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Valorization of glycerol/ethanol-rich wastewater to bioflocculants: recovery, properties, and performance

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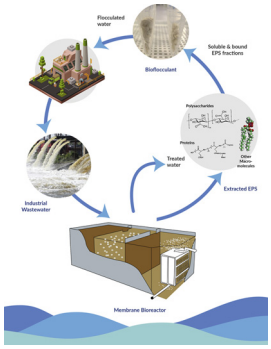
GRAPHICAL ABSTRACT



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ABSTRACT

Microbial extracellular polymeric substances (EPS) were produced in two membrane bioreactors, each separately treating fresh and saline synthetic wastewater (consisting of glycerol and ethanol), with the purpose of applying them as sustainable bioflocculants. The reactors were operated under nitrogen-rich (COD/N ratios of 5 and 20) and limited (COD/N ratios of 60 and 100) conditions. Under both conditions, high COD removal efficiencies of 87–96% were achieved. However, nitrogen limitation enhanced EPS production, particularly the polysaccharide fraction. The maximum EPS recovery (g EPS – COD/g $COD_{influent}$) from the fresh wastewater was 54% and 36% recovery was obtained from the saline (30 g NaCl/L) wastewater. The biopolymers had molecular weights up to 2.1 MDa and anionic charge densities of 2.3–4.7 meq/g at pH 7. Using kaolin clay suspensions, high flocculation efficiencies of 85–92% turbidity removal were achieved at EPS dosages below 0.5 mg/g clay. Interestingly, EPS produced under saline conditions proved to be better flocculants in a saline environment than the corresponding freshwater EPS in the same environment. The results demonstrate the potential of glycerol/ethanol-rich wastewater, namely biodiesel/ethanol industrial wastewater, as suitable substrates to produce EPS as effective bioflocculants.

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1. Introduction

One of the challenges faced in the water sector with respect to particle removal is the replacement of synthetic flocculants with effective and eco-friendly flocculants such as natural flocculants, and for this reason, bioflocculants have gained increasing attention [1–3]. They are potential alternatives to oil-based synthetic flocculants, which are largely non-biodegradable and may cause harmful pollution [3,4]. A class of promising bioflocculants yet to be fully exploited are microbial extracellular polymeric substances (EPS) [5,6]. EPS comprise high molecular weight compounds (namely polysaccharides and proteins) that make them attractive flocculants. Being anionic polyelectrolytes, they can interact with negatively charged particles in the presence of divalent cations, forming divalent cation bridges between particles and polymers which lead to bridging flocculation [7].

EPS are often produced by employing pure cultures [1,5,6]. However, this approach requires sterile conditions as well as expensive and unsustainable carbon sources such as glucose [1]. As a more sustainable alternative, we demonstrate a mixed-culture strategy that involves non-sterile cultures and feedstock such as organic wastewater. Such an approach not only yields cheap and environmentally friendly flocculants but also saves on wastewater treatment costs. Recently, we showed the possibility of producing EPS with excellent flocculation performance from wastewater [8], but the yield of 6–8% based on wastewater COD was relatively low.

Although conflicting results have been obtained, nitrogen limitation is often reported to enhance microbial EPS production [9–13]. This seems a functional strategy to produce bioflocculants from wastewater as many wastewaters such as biodiesel and paper industry wastewaters are rich in organic carbon but low in nitrogen.

This study investigates the effect of nitrogen limitation as an approach to enhance EPS yield during the treatment of biodiesel/(bio) ethanol wastewater mixture, and to explore the effect of nitrogen limitation on EPS properties and flocculation performances. In addition, EPS obtained from saline wastewater were compared to EPS from fresh wastewater as it was hypothesized that the former might also give a better flocculation performance under saline conditions.

2. Materials and methods

2.1. Wastewater treatment reactors

Two membrane bioreactors (MBRs, 3.3 L effective volume) were operated in parallel, each separately treating fresh and saline synthetic wastewater. The MBRs were equipped with a PVDF submerged flat sheet membrane with a nominal pore size of 0.2 µm [8]. The freshwater (FW) MBR was inoculated with non-saline aerobic sludge (180 ± 60 mg Na^+/L) from a municipal wastewater treatment plant (WWTP) located in Leeuwarden, the Netherlands. The saline (Sal) MBR was inoculated with aerobic sludge obtained from a saline industrial WWTP (located in Delfzijl, the Netherlands) which treats wastewater from local chemical industries (sludge salinity 11 ± 3 g Na^+/L). Both MBRs were operated at a room temperature of 20 ± 1 °C, a pH of 7.5 ± 0.3, a dissolved oxygen concentration of 2.5 ± 1.5 mg O_2/L, and a solids retention time (SRT) of 3 days. The hydraulic retention time (HRT) varied between 7.3 h and 13.9 h, depending on the membrane fouling (Table 1).

2.2. Wastewater composition

Wastewater influent COD was 1000 ± 25 mg/L, mainly provided from glycerol (410 ± 10 mg/L) and ethanol (240 ± 5 mg/L) in a 1:1 COD ratio, simulating biodiesel and bioethanol wastewater. These substrates make up more than 60% of the wastewater COD of Delfzijl WWTP. The main nitrogen source was NH_4Cl, and its concentration varied based on the utilized COD/N ratio. The nutrient medium composition per liter of tap water comprised 200 mg $MgCl_2{\cdot}6H_2O$, 150 mg $CaCl_2{\cdot}2H_2O$, 15 mg K_2HPO_4, 25 mg KH_2PO_4, and 2 mL trace elements solution (detailed composition in Ajao et al. [8]). Yeast extract was also added to the nutrient medium, and the COD of the medium was considered in the total influent COD. The COD/N ratio was calculated based on the ratio of total influent COD to total nitrogen (N), measured in mg/L. The COD/N ratios employed were 5, 20, 60 and 100. The amount of NH_4-N and yeast extract required for each COD/N ratio is shown in Table 1.

Table 1
Hydraulic retention time (HRT) of each reactor under freshwater (FW-MBR) and saline (Sal-MBR) conditions, and amount of NH_4-N and yeast extract in the feed.

