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Critical review of EPS production, synthesis and composition for sludge flocculation

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ABSTRACT

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

Contents

Abbreviations: C/N, carbon to nitrogen molar ratio; CER, cation exchange resin; EDTA, *Ethylene di amine tetra acetate* group; EPS, exopolysaccharide or extra cellular polymeric substances; FTIR, *Fourier Transform Infrared* spectroscopy technique; GDP, Guanosine di phosphate; GT, Glucosyltransferase; MBR, membrane bioreactor; SEC, size exclusion chromatography; SS, suspended solids; UDP, uridine diphosphate; VSS, volatile suspended solids; WWTP, waste water treatment plant or wastewater treatment process.

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Introduction

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

The main characteristic of EPS is to enhance aggregation of bacterial cells and suspended solids (SS). Adhesion and cohesion occur between EPS and the biomass along with suspended solids by complex interactions such as London forces, electrostatics interactions and hydrogen bonding, which leads to the formation of flocs. These EPS properties make them suitable for many applications such as sludge flocculation, settling, dewatering, metal binding and removal of toxic organic compounds (Chien et al., 2013; Jia et al., 2011; Nouha et al. 2016; Solís et al., 2012).

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

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

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

1. Composition of EPS

The chemical structure of polymeric substances secreted by the microbial cells depends on the different environmental conditions they grew, which are highly diversified. The most investigated components of EPS are polysaccharides and proteins (More et al., 2012; Nouha et al. 2016; Subramanian et al., 2010). The presence of humic substances and nucleic acids as part of EPS extracted from sludge were also reported

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in some of the previous studies (Nguyen et al., 2016; Nouha et al. 2016; Sutherland, 2001).

1.1. Polysaccharides (carbohydrates)

Most EPS produced by microorganisms contains carbohydrate or polysaccharides. Microbial exopolysaccharides are comprised of either homopolysaccharides or heteropolysaccharides (Monsan et al., 2001). Homopolysaccharides are composed of only simple sugars and heteropolysaccharides contain repeated units of various monosaccharides such as D-glucose, D-galactose, L-fructose, L-rhamnose, D-glucuronic acid, L-guluronic acid and D-mannuronic acid. For example, alginate is a heteropolysaccharide produced by *Pseudomonas aeruginosa* and *Azotobacter vinelandii*, which is composed of D-mannosyl and L-glucuronosyl residues. However, dextran, a homopolysaccharide consisting only dextrose (glucose) units, is produced by *Leuconostoc* sp. and *Streptococcus* sp. (Rehm, 2010). The carbohydrate content of EPS can get affected by various factors during the production and extraction of EPS. The major factors that significantly affect the carbohydrate content of EPS are the microorganism, carbon substrate, nutrients (N, P) and the extraction method utilized for extraction.

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

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

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

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

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

1.2. Protein

Ton-That et al. (2004) stated that the protein was the principal component of the EPS matrix in the activated sludge and EPS (protein) production was not hugely affected by the type of substrates used for microbial growth. These results were in agreement with the observations of Frolund et al. (1995) and Liu et al. (2007) who also reported a consistent protein content (in activated sludge EPS), when microbe was supplied with different types of carbon sources (glucose, sodium acetate). Hoa et al. (2003) investigated the effect of nitrogen supplementation and reported that the protein content of EPS could be affected by nitrogen (NH_4Cl) limiting situations, which result in an increase of protein content of EPS (1.25 to 8.56 mg protein/g SS). It was found that the protein content of EPS was inversely proportional to nitrogen content in the activated sludge, while it remains unaffected by phosphorus.

1.3. DNA and humic substances

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

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

As evident from the discussion above, EPS biochemical composition is affected by many factors like microbial species, carbon source, nutrient supplementation and the downstream extraction methods. The composition of the EPS molecule is very important as it determines ultimately the functional property of the molecule as bioflocculant. The chemical composition of the EPS thus produced can determine its suitability for various kinds of applications. Among the various novel applications that EPS can be used for, metal removal is the most prominent one. EPS as carbon substrate has drawn the significant attention of researchers and the subsequent section is dedicated to these two applications of EPS.

2. EPS biosynthesis

Extracellular polysaccharide synthesis by microorganisms is accomplished by a specific secreted enzyme (polymerization and precursor synthesis enzymes), and synthesis can occur

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either outside the cell or within the cell wall (Roger, 2002). Table 1 present the several classes of polymers and their diverse characteristics.

The EPS biosynthesis pathway can be divided into three major steps: (i) synthesis of precursor substrate, (ii) polymerization and cytoplasmic membrane transfer and (iii) export through the outer membrane (Fig. 1). These three steps vary with carbon source used, from one microorganism to the other and specifically depends on polymers classes.

2.1. Synthesis of precursor substrate

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

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

2.2. Polymerization and cytoplasmic membrane transfer

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

The biosynthetic pathway of xanthan (Fig. 1 as an example of polymer biosynthesis) has been explored by (Rosalam and England, 2006). The synthesis of Xanthan starts with the assembly of repeating pentose units (GDP–mannose and UDP–glucoronate). These units are then polymerized by GumE, which is the catalytic subunit of the xanthan polymerase, localized in the cytoplasmic membrane and then produces the macromolecule xanthan (Fig. 1). Once xanthan is synthesized, it is exuded into the extracellular environment.

In the case of Alginate, this step requires the transfer of the cytosolic precursor GDP-mannuronic acid across the cell membrane and the polymerization of the monomers to

Table 1 – Metabolic characteristic of Bacterial polymers and their fermentation conditions.

