Research Paper

Microbial lipid and biodiesel production from municipal sludge fortified with crude glycerol medium using pH-based fed-batch strategy

The corrections made in this section will be reviewed and approved by a journal production editor.

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Editor: ~~<V. Victor>~~V. Victor

~~ABSTRACT~~Abstract

To achieve a good performance in fed-batch fermentation, the feeding strategy is essential and important. In this study, pH-based (pH 6.0) fed-batch fermentation has been conducted on municipal sludge fortified with crude glycerol medium using *Y. lipolytica* SKY7 (YL). No additional nitrogen source and trace elements had to be added as secondary municipal sludge had sufficient nitrogen and trace elements to support growth of YL. At 72 h of fed-batch fermentation, high biomass (59.67 g/L) and lipid concentration (31.44 g/L) was obtained. The biodiesel produced using sludge cultivated YL had similar fatty acid ester profile as that of vegetable oil. It was revealed from energy balance that biodiesel production using pH-based fed-batch strategy was energetically favorable and has unit production cost of 0.67 $/L B10.

Keywords: pH-based fermentation; Municipal sludge; Crude glycerol; Microbial ~~Lipid~~lipid; Biodiesel

1 Introduction

Lipids (or microbial oils), which are accumulated in oleaginous microorganisms have shown great potential to replace plant seed oils for biodiesel production [1–3]. Great interest has been paid to biodiesel production from lipids [4]. Hence, renewable carbon sources have been explored for lipid production [5–7]. Crude glycerol is a by-product of biodiesel industry and cheaply available [8]. Wastewater sludge is also a low-cost substrate and is rich in the necessary nutrients, required for the growth of microorganisms [8]. Thus, wastewater sludge and crude glycerol can be applied as raw materials for oleaginous microorganisms' cultivation to produce lipids [8,9].

For lipid production, fed-batch fermentation gives better performance than batch fermentation [10]. Fed-batch fermentation is a biotechnological process during which substrate is fed into the reactor multiple times. To achieve enhanced productivity in fed-batch fermentation, the feeding strategy is essential and important. The feeding strategy can be based on substrate consumption, time, DO (dissolved oxygen) or pH. A high biomass (65.63 g/L) and lipid (35.79 g/L) concentration have been achieved from the pH-based (pH 5.0) fed-batch fermentation using *T. oleaginosus* cultivated on crude glycerol medium [11]. However, in the reported study, trace elements and nitrogen source were

supplied at 0 h to assist cell growth. To reduce the cost of fermentation, commercial trace elements and nitrogen source can be replaced with wastewater sludge as it is rich in nutrients [8].

The pH-based fed-batch fermentation can be widely applied with a minor adjustment in the control precision and pH of control agent. In the present study, pH-based fed-batch fermentation has been conducted on sludge fortified with crude glycerol medium using oleaginous yeast *Y. lipolytica* SKY7 (YL). Due to decrease in pH during fermentation, crude glycerol (basic pH) was used as feed and pH-control agent. Energy balance for biodiesel productioned fromusing microbial lipid obtained withfrom pH-based fed-batch fermentation has been conducted for the industrial feasibility.

2 Materials and ~~Methods~~methods

2.1 Materials

The crude glycerol was obtained from Canadian biodiesel producing company BIOCARDEL. BIOCARDEL crude glycerol was high on sodium concentration as sodium hydroxide was used as catalyst during trans-esterification. The characterization for crude glycerol was performed using protocols given in [10]. The composition of crude glycerol is mentioned in Table 1.

alt-text: Table 1

Table 1

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Characteristics of BIOCARDEL crude glycerol.

Parameter	BIOCARDEL
Density (g/L)	877.00
Glycerol concentration (g/L)	120.00
Soap (g/L)	263.10
Water (g/L)	219.25
Methanol (g/L)	263.10
pH	9.0
Al (mg/L)	1.94
B (mg/ L)	0.37
Ca (mg/ L)	77.18
Co (μg/ L)	–
Cr (mg/ L)	1.40
Cu (mg/ L)	3.03
Fe (mg/ L)	199.00
K (g/L)	0.20
Mg (mg/L)	52.62
Mn (mg/ L)	1.64
Mo (μg/ L)	87.70
Na (g/ L)	12.80
Ni (mg/ L)	0.26
P (g/ L)	0.39

S (g/L)	3.20
Si (mg/ L)	12.54
Sn (mg/ L)	350.00
Sr (µg/ L)	438.50
Ti (µg/ L)	87.70
Zn (mg/ L)	12.72

The sludge was collected from the secondary sedimentation tank of a municipal wastewater treatment plant, Communauté ~~Urbain~~Urbaine de Québec (CUQ) in Québec, Canada. The sludge was first allowed to settle by gravity at ~~4 °C~~ 4 °C for ~~24~~24 h, and then the suspended solids concentration (SS) of the resulting solution was measured. The sludge characterization is mentioned in Table 2. Thereafter, settled was centrifuged at ~~8000~~8000 g for ~~10~~10 min to obtain a concentrated sludge of ~~61.5~~61.5 g/L SS. Concentrated sludge was washed with tap water ~~(30~~(30 L/ kg sludge solids) to remove the heavy metals (Fe, Mg, Mn, Ni, Zn. Cu) sticking to the sludge solids, which could be inhibitory for microbial cell growth [12].

alt-text: Table 2

Table 2

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Characteristics of municipal secondary sludge.

Characteristics	Concentration	Units
Total solids (TS)	29.23	g/L
Total suspended solids (TSS)	21.86	g/L
Volatile solids (VS)	17.35	g/L
Total carbon (TC)	420.46	g/kg
Total nitrogen (TN)	54.05	g/kg
Total phosphorus (TP)	29.08	g/kg
pH	6.45	–

2.2 Micro-organism

Y. lipolytica SKY7 isolated in INRS laboratory was used in this study. *Y. lipolytica* SKY is a wild strain which can accumulate up to 50% lipid (w/w) and the lipids produced by the strain has a close resemblance with vegetable oil and could serve as a feedstock for biodiesel production [13].