Reactor	HRT(h) (FW-MBR/Sal-MBR)	COD/N (g/g)	NH_4-N (mg/L)	Yeast extract (mg/L)	Total nitrogen (mg/L)
COD/N 5	7.3/8.6	5	189.5	100.0	200.0
COD/N 20	7.9/9.2	20	39.5	100.0	50.0
COD/N 60	9.9/13.9	60	6.1	100.0	16.7
COD/N 100	9.9/13.9	100	4.7	50.0	10.0

2.3. Wastewater treatment performance analysis

COD, total nitrogen, and NH_4-N concentrations were determined using Dr. Lange test kits (LCK, Hach Lange, UK). The nitrite and nitrate concentrations of reactor effluents were analyzed using ion chromatography (Metrohm IC Compact 761): the stationary phase was a packed polyvinyl alcohol with quaternary ammonium groups and the mobile phase was an aqueous solution of 1 mM $NaHCO_3$ and 3.2 mM Na_2CO_3.

2.4. EPS extraction

For each COD/N ratio, the MBRs were operated for a period of at least three times the SRT, after which EPS were extracted from the reactor content. The detailed extraction technique has been described by Ajao et al. [8], but was slightly modified. In summary, 400 mL sludge was centrifuged at 17,000*g* and 4 °C for 30 min. The sludge supernatant containing soluble (*S*)-EPS was dialyzed successively against demineralized water at least eight times. From the sludge pellets, bound (B)-EPS were extracted into a phosphate buffer saline solution (PBS, pH 7.4) using a cation exchange resin (Sigma-Aldrich's DOWEX Marathon C, sodium form) at a dosage of 70*g*/g VSS for 2 h. After extraction, the supernatant containing the B-EPS was centrifuged at 12,000*g* and 4 °C for 15 min and dialyzed as explained above. Dialyzed soluble and bound EPS fractions were frozen at −80 °C and freeze-dried to obtain dry solids. Dried EPS were weighed and measured for COD content. EPS recovery was based on the influent COD using the equation:

$$EPS\ recovery\ (\%) = \frac{Q_{was}{*}C_{EPS}}{Q_{inf} * COD_{inf}} * COD(EPS) \quad (1)$$

where Q_{was} and Q_{inf} are the flow rates (L/d) of the waste sludge and influent feed respectively, C_{EPS} is the extracted EPS concentration (g/L), COD_{inf} is the influent COD (g/L), and *COD(EPS)* is the COD of extracted EPS (g COD/g EPS).

2.5. EPS characterization

2.5.1. Viscosity

As a quick tool to monitor EPS production in the reactors, viscosities of the sludge supernatants (after centrifugation at 17,000*g* for 30 min.) were measured at 10, 12, 20, and 30 rpm at room temperature (21 °C) using a HAAKE Viscotester 6 L plus (Thermo Electron, Germany).

2.5.2. Functional group determination

Fourier transform infrared spectroscopy (FTIR) was carried out on the freeze-dried EPS samples using an FTIR-8400S spectrometer (Shimadzu, Japan) with a scanning range of 4000 - 650 cm^{-1} for 40 scans at a spectral resolution of 2 cm^{-1}.

2.5.3. Total protein and polysaccharide quantification

Total protein content of the EPS was determined using a bicinchoninic acid (BCA) assay kit (Thermo Scientific, USA). The assay was performed in a microplate, where 25 µL of EPS solution (dissolved in PBS, pH 7.4) or standard bovine serum albumin was mixed with 200 µL of BCA working reagent and incubated at 37 °C for 30 min. Afterward, the absorbance was measured at 570 nm using a spectrophotometer (Victor3 1420 Multilabel Counter, Perkin Elmer, USA).

Total polysaccharides/carbohydrates were quantified using the phenol-sulphuric acid method described by Dubois et al. [14] using glucose as the standard sugar. Absorbance was measured at 490 nm in the spectrophotometer mentioned above.

2.5.4. Molecular weight determination

A solution of EPS was first passed through 0.45 µm polytetrafluoroethylene filter before molecular weight determination using liquid chromatography-organic carbon detection (LC-OCD -model 8, DOC-LABOR, Germany) with a built-in Siemens Ultramat 6^E Non-Dispersive Infra-Red detector, coupled with an Agilent 1260 organic nitrogen detector (UV 220 nm) and a UV detector (254 nm). The column used was a tandem setup with an Agilent BioSec5 5 µm 1000 A (7.8 mm*300 mm) and a Toyopearl HW-50S 30 µm (20 mm*250 mm). The mobile phase was a phosphate buffer (28 mmol, pH 6.6). EPS molecular weights were determined using pullulan standards obtained from PSS, Germany.

2.5.5. Charge density

Charge density was determined by colloid titration using a Mütek Particle Charge Detector (PCD03, Germany) described by Tan et al. [15] and a titration procedure explained by Ajao et al. [8]. The charge density was calculated from the titrant (poly-diallyldimethylammonium chloride, pDADMAC) consumption according to:

$$q = \frac{c * V}{m} \quad (2)$$

where q is the specific charge quantity (eq/g), c is the titrant concentration (eq/L), V is the consumed titrant volume (L), and m is the mass of the PE sample (g). Charge density was determined at the initial pH of sample solution (5.0 ± 0.2) and at pH 7.0 ± 0.1 (since most waters are flocculated at close to neutral pH) using 1 mM NaOH for pH adjustment.

2.6. Flocculation tests

Flocculation tests were carried out on a jar test flocculation unit as described by Ajao et al. [8]. Naturally-occurring kaolin clay (Sigma-Aldrich natural kaolinite, $Al_2O_3{\cdot}2SiO_2{\cdot}2H_2O$), possessing a net negative charge, was used as a model for water particles [5,16]. We investigated the potentials of different EPS fractions produced under different COD/N ratios as natural flocculants. For this purpose, two model suspensions were prepared: (i) saline kaolin suspension: 5 g kaolin/L and 30 g NaCl/L (to mimic saline water), and (ii) non-saline kaolin suspension comprising only 5 g kaolin/L. Both were prepared in Milli-Q water. EPS solutions were made by dissolving freeze-dried EPS in Milli-Q water. For the flocculation of the non-saline kaolin suspension, 100 mg Ca^{2+}/L ($CaCl_2{\cdot}2H_2O$) was added as a coagulant. Supernatant turbidity after flocculation was measured with a turbidimeter (2100N IS, Hach) in Nephelometric Turbidity Units (NTU). Flocculation efficiency was calculated as follows:

Table 2
COD removal efficiency, effluent COD and NH_4-N concentrations in both freshwater (FW) and saline (Sal) membrane bioreactors (MBRs) operated at different COD/N ratios. Data expressed as the mean ± standard deviation.

