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Kinetics of lipid production at lab scale fermenters by a new isolate of *Yarrowia lipolytica SKY7*

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Highlights

- A glycerol concentration of 85.5g/L and C/N ratio of 75 with inoculum volume of 6.25%v/v found optimal
- Biomass concentration of 29.5g/L with 50%w/w lipid accumulation was obtained in 15L scale
- The biomass and lipid yield was reached to 0.332g/g and 0.179g/g respectively

Abstract

The objective of this work was to study the kinetics of lipid production at lab scale fermenters by a new isolate of *Yarrowia lipolytica* SKY7. The model terms glycerol concentration inoculum and C/N ratio with inoculum were found to be significant for lipid production. Lipid production was found to be higher in glycerol 82.5g/L, C/N ratio 75 and inoculum volume 6.25%. Optimized culture conditions were tested at 15L bench scale reactor. The biomass concentration and lipid content obtained was 29.5g/L and 50% (w/w), respectively. The yield coefficients were calculated and found to be 0.332g/g (g biomass/g of glycerol) of biomass and 0.179g/g (g lipid /g glycerol consumed) for lipid. Observed rates of lipid production show lipid production from 30h of fermentation. Out of the total glycerol consumed, 41.1% glycerol was converted into biomass, lipid, and citric acid.

Keywords: Microbial lipids, *Yarrowia lipolytica*, Fermentation, RSM optimization, Biomass

1. Introduction

Bio-diesel is the clean, biodegradable, renewable, and non-toxic fuel produced by bacteria, yeast, fungi and algae (Li et al., 2008; Okamura et al., 2015; Poli et al., 2014; Ruan et al., 2014). Biodiesel is considered as the green fuel since it does not release excess CO_2 and sulfur (Gustone, 2009). At present, identification and effective utilization of low-cost raw materials such as starch, crude glycerol, lignocellulosic, volatile fatty acids and other waste materials for biofuel product are a matter of active research(Li et al., 2015; Liu et al., 2015; Vajpeyi & Chandran, 2015; Xue et al., 2010). Currently, biodiesel is produced from vegetable oil, animal fat and used cooking oil; however, this practice is expensive and created food versus fuel competition (Demirbas, 2005; Gui et al., 2008). The lack of availability in sufficient quantity and high sulfur content and other impurities makes cooking oil as unsuitable raw material for the biodiesel application. Even though extensive research has been conducted to find a better raw material for microbial lipid (or oil) production; crude glycerol gained special importance among the researchers because it is available as a by-product at the biodiesel production site at low cost (Yazdani & Gonzalez, 2007). The other principle industries that produce glycerol as a by-product are petrochemical industry and soap and saponification industry (Christophe et al., 2012). However, the microbial conversion of crude glycerol into lipids are not economical due to the following reasons. Primarily the capacity of glycerol tolerance and optimum concentration of glycerol required for lipid production varies based on the type of microbial strain. Tolerance of different inhibitors presents in the crude glycerol and adaptation to batch to batch variation of glycerol and inhibitors. Secondly the influence of raw material. That is the chemical composition of the crude glycerol, which varies based on the catalyst used in the transesterification process. This includes the glycerol content of crude glycerol solution (ranges from 36-96%w/w) and other major by-products such as methanol, soap, catalyst salt, non-

glycerol organic matters, and water (Ayoub & Abdullah, 2012; Chatzifragkou & Papanikolaou, 2012).

The increased interest in microbial oil production initiated the bio-diesel industry to enter into a new generation fuel production. Substantial research has been conducted and varying lipid accumulation capacities of different microbial strains ranging 20 to 70% w/w have been reported. Among the microbes bacteria, yeast and fungi are suitable for utilizing crude glycerol as the sole carbon source to produce lipid (André et al., 2010; Okamura et al., 2015; Poli et al., 2014).The main advantage of microbial oil production over oil crops is the fast growth, high lipid content and non-seasonal(Dias et al., 2015). However, a complete bioprocess for lipid production from microbes at low cost has not been developed.

Apart from high lipid accumulating capacity of a microbial strain, the second most important factor is the macro and micronutrients required for cell growth. The biochemical pathways of lipid production clearly dictate that the citrate is the precursor for the triacylglycerols (TAG) biosynthesis (Ratledge & Wynn, 2002). The limitation of nitrogen availability in the media triggers the pathway towards the lipid biosynthesis. Therefore, a limited supply of nutrients is one of the major concerns in the microbial lipid synthesis. Considering the fact of high lipid production in a feasible way requires primarily a well-optimized process that could flexible to adapt the variation in media composition and produces the end product without any huge variation. Yet, in this case, more than one culture parameter determines the biomass and lipid production. Due to the complex nature of the process, a conventional way of optimizing the process marks the research laborious and time-consuming. Statistical tools are (RSM-Response Surface Methodology) well used in optimizing the culture growth media and to study the interaction between the various growth parameters.