2.3 Pre-culture and inoculum preparation

The dormant pure culture of *Y. lipolytica* (4ºC) was revived by cultivating in a pre-culture 1 (or PC1) synthetic media (Yeast extract peptone dextrose broth/ YPD: 20 g/L glucose, 20 g/L peptone and 10 g/L yeast extract) for 24 h in shaking incubator with agitation 180 rpm and temperature of 28ºC. PC1 volume of 6.25% v/v was used to produce pre-culture 2 (PC2). Pre-culture 2 was prepared in a medium containing 20 g/L SS of washed sludge fortified with sterilized crude glycerol (equivalent to 5 g carbon/L). Methanol present in crude glycerol was evaporated during sterilization while soap (in crude glycerol) was converted to free fatty acids (FFA) by adjusting pH to 6.0 before being used in PC2. Before using washed sludge as growth medium, the sludge was fortified with 4 M NaOH to bring pH to 12 and was sterilized at 121 °C~~.~~ for 30 min. After cooling, pH was brought at 6.0 using 4 M H_2SO_4 [12]. PC2 was

cultivated to acclimatize YL in sludge medium. PC2 was grown at 180 rpm and 28ºC for 36 h before being transferred to the production fermenter.

2.4 Fermentation

2.4.1 Operation

Fermentation was carried out in stirred tank fermenter~~s~~ (INFORS AG Fermenter) equipped with accessories and programmable logic control (PLC) system. The inoculum size of 6.25% (v/v) was chosen for *Y. lipolytica* SKY7 based on reported study [14]. During 6–12 h, DO decreases from 95% to 40% and later it was maintained in the range of 25%–40% by manually adjusting agitation rate (400–600 rpm) and air flow rate (1–1.3 L/min). The limitation of DO favors lipid production [15]. The temperature was maintained at 28 °C by circulating water through the fermenter jacket. Dissolved oxygen and pH were continuously monitored by dissolved oxygen probe and pH sensor (Mettler-Toledo, USA), respectively.

2.4.2 Establishment of fermentation system

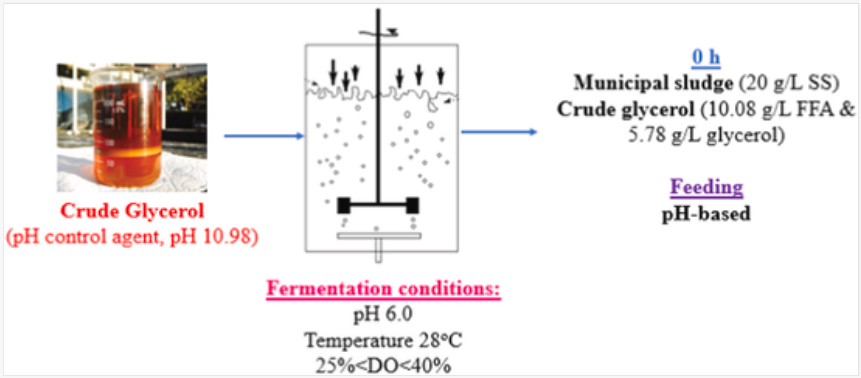
Fermentation was conducted using sludge fortified with crude glycerol. Before using sludge as growth medium, the sludge was pre-treated as mentioned in Section 2.3. The fermentation was started with ~~20~~20 g/L SS of washed sludge and crude glycerol ~~(10.08~~(10.08 g/L FFA with ~~5.78~~5.78 g/L glycerol) at ~~0~~0 h with no addition of trace elements and nitrogen. The selection of ~~20~~20 g/L SS for YL cultivation was based on study conducted by Ram, Tyagi and Drogui [16]. During YL fermentation, pH continuously decreases due to secretion of organic acids in the medium and alkaline pH is required to adjust the pH. Hence, pH adjusted crude glycerol solution (pH 10.98) was used for pH-control. Crude glycerol was sterilized before being used for pH-control and feed, in order to prevent the overdose of methanol (present in crude glycerol). There was no residue of methanol or composition change in crude glycerol after sterilization [10]. Soap (present in crude glycerol) could affect microbial growth by impairing their orientation, decreasing their motility parameters and changing their morphology [17,18]. Soap conversion to readily utilized FFA is governed by following equation:

$$C_{17}H_{35}COO^{-}\ (\text{soap}) + H_2O \leftrightarrow C_{17}H_{35}COOH\ (\text{FFA}) + OH^{-} \quad (1)$$

At acidic pH, the above reaction tends to move towards forward direction leading to formation of FFA. At pH 5.0, 6.0, 7.0 the conversion efficiency of soap to FFA was 98.71%, 87.01% and 0%, respectively [11]. However, at pH 5.0, growth of YL was inhibited. Hence pH 6.0 was chosen for pH-based fed-batch fermentation. Fig. 1 depicts fermentation system for microbial lipid production. Samples were withdrawn after every ~~8~~8 h to determine concentration of biomass, lipid, glycerol and organic acids in the sample~~s~~.

alt-text: Fig. 1

Fig. 1



Fermentation system for microbial lipid production.

2.5 Analytical ~~Techniques~~techniques

Biomass and lipid concentration were determined as reported by Zhang, Chen, Yan, Tyagi, Surampalli and Li [19]. Glycerol was measured according to the method by Bondioli and Della Bella [20]. FFA was measured by titration method as described in [21]. The volume of crude glycerol (containing FFA and glycerol) pumped into the fermenter for pH-control was continuously monitored. The consumption of FFA and glycerol at time t was calculated by below equation:

$$\text{Consumption (g/L)} = \text{con[Instruction: Please add space between 'conc.' and 'in']c·\_in f[Instruction: Please write 'fermentation medium' instead of 'fermenter' here]ermenter at 0 h (g/L)} + \text{conc·a[Instruction: Please add space between 'conc.' and 'added']dded in fermenter due to pumping (g/L)} - \text{concentration in fermentation medium (g/L) at t} \quad (2)$$

Where 'concentration added in fermenter due to pumping' was estimated from the density and composition of crude glycerol.

Organic acids in the supernatant of the samples were analyzed using LC-MS~~-MS~~ (Liquid chromatography-mass spectrometry). Elemental concentration in samples were determined by inductively coupled plasma mass spectroscopy (ICP-MS) after acid digesting the samples (model DRE, Leeman Labs Inc).

2.5.1 Lipid characterization

Lipid obtained was trans-esterified and characterized as per the protocol given in [21].

All samples were analyzed in duplicates.