Reactor	COD/N g/g	Effluent COD mg/L	COD removal %	Effluent NH4-N mg/L
FW-MBR	5	58 ± 9	94.0 ± 1.0	127 ± 11
	20	50 ± 6	94.8 ± 0.6	9 ± 6
	60	36 ± 8	96.2 ± 0.8	0.05 ± 0.05
	100	36 ± 10	96.0 ± 1.0	0.008 ± 0.006
Sal-MBR	5	57 ± 12	94.0 ± 1.2	170 ± 10
	20	50 ± 5	94.9 ± 0.6	8 ± 4
	60	48 ± 6	94.9 ± 0.6	0.3 ± 0.3
	100	130 ± 50	86.7 ± 5.2	0.2 ± 0.1

$$\textit{Flocculation efficiency}\ (\%) = \frac{NTU_{control} - NTU_{test}}{NTU_{control}} \quad (3)$$

where $NTU_{control}$ is the turbidity value of the control experiment (without EPS addition), and NTU_{test} is the turbidity value of test experiment (with EPS as flocculant).

3. Results and discussion

3.1. Wastewater treatment performance

During the operational period of at least three times the SRT, similar COD removal efficiencies (93–97%) were observed for all the COD/N ratios (except Sal-MBR at COD/N 100) irrespective of the wastewater salinity (Table 2). COD concentrations in the effluent varied between 36 and 58 mg/L. Only at a COD/N ratio of 100 did the Sal-MBR give an average removal efficiency of 87% and an effluent concentration of 130 mg/L.

The high COD removal efficiencies at COD/N of 60 and 100 were rather surprising because nitrogen was clearly limiting under these conditions (effluent NH_4-N concentrations below 0.3 mg/L). To exclude a temporary effect, FW-MBR operation at COD/N 100 was continued for another 105 days, but a high COD removal efficiency of at least 96% could still be maintained. Under the nitrogen-limited condition, excellent COD removals were achieved because influent soluble COD was converted to EPS − COD which was retained by the membrane. Moreover, the COD mass balances (Fig. S1, see supplementary material) reveal that by limiting nitrogen, mineralization and biomass production from influent COD were substituted by EPS production. At COD/N 100, 54% of the wastewater COD was converted to EPS and 17% for biomass growth. Mineralization of wastewater COD, calculated as a closure of the COD mass balance, was only 16%, implying that the oxygen demand of the process was very low.

3.2. EPS production

The COD/N ratio had a noticeable effect on the sludge viscosity in both the FW- and Sal-MBRs. The sludge viscosity increased at COD/N 60 and 100, especially the FW-MBR sludge which was slimy and sticky (Fig. 1A and B). The associated decrease of S-EPS viscosity with shear rate (Fig. 1C) reveals its non-Newtonian pseudoplastic fluid behavior, a property usually found in exopolysaccharides [17]. This pseudoplastic behavior was more evident with FW-EPS than with Sal-EPS. Furthermore, the sludge viscosity is proportional to the S-EPS concentration and can therefore be used as a quick tool to monitor S-EPS production in bioreactors (Fig. 1D).

The EPS concentrations, particularly those of the S-EPS, generally increased with increasing COD/N ratio (Fig. 2). At COD/N 100, the total EPS (soluble and bound) concentration was 2.32 g/L for the FW-MBR and 1.86 g/L for the Sal-MBR, from which the soluble fractions accounted for 77 and 67% respectively. The highest concentration of B-

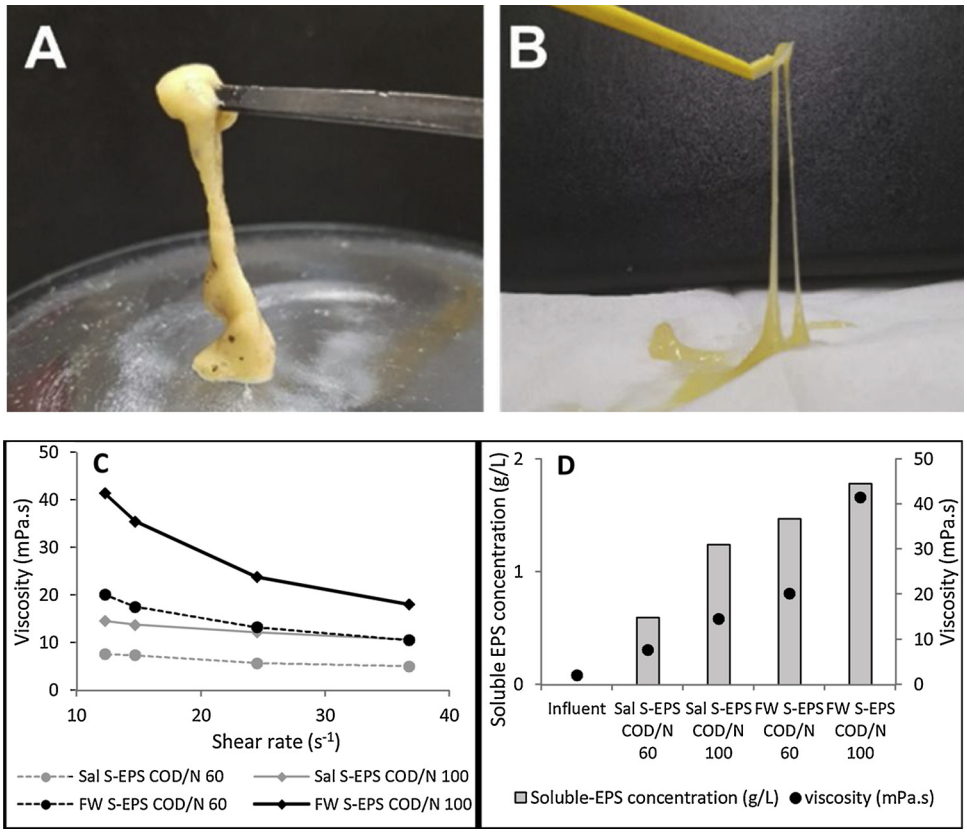

Fig. 1. (A, B) - Viscous sludge produced from freshwater-MBR at COD/N ratio 60 (A) and 100 (B). (C) - Soluble-EPS viscosity as a function of shear rate at room temperature (21 °C). (D) - Soluble-EPS concentration *versus* viscosity at a shear rate of 12.2 s^{-1}. Viscosity of the influent was obtained at a shear rate of 244.8 s^{-1}. Sal S-EPS: saline soluble-EPS, FW S-EPS: freshwater soluble-EPS.

