Original Article

Biodiesel production from microbial lipid obtained by intermittent feeding of municipal sludge and treated crude glycerol

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Abstract

In the present study, municipal secondary sludge and purified glycerol (obtained after acid treatment of crude glycerol) were used together for lipid production using intermittent feeding strategy. Intermittent sludge feeding strategy (sludge SS 30 g/L) resulted in a higher biomass (54.99 g/L) and lipid concentration

(25.35 g/L) at 96 h when compared to 35 g/L SS single sludge feeding or control strategy (45.67 g/L biomass & 19.16 g/L lipid). Moreover, the intermittent sludge feeding strategy significantly reduced foaming and requirement of anti-foam during fermentation when compared to control strategy. The energy balance of biodiesel production from lipid obtained by intermittent sludge feeding strategy (30 g/L SS) was energetically favorable. It was also revealed from yield coefficients and energy balance that sludge had an important contribution in microbial lipid and biodiesel production.

Keywords

Intermittent feeding
Municipal Sludge
Purified glycerol
Microbial lipid
Biodiesel

Supplementary Information

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Introduction

Domestic wastewater sludge (or municipal sludge or sewage sludge) is a by-product of municipal wastewater treatment. For a long time, wastewater sludge has been considered to be a waste and its treatments are mainly focused on disposal or reduction [1]. However, neither disposal nor reduction can efficiently or completely eliminate the environmental threat associated with wastewater sludge [2, 3]. Recently, wastewater sludge has been recognized as a bioresource due to its high organic content, which is mainly composed of the microbial biomass [4]. Also, wastewater sludge is rich in carbon, nitrogen and nutrients [5]. Thus, wastewater sludge can be applied as raw material for oleaginous microorganisms' cultivation to produce lipids [6, 7]. The lipids accumulated in oleaginous microbial cells can be utilized to produce biodiesel through trans-esterification reaction.

Since suspended solids of wastewater sludge has only 30–40% carbon, other substrates, rich in carbon sources such as crude glycerol, are fortified to the wastewater sludge for oleaginous microorganisms' cultivation [1, 5]. However, to increase the sludge valorization, the maximum amount of sludge suspended solids

should be utilized for microbial lipid production. A study has been conducted where 35 g/L suspended solids (SS) concentration of washed sludge and 40 g/L crude glycerol were utilized for lipid production in batch fermentation [8]. However, excess foaming was observed during fermentation due to high sludge SS concentration and the operation had to be stopped at 48 h. Also, the impurities present in crude glycerol affects the cellular metabolism for lipid production [9, 10].

The novelty of the present study lies in use of intermittent feeding of municipal sludge and purified glycerol (obtained after acid treatment of crude glycerol) for lipid production. Purification of crude glycerol will reduce the impact of impurities present in crude glycerol on lipid production [11]. The problem of excess foaming and lower lipid productivity during fermentation can be resolved through intermittent sludge feeding strategy. Energy balance for biodiesel produced using microbial lipid produced from intermittent sludge feeding strategy was also conducted.

Materials and methods

Materials

The crude glycerol was obtained from Canadian biodiesel producing company BIO-LIQ INC. BIOLIQ crude glycerol was high on potassium concentration as potassium methoxide was used as catalyst during trans-esterification. Since BIOLIQ crude glycerol was high on potassium, it was purified using phosphoric acid (using pH adjustment to 2) followed by batch centrifugation at 6000 rpm for 10 min. The characterization for crude and purified glycerol was performed using protocols given in [12]. The composition of crude and purified glycerol is mentioned in Table 1.

Table 1

Characteristics of crude and purified glycerol

Parameter	Crude glycerol	Purified glycerol
Density (g/L)	1385.00 ± 10.00	1242.00 ± 10.00
Glycerol concentration (g/L)	453.00 ± 5.00	473.00 ± 5.00
Water (g/L)	132.96 ± 5.00	232.25 ± 5.00
pH	13.50 ± 0.10	2.00 ± 0.05
Al (g/L)	2.58×10^{-3}	2.83×10^{-3}

Parameter	Crude glycerol	Purified glycerol
Ca (g/ L)	1.30×10^{-2}	2.08×10^{-2}
Cr (g/ L)	1.00×10^{-4}	1.80×10^{-4}
Cu (g/ L)	1.72×10^{-2}	1.34×10^{-2}
Fe (g/ L)	8.10×10^{-3}	4.20×10^{-3}
K (g/L)	73.04 ± 2.20	10.56 ± 0.5
Mg (g/L)	7.00×10^{-3}	9.20×10^{-3}
Mn (g/ L)	1.70×10^{-4}	1.80×10^{-4}
Na (g/ L)	0.38 ± 0.01	0.38 ± 0.01
Ni (g/ L)	–	2.00×10^{-4}
P (g/ L)	0.18 ± 0.00	23.70 ± 1.00
Pb (g/L)	0.80 ± 0.01	1.00 ± 0.05
S (g/L)	1.20×10^{-2}	2.10×10^{-2}
Sn (g/ L)	3.76×10^{-2}	2.62×10^{-2}
Zn (g/ L)	5×10^{-3}	3.88×10^{-3}

The sludge was collected from the secondary sedimentation tank of a municipal wastewater treatment plant, Communauté Urbain de Québec (CUQ) in Québec, Canada. The sludge was first undergone through gravity settling at 4 °C for 24 h, and then SS concentration of the resulting solution was measured (around 22 g/L). The settled sludge was then stored at 4 °C for further utilization. The protocols for sludge characterization has been reported in [13]. The sludge characterization is mentioned in Table 2. Thereafter, settled was centrifuged at 8000 *g* for 10 min to obtain a concentrated sludge of 41.5 g/L SS. Concentrated sludge was washed with tap water (30 L/ kg sludge solids) to remove the heavy metals (Fe, Mg, Mn, Ni, Zn. Cu) sticking to sludge solids, which could be inhibitory for microbial cell growth [8].

Table 2

Characteristics of municipal secondary sludge

Characteristics	Concentration	Units
Total solids (TS)	29.23 ± 1.76	g/L

Characteristics	Concentration	Units
Total suspended solids (TSS)	21.86 ± 1.49	g/L
Volatile solids (VS)	17.35 ± 0.94	g/L
Total carbon (TC)	420.46 ± 11.57	g/kg
Total nitrogen (TN)	54.05 ± 3.85	g/kg
Total phosphorus (TP)	29.08 ± 0.95	g/kg
pH	6.45 ± 0.01	–

Micro-organism

Y. lipolytica SKY7 (YL) isolated in INRS laboratory was used in this study [14]. YL 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 [14].

Pre-culture and inoculum preparation

The dormant pure culture of *Y. lipolytica* (4 °C) 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 28 °C. 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 sludge fortified with 10 g/L purified glycerol. Before using sludge as growth medium, washed 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 6.5 using 4 M H_2SO_4 [8]. PC2 was grown at 180 rpm and 28 °C for 36 h before being transferred to the production fermenter. PC2 was cultivated to acclimatize *Y. lipolytica* in sludge medium.

Fermentation

Operation

Fermentations were carried out in stirred tank fermenters (SARTORIUS BIOSTAT & INFORS AG Fermenter) equipped with accessories and programmable logic control (PLC) system. The inoculum size of 6.25% (v/v) was chosen as it is the optimum value reported for *Y. lipolytica* SKY7 [15]. During 6–12 h of fermentation, DO decreases from 95 to 40% and later it was maintained in the range of 25%-40%

by manually adjusting agitation rate (250–600 rpm) and air flow rate (1–2 L/min). The limitation of DO favors lipid production [16]. The temperature was maintained at 28 °C by circulating water through the fermenter jacket. Fermentation pH was controlled automatically at 6.5 ± 0.1 by the addition of pH control agents: 4 M NaOH or 4 M H_2SO_4. Dissolved oxygen and pH were continuously monitored by means of a polarographic dissolved oxygen probe and of a pH sensor (Mettler-Toledo, USA), respectively.

