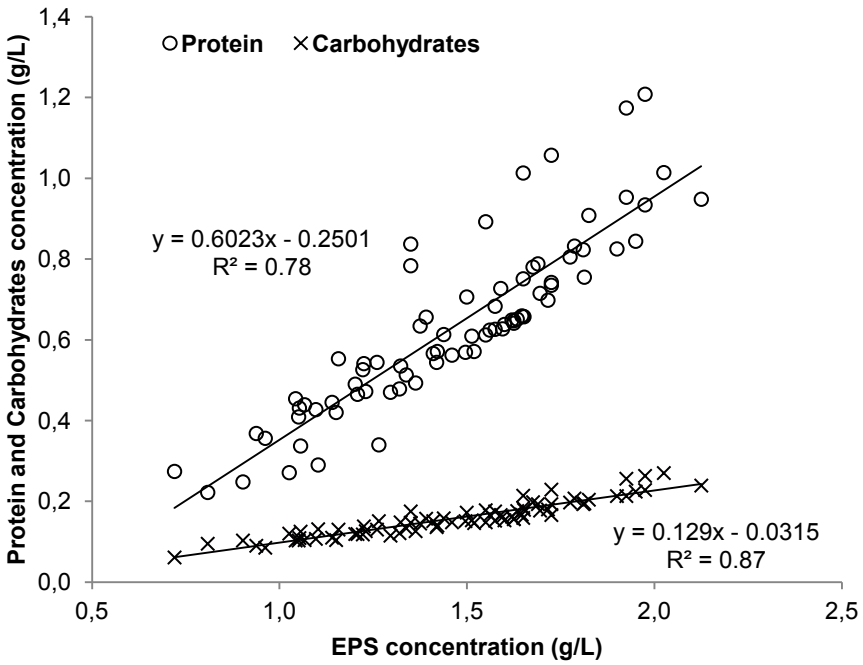


Figure A2.4: Proteins and Carbohydrates concentrations observed at different B-EPS concentrations, irrespective of the bacterial strains.

