Concomitant Extracellular Polymeric Substance and Lipid Production by Cloacibacterium Normanense via Fermentation of Sterilized Activated Sludge Fortified with Crude Glycerol

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Abstract: Exopolymeric substances (EPS) are produced by numerous microorganisms as their defense mechanism, in response to hostile conditions. Recently EPS have been reported to be used as a potential bioflocculant for settling and dewatering of solid waste generated in wastewater treatment plants. In our previous study (Klai et al., 2015) we reported the production of EPS by newly isolated strain Cloacibacterium normanense NK6. In this study, we report the simultaneous production of EPS and lipid by C. normanense. The EPS production and lipid accumulation by the bacterium was enhanced by varying the carbon-nitrogen (C/N) ratio in sterilized activated sludge media supplemented with crude glycerol as additional carbon source. Sterilized activated sludge was inoculated with 5% (v/v) of C. normanense. At low C/N ratio, lipid content was found to be low, but EPS concentration was significantly high while at high C/N ratio, an increase in lipid % (g/g cell dry weight) and a decrease in EPS concentration was observed. The best results were obtained using C/N 25. C. normanense accumulated a lipid content of 27.6% (w/w) and 22 g/L of EPS in 72 h. Therefore, *Cloacibacterium sp.* appears to be a suitable candidate for fermentation processes to produce EPS and lipid from renewable resources.

Keywords: Wastewater solid sludge, crude glycerol, Cloacibacterium normanense, EPS, microbial lipid.

Abbreviations

B-EPS	Broth EPS
C	Carbon
C-EPS	Capsular EPS
CFU	Colony forming units
C.G	crude glycerol
CHNS	Carbon Hydrogen Nitrogen Sulfur – Analysis instrument
C. normanense	*Cloacibacterium normanense*
C/N:	Carbon /Nitrogen
CUQ	Communauté Urbaine du Québec, waste water treatment plant
EPS	Extracellular polymeric substances
FA	Flocculation activity
FAMEs	Fatty acid methyl esters
FFA	Free fatty acid
g CDW/g Glyc	cell dry weight per gram of glycerol
GC-FID	Gas Chromatography-Flame Ionization Detector
Glyc	Glycerol
MeOH	methanol
mg EPS/g of kaolin	Mass of EPS per gram of kaolin
N	nitrogen
pep	peptone
S-EPS	Slime EPS

SS	Suspended solids
T. oleaginosus	Trichosporon oleaginosus
TC	Total Carbon
TN	Total Nitrogen
TSB	Tryptic soy broth

1 Introduction

Extracellular polymeric substances (EPS) are complex mixture of high molecular weight biopolymers like polysaccharide, protein, nucleic acids, and humic substances. They can protect the cells from the external environment and provide energy and carbon source when the substrate is in short supply (Wingender et al., 1999). It has been reported that EPS produced by bacteria can play a significant role in controlling the flocculation and floc properties, including settling and dewatering (More et al., 2012, Klai et al., 2015). EPS composition is very complex, and its composition and content vary with many factors such as the microbial strain, cultivation time, carbon source, and growth state (Sheng and Yu, 2006). For many microorganisms, EPS synthesis is stimulated by the abundance of carbon availability and limitation of other nutrients, such as nitrogen, oxygen or phosphorus (Kumar et al., 2007). Low C: N ratios (10-30) are commonly required for attaining high yield of EPS synthesis (Rosalam et al., 2006) whereas high C: N ratio is reportedly required for lipid synthesis. At high C: N nitrogen availability is limited, and thus it is unfavorable for biomass growth. Hence, a compromise in the C: N ratios are required to promote cell growth while favoring EPS synthesis. Nevertheless, for most EPS-synthesizing microorganisms, the highest polymer productivities are usually achieved for low nitrogen concentrations (Farhadi et al., 2012).

Accordantly, lipid accumulation is receiving considerable attention because of their potential as a source of feedstock for biofuel production. Numerous studies have successfully transferred fourth generation microbial oil to biodiesel (Meng et al. 2009; Gao et al. 2014). Lipids serve as storage materials in some lipid accumulating yeasts and bacteria. According to many studies (Zhang et al., 2014), under nitrogen limiting and carbon-excess conditions, organisms tend to store lipids. Therefore, the limitation of nitrogen source could be the key to the simultaneous production of EPS and lipid.

Lipid and EPS are produced respectively inside and outside the cell by microorganisms. They can serve as energy and carbon source when microorganisms are under starvation conditions. Thus, it is essential to understand the relationship between the intracellular and extracellular product by the same microorganism.

C. normanense NK6 has been reported to produce high EPS concentration, using activated sludge and crude glycerol as the sole carbon source (Nouha et al., 2016). The crude glycerol feeding was used for EPS synthesis. High and reproducible EPS productivity (0.28 g/L.h) were achieved with initial glycerol concentrations of 20 g/L. The EPS produced is composed of galactose (67 mol %) and was poor in other sugars like glucose (13 mol %), xylose (9 mol %), sucrose (8 mol %) and lactose (3 mol %). Pyruvyl, succinyl, and acetyl substituent groups accounted for 0.4–3.3 wt% of the EPS dry weight.

The impact of nitrogen concentration on the balance between growth and polymer synthesis by C. normanense was not previously assessed nor their influence on the polymer's production or flocculation properties. EPS can allow microorganisms to live at a high-cell-density in stable mixed population communities and significantly influence their topographical properties, which are of considerable importance in governing bacterial flocculation and adhesion (Sheng and Yu, 2006). Thus, elucidation of the relationship between EPS, Lipid, and biomass growth would benefit for the lipid accumulation by C. normanense. Moreover, information about the lipid production by C. normanense is never studied. Therefore, in this study, the simultaneous production of EPS and lipid by this strain was investigated at optimal C/N ratio. The results obtained in this study will be helpful in understanding the biosynthesis of the extracellular and intracellular product by C. normanense.

