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Fourier Transform Infrared Spectroscopy and Liquid Chromatography – Mass Spectrometry Study of Extracellular Polymer Substances Produced on Secondary Sludge Fortified with Crude Glycerol

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Abstract

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

Keywords: Cloacibacterium normanense; EPS; Activated sludge; Crude glycerol; FTIR; Rheology; Bioflocculation

Abbreviations: CAN: Acetonitrile; ANOVA: Analysis of Variance; B-EPS: Broth EPS; BSA: Bovine Serum Albumin; C-EPS: Capsular EPS; CDW: Cell Dry Weight; CHNS: Carbon, Hydrogen, Nitrogen and Sulphur; CFU: Colony Forming Units; C/N: Carbon/Nitrogen Ratio; CUQ: Communauté Urbaine du Québec; DLVO: Derjaguin-Landau-Verwey-Overbeek; EPS: Extracellular Polymeric Substances; FA: Flocculation Activity; FTIR: Fourier Transform Infrared Spectroscopy; KBr: Potassium Bromide; LC/MS: Liquid Chromatography/Mass Spectrometry; mg EPS/g of kaolin: mg of EPS added per gram of kaolin suspension in water; NTU: Nephelometric Turbidity Unit; S-EPS: Slime EPS; SEM: Scanning Electron Microscopy; SS: Suspended Solids; TSB: Tryptic Soy Broth; (v/v): Volume per Volume; ζ-potential: Zeta potential

Introduction

Microbial polysaccharides have been demonstrated with a vast range of functional properties and applications including food products, pharmaceuticals, bioemulsifiers [1], bioflocculants [2], chemical products [3] and the biosorption of heavy metals [4]. The chemical compositions of EPSs produced by different bacterial strains are very diverse. The main constituents of EPS are sugars such as galactose, glucose and rhamnose. Acetate and puryvate are characteristic substituents. Whilst the structures are very complex, consist of branched repeating units and different linkages that exist between the monosaccharides [5].

Depending on their specific composition, EPSs have different cellular functions, including accumulation of nutrients, diffusion barrier for toxins and heavy metals, cell motility, attachment to surfaces, protection against desiccation. Thus, several researchers have discussed recent advancements in the understanding of the structure–function relationships, i.e., to relate EPSs structure with their properties (bioflocculant, bioemulsfiers) in order to improve EPSs synthesis and applications [6]. Therefore, there is a need to develop an understanding of structure–function relationships to relate EPS structure, chemical composition and molecular weight with bioflocculation properties, especially for the EPS produced in wastewater sludge by new isolated bacterial strain.

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

Methods

EPS production

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

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glycerol. During the experiments, samples of the broth were collected every 24 h to determine EPS concentration, glycerol concentration and cell population. The crude glycerol solution also contains other components (soap and methanol), which can act as carbon source. Therefore, consumption of soap and methanol was determined during the fermentation [7].

EPS extraction and dry weight

The centrifugation method was used to extract S-EPS (slime EPS) and C-EPS (capsular EPS). Broth EPS (B-EPS) consists of both S-EPS and C-EPS, or the fermented broth is named as B-EPS. The fermented broth was centrifuged at 9000 g, 4°C for 20 min to obtain supernatant (containing S-EPS). The biomass pellet was re-suspended in deionized water equal to the initial volume and then heated at 60°C for 20 min followed by centrifugation at 9000 g, 4°C for 20 min to extract capsular EPS [8].

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

The EPS concentration was estimated by the following formula:

$$[\mathrm{EPS}](\mathrm{g/L}) = \frac{W_2 - W_1}{V}$$

Where, W_1: Initial dry weight of the empty aluminium dish without a sample (g)

W_2: Dry weight of the aluminum dish with dried sample (g)

V: volume of the sample (L)

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

Analytical methods

Glycerol concentration in the cell-free supernatant, methanol and soap content was determined according to Hu et al. [7]. The growth was measured based on the dry weight per volume of the culture. The cell dry weight (CDW) or biomass was determined by centrifugation (8000 g, 4°C, 15 min) and after the C-EPS extraction, followed by overnight drying the sample to a constant weight in an oven at 60°C. The cell concentration of all the samples (diluted with saline solution), was measured as CFU employing standard agar-plate technique. Total nitrogen and organic carbon in the samples collected at various times of fermentation were measured by the CHNS analyzer (Shimadzu VCPH).

EPS characterization

Chemical composition of the EPS: Glycosyl composition analysis was performed by combining Liquid Chromatography/Mass Spectrometry (LC/MS) with Hypersil Gold column (100*2.1 mm ID), using 85% Water, 0.1% formic acid (Phase A)/ 15% acetonitrile (ACN), 0.1% formic acid (Phase B), as eluent, at a flow rate of 0.4 mL/min and a temperature of 30°C [9]. EPS sample 20 µl was used for the identification and quantification of acyl group and monosaccharides present in the purified EPS (the ethanol precipitated EPS). The monosaccharides were identified by their retention times in comparison to standards.

FT-IR spectroscopy: Precipitated EPS were collected by centrifugation at 4000 g, 30 min at 4°C and dried at 60°C. The purified and dry S-EPS (0.1-0.2 mg) and 100 mg of potassium bromide (KBr) were mixed and pressed in a die (at five tons and one minute) to form a pellet. Afterwards, the pellet was immediately put into the sample holder and FT-IR spectra were recorded. The transmission FT-IR spectra were obtained using a Perkin Elmer 2000 FT-IR spectrometer. FT-IR scanning was conducted in ambient conditions. The resolution was set to 4 cm^{-1} and the operating range was 400 to 4000 cm^{-1} [10].

EPS properties

Rheology: To investigate the stability of the EPS at different temperatures, the crude EPS solution was incubated for 10 min at 80°C to 200°C in an incubator. The viscosity of the above EPS solution (20 ml) was measured using a ULA S 34 spindle (Digital Viscometer, DV-II+ Pro, Brookfield), at 60 rpm and room temperature.