EPS	Polymer localization	Precursors	Polymerization enzyme	Fermentation conditions	Microorganisms	EPS (g/L)	Reference
Cellulose	Extracellular	UDP–d-glucose	Cellulose synthase (BcsA)	Glucose/fructose pH = 4–5; 30°C; 40 hr	*Acetobacter xylinum*	7–23.9	Hwang et al. (1999)
Dextran	Extracellular	Saccharose	Dextransucrase (DsrS)	Sucrose pH = 5.5; 35°C; 100 kPa; 5 days	*Leucomostoc* sp.	8–17	Santos et al. (2000)
Xanthan	Extracellular	UDP–glucose, GDP–mannose and UDP–glucuronate	Xanthan polymerase (GumE)	Molasse pH = 7; 28°C; 100 kPa; 24 hr	*Xanthomonas campestris*	50	Kalogiannis et al. (2003)
Alginate	Extracellular	GDP–mannuronic acid	Glycosyl-transferase (Alg8)	Glycerol + ethanol pH = 5.8–6.5; 28°C; 150 r/min; 48 hr	*Pseudomonas* sp.	15.2	Hay et al. (2010)
Pullulan	Extracellular	UDP–d-glucose	–	Sucrose pH = 4–4.5; 30°C; 100 hr	*Aureobasidium pullulans*	1.3–5 2.5	Jiang (2010)
Curdlan	Extracellular	UDP–glucose	Curdlan synthase (CrdS)	Glucose/sucrose pH = 5.5; 22–26°C; 3–4 days	*Rhizobium* spp.	1–5	Pavlova et al. (2005)
Others EPS				Glycérol/glucose 6–18 pH = 7; 30°C, 4 days	*Enterobacter* sp.	6–18	Alves et al. (2010)
				Sucrose/maltose pH = 6.8–9.8; 54–87°C	*Geobacillus* sp.	0.1–14	Kambourova et al. (2009)
				Sucrose/glucose pH = 7; 32–37°C	*Halomonas* sp.	1.6–4.5	Béjar et al. (1998), Poli et al. (2009)

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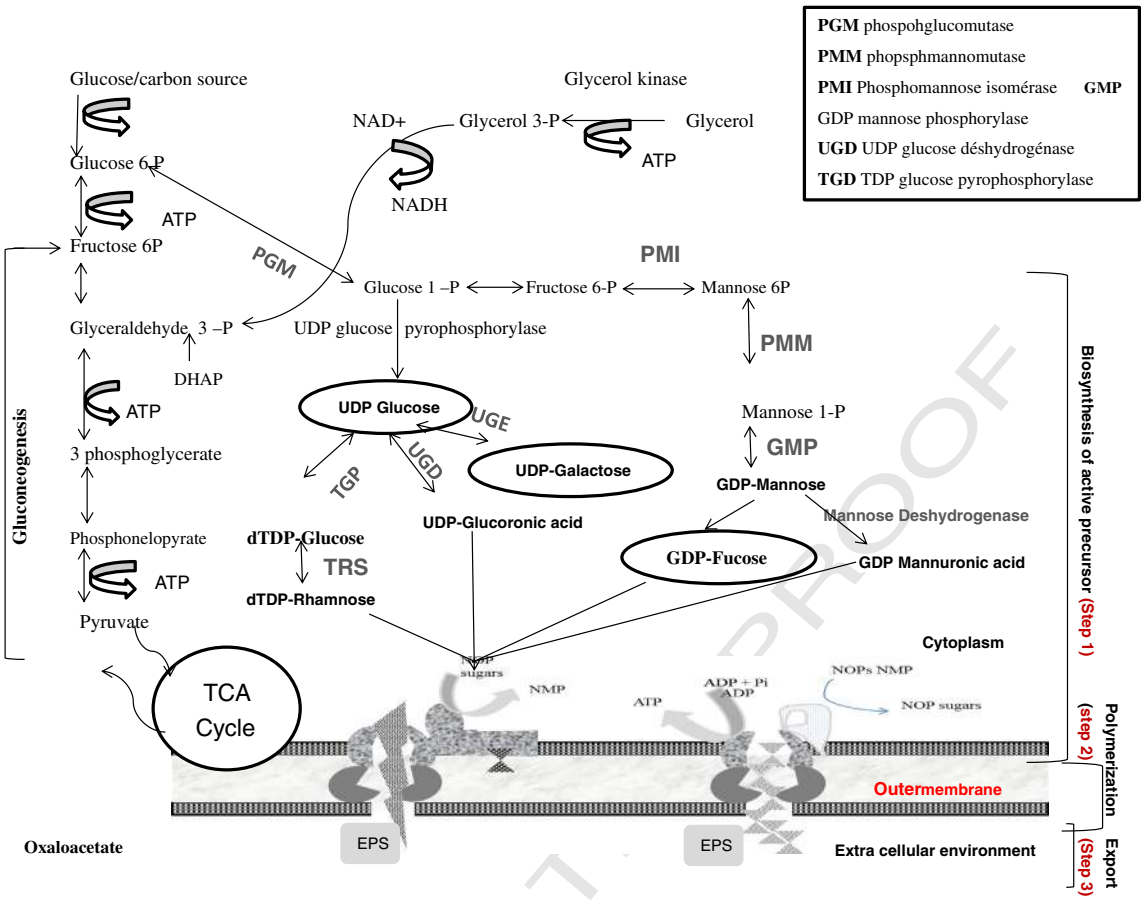

Fig. 1 – Biosynthesis pathway steps of bacterial polymers.

polymannuronate using Glycosyl-transferase (Alg8) (Figs. 1 and 2) (Rehm, 2010).

2.3. *Export through the outer membrane*

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

In EPS synthesis, lipid transporters provide an anchor to the extracellular membrane, which facilitates the precise and orderly formation of the carbohydrate chain proceeded by the transport of the chain through the cell membrane. Polysaccharides are polymerized on the inner side of the cytoplasmic membrane and then directly exported through the intermediary of a lipid transporter. These transporters are long-chain phosphate esters and isoprenoid alcohols, identical to those described in the biosynthesis of lipopolysaccharides and peptidoglycans (Sutherland, 1999). They play an important role in heteropolysaccharide synthesis, which is combined with the EPS excretion. After excretion, the intervention of an enzyme specific to the EPS may liberate the polymer. Table 1 shows further categorization dividing the polysaccharides into repeated unit polymers and non-repeating polymers, presenting their main compounds, precursors and polymerizing enzymes.

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

3. Engineering strategies for bacterial polysaccharides biosynthesis

Several studies were performed to genetically engineer the EPS-producing microbes to produce novel polymer variants while improving the production. As presented in the previous section (Section 2), various enzymes are involved in all three Q10 stages of the metabolic pathway of EPS biosynthesis. Gene-encoded enzymes regulate the formation of nucleotide sugar metabolite, chain length determination, repeat unit assembly, polymerization and export of polymers (Figs. 1 and 2) (Broadbent et al., 2003).