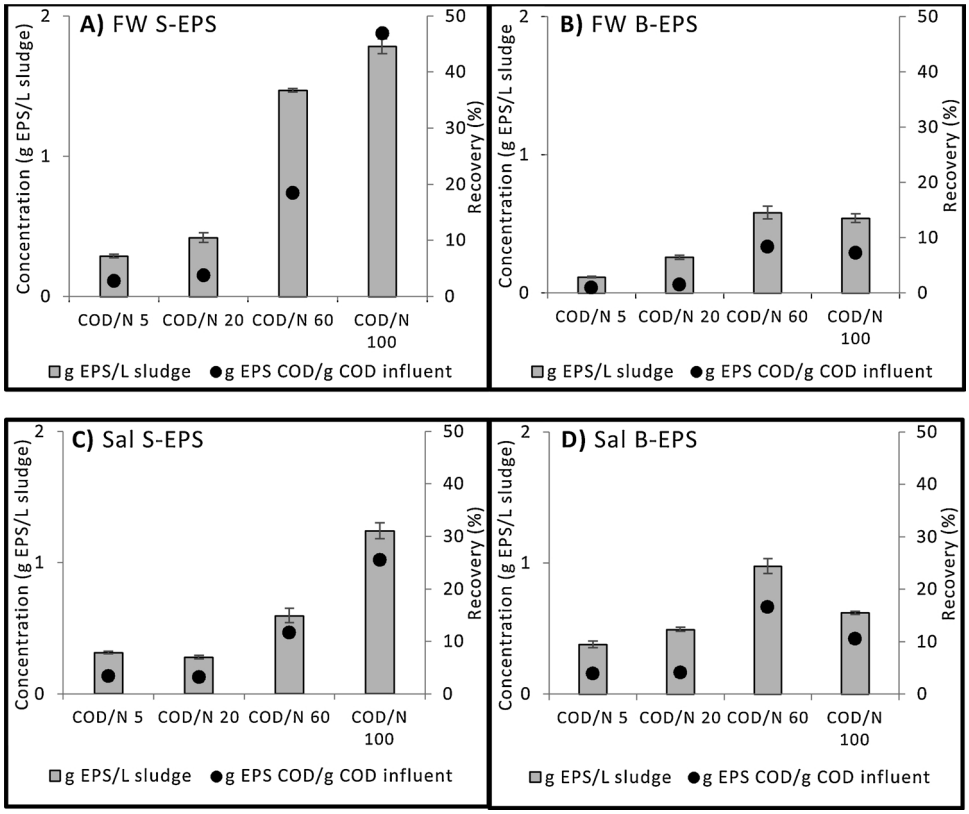

Fig. 2. EPS production at different COD/N ratios. (A) FW S-EPS: freshwater soluble-EPS, (B) FW B-EPS: freshwater bound-EPS, (C) Sal S-EPS: saline soluble-EPS, and (D) Sal B-EPS: saline bound-EPS.

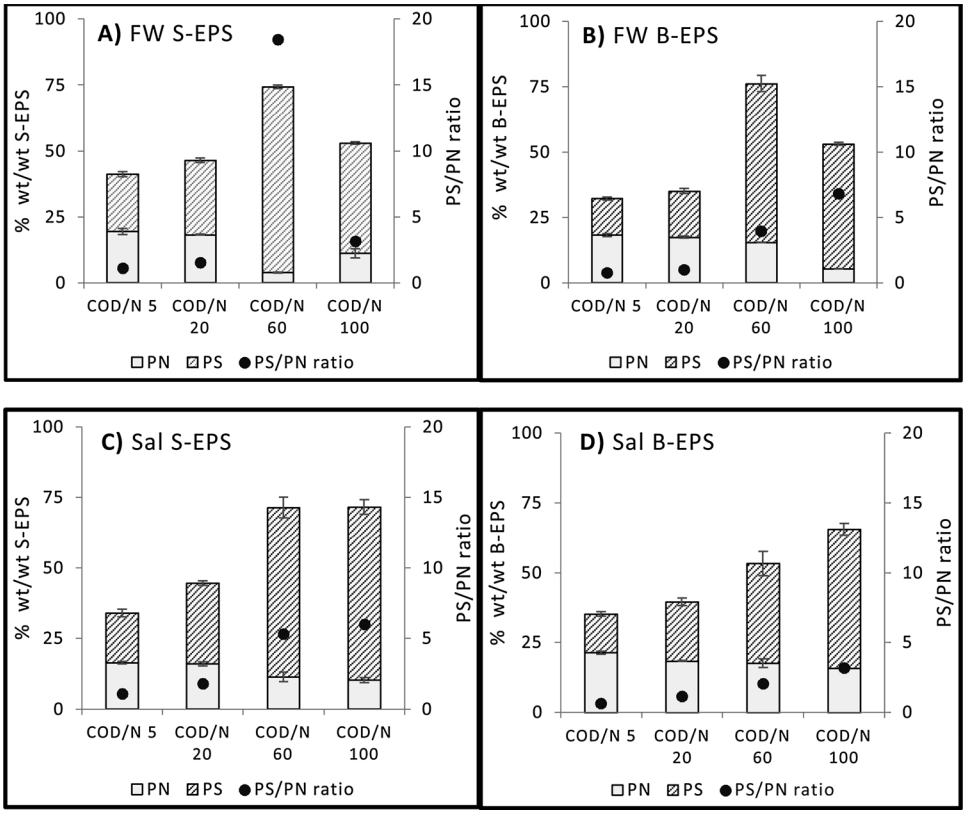

Fig. 3. Total proteins (PN) and polysaccharides (PS) content of EPS fractions from COD/N ratios 5, 20, 60, and 100.

EPS was observed at COD/N 60, with values of 0.58 g/L (FW) and 0.98 g/L (Sal) (Fig. 2B and D).

