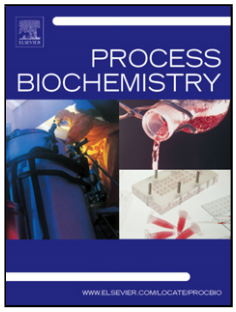

Purified crude glycerol by acid treatment allows to improve lipid productivity by *Yarrowia lipolytica* SKY7

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Purified crude glycerol by acid treatment allows to improve lipid productivity by *Yarrowia lipolytica* SKY7

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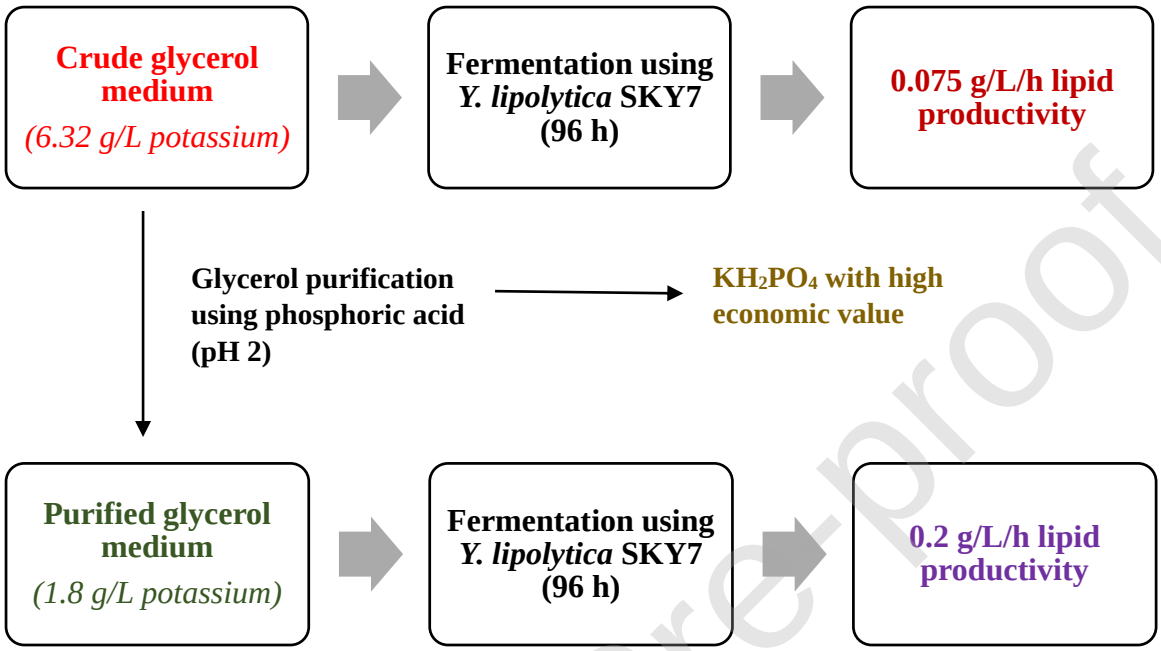
Graphical abstract



Highlights

- Crude glycerol (with high potassium concentration) was purified and used for lipid production
- Purified glycerol resulted in higher lipid and biomass productivity
- Lipid yield on purified and pure glycerol as substrate was comparable
- Potassium salt with high economic value was produced during purification

ABSTRACT

In this study, crude glycerol with high potassium concentration was purified using acid treatment and used as carbon source for lipid production using *Yarrowia lipolytica* SKY7. The crude glycerol was purified using phosphoric acid (pH 2) followed by centrifugation. When purified glycerol was

used as carbon source for fermentation, higher biomass productivity (0.54 g/L/h) and lipid productivity (0.2 g/L/h) was observed at 96 h compared to crude glycerol. Results indicated that 6.32 g/L potassium in crude glycerol medium was inhibitory for cell growth and lipid production by *Y. lipolytica*. Yield coefficients, productivities and specific growth rates were calculated for each glycerol medium. The process performance with purified glycerol medium was comparable to that of pure glycerol medium. A higher lipid yield was obtained in purified glycerol medium (0.21 g/g glycerol) than crude glycerol medium (0.124 g/g glycerol). This study provides a way for valorization of crude glycerol with high potassium content for microbial lipid production.