In this study, Box-Behnken design was used to understand the significance of principle parameters such as glycerol concentration, C/N molar ratio and inoculum volume for biomass production and lipid synthesis. In the case of *Y.lipolytica*, lipid accumulation was reported mostly with high glycerol concentration (100g/L) with high initial C/N ratio. However, in most of the cases, the glycerol was not completely utilized even after 5 days (120h) of fermentation (Kuttiraja et al., 2015). This could lead to a high waste of glycerol. At the same time, the high concentration of glycerol can influence the growth and finally reduces the yield of lipid production(Tchakouteu et al., 2015). Since the strain, *Y.lipolytica* SKY7 is a new isolate it is highly important to determine the optimum culture parameters to obtain a high biomass and lipid to develop an economically feasible process. Due to the above reason, a statistical method was introduced to know the significance of the important variables and its influence on biomass and lipid production. Meanwhile, the flask scale studies (statistical optimization) has limitation to draw a complete conclusion of process kinetics. To evaluate the RSM optimized culture parameters a bench scale fermentation was also conducted to assess the optimal kinetic parameters of lipid production in crude glycerol as a growth medium.

2. Materials and method

2.1.1. Culture and fermentation condition

The pure culture of *Y.lipolytica* SKY7 was maintained at YPD slants (Yeast extract 10g/L, Peptone 10g/L dextrose 20g/L and agar 20g/L). The inoculum was prepared in YPCG (yeast extract 10g/L, peptone 10g/L, crude glycerol 20g/L) media. A loop full of pure colony was aseptically transferred to the media and it was incubated at 28^0C in an agitating shaker at 180 rpm for 24h. Well grown inoculum was transferred to a sterile centrifuge tube (500mL) and centrifuged at 6000 rpm for 10 minutes and the supernatant was discarded under the sterile

condition and then the cells were re-suspended in 250mL of sterile distilled water. This suspension was used as the inoculum for all the study.

The crude glycerol used in the present study was kindly provided by a biodiesel producing industry in Quebec region, Canada. In all the cases the crude glycerol was used without any treatment. The glycerol solution used in this study contains 78%w/w glycerol, methanol 1.28% w/w, soap 2.4%w/w, water 2.48% w/w and catalysts 0.12%w/w.

2.1.2. Optimization of culture parameters (Box-Behnken design)

The glycerol concentration was varied from 75 to 100g/L. The glycerol stock solution was diluted according to the required glycerol concentration in the medium. The C/N molar ratio was calculated based on the carbon available in glycerol. Though several other components in the glycerol solution such as methanol, free fatty acids (FFA) and soap can contribute carbon, in the present investigation only glycerol was taken into account because the other component were comparatively low in concentration. In all experiments, 1g/L yeast extract was added to meet the trace elements requirement. $(NH_4)_2 SO_4$ was used as the nitrogen source. The required C/N molar ratio was adjusted by varying the $(NH_4)_2 SO_4$ concentration. Based on the above information a full-fledged factorial design was made with the design expert software (Design Expert 8- USA) and the process was optimized to obtain maximum biomass and lipid concentration. The range of process parameters was selected based on previous lab experiments. The C/N molar ratio was selected in the range of 75 to 150, glycerol concentration from 75g/L to 100g/L, inoculum volume 2.5 to 10% v/v. The initial pH was adjusted to 6.5 and the other media components were same as described in previous sections.

2.1.3. Validation of design

The validation of the parameters values obtained was conducted by comparing the values given by the software and the correlation of the predicted versus experimental values were performed.

2.2. Fermenter Studies

The validated experimental conditions were used further to verify the process in 15L bioreactors with working volume 10L (Biogenie, Quebec, Canada) equipped with programmable logic control (PLC) system, which included a dissolved oxygen (DO) probe, antifoam, mixing, aeration, temperature, and pH. The above parameters were maintained using the program ifix 3.5, Intellution, USA. The pH meter (Mettler Toledo, USA) was calibrated using buffers pH 4 and 7.0 (VWR, Canada). The DO probe was calibrated to zero using sodium sulfate and to 100% by purging air in distilled water. The fermentation media was fortified with 85.5g/L crude glycerol and the C/N molar ratio was maintained at 75. Well, grown (24h) inoculum was transferred aseptically. The fermentation was conducted up to 72h and the samples were collected at every 6h intervals and analyzed for biomass concentration, biomass lipid content, and residual glycerol. Citric acid was measured at every 24h intervals.

2.3. Analytical methods

Fermented broth (25 mL) was used for biomass and lipid quantification. The collected samples were centrifuged at 4000 rpm for 10 minutes and the supernatant was collected in a clean tube. The biomass was washed twice with distilled water and the cell pellets were collected by centrifugation. The samples for biomass quantification were transferred to a clean pre-weighed aluminum pan and it was kept at 105^0C until it reached a constant weight. A chloroform-methanol based conventional method of lipid extraction was employed for lipid quantification (Folch et al., 1957). Wet sample was mixed with 15 mL mixture of 2:1 chloroform: methanol and zirconium beads (0.7mm) were added to the mixture and the cells were disrupted by a bead

beater for 3 minutes (BioSpec Products, Bartlesville, OK, USA). The samples after bead beating were filtered through Whatman filter paper with a pore size of 0.45µm. The solids thus obtained were re-suspended in 1:1 chloroform-methanol mixture and the procedure was repeated. The resulting filtrates were pooled in a pre-weighed glass tube and the solvent was evaporated under low nitrogen pressure. The residual samples were further dried at 60^0C in a hot air oven until the sample reached a constant weight. The weight thus recorded represented an amount of lipids in that sample.