A notable observation is the distribution of soluble and bound EPS (Fig. 2A–D). The contribution of S-EPS to the total EPS (soluble and bound) was significantly higher in FW-EPS (62–77%) than in Sal-EPS (36–45%, but 67% at COD/N 100). Considering the S-EPS are likely sheared-off products of cell-bound EPS [18], this implies that EPS produced under saline conditions are more strongly attached to the microbial cells/flocs than FW-EPS, possibly due to the function of EPS to protect against salt stress [19].

The amount of influent COD recovered as EPS – COD Eq. (1) is also shown in Fig. 2. A relatively low amount of total EPS (soluble and bound) could be recovered (< 8%) under nitrogen-rich conditions (COD/N 5 and 20). However, a huge increase in EPS recovery was observed when the COD/N ratio increased from 20 to 60, with respective recoveries of 5–27% for the FW-EPS, and 7–28% for the Sal-EPS. At COD/N 100, the highest recoveries were obtained with a total of 54% under freshwater condition (S-EPS 47%, B-EPS 7%), and 36% under saline condition (S-EPS 25%, B-EPS 11%). These high recoveries clearly demonstrate the key role of nitrogen limitation in EPS production.

Microbial EPS synthesis can be considered a secondary metabolism that is uncoupled from growth. Russell [20] defined this as 'energy spillage' - non-growth dissipation of excess energy by microorganisms. This excess energy under nitrogen-limited condition is converted to extracellular polymers, especially polysaccharides (which do not contain the growth-limiting nutrient) [21], possibly as a means to store carbon under unbalanced carbon to nitrogen ratios [22]. The COD mass balance in Fig. S1 demonstrates that at high COD/N ratios, EPS production is favored over biomass growth and *vice versa* at low COD/N ratios.

Aside from nitrogen limitation as a strategy to increase EPS yield, the carbon/energy source also plays a crucial role in EPS biosynthesis. Studies using pure cultures fed with a mixture of glycerol and ethanol/methanol reported higher EPS production and yields compared to single sources such as glucose, sucrose and soluble starch [5]. Moreover, fortification of bacterial growth medium with glycerol (in the presence of ethanol or methanol) was demonstrated to increase EPS yield [23], and in some cases more than two-fold [24]. Yields (g EPS/g substrate COD) as high as 51.2 and 57.4% have been reported by Buthelezi et al. [25] and Nouha et al. [23] respectively from *Klebsiella terrigena* and *Cloacibacterium normanense* using substrates of glycerol and ethanol mixture in batch cultures. Our study also demonstrates the possibility of very high EPS recoveries from a glycerol/ethanol mixture (54% from fresh wastewater and 36% from saline wastewater), though with mixed cultures (see S2, supplementary material) and in a continuous wastewater treatment process. One possible explanation for the high EPS yield is that glycerol has a shorter pathway towards EPS synthesis compared to most sugar substrates [21]. Another reason may be the combined effects of energy non-equivalent substrates [26,27]. According to this bioenergetic concept, all growth substrates are either energy excessive or deficient based on the amount of ATP and reducing equivalents formed in the synthesis of phosphoglyceric acid – the key precursor for the synthesis of all cell components. On this basis, glycerol, glucose, and sucrose are energy-deficient substrates, while ethanol and methanol are energy-excessive substrates that can be used as auxiliary substrates [26]. Although the underlying metabolic mechanism is yet to be understood, a combination of these two substrate classes (the former utilized for carbon conversion and the latter as an energy source) at the optimum ratio has been reported to increase carbon conversion efficiencies towards the production of secondary metabolites such as EPS [24,27]. Further studies using different energy non-equivalent substrate mixtures in a continuous system would be justified as this would give insights into the combination of industrial wastewater types needed as best substrates for EPS production.

3.3. EPS properties

3.3.1. Composition

In general, the total polysaccharide (PS) content of the produced EPS increased with increasing COD/N ratios (Fig. 3). In the FW-MBR, the trend of increasing PS content was observed from COD/N 5 to 60 (21.7–70.4 wt% for the S-EPS, and 14.0–60.7 wt% for the B-EPS). At COD/N 100, the values decreased to 41.8 and 47.8 wt% for the S-EPS and B-EPS, respectively. In the Sal-MBR, the PS content increased steadily from COD/N 5 to 60/100 (17.6–61.2 wt% for the Sal S-EPS, and from 13.8 to 49.8 wt% for the Sal B-EPS). In contrast to the PS content, the nitrogen (PN) content decreased or remained rather constant with increasing COD/N ratio, revealing that the carbohydrate fraction was the main EPS component under nitrogen-limited conditions.

It should be noted that, with the colorimetric assays, no distinction could be made between free or bound proteins such as glycoproteins. To further investigate the presence of glycoproteins in the produced EPS, SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis – see supplementary material) of EPS produced at COD/N 100 was carried out. Fig. S3 reveals that the protein fraction of the produced EPS is covalently bonded with carbohydrates to form neutral and acidic glycoproteins in both the FW- and Sal-EPS fractions.

3.3.2. Molecular weight

Molecular weight (MW) is an important property of EPS if they are to be applied as flocculants. The longer the polymeric chain, the better they can extend into solution (if the chain has a hydrophilic property) to aggregate particles. EPS extracted from COD/N 20 (nitrogen-rich) and 100 (nitrogen-limited) were selected for MW determination. Typical size exclusion chromatograms are shown in Fig. S4, and Table 3 presents the average MW of the EPS biopolymer fractions. The EPS fractions showed two biopolymer chromatographic peaks – one with high (< 1000 kDa) or medium (1000 -~100 kDa) MW, and the other with low (< 100 kDa) MW, except for the FW-EPS at COD/N 20 where only one peak was detected.

Higher molecular weight EPS were produced under nitrogen-limited conditions compared to EPS from nitrogen-rich sources. This is more evident for the saline EPS where the polysaccharide fractions at COD/N 100 are much higher than the polysaccharide fractions at COD/N 20 (Fig. 3C and D). This demonstrates that nitrogen limitation not only stimulates microorganisms to excrete large quantity of EPS, it also builds longer chain biopolymers compared to the nitrogen-rich counterparts. Another observation is that the soluble and bound fractions under the different conditions had similar molecular weights (except for saline EPS from COD/N 20), suggesting, as was mentioned earlier, that S-EPS were sheared-off products of cell-bound EPS. However, as will be explained below, their charge densities do show differences.