Fed-batch strategy

Fermentations were conducted using sludge fortified with purified glycerol. Before using sludge as growth medium and feed, the sludge was pre-treated as mentioned in previous section. The production fermenters were operated at temperature 28 °C and pH 6.5 for *Y. lipolytica* as reported in [17]. The fermentations were started with SS 20 g/L of washed sludge and 20 g/L glycerol (purified) at 0 h with no addition of trace elements and nitrogen. The sludge solids were fed at different time of fermentation in case of intermittent sludge feeding as shown in Table 3. One run was conducted with a single sludge feed of SS 35 g/L at 0 h of fermentation and was considered as control. The control fermenter run was conducted to compare the single sludge feed strategy with intermittent sludge feeding strategy. Purified glycerol was also fed to the fermenters based on consumption. Other than control, each fermentation run was started with 20 g/L SS of washed sludge and 20 g/L glycerol (purified glycerol). When glycerol concentration in the medium reached below 5 g/L, a feed of purified glycerol was added so that the glycerol concentration in the medium reaches around 20 g/L. The purified glycerol feeding details are presented in Table 4. The schematic of the experimental setup for single sludge feeding strategy and 30 g/L intermittent sludge feeding strategy has been provided in Fig. 1. Samples of 50 mL volume were drawn after every 8 h to determine colony-forming unit (CFU) count in the medium, biomass as suspended solids, lipid concentration and concentration glycerol and organic acids in the supernatant.

Table 3

Sludge feeding strategy for intermittent and single sludge feeding strategy

~~Strategy s~~Sludge SS conc (g/L)	**24 (intermittent feed)**		**27.6 (intermittent feed)**			**30 (intermittent feed)**			**35 (Control, single feed)**
Feeding time (h)	0	48	0	48	72	0	48	72	0

~~Strategy s~~Sludge SS conc (g/L)	24 (intermittent feed)		27.6 (intermittent feed)			30 (intermittent feed)			35 (Control, single feed)
Sludge solids added (g)	70	35	40	20	25	40	30	40.5	140
Total sludge suspended solids added in 96 h (g)	105		85			110.5			140
Final working volume (L)	4.34		3.08			3.7			4

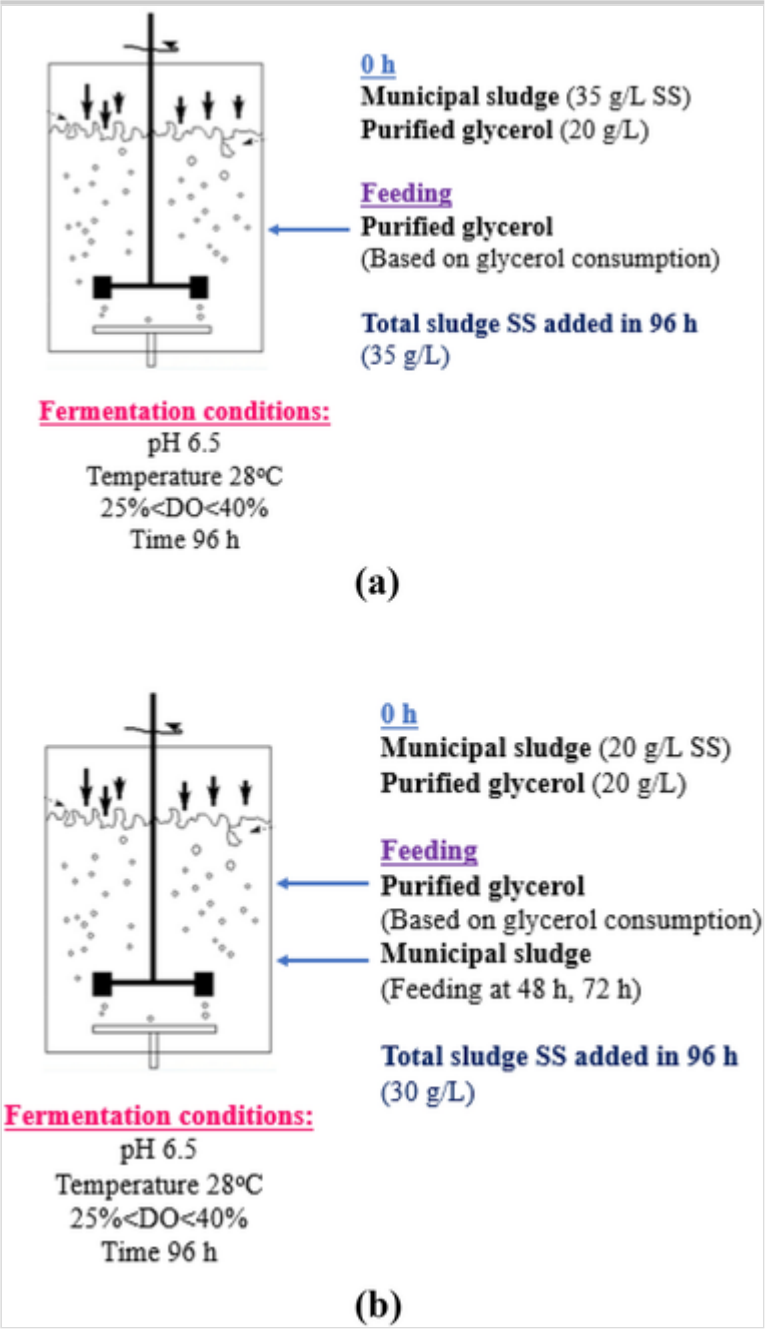
Table 4

Purified glycerol feeding strategy for intermittent and single sludge feeding strategy

Sludge SS conc (g/L)	24 (intermittent feed)		27.6 (intermittent feed)			30 (intermittent feed)			35 (Control, single feed)		
Feeding time (h)	0	40	0	16	48	0	16	48	0	32	80
Glycerol added (g/L)	20	17.85	20	15.8	19.11	20	16.5	17.3	20	16	18
Carbon added as glycerol (g/L)	8	7.14	8	6.32	7.64	8	6.6	6.92	8	6.4	7.2
Total carbon added from glycerol	15.14 g/L		21.96 g/L			21.22 g/L			21.6 g/L		

Fig. 1

Schematic of the experimental setup for **a** single sludge feeding strategy and **b** 30 g/L intermittent sludge feeding strategy



Analytical techniques

Biomass and lipid concentrations were determined as reported by [12, 18]. Glycerol was measured according to the method by [19]. Citric acid estimation was carried out according to the method reported in [20]. Elemental concentration in samples was determined by inductively coupled plasma mass spectroscopy (ICP-MS) after acid digesting the samples (model DRE, Leeman Labs Inc) [21].

Lipid characterization: Lipid obtained was trans-esterified and characterized as per the protocol given in [17]. All samples were analyzed in duplicates.

Determination of kinetic parameters

The specific cell growth rate (μ, h^{-1}) was calculated with Eq. 1:

$$\mu = ln\left(CFU2/CFU1\right)/t2 - t1 \quad (1)$$

where CFU1 and CFU2 were the CFU concentration of the sample at time t1 and t2, respectively.