2 Material and Methods

2.1 Preparation of C. Normanense Culture

For inoculum preparation, Cloacibacterium sp. (NK6, accession number KF675202) was inoculated into an Erlenmeyer flask (150 mL) containing 50 mL TSB (Tropic soy broth) media and it was incubated for 48 h at 30 °C, 180 rpm to logarithmic phase. Further, 5 ml of the TSB pre-grown broth was transferred to 100 mL of sterilized sludge (25 g/L suspended solids or SS) media and incubated for 24 h.

2.2 Culture Conditions for Flask Fermentation

For fermentation, 5% (v/v) pre-culture was added to the flask (500 mL) containing 150 mL sterilized sludge. The pH was adjusted to 7. Crude glycerol and peptone were used as carbon and nitrogen source respectively for EPS production and Lipid accumulation. Different initial C/N ratios (18, 25, 30, 50 and 100) were adjusted by changing the nitrogen quantity to determine the optimum C/N (carbon/nitrogen) ratio for high EPS production and lipid accumulation yield. The initial concentration of 40 g/L of crude glycerol was used as supplemented carbon source in sterilized sludge. This concentration was optimal for high lipid accumulation yield (Zhang et al., 2014). The crude glycerol utilized contains other compounds such as soap, methanol, and free fatty acid (FFA) (Table 1). These components can act as a carbon source for growth and product formation. The composition of crude glycerol was determined according to Hu et al. (2012). The peptone content was determined by calculating the total carbon as given in Table 2. The nitrogen requirements were calculated as per following equations:

Total Carbon (TC) = C-Glycerol + C-Soap + C-Methanol all in grams of C

Total Nitrogen required (TN) = TC/ (C: N ratio required)

Peptone Required (Pep) = TN/ N content in peptone

The nitrogen content of peptone is assumed to be 10% (w/w). The carbon and nitrogen content available from sludge is assumed negligible as compared to the supplemented carbon source.

2.3 Estimation of Bacterial Growth and Analytical Methods

During the fermentation, samples were withdrawn at each 12 h interval to measure the cell concentration and biomass concentration. Cell concentration was measured as CFU (colony formation unit) per mL employing the standard serial dilution technique on an agar plate. 25 mL of culture was centrifuged at 6000 g at 4°C for 20 min for biomass determination. After C-EPS extraction, as described by Nouha et al. (2016), the residual biomass pellet was dried at 105°C to get the dry weight of the biomass. Glycerol concentration, methanol and soap content in the cell-free supernatant were determined according to Hu et al. (2012). Total carbon and nitrogen in the samples were measured by the CHNS analyzer (Shimadzu VCPH).

Table 1. Crude glycerol (C.G.) stock composition, initial media composition, and Fermented broth composition after 72 hours.

Component	Stock C.G. (% w/w)	0th hour (g/L)	72 hours (g/L)
Glycerol	13.4	40.0	14.45
Methanol	4.6	13.6	0.35
soap	23.5	70	41.2
Peptone	NA	28.6	0
Biomass	NA	ND	29.28
Density	0.845		ND
pH	6.8		8.7
Ash	1.5		ND

NA: Not applicable, ND: Not determined

Table 2. Flask fermentation conditions to maintain C/N ratio.

	C/N 18			C/N 25			C/N 30			C/N 50			C/N 100		
	Soap	Glyc	MeOH	Soap	Glyc	MeOH	Soap	Glyc	MeOH	Soap	Glyc	MeOH	Soap	Glyc	MeOH
Concentration of C.G. components in 44.77 mL (g/L)	10.5	6.0	2.0	10.5	6.0	2.0	10.5	6.0	2.0	10.5	6.0	2.0	10.5	6.0	2.0
Gram of Carbon	7.8	2.3	0.8	7.8	2.3	0.8	7.8	2.3	0.8	7.8	2.3	0.8	7.8	2.3	0.8
Total grams of Carbon		10.9			10.9		10.9				10.9			10.9	
Peptone N content (g/150 mL)	6.04			4.3			3.6			2.2			1.1		
Initial concentration of C.G. components (g/L)	70.0	40.0	13.6	70.0	40.0	13.6	70.0	40.0	13.6	70.0	40.0	13.6	70.0	40.0	13.6

MeOH: Methanol, Glyc: Glycerol

2.4 EPS and Lipid Extraction

EPS was extracted by centrifugation method. Samples were centrifuged at 9000 g, 4 °C for 20 min to obtain supernatant (containing slime EPS, termed as S-EPS). The pellets were re-suspended to the initial volume and then heated at 60 °C for 20 min to obtain capsular EPS (C-EPS) (Nouha et al., 2015). Broth EPS (B-EPS) contains both S-EPS and C-EPS. The supernatant obtained after centrifugation (crude S-EPS) was mixed with two volumes of chilled ethanol (95% v/v (volume/volume)) and incubated at -20°C to get the pellets S-EPS. After precipitation, the pellets were separated by centrifugation. The pellet was further dried at 60°C to determine S-EPS dry weight.

Lipid was extracted using standard method (Folch et al., 1957, Vicente et al., 2009). The washed pellet (2.6 ± 0.1g) was mixed with 15 mL solvents of chloroform-methanol (2:1 v/v), and 5 mL zirconium beads (0.7mm diameter) were added to the mix. The cells were disrupted by a bead beater for 12 h (BioSpec Products, Bartlesville, OK, USA). The mixture was separated into three different layers by 10 min centrifugation. The residual biomass was present in the bottom layer; intermediate phase was lipid dissolved in chloroform, and top phase was methanol and water. The chloroform containing lipid was taken out and transferred into a pre-weighed glass tube (W1). The rest of the solution (containing cell debris, methanol, and water) was again supplemented with a 15mL chloroform-methanol (1:1 v/v) solvent and kept for agitation. After 12 hours of agitation, the solution was filtered using Whatman filter paper. The filtrate was mixed with a previously extracted solution (chloroform solution containing lipid), and the mix was allowed to settle for phase separation. The bottom phase containing lipid in chloroform (the other phase was water and methanol) was collected and subjected to overhead nitrogen sparging until total chloroform was evaporated completely. The remaining samples were further dried at 60°C in a hot air oven until the sample reached a constant weight (W2). The weight, thus recorded, represents the amount of lipid (or lipid-like cellular components, which were soluble in chloroform) in that sample. The dried extract is trans-esterified and analyzed by GC (gas chromatography) in the form of FAMEs (Fatty acid methyl esters) to quantify the actual lipid. The chloroform extract (crude lipid) content of the biomass was calculated as crude lipid content= (W2-W1)/dry biomass weight × 100%, where W1 expresses the pre-weighed glass tube, and W2 denotes the oven dried microbial crude lipid in a pre-weighed glass tube.