Recently intensive researches have been performed by focusing on the underlying mechanisms behind bacterial exopolysaccharide biosynthesis pathways, on different operons, promoters and the expression of regulatory gene segments. The

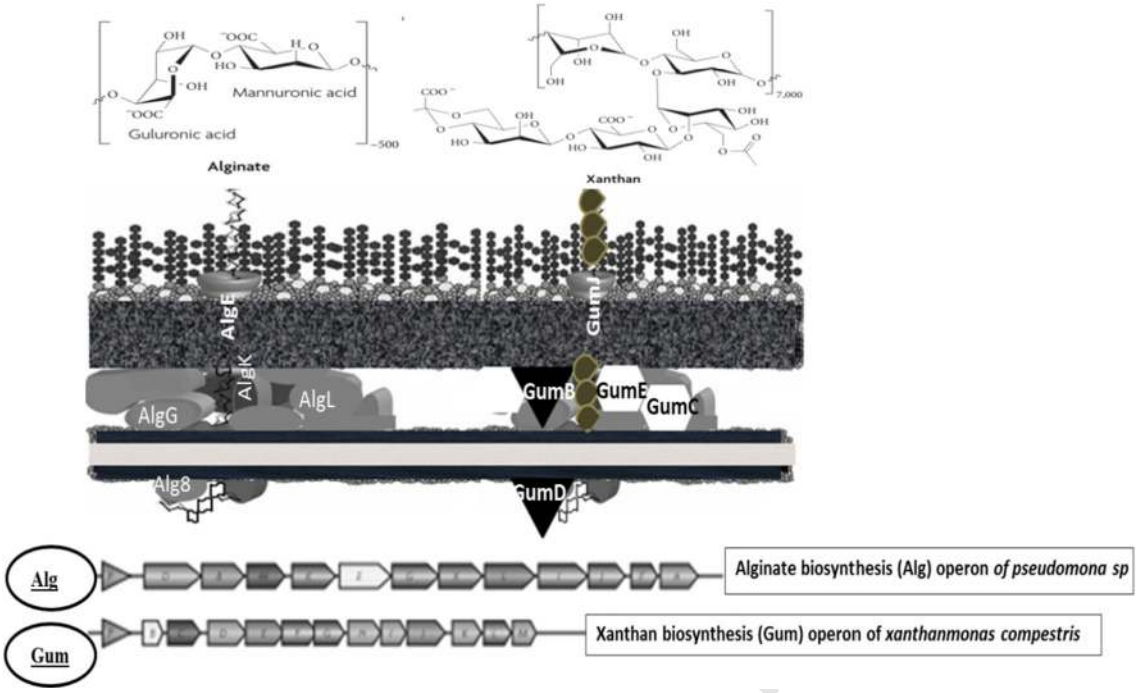

Fig. 2 – Model of alginate and xanthan biosynthesis and secretion mechanisms.

variability of sugar precursors, protein structure analysis, and new bioinformatics tools provide new avenues to enhance the EPS biosynthesis and understand the principal engineering strategies of EPS formation.

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

Researchers (Huang et al., 2013; Schatschneider et al., 2013; Wu et al., 2014) studied *Xanthanmonas campestris* EPS production in detail and found that it possess a series of 12 genes embedded in tandem. This operon includes seven genes needed for monosaccharide transfer and acylation of lipid intermediate to form the completely acylated repeating unit (Figs. 1 and 2). It has been suggested that alteration in promoters related to this operon can yield higher precursor metabolites (Galindo et al., 2007).

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

Schatschneider et al. (2013) studied most sensitive gene segments that significantly affect EPS synthesis. The study demonstrated that the overexpression of gumD is the key enzyme involved in the EPS assembly construction and precursor conversion (Fig. 2). Thus, it was suggested to clone the entire gumD gene cluster of a 16 kb chromosomal fragment with high copy number in *X. campestris*. The results indicated elevated expression of all biosynthetic eps gene, which could be achieved by cloning them on a high copy number plasmid (Janczarek et al., 2009).

In another strategy, the idea was to engineer the EPS molecules at the molecular level for having the desired behavior and material characteristics of the final polymer while improving the property as bioflocculant. For example, this molecular alteration can be deleting substituents or monomeric sugar residue from the side chain.

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

Many efforts were done in engineering the degree of acetylation and pyruvylation of various polymers (alginate and xanthan), to control their rheological properties (Donati and Paoletti, 2009). The level of O-acetylation and pyruvylation can be controlled reasonably well using specific strains/mutants or altering the growth media and controlling cultivation conditions

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such as aeration, pH and temperature (Gaytán et al., 2012; Peña et al., 2006).

Interesting insights were given by Rehm (2010) and Galván et al. (2013) regarding the general structure and functional relationships. The extent of acetylation and pyruvylation has antagonistic effects on viscosity. The GumL enzyme incorporates pyruvyl residues to the external β-mannose residues, while the acetyl residues are incorporated into internal α-mannose units by GumF, and external β-mannose by GumG (Becker et al., 1998). A high degree of pyruvylation by increasing the transcription of gumL (cloning an additional promoter upstream gumL) resulted in higher viscosity, whereas more acetyl group decreased the viscosity of the resulting EPS (Gaytán et al., 2012; Rehm, 2015).

Taxonomically different microbes can produce the same types of extracellular compounds with different concentrations. *P. aeruginosa* and *A. vinelandii*, despite having most of the genes involved in the biosynthesis, their organization, regulations and genetic switch clusters identical have differences at transcriptional and functional level expression of EPS. In *P. aeruginosa* and *A. vinelandii* a cluster of 13 structural and five regulatory genes (Hay et al., 2010) involved in EPS biosynthesis (Fig. 2). In *P. aeruginosa* the transcriptome is regulated by algR, algB, algC and algD gene segment that are algT dependent whereas in *A. vinelandii,* these genes were independent of algT. The regulator gene algT encodes for the regulatory expressions of sigma factor (σ^{22}) which could explain the variation in alginate concentration (Ahmed, 2007).

Though literature exists for genomic and proteomic level engineering to overproduce classical biopolymers, recombinant molecular engineering techniques are near to inexistent for EPS over production. Molecular techniques can be applied for transcriptional overexpression of RNAs involved in biosynthetic pathways, translational overexpression of the proteome involved in EPS biosynthesis. The most generic approach is an in-frame insertion of a strong promoter upstream of EPS operon to have more than basal level of constitutive or inductive expression of genome and proteome level. Overexpression of EPS can be induced when the carbon flux channeling is streamed favorably toward the generation of precursor molecules like *nucleotide diphosphate-Glucose* conjugates by gene silencing, which divert carbon flux away from EPS synthesis without compromising the survival of the organism. Similarly, overexpressing enzymes involved in irreversible synthesis is a key mechanism and can be used as control points to induce diversion of this excess carbon pool toward EPS biosynthesis.

