Effect of Sludge Concentration and Crude Glycerol Matrix as a Substrate on the Production of Single-Cell Oil by Oleaginous Yeast *Yarrowia lipolytica SKY7*

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Abstract: The disposal of excess crude glycerol produced by the booming biodiesel industry and wastewater sludge solid waste has become a severe problem, and alternate routes of use and valorization of these waste byproducts are needed. The use of cheaply available wastewater sludge solids in fermentation media is very much desirable to reduce the cost of production. The strains of *Yarrowia lipolytica* can assimilate a wide array of waste substrates, such as crude glycerol, waste cooking oil, starch wastewater, and cellulosic. This study optimized the concentration of wastewater sludge solids (5–35 g/L) to be used with crude glycerol in fermentation media to produce microbial oil as feedstock for biodiesel production. The results indicated that 20 g/L of sludge solids with 40 g/L of crude glycerol resulted in highest lipid content of 29.35% in 96 h. Further, assuming wet extraction of lipids, it was found that at least 11.2% or higher lipid content is required for this process to have an overall positive net solid waste reduction. Insignificant inhibition was observed by the crude glycerol used in this study as compared to pure glycerol, which proves it to be an adequate source of carbon substrate for lipid production.

Keywords: crude glycerol; *Yarrowia lipolytica*; lipid; biodiesel; municipal wastewater sludge; single cell oil

1. Introduction

Global energy demands are increasing day by day, but the non-renewable fuel resources are depleting rapidly and will be exhausted in the near future. Therefore, it is imperative to find alternate renewable and sustainable fuel resources [1]. Biodiesel produced from microbial feedstock oil has proven itself to be a strong candidate to replace petrochemical fuels. The biodiesel industry is rapidly expanding due to increasing awareness towards the alarming situation of global warming and the "food versus fuel debate" among policymakers, stakeholders, and the scientific community, giving rise to rejuvenated interest to develop a green biotechnological process for biodiesel feedstock microbial oil.

Despite its tremendous potential and ecological advantage, biodiesel production using microbial oil has been limited by the significant cost incurred by expensive carbon sources, such as glucose, to produce single-cell oil (SCO). According to various techno-economic reports, over 50% of the operational cost is realized due to the raw material (carbon and nitrogen sources) used in fermentation [2]. It is essential to find cheaper carbon and nitrogen sources to make the SCO production process economical.

Due to the increasing global population, the wastewater treatment sector is facing a monotonously increasing problem of an unmanageable volume of wastewater sludge for disposal. Different researchers

have developed many different processes to valorize the nutritional components present in wastewater sludge to produce bioplastics, biodiesel, a biopesticide, and extra polysaccharides [3–7].

The biodiesel industry has been exponentially expanding due to the increasing amount of awareness about global warming, the preservation of ecology, and sustainable development. While biodiesel provides a green alternative to current existing petrochemical fuel resources, due to the expansion of the biodiesel industry, an increasing amount of crude glycerol, a waste product of the biodiesel industry, is also generated [1,8]. Researchers have also tried to valorize the crude glycerol generated by biodiesel industry to produce other value-added products [3,7–10].

Recently, Zhang et al. [7] attempted to solve these two problems by developing a process for the production of single-cell oil using *Trichosporon oleaginosus.* The study simultaneously used crude glycerol and municipal sludge as a source of carbon and other nutrients. Deriving the concept of the utilization of crude glycerol and sludge together from Zhang et al.'s [7] study, when the combination of crude glycerol and sludge solids were applied (without any further supplementation of minerals or other nutrients) to the yeast *Yarrowia lipolytica SKY7*, the fungal strain was strongly inhibited by the media. A bioreactor run (10 L working volume) was effectuated with 35 g/L of sludge suspended solids as nutrient media, and it was supplemented with the crude glycerol used in this study as a carbon source in fed-batch mode (data not presented). When the media was inoculated with an actively growing culture of *Yarrowia lipolytica* (used in this study), no lipid accumulation occurred. In fact, a monotonously decreasing concentration of suspended solids was observed. Since glycerol was fed at different intervals (every 12 h) at a maximum concentration ranging between 10 and 20 g/L, no substrate inhibition was suspected. Under the microscope, the cells were actively viable. No increase in suspended solids (SS) was attributed to either a lack of minerals or an inhibitory effect of sludge hydrolysate components on microbial growth of *Y. lipolytica*. The SS decreased from 24 to 5 g/L monotonously in 96 h. Therefore, this study attempts to optimize the suspended solid concentrations of the sludge solids (5–35 g/L) to be used to produce single-cell oil using yeast *Yarrowia lipolytica*. Different sludge solid concentrations were used to prepare sludge media and were tested in shake flasks. To avoid any skepticism accounting for the limitation of nutrients, the media was supplemented with minerals and trace elements to see the independent effect of suspended solid concentrations.

2. Materials and Methods

2.1. Sludge Source

Secondary sludge was collected from the municipal wastewater treatment plant, CUQ (Communauté Urbaine de Québec), Quebec City on Monday, 8 December 2017. The sludge concentration was ranging between 5 and 10 g/L at the source. Therefore, the sludge was settled for 30 min at the site during collection. The sludge was concentrated by settling to 24 g/L; thereafter, it was centrifuged ($9000\times g$ for 15 min) to obtain a concentrated slurry of 47 g/L. The characteristics of the raw sludge are presented in Table 1.

Table 1. Characteristics of raw municipal wastewater sludge.

Characteristic of Sludge Solids	Value
Suspended solids (g/L)	24.32
Volatile suspended solids (g/L)	17.81
Total Carbon (mg/g)	401
Total Nitrogen (mg-N/g)	52.01
Total ammonical Nitrogen (mg-NH_4/g)	0.637
Total Phosphorus (mg/g)	1.23
pH	6.32

2.2. Oleaginous Yeast

The oleaginous yeast *Yarrowia lipolytica SKY7*, isolated from a forest soil sample (Forest Canada) by the previous researcher Kuttiraja et al. [3], was used. The seed culture was revived in yeast extract-peptone-dextrose (YPD) media and adapted in sludge media before being used as the inoculum in the experimental tests. A single colony of *Y. lipolytica* was inoculated in 30 mL of YPD media and incubated for 24 h at 28 °C in a rotary shaker incubator with continuous agitation of 200 rpm. The inoculum was prepared in sludge media supplemented with crude glycerol (40 g/L).