Enzymatic digestions test: Proteinase K was used as the proteolytic enzyme and 80 units of the enzyme were added to 200 ml sample. Proteinase K is a non specific enzyme that hydrolyses proteins at a number of cleavage sites. Cellulase (β glucosidase) was used as an extracellular polysaccharide degrading enzyme and 150 active units were added to 200 mL sample of B-EPS. Cellulase hydrolyses the polysaccharides present in EPS [1]. Enzymes were added to each 200 mL sample of the fermented broth collected at 48h and the enzymatic digestion was conducted for 36h at 30°C. The samples, each 20 ml, were collected at every 12 h to measure ζ-potential, viscosity and turbidity index. All the measurements were carried out in duplicates and the average result was presented.

Scanning electron microscopy (SEM): Samples of enzymatic digestion test were collected 6 hr after enzyme addition. Broth sample of 10 µl was taken on the glass slide and the cells were fixed with 3% glutaraldehyde. After fixing, the cells were washed with ethanol solution of different concentration (30–100% v/v) and air dried between each wash to completely remove the water adhered to the cells. After final washing, cells were subjected to overnight drying. The dried samples were mounted on conventional 12.7 mm or 25.4 mm diameter aluminium stubs using double sided adhesive carbon discs and coated with gold film to a thickness of 10-20 nm using a sputter coater (SPI™ sputter coater module) to examine their morphology under scanning electron microscopy (Model: Carl Zeiss EVO®50 smart SEM) [11].

Zeta potential: The charge of B-EPS after enzymatic digestion was determined by adding 50-1000 µL of individual EPS sample to 100 ml of deoinzed water. Characterization of charge (zeta potential) was implemented using Zetaphoremeter (Zetaphoremeter IV, Zetacompact Z8000, CAD Instrumentation, France) with the application of the Smoluckowski equation. Surface charge of the wastewater sludge was also measured. The zeta potential values were obtained from an average of around 10 measurements, the average values are presented with its half-width confidence interval at 95% confidence level.

Measurement of turbidity index: Kaolin clay was used as a test material to measure the flocculation activity or the turbidity index of EPS samples digested by enzymes. Flocculation activity or turbidity index of EPS was carried out through the jar test method [8]. Kaolin with concentration of 5 g/L was suspended in distilled water, 150 mg/L of Ca^{2+} was added to the kaolin suspension and pH was adjusted to 7.5 after addition of Ca^{2+}. The samples collected at different times of (6 h, 12 h, 24 h and 36 h) after adding enzymes, were added in different volumes (corresponding to desired concentrations of EPS, which was calculated through dry weight and the volume of EPS solution required) to kaolin suspension and rapidly mixed at 100 rpm for an initial 5 min

then slowly mixed for an additional 30 min at 70 rpm. After 35 min of mixing, samples were transferred to a 500 ml cylinder and allowed to settle for 1 h. The supernatants of the settled samples were collected to measure the turbidity using turbidimeter (Micro 100 turbidimeter, Scientific Inc.). The flocculation activity (FA) was determined using the formula [100*(B-A)/B]; where 'A' is the turbidity of the sample (treated with EPS; Slime, Capsular or Broth-EPS) and 'B' is the turbidity of the control (in which equal volume of EPS solution was replaced with distilled water).

Statistical analysis

All analysis reported in this manuscript was performed in triplicate, and the results are presented as the mean values. The results were analysed by analysis of variance (ANOVA), using Excel's Analysis ToolPak.

Results and Discussion

EPS production

The variation of biomass, nitrogen concentrations and C/N ratio (with and without adding crude glycerol) during the fermentation is presented in Figure 1 and Table 1. Cell count, glycerol and EPS concentrations are depicted in Figure 2. The C/N ratio was calculated as the concentration ratio (i.e. concentration of organic carbon/concentration of total nitrogen). Different C/N ratio (C/N 10, C/N 25 and C/N50) have been studied before to determine the optimum C/N ratio (Data not shown). Thus, C/N 25 was chosen in present study.

The crude glycerol was added at 24 h fermentation as an extra carbon source to aid EPS production by *Cloacibacterium normanense*, which increased the C/N ratio to 25 compared to 11 in the control or without glycerol (Table 1). The maximum EPS production (about 17.5 g/L) was observed at 72 h of fermentation with glycerol fortification (C/N 22, Table 1), whereas EPS concentration of 13.3 g/L was observed at 48 h (C/N reached 14) fermentation time without glycerol fortification. Without glycerol, the C/N ratio initially increased (0 to 48 h) and was due to higher consumption of nitrogen than carbon, both required for growth (Figure 2). Further, decrease in the C/N ratio after 48 h could be due to higher consumption of carbon required for maintenance of cells, because EPS did not increase during this time. Corresponding to results found C/N we suggest that C/N 22 is considered to be the most favourable for EPS production by *Cloacibacterium normanense* in present study. However, Miqueleto et al. [12] have reported that C/N 10 was recommended for high EPS production in an anaerobic sequencing batch biofilm reactor. In the study of Liu et al. [13], the suggested C/N ratio was 12 for maximum EPS concentration (8.90 g/L) produced by *Zunongwangia profunda* SM-A87. Nevertheless, for most EPS-synthesizing microorganisms, the highest polymer productivities are usually achieved at high C/N ratio and is microbial strain dependent.

With glycerol addition, EPS concentration steadily increased until 72 h, whereas C/N ratio first increased to 25 (at 24 h due to the addition of extra glycerol) and then decreased to 22 at 72 h. Most of the added glycerol was consumed at 48 h (residual glycerol 2.0 g/L, Figure 2); however, EPS substantially increased from 48 to 72 h. Therefore, the microbe used carbon from sludge for synthesis of EPS, thus decreased the C/N ratio (even in spite of nitrogen decrease, Figure 1).