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

4. EPS production methodology

4.1. EPS extracted directly from sludge

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

Further, researchers (Peng et al., 2012; Sponza, 2003) examined the EPS extracted directly from activated sludge treating various types of wastewaters. They found that the protein content of EPS is higher in the sludge treating winery and municipal wastewater than that of sludge produced in treating pulp-paper, textile and petrochemical wastewaters. Thus the EPS concentration varied accordingly to the type of wastewater treated in WWTPs. Simon et al. (2009), reported that the nucleic acid content of EPS could be affected by the type of sludge from different municipal WWTP. High nucleic acid (7 mg/g EPS DW) concentration was observed in Eerbeek municipal WWTP sludge as compared to 4 and 1 mg/g EPS DW in Emmtec and Revico WWTP, respectively.

4.2. EPS production using pure carbon sources

Although some researchers are convinced that the nature of the substrate cannot influence the composition of the EPS produced, however, many others have stated that medium composition either carbon source or nitrogen sources are important parameters in EPS biosynthesis and production (Simon et al., 2009).

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

The effect of substrate and its suitability depends on upon the organisms. Different bacteria may produce different bioflocculants in composition and structure. For example, *Bacillus licheniformis* (Shih et al., 2001) and *Nocardia amarae* (Takeda et al., 1992) produce protein bioflocculants whereas *Bacillus subtilis* (Yokoi et al., 1995) produce polysaccharide bioflocculant, and *glycoproteins* were produced by *Arcuadendron* sp. and *Arathrobacter* sp. (Wang et al., 1995). Van Geel-Schutten et al. (1998) reported that the *Lactobacillus* strains produce

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varying amounts of EPS with different sugar compositions when they are grown on sucrose, raffinose and lactose as a carbon source. The EPS produced by *Lactobacillus casei* with glucose as carbon source was reported to be different from that produced on lactose (Pham et al., 2000). Vijayendra et al. (2003) investigated the effect of different hexose sugars (glucose, sucrose and lactose) on EPS production by *Agrobacterium, Alcaligenes, Pseudomonas* and *Xanthomonas*. Lactose was found to be the best carbon source for EPS production by *Pseudomonas* sp. whereas other bacterial strains favored sucrose.

Yuksekdag and Aslim (2008) studied the impact of various carbon sources on EPS production by *L. delbrueckii*, *Lactobacillus bulgaricus* and *Streptococcus thermophiles*. For all the strains, glucose was the most efficient carbon source and EPS concentration of 211 mg/L produced by *L. delbrueckii sub* sp. was the highest concentration. The effect of carbon source on EPS synthesis by the marine bacterium *Saccharophagus degradans* was also studied by González-García et al. (2015). *S. degradans* was able to grow in the mineral medium while producing EPS concentration depending on the carbon source: with glucose or starch, EPS 1.5 g/L; with galactose, sucrose, or xylose, EPS 0.7 g/L and with fructose, EPS 0.3 g/L. The results were in agreement with the recent studies (Qin et al., 2015; Mane and Hamde, 2015). Lactose gave the maximum EPS concentration of 6.9 g/L as compared to 0.9 g/L when glucose was used (Qin et al., 2015). According to Mane and Hamde (2015), glucose gave maximum EPS concentration of 750 mg/L as compared to other substrates (lactose 390 mg/L; sucrose 670 mg/L) used.

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

In another case, the biosynthesis of extracellular polysaccharides in *A. xylinum* was improved by using galactose and xylose as carbon source compared to fructose, sucrose and starch in the medium (Sutherland, 2001). Recently, EPS from *Pseudomonas fluorescens* was produced using different concentrations of sucrose and sugarcane molasses as the carbon substrates (Razack et al., 2013). Maximum EPS concentration of 2843 mg/L was obtained at 5% (*w/v*) sugarcane molasses concentration in the media. The sugarcane molasses as carbon source gave a higher EPS concentration than sucrose with 1389 mg/L of EPS concentration (Razack et al., 2013).

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

4.3. Use of sludge as nutrient source for EPS production

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

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

5. EPS extraction

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

5.1. Physical methods

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

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

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However, some studies (Frolund et al., 1995; Liu and Fang, 2002) proposed that the high EPS extraction efficiency by the heating method may be caused by significant cell lysis and disruption, which may reveal results into high protein content in EPS.

5.2. Chemical methods

As per literature, many methods have been proposed and applied to extract EPS from pure or undefined mixed cultures. The extraction efficiencies of chemical methods, such as cation exchange resin (CER), EDTA (Ethylene diamine tetra-acetic Acid) and NaOH methods have been studied (Frolund et al., 1995; Liu and Fang, 2002; Sheng et al., 2005).

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

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

Comparative study of chemical extraction methods has been exhaustively investigated. Tapia et al. (2009) conducted a comparative study of chemical and physical methods to understand the principal role of the different extraction methods on EPS composition and concentration. The concentration of EPS from bacterial culture of *Acidiphilium* sp. was higher when extracted by centrifugation (600 mg carbohydrate/g DW, 220 mg protein/g DW, 820 mg EPS/g DW) than extracted with NaOH (430 mg carbohydrate/g DW, 170 mg protein/g DW, 650 mg EPS/g DW). However, in the study of Sheng et al. (2005) the concentration of EPS extracted by NaOH was higher 159.2 mg/g-DW in comparison to centrifugation and ion exchange resin (Table 2). These results have also been confirmed by using more complex microbial consortia such as activated sludge (Table 2). Liu and Fang (2002) reported extractions of EPS ranging between 25.7 mg EPS/g volatile suspended solids (VSS) by centrifugation and 164.9 mg EPS/g-VSS by chemical methods (NaOH and formaldehyde). A significant difference (>60%) of EPS extracted was observed between the two methods when EPS was extracted from the same culture. However, it appears that the chemical extraction becomes more effective when it is combined with physical methods. Liu and Fang (2002) investigated high proteins, carbohydrates, humic acid and DNA content extracted by combined methods of formaldehyde and ultrasonication compared to that obtained by only formaldehyde. Comte et al. (2006a) also proved that sonication and CER as combined EPS extraction method were more effective to extract high proteins, carbohydrate and nucleic acid content comparing to CER or sonication alone.

5.3. Chemical methods vs physical methods

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

Liu and Fang (2002) and Comte et al. (2006a) indicated a constant nucleic acid content of EPS using different physical extraction methods (Cation-exchange resin, centrifugation, sonication and heating). This study indicated that the physical methods resulted in lower extent of cell lysis than chemical extraction methods.

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

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

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

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Table 2 – Comparison of total EPS (carbohydrate and protein) extracted from various cultures and extraction methods.