2.3. Media Preparation

The sludge media was prepared using different sludge solid concentrations (5, 10, 20, 30, and 35 g/L). Sludge was sterilized in an autoclave at 121 °C for 30 min. The sludge's thermal hydrolysis was assisted with the addition of base (NaOH, 0.11 g/g-sludge). The composition of sludge media used was (in g/L), sludge solids 5–35; Na_2PO_4 3.2; K_2HPO_4 8; $CaCl_2$ 2; $MgSO_4 \cdot 7H_2O$ 2; NH_4Cl 0.74; Glycerol 40; and 1000 µL of 1000× trace elements solution. The 1000× trace element solution comprises (in g/L) $FeSO_4$ 5.5; $MnSO_4$ 0.5; $CuSO_4$ 1; $ZnSO_4$ 2; $MoCl_3$ 0.2; the trace element solution was acidified by using hydrochloric acid resulting a 0.5 M HCl solution. Assuming an insignificant contribution of carbon and nitrogen from the sludge solids, the final C:N ratio of the sludge media is 100 (moles-C/moles-N). The crude glycerol used in this experiment was obtained from Bio-Liq, Quebec, Canada. The crude glycerol contained 675 g/L of glycerol. All the other components, such as methanol, free fatty acids (FFA), catalyst, and soap, were present in trace (<1–2 g/L) quantities (Table 2).

Table 2. Composition of crude glycerol.

Characteristic	Value
Density (g/mL)	0.909
Glycerol (g/L)	675
Methanol (g/L)	58
Soap (g/L)	ND
Catalyst (% *w/w*)	0.8
FFA (g/L)	ND
Water (g/L)	190
pH	9.01
Source	Bio-Liq, Saint-Marc-des-Carrières, QC, Canada

ND: Not detected; FFA: free fatty acid.

2.4. Experimental Plan

Five different flasks were prepared with different sludge-suspended solid concentrations, namely SS05, SS10, SS20, SS30, and SS35. The flasks contained 5, 10, 20, 30, and 35 g/L of sludge solids, respectively. Additionally, two more flasks SSCG and SSPG (without sludge) were prepared using crude glycerol and pure glycerol, respectively. The flasks SSPG and SSCG were used to understand the effect of the components of the crude glycerol matrix on growth and lipid production by the oleaginous yeast. The pH of the flasks was initially adjusted to 6.8; thereafter, the flasks were inoculated with 10% *v/v* inoculum of yeast *Yarrowia lipolytica*. The flasks were incubated at 28 °C for 4 days in a rotary shaking incubator with an agitation of 200 rpm. Samples were collected every 24 h and were analyzed further.

2.5. Analytical Procedure

Periodic samples (V mL) collected from the flasks were analyzed for CFU (colony forming unit), pH, suspended solids, lipid, citric acid, and glycerol concentration. The samples were collected in pre-weighed (W_i) falcon tubes. The samples were centrifuged in differential centrifuge at 5000× *g* for 20 min to collect clear biomass-free supernatant. The supernatant was collected in a fresh falcon tube

for further analysis. The obtained biomass was dried at 80 °C. The final weight of the falcon tubes (W_f) was noted down, and the biomass concentration was calculated according to the Equation (1).

$$SS = \frac{W_f - W_i}{V} \times 1000 \tag{1}$$

The collected supernatant was used to estimate the pH using pH-Probe. A small fraction of fermentation broth was serially diluted in 0.85% NaCl solution and used to estimate the CFU/mL by using the spread plating method. Lipid analysis was performed by extracting the lipid from the suspended solids using the chloroform-methanol extraction method [11]. The residual glycerol present in the supernatant fraction of the sample was estimated by using the spectrophotometer method as used in Kuhn, et al. [12]. All analyses were performed in duplicate and average values have been presented in this study.

The kinetic parameters were calculated using the experimental data points. For any two data points, for example, at time t_1 (X_1, S_1, P_1) and t_2 (X_2, S_2, P_2), the specific rates were calculated as per the following equations (Equations (2)–(7)).

$$t_{avg} = (t_1 + t_2)/2 \tag{2}$$

$$X_{avg} = (X_1 + X_2)/2 \tag{3}$$

$$\mu = \frac{1}{X} \times \left(\frac{dX}{dt}\right) = \frac{1}{X_{avg}} \times \frac{(X_2 - X_1)}{(t_2 - t_1)} \tag{4}$$

$$\check{r} = \frac{1}{X} \times \left(\frac{dP}{dt}\right) = \frac{1}{X_{avg}} \times \frac{(P_2 - P_1)}{(t_2 - t_1)} \tag{5}$$

$$\check{S} = \frac{1}{X} \times \left(\frac{dS}{dt}\right) = \frac{1}{X_{avg}} \times \frac{(S_2 - S_1)}{(t_2 - t_1)} \tag{6}$$

$$Y_{\frac{X}{S}} = \frac{(X_2 - X_1)}{(S_2 - S_1)} \tag{7}$$

Here X, S, and P are the biomass, the substrate, and the product, respectively; μ is the specific biomass growth rate; $\check{S}$ is the specific substrate consumption rate; $\check{r}$ is the specific product formation rate; and $Y_{X/S}$ is (biomass yield or lipid) w.r.t the substrate.

2.6. Statistical Analysis

To evaluate the statistical significance of the variation pattern and the effect of changing suspended solid concentrations on the microbial growth of the yeast *Y. lipolytica*, one factor analysis of variance (ANOVA) was performed using the MS-Excel 2013 Data analysis tool. The test was performed with the null hypothesis that no effect of changing the sludge solid concentration will be observed on microbial growth. The test confidence value was set to 95% with $\alpha = 0.05$. The validation of the hypothesis was performed using the standard Fisher ratio (F-test) for statistical variance.

3. Results

3.1. Biomass Growth

The normalized suspended solids (NSS) profiles in the case of all of the flask fermentations with different sludge solid concentrations are presented in Figure 1. In this context, normalized suspended solids (NSS) is the net increase in solid concentration obtained by subtracting the 0th-hour suspended solid concentration (after basic thermal sterilization) from the suspended solid concentration at any time, t.
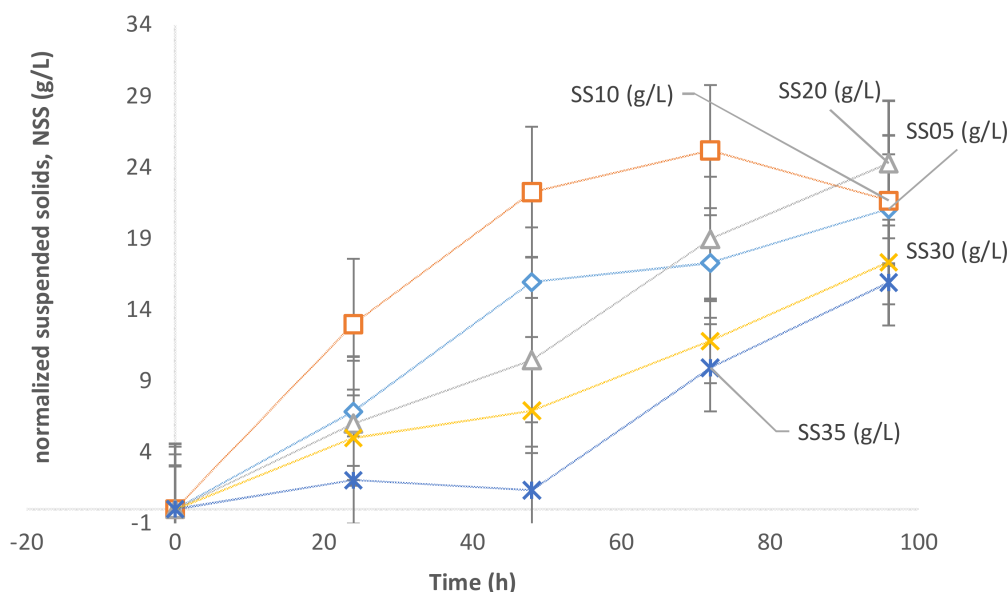
Figure 1. Net suspended solids (NSS) profiles for flask fermentation with different sludge solid concentration 5, 10, 20, 30, and 35 g/L.