Glycerol in the centrifuged supernatant was estimated based on the chemical method. Pure glycerol (Sigma-Aldrich, Canada) was used as the calibration standard (Bondioli & Della, 2005). Citric acid estimation was carried out according to the method described by (Marier & Boulet, 1958). Appropriately diluted samples were mixed with pyridine and acetic anhydride. The reaction mixture was incubated in a water bath preset at 60^0C. After five minutes of incubation, the reaction mixture was cooled to room temperature and the OD was recorded at 510nm (Varian Cary 500, USA). Citric acid (Fisher, Canada) was used as the calibration standard.

2.4. Determination of yield constants

The rate of lipid and biomass production was determined by the following equations. The specific growth rate (µ) was calculated according to the following equation

$$\mu = \frac{1}{X} \frac{dX}{dt} \quad \text{(Equn 1)}$$

Where X is the biomass concentration (g/L) at time t (h)

The biomass yield *(Yx/s, g/g)* was calculated according to Equation (2), where $\Delta X$the biomass (g/L) is produced and ΔS is the substrate consumed to generate the biomass at time t (h^{-1}).

$$Y_{X/S} = \frac{\Delta X}{\Delta S} \quad \text{(Equn 2)}$$

The lipid yield *(Yp/s, g/g)* was calculated based on Equation 3, where ΔP is the lipid produced (g/L^{-1}) and ΔS is the substrate consumed to generate the biomass at time t (h^{-1})

$$Y_{P/S} = \frac{\Delta P}{\Delta S} \quad \text{(Equn 3)}$$

The lipid content *(Yp/x, g/g)* was calculated from Equation (4)

$$Y_{P/X} = \frac{\Delta P}{\Delta X} \quad \text{(Equn 4)}$$

The specific lipid production rate (U) was calculated based on the Equation (5), were U is specific lipid production rate.

$$\mathrm{U} = \frac{1}{X}\frac{dP}{dt} = Y_{P/X}\,\mu \text{ (Equn 5)}$$

3. Results and discussion

3.1. Optimization of culture parameters (Box-Behnken design)

RSM (Response Surface Methodology) study was designed based on the previous lab data and the results obtained from 15L fermenter under controlled conditions. *Y lipolytica* SKY7 was found to tolerate a glycerol concentration of 150g/L. meanwhile, the current literature also supports the fact that *Y lipolytica* can grow and produce a high concentration of lipid when initial glycerol concentrations were high. ((Dobrowolski et al., 2016; Kuttiraja et al., 2016). The time of incubation was reduced to 72h since the lipid production was noted to substantially decrease 227after 60h. The lipogenic phase in *Y.lipolytica* was reported from 30 to 60h. The inoculum age was 24h because the exponential growth was observed from 12 to 24h in previous reactor studies. Nitrogen source (YE) was replaced with $(NH_4)_2SO_4$ because YE furnishes un-utilizable

nitrogen, which cannot be assimilated (Pawlak & Bizukojć, 2012). This can be further explained by the fact of yeast assailable nitrogen (YAN) (Bely et al., 1990). That is when the nitrogen concentration goes below the assimilable limit; it will not be utilized by the microbes. From the current results and previous experiments, a nitrogen concentration below 70mg/L was not utilized by *Y lipolytica* SKY-7. After a certain time, when all assimilable nitrogen of YE is exhausted, glycerol will be used for lipid production. This will result in a decrease of C/N ratio towards the end of fermentation as observed in previous work i.e. after 66h of fermentation the C/N ratio was decreased from 400 to 250 (after 24h of fermentation the C/N ratio was decreased from 310 to 136 in the present study). In order to know the impact of yeast extract on lipid accumulation the organic nitrogen source was replaced by inorganic nitrogen ($(NH_4)_2SO_4$). As expected the biomass concentration decreased from 15g/L to 10g/L at 72h in shake flask experiments (Table 1).This phenomenon was also observed by several other studies that the inorganic nitrogen favors mostly product formation (lipid in the present case) rather than the biomass, whereas the organic nitrogen favors the biomass and product (lipid) accumulation (Kalam et al., 2014). Due to this reason in the present study, both organic (yeast extract) and inorganic nitrogen ($(NH_4)_2SO_4$) sources were used.

3.2. Interaction of model terms in Biomass production.

Glycerol concentration, C/N ratio, and inoculum concentration were evaluated to produce maximum biomass concentration. The actual biomass and lipid produced for each combination of experiments were presented in Table 1. Higher biomass was produced for 75g/L glycerol irrespective of C/N molar ratio and inoculum volume. The average biomass concentration at 75g/L glycerol was 10g/L and at 87.5g/L glycerol the biomass concentration varied from 9 to 9.84 g/L. A lower biomass concentration (7.2 to 9.12g/L) was observed at 100g/L glycerol

concentration. This indicates that the low glycerol concentration favors the biomass production, whereas the C/N molar ratio and inoculum volumes are ineffective in the range studied. To confirm the above claims, interactions between the culture parameters were analyzed with quadratic model and the results are discussed in the following sections.