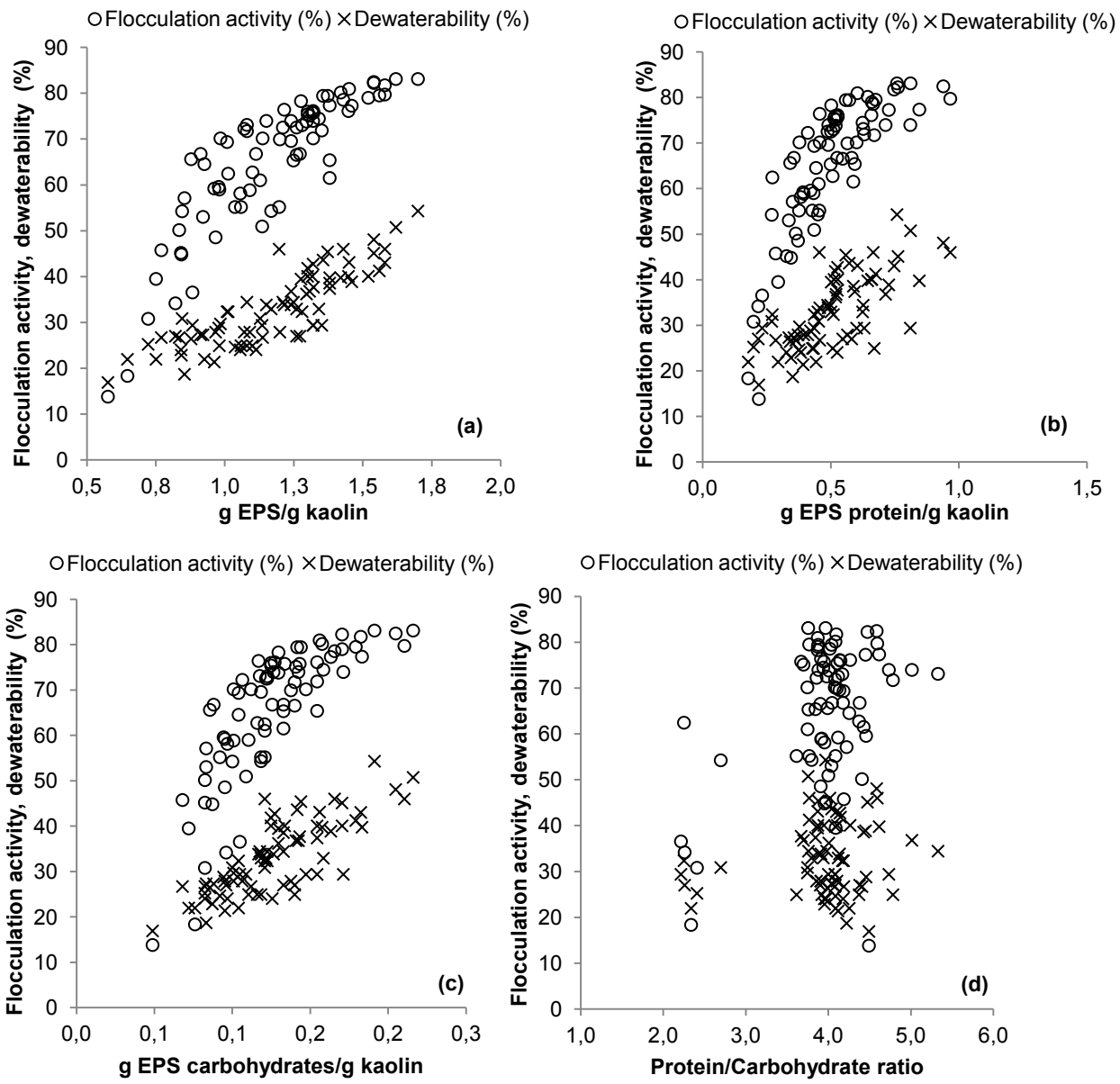


Figure A2.5: Flocculation activity and dewaterability (%) observed for (a) g EPS/g kaolin, (b) g EPS protein/g kaolin, (c) g EPS carbohydrates/g kaolin, (d) protein/carbohydrates, irrespective of the bacterial strains.

Annexe III

Données

Bacterial polymer production using pre-treated sludge as raw material and its flocculation and dewatering potential