Productivity (g/L/h) is defined as g product produced per unit volume at a particular time t. productivity will be calculated by Eq. 2

$$Lipid\ Productivity\left(\frac{g}{L.h}\right) = \frac{Lipid\ produced(g)}{(Volume\ x\ time)} \quad (2)$$

Product yield coefficient ($Y_{P/S}$, g/g) is defined as the amount of product produced per g of substrate consumed. Lipid yield will be determined by the Eq. (3):

$$Y_{l/S} = dL/dS \quad (3)$$

Energy balance

Energy balance was computed based on per tonne of biodiesel production. The energy balance have been performed according to [22]. The positive net energy balance (energy output-energy input) or energy ratio (energy output/energy input) higher than 1 are required to make the process energetically favorable.

Results

Fermentation using different feeding strategies

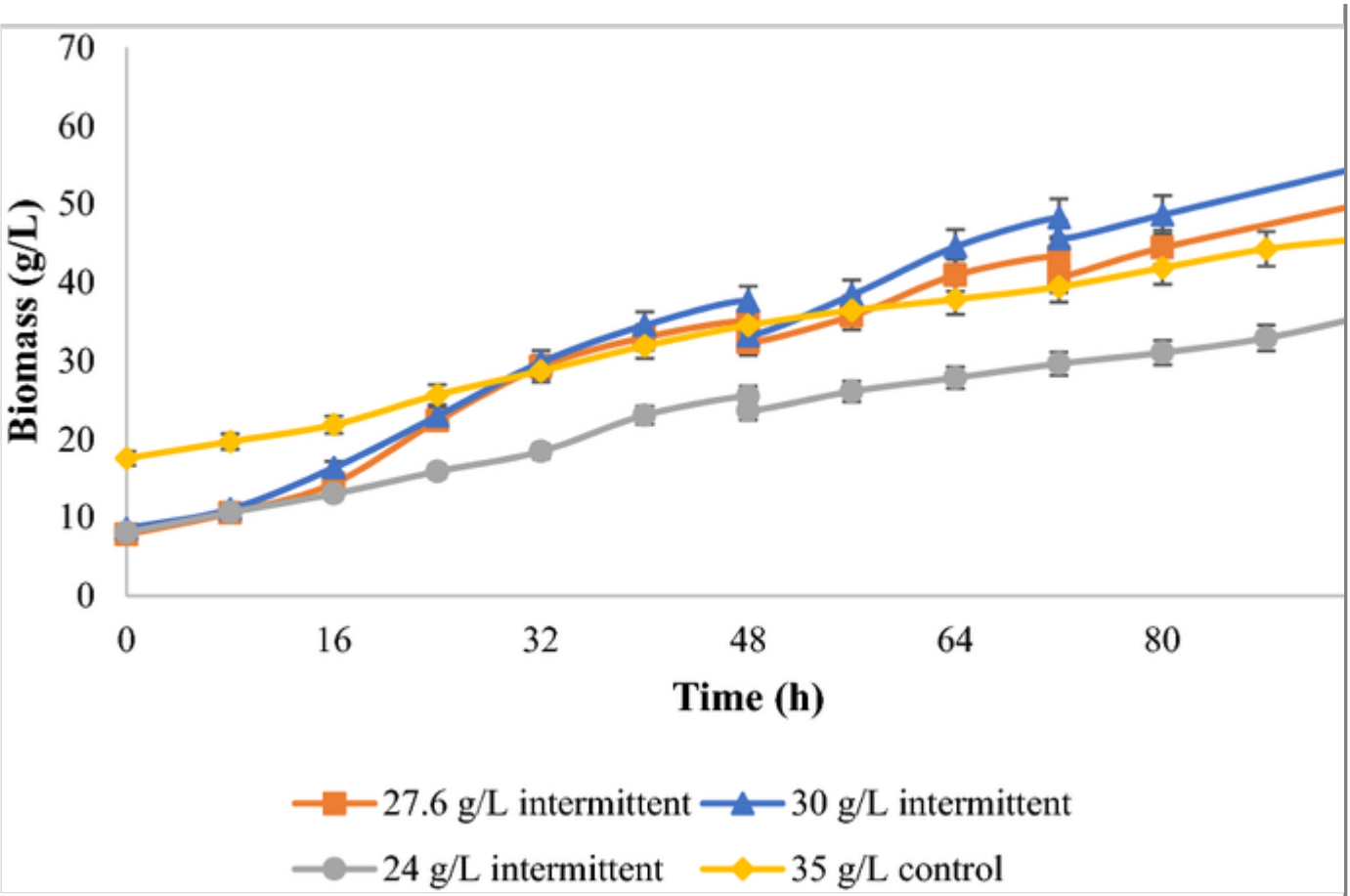
Variation in biomass concentration

Variation of biomass concentration in different sludge feeding strategies is highlighted in Fig. 2. Reduction in biomass concentration was observed in intermittent sludge feed strategies at 48 h and 72 h because when the sludge feed was added at these intervals, it had a substantial volume (500–700 mL) that changed the working volume of the reactor while changing the biomass concentration too. No reduction in biomass concentration or a continuous curve of biomass concentration was observed in 35 g/L SS control feed strategy as intermittent sludge feeding was not added. However, no reduction in biomass

concentration was observed while feeding glycerol at different intervals because glycerol feed has a volume of 80–100 mL that did not change the working volume of the reactor significantly. Online resource (Table S1) indicates chemical elements and nitrogen present in the media (supernatant or soluble phase of the medium) for all the intermittent feeding strategies. Sludge media had all necessary trace elements (P, K, Mn, Mg, Na, Zn, Ca, Fe) and nitrogen required for cell growth. There was a decrease in the concentration of chemical elements and nitrogen during 0–48 h (before adding the sludge feed) in all three intermittent feeding strategies. It is due to consumption of these elements by YL for cell growth and lipid production. When the sludge feed was added at 48 h (‘ + ’ sign), there was an increase in the concentration of chemical elements and nitrogen. This is because of imparting of sludge feed in the reactor, the concentration of these elements in the medium increased as sludge contains these elements and nitrogen. Again, there was a decrease in elemental and nitrogen concentration between 48^{+} and 72^{-} h for 27.6 g/L and 30 g/L sludge SS feeding strategies. The nutrients were being utilized by YL for microbial biomass build-up and lipid production. And when sludge was fed at 72 h (‘ + ’ sign) for 27.6 g/L and 30 g/L sludge SS feeding strategies, the concentration of elements and nitrogen increased again. The decrease in elemental concentration between 72^{+} and 96 h for 27.6 g/L and 30 g/L sludge SS feeding strategies was again due to the consumption of these elements by YL. Similar reason applies for the decrease in the concentration of elements and nitrogen between 48^{+} and 96 h for 24 g/L intermittent sludge feeding strategy. There was no additional sludge feed (besides at 0 h) in 35 g/L SS (control run) strategy. The decrease in elemental concentration during 0–96 h for the control strategy is due to cell growth, biomass buildup and lipid accumulation. Throughout the fermentation, sufficient trace elements and nitrogen were present in each medium and did not get completely exhausted as intermittent sludge feeding was provided. Hence, there was no requirement of additional trace elements and nitrogen [8]. The high Na concentration in each medium is because of sludge treatment with 4 M NaOH before sterilization. As reported in previous study, Na concentration of 6–8.3 g/L slows the cell growth of YL [21]. However, in this study, Na concentration was around 2–4 g/L in each medium, which was lower than the inhibition level (Online Resource Table S1).