3 Results and discussion

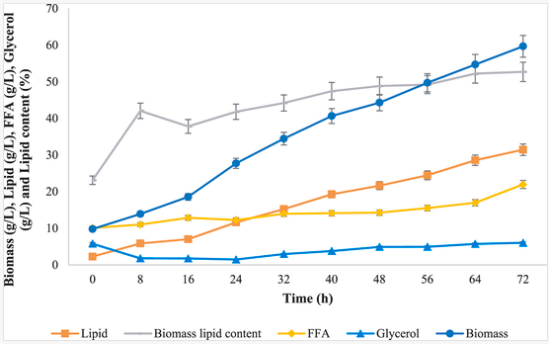
3.1 pH-based fed-batch fermentation

3.1.1 Variation of biomass, lipid and substrate concentration

The variation of biomass, lipid and substrate concentration during pH-based fed-batch fermentation is highlighted in Fig. 2. It can be observed that during initial ~~1~~6 h, decrease in glycerol concentration was observed from ~~5.78~~5.78 g/L to ~~1.76~~1.76 g/L. However, an increase in FFA concentration was observed from ~~10.08~~10.08 g/L to ~~12.85~~12.85 g/L. This indicates that during initial ~~1~~6 h, the strain had~~s~~ preference for glycerol over FFA. It is also evident from Fig. 3 that during initial ~~1~~6 h, glycerol consumption was higher than FFA consumption. However, after ~~1~~6 h, FFA consumption increased and was higher than glycerol consumption. From Figs. 2 and 3, it can be concluded that it is necessary to provide supplement quantity of FFA and glycerol at ~~0~~0 h. Total glycerol and FFA consumption during ~~72~~72 h fermentation were ~~22.26~~22.26 g/L and ~~40.41~~40.41 g/L respectively. Table 3 indicates chemical elements present in the fermentation medium (supernatant or soluble phase) at the beginning and at the end of fermentation. It can be observed that ~~20~~20 g/L sludge SS provided sufficient trace elements for growth of YL. Moreover, nitrogen in the medium furnished by ~~20~~20 g/L sludge SS was abundantly present in the fermentation medium. Due to which, high biomass concentration ~~(59.67~~(59.67 g/L) was observed at ~~72~~72 h. A decrease in elemental composition was observed in ~~72~~72 h of fermentation (Table 3) except Na. The decrease in elemental composition is due to utilization of these elements for cell growth. However, increase in concentration of Na in the medium was~~is~~ due to regular pumping of crude glycerol into the fermenter which contains high Na concentration (Table 1). The fermentation was stopped at ~~72~~72 h. First reason for ending fermentation at ~~72~~72 h was reduced oxygen transfer rate due to high cell concentration and increased viscosity of the medium [22,23]. High oxygen demand and low oxygen transfer rates made the DO maintenance difficult. Agitation reached maximum value of ~~800~~800 rpm for maintaining DO in the range of ~~25–30%~~25–30% but DO was in the range of ~~5–7%.~~5–7%. Second reason was that FFA was not being utilized for cell growth and lipid production. It is also evident from Fig. 3 that during ~~56–72~~56–72 h, FFA consumption increased by ~~2.79~~2.79 g/L only. High Na concentration ~~(6.25~~(6.25 g/L, Table 3) in the medium might be the reason for slow utilization of substrate because Na concentration between ~~6–8.2~~6~~g/L~~ and 8.2 g/L slows down the cellular activities of YL [21]. After ~~56~~56 h, the pH-based fed-batch strategy can be shifted to regular pH control with NH_4OH until excess carbon source is depleted. At ~~72~~72 h,~~h, 31.44~~ 31.44 g/L lipid was obtained with 52.69% biomass lipid content. The biomass concentration ~~(59.67~~(59.67 g/L) obtained at ~~72~~72 h was~~is~~ not real biomass concentration resulting from microbial growth. The municipal secondary sludge also contains ~~45–50%~~45–50% non-biodegradable material [12].

alt-text: Fig. 2

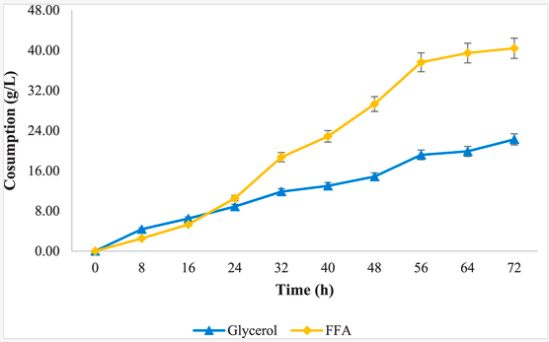
Fig. 2



Variation in biomass, lipid and substrate concentration during pH-based fed-batch fermentation.

alt-text: Fig. 3

Fig. 3



Glycerol and FFA consumption during pH-based fed-batch fermentation.

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Table 3

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Chemical elements present in sludge medium (supernatant or soluble phase) at beginning and end of fermentation.