2.5 EPS Flocculation Activity

The sludge flocculation activity of EPS was determined by modified jar test method (Klai et al., 2015). Municipal wastewater (5 g/L) sludge sample was used for the test. 600 mg/L of Alum (Al2 (SO4)3) was added to the sludge sample. In this study, instead of Ca2+, alum was used as a coagulant to compare the results with the actual treatment condition in wastewater treatment plant. Further, Ca2+ as a coagulant is not sufficiently active with municipal sludge as concluded by our preliminary studies (data not shown). In fact, the alum concentration (600 mg/L) is used to simulate the treatment plant scenario (Communauté Urbaine du Québec, CUQ, Quebec) where the same concentration is used. The pH of sludge was changed to 7 after it has been optimized. S-EPS produced by C. normanense was selected because of high flocculation activity observed in kaolin solution. The supernatant of the fermented broth

without ethanol precipitation was used as S-EPS. The samples were mixed at 120 rpm for first 5 min after addition of 600 mg/L of alum (which enables the neutralization) followed by addition of EPS (different concentration) with mixing at 50 rpm for 25 min allowing floc formation (Li and Yang, 2007; Nouha et al., 2015).

The well-mixed sludge samples (1L, 5g/L SS) were transferred into beakers and 600 mg/L of Al2 (SO4)3) was added along with a different dosage of S-EPS. Another two beakers served as positive control (addition of Zetag in place of S-EPS) and negative control (without the addition of Alum, EPS or Zetag). Zetag is the cationic chemical polymer, used by municipal wastewater treatment plant (CUQ, Québec). It was utilized to compare its flocculation efficiency with that of EPS. After addition of S-EPS, each mixed sludge sample was then transferred into 1L graduated measuring cylinder for turbidity measurement (Nouha et al., 2015). After 30 minutes, the supernatant of each sample was then collected to measure the turbidity using turbidimeter (Micro 100 turbidimeter, Scientific Inc.).

2.6 Lipid Transesterification

For transesterification, lipid was dissolved in hexane (25 mL/ gram lipid), then mixed with methanol (in 6:1 (mol/mol) ratio or 0.4 mL methanol per gram lipid) containing 1% w/w (1 g NaOH/ 100g oil) sodium hydroxide. Afterward, the mixture was heated to 55 °C for 12 h. After the reaction, NaCl solution was added (50 mL of 5% w/v NaCl solution per gram lipid) and the solution was allowed to stand for 15 min. FAMEs were extracted with hexane (top) phase. The bottom phase was again mixed with hexane (25 mL per gram lipid, to remove non-recovered FAMEs) and FAMEs were then mixed with the fraction separated earlier. The FAMEs in hexane were washed with sodium bicarbonate solution (10 mL of 2% w/v solution per gram lipid), and the top hexane layer was then dried at 60 °C in an oven (Halim et al., 2011). The FAMEs were dissolved in hexane (0.01 g lipid/10 mL hexane) and analyzed using a Gas Chromatography linked with FID (Flame Ionization Detector) (GC-FID) (Perkin Elmer, Clarus 500). 1µl of the trans-esterified sample was injected with an automated sample injector, and the sample analysis was performed with Agilent Chem Station module software from Agilent Technologies. The calibration curve was prepared using a standard mix of 37 FAMEs (47885-U, 37 Component FAME Mix; Supelco, Bellefonte, PA, USA). 1, 3- Dichlorobenzene was used as an internal standard with a concentration of 50 ppm.

2.7 Kinetic Parameters Calculation

The maximum specific growth rate (µmax, h−1) was determined using equation 1:

$$\ln\left(\frac{x}{x0}\right) = \mu_{\max} \times t \tag{1}$$

Here X_0 (g/L) is the initial cell concentration. The yields of biomass (Yx/s, gCDW (dry cell weight)/gGlyc) and EPS (Yp/s, gEPS/gGlyc; gLipid /gGlyc) on glycerol (Glyc.) as substrate were determined using Eq. (2) and (3),

$$\Delta X = final\ \ biomss\left(Xf\right) - Initial\ \ Biomass\left(Xi\right)$$

$$\Delta S = final\ \ Substrate\left(Sf\right) - Initial\ \ Substrate\left(Si\right)$$

$$\Delta P = final\ \ Product\left(Pf\right) - Initial\ \ Product\left(Pi\right)$$

$$\mathrm{Y}x/s = \frac{\Delta x}{\Delta s} \tag{2}$$

$$\mathrm{Y}p/s = \frac{\Delta p}{\Delta s} \tag{3}$$

where $\Delta x(\mathrm{g/L})$ and $\Delta p(\mathrm{g/L})$ are the biomass and EPS produced, respectively, and $\Delta s(\mathrm{g/L})$ is the substrate up taken during the same cultivation time. The EPS volumetric productivity (rp, g/L h) was determined as follows:

$$r_p = \frac{\Delta p}{\Delta t} \tag{4}$$

where p corresponds to the concentration of the product, EPS (g/L) or lipid (g/L) at time t (h). The EPS specific productivity (qp, gEPS/gCDW.h) was determined using Equation 5.

$$q_p = \frac{rp}{\Delta x} \tag{5}$$

3 Results and Discussion

3.1 Effect of C/N Ratio

In the present study, five set of experiments were tested. The initial glycerol concentration used was fixed at 40 g/L with a variation of nitrogen concentration (peptone) to adjust the desired C/N ratio (18, 25, 30, 50 and 100). As presented in Table 1 (crude glycerol composition) the methanol concentration is very high, which could affect the C. normanense growth. The bacteria could tolerate 6% (v/v), therefore the concentration of methanol in the broth was maintained < 6% (v/v) or 4.6% (w/v).