Fig. 2

Biomass profile for different sludge feeding strategies. Biomass concentration refers to that of combined yeast and sludge



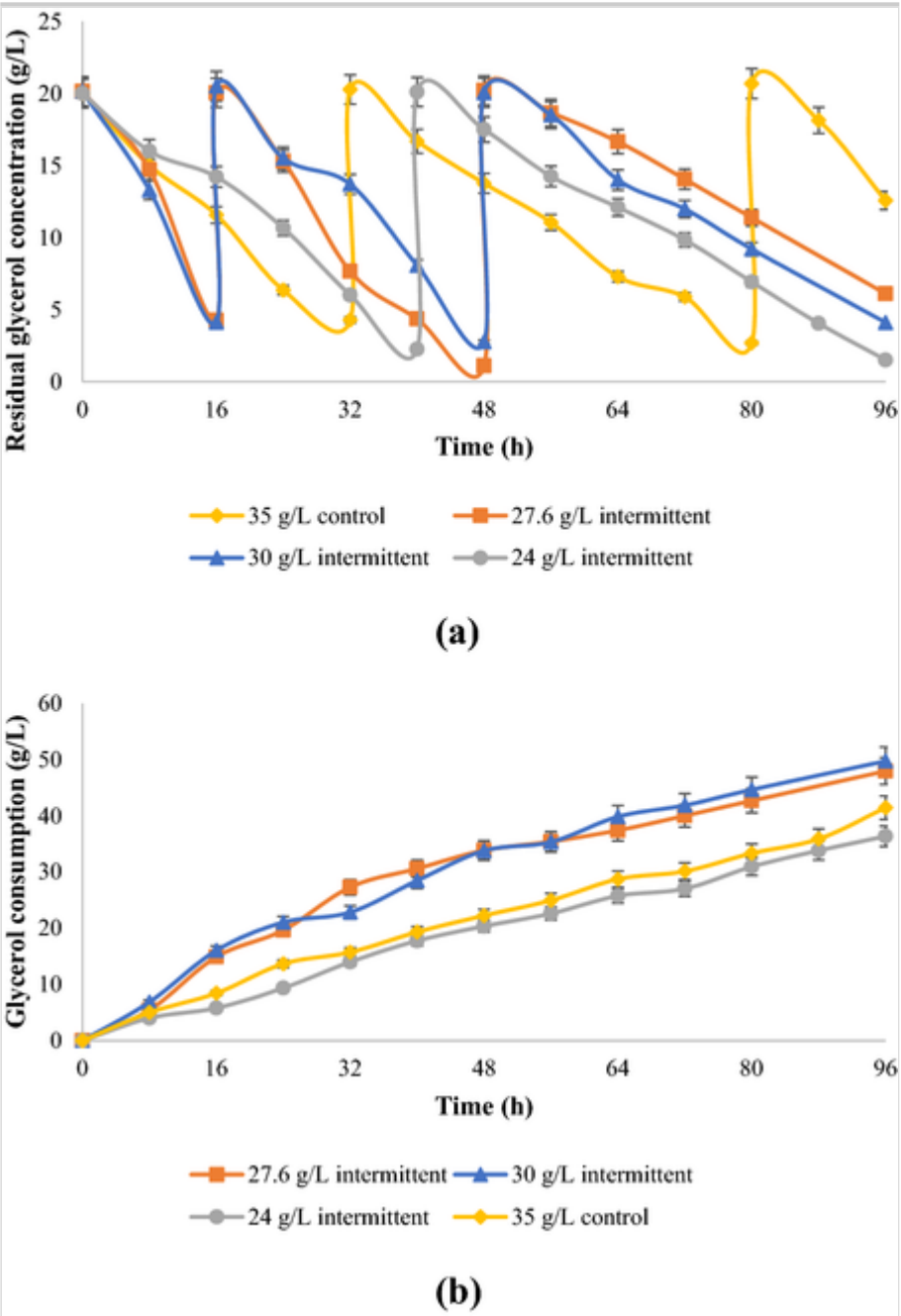
The SS concentration at 96 h for the feeding strategy of sludge SS 30 g/L, 27.6 g/L, 24 g/L and 35 g/L (control) was 54.99 g/L, 50.22 g/L, 35.84 g/L and 45.67 g/L, respectively. However, this SS concentration is not real biomass concentration resulting from microbial growth because municipal secondary sludge contains 45–50% non-biodegradable solids [8]. Some of these non-biodegradable solids are solubilized during sludge pre-treatment and the other stay with suspended solids and are included in SS measurement.

Variation in glycerol consumption

Residual glycerol concentration and glycerol consumption in the fermenter for different sludge feeding strategies is highlighted in Fig. 3a, b. Maximum glycerol consumption at 96 h for feed strategy of sludge SS 30 g/L, 27.6 g/L, 24 g/L and 35 g/L(control) and 24 g/L strategy was 49.73 g/L, 47.95 g/L, 41.43 g/L and 36.35 g/L, respectively. Continuous utilization of glycerol was observed throughout fermentation in all sludge media.

Fig. 3

a Residual glycerol concentration in fermenter and **b** glycerol consumption for different sludge feeding strategies