3.3. Interaction of glycerol concentration, C/N molar ratio and inoculum volume on biomass production

At low glycerol concentration and low C/N molar ratio a high biomass concentration was observed compared to other combinations (Table 1). The biomass concentration decreased with the increase in glycerol concentration or C/N molar ratio. The low biomass concentration (7.2g/L) was observed at glycerol concentration 100g/L and C/N ratio 150. The highest biomass in this experiment was (10.44g/L) obtained with glycerol concentration 75 g/L and C/N molar ratio 75. Under the studied inoculum volumes, the highest biomass was obtained in 10%v/v inoculum supplemented experiment. However, a moderate biomass (9 to 10g/L) was accumulated in experiments with 6.2% v/v inoculum, which revealed a close significance with 10% v/v inoculum supplemented experiments. A trend of decrease in biomass concentration was noticed with a decrease in inoculum volume and increase in glycerol concentration. Meanwhile, low C/N ratio with high inoculum volume showed high biomass production. However, the study shows that there was no significant variation in biomass concentration when C/N ratio and inoculum concentration was changed. Thus, the biomass concentration strongly depends on the glycerol concentration, C/N molar ratio, and inoculum concentration. Based on the data obtained the Fischer's test for the analysis of variance (ANOVA) was performed. The final equation for improving the biomass concentration thus obtained is given below

$$Y= 9.38-1.08A-0.22B+0.37C-0.19AB+0.46AC-0.24BC-0.16A^2-0.023B^2+0.067C^2$$

Where A, B, and C are the coded values for glycerol concentration, C/N molar ratio, and inoculum volume, respectively. Based on the ANOVA test, the model terms glycerol concentration (A), inoculum volume (C) and coded term (AC), glycerol concentration and inoculum volume are found to be significant with a p-value of 0.0001 and 0.01. Further, the results showed that the selected model terms are significant with a p-value of 0.0014. However, as mentioned above the model terms AB and BC were not significant. This could be explained by taking into account the nitrogen added to the medium. The actual nitrogen concentration added to the medium ranged from 0.2g/L to 0.4g/L, which is low compared to the concentration added in common fermentation medium (0.36 to 0.6g/L). Hence, the biomass concentration observed was also low.

3.4. Glycerol concentration, C/N molar ratio, and inoculum volume interaction with lipid accumulation

High lipid content was attained when the glycerol concentration was 100g/L with a C/N ratio of 150 and inoculum volume of 6.5 % (v/v). This shows that an increase in C/N molar ratio will result in the high content of lipid in the biomass; however, high C/N ratio reduces the biomass production. The low biomass production on high C/N ratio could be the result of low nitrogen content in the media. Low biomass lipid content was noted with 75g/L glycerol concentration, C/N ratio 112.5 with an inoculum volume of 2.5% v/v. In this case high C/N ratio and low inoculum volume resulted in only biomass accumulation instead of lipid. When glycerol concentration was 87.5g/L with C/N ratio 112.5 and 6.25%v/v inoculum an average lipid content of 40.5 to 45.3% w/w was attained. Thus, in order to achieve a high biomass lipid content, glycerol concentration and C/N ratio needs to be high (Table 1).

A significant increase in lipid concentration was noted when the inoculum and glycerol concentration increased. An average lipid concentration was obtained with a glycerol concentration of 87g/L with 6.2%v/v inoculum. Under above conditions, the increase in lipid concentration was about 0.4g/L. This indicates that when the glycerol concentration is 87.5g/L an inoculum volume of 6.2% would be ideal for lipid accumulation. Further, the interaction of glycerol concentration and C/N molar ratio with lipid accumulation was evaluated. As perceived in the case of lipid production, C/N ratio was noted to be insignificant in lipid accumulation. It is evident that when the C/N ratio is low the glycerol concentration needs to be optimum in order to accumulate high lipid content. In this case, at 87.5g/L of glycerol concentration, the lipid accumulation was similar with respect to all C/N ratios. However, when the glycerol concentration was high (100g/L) the lipid accumulation decreased at low C/N ratio (75). It is evident that C/N ratio of 75 with 87.5g/L glycerol could be optimum for batch cultures. Moreover, the change in C/N ratio during the course of batch fermentation may lead to a change in lipid accumulation. Here the only factor that affects the lipid accumulation is the glycerol (carbon) concentration since lipid synthesis requires excess carbon with low. The interaction of C/N molar ratio and inoculum volume with lipid concentration is evaluated. A high inoculum with low C/N molar ratio was noted to be better for lipid accumulation. This can be explained by the assimilation of nitrogen by the yeast cells when a high concentration of inoculum is used in the fermenter. A rapid depletion of nitrogen in the fermentation media coerces the cell biomass to route the available glycerol towards lipid synthesis. Here in the present case, this condition was observed when 6.25%v/v inoculum was used. The Fischer's test for the analysis of variance (ANOVA) was performed based on the data obtained. Thus, the final equation obtained for improving the biomass lipid content was given below.