Table 2 presents the culture condition such as the carbon source and nitrogen source used in flask fermentation to maintain the desired C/N ratio. In the five flask fermentation experiments, C/N ratio was set by changing only nitrogen concentration while the total carbon concentration was fixed to 10.9 g/L in the media.

EPS production seems to be affected by variable C/N ratios (Table 3, Figure 1). EPS production level decreases when the C/N ratio increases. For C/N 18, the EPS concentration was 22.5 g/L compared to 4.9 g/L in case of C/N ratio 100. In contrast, lipid accumulation steadily increased at higher C/N ratio until C/N ratio reached 100; concomitant with biomass concentration (Figure 1). The maximum lipid concentration was measured at C/N ratio of 100 with 14.1 g/L.

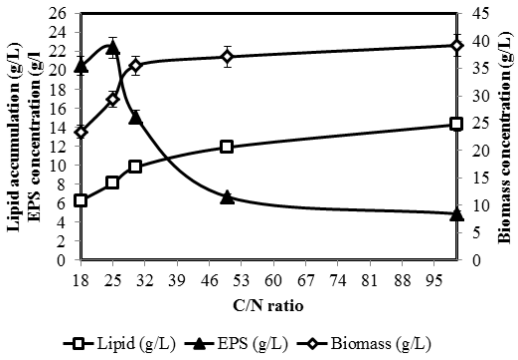

Figure 1. Effect of different C/N ratios on biomass, lipid accumulation and exopolysaccharide production in shake flask cultures of C. normanense.

Table 3. Kinetic parameters obtained in flask fermentation experiments using fixed glycerol concentration and different nitrogen concentrations.

C/N ratio	Initial concentration (g/L)		Cultivation time (h)	CDW (g/L)	μmax h-1	EPS (g/L)	Lipid (g/L)	YX/S (g CDW/g Glyc)	Yp/s		rp (g/L.h)	
	Glycerol	Peptone							g EPS/g Glyc	g Lipid/g Glyc	EPS	Lipid
18	40	40.3	72	23.4	0.076	22.5	5.8	0.6	0.96	0.26	0.31	0.08
25	40	28.6	72	29.4	0.070	22.4	7.8	0.7	0.76	0.27	0.31	0.11
30	40	23.9	72	35.5	0.064	15.1	9.8	0.8	0.42	0.28	0.21	0.14
50	40	14.5	72	37.1	0.046	6.7	11.9	0.9	0.18	0.32	0.09	0.16
100	40	7.25	72	39.2	0.035	4.9	14.1	1.0	0.12	0.36	0.07	0.20

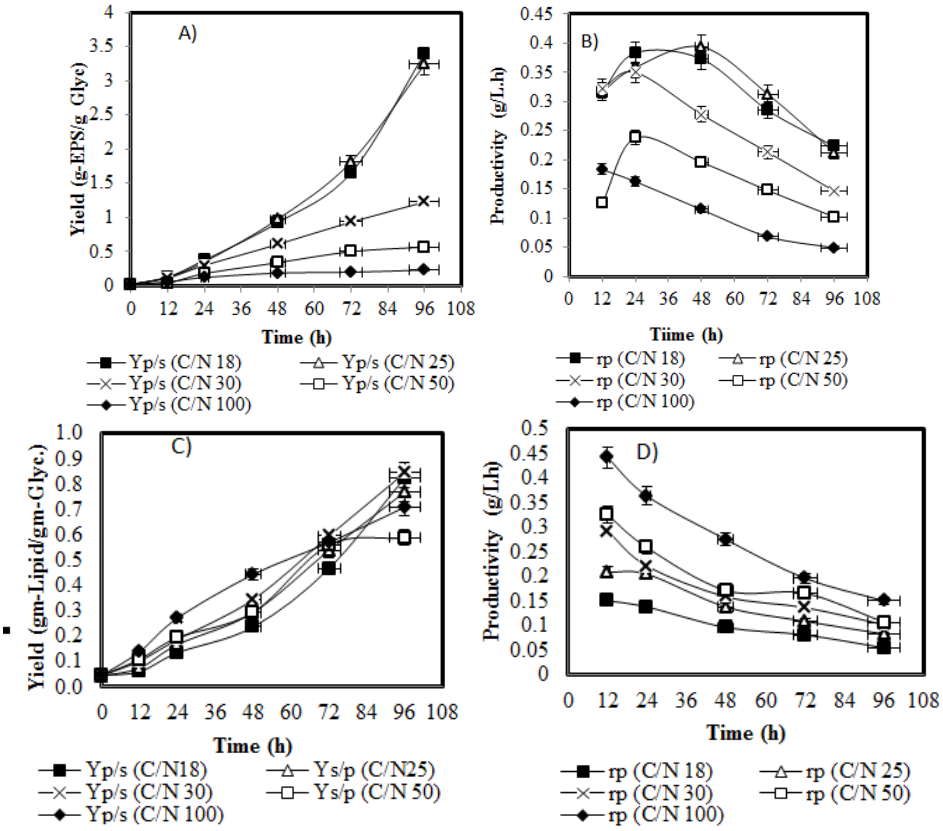

Figure 2. A) Yield of EPS per unit gram of glycerol consumed during the fermentation B) EPS formation rate (g/L.h) during the fermentation C) Yield of lipid per unit gram of glycerol consumed during the fermentation D)Lipid formation rate (g/L.h) during the fermentation.