3.3.3. Charge density

In addition to molecular weight, charge density is another crucial property for flocculation. The charge density of EPS is due to the presence of carboxyl and amino groups as indicated in the FTIR spectra in Fig. S5 (see supplementary material). Table 4 shows the anionic charge density of EPS extracted at COD/N 20 and 100 at the initial pH of the sample solution (5) and neutral pH (7).

Table 3
Average molecular weights (MW) of the EPS biopolymer fraction.

EPS fraction	MW at COD/N 20	MW at COD/N 100
Freshwater soluble-EPS	1.7 MDa	1.9 MDa, 24.5 kDa
Freshwater bound-EPS	1.6 MDa	2.1 MDa, 24.6 kDa
Saline soluble-EPS	202.3 kDa, 22.5 kDa	1.0 MDa, 22.7 kDa
Saline bound-EPS	98.4 kDa, 39.6 kDa	1.0 MDa, 22.7 kDa

Table 4
Anionic charge density of EPS (meq/g EPS) extracted from COD/N 20 and 100 reactors.

COD/N	pH	Freshwater EPS		Saline water EPS	
		Soluble	Bound	Soluble	Bound
20	5	2.14 ± 0.13	1.94 ± 0.01	3.11 ± 0.02	1.60 ± 0.16
	7	3.50 ± 0.10	3.02 ± 0.01	4.68 ± 0.05	2.69 ± 0.12
100	5	2.25 ± 0.18	2.72 ± 0.13	1.96 ± 0.16	1.40 ± 0.01
	7	3.25 ± 0.07	3.48 ± 0.01	3.06 ± 0.22	2.25 ± 0.00

As expected, the charge density at an elevated pH (7) was higher because of the functional groups in a largely deprotonated form, *i.e.* $-COO$ and $-NH_2$, compared to $-COOH$ and $-NH_3^+$. These values can even go as high as 5–6 meq/g at higher pH values such as 10 [8]. At both studied pH values, charge densities of the S-EPS were higher than those of the corresponding B-EPS (except for the FW-EPS at COD/N 100), likely due to the higher polysaccharide content in the soluble fractions compared to the bound fractions (Fig. 3). In the case of the FW-EPS produced at COD/N 100, where a higher polysaccharide content was in the B-EPS than in the S-EPS (Fig. 3A and B), the charge density was also higher in the B-EPS than in the S-EPS. These findings imply that polysaccharides (with functional groups OH and $-COO^-$) give a higher contribution to the anionic charge density than proteins (with functional groups NH_2 and $-COO^-$), which is consistent with the results of Durmaz and Sanin [9].

3.4. EPS flocculation performance

Flocculation tests were focused on the EPS produced at COD/N 20 and 100. First, the flocculation performances of the EPS fractions on non-saline kaolin suspension were tested, with the addition of 100 mg/L Ca^{2+} to facilitate bridging between the anionic particles and EPS (Fig. 4). The maximum flocculation efficiencies that were obtained varied between 81 and 91%, with no significant difference between the performances of FW-EPS and Sal-EPS at COD/N 100. The maximum efficiency (91%) and optimum dosage (0.2 mg/g) are comparable to that of a commercial synthetic flocculant (anionic polyacrylamide –93% efficiency at 0.1 mg/g) operated under similar flocculation conditions [8].

The three best dosages (0.1, 0.2 and 0.5 mg/g) were also utilized to flocculate saline kaolin suspension (30 g/L NaCl) without Ca^{2+} addition. A similar flocculation performance pattern was also observed like in Fig. 4 (see supplementary materials, Fig. S6), with maximum flocculation efficiencies between 87 and 92%. However, under the saline conditions, Sal-EPS consistently gave a better performance (by 6–13%) than the corresponding EPS produced under freshwater conditions. Although this cannot be directly related to observed differences in EPS properties such as composition, molecular weight and charge, the implication may be that, for flocculation in saline environments, it is more attractive to use EPS that were produced from saline wastewater.

Overall, three flocculation patterns were seen in the non-saline and saline kaolin experiments. First, the general trend was an increase in flocculation efficiency with increasing EPS concentration in the range of 0.1 – 0.2/0.5 mg/g kaolin, but reduced efficiency at higher concentrations (Figs. 4 and S6). A similar pattern has also been reported with synthetic anionic flocculants and in sludge bioflocculation with EPS [28–30]. This effect is known to be caused by restabilization of the clay particles at concentrations above the optimum [31]. Secondly, FW and Sal EPS produced from COD/N 100 generally showed better flocculation performances than corresponding EPS from COD/N 20. This is likely due to the higher MW biopolymers in EPS produced under nitrogen-limited conditions (Table 3). It is well established that a higher MW and longer polymer chain facilitates the formation of adsorbed 'loops' and 'tails' that can extend into the solution, thereby enhancing