$$Y= 4.07+0.27A+0.038B+0.33C+0.065AB+ 0.29AC-0.44BC-0.55A^2+0.050B^2-0.38C^2$$

Where A, B, and C are the coded values for glycerol concentration, C/N molar ratio, and inoculum volume, respectively. From the ANOVA test, it is clear that the model selected for the study was significant (p-0.0165). The model terms glycerol concentration (p-0.0373 0) and inoculum volume (p-0.0172) were the significant parameters with respect to lipid synthesis. The model term C/N molar ratio was non-significant with the p- the value of 0.734. In the coded terms BC, $A^{2,}$ and C^2 were found to be significant.

3.5. Validation of the model

Based on the analysis, 10 different solutions were predicted by the software with reference to accumulate high biomass concentration with high lipid content. Based on the high (0.95%) desirability values 4 experimental conditions were chosen to conduct the experiments. The obtained results are presented in Table 2. A correlation analysis of predicted versus experimental data was conducted and it shows a correlation coefficient value of 0.85 for biomass and 0.92 for lipid. This indicates that the predicted experimental conditions are suitable to produce a high concentration of biomass and lipid. Based on the validated data glycerol concentration of 85g/L, C/N ratio 75 and inoculum volume of 6.25%v/v was chosen to further verify the conclusion at 15L bench scale fermenter, as described below.

3.6. Fermenter studies

The fermentation media was modified according to the optimal media composition obtained through RSM studies. In order to maintain the steady growth, the initial aeration was maintained at 0.35vvm (volume/volume/minute) and pH of the fermentation was controlled by adding 4N NaOH solution. The mixing was varied according to the DO (dissolved oxygen) requirement and the temperature was maintained at 28^0C. The DO value in the fermenter decreased to 30% saturation within 12h of fermentation, which indicated an active microbial growth. When growth

decreased, the DO started to increase. When DO reached 60% of saturation, the mixing was reduced to 400 rpm and then the aeration was reduced to 0.325vvm in order to maintain the DO at about 30% of saturation.

The biomass, lipid, citric acid production and glycerol consumption during the fermentation process is described in Figure 1. The biomass concentration reached 29.5g/L at 72h of fermentation with a lipid concentration of 15.16g/L. The final lipid content in the biomass was 54.1% (w/w) at 72h of fermentation. From 0 to 6 h only 0.82g/L of glycerol was consumed by the cells and the biomass produced was 1.7g/L. However, the biomass produced during this time period was higher than the carbon assimilated in the form of glycerol. (Carbon content of glycerol= 0.39g/g, 1gm of yeast cell constitutes 0.4-0.5g/g carbon (Teixeira & Vicente, 2013; Wagner et al., 2012), therefore 1.7 g of cells requires 0.81g of carbon). However, only 0.82g of glycerol was consumed by the cells during the first 6h of fermentation, which can only provide 0.32g of carbon. Therefore, the rest of the carbon required for biomass production must be supplied by other carbonations components (yeast extract, soap, methanol) available in the fermentation medium (Holwerda et al., 2012). After a short lag period of 0-6h, 5.53g/L biomass was produced during 6 -12 h of fermentation and then the production of biomass was decreased due to the nutrient depletion.

At initial stage (12h) of fermentation, the lipid production was observed higher. When fermentation precedes the lipid production dropped and the biomass production was noticed to be increased until 30h of fermentation. The lipid produced during this time could be contributed by cell wall lipids and a small quantity of TAGs. From 36h, the lipid production was observed to be stable and the lipid concentration at 36h of fermentation was 4.9g/L with the biomass lipid content 24.98%w/w. The maximum lipid concentration reached was 15.16g/L at 72h.

The citric acid concentration reached 2.52g/L at 72h. In previous studies under pH control condition citric acid concentration up to 11.32, g/L was observed using same strain (Kuttiraja et al., 2016 under review). A low citric production in the present case may be due to the fact that the synthesized citric acid is used by the strain and converted to lipids efficiently under optimized culture conditions; it has already been reported that the citric produced can be converted to lipid (Ratledge & Wynn, 2002). Meanwhile, the citric acid production is also varies based on carbon concentration (high initial sugar concentration 10 to 14%(Xu et al., 1989), fermentation time (4-8days)(Maddox et al., 1985) and the amount of available nitrogen.

The biomass yield (Yx/s) shows a slow increase from 24 to 72h (Figure 2), which is due to the cumulative increase of biomass and lipid content of the biomass. An average biomass production of 0.321g/g was observed from 12 to 72h. To assess the actual biomass produced, the yield of lipid-free biomass was calculated (Figure 2), which decreased with the fermentation time. The above observation helps conclude that most of the assimilated carbon is converted to lipid synthesis rather than biomass production. The lipid yield (Yp/s, g lipid produced/g glycerol consumed) initially decreased from 0.129g/g to 0.078g/g during 12 to 36h. From 36h onwards more substrate was transformed into lipid and, therefore, Yp/s increased until 72h (0.179g/g). The trend of Yp/s and Yp/x were noted to decrease initially and then increase with time of fermentation. The decrease in yield coefficients (Yp/s and Yp/x) until 36h was due to the diversion of most of the glycerol carbon for biomass production. After 36 h the depletion in nitrogen content in the medium limited the biomass production and diverted the carbon available for lipid production, which increased the lipid yield coefficients. The lipid free biomass yield was decreased from 30 to 72h, which confirms the substrate conversion to lipids.