The results obtained show that increasing the initial nitrogen concentration (decrease in C/N ratio) led to higher EPS synthesis. The highest EPS productivity gained was at peptone concentration of 40.3 g peptone/L for C/N ratio 18 at 72h, (Figure 2 A, B). The nitrogen source is used mainly for cell growth and enzyme production for catabolic and anabolic pathways. Several authors (Vincente Garcia et al., 2004; Liu et al., 2010) reported that EPS-producing bacteria need a specific C/N ratio to promote EPS synthesis. In the case of balanced optimum nitrogen availability, the nitrogen is efficiently used to generate necessary cell machinery (enzymes) and simultaneously process the excess carbon for extracellular polymers. The EPS concentration was mainly affected by a decrease in peptone concentration in the event of limiting nitrogen source condition during which the strain starts to store lipid (Gao et al. 2014; Zhang et al., 2014) (Figure 1).

The reduction of EPS polymer production at high C/N ratios might have caused by the low nitrogen concentration used for cultivation. The limitation of nitrogen disrupts the normal anabolism of the cell thereby the tendency of the cell is to accumulate the available carbon in the form of intracellular products (lipid) rather than to metabolize it in usual ways. The extracellular polysaccharides are primary functional products of the microbe to serve as protection matrix of the microbes, and they are produced in normal balanced growth situation with a little excess of carbon. In high C/N ratio, the scarcity of nitrogen disables this standard functionality of the cell. On the other hand with high C/N ratio when growth metabolism is disrupted the carbon availability in the cell increases for lipid accumulation and microbes tend to preserve it in the form of polymeric substances like lipid.

The yields and productivities for EPS are listed in Table 3, when the C/N ratio was 18- 30, the yield of EPS (YP/S, gEPS/gGlyc) increases with the fermentation time; however, the productivity of EPS rp (g/L.h) decreases drastically (Figure 2A, B). According to C/N ratio, the productivity of EPS is higher (0.31 g/L.h) at lower C/N 18 comparing to 0.07 g/L.h at C/N 100 at 72 h. These results were in

agreement with Torres et al. (2012). These authors reported that when the nitrogen feed concentration was increased from 4.5 g/L to 45 g/L, the productivity and yield of EPS increased from 2.04 (g-EPS/L.d) and 0.17(g-EPS/g-Glycerol) to 5.6(g-EPS/L.d) and 0.23(g-EPS/g-Glycerol), respectively.

Table 4. Comparison of critical parameters between T. oleaginosus and C. normanense.

Parameter	Nouha et al. 2016	Xiaolei et al. 2015
Organisms	Cloacibacterium normanense	Trichosporon oleaginosus
Carbon	Sludge (municipal), Crude glycerol	Sludge (municipal), Crude glycerol
Nitrogen	Peptone	Peptone
C/N	25	30
Yield biomass	0.7 g-CDW/g-glycerol	NA
Yield Lipid	0.305 g-Lipid/g-glycerol	0.26 g-Lipid/g-glycerol
Yield EPS	0.87 g-Lipid/g-glycerol	NA
Lipid Productivity	0.108 g/L/h	0.113 g/L/h
EPS Productivity	0.31 g/L/h	NA
Lipid content	0.27 g-Lipid/g-CDW	0.4 g-Lipid/g-CDW
Time of fermentation	72 hours	84 hours
Max lipid concentration	7.8 g/L	16.4 g/L (4.43 g/L from sludge)
Max EPS concentration	22.4 g/L (extracellular)	NA
Glycerol consumed	40g/L to 14.45 g/L	40 g/L to 1.26 g/L

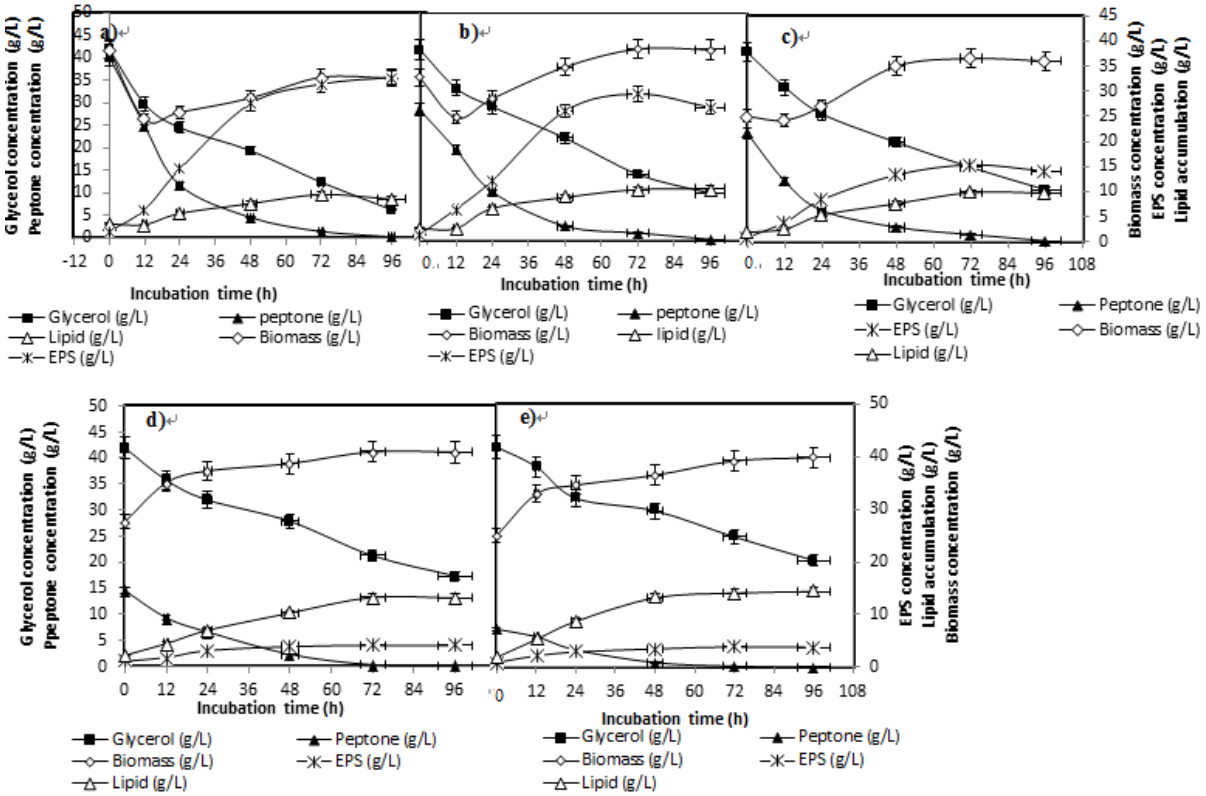

Figure 3. Cultivation profile of C. normanense with initial glycerol and different nitrogen concentrations a) Run 1C/N 18 b)Run 2 C/N 25 c) Run 3 C/N 30 d) Run 4 C/N 50 e) Run 5 C/N 100.