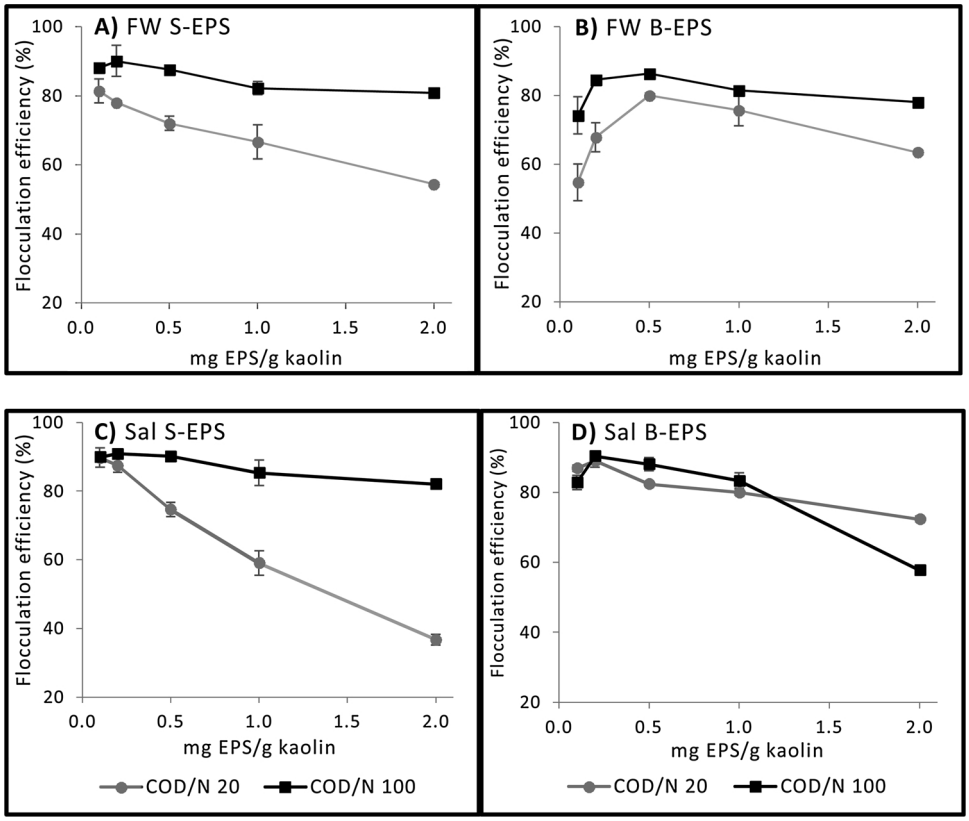

Fig. 4. Non-saline kaolin clay flocculation efficiency (%) obtained using EPS fractions of COD/N ratio 20 and 100, with the addition of 100 mg Ca^{2+}/L.

the ability to attach to other clay particles [4]. The third trend is that S-EPS generally had higher maximum flocculation efficiencies than the corresponding B-EPS, which is consistent with the findings of Bala Subramanian et al. [32] and Nouha et al. [33]. From our study, this is possibly related to the higher charge densities of S-EPS compared to the corresponding B-EPS (except for freshwater EPS of COD/N 100 – Table 4), since both fractions have similar MWs. A higher charge density implies more repulsion between charged segments and a wider expansion of the polymer chain into the solution, increasing the ability to adsorb and bridge between particles [4].

3.5. Practical implications and outlook

The advantage of such mixed EPS (mixed composition and MW) over single-type EPS and single synthetic flocculants could be their resilience and robustness under practical conditions such as different particle type, size, and concentration. However, further research is needed to substantiate this hypothesis.

The use of non-synthetic glycerol/ethanol-rich wastewater is also expected to afford high EPS yield if influent nitrogen is limited. However, the impact of this non-synthetic wastewater on the produced EPS properties and flocculation acitvity, though not expected to differ much from the findings of this study, still needs to be investigated. Also, additional studies should be carried out on (non-synthetic) wastewater mixtures of varying compositions and how these may affect the reproducibility of the produced EPS. Finally, while EPS production from wastewater may have the additional advantage of much lower oxygen demand, the consequent membrane fouling due to a higher sludge viscosity is a challenge that still needs to be addressed.

4. Conclusions

- It is possible to continuously treat glycerol/ethanol-rich wastewater while simultaneously producing, with a mixed microbial population, large amounts of extracellular polymers that can be utilized as bioflocculants.
- Under nitrogen-limited (COD/N 100) and freshwater conditions, up to 54% of the wastewater COD could be recovered as EPS.
- Under saline conditions, EPS recovery was as high as 36%.
- High flocculation efficiencies were obtained, which can be explained by the relatively high molecular weight (up to 2.1 MDa) and medium charge densities (2–5 meq/g) of the flocculants.
- In the flocculation tests under saline conditions, polymers that were produced under saline conditions gave a consistently higher (6–13%) flocculation performance than polymers produced under freshwater conditions.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the

online version, at doi:https://doi.org/10.1016/j.jhazmat.2019.05.009.

References

[1] H. Salehizadeh, N. Yan, Recent advances in extracellular biopolymer flocculants, Biotechnol. Adv. 32 (2014) 1506–1522, https://doi.org/10.1016/j.biotechadv.2014.10.004.

[2] F. Renault, B. Sancey, P.M. Badot, G. Crini, Chitosan for coagulation/flocculation processes– an eco-friendly approach, Eur. Polym. J. 45 (2009) 1337–1348, https://doi.org/10.1016/j.eurpolymj.2008.12.027.

[3] C.S. Lee, J. Robinson, M.F. Chong, A review on application of flocculants in wastewater treatment, Process Saf. Environ. Prot. 92 (2014) 489–508, https://doi.org/10.1016/j.psep.2014.04.010.

[4] B. Bolto, J. Gregory, Organic polyelectrolytes in water treatment, Water Res. 41 (2007) 2301–2324, https://doi.org/10.1016/j.watres.2007.03.012.

[5] T.T. More, J.S.S. Yadav, S. Yan, R.D. Tyagi, R.Y. Surampalli, Extracellular polymeric substances of bacteria and their potential environmental applications, J. Environ. Manage. 144 (2014) 1–25, https://doi.org/10.1016/j.jenvman.2014.05.010.

[6] M. Shahadat, T.T. Teng, M. Rafatullah, Z.A. Shaikh, T.R. Sreekrishnan, S.W. Ali, Bacterial bioflocculants: a review of recent advances and perspectives, Chem. Eng. J. 328 (2017) 1139–1152, https://doi.org/10.1016/j.cej.2017.07.105.

[7] D.C. Sobeck, M.J. Higgins, Examination of three theories for mechanisms of cation-induced bioflocculation, Water Res. 36 (2002) 527–538, https://doi.org/10.1016/S0043-1354(01)00254-8.

[8] V. Ajao, H. Bruning, H. Rijnaarts, H. Temmink, Natural flocculants from fresh and saline wastewater: comparative properties and flocculation performances, Chem. Eng. J. 349 (2018) 622–632, https://doi.org/10.1016/j.cej.2018.05.123.

[9] B. Durmaz, F.D. Sanin, Effect of carbon to nitrogen ratio on the physical and chemical properties of activated sludge, Environ. Technol. 24 (2003) 1331–1340, https://doi.org/10.1080/09593330309385677.

[10] A.P. Miqueleto, C.C. Dolosic, E. Pozzi, E. Foresti, M. Zaiat, Influence of carbon sources and C/N ratio on EPS production in anaerobic sequencing batch biofilm reactors for wastewater treatment, Bioresour. Technol. 101 (2010) 1324–1330, https://doi.org/10.1016/j.biortech.2009.09.026.

[11] S. Feng, N. Zhang, H. Liu, X. Du, Y. Liu, H. Lin, The effect of COD/N ratio on process performance and membrane fouling in a submerged bioreactor, Desalination. 285 (2012) 232–238, https://doi.org/10.1016/j.desal.2011.10.008.