The solid sludge concentrations depicted in Figure 1 (NSS) are solid concentrations, including intracellular lipids. In Figure 1, the suspended sludge concentration has a significant impact on the microbial growth of the yeast *Yarrowia lipolytica*. The maximum biomass was generated with a sludge concentration of 20 g/L (SS10). The net biomass generated was 24.29 g/L. The biomass, in this case, increased from an initial SS of 10.57 g/L (after sterilization) to a final biomass concentration of 34.86 g/L (Table 3). With further increase in sludge concentration beyond 20 g/L the net increase in biomass concentration decreases. Subsequently, the net biomass generated was in the order of (case of) 20 g/L > 10 g/L > 5 g/L > 30 g/L and 35 g/L with a net biomass value of 24.29, 21.66, 21.08, 17.34, and 15.93, respectively. The results indicate a clear inhibition to the microbial growth of the yeast *Y. lipolytica* at concentrations higher than 20 g/L.

Table 3. Suspended solids (SS) concentration for fermentations performed with different sludge solid concentrations.

Time (h)	SS05 (g/L)	Stdev.	SS10 (g/L)	Stdev.	SS20 (g/L)	Stdev.	SS30 (g/L)	Stdev.	SS35 (g/L)	Stdev.
−1	5.84	0.742	9.98	1.160	18.84	1.103	29.62	0.488	33.68	0.226
sterile 0	4.57	0.948	6.07	0.283	10.57	0.403	18.87	0.559	27.34	0.721
24	11.43	1.308	19.06	0.233	16.59	0.516	23.87	1.011	29.34	0.997
48	20.51	1.273	28.34	1.174	21.03	0.184	25.78	0.523	28.64	0.042
72	21.84	1.202	31.26	0.410	29.53	0.573	30.67	1.117	37.23	0.580
96	25.65	0.028	27.73	0.424	34.86	0.417	36.21	0.792	43.24	1.047

As is evident from Figure 2, the biomass growth in the presence of pure glycerol is relatively higher than that in crude glycerol. The maximum biomass concentration achieved in the case of crude glycerol is 20.36 g/L after 96 h of fermentation, while in the case of pure glycerol the maximum biomass concentration observed was 25.45 g/L. Papanikolaou and Aggelis [13] in their study found that crude glycerol (65% *w/w* glycerol content) poses some inhibition to the microbial growth of *Y. lipolytica* due to its matrix composition. Despite the probable matrix inhibition, the growth with crude and pure glycerol was comparable. This can be attributed to the fact that the strains of *Y. lipolytica* have higher tolerance towards the major impurities found in crude glycerol-like methanol, free fatty acids (FFA), basic salts (NaOH, KOH, and methoxides), and soap.
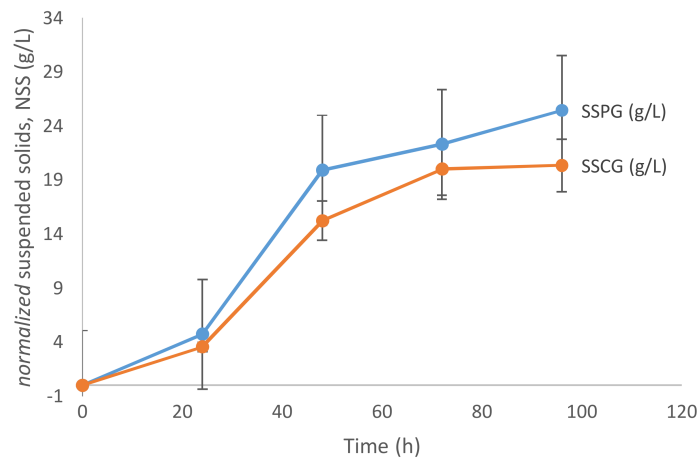
Figure 2. Net suspended solids (NSS) profile for the flask fermentation without sludge using pure glycerol (SSPG) and crude glycerol (SSCG).

Evidently, the presence of sludge imposes some inhibition on the microbial growth of the yeast. The results were further confirmed by CFU analysis data. In Figure 3A,B, it is apparent that the values of Log (CFU/mL) in the presence of sludge were in the order of 10–10.4 while in the case of flask fermentations without sludge these values were in the order of 11.5. Further slight inhibition was observed due to crude glycerol. The CFU/mL in the case of pure glycerol was found to be higher than that of crude glycerol (Figure 3A).
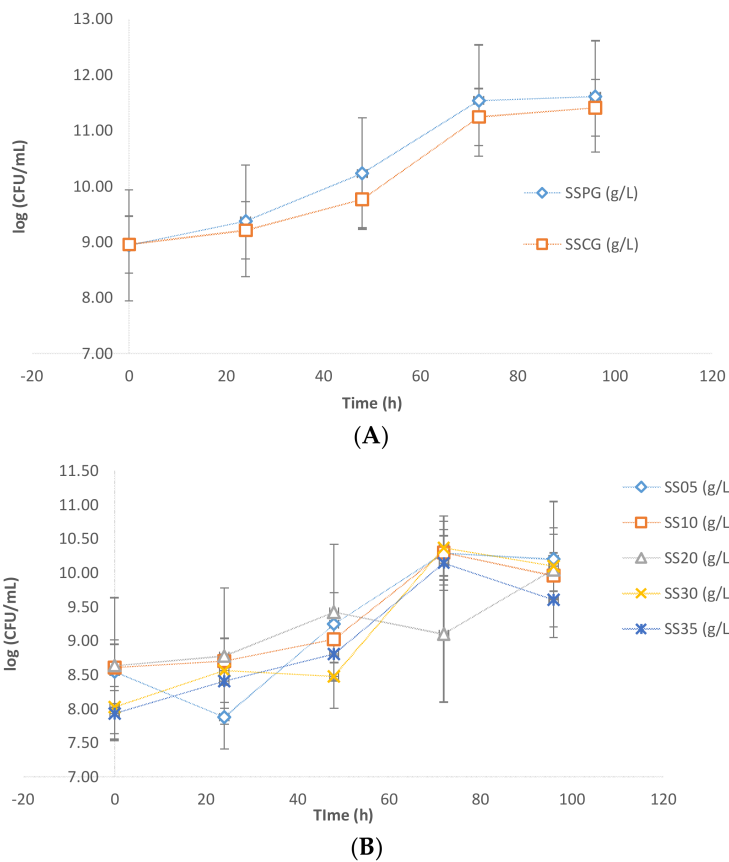