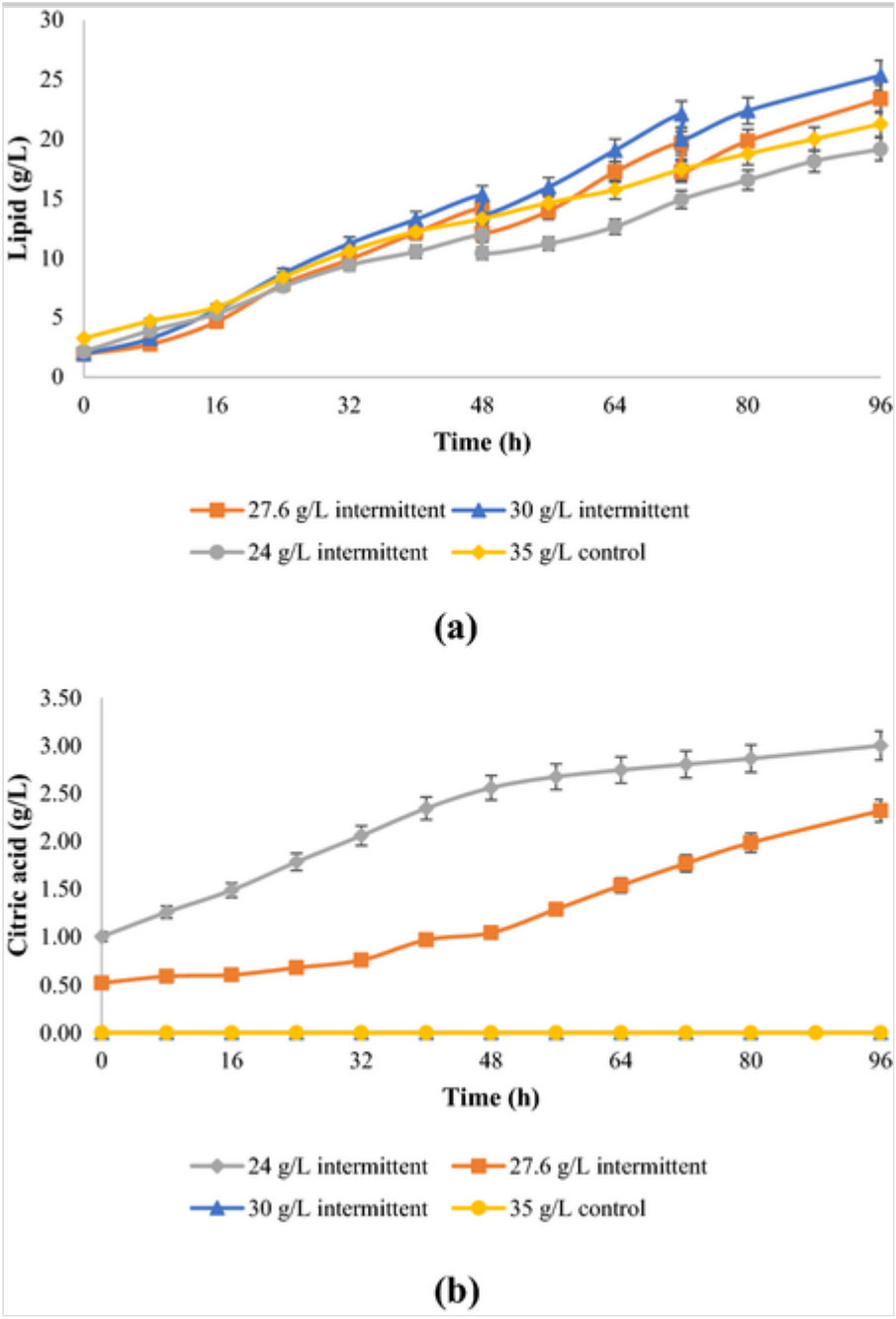
Variation in lipid production

Variation of lipid concentration for different sludge feeding strategies is highlighted in Fig. 4a. The initial lipid concentration (at 0 h) due to sludge suspended solids has around 10% inherent lipid content [8, 18]. In all sludge media, the initial increase in lipid concentration (during first 16 h) occurred due to the development of cell membrane lipids (cell growth phase) and from 24 h onwards, an increase in lipid concentration was observed due to cytosolic lipid production [11]. Reduction in lipid concentration was observed at sludge feeding intervals due to the medium dilution as mentioned in previous ~~S~~section~~. 3.1.1~~. The lipid concentration at 96 h

for the sludge feed strategy of SS 30 g /L, 27.6 g /L, 24 g /L and 35 g /L (control) was 25.35 g/L, 23.38 g/L, 19.16 g/L and 21.28 g/L, respectively. The high lipid concentration at the sludge feeding strategy of SS 30 g/L is because it provided higher carbon and other nutrients for lipid production compared to SS 24 g/L and 27.6 g/L intermittent sludge feeding strategies. In another study, where YL was cultivated on purified glycerol medium (without sludge) containing 2.7 g/L KH_2PO_4, 0.95 g/L Na_2HPO_4, 0.2 g/L $MgSO_4.7H_2O$ and 9.5 g/L peptone (nitrogen source) the biomass concentration of 51.67 g/L and lipid concentration of 19.47 g/L was observed at 96 h of fed-batch fermentation [11]. Higher biomass (54.99 g/L) and lipid concentration (25.35 g/L) in 30 g/L SS intermittent sludge feeding strategy indicate that sludge can replace the commercial trace elements and nitrogen source for cell growth and lipid production. In addition, sludge provides a carbon source for biomass and lipid accumulation. The contribution of sludge (used during fermentation) in lipid and ultimately biodiesel production is also highlighted in next ~~S~~section~~. 3.3.2~~ through energy balance.

Fig. 4

a Lipid production and **b** Citric acid production for different sludge feeding strategies



Variation in organic acids production

Y. lipolytica is a well-known industrial scale citric acid producer. Variation of citric acid production in different sludge feeding strategies is highlighted in Fig. 4b. Nitrogen concentration in the medium is important for deciding the pathway for citric acid production or de-novo lipid accumulation. The absence or minimum concentration of nitrogen in the medium favors citric acid production [23]. The presence of nitrogen in the sludge medium is responsible for very low citric acid production in all cases. In the case of sludge SS 35 g/L (control), nitrogen concentration was abundant in the medium from the beginning of the fermentation

due to high sludge SS concentration at 0 h. Hence no citric acid production was observed in the control strategy. Similarly, no citric acid was produced in 30 g/L SS (high nitrogen concentration) intermittent sludge feeding strategy due to higher sludge SS concentration as compared to 24 g/L and 27.6 g/L feed strategies. Citric acid produced at 96 h for 24 g/L and 27.6 g/L intermittent sludge feeding strategy was 3.11 g/L and 2.32 g/L, respectively. Other organic acids produced during this study were pyruvic acid, glutamic acid and malic acid (Online Resource Table S2). Although, these organic acids have commercial applications [24], but the concentration of these acids in the medium is very low (< 50 mg/L) and may not be economical to recover them. For feeding strategies 24 g/L SS and 27.6 g/L SS, significant amount of lipid, citric acid and pyruvic acid are produced, whereas the amount of malic acid is significantly low when compared to the rest of the strategies. In oleaginous yeast, NADPH is continuously produced via the conversion of Malic acid into pyruvate due to the action of malic enzyme [17]. Additionally, the AMP is directly linked with the activity of isocitrate dehydrogenase. So, under nitrogen limited condition, the citric acid goes up and then channeled into cytoplasm to produce acetyl-CoA, a major precursor for fatty acids [17].

Impact of feed strategies on foaming

When sludge media is employed, foaming is observed. The foam production in sludge media occurs for two reasons: (1) due to high protein concentration in complex sludge media or protein precipitated during sludge sterilization leads to foaming and (2) owing to protein release from cell autolysis or some other property of the culture itself [25]. The foaming due to cell autolysis occurs once (after 24 h) and is easier to control. The foaming in the 1st case is due to the property of sludge medium and occurs at regular intervals [25]. The consumption of antifoam agent for different sludge feeding strategies is presented in Online Resource Table S3. Higher quantity of antifoam consumed at 35 g SS/L (control strategy) indicates higher and continuous foaming during the fermentation. Higher foaming in control strategy is due to higher sludge SS concentration fed at one time (in the beginning of the fermentation process), which has a high concentration of proteins and foam producing substances in the medium. Intermittent sludge feeding reduces the foaming problem as the proteins and foam producing substances are present in relatively lower concentration at a particular time. The amount of antifoam utilized per L broth has been reduced significantly in intermittent sludge feeding strategies. Considering the bulk price of antifoam to be 0.2 $/L, the antifoam utilization cost in 35 g/L SS control strategy is 0.026 $ for 4 L working volume. Although, the cost of