Keywords: *Yarrowia lipolytica* SKY7; Glycerol Purification; Fed-batch fermentation; Microbial lipid; Citric acid

1. Introduction

Net energy production and carbon dioxide emission are the two most important factors for evaluating the sustainability of new energy sources. Biodiesel is a promising new energy source and is safe, renewable, non-toxic, and biodegradable. It is produced mainly from vegetable oils and animal fats [1]. The increasing price of edible oil and limited feedstock sources leads to unaffordable biodiesel production. Crops like rapeseed, jatropha, and canola are used for biodiesel production, but they have disadvantages such as, dependency on land and climatic conditions, removal of rain forest, high labour and energy intensive process [2]. The lipid, which is derived from microorganisms, also known as microbial oil, can be quickly synthesized and accumulated

in cells; its fatty-acid composition is highly similar to that of vegetable oil [3, 4]. However, cost assessment revealed that the unit production cost of microbial biodiesel produced using commercial substrate was estimated to be $5.9/kg biodiesel while market price of biodiesel is around $1/L [5]. Hence, renewable and cheap carbon sources are being explored for lipid production to reduce its production cost [6-8]. Therefore, crude glycerol has attained researchers' attention in past years as it is a by-product of biodiesel industry and using crude glycerol for lipid production will help maintaining circular economy [9].

However, crude glycerol has several impurities depending on the trans-esterification process: catalyst, methanol, soap, free fatty acids, metals and salts [10] [11]. The consideration for purification of glycerol is largely dependent on the usage of glycerol. Acidification is an effective technique for removal of soap, metals and salts from the crude glycerol [9, 12, 13]. Purified glycerol, thus obtained, can be used as a carbon source in fermentation process for lipid production. Thus, objective of this study was to purify crude glycerol with high potassium content and use it as a carbon source for microbial lipid and citric acid production using *Yarrowia lipolytica* SKY7 (YL). A comparison between purified and unpurified glycerol was made in terms of specific growth rate, product productivity and product yield coefficient. Moreover, a mass balance was made for purification of 1 L of crude glycerol and chemicals cost involved in in the purification process has been discussed. This study provides a way for valorization of crude glycerol with high potassium content for microbial lipid production.

2. Methodology

2.1 Crude glycerol purification

The crude glycerol was obtained from Canadian biodiesel producing company BIOLIQ-INC. BIOLIQ has high on potassium concentration as potassium methoxide was used as catalyst during trans-esterification. The crude glycerol contained potassium methoxide which is highly toxic compound as it is an alkoxide of methanol (strong base). For crude glycerol purification, phosphoric acid was added to crude glycerol (neutralization using pH adjustment to 2) followed by centrifugation at 6000 rpm for 10 minutes. The characterization for crude and purified glycerol was performed using protocols given in [14]. The composition of original and purified glycerol is mentioned in Table 1. Although potassium concentration has been reduced in purified glycerol but phosphorus concentration has increased in purified glycerol due to use of phosphoric acid.

2.2 Strain Used

Y. lipolytica SKY7 (YL) isolated in INRS laboratory was used in this study. *Y. lipolytica* SKY is a wild strain, which can accumulate up to 50% lipid (w/w) and the lipids produced by the strain has a close resemblance with vegetable oil and could serve as a feedstock for biodiesel production. The strain also produces citric acid, which could be recovered and utilized by the food and/or chemical industry since the organism is safe to use at the industrial level [15].

2.3 Pre-culture and inoculum preparation

The dormant pure culture of *Y. lipolytica* (4ºC) was revived by cultivating in a pre-culture 1 (or PC1) synthetic media (Yeast extract peptone dextrose broth/ YPD: 20 g/L glucose, 20 g/L peptone and 10 g/L yeast extract) for 24 h in shaking incubator with agitation 180 rpm and temperature of 28ºC. PC1 volume of 6.25% v/v was used to produce pre-culture 2 (PC2). Pre-

culture 2 was prepared in a medium containing 20 g/L purified glycerol fortified with 50 g/L YPD. This was done to acclimatize *Y. lipolytica* in purified glycerol medium. PC2 was grown at 180 rpm and 28ºC for 36 h before being transferred to the production fermenter.