Based on the fermentation profile the glycerol concentration was 5.78g/L at 66h. Further increase in fermentation time may lead depletion of glycerol to a low critical level, which may result in lipid degradation. This could be further explained by assessing the variation in specific lipid production rate. The specific lipid production rate was noted to decrease from 0.007 h^{-1} to 0.006 h^{-1} at 60 to 72h (Figure 3).

Based on glycerol conversion to biomass, lipid, and citric acid, a simple mass balance was made. It was found that 41.1% of the total assimilated glycerol, 18.38% was converted into biomass, 19.49% into lipids and 3.23% citric acid at the end of fermentation. The rest of the glycerol consumed could be mainly converted to other metabolic byproducts such as CO_2 or other organic acids, sugar alcohols, and carbohydrates and for maintenance metabolism (Tomaszewska et al., 2014). When it is compared with the un-optimized fermentation condition (same strain) lipid production was improved 2% and citric acid production was reduced to10%.

The maximum specific growth rate 0.15h^{-1} was attained at 12h (Figure 3). From 12h onwards the specific growth rate declined and reached 0.042h^{-1} at the end of fermentation (72h). The specific lipid production rate attained maximum value at 12h of fermentation (Figure 3). A decreasing trend of specific lipid production was found from 12h to 30h, which correlated with the decreasing trend of yield coefficients (Yp/x and Yp/s) (Figure 2). After 36h, the specific lipid production was approximately constant until the end of fermentation. As described above, the specific lipid production was proportional to specific growth rate at the initial stage of fermentation. However, it decreased with the declining specific growth rate at mid-point (12 to 30h) of fermentation. The μ value reached 0.1h^{-1} at 30h where the U start to climb again and this was denoted by lipogenic phase by different authors. The lipid accumulated in cells during 36 to 72h was 10.29g/L and 4.87g/L of lipid was accumulated at the early stage (6 to 30h) of

fermentation. To confirm the above statement the (lipid) point productivity was calculated by differentiating the lipid concentration data and presented in Figure 3. The point productivity was noted to decline from 6h to 36h of fermentation. Thus, it is assumed that most of the glycerol consumed during this time period were routed for biomass synthesis. From 36h onward the lipid productivity increased and attained 0.21g/L/h at 72h. This shows that the TAG was mainly synthesized by the cells during 36 to 72h fermentation (as is also clear from yield coefficients)

4. Conclusion

The current study shows a glycerol concentration of 85g/L, C/N ratio75, with an inoculum volume of 6.25%v/v was found to be ideal for biomass and lipid production. The maximum biomass and lipid concentration were attained at 72h with a biomass and lipid yield of 0.332g/g and 0.179g/g, respectively. At the initial stage of fermentation, the organism found to assimilate carbon from methanol, yeast extract, and free fatty acids. During growth at optimal culture conditions, the citric acid production was noticed to decrease significantly compared to non-optimized conditions. The specific growth rate was noted to be declined from 12h onwards and the specific lipid production was found to increase from 30h.

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Contents

Table 1 Box behnken design of experiments

Order	Run order	Glycerol conc (g/L)	C/N molar ratio	Inoculum volume (v/v)	Biomass (g/L)	Lipid (g/L)	% of lipid (w/w)
4	1	100	150	6.25	7.56	3.84	50.79
15	2	87.5	112.5	6.25	9	4.08	45.33
2	3	100	75	6.25	8.72	3.6	41.28
1	4	75	75	6.25	10.44	3.43	32.85
13	5	87.5	112.5	6.25	9.4	4.06	43.19
14	6	87.5	112.5	6.25	9.4	4.12	43.82
8	7	100	112.5	10	9.12	4.4	48.24
16	8	87.5	112.5	6.25	9.84	3.99	40.54
10	9	87.5	150	2.5	9.36	4.12	44.01
7	10	75	112.5	10	10.44	3.04	29.11
11	11	87.5	75	10	9.96	4.24	42.57
17	12	87.5	112.5	6.25	9.24	4.1	44.37
5	13	75	112.5	2.5	10.36	2.45	23.64
3	14	75	150	6.25	10.04	3.41	33.96
12	15	87.5	150	10	9.36	3.4	36.32
9	16	87.5	75	2.5	9	3.2	35.55
6	17	100	112.5	2.5	7.2	2.67	37.08