Pure culture	Extraction methods	EPS composition (mg/g DW)				EPS concentration (mg/g- DW)	Prot./carb. ratio	References
		Prot.	Carbo.	HA	DNA			
Rhodopseudomonas acidophilap		4.1	6.2	–	–	12.9	1.5	Sheng et al. (2005)
		6.5	58.5	–	–	70.3	9.0	
	Centrifugation	7.7	126.5	–	–	159.2	16.4	
	EDTA	10.3	37.3	–00	–	71.6	3.7	
	NaOH							
	Heating							
Acidiphilium sp.		600	220	–	–	820	0.3	Tapia et al. (2009)
		550	200	–	–	750	0.3	
		430	170	–	–	600	0.4	
		570	200	–	–	770	0.4	
Mixte culture								Liu and Fang (2002)
Aerobic		54.6	40.5	50.4	0.3	165	0.2	
		17.7	12.7	16.4	0.1	109	0.1	
		22.9	12.4	59.2	0.4	146	0.1	
	Formal + NaOH	20.4	28.8	18.9	0.1	78	0.1	
	CER							
	EDTA							
Acidogenic	Formal + ultrasound	110.9	25.8	0.1	15.1	179	4.3	
		38.7	6.2	0.1	3.0	58	6.2	
		41.7	6.5	0.2	15.9	105	4.6	
		71.6	10.8	5.0	0.05	100	6.6	
Mutagenic sludge								
		19.1	42.1	23.3	0.19	102	2.2	
		7.9	10.6	5.5	0.05	30	1.3	
		6.8	12.0	24.3	1.20	73	1.7	
		12.0	13.1	5.6	0.04	30	1.1	
Activated sludge								Zuriaga-Agusti et al. (2010)
	Sonication	140	343	62	46	200	2.4	Comte et al. (2006a)
	Heating	166	378	126	17	369	2.3	
	CER	16	4	–	–	24	4.2	
	Triton X 100	81	10	–	–	100	7.9	
Biofilm	Centrifugation	57.0	–	–	–	57	–	Pan et al. (2010)
	Ultrasonication	22.3	56.6	–	–	79	0.4	
	EDTA	1.7	3.2	–	–	45	0.5	
	Formaldehyde	8.0	25.2	–	–	33	0.3	
	Formal + NaOH	17.0	13.3	–	–	30	1.3	

Prot: Protein; Carb: Carbohydrate.

2184 ± 27 μmol/g of EPS as analyzed using two adsorption models, Chau and Rezic, respectively.

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

5.4. *Combination of different methods*

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

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

Each and every method has their advantages and disadvantages (Table 3). Although the physical methods cause less cell lysis, it also has low EPS extraction efficiency. The chemical methods generated high protein content, and these proteins

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Table 3 – Advantages and disadvantages of different extraction method used.

Extraction methods		Mechanism and Extraction conditions	Limitation	Advantages	References
Physical	Centrifugation	EPS separates from cell surface and dissolve to solution under the centrifugal force	(–) Low extraction efficiency (–) Bound EPS cannot be extracted.	(+) Simple method (+) No cell lysis	Liu and Fang (2002)
		• 20,000/600 g in 20 min	(–) Depend of shear rate	(+) No chemical addition	Comte et al. (2006a)
	Heating	Accelerates the EPS solubilization	(–) Cells disruption	(+) Extraction of bound EPS	Frolund et al. (1995)
		• 80°C, 1 hr	(–) Denaturation of EPS		
	Ultra-sonication	Act of applying sound energy or mechanic pressure to agitate particles in a sample	(–) High cell lysis in less time	(+) Dissociation of aggregates	Comte et al. (2006a, 2006b)
		• 40 W, 0–30 min	(–) High energy	(+) Break the hydrogen bound	
Chemical	CER	Removes the divalent cations, causing the EPS to fall apart	(–) Extract only the proteins and carbohydrates coupled to cations	(+) Avoid EPS pollution by chemical reagent	Liu and Fang (2002)
		• 70 g resin/g MVS, 600 r/min, 1 hr			
	EDTA	Remove the multivalent cations forming the bond between the charged compounds of EPS.	(–) EPS contamination by intracellular compounds (–) Interfere the proteins analysis	(+) No modification of EPS structure	Liu and Fang (2002) Comte et al. (2006a)
		150 mL of 2% EDTA added for 3 hr at 4°C	(–) Cost of chemical		
	NaOH	Ionization of carboxylic group	(–) Severe disruption in EPS composition	(+) Break the covalent disulfide binding in glycoproteins	Liu and Fang (2002)
		• 1N of NaOH, 3 hr at 4°C	(–) high damage of cell (–) Many charged groups results repulsion between the EPS (–) Alkaline hydrolysis of many polymers may take place		Comte et al. (2006a)
	Formaldehyde	Fix the cell and denature the EPS, linking the proteins and carbohydrate	(–) Modify EPS characteristics (–) Interferences in carbohydrates content	(+) Prevent the cell lysis	Liu and Fang (2002) Comte et al. (2006a)
		• 36%, 1 hr, 4°C	(–) Cost of chemical reagents		
Combined chemical and physical methods	NaOH-Heating CER-centrifugation Formaldehyde-sonication	–	(–) Extraction time (–) EPS need purification (–)Not economical	(+) High Extraction efficiency	D'Abzac et al. (2010)

can be originated from extracellular enzymes and or intracellular materials contaminations. A combination of two methods could affect the production cost and the efficiency of EPS extraction during an industrial application (Domínguez et al., 2010). There is no simple and single method that exists to extract 100% of total or complete EPS components from the microbial cell or activated sludge flocs. Each technique extraction efficiency depends on many factors mainly the origin of EPS. It is recommended that extraction is only performed after running a comparative study of various methods to select the best one for desired application. Furthermore, an extraction technique must be chosen and optimized for each case, taking into account many parameters (such as extraction time, cost and dosage of chemical used and evaluation of cell lysis), which could affect the cost and the properties of EPS.

5.5. *Effect of extraction methods on functional group and molecular weight of EPS*

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

Many researchers (Sheng and Yu, 2006; Tapia et al., 2009) proved the effect of the extraction methods applied on structural

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Table 4 – Effect of extraction method on functional groups of EPS.