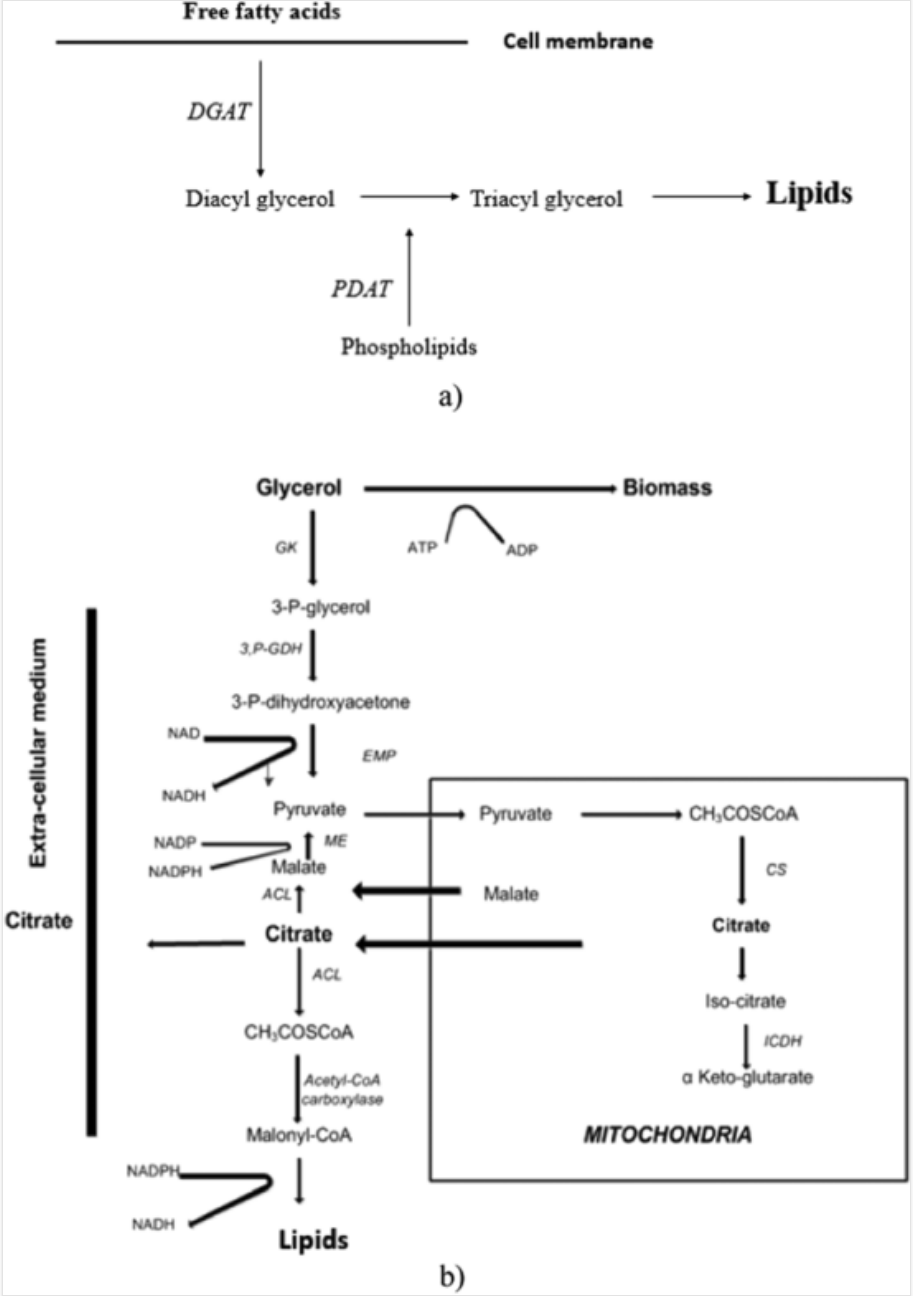
Metals	Requirement	~~0~~ 0 h	~~72~~ 72 h
Al (mg/L)	–	91.8	68.82
Ba (mg/L)	–	1.98	–
Ca (mg/L)	–	172.2	65.2
Cr (mg/L)	–	0.72	0.49
Cu (mg/L)	–	1.94	0.26
Fe (mg/L)	1	117	106.4
K (mg/L)	774	735	477
Mg (mg/L)	19.7	41.6	28.42
Mn (mg/L)	0.25	1.38	0.68
Na (mg/L)	154	3840	6251

Ni (mg/L)	–	0.28	0.086
P (mg/L)	207	474	137.5
S (mg/L)	26	2160	1545
Zn (mg/L)	0.23	3.86	1.34
N (mg/L)	–	1020	720

It can be observed that biomass lipid content was high (37.8%–42.06%), even during initial 24 h. BIOCARDEL crude glycerol contains FFA (hydrophobic substrate) besides glycerol. Lipid production from hydrophobic substrates by oleaginous microbes follow ex-novo lipid accumulation [24]. Hydrophobic substrates get transferred inside the microbial cell by means of active transport. In order to transfer these hydrophobic substrates from the medium to inside the cell, *Y. lipolytica* can generate protrusions (structural changes on the surfaces of cells), which increases the contact surface between the yeast and substrates [25]. Inside the microbial cell, fatty acids are then either used for cell propagation or transformed to new fatty acid profiles [24]. During ex novo lipid accumulation process, cellular lipids are accumulated and, at the same time, lipid-free biomass is generated. Therefore, ex-novo lipid accumulation is a perfectly growth-associated anabolic activity and is independent of nitrogen concentration in the medium. Utilization of glycerol (in crude glycerol) for lipid accumulation occurs through de-novo lipid accumulation process which is nitrogen dependent phenomenon. Fig. 4 depicts use of FFA and glycerol for lipid accumulation.

alt-text: Fig. 4

Fig. 4



a) Conversion of FFA to lipid using ex-novo lipid accumulation b) Conversion of glycerol to lipid using de-novo lipid accumulation (*DGAT* is DAG acyltransferase, *PDAT* is phospholipid: DAG acyltransferase, *ME* is malic enzyme and *ACL* is ATP citrate lyase).

YL was also cultivated on crude glycerol medium (without sludge) containing ~~2.7~~2.7 g/L KH_2PO_4, ~~0.95~~0.95 g/L Na_2HPO_4, ~~0.2~~0.2 g/L $MgSO_4$~~.7H~~•$7H_2O$ and ~~12.5~~12.5 g/L peptone (nitrogen source) where ~~38.1~~38.1 g/L biomass and ~~14.8~~14.8 g/L lipid was produced in ~~96~~96 h of fed-batch fermentation [21]. Higher biomass ~~(59.67~~(59.67 g/L) and

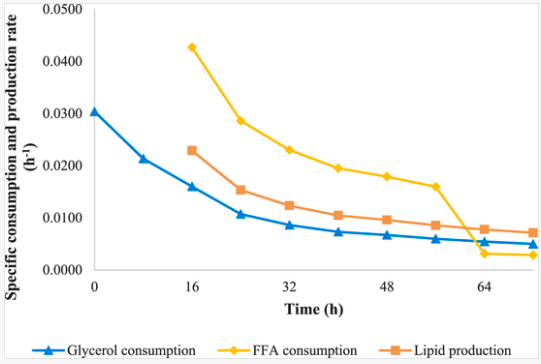
lipid concentration ~~(31.44~~(31.44 g/L) in~~on~~ municipal sludge fortified with crude glycerol medium (this study) indicates that sludge can replace the commercial trace elements and nitrogen source for cell growth. Moreover, sludge also provides additional carbon source for biomass and lipid accumulation. The contribution of sludge (used during fermentation) in biodiesel production is discussed in Section 3.2.2.

3.1.2 Kinetic parameters

Specific consumption rates (consumption rate divided by biomass concentration at particular time) for glycerol and FFA are highlighted in Fig. 5. Based on the specific FFA consumption rate and specific lipid production rate, the conversion rate of FFA to lipid was calculated to be 53.59% during initial ~~56~~56 h. However, glycerol (in crude glycerol) and sludge were~~are~~ also utilized for lipid production. It is evident from Fig. 5 that specific consumption rates for both FFA and glycerol were~~are~~ decreasing with time. Following could~~an~~ be reasons for the decrease in specific consumption rates: a) limitation of nutrients in the medium as ~~20~~20 g/L SS sludge was supplied at ~~0~~0 h only and not at later stages. To resolve this problem, ~~I~~intermittent sludge feeding can be combined with pH-based fed batch strategy to enhance the lipid production in the medium. b) substrate inhibition due to continuous addition of crude glycerol feed in the medium. However, the decreasing trend of specific rates is generally observed during fermentation [14] as biomass is continuously increasing with time.

alt-text: Fig. 5

Fig. 5



Specific consumption and production rates during pH-based fed-batch fermentation.