The CFU (colony forming units) count increased during the first 48 hours of fermentation (with the addition of glycerol) and nitrogen used was 37 mg/L, whereas between 48 and 72 h, the increase in CFU was relatively less but nitrogen consumed was almost 100 mg/L (Figure 1 and 2). The excess consumption of nitrogen (48-72 h) was routed toward formation of other unknown metabolites [14]. In this context, Duenas et al. [15] reported that EPS production by *Pediococcus damnosus* was mainly enhanced with an increase in glucose concentration, but not by an increase in nitrogen concentration.

In addition, the crude glycerol is source of soap and methanol,

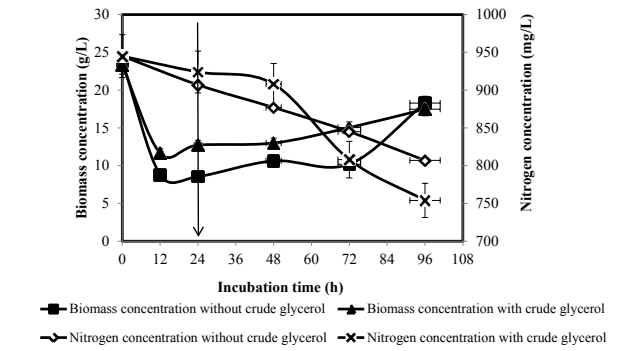

Figure 1: Variation of biomass and nitrogen concentration during fermentation with and without addition of crude glycerol as carbon source (Arrow represents the addition of glycerol at this point).

Experiments with crude glycerol addition	Incubation time (h)	0	24	48	72	96
	C/N ratio	3	25	21	22	16
	S-EPS (g/L)	1.4 ± 0.3	4.6± 0.3	13.9 ± 0.4	15.6 ± 0.5	4.3± 0.3
	B-EPS (g/L)	1.8 ± 0.3	5.2 ± 0.2	14.9± 0.2	17.5± 0.4	15.4± 0.6
	EPS/biomass ratio (g/g)	0.08 ± 0.01	0.4 ± 0.2	1.1 ± 0.3	1.1 ± 0.1	0.9 ± 0.4
Experiments without crude glycerol addition	C/N ratio	3	11	14	12	11
	S-EPS (g/L)	1.5 ± 0.3	3.9 ± 0.3	12.9 ± 0.5	12.2 ± 0.3	10.4± 0.2
	B-EPS (g/L)	1.9± 0.3	4.7± 0.3	13.3± 0.2	12.5± 0.1	10.8± 0.4
	EPS/biomass ratio (g/g)	0.08 ± 0.3	0.55 ± 0.2	1.25 ± 0.3	1.27± 0.1	0.60 ± 0.4

Table 1: Production of biomass and EPS produced with and without crude glycerol fortification during fermentation.

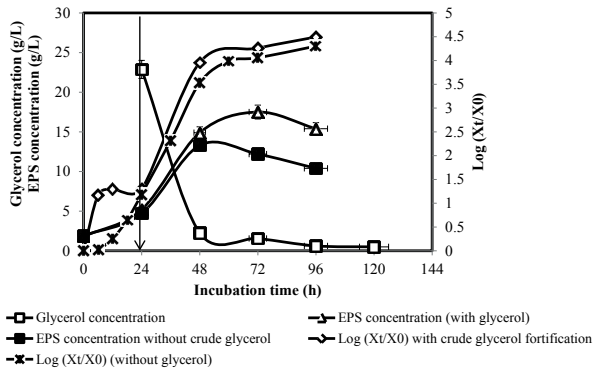

Figure 2: Variation of glycerol, cell and B-EPS concentration during fermentation. Xt and X0 are CFU at time t and t = 0, respectively (Arrow represents the addition of glycerol at this point).

which can be used as substrate for the EPS production. Figure 2 shows the decrease of crude glycerol content during the fermentation concomitant with the growth of biomass on semi log scale (X0 is the initial CFU and Xt is CFU at time 't'). Figure 3 illustrates the decrease of methanol and soap concentration during the fermentation process indicating that soap and methanol are degraded by the strain for growth and to generate EPS. Kumar et al. [16] have also reported that non sugar carbon sources (like methanol) could contribute to produce microbial EPS.

The carbon source was studied for the EPS and biomass production of *Cloacibacterium normanense*, determining the relative significance of two variables (crude glycerol added and sludge media without crude glycerol addition), using ANOVA Excel's Analysis ToolPak. The ANOVA analysis for EPS production and biomass is shown in Table 2. The value of regression coefficient R^2 was 0.9890 for biomass and 0.9723 for EPS production. The p-values of the models were 0.0002 and 1.3632E-05, in case of crude glycerol fortification, 4.27E-06 and 3.4637E-05 without crude glycerol for biomass and EPS production, respectively, indicating that the models were significant. Usually, a model term is considered to be significant when its value of "p-value" is less than 0.05 [17].

EPS physico-chemical characterization

The B-EPS obtained in the experiments were analysed for their sugar and acyl group composition (Figure 4). Five main constituent sugar residues were identified by the glycosyl composition analysis, namely, glucose, galactose, lactose, sucrose and xylose. Galactose was the most abundant monosaccharide, accounting for 67 mol % of the total carbohydrate content of the B-EPS. Glucose represented 13 mol %, but lactose, sucrose and xylose was present in only minor amounts i.e. 3, 8 and 9 mol %, respectively.