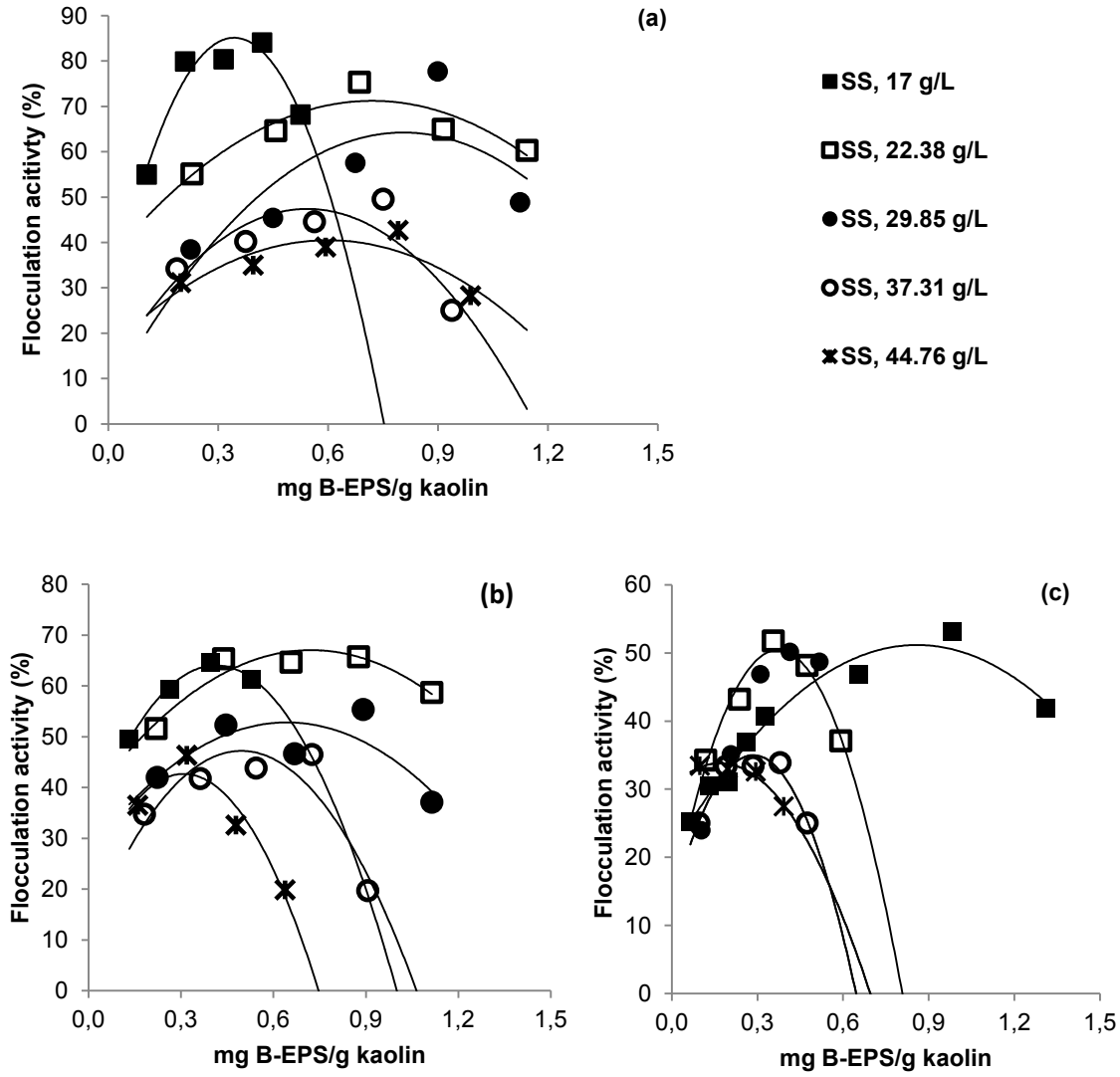


Figure A3.1: Flocculation activity of B-EPS produced in (a) sterilized sludge (b) alkaline-thermal treated sludge and (c) acid-thermal treated sludge at different sludge solids concentrations (SS: 17, 22.38, 29.85, 37.31 and 44.76 g/L).