As apparent in Figure 2C-D, the yield of lipid on glycerol (Yp/s) increases with fermentation time while contrarily the productivity (rp) decreases. According to C/N ratio, the productivity of lipid is higher at C/N 100 (0.2 g/L.h) than at C/N 18 (0.08 g/L.h) at 72 h. These results obtained were better than that achieved by Angerbaur et al. (2008). They observed lipid productivity of 0.0045g/L.h at C/N 100 while growing Lipomyces starkeyi in sewage sludge. The results obtained in this study are also in accordance with the results achieved by cultivating Trichosporon oleaginosus in sludge fortified with glycerol and peptone and obtained a lipid productivity of 0.11 g/L.h in our laboratory (unpublished data, Table 4).

As shown in Figure 3(a, b and c), there is an insignificant lipid accumulation during first 24 hours of fermentation by C. normanense in the case of C/N 18, 25 and 30, but significant EPS production of 9.2, 9.2 and 8.4 g-EPS/L in C/N 18,25 and 30 respectively were produced. A decrease of biomass concentration was observed in the first 24 hours of fermentation; this can be accounted by the fact that the insoluble fraction of sludge is metabolized by the bacteria to produce EPS, which is extracellular thus the production of EPS is not reflected in the SS of the samples taken during first 24 hours. Simultaneously, a large amount of glycerol was consumed during the first 24 hours, indicating that the carbon source was probably being directed towards the synthesis of EPS, thus making the carbon substrate unavailable for significant lipid accumulation rendering low lipid yield (Figure 2C).

Typically, lipid accumulation by Cloacibacterium sp. occurs mostly under nitrogen limiting conditions in the case of C/N 50 and 100. At higher C/N ratio, the biomass concentration increased at the beginning of fermentation indicating the lipid accumulation by this strain (Figure 3d and e).

Angerbauer et al. (2008) and Mulder et al. (1962) observed that under nitrogen limiting conditions and the presence of an excess carbon source, organisms start to store lipids. Therefore, a high carbon to nitrogen (C/N) ratio, around 100, is a basic requirement for the accumulation of lipids. However, some nitrogen is also furnished by sludge, which is not possible to exactly determine.

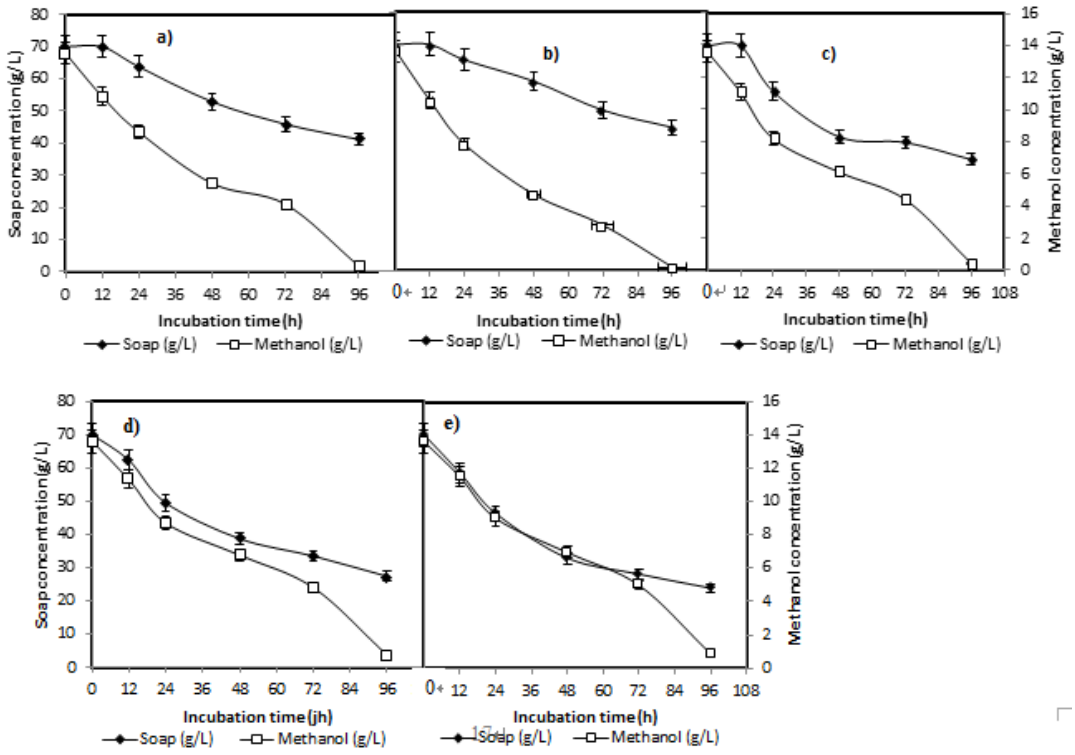

Figure 4. Methanol and soap consumption during the fermentation a) Run1 C/N 18 b)Run 2 C/N 25 c) Run 3 C/N 30 d) Run 4 C/N 50 e) Run 5 C/N 100.