Table 2 Validation of predicted values

Column1	Glycerol concentration g/L	C/N molar ratio	Biomass predicted (g/L)	Biomass obtained (g/L)	Lipid Predicted (g/L)	Lipid Obtained(g/L)
1	85.5	75	10.3	9.2	4.4	5.1
2	85.9	75	10.3	9.5	4.4	4.124
3	85.9	75	10.3	9.2	4.4	4.524
4	85.8	75	10.3	9.3	4.3	3.268

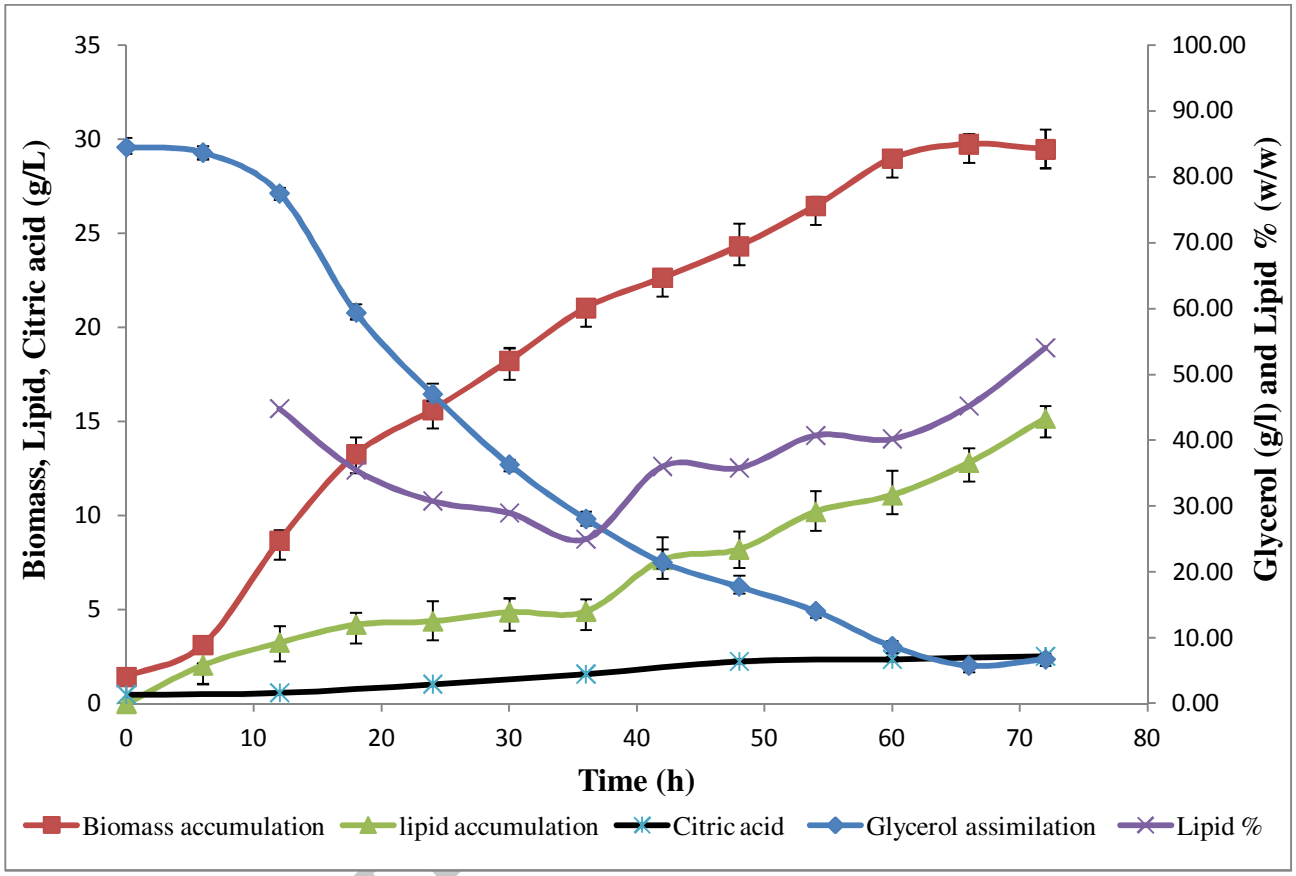

Figure 1- Variation of Biomass, glycerol, citric acid and lipid concentration during fermentation in 15L fermenter