EPS synthesized without the addition of crude glycerol was distinct from the EPS produced with glycerol addition. The EPS without glycerol addition was characterized by only one type of monosaccharide, i.e. eighty mol % of glucose. The appearance of galactose and lactose monosaccharide after adding crude glycerol was demonstrated. This can be explained by the fact that the strain *Cloacibacterium normamnense* converts glucose to galactose or/and glucose to lactose in the presence of glycerol. We can, therefore, anticipate that the addition of glycerol has influenced the level of activation of the enzymes necessary for the different metabolic and involved in the synthesis and assembling of the sugar nucleotides, thus resulting in the production of polymers with such a diverse sugar composition. Similar observations have been recorded by Yolunda et al. [18]. Polysaccharides containing galactose are produced by several bacteria, such as *Pseudomonas, Lactobacillus* and *Streptococcus* [9]. The data obtained in our study are in agreement with the findings reported by Freitas et al. [9]. They demonstrated high galactose content of the EPS produced by *Pseudomonas oleovarans* grown on pure glycerol.

The present results have also shown that the EPS contained (with or without glycerol addition) non-saccharide components, namely, acyl groups. Two different acyl groups were identified in small quantity. Pyruvate (0.014 wt%) appeared after addition of glycerol; however, succinate (0.057wt.%) was present in the absence of glycerol (Figure 4c and 4d). These components are frequently present in microbial EPS and notably influence polymer's properties, namely, solubility and rheology [19]. According to Freitas et al. [9], three acyl groups were identified using media containing pure glycerol, i.e. pyruvate (3.35 wt%), succinate (1.04 wt%) and acetate (0.38 wt%).

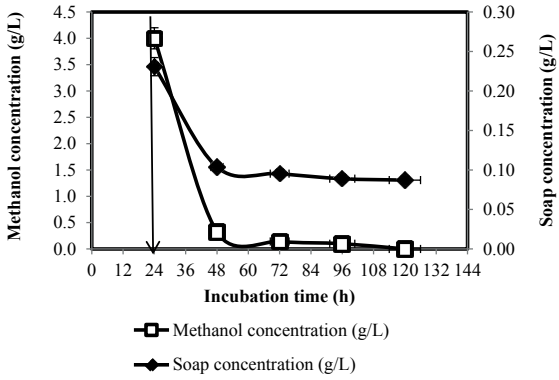

Figure 3: Soap and methanol concentration profile during fermentation (Arrow represents the addition of glycerol at this point).

Variables	Biomass[a]			EPS production[b]		
	coefficient estimate	F value	p value	coefficient estimate	F value	p value
Sludge + crude glycerol	0.93	0.07	0.0002[c]	1.01	0.013	1.3632E-05[c]
Sludge without crude glycerol	0.99	0.01	4.2769E-06[c]	1.0087	0.016	3.4637E-05[c]

[a]R^2 = 0.9890;
[b]R^2 = 0.9723;
[c]Model terms are significant

Table 2: Identification of significant variables for EPS production and Biomass of *Cloacibacterium normanense* using ANOVA of Excel.

FT-IR spectroscopy

The FTIR analysis of EPS (produced with or without crude glycerol) was presented in Figure 5a and 5b. The FT-IR spectrum of the exopolysaccharide is in agreement with the chemical analysis (LC/MS) described above. The main functional groups are hydroxyl, carboxyl, acyl and amino, which corresponds mostly to the presence of carbohydrates and proteins in the EPS.

In the two cases (with and without crude glycerol addition), it revealed a broad stretched peak at 3455 cm^{-1} (range 3600-3200 cm^{-1}), corresponding to the hydroxyl group. A weak absorption at 2925 cm^{-1} (range 300-2500 cm^{-1}) was assigned to an asymmetrical l C-H stretching vibration of the aliphatic CH_2 group, which represents the presence of organic substances like sugars and proteins. The amide II band at 1729 cm^{-1} originates from N-H bonding and C-N stretching vibrations in -CO-NH of proteins. Presence of an asymmetric stretching peak or vibration at 1643 cm^{-1} may correspond to the ring stretching of galactose as the LC/MS analysis indicated in the last section. Another peak at 1404 or 1455 cm^{-1} could be attributed to the symmetric stretching of the COO– group. The absorption peaks (1271 or 1233 cm^{-1}) ranging from (1500-1200 cm^{-1}) were designated to C-O-C and C=O, which indicates the occurrence of carbohydrates. A peak at 1058 cm^{-1} (1000-1125 cm^{-1} range) may be attributed to O-acetyl ester linkage bond of uronic acid. Absorption peak approximately in the range of 781-522 cm^{-1} corresponded to stretching of alkyl-halides. These findings are in agreement with the studies of [9,20,21].

However, the EPS produced with sludge supplemented with crude glycerol differs from the EPS produced without crude glycerol by having