For estimating lipid yield with respect to sludge, lipid yield with respect to crude glycerol was important. The value of lipid yield from crude glycerol (without sludge) was taken from previous experiments conducted on YL for lipid production using crude glycerol medium [21]. Using lipid yield from crude glycerol ~~(0.077~~(0.077 g lipid /g crude glycerol), lipid contribution from crude glycerol in sludge medium was calculated by multiplying lipid yield with~~from~~ crude glycerol consumed (including FFA and glycerol) in ~~72~~72 h of fermentation. It was revealed that for production of 1-tonne lipid, 9.39-tonnes crude glycerol and 0.64-tonne of sludge solids were~~are~~ required. When crude glycerol was~~is~~ used as sole carbon source for lipid production, 12.97-tonnes glycerol would~~ill~~ be utilized to produce 1-tonne lipid. However, the crude glycerol requirement to produce 1-tonne lipid decreases when crude glycerol is fortified with municipal sludge.

3.1.3 Organic acids production during fermentation

Y. lipolytica is well known industrial scale citric acid producer. However, citric acid production in this study was found be < 1 mg/L (undetectable). Generally, absence or minimal concentration of nitrogen in the medium favors the citric acid production [26]. However, abundant nitrogen was present in the medium throughout fermentation (Table 3). Hence, undetectable citric acid was obtained in the medium. Besides, citric acid, other organic acids produced during fermentation were pyruvic acid (500 mg/L), alpha-ketoglutaric acid (2 g/L), malic acid (70 mg/l) and glutamic acid (140 mg/L). Although the produced organic acids all have commercial applications [27], the concentration of these acids in the medium is very low to recover them.

3.2 Biodiesel production from microbial lipid

3.2.1 Lipid ~~Characterization~~characterization

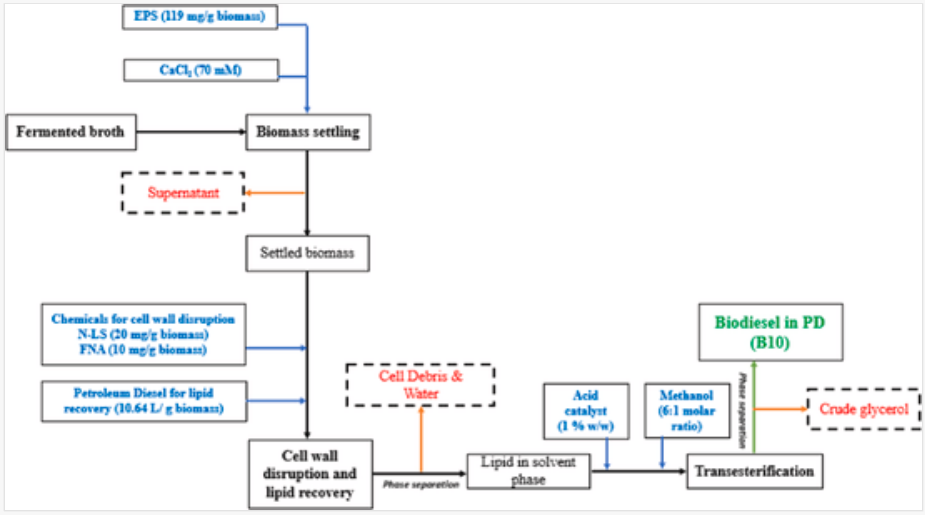
Characterization of lipid obtained from sludge cultivated YL revealed 17.65% oleic acid (C18:1), 32.36% linoleic acid (C18:2) and 30.22% Eicosenoic acid (C20:1) were~~are~~ produced in sludge fortified with crude glycerol medium. Oleic acid (C18:1) and linoleic acid (C18:2) are the major components in the case of SKY7 as reported in other studies [13,26]. The degree of saturation for biodiesel obtained from sludge medium~~a~~ was ~~between~~ 13.75%, which was in the range of biodiesel obtained from vegetable oil ~~(10–15%)~~(10–15%) [19]. Since, 80.23% of the extracted lipids were~~are~~ long chain unsaturated fatty acids, it cannot be a promising fuel in places of high temperature due to poor oxidative stability. However, oxidative stability of biodiesel can be improved by adding synthetic anti-oxidants like tertiary butylhydroquinone (TBHQ), pyrogallol (PY) and propyl gallate (PG) [8].

3.2.2 Energy ~~Balance~~balance for 1 tonne biodiesel production using pH-based fed-batch strategy

For performing energy balance for biodiesel production, downstream processing needs to be considered. The downstream processing for biodiesel production was chosen from Yellapu, Klai, Kaur, Tyagi and Surampalli [28]. This study was chosen as biomass settling was performed using bio-flocculant, extracellular polymeric substances (EPS) instead of expensive centrifugation. Slime EPS was produced by EPS producing bacterial strain BS4 (isolated in INRS laboratory) [29] using wastewater sludge as a raw material. EPS is reported to be cheaper than other bio-flocculants like coconut shell, chitosan, sodium alginate, amylopectin and okra-based but expensive than chemical flocculants [30]. However, EPS was used in this study as it is biodegradable and its monomer units are harmless to the ecosystem. Synthetic flocculants have drawbacks of being less biodegradable and producing carcinogenic monomers during degradation [30]. Positively charged calcium ions (from coagulant) interact with negatively charged yeast cell wall. EPS contains a relatively large number of hydroxyl ~~(–OH)~~(–OH) and carboxyl ~~(–COO–)~~(–COO–) groups. The presence of these groups is favorable for the flocculation process to provide the surface charges, which helps in further binding with suspended particles and causes floc formation [31]. Lipid was extracted from wet biomass using surfactant n-lauryl sarcosine (N-LS) and lipid was recovered using petroleum diesel (PD). This downstream process has already been proven energetically and economically favorable over conventional solvent based lipid extraction from dried biomass [32,33]. Biomass settling~~, which~~ was performed using ~~70~~70 mM calcium chloride followed by doses of EPS ~~(119~~(119 mg/g biomass) [28]. The biomass concentration (near ~~60~~60 g/L biomass) obtained during fermentation decide the concentration of EPS and $CaCl_2$. The settled sludge biomass ~~(177~~(177 g/L) was treated sequentially by free-nitrous acid ~~(10~~(10 mg FNA/ g biomass) and n~~N~~-lauryl sarcosine ~~(20 mg N-LS/ g~~ (20 mg N-LS/g biomass) [34]. Petroleum-diesel was used as solvent ~~(10.64~~(10.64 mL PD/g lipid) for lipid recovery at ~~70~~70 °C ~~o~~ for ~~20~~20 min [34]. One % loss of PD has been considered during lipid recovery and subsequent steps. The PD with lipid was separated from the cell debris through phase separation. The recovered lipid-PD mixture was reacted with methanol (6:1 molar ratio of methanol: lipid) for trans-esterification in presence of 1% (w/w of lipid) H_2SO_4 as catalyst. Acid catalyst was used during trans-esterification of sludge cultivated microbial lipid as it has free fatty acids ~~(> 1%~~ (≥ 1% w/w lipid), which lead to soap formation in presence of base catalyst [34]. The lipid extraction and trans-esterification efficiency were considered to be 92% and 97%, respectively [34]. The downstream process used for B10 biodiesel production has been depicted in Fig. 6.