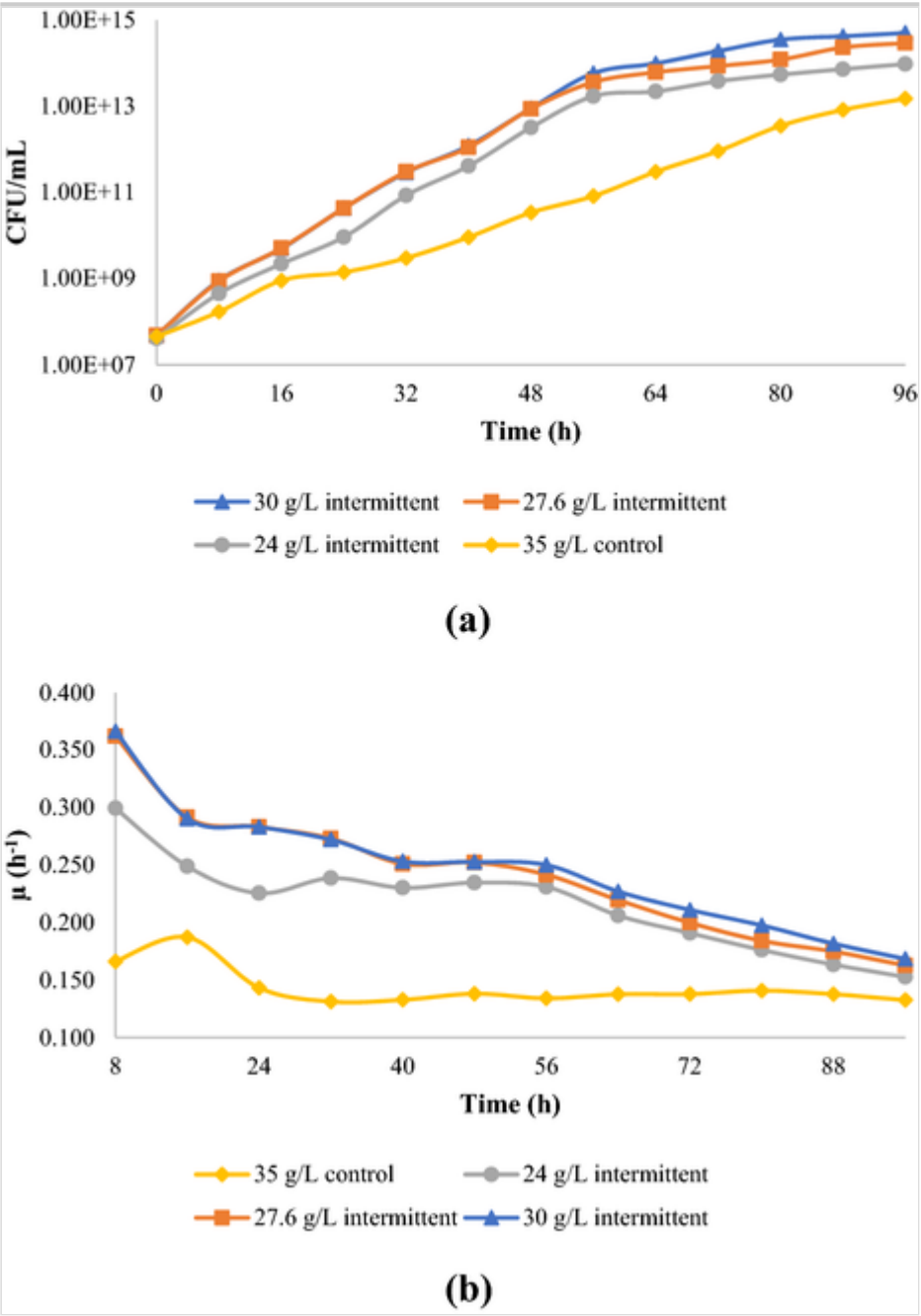
antifoam utilization in lab scale reactors appears low, the cost will increase with reactor size.

Kinetic parameters under different feeding strategies

The feeding strategy also has an effect on growth or CFU count. Throughout the fermentation, CFU count at 35 g/L SS control strategy was found lower than three intermittent sludge feeding strategies (Fig. 5a). The control strategy had higher suspended solid particles in the medium from the beginning of the fermentation. Higher suspended solids in the medium provide interference to oxygen and nutrient transfer to cells, hence impacting the cell growth. While intermittent sludge feeding strategies had lower suspended solids concentration at a particular time, providing less interference in oxygen and nutrient transfer rates. The CFU/ mL at 96 h for 30 g/L, 27.6 g/L, 24 g/L and 35 g/L SS strategies (control) was 5×10^{14}, 2.9×10^{14}, 9.5×10^{13} and 1.5×10^{13}, respectively. The specific growth rate (μ) for different sludge feeding strategies is shown in Fig. 5b. Specific growth rate in case of intermittent sludge feeding strategies was higher than the control strategy throughout the fermentation. It indicates that intermittent sludge feeding strategies provided better conditions for cell growth due to above mentioned reasons. The maximum specific growth rate observed for the SS concentration of 24 g/L, 27.6 g/L and 30 g/L (intermittent sludge feeding strategies) at 8 h was 0.299 h^{-1}, 0.362 h^{-1} and 0.366 h^{-1}, respectively.

Fig. 5

a CFU/mL and **b** specific growth rate (μ) of yeast for different sludge feeding strategies



Lipid yield coefficients for control and intermittent sludge feeding strategies was calculated (Table 5). For estimating lipid yield with respect to sludge, lipid yield with respect to glycerol was important. The value of lipid yield from only glycerol (without sludge) was taken from previous experiments conducted on YL for lipid production using pure glycerol medium [11]. Lipid contribution from purified glycerol in sludge medium was calculated by multiplying the lipid yield with glycerol consumed in 96 h of fermentation. When glycerol is used as the sole carbon source for lipid production, 4.55-tonne glycerol will be utilized to produce 1-tonne lipid. However, the glycerol requirement to produce 1-tonne lipid decreases when glycerol is fortified with municipal sludge (Table 5). Lipid yield with respect

to sludge was higher in the intermittent sludge feeding strategy than the control feed strategy, indicating sludge was better utilized for lipid production in intermittent feeding. Although sludge contains carbon in complex form, the strain takes time to assimilate carbon from sludge for biomass and lipid production. The overall lipid productivity for sludge SS 35 g/L (control) and sludge intermittent feeding with sludge SS 24 g/L, 27.6 g/L, 30 g/L strategy at 96 h was 0.22 g/L/h, 0.2 g/L/h, 0.24 g/L/h and 0.26 g/L/h, respectively. Thus, the intermittent sludge feeding strategy provides carbon and nutrients at specific intervals, increases lipid productivity and reduces the foam produced compared to single sludge feed strategy with high concentration (35 g/L SS).

Table 5

Yield coefficients for control and intermittent sludge feeding strategies

Parameters	**35 g/L (Control)**	**24 g/L (intermittent)**	**27.6 g/L (intermittent)**	**30 g/L (intermittent)**
Lipid concentration at 96 h (g/L)	21.28	19.16	23.38	25.35
Lipid yield wrt glycerol (without sludge) (g/g)	0.22	0.22	0.22	0.22
Glycerol consumed in 96 h (g/L)	41.43	36.35	47.95	49.73
Lipid contributed from glycerol in sludge medium, Lg (g/L)	9.11	8	10.55	10.94
Lipid contributed by sludge, Ls (g/L)	12.17	11.16	12.83	14.41
Lipid yield wrt sludge solids (g/g solids)	0.35	0.47	0.46	0.48
Glycerol required to produce 1-tonne lipid in sludge medium (tonne)	1.95	1.9	2.05	1.96
Sludge required to produce 1-tonne lipid in sludge medium (tonne)	1.64	1.25	1.18	1.18

Biodiesel production from microbial lipid

Lipid characterization

Lipid characterization in this study revealed that Oleic acid (C18:1), Linoleic acid (C18:2) and Arachidonic acid (C20:4) were the major fatty acids produced in every fermentation run. Oleic acid (C18:1) and linoleic acid (C18:2) are the major components in the case of SKY7 as reported in other studies [14, 17]. The degree of saturation for biodiesel obtained from sludge media was between 10–15%, which was comparable to biodiesel obtained from vegetable oil [18]. The presence of polyunsaturated fatty acids (PUFAs) makes the isolate *Y. lipolytica* SKY7 important for biodiesel production.

Energy balance for 1 tonne biodiesel production using intermittent sludge feeding strategy (30 g/L SS)

To verify the energy feasibility of lipid production process using intermittent sludge feeding of 30 g/L SS, energy balance needs to be performed for biodiesel production. The first step of the process is crude glycerol purification to minimize impurities so that the yield of lipid is high. In this research, since purified glycerol was used along with sludge for lipid production, the energy consumed in glycerol purification was taken into account. During the purification of crude glycerol using phosphoric acid, a precipitate of KH_2PO_4 is formed through following equation [11]:

$$RCOOK + H_3PO_4 \rightarrow RCOOH + KH_2PO_4\,(precipitate) \quad (4)$$

For performing complete energy balance, downstream process (lipid recovery, transesterification and final preparation of B10 biodiesel) reported by Yellapu, Klai, Kaur, Tyagi, Surampalli [26] was selected. Bio-flocculant was used for biomass settling after fermentation while to release the intracellular lipid biodegradable surfactant was used to disrupt the lipid containing yeast cells. Finally, the released lipid was separated (recovered) in petroleum diesel. The reported downstream process has economic and environmental advantages over traditional broth harvesting, which employs continuous centrifuge followed by biomass drying. The lipid from dry biomass is extracted using toxic solvents [27]. The biomass settling was performed using 52 mM calcium chloride and 39.9 mg EPS/g biomass of bio-flocculant (EPS- extra-cellular polymeric substances) [26]. The biomass concentration in the fermented broth (between 40–60 g/L) decides the concentration of EPS and $CaCl_2$ required for biomass settling. The settled sludge biomass (177 g/L) was treated sequentially by free-nitrous acid (10 mg FNA/ g biomass) and biosurfactant N-lauryl sarcosine (20 mg N-LS/ g biomass) [28]. Petroleum-diesel was used as solvent for lipid recovery (10 mL PD/g lipid) at 70 °C for 20 min [28].