Extraction methods	Wave number (cm^{-1})	Transmittance (%)	Vibration type	Functional type	Molecular weight range	References
EDTA	1550–1600	50	Stretching vibration of C═O Proteins	Proteins (peptide bond)	8 kDa	Comte et al. (2006a)
	1300	55	C – N stretching		150–200 kDa 5 kDa	Simon et al. (2009)
NaOH + Formaldehyde	2450	60	Specific band corresponding to a product of a formaldehyde and EPS	Carboxylic acids	–	Comte et al. (2006a)
	1750	40	Stretching vibration of C═O Stretching vibration of C═O			
	1400	10	Several bands visible	Carboxylates		
	800	30		Phosphorus/Sulfur functional group		
Gluraldehyde	1950	75	Specific band corresponding	–	–	Comte et al.
	1500	70	to a product of a glutaraldehyde and EPS reaction			(2006a)
Centrifugation	3200–3420	80	Stretching vibration of OH	OH into polymeric compounds	Low molecular weight	Comte et al. (2006a)
	2935	85	Stretching vibration of C═O and C–N (Amide I)		0.16–0.3 kDa	Simon et al. (2009)
	1630–1660	73	Stretching vibration of C – N and deformation vibration of N – H (Amide II)	Proteins (peptide bond)	0.7–2.7 kDa	
	1550–1560	83	Deformation vibration of CH_2 Stretching vibration of C═O Stretching vibration of OH, of polysaccharide		4.6–6 kDa	
	1450–1460	82	Several bands visible	Carboxylates	High molecular weight	
	1400–1410	85		Polysaccharides	16–190 kDa	
	1060–1100	75			270–275 kDa	
	<1000			Phosphor/Sulfur functional group		
Sonication	3200–3420	60	Stretching vibration of OH	OH into polymeric compounds	Low molecular weight	Comte et al. (2006a)
	2935	70	Stretching vibration of C═O and C–N (Amide I)		0.16–0.3 kDa	Simon et al. (2009)
	1630–1660	52	Stretching vibration of C–N and deformation vibration of N–H (Amide II)	Proteins (peptide bond)	0.7–2.7 kDa	
	1550–1560	60	Deformation vibration of CH_2 Stretching vibration of C═O Stretching vibration of OH, of polysaccharide		4.6–6 kDa	
	1450–1460	71	Several bands visible	Carboxylates	High molecular weight	
	1400–1410	65		Polysaccharides	16–190 kDa	
	1060–1100	63			270–275 kDa	
	<1000			Phosphorus or Sulfur functional group		
Sonication + resin	3200–3420	60	Stretching vibration of OH	OH into polymeric compounds	Low molecular weight 0.16–0.3 kDa	Comte et al. (2006a)
	2855	70	Stretching vibration of C═O and C–N (Amide I)	Proteins	0.7–2.7 kDa 4.6–6 kDa	Simon et al. (2009)
	1630–1660	52	Stretching vibration of C–N and deformation vibration of N–H (Amide II)	(peptide bond)		
	1550–1560	60	Deformation vibration of CH_2	Carboxylates		
	1450–1460	71	Stretching vibration of C═O	Polysaccharides	High molecular weight 16–190 kDa 270–275 kDa	

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t4.35 **Table 4** (*continued*)

t4.36 Extraction methods	Wave number (cm^{-1})	Transmittance (%)	Vibration type	Functional type	Molecular weight range	References
t4.34	1400–1410	65	Stretching vibration of OH, of polysaccharide	Phosphorus/sulfur functional group		
t4.35	1060–1100	63	Several bands visible			
t4.36	<1000					
t4.37 CER	3200–3420	60	Stretching vibration of OH	OH into polymeric compounds	Protein molecular weight	Comte et al. (2006a)
t4.38	1630–1660	70	Stretching vibration of C═O and C–N (Amide I)	Proteins (peptide bond)	45 to 670 kDa	Simon et al. (2009)
t4.39	1550–1560	52	Stretching vibration of C–N and deformation vibration of N–H (Amide II)			
t4.40	1450–1460	60	Deformation vibration of CH_2			
t4.41	1400–1410	71	Stretching vibration of C═O	Carboxylates	Polysaccharides	
t4.42	1060–1100	65	Stretching vibration of OH, of polysaccharide	Polysaccharides	<1 kDa	
t4.43	<1000	63	Several bands visible	Phosphorus/Sulfur functional group		
t4.44 Heating	3200–3420	30	Stretching vibration of OH	OH into polymeric compounds	Low molecular weight	Comte et al. (2006a)
t4.45	2935	40	Stretching vibration of C═O and C–N (Amide I)		0.16–0.3 kDa 0.7–2.7 kDa	Simon et al. (2009)
t4.46	1630–1660	20	Stretching vibration of C–N and deformation vibration of N–H (Amide II)	Proteins (peptide bond)	4.6–6 kDa	
t4.47	1550–1560	25	Deformation vibration of CH_2			
t4.48	1450–1460	45	Stretching vibration of C═O	Carboxylates	High molecular weight 16–190 kDa	
t4.49	1400–1410	33	Stretching vibration of OH, of polysaccharide			
t4.50	1060–1100	41	Several bands visible	Polysaccharides	270–275 kDa	
t4.51	<1000			Phosphorus or sulfur functional group		

t4.53 EPS: Extracellular polymeric substance; EDTA: Ethylene diamine tetra acetate group; CER: cation exchange resin.

properties of EPS and their molecular weight (Table 4). Fourier transform infrared spectroscopy (FTIR) was mostly used to identify the functional group of extracted EPS (Omoike and Chorover, 2004; Sheng and Yu, 2006). However, quantitatively this aspect has never been reviewed. Tapia et al. (2009) compared two FTIR spectra of EPS obtained from EDTA and centrifugation method. Significant peaks were visible in both the spectra corresponding to hydroxyl, carbonyl and peptide group bonding. However, the spectrum of EPS extracted with EDTA shows specific bands in the fingerprint region, especially the thick band at 1717 cm^{-1}. This band corresponds to the C═O asymmetric stretching vibration of carboxylic acids of EDTA. It has been a general observation by many studies that during extraction with a chemical method the final EPS gets contaminated by the chemical reagent (Comte et al., 2006a). Similar contaminations were observed in other methods like NaOH-formaldehyde (Comte et al., 2006a; Pervaiz and Sain, 2012; Sheng et al., 2005).

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

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

Table 5 – Metal-binding potential of bacterial EPS and wastewater sludge EPS.