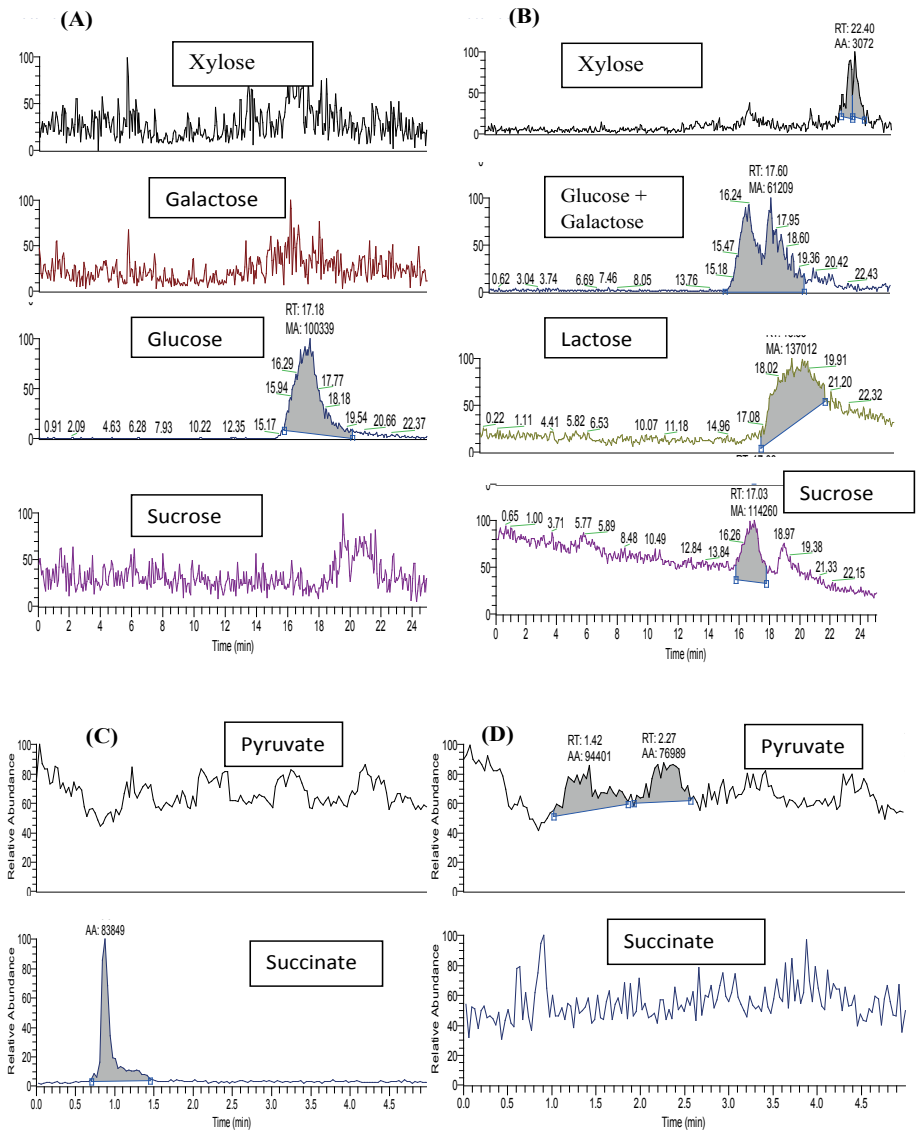

Figure. 4. LC/MS analysis of the EPS produced by Cloacibacterium normanense : (A) Monosaccharide analysis without crude glycerol, (B) Monosaccharide analysis with crude glycerol, (C) Acyl group analysis without crude glycerol, (D) Acyl group analysis with crude glycerol.

additional peaks at different regions. IR spectra of EPS produced in the case of crude glycerol addition (Figure 5a) show particular bands, which do not appear for EPS produced in the absence of crude glycerol (Figure 5b). Three bands around 2855 cm^{-1} (symmetric stretching vibration of CH_2, C = O stretching), 1678 cm^{-1} (deformation vibration of N-H or Amide I and C-N stretching) and 1578 cm^{-1} (deformation vibration of N-H or Amide II) appeared when crude glycerol was added to the production medium. These characteristic bands can be attributed to protein and polysaccharide functional groups. By the presence of these peaks, FTIR of EPS (with crude glycerol) demonstrates relatively higher quantity of hydroxyl (-OH), amide (-CO-NH), carboxyl (-COO-), and primary amine ($-NH_2$) groups compared with those observed without crude glycerol. The abundance of these groups in EPS produced with supplementation of crude glycerol in the medium may contribute to the difference in flocculation activity (93.4%) compared to flocculation activity (90.2%) of EPS produced without crude glycerol fortification. Further, the presence of carboxyl, hydroxyl, and amine groups is very important for bioflocculation with relatively quantity, providing surface charges, which serve as the binding sites for suspended particles causing aggregation or floc formation as also discussed by Li et al. [22] and Kavita et al. [20]. However, the excess of these groups may increase the negative charge causing a repulsion of particles [20].

The difference in the occurrence, position and frequency of these groups could affect the flocculation activity and that could be the reason of high flocculation activity of the EPS obtained in this study (with or without fortification of crude glycerol) compared to those reported by other researchers. The flocculation activity of EPS synthesised by *Pseudomonas* SM9913 was 49.3% [22] and 40% of the EPS produced by *Oceanobacillus iheyensis*. These groups (C-O-C; O-H; C = O…etc.) were either absent or present in insufficient quantity in EPSs as described in Table 3. Table 3 recaps the functional groups corresponding to bands observed in the IR spectra of B-EPS produced by the strain with or