The PD containing lipid was separated from the cell debris through phase separation. Loss in PD of 1% has been considered in lipid extraction and subsequent steps. The recovered PD-lipid mixture was reacted with methanol (6:1 molar ratio of methanol: lipid) for trans-esterification in the presence of 1% (w/w of lipid) H_2SO_4 as catalyst. The lipid extraction and trans-esterification efficiency was considered to be 92% and 97%, respectively [28]. After lipid extraction, the left waste is cell debris containing structural components of cell mass, including carbohydrates, humic substances, and proteins, which are released during the breakdown of cell wall. The cell debris can be used as fertilizer [29] or as animal feed supplement [27].

Mass and energy balance have been performed to produce 1 tonne FAMEs (Fatty acid methyl esters) or 10 tonnes blended biodiesel B10 using intermittent sludge feeding (30 g/L SS) strategy (Table 6). It was assumed that the fermentation would take place near a waste treatment plant; thus, no energy input has been considered for sludge transportation. No energy input has been assumed for sludge as it is considered a waste and normally energy is consumed for its treatment. The energy input due to chemical addition was taken from literature [18, 30, 31]. The energy consumed during the agitation, centrifugation and mixing were taken from the literature [22, 27, 32]. The mass of chemicals required for the biodiesel production is in the column ‘amount supplied’. For purification of 6490 kg crude glycerol, 1427 kg phosphoric acid is required resulting in 4073 kg purified glycerol and 3844 kg KH_2PO_4 (by-product). Energy consumed during glycerol purification was 18.4 GJ/ tonne FAMEs where crude glycerol and phosphoric used for glycerol purification were main energy imparting component. The sludge was concentrated using centrifugation to obtain desired SS concentration and washed with tap water. The total energy input in sludge washing and concentration step was 0.49 GJ/ tonne FAMEs where centrifugation (1 kWh/m^3) was the major energy contributing factor. The total energy input for lipid production (main fermenter) was 19.94 GJ/ tonne FAMEs. Out of which, aeration provided for cell growth was a major contributing factor. NaOH and H_2SO_4 used for sludge pre-treatment were second and third most contributors in lipid production, respectively. For seed fermentation, energy input was calculated to be 1.25 GJ (6.25% of production fermenter) based on inoculum size. During biomass settling, the energy input was calculated to be 4.21 GJ where the energy content of EPS and calcium chloride were the contributing factors. During lipid recovery, 1% loss in PD has been taken as an energy input (Table 6). The remaining (99%) diesel used during lipid recovery remained as fuel in the blended biodiesel (B10). The total energy input during lipid extraction was 4.45 GJ/ tonne FAMEs where loss of PD during lipid recovery was a major contributing

factor. The energy input for trans-esterification was 7 GJ/ tonne FAMEs where methanol was a major contributing factor.

Table 6

Mass and energy balance for 1 tonne biodiesel (10 tonne B10) production with 30 g/L intermittent sludge feeding with 49.73 g/L glycerol feed strategy

Step	Items	Unit energy input	Amount supplied	Total energy input (MJ)	Energy input (%)
Glycerol purification	Crude glycerol (MJ/kg)	1.67	6490 kg	10,838	19.79
	Mixing (W/m^3)	7.3	6.09 m^3	0.16	0.00
	H_3PO_4 (MJ/kg)	5.3	1426.7 kg	7562	17.22
	Energy input in glycerol purification (MJ)			18,400	33.6
Sludge concentration & washing	Centrifuge (kWh/m^3)	1	132 m^3	475	0.87
	Tap water (MJ/m^3) for washing	0.04	39.78 m^3	2	0.00
	Agitation (W/m^3)	7.3	50.38 m^3	13	0.02
	Energy input in~~for~~ sludge washing (MJ)			490	0.89
Production fermenter	Sterilization (MJ/kg steam)	26	4.86 kg	126	0.23
	NaOH (MJ/kg)	18.5	154.70 kg	2862	5.23
	H_2SO_4 (MJ/kg)	7.1	221 kg	1569	2.87
	Agitation (W/m^3)	7.3	44.2 m^3	112	0.2
	Aeration (kW/m^3)	1	44.2 m^3	15,276	27.9
	Energy input in Lipid Production (MJ)			19,944	36.43
Seed Fermenter	6.25% of Production fermenter (MJ)			1247	2.28
Biomass settling	EPS (MJ/kg)	14.36	97.22 kg	1396	2.55
	$CaCl_2$ (MJ/kg)	7.2	252 kg	1814	3.31
	Energy input in Biomass settling (MJ)			3210	5.86

Step	Items	Unit energy input	Amount supplied	Total energy input (MJ)	Energy input (%)
Lipid extraction	N-LS (MJ/ kg)	5.76	48.6 kg	280	0.51
	FNA (MJ/ kg)	3.2	24.3 kg	78	0.14
	Loss in PD (MJ/ kg)	45	90 kg	4050	7.4
	Agitation (W/m^3)	7.30	12.33 m^3	0.32	0.00
	Heating (kW/m^3)	2.72	12.33 m^3	40	0.07
	Energy input in Lipid Extraction (MJ)			4448	8.12
Trans-esterification	Methanol (MJ/kg)	20	326.6 kg	6532	11.93
	Sulfuric acid (MJ/kg)	7.10	19 kg	135	0.25
	Mixing (kWh/ kg biodiesel)	0.03	1000 kg	108	0.20
	Heating (kJ/kg biodiesel)	240	1000 kg	240	0.44
	Energy input in Trans-esterification (MJ)			7015	12.81
Total energy input (MJ)				54,754	
Total energy input (GJ)				54.75	
Energy output from KH_2PO_4 (GJ, by-product)				39.59	
Energy output from FAMEs (GJ)				37.80	
Total energy output (GJ)				77.4	
Net Energy gain (GJ)				22.65	
Energy Ratio				1.41	

The main contributing step in B10 production process was lipid production in fermentation (36.43%) followed by crude glycerol purification (33.6%) and trans-esterification (12.81%). The total energy input in the process was 54.75 GJ/tonne FAMEs. Energy output for 1 tonne FAMEs/ biodiesel was 37.80 GJ/tonne FAMEs (37.8 MJ/ kg FAMEs). However, 3844 kg KH_2PO_4 was produced during glycerol purification, which has commercial applications (buffering agent and fungicide)

and energy density of 10.3 MJ/kg. Taking account of the additional energy output KH_2PO_4 (39.59 GJ), total energy output was 77.4 GJ/tonne FAMEs with a net energy gain (total energy output—total energy input) of 22.65 GJ/tonne FAMEs and energy ratio (total energy output/ total energy input) of 1.41. Positive net energy gain and energy ratio of greater than 1 make the process energetically favorable.