(**A**)

(**B**)

Figure 3. (**A**) CFU profiles comparison between pure glycerol (SSPG) and crude glycerol (SSCG); (**B**) CFU profiles for flask fermentation containing different concentrations of sludge solids.

Statistical Analysis of the net biomass generation using single factor ANOVA was performed using the MS-Excel 2013 data analysis tool. The duplicates data for the net biomass generation was used for the statistical analysis. The results are summarized in Table 4. According to the test results, the F ratio (of variances) value was 13.899, which was higher than the F critical value of 5.19217 as recommended by the Fisher's F test table for statistical significance for 95% confidence for the F(4,5) degree of freedom data set. Further, the probability value (p value) of the test was 0.006 < 0.05, less than the set p value for this test. Therefore, the results indicate that the null hypothesis can be easily rejected to conclude that the variation in sludge suspended solid concentrations have a significant effect on the biomass growth of the microbe.

Table 4. Single factor ANOVA analysis for net biomass generation data.

Summary							
Groups	**Sample**	**SS**	**Avg.**	**Variance**			
SS05	2	42.16	21.08	0.0032			
SS10	2	43.32	21.66	0.72			
SS20	2	48.58	24.29	0.6962			
SS30	2	34.68	17.34	2.5088			
SS35	2	31.8	15.9	4.3808			
Analysis of Variance							
Source of Variance	**Sum of Squares**	**dF**	**MSS**	**F**	**P**	**F Critical**	
Between groups	92.39424	4	23.09856	13.8997232	0.00644096	5.19216777	
Within groups	8.309	5	1.6618				
Total	100.70324	9					

3.2. Lipid Production

The maximum lipid content (29.35% w/w) was observed with the sludge solid concentration of 20 g/L, whereas the lowest lipid content was observed with the 5 g/L sludge solid concentration (Figure 4). The lipid content in the cases of the 10, 30, and 35 g/L sludge concentrations were 20–24% w/w. A possible account for such variation is the (carbon and nitrogen) nutrients furnished by the sludge. If the nitrogen concentration is too low, then the lipid accumulation process is negatively affected by virtue of the fact that a minimal concentration of nitrogen is required for basal cell metabolism and accumulation activity. On the other hand, if an excess of nitrogen is available (low C:N ratio), the cell favors growth metabolism as against the accumulation of carbon as lipid reserves [3]. This suggests that an optimum level of nitrogen is required for the microbes to perform lipid accumulation. The presence of different concentrations of sludge solids furnishes different concentrations of the nutrients (carbon and nitrogen), resulting in different sub-optimal C:N ratios for lipid accumulation.

In experiments performed without sludge to compare pure glycerol and crude glycerol, the lipid contents were 35% and 28% for crude glycerol and pure glycerol, respectively (Figure 4B). This result is in agreement with the results obtained by previous studies [3,8,13,14]. Similar to biomass, inhibition of lipid accumulation was also observed by the presence of sludge (Figure 4A,B). Maximum lipid content with sludge (in case of 20 g/L) was 29.35% (w/w), while without sludge the maximum lipid content was 37% (w/w). Further, crude glycerol is approvingly a favorable source for lipid accumulation using *Y. lipolytica*. A comparison between lipid content and the yield of previous studies and this study has been presented in Table 5.
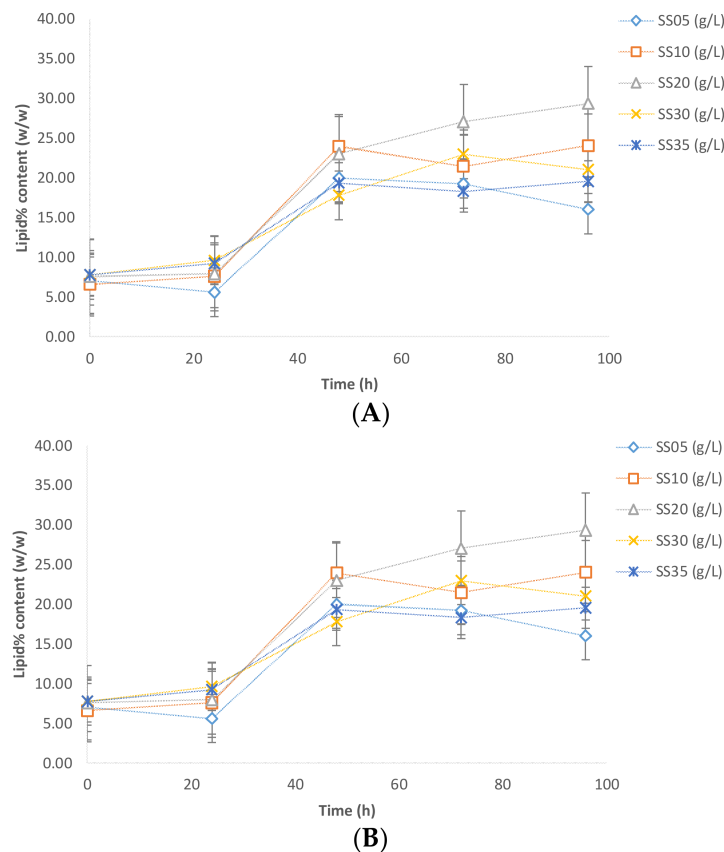
Figure 4. Lipid % (*w/w*) content data for (**A**) Flask fermentations at different sludge solid concentrations; (**B**) Flask fermentation without sludge to compare pure glycerol (SSPG) and crude glycerol (SSCG).

Table 5. Lipid production by different strains of *Yarrowia lipolytica* using crude glycerol from previous studies.