alt-text: Fig. 6

Fig. 6



Process flow diagram for downstream process used for B10 biodiesel production.

Mass and energy balance have been performed to produce 1 tonne FAME (Fatty acid methyl esters) or 10 tonnes blended biodiesel B10 using microbial lipid~~oil~~ produced pH-based strategy (Table 4). Two scenarios were considered for this energy balance: Case A: Crude glycerol with no energy input and Case B: Crude glycerol with energy input ({}, Table 4). The energy input from chemical addition was the energy consumed to produce the amount of chemicals [35–37]. The energy consumed during the agitation, centrifugation and mixing were also taken from the literature [32]. The mass of chemicals required for the biodiesel production was in the column 'amount supplied'. The total energy input in sludge washing and concentration step was ~~266 MJ/ tonne~~ 266 MJ/tonne FAMEs where centrifugation ~~(1 kWh/m~~(1 kWh/m^3) for sludge concentration was main energy consuming factor. During lipid production (fermentation), the total energy input was ~~11.25 GJ/ tonne~~ 11.25 GJ/tonne FAMEs (Case A, Table 4). Out of which aeration provided for microbial growth was main contributing factor. The seed fermentation energy input was calculated to be ~~703 MJ~~ 703 MJ (6.25% of production fermenter) as 6.25% (v/v) was the inoculum size. During biomass settling, the total energy input was ~~5.61 GJ~~ 5.61 GJ where the energy content of EPS and calcium chloride were the contributing factors. Loss of PD (1%) during lipid recovery and subsequent steps was taken as an energy input (Table 4). The remaining (99%) diesel used during lipid recovery remained as fuel in the blended biodiesel (B10). The total energy input during lipid extraction was ~~4.4 GJ/ tonne~~ 4.4 GJ/tonne FAMEs where loss of PD was a major contributing factor. The total energy input for trans-esterification process was ~~7 GJ/ tonne~~ 7 GJ/tonne FAMEs where methanol was major contributing factor. The main contributing step in B10 production process was lipid production in fermentation (38.43%) followed by trans-esterification (23.96%) and biomass settling (19.27%). The net energy input for the process (Case A, Table 4) is ~~29.28 GJ/ tonne~~ 29.28 GJ/tonne FAMEs while energy output was ~~37.80 GJ/ tonne~~ 37.80 GJ/tonne FAMEs with a net energy gain (energy output – net energy input) of 8.52 GJ/ tonne FAMEs and energy ratio (energy output/ energy input) of 1.29 (Case A, Table 4). Positive net energy gain and energy ratio of greater than 1 make the process energetically favorable. If energy input of crude glycerol ~~(8.29 MJ/kg)~~ (8.29 MJ/kg) was~~is~~ accounted in energy balance (Case B, Table 4), energy input in production fermenter would increase~~s~~ from 11.25 GJ/ tonne FAMEs to ~~98.71 GJ/ tonne~~ 98.71 GJ/tonne FAMEs. For the complete process, total energy input would increase~~s~~ from ~~29.28 GJ/ tonne~~ 29.28 GJ/tonne FAMEs to ~~122.2 GJ/ tonne~~ 122.2 GJ/tonne FAMEs while net energy gain would be~~is~~ ~~– 84.4 GJ/tonne~~ – 84.4 GJ/tonne FAMEs and energy ratio would be~~is~~ 0.31 (Case B, Table 4). If energy input of crude glycerol was~~is~~ accounted, it would make~~s~~ the process energetically unfavorable.

alt-text: Table 4

Table 4

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Q1 Mass and energy balance for 1 tonne FAME (10 tonne B10) production using pH-based strategy on sludge fortified with crude glycerol medium.

Step	Items	Unit energy input	Amount supplied	Total energy input (MJ)	Energy input (%)
Sludge concentration	Centrifuge (kWh/m^3)	1	71.28 m^3	257	0.88
	Tap water (MJ/m^3) for washing	0.04	21.38 m^3	0.86	0.00
	Agitation (W/m^3)	7.3	27 m^3	9	0.02
	Energy input for sludge washing and conc. (MJ)			**266**	**0.91 {0.22}**
Production fermenter	Sterilization (MJ/kg steam)	26	3.9 kg	101	0.35
	Crude glycerol (MJ/kg)	0 {8.29}	10,550 kg	0 {87460}	0 {71.57}
	NaOH (MJ/kg)	18	73.58 kg	1361	4.65
	H_2SO_4 (MJ/kg)	7.1	68.07 kg	483	1.65
	Agitation (W/m^3)	7.3	35.64 m^3	67	0.23
	Aeration (kW/m^3)	1	35.64 m^3	9238	31.55
	Energy input in Lipid Production (MJ)			~~11251~~ 11,251 **{98711}**	**38.43 {80.78}**
Seed fermenter	**6.25% of Production fermenter (MJ)**			**703 {6169}**	**2.4 {5.05}**
Biomass settling	EPS (MJ/kg)	14.36	255.20 kg	3665	12.52
	$CaCl_2$ (MJ/kg)	7.2	274.43 kg	1976	6.75
	Energy input in Biomass settling (MJ)			**5641**	**19.27 {4.62}**
Lipid extraction	N-LS (MJ/kg)	5.76	42.43 kg	244	0.83
	FNA (MJ/kg)	3.2	21.27 kg	68	0.23
	Petro-diesel (MJ/kg)	45	90 kg	4050	13.83
	Agitation (W/m^3)	7.3	12.33 m^3	0.32	0.00
	Heating (kW/m^3)	2.72	12.33 m^3	40	0.14
	Energy input in Lipid extraction (MJ)			**4403**	**15.04 {3.6}**
Trans-esterification	Methanol (MJ/kg)	20	326.58 kg	6532	22.31
	Sulfuric acid (MJ/kg)	7.1	19 kg	135	0.46
	Mixing (kWh/kg biodiesel)	0.03	1000 kg	108	0.37
	Heating (kJ/kg biodiesel)	240	1000 kg	240	0.82
	Energy input in Trans-esterification (MJ)			**7015**	**23.96 {5.74}**
Total energy input (MJ)				~~29278~~ 29,278 **{122204}**	
Net energy input (GJ)				**29.28 {122.2}**	
Net energy output (GJ)				**37.80**	
Net Energy gain (GJ)				**8.52** ~~{-84.4}~~ {– 84.4}	
Energy Ratio				**1.29 {0.31}**	