C. normanense used glycerol, methanol, and soap as a carbon source for EPS production and lipid storage. Figure 3 and Figure 4a, b, and c indicate that the glycerol, methanol, and soap were consumed

during the fermentation. Increasing the C/N ratio, the rate of consumption of glycerol decreases (Figure 3); however, the global rate (after 72 hours) of soap consumption increases particularly at C/N 50 and 100, which could explain the low utilization of glycerol by C. normanense. The soap is metabolized favorably for lipid accumulation (at C/N $\geqslant$ 30) as it goes through direct assimilation by the microbe for forming lipid molecules (Figure 4). The soap was significantly consumed by the strain, as presented in Figure 5, in the case of C/N ratios 30, 50 and 100 as compared to C/N ratio 18 and 25 where the soap concentration decreases only after 12 hours. Cloacibacterium sp. starts to consume soap at the beginning of fermentation (0h) in the case of C/N 50 and 100, which led to lipid production via the ex-novo mechanism of lipid production. Soap formation in the trans-esterification reaction depends on the catalyst used in the reaction. When alkali such as NaOH or KOH is used as a catalyst for transesterification, the oleic acid present in lipid fraction is converted to sodium or potassium oleate during FAMEs generation. Soap is reported to enhance the lipid accumulation by microbes. A significant consumption of sodium oleate (representative soap) was observed by biomass for lipid production as reported by (Xu et al., 2012). Increasing the concentration of sodium oleate from 0.5 g/L to 2 g/L increased the lipid content of biomass from 34% (w/w) to 59% (w/w).

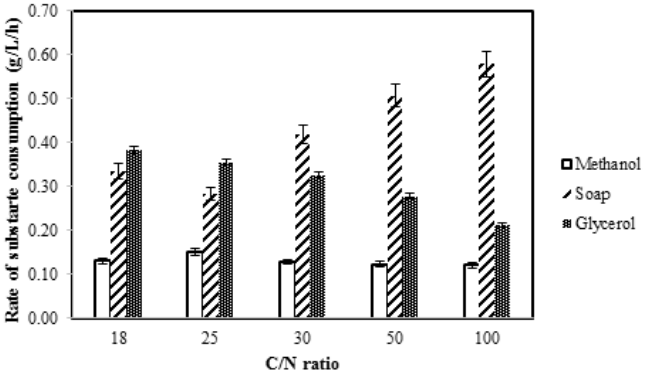

Figure 5. Rate of substrate consumption for glycerol, methanol and soap after 72 hours of fermentation for various C/ N ratio.

These results conclude that the optimum C/N ratio for the accumulation of lipid by C. normanense was C/N ratio 100, which leads to a lipid content 36.5% (w/w) equivalent to a concentration of 13.4 g/L while C/N 18 was optimum for EPS production by this strain.

Lipid production by Lipomyces starkeyi was evaluated in the media containing sewage sludge, in which sludge pre-treatment with ultrasound resulted in lipid accumulation values greater than 1 g/L with lipid production 35.6 % (w/w) (Angerbauer et al. 2008). In fact the lipid percentage obtained in present study (36.5 % w/w) is almost the same as obtained by Angerbauer et al. study; however, the productivity of lipid (0.11 g/L/h, Table 3) at 72 h of fermentation was 5 times higher than obtained (0.02 g/l/h) at 220 h by Angerbauer et al. (2008). Further, in the present study, two co-products (EPS and lipid) are simultaneously generated, which is not the case in previous studies. In addition, not all bacteria accumulate large quantities of fatty acids. Bacterial strains Nocardioides sp., Sphingomonas sp., Oceanicaulis alexandrii sp. and Micrococcus sp. isolated from marine living cells, contain a fatty acid (FA) content from 0.3 to 4% (w/w) dry weight (Zabeti et al., 2010).

3.2 Properties of EPS Produced By C. Normanense as Bioflocculant

The results of sludge flocculation activity (FA) using S-EPS are presented in Figure 6A. The turbidity decreased with the addition of 13 mg S-EPS/g sludge. For the conditions tested, the exopolysaccharide shows a higher flocculating capacity (72 $\pm$ 1.3 %) compared to 66 $\pm$1.5 % using 10 mg Zetag /g sludge. Increasing the S-EPS concentration beyond 13 mg S-EPS/gm sludge had no significant improvement in supernatant turbidity; therefore, 13 mg S-EPS/g sludge was selected as optimum concentration. In

practice at wastewater treatment plant (CUQ), Zetag was used as chemical flocculants to concentrate the secondary sludge. After addition and mixing of the flocculants at a concentration of 0.5 mg-Zetag/ g-SS, the settling time required in the actual plant is 42 minutes (personal communication) while in our study it was shown that after addition of optimum concentration of S-EPS (13 mg-S-EPS/g-SS), only 20 minutes settling is required to achieve same separation efficiency. The difference in the dosage value can be because of the reactor dimension, agitation profiles, and rheology that affect the binding of the flocculant with the sludge particles. In order to compare Zetag with EPS, a pilot scale comparison study is required to estimate the real potential of S-EPS. Figure 6B presents the beakers used in the laboratory test for the sludge flocculation experiments. It is apparent from the figure that the turbidity of S-EPS is comparable to that of Zetag. Further, it is evident from the picture that the clarity of the supernatant for 13 mg S-EPS/ g SS is similar to that of 18 mg S-EPS/ g SS.

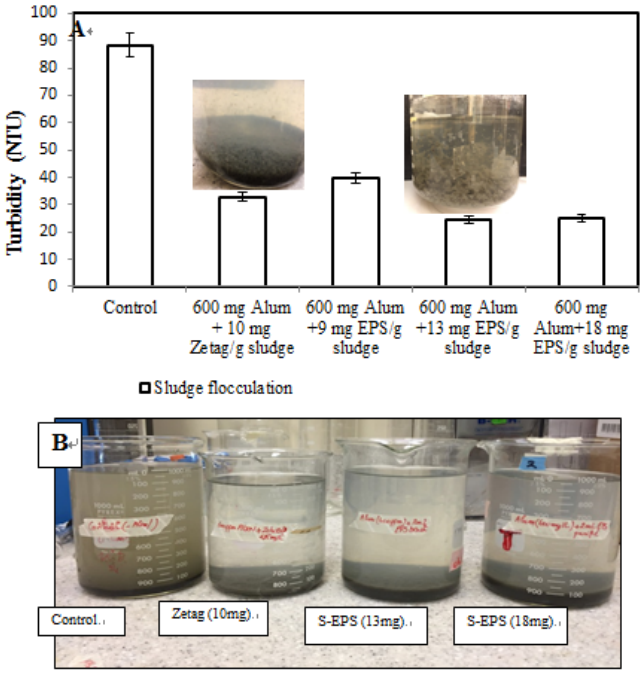

Figure 6. (A)Comparative study of sludge flocculation using Zetag and EPS produced by C. normanense (B) Jar test pictures of secondary municipal sludge flocculation by control (-), Zetag and S-EPS.