[12] F. Ye, Y. Ye, Y. Li, Effect of C/N ratio on extracellular polymeric substances (EPS) and physicochemical properties of activated sludge flocs, J. Hazard. Mater. 188 (2011) 37–43, https://doi.org/10.1016/j.jhazmat.2011.01.043.

[13] Z. Wang, M. Gao, S. Wang, Y. Xin, D. Ma, Z. She, Z. Wang, Q. Chang, Y. Ren, Effect of C/N ratio on extracellular polymeric substances of activated sludge from an anoxic–aerobic sequencing batch reactor treating saline wastewater, Environ. Technol. 35 (2014) 2821–2828, https://doi.org/10.1080/09593330.2014.924563.

[14] M. DuBois, Ka. Gilles, J.K. Hamilton, Pa. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (1956) 350–356, https://doi.org/10.1021/ac60111a017.

[15] W.F. Tan, W. Norde, L.K. Koopal, Humic substance charge determination by titration with a flexible cationic polyelectrolyte, Geochim. Cosmochim. Acta 75 (2011) 5749–5761, https://doi.org/10.1016/j.gca.2011.07.015.

[16] S.J. Yuan, M. Sun, G.P. Sheng, Y. Li, W.W. Li, R.S. Yao, H.Q. Yu, Identification of key constituents and structure of the extracellular polymeric substances excreted by bacillus megaterium TF10 for their flocculation capacity, Environ. Sci. Technol. 45 (2011) 1152–1157, https://doi.org/10.1021/es1030905.

[17] P.L.L. Ribeiro, M.I. Campos, J.I. Druzian, Novel extracellular polymeric substances produced by Cupriavidus necator IPT 027 grown on glucose and crude glycerol originated from biodiesel, Polym. Adv. Technol. 28 (2017) 549–556, https://doi.org/10.1002/pat.3957.

[18] C.S. Laspidou, B.E. Rittmann, A unified theory for extracellular polymeric substances, soluble microbial products, and active and inert biomass, Water Res. 36 (2002) 2711–2720, https://doi.org/10.1016/S0043-1354(01)00413-4.

[19] Z.-J. Zhang, S.-H. Chen, S.-M. Wang, H.-Y. Luo, Characterization of extracellular polymeric substances from biofilm in the process of starting-up a partial nitrification process under salt stress, Appl. Microbiol. Biotechnol. 89 (2011) 1563–1571, https://doi.org/10.1007/s00253-010-2947-y.

[20] J.B. Russell, The energy spilling reactions of bacteria and other organisms, J. Mol. Microbiol. Biotechnol. 13 (2007) 1–11, https://doi.org/10.1159/000103591.

[21] D.W. Tempest, O.M. Neijssel, Eco-physiological aspects of microbial growth in aerobic nutrient-limited environments. In advances in microbial ecology, Adv. Microb. Ecol (1978) 105–153, https://doi.org/10.1007/978-1-4615-8222-9.

[22] H.-C. Flemming, J. Wingender, The biofilm matrix, Nat. Publ. Gr. 8 (2010) 623–633, https://doi.org/10.1038/nrmicro2415.

[23] K. Nouha, N.V. Hoang, R.D. Tyagi, Fourier transform infrared spectroscopy and liquid chromatography – mass spectrometry study of extracellular polymer substances produced on secondary sludge fortified with crude glycerol, J. Mater. Sci. Eng. 5 (2016) 1–10, https://doi.org/10.4172/2169-0022.1000240.

[24] J. Lin, C. Harichund, Production and characterization of heavy-metal removing bacterial bioflocculants, African J. Biotechnol. 11 (2012) 9619–9629, https://doi.org/10.5897/AJB11.1698.

[25] S.P. Buthelezi, A.O. Olaniran, B. Eillay, Production and characterization of bioflocculants from bacteria isolated from wastewater treatment plant in South Africa, Biotechnol. Bioprocess Eng. 15 (2010) 874–881, https://doi.org/10.1007/s12257-009-3002-7.

[26] W. Babel, R.H. Muller, Mixed substrate utilization in micro-organisms: biochemical aspects and energetics, Microbiology 131 (1985) 39–45, https://doi.org/10.1099/00221287-131-1-39.

[27] T.P. Pirog, M.A. Kovalenko, Y.V. Kuz'minskaya, Intensification of exopolysaccharide synthesis by Acinetobacter sp. on an ethanol-glucose mixture: Aspects related to biochemistry and bioenergetics, Microbiology. 72 (2003) 305–312, https://doi.org/10.1023/A:1024247915758.

[28] M.S. Nasser, A.E. James, The effect of polyacrylamide charge density and molecular weight on the flocculation and sedimentation behaviour of kaolinite suspensions, Sep. Purif. Technol. 52 (2006) 241–252, https://doi.org/10.1016/j.seppur.2006.04.005.

[29] B.J. Lee, M.A. Schlautman, E. Toorman, M. Fettweis, Competition between kaolinite flocculation and stabilization in divalent cation solutions dosed with anionic polyacrylamides, Water Res. 46 (2012) 5696–5706, https://doi.org/10.1016/j.watres.2012.07.056.

[30] J.I. Houghton, J. Quarmby, T. Stephenson, Municipal wastewater sludge dewaterability and the presence of microbial extracellular polymer, Water Sci. Technol. 44 (2001) 373–379, https://doi.org/10.2166/wst.2001.0792.

[31] J. Gregory, S. Barany, Adsorption and flocculation by polymers and polymer mixtures, Adv. Colloid Interface Sci. 169 (2011) 1–12, https://doi.org/10.1016/j.cis.2011.06.004.

[32] S. Bala Subramanian, S. Yan, R.D. Tyagi, R.Y. Surampalli, Extracellular polymeric substances (EPS) producing bacterial strains of municipal wastewater sludge: isolation, molecular identification, EPS characterization and performance for sludge settling and dewatering, Water Res. 44 (2010) 2253–2266, https://doi.org/10.1016/j.watres.2009.12.046.

[33] K. Nouha, N. Hoang, Y. Song, R. Tyagi, S. RY, Characterization of extracellular polymeric substances (EPS) produced by cloacibacterium normanense isolated from wastewater sludge for sludge settling and dewatering, J. Civ. Environ. Eng. 5 (2015) 1–8, https://doi.org/10.4172/2165-784X.1000191.