2.4 Fermentation

2.4.1 Operation

Fermentations were carried out in stirred tank fermenters (SARTORIUS BIOSTAT 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 [2]. During fermentation, DO was maintained between 25-40% saturation by manually adjusting agitation rate (250–600 rpm) and air flow rate (1–2.5 L/min). The limitation of DO favors lipid production [16]. During 6-12 h, DO decreases from 90% to 35% and later it was maintained in the range of 25%-40%. 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: 4M NaOH or 4M 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.

2.4.2 Fed-batch strategy

Fermentations were conducted using purified glycerol medium and crude glycerol medium. One fermenter with pure glycerol medium was used as control. The fermenters were

operated under fed-batch mode to avoid both substrate limitation. The production fermenters were operated at temperature 28ºC and pH 6.5 for *Y. lipolytica* as reported by Kuttiraja, Dhouha and Tyagi [17]. The production fermenter was started with 10 g/L carbon (25 g/L glycerol), and C/N ratio (molar) of 10 at 0 h. The additional trace elements were added in the medium at 0 h which had composition of 2.7 g/L KH_2PO_4, 0.95 g/L Na_2HPO_4, 0.2 g/L $MgSO_4.7H_2O$, 0.04 g/L $CaCl_2.2H_2O$, 0.0055 g/L $FeSO_4.7H_2O$, 0.001 g/L $ZnSO_4.7H_2O$ and 0.00076 g/L $MnSO_4.H_2O$. The feeding strategy was based on glycerol consumption. The fermentations with different type of glycerol solutions were started with 25 g/L glycerol (10 g/L carbon) and when glycerol concentration in the medium reached below 5 g/L, a glycerol feed was added so that glycerol concentration in the medium reaches 20-25 g/L. Peptone was added at 0 h only to maintain initial C/N ratio in the fermenter medium. However, no nitrogen was imparted during feed at later stages. The reason for it is that high initial nitrogen concentration or low initial C/N ratio will help in build of high biomass (lipid free) during early stages of fermentation and once nitrogen concentration will become limiting, the addition of glycerol feed will increase C/N ratio in the medium resulting in high lipid accumulation [14]. Feeding details are presented in Table 2. Samples were withdrawn after every 8 h to determine the biomass, lipid, glycerol and organic acids in the sample.

2.5 Analytical Techniques

Biomass and lipid concentration were determined as reported by Chen, Zhang, Yan, Tyagi and Drogui [14], [18]. Glycerol was measured according to the method by Bondioli and Della Bella [19]. Citric acid estimation was carried out according to the method by Marier and Boulet [20]. Elemental concentration in samples were determined by inductively coupled plasma mass spectroscopy (ICP-MS) after acid digesting the samples (model DRE, Leeman Labs Inc).

Lipid characterization: A lipid sample of 25 mg was trans-esterified using acidified methanol. Decahexanoic acid was used as the internal standard. The trans-esterified lipid fraction was extracted using hexane and the samples were further characterized by GC (Agilent 7890B) equipped with flame ionization detector. Column length was 60 m (Agilent J&W); the carrier gas was helium at a flow rate of 1.18 mL/min with the oven temperature 230 °C. Trans-esterified sample (1 µL) was injected with an automated sample injector and the sample analysis was performed with Agilent GC chem station software. A 37 components FAME mixture from Supelco was used as the calibration standard at different concentrations.

All samples were analyzed in duplicates and their standard deviation was less than 5%.