EPS producer	EPS concentration mg/L	Metal biosorbed	References
Methylobacterium organophilum	184.2	Pb(II)	Kim et al. (1996)
	200.3	Cu(II)	
Pseudomonas aeruginosa cur	320	Cu(II)	Kazy et al. (2008)
Rhizobium etli M4	67	Mn(II)	Pulsawat et al. (2003)

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

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

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

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

6. Potential applications of EPS

6.1. EPS as adsorbent

Heavy metal removal from a polluted environment is a major challenge in bioremediation, which has been studied extensively using microbes. EPS produced by many microorganisms are of particular significance to the bioremediation process because of their metal ions binding ability from solutions (Kachlany et al., 2001). The use of purified biopolymers in biosorption phenomenon is a cost-effective and useful approach than using methods like precipitation, coagulation, ion exchange, electrochemical and membrane processes used for metal removal (Gutnick and Bach, 2000). EPS and other biopolymers exhibit excellent metal-binding properties with varying degrees of specificity and affinity (Gutnick and Bach, 2000). The effect of and EPS bridging occur by electrostatic interactions with negatively charged functional groups such as uronic acids and phosphoryl groups of carbohydrates or carboxylic groups of amino acids in protein moiety. Besides, there may also be anionic binding by positively charged polymers or coordination with hydroxyl groups. EPS were able to chelate some metals (like Th^{4+} and Al^{3+}) and bind them to the cell surface (Santamaría et al., 2003).

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

Numerous bacteria have been shown to produce exopolymeric substances. Several studies (Forster and Clarke, 1983; Prado Acosta et al., 2005) have compared and evaluated the metal binding potential of microbial biofilms obtained from activated sludge and purified exopolysaccharides (Table 5). Over 90% of metal adsorption was achieved at an EPS concentration (67 and 160 mg/L) using different bacterial strain such as *Rhizobium etli* M4 and *Paenibacillus polymyxa* (P13), respectively. The cells and EPS showed a strong ability to bind Mn, Pb and Cu ions (Forster and Clarke, 1983; Nouha et al. 2016; Prado Acosta et al., 2005; Salehizadeh and Shojaosadati, 2003).

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

EPS hydrophobicity is another significant factor, which favors the sludge flocculation and settling. EPS hydrophobicity can be rendered by EPS-proteins produced by the microbial communities. According to Geyik et al. (2016), higher protein content or protein to carbohydrate (P/C) ratio gives higher EPS hydrophobicity which is correlated with the substrate provided to the microbial communities. EPS hydrophobicity is significantly affected by the functional groups in its protein fraction. The hydrophobicity is an important factor when EPS is intended to use in organic pollutant removal (Flemming et al., 2000). A strong correlation was demonstrated by Zita and Hermansson (1997) between sludge particle adhesion and EPS hydrophobicity.

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The pH of the surrounding environment affects the deprotonation state of the side residues of the protein fractions present in EPS. At lower pH, the acidic residues are protonated to have higher hydrophobicity. Thus this allows dense intramolecular hydrogen bonding between flocculating particles and further improve sludge compactness and settling efficiency (Wang et al., 2012).

6.2. EPS as carbon source

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

The EPS biodegradability studies were performed by various authors (Wu and Ye, 2007). Zhang and Bishop (2003) observed that carbohydrate fraction of the supplemented EPS as carbon source was consumed more rapidly than the protein fraction. Pannard et al. (2016) investigated and confirmed the biodegradability of EPS by bacteria for growth under nitrogen (or phosphorus) and carbon limiting conditions.

The susceptibility of EPS toward degradation depends on the hydrolyzing agent, which leads to breaking the polymer chains, and also depends on the chemical nature of the polymer (Wingender et al., 1999). Many reports (Neyens et al., 2004; Watson et al., 1987; Watson et al., 2014) have been presented on the influence of sludge treatment in EPS degradation.

Watson et al. (1987) found that protein and carbohydrates, the main component of EPS in activated sludge are degraded or hydrolyzed by heat treatment. Neyens et al. (2004) revealed that heat treatment (120°C) alters the structure of the sludge in term of proteins and carbohydrate moieties of EPS and transforming some of EPS from less degradable to easily degradable. Acid-thermal and alkaline-thermal hydrolysis were also used by Neyens et al. (2004). They indicated an increase in the sludge filtration rate (capillary suction time, CST). Extreme pH (acidity or alkalinity) also causes EPS proteins to lose their natural shapes thus improved their degradability. Polysaccharides and the other components of EPS, are unstable in strong acids, which lead to acid hydrolysis of the glycosidic linkages (Fig. 3) (Wingender et al., 1999). The strong alkali may solubilize gels not only because of chemical degradation but also because of the ionization of the carboxylic groups, which leads to subsequent solubilization of EPS (Wingender et al., 1999). As the EPS is a complex molecule, different treatments could interfere with their chemical structure and even could form a novel compounds. Fig. 3 presents alginate as an example of polymers and their transformation due to several different treatment methods.

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

6.3. Effect of functional group and molecular weight on flocculation activity

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

No consensus exists on the role of (importance) carbohydrate and protein content of EPS for flocculation. Deng et al. (2005) concluded in his study that EPS containing 76.3% of sugar and 21.6% of protein gave high flocculating abilities of 98.1%.

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

A more functional level analysis of flocculating abilities was performed by various studies (Deng et al., 2005; Kavita et al., 2014) to understand the importance of functional groups on flocculation ability. They investigated that cations stimulate flocculation by neutralization and stabilization of residual negative charges of the carboxyl group of a bioflocculant forming bridge that binds kaolin particles to each other. Further, the negatively charged carboxyl group (COO^-) of the bioflocculant could bind with the positively charged site of the suspended kaolin particles.

Although flocculation ability of EPS seems to be sensitive to the carbohydrate content (Shin et al., 2001), a study conducted by Yu et al. (2009) concluded that protein fraction of EPS is the most important parameter for flocculation activity. Researchers advocating for protein suggest that negatively charged amino