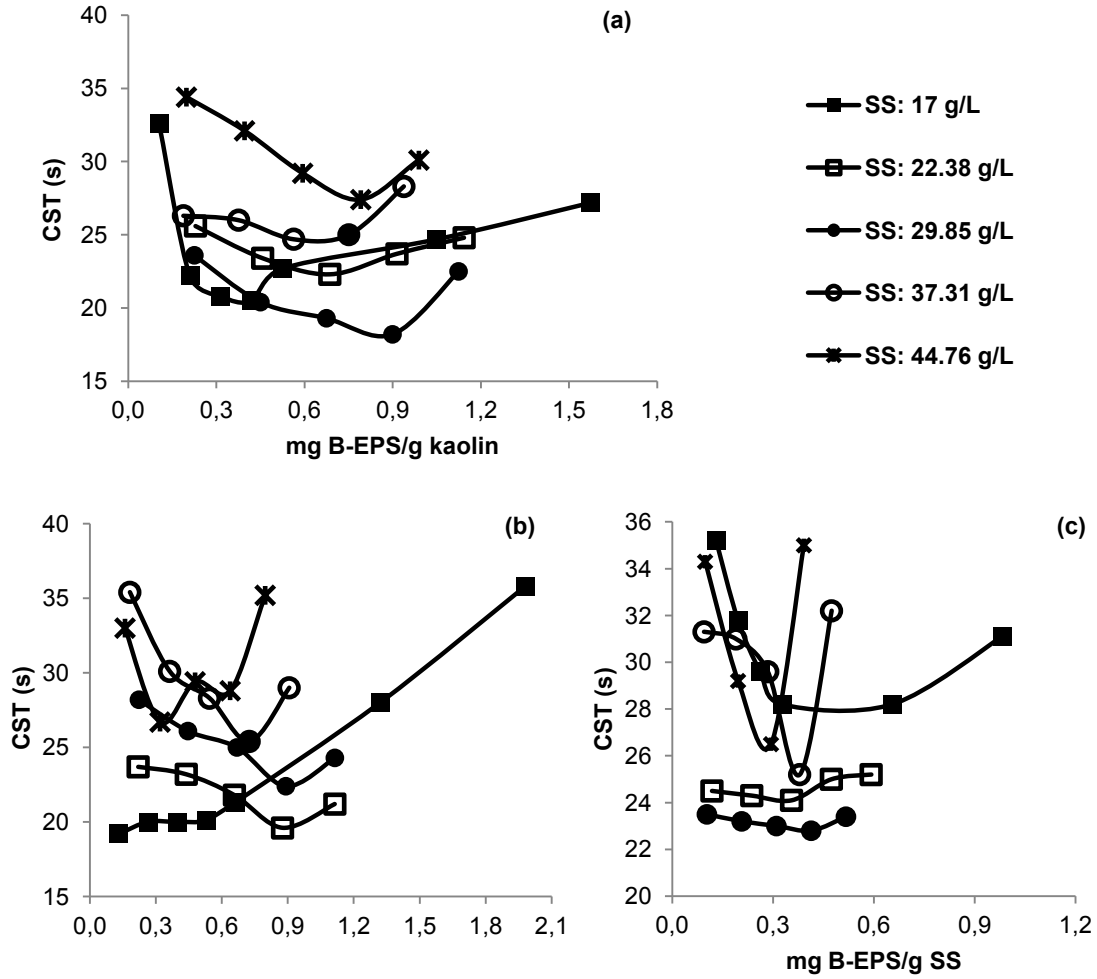


Figure A3.2: Change in CST of the B-EPS produced in (a) sterilized sludge, (b) alkaline-thermal treated sludge and (c) acid-thermal treated sludge at different sludge solids concentrations (SS, 17, 22.38, 29.85, 37.31 and 44.76 g/L).