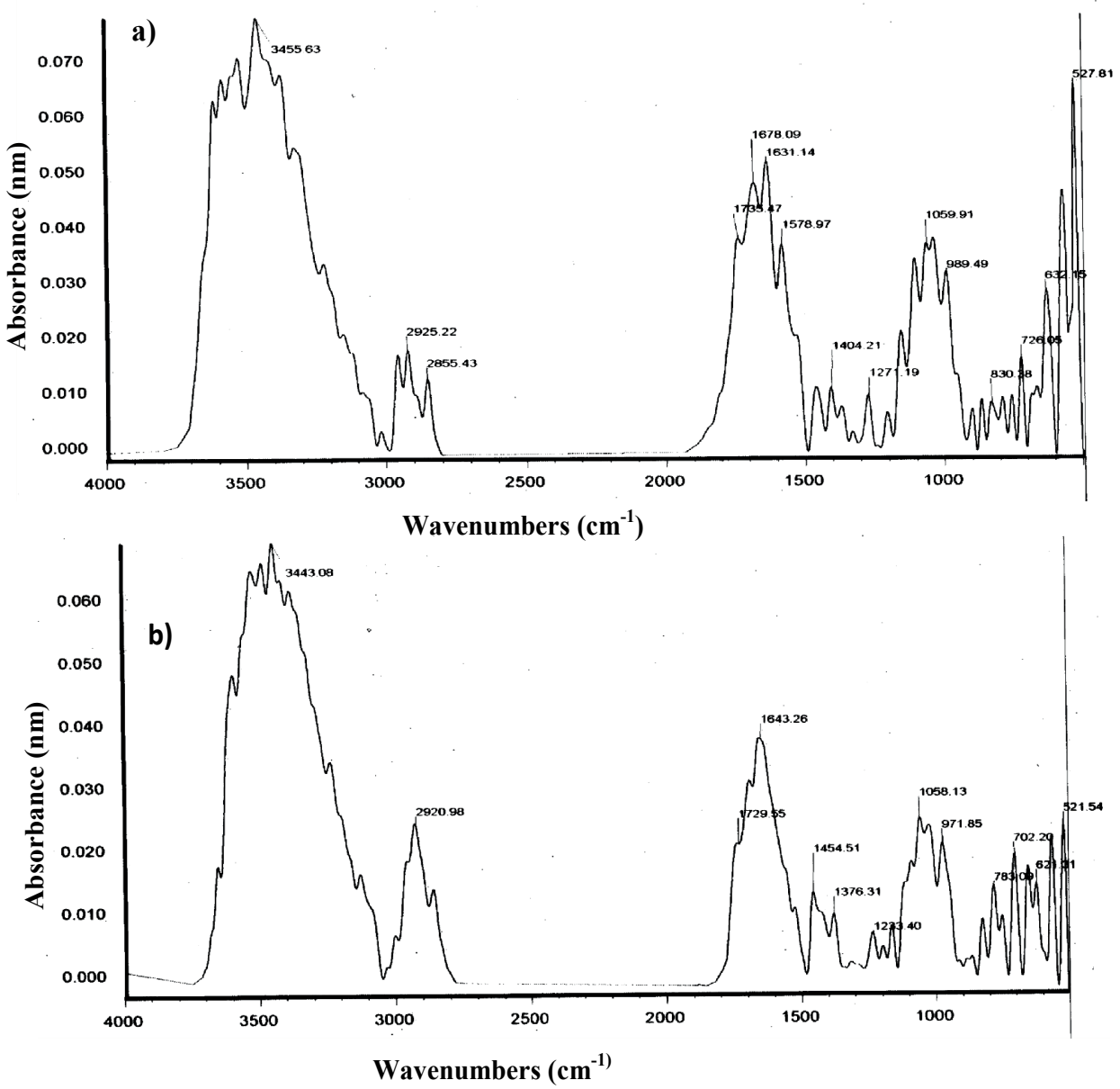

Figure 5: IR spectra of the S-EPS produced in sludge by pure culture of *Cloacibacteriun normanense* grown: a) with crude glycerol and b) without crude glycerol addition.

without glycerol fortification of sludge as well as those available in the literature.

In general, flocculation activity is due to the interaction between the cations and the EPS functional groups and the particles to be flocculated. Several researchers [23] have performed experiments that support the DLVO theory, as London force, for the mechanism of bioflocculation. London forces are present between all chemical groups. The source of this type of interaction is the spontaneous formation of transient dipoles due to fluctuations in the electron distribution within the molecule. This temporary dipole polarises the molecule and thus creating dipolar attraction forces. It represents the main cohesive force between hydrocarbon chains.

The carboxyl functional group (–COOH) prefers to lose the H+ ion in to the solution and the $–COO^-$ ion can be formed. These results were confirmed by Li et al. [22]. They proposed that a large number of carboxyl groups of EPS can also serve as binding sites for divalent cations (Ca^{2+}). When bridged by cations, the negatively charged EPS combines the flocs together. This enables EPS to serve a key role in the flocculation of activated sludge. The 1678 cm^{-1} band appeared in the IR spectrum of EPS produced in the presence of crude glycerol, whereas it was absent in the IR spectrum of EPS without glycerol (Figure 5a and 5b). This band represents the carboxyl group, which could bind to cations and form flocs by bridging mechanism. In relation to the carboxyl group, the EPS produced by *Pseudoalteromonas* sp. SM991 [22] exhibited low FA of 37.7% compared to 93.4% FA of EPS produced in this study with crude glycerol supplementation. The low activity can be related to a relatively smaller strength (15.4%) of the band at 1678

Wave number (cm^{-1})	Absorbance (nm)							Vibration type	Functional type
	EPS with glycerol (present study)	**EPS without glycerol** (present study)	**EPS by *Pseudoaltermonas* SM9913** (Li et al. [22])	**EPS by *Oceanobacillis iheyensis* BK6** (Kavita et al. [20])	**EPS by *Klebsiella pneumoniae*** (Nie et al. [24])	***EPS by Cobetia* sp. and *Bacillus* sp.** (Ugbenyen et al. [25])	**EPS by *Bacillus megaterium* TF10** (Yuan et al. [26])		
3200-3420	0.05	0.065	0.3	0.4	0.5	0.6	0.3	Stretching vibration of OH	OH into polymeric compounds
2930-2935	0.025	0.02	0.3	0.3	0.4	0.5	0.2	Asymmetric stretching vibration of CH_2	Proteins (peptidic bond)
2850-2865	0.015	-		-	-	-	-	Symmetric stretching vibration of CH_2	Proteins (peptidic bond)
1678	0.05	-		-	-	-	-	Stretching vibration of C = O and C-N (Amide I)	Proteins (peptidic bond)
1630-1660	0.06	0.04	0.8	0.3	0.4	0.3	0.3	Stretching vibration of C = O and C-N (Amide I)	Proteins (peptidic bond)
1550-1580	0.04	-	-	-	-	-	0.02	Stretching vibration of C-N and deformation vibration of N-H (Amide II)	Proteins (peptidic bond)
1450-1460	-	0.01	-	-	-	0.6		Deformation vibration of CH_2	phenols
1400-1410		-	0.7	0.3	0.2	-	0.2	Stretching vibration of C = O / Deformation vibration of OH	Carboxylates
1235-1245	0.015	0.01	-	-	0.3	-	0.09	Deformation vibration of CH_2/ Stretching vibration OH	Alcohols and phenols
1130-1160	0.04	0.04	-	0.3	-	-	-	Stretching vibration C-O-C	phenols
1060-1100	0.03	0.03	0.01	-	0.4	0.6	0.4	Stretching vibration of OH	polysaccharides
< 1000	0.06	0.06	-	0.2	-	0.2	0.02	Several bands visible	Phosphorus or sulphur functional group

Table 3: Main functional group observed from IR spectra of broth EPS (B-EPS) with and without crude glycerol fortification

cm^{-1} in EPS produced by Li et al. than 40% strength in EPS obtained in the present study (with crude glycerol).