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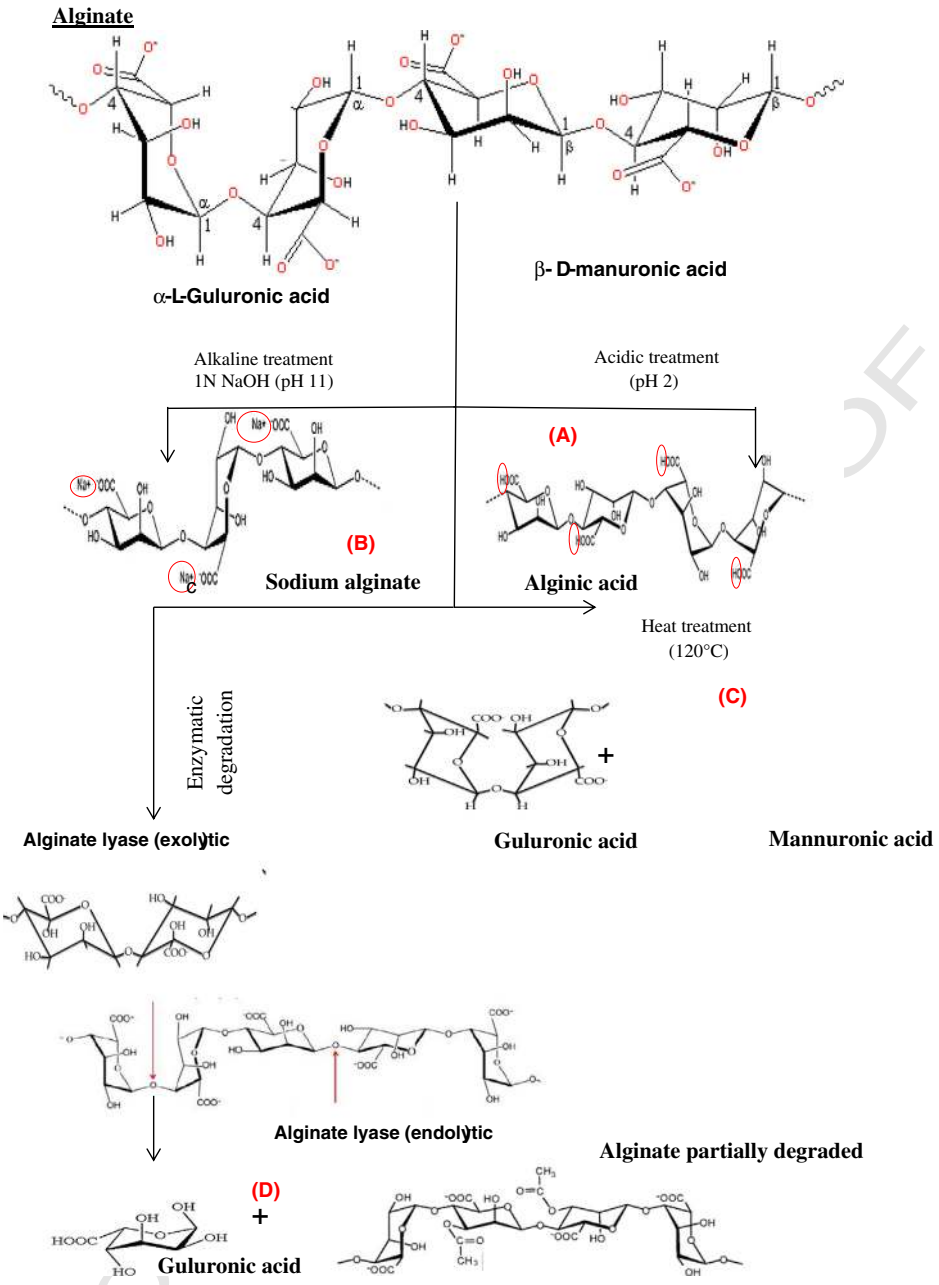

Fig. 3 – Alginate degradation: A) acidic treatment; (B) alkaline treatment; (C) heat treatment and (D) enzymatic degradation.

acid contribute to flocculation abilities. The hydrogen bonds are present frequently in proteins and they could affect the capacity of bioflocculant to agglomerate.

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

High molecular weight (2.6x10^{6}Da) bioflocculant produced by *Bacillus mucilaginosus* revealed high flocculation activity (99%) compared to low MW (10^5 Da) obtained from *Corynebacterium glutamicum* (80% of flocculation activity) (Deng et al., 2003; He et al., 2002). Furthermore, the bioflocculant produced by *Bacillus subtilis* DYU500 (3.20 × 10^6 Da) in the study of Wu et al. (2014) Q14 seems to favor the performance of flocculation (97%) comparing to 90% obtained by *Gyrodinium impudicum* KG03 (MW 1.58 × 10^6 Da) (Yim et al., 2007).

Recently, Tang et al. (2014) discovered a new bioflocculant produced by *Enterobacter* sp. ETH-2. The MWs of ETH-2 ranges

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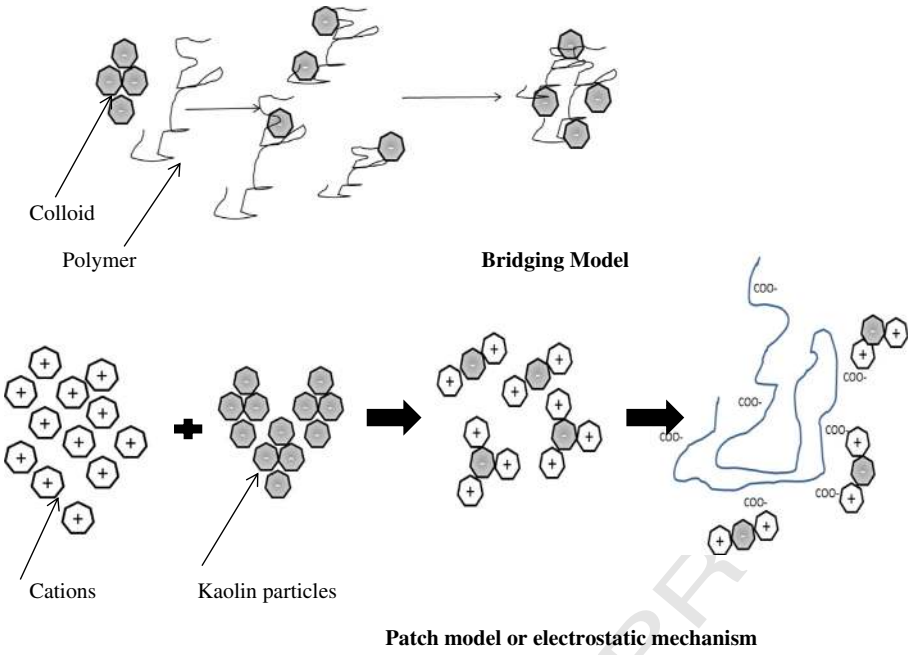

Fig. 4 – Flocculation mechanism of bioflocculant.

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

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

7. Conclusion and recommendations

As evident from the review that sludge EPS remains to be an unexplored field of study with a plethora of research opportunities for many industrial and eco-friendly applications. EPS is composed of mainly carbohydrates and proteins, and they play very significant role in determining their functionality. The presence of nucleic acid and humic acid substances as a resultant of cellular lysis during post-production processing can further contribute to enhance the functional properties of EPS. The microorganism, carbon substrate, and other growth conditions play very significant role in determining EPS composition. EPS has great potential to be used as metal removing agent in mining industries, as a flocculating agent in WWTP for sludge dewatering and as a carbon source for biotechnological production of other metabolites.

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

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

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