The efficiency of EPS produced by C. normanense as bioflocculant for secondary sludge settling was studied by Nouha et al. (2015). The high flocculation activity of EPS was due to structure–function relationships. The EPS structure and chemical composition rich in proteins and carbohydrates could explain the highest flocculation activity obtained by EPS-producing C. normanense.

3.3 Profiles of the Accumulated Lipids

The efficiency of lipid conversion to FAMEs is 36.5 % (w/w) of total lipids in the case of C/N 100 at 72 h (Table 5). Lipid mainly contains C16, C18, and C20 fatty acid methyl ester, which is an indication that the lipids produced are suitable for the production of biodiesel with excellent burning characteristics (Fan et al., 2013). This can be accounted by the fact that the esterification reaction was catalyzed by the base (NaOH) and the lipid obtained in this study contains a high content of free fatty acids (FFAs, 11% w/v data not shown) and principally all the FFA was saponified to soap, which may yield low transesterification. The FFA can be contributed by the residual crude glycerol or from thermal hydrolysis of sludge which was used as carbon source. Further, the lipid extraction was conducted by chloroform. Thus the crude chloroform extract may include cellular bodies which are soluble in chloroform-like FFA, triacylglycerides, PHA, etc.

Table 5. Lipid profile of C. normanense grown in activated sludge in different culture conditions.

C/N ratio/fermentation time	The efficiency of lipid conversion to FAMEs (% w/w)	Fatty acids	Relative amount of total fatty acid (% w/w)
25/ 48 h	24.8	Palmitic (C16:0)	14.85
		Palmitoleate (C16:1n7	1.27
		Stearic (C18:0)	4.19
		Elaidic (C18:1n9t)	38.60
		Linoleic (C18:2n6c)	36.69
		Linolenate (C18:3n3)	4.40
25/ 72 h	27.6	Palmitic (C16:0)	15.87
		Palmitoleate (C16:1n7	2.07
		Stearic (C18:0)	4.59
		Elaidic (C18:1n9t)	42.45
		Linoleic (C18:2n6c)	35.02
100/ 48h	23.9	Palmitic (C16:0)	18.44
		Palmitoleate (C16:1n7	2.90
		Stearic (C18:0)	5.65
		Elaidic (C18:1n9t)	50.57
		Linoleic (C18:2n6c)	21.09
		Eicosenoate (C20:1n9)	0.65
100/ 72 h	36.5	Palmitic (C16:0)	18.53
		Palmitoleate (C16:1n7	2.90
		Stearic (C18:0)	5.49
		Elaidic (C18:1n9t)	53.49
		Linoleic (C18:2n6c)	19.59

Table 5 presents the lipid composition of C. normanense (NK6) for C/N 25 and 100 at 48 and 72 h of fermentation. We choose these two ratios due to the optimum concentration of lipid accumulated (C/N 100) and the significant results of both lipid and EPS concentration in case of C/N 25.

The composition of lipid considerably depends on the C/N ratio of the medium. There are several studies which showed that the lipid profile changed with fermentation time (Papanikolaou et al., 2004). Elaidic (C18:1n9t) was found to be the major component in all cases. C. normanense produced the following unsaturated fatty acids in high quantity: Linoleic (C18:2n6c) and Palmitic (C15:0) (Table 5), Palmitoleate (C16:1n7), Linoleic acid (C18:2n6c), stearic (C 18:0) and Eicosenoate (C20:1n9) in minor quantities. Elaidic acid fraction increased from 38.6% (C/N 25) at the 48th hour to 42.45 % (C/N 25) at 72nd hour. Similarly, for C/N 100, the Elaidic acid fraction increases from 50.5% w/w (C/N 100) at 48th h of fermentation to 53.49% w/w (C/N100) at 72nd hour. In both cases (C/N 25 and 100), Elaidic acid increases with fermentation time (Table 5). For other fatty acids (stearic, palmitic, linoleic and palmitoleate), the FFA fractions remained constant with fermentation time.

Patil et al. (2010) investigated the effect of the C/N ratio on lipid production and on fatty acid composition of lipids in L. starkeyi cultivated under different operating conditions. In his study lipid, content in cells increased from 19 to 30%. As the C/N ratio rose from 20 to 61, the fatty acid composition of the lipid was also increasing for C16:0 (38.7 to 44.8%), C18:1 (40.7 to 50.2%) and small amounts of C16:1 (5.9 and 14.5%) were also detected. Hu and Gao, (2003) found that the fatty acid composition was influenced by the concentration of the nitrogen. The nitrogen content present in the medium significantly altered the saturated and unsaturated fatty acid compositions. Similar observations were reported by Huang et al. (2010).

4 Conclusion

This study reports for the first time, the simultaneous production of EPS and lipid. The C/N ratio is a crucial parameter for the EPS production and the accumulation of lipids by C. normanense. The study elucidates the importance of C/N ratio; the highest amounts of lipids and EPS concentration were

obtained in activated sludge using C/N 25. Further, a comparison of S-EPS produced by the microbe was compared with commercial flocculants, and it was found that S-EPS has comparable flocculating efficiency in lab experiments. The fermentation produces simultaneously two products, microbial oil (7.8 g/L) and EPS (22.4 g/L) using C/N ratio 25. The lipids obtained can be used for biodiesel production while EPS can be used to replace chemical flocculants after pilot scale testing.

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