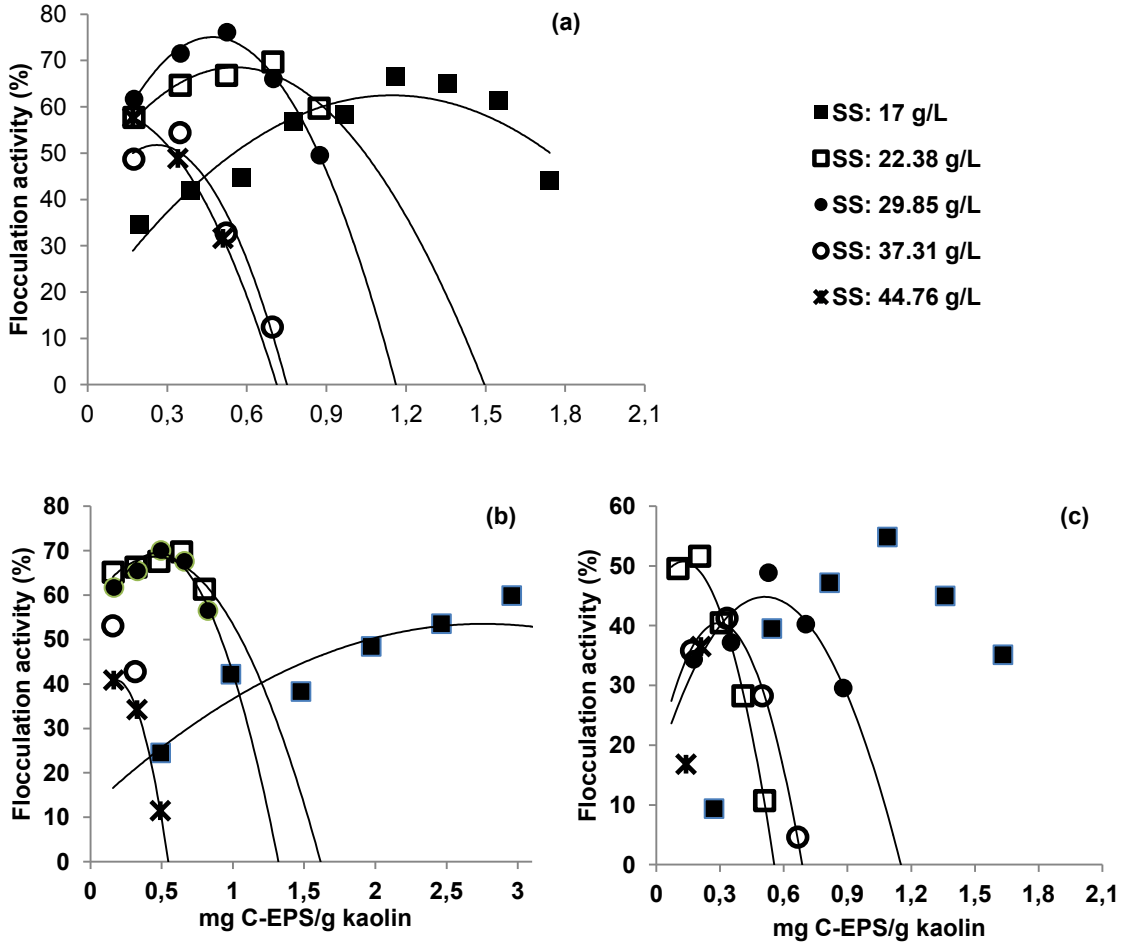


Figure A3.3: Flocculation activity of the B-EPS produced in (a) sterilized sludge, (b) alkaline-thermal treated sludge and (c) acid-thermal treated sludge at different sludge solids concentrations (SS: 17, 22.38, 29.85, 37.31 and 44.76 g/L).