{} indicates values incorporating energy input of crude glycerol – Case B

To calculate the sludge contribution in lipid production, it was~~is~~ important to compare the energy balance for biodiesel production using crude glycerol medium (without sludge). In a previous study, ~~38.1~~38.1 g/L and ~~14.8~~14.8 g/L lipid was produced at ~~96~~96 h cultivating YL on crude glycerol medium (BIOCARDEL, without sludge) containing ~~2.7~~2.7 g/L KH_2PO_4, ~~0.95~~0.95 g/L Na_2HPO_4, ~~0.2~~0.2 g/L $MgSO_4$~~.7H~~•$7H_2O$ and ~~12.5~~12.5 g/L peptone [21]. For downstream processing, same process was used as mentioned above except concentration of EPS and $CaCl_2$ used in biomass

settling and type of catalyst employed in trans-esterification. The concentration of $CaCl_2$ ~~(52~~(52 mM) and EPS ~~(39.9~~(39.9 mg/ g biomass) used in the process is dependent on the biomass concentration (near ~~40~~40 g/L biomass) [28]. In this process, NaOH (1% w/w lipid) was used instead of H_2SO_4 during trans-esterification. Base catalyst was used for glycerol cultivated microbial lipid as it has ~~FFA < 1%.~~ FFA < 1%. Mass and Energy balance for 1 tonne biodiesel (or 10 tonne B10) production using crude glycerol medium has been tabulated in Table 5. Two scenarios were considered for this energy balance: Case A: Crude glycerol with no energy input and Case B: Crude glycerol with energy input ({}, Table 5). It can be observed that energy input for production fermenter for crude glycerol medium is ~~44.48 GJ/ tonne~~ 44.48 GJ/tonne FAMEs (Case A, Table 5) while it was ~~11.25 GJ/ tonne~~ 11.25 GJ/tonne FAMEs in sludge based (Case A, Table 4). The nitrogen source (peptone) and trace elements supplied to assist cell growth in crude glycerol medium increased the energy input while this energy is saved in sludge-based fermentation where sludge provided nutrients and nitrogen for cell growth. Another reason for increased energy input in production fermenter for crude glycerol medium is lower lipid concentration ~~(14.8~~(14.8 g/L) obtained at ~~96~~96 h when compared to sludge fortified with crude glycerol medium ~~(31.44~~(31.44 g/L lipid at ~~72~~72 h). This led to increase in reaction volume in crude glycerol medium increasing energy input for aeration. This indicates that sludge not only provides nutrients and nitrogen but also provides additional carbon for lipid production. The total energy input for biodiesel production using crude glycerol medium is ~~63.95 GJ/ tonne~~ 63.95 GJ/tonne FAMEs (Case A, Table 5) while it was ~~29.28 GJ/ tonne~~ 29.28 GJ/tonne FAMEs (Case A, Table 4) in sludge fortified with crude medium. The energy ratio for biodiesel production using crude glycerol (without sludge) was~~is~~ 0.59 (Case A, Table 5), making the process energetically unfavorable. Hence, sludge had~~s~~ important contribution in microbial lipid and biodiesel production. If energy input of crude glycerol ~~(8.29 MJ/kg)~~ (8.29 MJ/kg) was~~is~~ accounted in energy balance for biodiesel production using crude glycerol (Case B, Table 5), energy input in production fermenter would increase~~s~~ from ~~44.84 GJ/ tonne~~ 44.84 GJ/tonne FAMEs to ~~161.27 GJ/ tonne~~ 161.27 GJ/tonne FAMEs. For the complete process, total energy input would increase~~s~~ from ~~63.95 GJ/ tonne~~ 63.95 GJ/tonne to ~~188.04 GJ/ tonne~~ 188.04 GJ/tonne FAMEs while net energy gain would be~~is~~ ~~–150.24 GJ/tonne~~ – 150.24 GJ/tonne FAMEs and energy ratio would be~~is~~ 0.2 (Case B, Table 5).

alt-text: Table 5

Table 5

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Mass and energy balance for 1 tonne FAME (10 tonne B10) production using BIOCARDEL crude glycerol medium (without sludge).

Step	Items	Unit energy input	Amount supplied	Total energy input (MJ)	Energy input (%)
Production Fermentation	Sterilization (MJ/kg steam)	26.00	8.09 kg	210	0.33
	Crude glycerol (MJ/kg)	0 {8.29}	14,088 kg	0 {116790}	0 {62.11}
	KH_2PO_4 (MJ/kg)	10.30	198.53 kg	2045	3.2
	Na_2HPO_4 (MJ/kg)	8.21	69.85 kg	573	0.9
	Peptone (MJ/kg)	17.30	919.13 kg	15,901	24.86
	$MgSO_4 \cdot 7H_2O$ (MJ/kg)	10.65	14.71 kg	157	0.24
	Agitation (W/m^3)	7.30	73.53 m^3	186	0.29
	Aeration (kW/m^3)	1.00	73.53 m^3	25,412	39.74
	Energy input in Lipid Production (MJ)			**~~44484~~ 44,484 {161273}**	**69.56 {85.77}**
Seed Fermenter	**6.25% of Production fermenter (MJ)**			**2780 {10080}**	**4.35 {5.36}**
Biomass settling	EPS (MJ/kg)	14.36	111.50 kg	1601	2.5
	$CaCl_2$ (MJ/kg)	7.20	424.42 kg	3056	4.78