Organism	Glycerol Content % *w/w*	Gly. Conc. (g/L)	Time (h)	Biomass (g/L)	Lipid Content (% *w/w*)	Yield (g-Lipid/g-Glycerol)	Ref.
Y. lipolytica ACA-YC 5030	81	100	216	20.8	15–20	-	[15]
Y. lipolytica SKY7	78.3	112.5	96	14.08	44.60	0.192	[3]
Y. lipolytica A101	80	50	120	6.87	24.9	0.035	[16]
Y. lipolytica SKY7	67.5	40	96	20.36	34.99	0.295	This study
Y. lipolytica SKY7	67.5	40 (with sludge)	96	24.29	29.35	0.253	This study

3.3. Glycerol Consumption

Many researchers have studied the effect of crude glycerol composition and found that crude glycerol obtained from the biodiesel industry is a very suitable carbon source for lipid production [16]. In this study, crude glycerol obtained from the biodiesel industry via Bio-Liq, Saint-Marc-des-Carrières, QC, Canada was used. An initial C:N ratio of 100 (mol-C/mol-N) was maintained in all of the experiments as a C:N ratio of 75–100 has been reported to be optimum for lipid production [3,16]. The glycerol consumption profile is presented in Figure 5, and it is observed that glycerol was rapidly consumed in the first 24 h of fermentation in all of the different sludge solid concentrations. After 96 h of fermentation, the residual glycerol concentration was found to be in the range of 3–9 g/L. Similar glycerol consumption profiles were observed in case of experiments performed without sludge solids in the media. The residual glycerol concentration in the case of zero sludge solids was also in

the range of 7.5–9.9 g/L. As seen earlier in the SS and lipid data, the crude glycerol used in this study apparently poses insignificant inhibition due to the traces of impurities. Also, no significant difference in substrate consumption rates was observed between pure glycerol and crude glycerol (Figure 5B). An untraceable amount of citric acid was produced in these experiments, which can be attributed to the composition of the crude glycerol matrix and evolving C:N ratio. These results conform with the findings of previous studies [15].

(**A**)

(**B**)
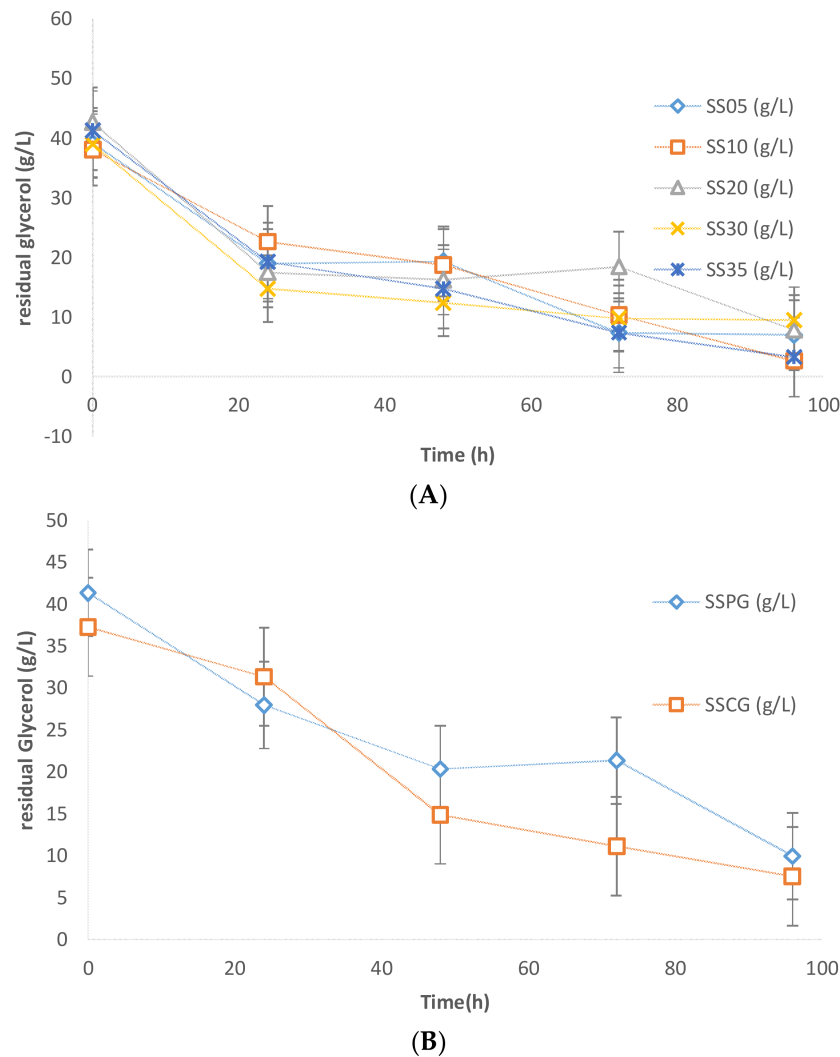
Figure 5. Glycerol consumption profile for (**A**) Flask fermentations at different sludge solid concentrations; (**B**) Flask fermentation without sludge to compare pure glycerol (SSPG) and crude glycerol (SSCG).

3.4. Yield and Productivity Analysis

The yield and productivity data for different suspended solid concentrations have been presented in Figure 6. As is evident from the graphics, with an increase in sludge suspended solid concentration higher than 20 g/L in the media, the total biomass (including lipid) yield w.r.t glycerol decreases monotonously. This, as discussed earlier, is the clear indication of the growth inhibition caused by sludge solids.
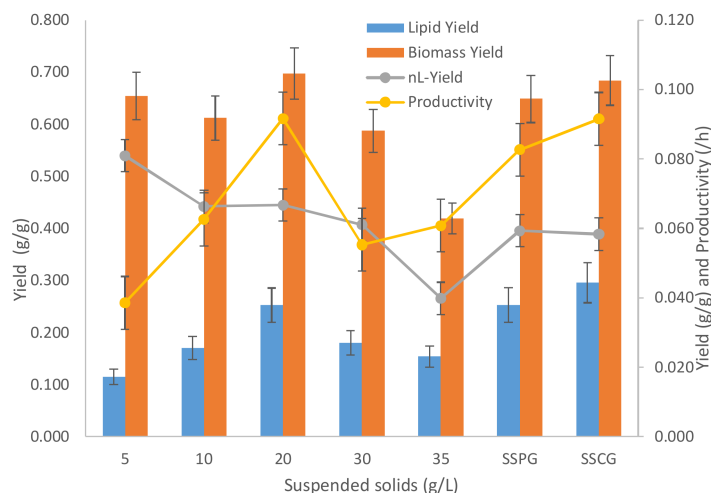
Figure 6. Yields and productivity comparison for flask fermentations at different sludge solid concentrations.