The bands (1678, 1630-1660, and 1550-1580) are characteristic of C-N stretching and deformation vibration of N-H (Amide II). Hydrogen bonds may contribute to the active reaction between water molecules and amide group (N-H), which is present frequently in protein moiety of EPS (with crude glycerol). The researchers also suggested that higher content of protein moiety in EPS, as indicated by the presence of peptide bond in the IR spectrum of EPS obtained with supplementation of crude glycerol (corresponding to wave numbers 1678, 1630-1660, and 1550-1580 in Table 3 and Figure 5a), could bring more negatively charged amino groups, thus strengthening electrostatic interaction with cations. This plays an important role in flocculation. Further, the large band of Amide II (wave number 1550-1580, Table 3) is present in the EPS (produced with glycerol) and absent in the EPS produced without glycerol. This implies a high concentration of protein moiety in EPS with glycerol, which leads to a high flocculation activity. Furthermore, the EPS obtained in the present study revealed better flocculation comparing to those produced by *Klebsiella pneumoniae* strain NY1 (85.3%) or the consortium of *Cobetia* sp. and *Bacillus* sp. MAYA (90.2%) due to the absence of the amide band (in the latter), where the bioflocculant structure was a polysaccharide (Table 3) [24,25].

The symmetric vibration of CH_2 group was observed in the EPS obtained in the present study (with glycerol case, corresponding to wavenumber 2850-2865, Table 3). This type of band was absent in the other IR spectrum of EPS obtained by other studies [22,24-26] as indicated in Table 3. This band could offer the covalent C = C bond, which is more effective in playing an important role in the aggregation of flocs compared to any other type of main interactions (such as hydrogen bond, London force…) [26].

EPS properties

Effect of temperature on EPS viscosity and FA: The high temperature resistance of the polymers or EPS is important for two reasons: Firstly, the EPS will be produced as powder product using atomiser, which is prepared by spray drying at high temperature (more than 80°C). Secondly, there are many other applications of EPS (chemical,

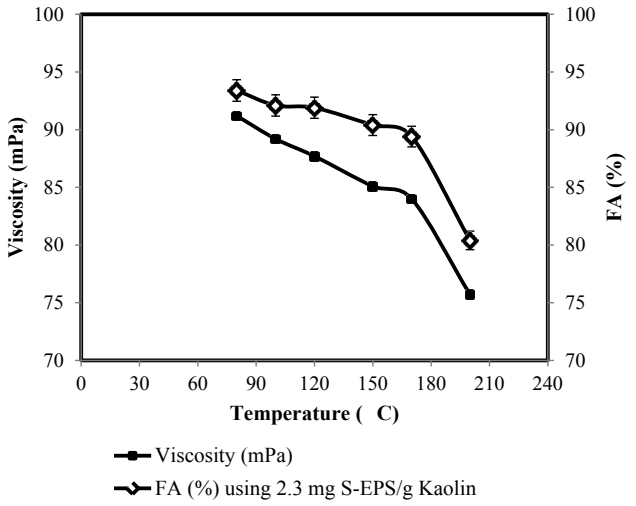

Figure 6: Variation of viscosity and floculation activity of S-EPS at ddifferent temperatures.

food and medical industries) where high temperature resisting polymers are used [27,28]. For food and medical requirement where high purity of EPS is required, the pure EPS product could be produced by growing high EPS yielding pure strain (*Cloacibactérium normanense* NK6) in a synthetic medium.

In the present study, the stability of S-EPS produced by *Cloacibactérium normanense* (NK6) was investigated by exposing the EPS for 10 minutes at different temperatures (from 80 to 200°C) and then measuring their viscosity. The S-EPS of the sample collected at 72 h of fermentation with glycerol addition to sludge and possessing highest EPS concentration was used. Figure 6 presents the impact of temperature on the flocculation activity and viscosity of the EPS, which revealed that the S-EPS from *Cloacibacterium normanense* strain exhibited a good stability at high temperature. The viscosity starts to decrease slowly from 80°C until 150°C and then decreased rapidly at 200°C. The flocculation activity decreased in a similar way as the viscosity (Figure 6). The decrease in viscosity could be attributed to the polymer degradation due to the cleavage of glycosidic bonds within the polysaccharide structure [9].

Past studies have discussed the degradation of polymers in aqueous and organic solutions, which was accelerated by strong acids, certain oxidizing agents, ultraviolet light and temperature [29]. They observed various reaction mechanisms and revealed that redox reactions involving free radicals were probably the cause of polymer degradation and concomitant viscosity losses, which could affect their ability to flocculate.