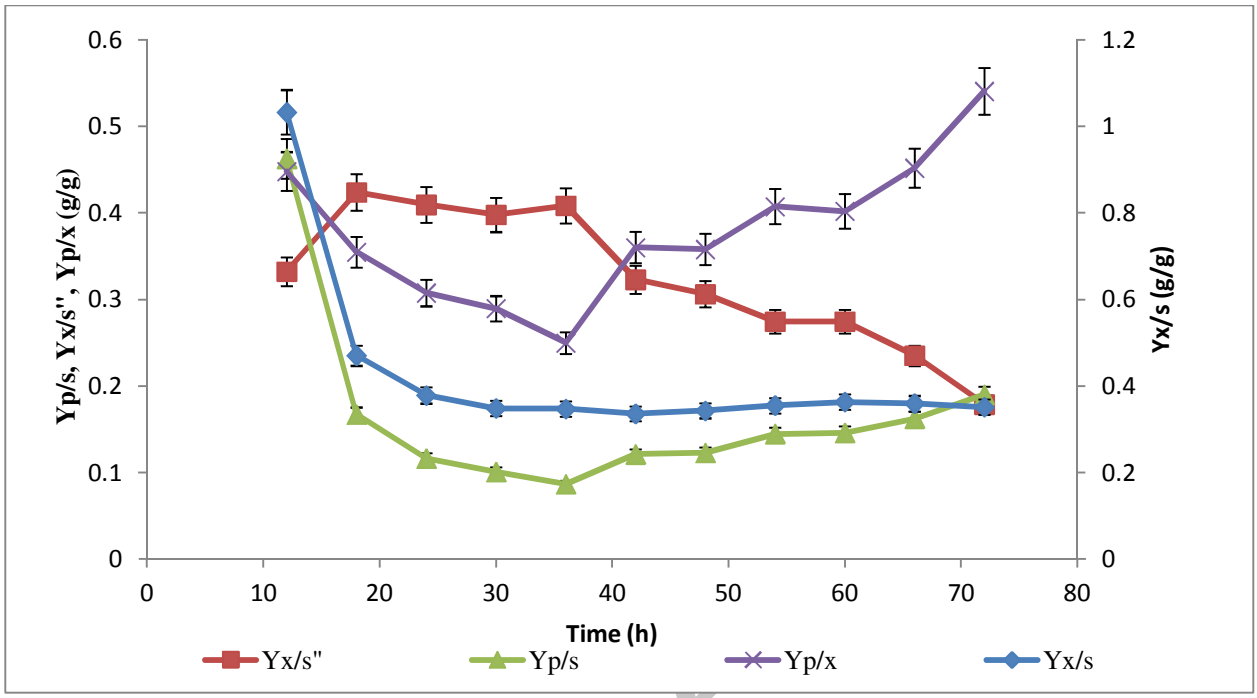

Figure 2- Variation of the yield of biomass (Yx/s), lipid free biomass (Yx/s"), the yield of lipid (Yp/s) and yield of lipid content per gram of dry biomass (Yp/x) with fermentation time. The yield was calculated based on the average biomass and lipid obtained on duplicates.

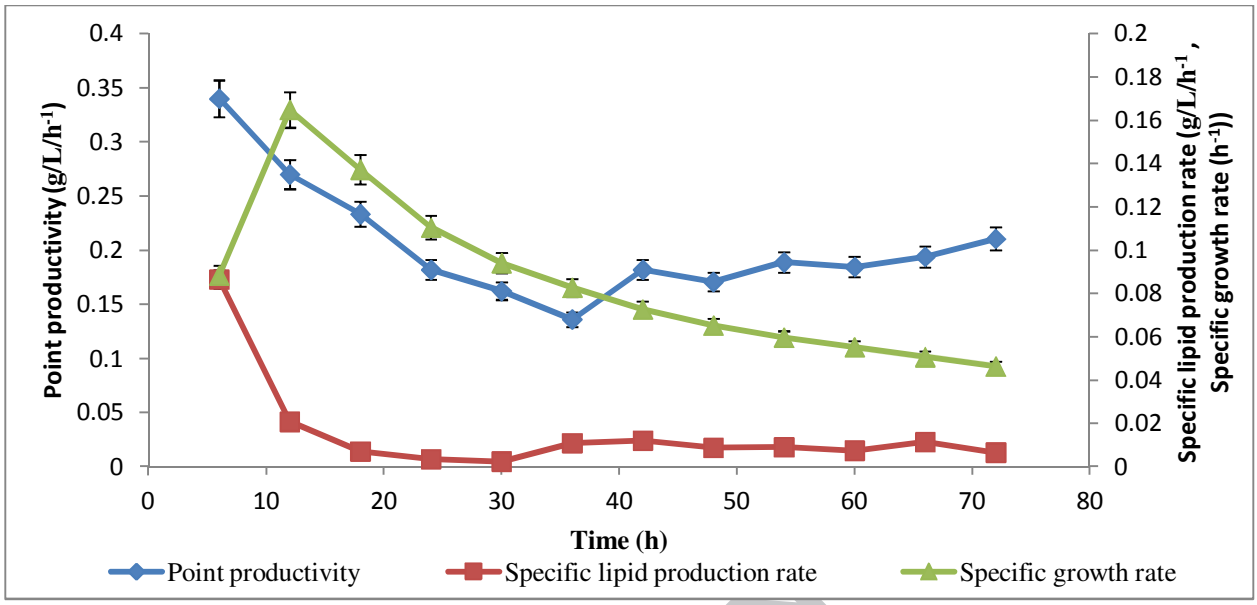

Figure 3- Variation of specific growth rate and specific lipid production rate and lipid productivity with fermentation time.

Highlights

- A glycerol concentration of 85.5g/L and C/N ratio of 75 with inoculum volume of 6.25%v/v found optimal
- Biomass concentration of 29.5g/L with 50%w/w lipid accumulation was obtained in 15L scale
- The biomass and lipid yield was reached to 0.332g/g and 0.179g/g respectively