To know the sludge contribution in lipid production, it is important to compare the energy balance for biodiesel production using intermittent sludge feeding with the purified glycerol medium. In a study conducted in INRS group, 51.67 g/L and 19.47 g/L lipid was produced at 96 h cultivating YL on purified glycerol medium (without sludge) containing 2.7 g/L KH_2PO_4, 0.95 g/L Na_2HPO_4, 0.2 g/L $MgSO_4.7H_2O$ and 9.5 g/L peptone [11]. For downstream processing, the same process was used as mentioned above except the catalyst employed in trans-esterification. In this process, NaOH (1% w/w lipid) was used instead of H_2SO_4 during the trans-esterification. Acid catalyst is used in sludge cultivated microbial lipid as it has free fatty acids (> 1%), which lead to soap formation in the presence of base catalyst during the trans-esterification. While the base catalyst is used for glycerol cultivated microbial lipid as it has FFA < 1%. Mass and Energy balance for 1 tonne biodiesel (or 10 tonnes B10) production using purified glycerol medium has been tabulated in Table 7. It can be observed from Table 7 that energy input for production fermenter for purified glycerol medium is 30.9 GJ/tonne FAMEs while it was 19.94 GJ/tonne FAMEs in intermittent sludge feeding strategy (Table 6). The nitrogen source (peptone) and trace elements supplied to assist cell growth in the purified 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 the production fermenter for purified glycerol medium (without sludge) is lower lipid concentration (19.47 g/L) obtained at 96 h when compared to sludge fortified with purified glycerol (25.35 g/L lipid). This led to increase in reactor volume in purified glycerol medium increasing energy input for aeration. This indicates that sludge not only acts as nutrient and nitrogen source, but also provides additional carbon for lipid production. The total energy input for biodiesel production using purified glycerol medium is 72.7 GJ/tonne FAMEs (Table 7) while it was 54.75 GJ/tonne FAMEs (Table 6) in sludge based medium. Hence, sludge has an important contribution in microbial lipid and biodiesel production.

Table 7

Mass and energy balance for 1 tonne biodiesel (10 tonne B10) production using purified glycerol medium (without sludge)

Step	Items	Unit energy input	Amount supplied	Total energy input (MJ)	Energy input (%)
Glycerol purification	Crude glycerol (MJ/kg)	1.67	8227 kg	13,739	18.9
	Mixing (W/m^3)	7.3	6.88 m^3	0.18	0.00
	H_3PO_4 (MJ/kg)	5.3	1906 kg	10,102	13.9
	Energy input in glycerol purification (MJ)			23,841	32.79
Production Fermenter	Sterilization (MJ/kg steam)	26	6.15 kg	160	0.22
	KH_2PO_4 (MJ/kg)	10.3	150.84 kg	1554	2.14
	Na_2HPO_4 (MJ/kg)	8.21	53.07 kg	436	0.60
	Peptone (MJ/kg)	17.3	530.73 kg	9182	12.63
	$MgSO_4.7H_2O$ (MJ/kg)	10.65	11.17 kg	119	0.16
	Agitation (W/m^3)	7.3	55.87 m^3	141	0.19
	Aeration (kW/m^3)	1	55.87 m^3	19,309	26.56
	Energy input in Lipid Production (MJ)			30,899	42.5
Seed Fermenter	6.25% of Production fermenter (MJ)			1931	2.66
Biomass settling	EPS (MJ/kg)	14.36	115.17 kg	1654	2.27
	$CaCl_2$ (MJ/kg)	7.20	322.46 kg	2322	3.19
	Energy input in Biomass settling (MJ)			3976	5.47
Lipid extraction	N-LS (MJ/ kg)	5.76	115.46 kg	665	0.91
	Loss in PD (MJ/kg)	45	90 kg	4050	5.57
	Agitation (W/m^3)	7.3	13.63 m^3	0.18	0.00
	Heating (kW/m^3)	2.72	13.63 m^3	33	0.05
	Energy input in Lipid extraction (MJ)			4749	6.53

Step	Items	Unit energy input	Amount supplied	Total energy input (MJ)	Energy input (%)
Trans-esterification	Methanol (MJ/kg)	20	327.36 kg	6547	9.01
	NaOH (MJ/kg)	18.5	22.01 kg	407	0.56
	Mixing (kWh/ kg biodiesel)	0.03	1000 kg	108	0.15
	Heating (kJ/kg biodiesel)	240.00	1000 kg	240	0.33
	Energy input in Trans-esterification (MJ)			7302	10.04
Total energy input (MJ)				72,698	
Total energy input (GJ)				72.70	
Energy output from KH_2PO_4 (GJ, by-product)				50.13	
Energy output from FAMEs (GJ)				37.80	
Total energy output (GJ)				87.97	
Net Energy gain (GJ)				15.27	
Energy Ratio				1.21	

Discussion

The previous studies on use of sludge media for microbial lipid production were performed on *T. oleaginosus* [8, 18]. When batch fermentation was conducted using only unwashed sludge (35 g/L SS) as a growth medium, lipid concentration was observed to decrease after 36 h of fermentation due to exhaustion of substrate [18]. When unwashed sludge (35 g/L SS) was supplied with additional carbon crude glycerol (40 g/L) in batch fermentation, the lipid accumulation phase was prolonged to 48 h and lipid concentration obtained was 10.35 g/L [18]. However, it was revealed that unwashed sludge contains a high concentration of heavy metals, which could inhibit microbial growth. Hence, washing of sludge was performed to reduce the concentration of heavy metals and washed sludge (35 g/L SS) fortified with 40 g/L crude glycerol resulted in 17.37 g/L lipid at 48 h of batch fermentation [8]. However, 35 g/L sludge SS gave consistent foaming in reactor throughout the fermentation. In the present study, fermentation time was prolonged to 96 h due to intermittent feeding of sludge (30 g/L SS) and purified glycerol (49.73 g/L) which resulted in 25.35 g/L lipid.

Several processes have been reported for biodiesel production from microbial lipid. The process employing commercial substrates (glucose, peptone and yeast extract) for fermentation and energy intensive solvents (chloroform and methanol) during lipid extraction was energetically unfavorable [27]. Lipid extraction step contributed 70.5% of total process energy input due to the high volume and energy content of chloroform and methanol [27]. Another study reported on lipid and biodiesel production using *T. oleaginous* cultivated on washed sludge (35 g/L SS) fortified with crude glycerol (40 g/L) in batch fermentation [8] had a net energy gain of 3.05 GJ/ tonne FAMEs with an energy ratio of 1.09 [27]. The energy savings in the reported process [27] is due to replacement of commercial substrates with waste substartes and replacement of solvent based lipid extraction with PD based recovery. In the present study, crude glycerol (additional carbon source) was purified using acidic treatment before being used during fermentation as it had a high potassium concentration and KH_2PO_4, produced during glycerol purification, was used as energy credits. Due to this, values of net energy gain (22.65 GJ/ tonne FAMEs) and energy ratio (1.41) are high.

Conclusion

In this study, wastewater sludge and purified glycerol (obtained after acid treatment of crude glycerol) were used together for lipid production using an intermittent feeding strategy. Intermittent sludge feeding strategy (30 g/L SS) resulted in a higher biomass (54.99 g/L) and lipid concentration (25.35 g/L) and lower requirement of antifoam (14.9 mL/L) when compared to single sludge feed strategy (45.67 g/L biomass, 19.16 g/L lipid and 32.5 mL/L antifoam, respectively). Sludge had important contribution in lipid production as it can provide nitrogen and nutrients for cell growth besides providing carbon for lipid production. Energy balance revealed that biodiesel production using intermittent sludge feeding strategy was energetically favorable.

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Availability of data and material

All data generated or analyzed during this study are included in this article and its supplementary information files.

Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Supplementary Information

Below is the link to the electronic supplementary material.

Supplementary file1 (DOCX 21 kb)

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