The significant decrease in biomass yield can be accounted for with the hindrance caused by the sludge solids at higher concentrations. At higher concentrations of sludge solids, any inhibitory compound furnished by sludge solids will also be present in higher concentrations, which can affect the microbial growth. Further, a higher solid concentration can cause mass transfer limitations for nutrients and oxygen. A higher solid concentration can cause inhomogeneity resulting in anaerobic pockets in the flask fermentation, which can severely affect the microbial growth by creating hostile and stressful conditions [17]. Under stressful conditions, cells invest a significant portion of their resources in the form of maintenance energy [18]. Retarded microbial growth is beneficial for any kind of storage molecule accumulation as can be seen in this study that lipid yield increases when the sludge solid concentration is increased from 5 to 20 g/L (Figure 6). Increasing the sludge solid concentration beyond 20 g/L decreases the lipid yield as the inhibitory effect of sludge solids scale the complete cellular metabolism causing neither growth (non-lipid biomass) nor accumulation of lipids.

The maximum lipid accumulation yield of 0.253 g-lipid/g-glycerol was found with 20 g/L of sludge solid concentration. The maximum productivity with 20 g/L sludge concentration was 0.092 g-lipid/L.h. The results of this study are comparable with previous studies done with this strain without sludge (Table 5).

Comparing the lipid accumulation in the presence of the optimum sludge solid concentration (of 20 g/L) with experiments performed without sludge (SSCG, using crude glycerol), it is evident that while using municipal secondary sludge in the media the lipid yield and productivity are comparably invariant as compared to fermentation performed in absence of sludge (SSCG). When sludge was used, the biomass and n-Lipid biomass yields were 0.697 and 0.445 g/g, respectively. On the other hand, the biomass and n-Lipid biomass yields were 0.684 and 0.389 g/g, respectively, when sludge was not used (SSCG). In both the cases, the lipid content and glycerol usage were not compromised significantly. The lipid yield with sludge (20 g/L) was 0.253 g-lipid/g-glycerol, while the one without sludge (SSCG) was 0.295 g-lipid/g-glycerol. A comparison between the two cases has been presented in Table 6.

Table 6. Summary of lipid yield, biomass yield, productivity, lipid content, and biomass concentration of this study.

SS (g/L)	Lipid Yield (g/g)	Biomass Yield (g/g)	nL-Yield (g/g)	Productivity g-Lipid/L.h	Lipid Content (% *w/w*)	Net Biomass Conc. (g/L)	n-Lipid Biomass (g/L)
5	0.115	0.655	0.540	0.039	16.02	21.08	17.38
10	0.170	0.612	0.442	0.063	24.05	21.66	15.65
20	0.253	0.697	0.445	0.092	29.35	24.29	15.49
30	0.180	0.587	0.407	0.055	21.04	17.34	12.03
35	0.154	0.419	0.266	0.061	19.57	15.93	10.09
SSPG	0.253	0.649	0.396	0.083	28.13	25.45	17.51
SSCG	0.295	0.684	0.389	0.092	34.99	20.36	11.57

Thus, it can be concluded that sludge at a higher concentration causes significant inhibition to microbial metabolism. If the optimum concentration of sludge solid is used for fermentation, then the lipid productivity and lipid content is not compromised as compared to its negative control (SSCG) where fermentation was performed without sludge. Use of crude glycerol (SSCG) as compared to pure glycerol (SSPG) is also beneficial in all aspects as it gives comparable lipid yield and higher lipid productivities. The productivity of crude glycerol (SSCG) is 0.092 whereas lipid yield for pure glycerol is 0.083 g-lipid/L.h. Therefore, crude glycerol is a preferable substrate which enhances the lipid production with a slight inhibition to microbial growth.

3.5. Specific Rates and Kinetics Analysis

The specific rate of biomass formation, lipid accumulation, and glycerol consumption have been presented in Figure 7 for various sludge solid concentrations. The specific biomass growth rate for 5 and 10 g/L solid concentration was monotonously decreasing throughout the fermentation. On the other hand, the specific biomass growth rate increased for the cases of 20, 30, and 35 g/L, where the SS of 20 g/L sludge fermentation has the highest specific rates of biomass growth (Figure 7A).

In the case of lipid accumulation, during the 48 h of fermentation in the cases of 5, 10, and 20 g/L sludge SS, the specific lipid accumulation reached a maximum and started decreasing thereafter (Figure 7B). At the 60th hour, the sludge fermentation with 20 g/L of sludge solids had the highest specific lipid accumulation rate while in the cases of 5 and 10 g/L sludge solids, the lipid accumulation ceased. A major fraction of lipid accumulation occurred after 30 h of fermentation. This is probably due to the fact that during fermentation, when carbon and nitrogen are consumed dynamically (in disproportionate quantities), the C:N ratio of the media changes inducing a metabolic shift to lipogenesis phase [16,19]. The specific glycerol uptake rates were similar in all cases of sludge solid concentration. At the beginning of the fermentation, the specific uptake rates were high mainly because of the active biomass synthesis that takes place during this period (Figure 7C). Thereafter, the rate of glycerol consumption is significantly lower in all of the cases of sludge solid concentration.
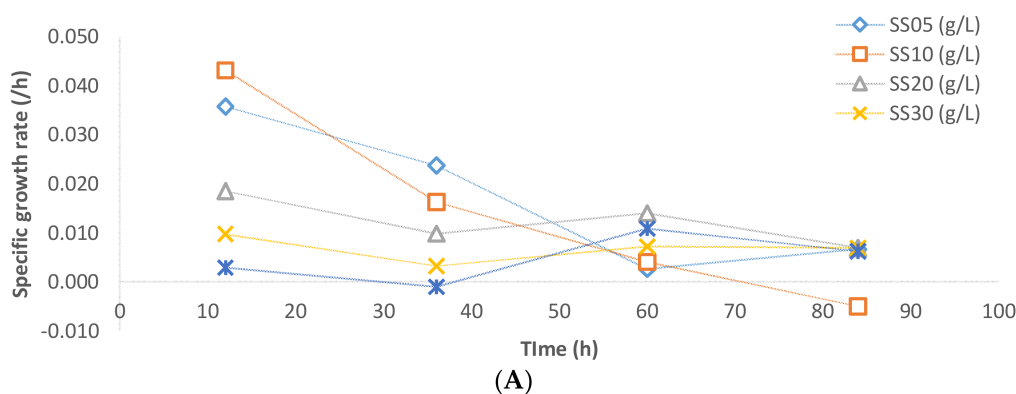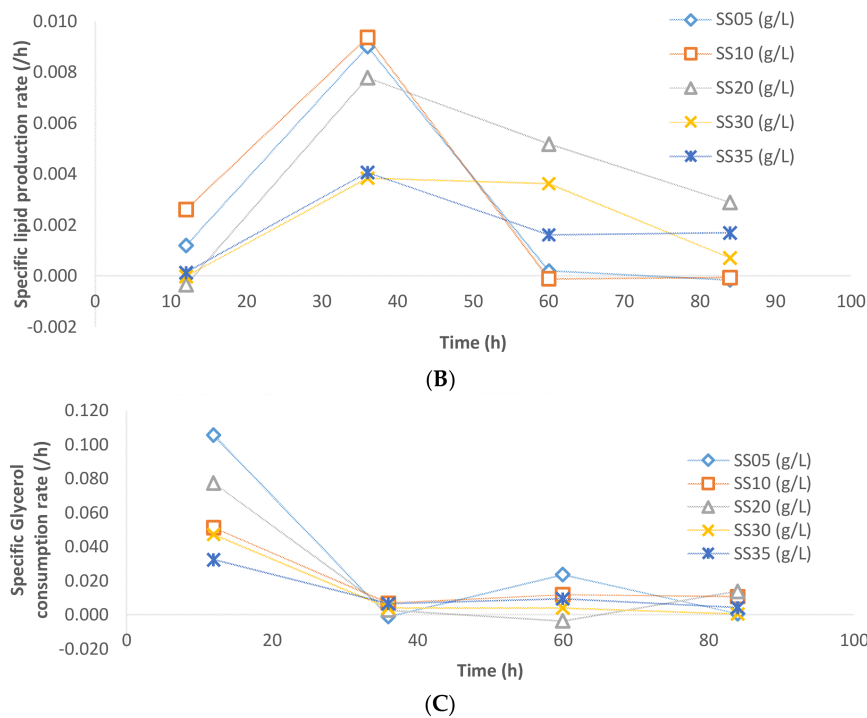