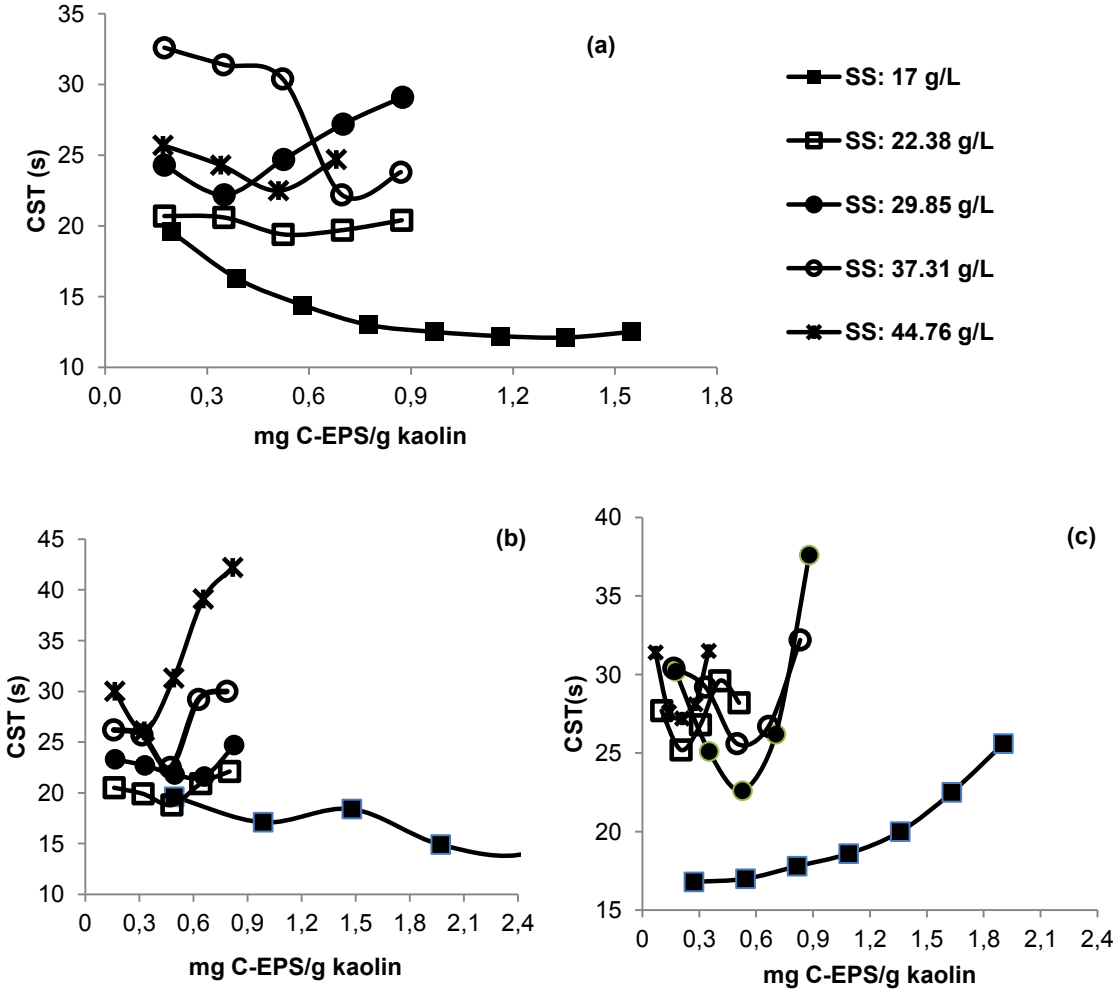


Figure A3.4: Change in CST of the B-EPS produced in (a) sterilized sludge, (b) alkaline-thermal treated sludge and (c) acid-thermal treated sludge at different sludge solids concentrations (SS, 17, 22.38, 29.85, 37.31 and 44.76 g/L).

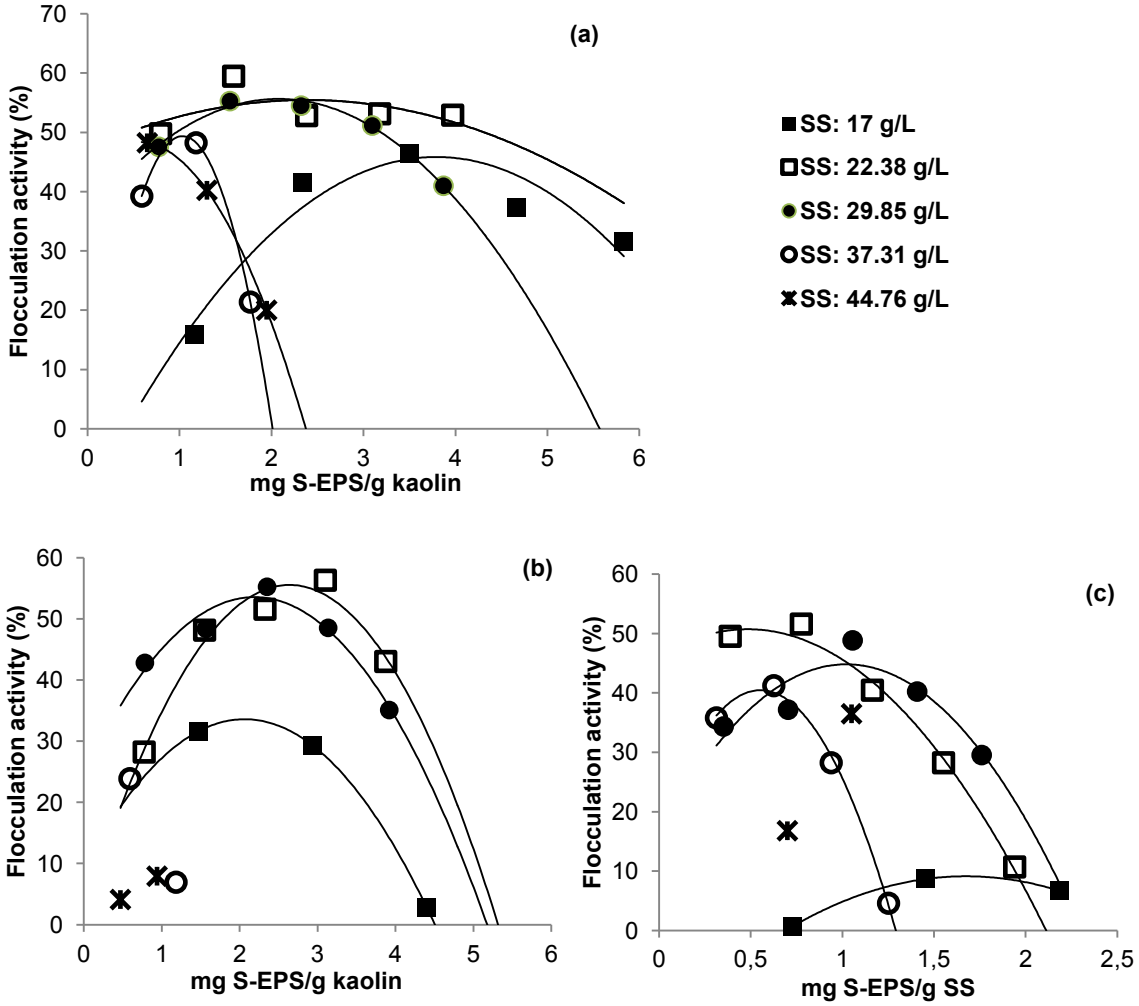


Figure A3.5: Flocculation activity of the S-EPS produced in (a) sterilized sludge, (b) alkaline-thermal treated sludge and (c) acid-thermal treated sludge at different sludge solids concentrations (SS, 17, 22.38, 29.85, 37.31 and 44.76 g/L).