	Energy input in Biomass Settling (MJ)			**4657**	**7.28 {2.48}**
Lipid extraction	N-LS (MJ/kg)	5.76	111.77 kg	644	1.01
	Loss in PD (MJ/kg)	45.00	90 kg	4050	6.33
	Agitation (W/m^3)	7.30	12.33 m^3	0.16	0.00
	Heating (kW/m^3)	2.72	12.33 m^3	30	0.05
	Energy input in Lipid extraction (MJ)			**4724**	**7.39 {2.51}**
Trans-esterification	Methanol (MJ/kg)	20.00	327.52 kg	6550	10.24
	NaOH (MJ/kg)	18.50	22.02 kg	407	0.64
	Mixing (kWh/kg biodiesel)	0.03	1000 kg	108	0.17
	Heating (kJ/kg biodiesel)	240	1000 kg	240	0.38
	Energy input in Trans-esterification (MJ)			**7306**	**11.42 {3.89}**
Total energy input (MJ)				~~**63951**~~ **63,951 {188040}**	
Net energy input (GJ)				**63.95 {188.04}**	
Net energy output (GJ)				**37.80**	
Net Energy gain (GJ)				~~**-26.15 {-150.24}**~~ **– 26.15 {– 150.24}**	
Energy Ratio				**0.59 {0.2}**	

{} indicates values incorporating energy input of crude glycerol – Case B

Table 6 compares the energy balance and unit production cost for different biodiesel processes reported in literature. The process employing commercial substrates for fermentation and energy intensive solvents (chloroform and methanol) during lipid extraction was~~is~~ energetically unfavorable where lipid extraction contributed 70.5% of total process energy input due to high volume and energy content of chloroform and methanol [33]. Another study reported on lipid and biodiesel production using *T. oleaginosus* cultivated on washed sludge (35 g/L) fortified with crude glycerol (40 g/L) in batch fermentation [12] had net energy gain of 3.05 GJ/tonne FAMEs with energy ratio of 1.09 [33]. The crude glycerol used during the fermentation had composition (w/w) of 78.22% glycerol, 2.63% soap, 2.52% ash, 12.15% methanol and 1.56% water [12]. In the present study, crude glycerol with high soap content was employed with different cultivation mode and microbe. Due to high lipid productivity (0.44 g/L/h) in fermentation in the present study, it had lowest total energy input and highest energy ratio among all fermentation based process. For 20 million L plant capacity, B10 unit production cost for the present study is 0.67 $/L (Table 6) which is lower than reported for previous study, 0.72 $/L [33]. The unit production cost is lower in this study due to enhanced lipid productivity in the fermenter. Lipid productivity in fermenter impacts capacity of fermenter, amount of substrate used (as capacity of fermenter changes, substrate requirement also changes), amount of product produced annually and time to process one batch.

alt-text: Table 6

Table 6

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Comparison of cost and energy balance results obtained in this study with those reported in the literature.

Substrate used	**Glucose, peptone & yeast extract**	**Washed sludge (35 g/L SS) & crude glycerol (40 g/L)**	**Washed sludge (20 g/L SS) with crude glycerol (high soap content)**
Microbe Used	*R. toruloides*	*T. oleaginosus*	*Y. lipolytica* SKY7

Cultivation mode	Fed-batch	Batch	pH-based fed-batch
Lipid Productivity (g/L/h)	0.54	0.36	0.44
Downstream process used	Centrifugation (harvesting) and solvent extraction (cell disruption and lipid recovery)	Bio-flocculant (settling), Biodegradable surfactant (cell disruption) & PD (lipid recovery)	Bio-flocculant (settling), Biodegradable surfactant (cell disruption) & PD (lipid recovery)
Total energy input (GJ/ tonne B10)	140.6	34.75	29.28
Credits (GJ/ tonne B10)	3.85 (crude glycerol)	–	–
Net energy gain (GJ/ tonne B10)	-98.95	3.05	8.52
Energy Ratio	0.28	1.09	1.29
Unit production cost ($/L B10)	6.78	0.72	0.67
Reference	[33]	[33]	This study

4 Conclusion

In this study, microbial lipid was produced from municipal sludge fortified with crude glycerol medium where crude glycerol (pH 10.98) was used as substrate and pH-control agent. High biomass ~~(59.67~~(59.67 g/L) and lipid concentration ~~(31.44~~(31.44 g/L) was obtained at ~~72~~72 h of fed-batch fermentation. Secondary m~~M~~unicipal sludge ~~(20~~(20 g/L SS) which was added as additional carbon source had sufficient nitrogen and trace elements to support growth of YL. Energy balance revealed that lipid production from sludge fortified with crude glycerol medium was more energetically favorable than with crude glycerol medium alone. This study provides an alternative route for disposal of waste streams (municipal sludge and crude glycerol) through their utilization in biodiesel production.

CRediT authorship contribution statement

Lalit R. Kumar: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing - original draft. **Sravan K. Yellapu:** Resources, Validation. **R.D. Tyagi:** Supervision, Writing - review & editing, Funding acquisition, Project administrator, Supervision, Writing - review & editing. **Patrick Drogui:** Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Q6 Acknowledgements

The authors would like to acknowledge the Natural Sciences and Engineering Research Council of Canada (grant A4984) for financial support. We are grateful to the technical staff of INRS-ETE (Mr. Stephane Moise and Mr. Stefane Premont) for their timely help to analyze the samples on LC-MS and ICP-MS.

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The corrections made in this section will be reviewed and approved by a journal production editor. The newly added/removed references and its citations will be reordered and rearranged by the production team.

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Graphical ~~abstract~~Abstract

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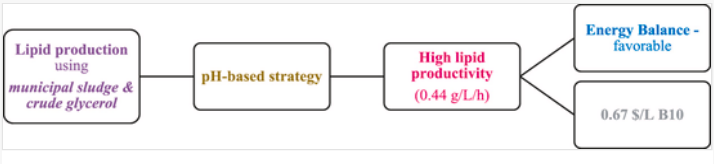

Highlights

- Microbial lipid produced using municipal sludge fortified with crude glycerol.

- pH-based fed-batch strategy resulted in ~~0.44~~0.44 g/L/h lipid productivity.
- Energy balance for biodiesel production was favorable.

Queries and Answers

Q1

Query: Please provide a definition for the significance of bold in the "Tables 4, 5".

Answer: Bold are either unit operations in the process or important parameters

Q2

Query: Please confirm that given names and surnames have been identified correctly and are presented in the desired order, and please carefully verify the spelling of all authors.

Answer: Yes, correct

Q3

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Q4

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Q6

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Answer: Yes