(A)

Figure 7. *Cont.*

(B)

(C)

Figure 7. (**A**) Specific growth rate, (**B**) specific lipid production rate, and (**C**) specific glycerol consumption rate comparison between fermentations performed with different concentrations of sludge solids.

A comparison of the specific growth rate, product formation rate, and glycerol uptake rate between the cases of sludge solids, (20 g/L), crude glycerol, and pure glycerol is presented in Figure 8A. As is apparent from Figure 8A, the pure glycerol (without sludge) resulted in the highest specific biomass growth rate followed by crude glycerol and then sludge (optimized conc. 20 g/L). Exceptionally near zero specific biomass growth rate in the first 24 h of fermentation was observed with higher suspended solid concentrations (30 and 35 g/L, Figure 7A). The stagnant biomass concentrations can be the results of the microbial action on the metabolizable suspended solids that are accompanied by sludge-like humic substances, loosely bound extra polysaccharides, nucleic acids, and other organics. The biomass growth of the microbe is compensated for by the decrease in sludge suspended solids concentration, which does not necessarily become completely assimilated (intracellularly) by the microbe. Similar observations were made by previous researchers [5,20].

The specific lipid accumulation rates were also highest for pure glycerol until the 36th hour of fermentation. Thereafter, the specific lipid accumulation rates decreased monotonously for the case of sludge and pure glycerol, but in the case of crude glycerol the specific lipid accumulation rates lagged at the 36th hour and major accumulation occurred after 50 h of fermentation (Figure 8B). The specific glycerol uptake rates followed the trend of the specific lipid accumulation rate, where crude glycerol showed lower (but steady) consumption rates until the 36th hour of fermentation before it started decreasing from the 36th hour onward. In the case of sludge and pure glycerol, the glycerol uptake rates decreased continuously until 40 h of fermentation and then remained insignificantly low until the end of the fermentation.
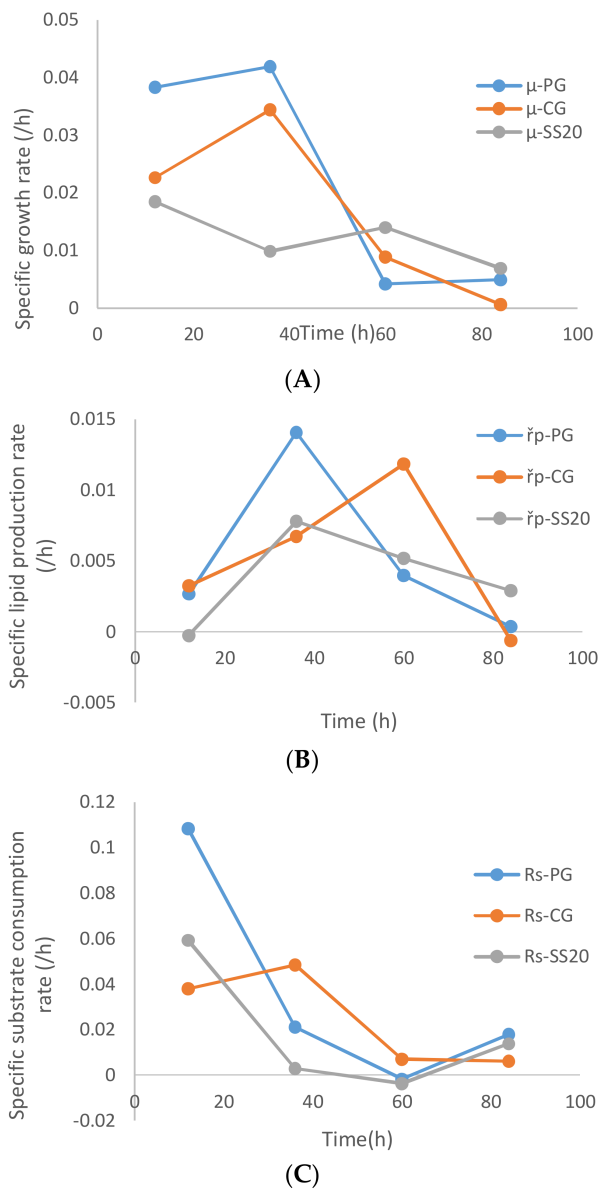
Figure 8. (**A**) Specific growth rate, (**B**) specific lipid production rate, and (**C**) specific glycerol consumption rate comparison between fermentations performed with sludge (SS20, 20 g/L), crude glycerol (CG), and pure glycerol (PG).

3.6. Degree of Sludge Disposal Problem

During the process when the sludge is integrated into the media with the expectation that it will provide an alternate solution for sludge disposal toward the end, it is essential to analyze to what extent the problem is solved. A simple mass balance over the amount of solids available for disposal is used to evaluate the degree of sludge disposal (DSD) problem.

$$DSD\ (\%) = \frac{Solid\ waste\ to\ dispose\ after}{solid\ waste\ to\ dispose\ before} \times 100 \tag{8}$$

At the zeroth hour, the sludge to be disposed of is the sludge used in the process. For example, in the case of fermentation conducted with 20 g/L of sludge, at the zeroth hour 18.84 g of sludge was fed into the process (Figure 9). Thus, the sludge to dispose of at the zeroth hour (SDZ) is 18.84 g. After the process is over, 34.86 g of solids are left which contain 10.23 g of lipids (lipid content 29.35%).