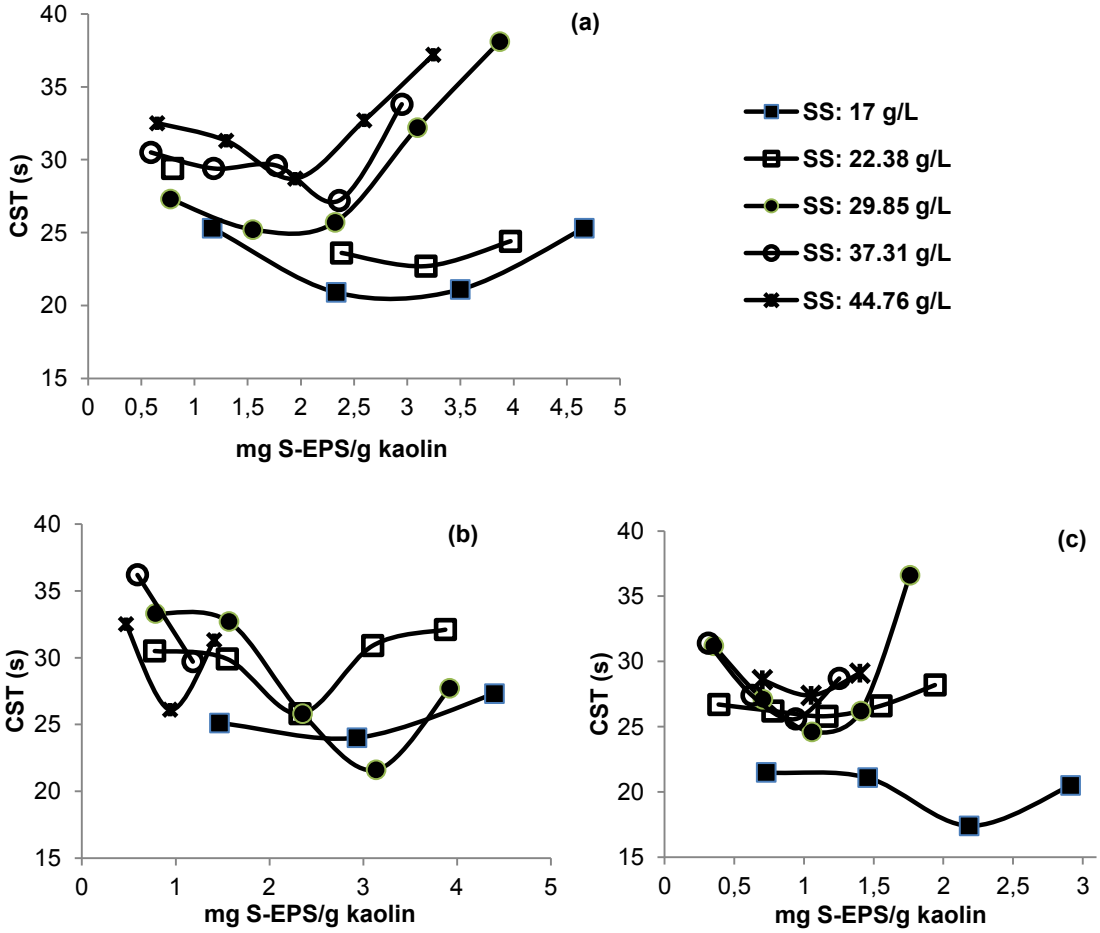


Figure A3.6: Change in CST of the B-EPS produced in (a) sterilized sludge, (b) alkaline-thermal treated sludge and (c) acid-thermal treated sludge at different sludge solids concentrations (SS, 17, 22.38, 29.85, 37.31 and 44.76 g/L).