Enzymatic study of S-EPS: Protein and carbohydrate are the main components of EPS. Many previous reports proposed that protein was more important than carbohydrate to floc formation and demonstrated that activated sludge deflocculated after incubation with a proteolytic enzyme [30,31]. In order to better understand the role of physicochemical properties, as well as the role of protein and carbohydrate in bioflocculation, enzymes were used in this study to degrade biopolymers of S-EPS produced by *Cloacibacterium normanense*. Viscosity, surface charge and turbidity index were measured at different incubation time. Proteinase K was used to degrade extracellular proteins moiety and cellulase was used to degrade extracellular polysaccharide moiety of the EPS. As shown in Figure 7,

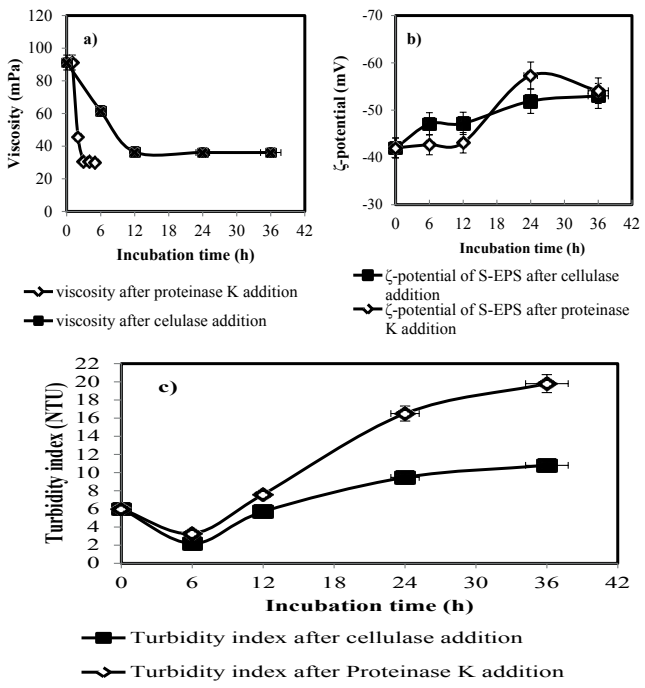

Figure 7: Change of (a) Viscosity, (b) ζ-potential and (c) Turbidity index of B-EPS after addition of cellulase or Proteinase K to the EPS solution.

the viscosity decreased after the addition of both enzymes. Treatment of EPS with cellulase caused the viscosity decrease by 60% (decreased from 91.2 mPa to 36.4 mPa) of the original value after 12 h; after that the viscosity remained stable.

Proteinase K treatment caused a rapid viscosity drop by 67% (decreased from 91.2 mPa to 30 mPa) in three hours. This clearly demonstrated that protein in S-EPS is more important to viscosity drop than carbohydrate. With the degradation of the carbohydrate and protein in S-EPS, the cell surface charge decreased (Figure 7b). The ζ-potential values decreased from −41 mV to around −57.3 mV and −47.1 mV after adding cellulose and Proteinase K, respectively. Increased negative surface charge would cause deterioration in flocculation activity of the EPS [32].

Six hours after the Proteinase K treatment (Figure 7c), turbidity index was measured by adding EPS to kaolin solution (2.3 mg S-EPS/g Kaolin). During the first six hrs, the turbidity decreased and then increased rapidly reaching 19.8 NTU after 36 hours. There was no sign of turbidity recovery with an increase in incubation time. Cellulase addition gave almost the same trend. Turbidity decreased, followed by a relatively slow increase (compared with proteinase K results) with incubation time. These findings suggested that hydrolysis of carbohydrate and protein moieties in S-EPS decreased the viscosity and increased the surface charge, which caused deflocculation. Thus, these results established that both protein and carbohydrate moiety of the EPS play an important role in floc formation. Moreover, the increase in turbidity (or deterioration of FA) was higher and rapid in case of proteinase treatment of EPS, which establishes major role of the protein moiety of EPS in determining flocculation activity.

Figure 8 demonstrates SEM photos presenting the state of bacterial

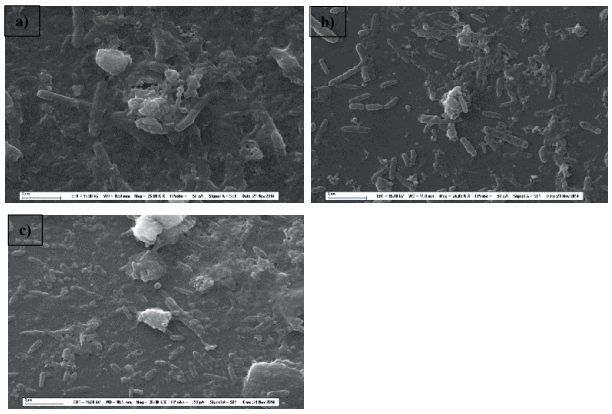

Figure 8: Scanning electron micrograph of *Cloacibacterim normanense:*(a) Control or without enzyme treatment; (b) 6 hrs after treated with Cellulase; (c) 6 hrs after treated with Proteinase K.

aggregate before and after enzyme treatment. Bacterial flocs before enzymes digestion were more visible than those treated with enzymes for six hours (Figure 8a-8c). This suggested that the enzymes destroyed the EPS structure and the cells were dispersed.

Conclusion

This research investigated the carbon and nitrogen content influence on EPS production, the chemical characterisation of EPS produced and their degree of stability as bioflocculant. The following conclusions could be drawn from the foregoing research:

- 17.5 g/L EPS was produced by *Cloacibacterium normannese.*
- LC/MS/MS results analysis demonstrated that the EPS obtained had higher galactose contents (67 mol%) and lower content of glucose (13 mol%), xylose (9 mol%), sucrose (8 mol%) and lactose (3 mol%).
- IR spectra revealed distinct functional groups in EPS produced with or without fortification of glycerol.
- The degree of EPS stability decreased under high temperature (150°C).
- Deflocculation was induced due to digestion of protein and carbohydrate moieties of the EPS by proteinase K and cellulase enzymes, respectively.
- Detailed structural analysis using SEM photos revealed that both protein and carbohydrate moieties of EPS are important factors to impact surface properties and bioflocculation ability of the EPS.

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