Assuming 90% of these solids are recovered as microbial lipids, then the solids that remain to dispose of are 25.65 g-lipids. There are dry and wet methods of lipid extraction available in the literature. In the case of dry lipid, no further reduction is possible for these disposable solids, but in the case of wet extraction methods using detergents [21], it is possible to dissolve a significant portion of de-fatted biomass by the chemical action of detergents. This soluble fraction in major part includes the proteins, carbohydrate, and nucleic acid (structural) components of the cell. Assuming 40% of the mass is lost during the extraction, the solids disposable after the process (SDP) will be 15.39 g [22,23]. Therefore, the DSD value for this case will be 81.69%. This indicates that there was a net reduction in the amount of solid waste to be disposed of. The lipid content is a very crucial parameter in determining the DSD of the process.

When the lipid content was varied from 5 to 70%, it was observed that at least 11.2% of lipid content is essential so that the situation remains neutral (with no net increase of solid waste) if not better off (Figure 10). When the lipid content is increased, the amount of sludge available to dispose of after the process reduces further.
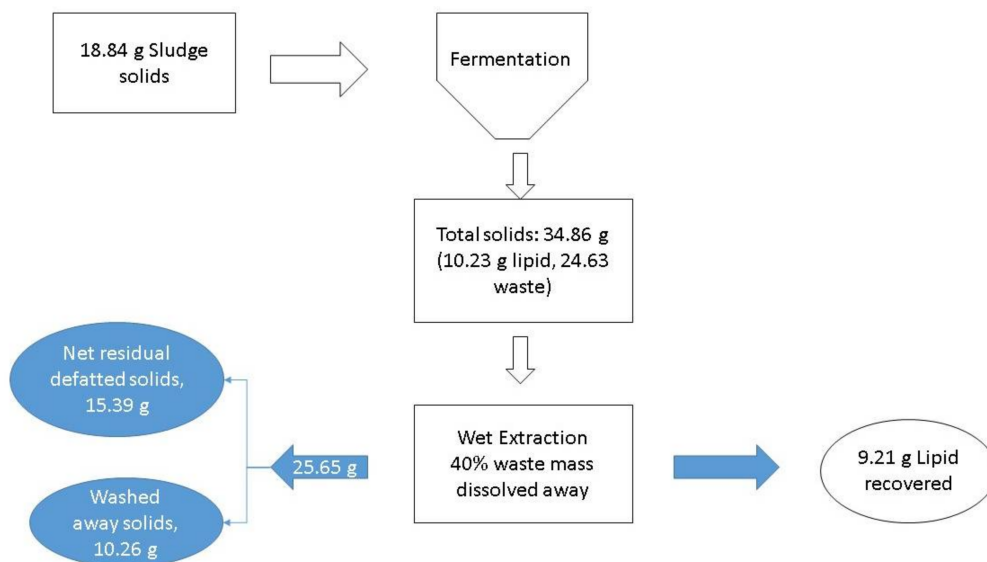

Figure 9. Mass balance illustration for the process using 20 g/L sludge solids (optimized concentration).
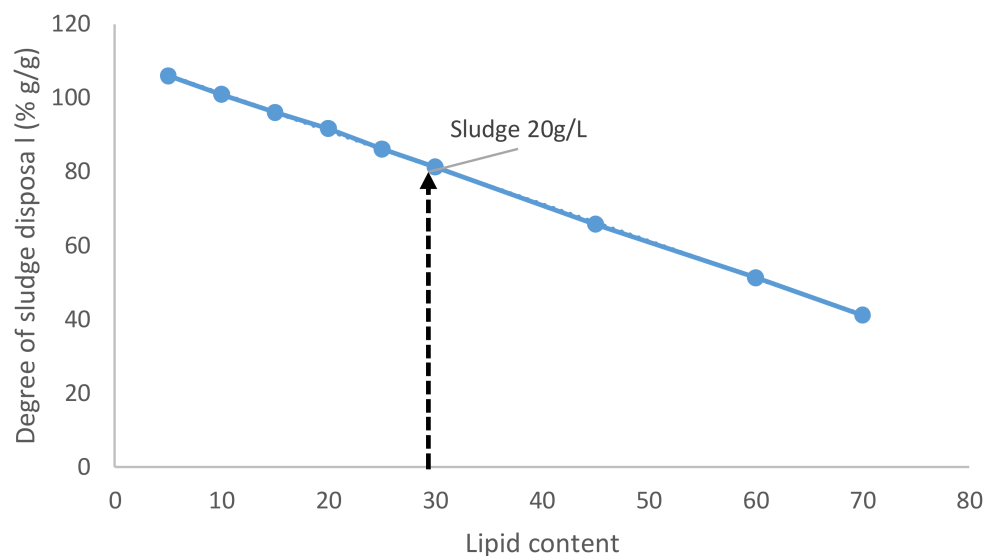

Figure 10. Degree of sludge disposal reduction variation with lipid content.

4. Conclusions

In this study, the SS concentration of municipal wastewater sludge solids (to be used along with crude glycerol as media) to produce microbial lipids using the oleaginous yeast *Y. lipolytica* was optimized. The results indicate that sludge renders a significant inhibition to microbial growth at higher concentrations and 20 g/L of sludge solid concentration is the optimum concentration for maximum lipid accumulation by the yeast. Further, the industrial crude glycerol used in the study imposed a slight inhibition to the microbial growth due to the matrix composition of crude glycerol as compared to pure glycerol and it is a very suitable source of carbon for lipid production. The use of optimum concentrations of sludge gives comparable lipid production yield and productivity as with crude glycerol only. The maximum biomass concentration, lipid content, lipid yield (w.r.t. glycerol), and productivity with 20 g/L sludge solids were 34.86 g/L, 29.35% (*w/w*), 0.295 g/g, and 0.092 g/L.h, respectively. Using sludge in growth media solves the problem of sludge disposal, and the degree of sludge disposal depends upon the lipid content and downstream extraction methods. It was found that a minimum of 11.2% (*w/w*) lipid content is recommended in this process to have no net increase in solid waste to dispose before and after the process. This study demonstrates the possibility of using municipal sludge solids (at an optimum concentration) as a component in growth media along with crude glycerol without compromising the lipid productivities. Further research is required to reduce the minerals and trace elements used in this study to obtain a minimal media composition to reduce the overall cost of production of microbial lipids.

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