2.6 Determination of kinetic parameters

Productivity is defined as g product produced per unit volume at a particular time t. Unit of productivity is g/L/h. Lipid and citric acid productivity will be calculated by equation 1.

$$Productivity\ \left(\frac{g}{L.h}\right) = \frac{product\ produced\ (g)}{(Volume\ x\ time)} \quad (1)$$

Biomass yield coefficient ($Y_{x/s}$) is defined as g of total biomass produced per g of substrate consumed. Biomass yield will be determined by the equation (2):

$$Yx/s = dX/dS \quad (2)$$

Product yield coefficient ($Y_{P/S}$) is defined as the amount of product produced per g of substrate consumed. Its will be determined for lipid and citric acid by the equation (3a) and (3b):

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

$$Yc/s = dCA/dS \quad (3b)$$

Specific growth rate (μ, h^{-1}) was determined by the equation (4):

$$\mu = dX/(X.dt) \quad (4)$$

Where X represents biomass concentration at particular time t

Biomass yield, productivity and specific growth rate have been calculated with respect to total biomass concentration including intracellular lipids.

3. Results and Discussion

3.1 Comparison of purified and crude glycerol medium using Y. lipolytica (fed-batch fermentation)

3.1.1 Variation of biomass concentration

Variation of biomass concentration in the medium with pure, purified and crude glycerol as carbon source is highlighted in Figure 1. It is clear that purified glycerol performed better than crude glycerol with biomass concentration of 51.67 g/L and 20.36 g/L, respectively at 96 h. Lower biomass in the medium with crude glycerol could be due to higher potassium concentration (4.8 g/L, Table 2) in the beginning of the process.

After adding feed in the medium with crude glycerol at 24 h, the potassium concentration in the medium increased to 6.32 g/L (Table 2) consequently cell growth further decreased (or nearly ceased) (Figure 1). Thus, it can be concluded that potassium concentration of 6.32 g/L in the medium further decreased growth rate (Table 4, discussed later).

It has been reported that yeast cells need to maintain optimum intracellular sodium/potassium ratio, which is required for maintaining cellular activities. A high extracellular potassium concentration leads to hyperosmotic stress conditions that can hamper the cellular activities required for the cell growth [21]. In the medium with purified glycerol, the potassium concentration in comparison to medium with crude glycerol was relatively very low (Table 2), the inhibition due to potassium was alleviated and therefore higher biomass concentration was obtained. Moreover, the biomass concentration in the medium with purified glycerol (51.67 g/L) was comparable to that observed in pure glycerol medium (54.27 g/L) at 96 h. This indicated that a significant inhibition was removed in the medium with purified glycerol and it was due to decreased concentration of potassium.

3.1.2 Variation of glycerol consumption

The residual glycerol concentration in the medium and variation of glycerol consumption for three types of glycerol is depicted in Figure 2. Maximum glycerol consumption for crude glycerol, purified glycerol and pure glycerol at 96 h of the process was 58.04 g/L, 92.43 g/L and 80.79 g/L, respectively. Due to inhibition of potassium in crude glycerol medium, glycerol consumption was lower as compared to purified glycerol medium as well as pure glycerol medium. When glycerol concentration in the medium reached <5 g/L, additional crude glycerol solution feed was added at 24 h of the fermentation process, which increased potassium concentration in the medium to 6.32 g/L (Table 2). Thus, a high concentration of potassium in crude glycerol media led to further inhibition of cell growth after the feed, which resulted in lower glycerol consumption.

3.1.3 Variation of lipid production

Variation of lipid concentration in purified and the crude glycerol medium is highlighted in Figure 3a. At 96 h, the maximum lipid concentration in the medium obtained with crude glycerol and purified glycerol was 7.21 g/L and 19.47 g/L, respectively. The biomass lipid content was almost similar for purified (0.377 g lipid/ g biomass) and crude glycerol medium (0.354 g lipid produced/g biomass) or meagrely different. Therefore, lower lipid concentration observed in the medium with crude glycerol was due to lower biomass concentration, which in turn could be attributed to a lower growth rate of YL (low growth rate due to high potassium concentration). For high lipid production, a large concentration of biomass is needed so that the empty cells can be filled with microbial lipid. Further, when the nitrogen concentration in the medium started decreasing and C/N ratio started increasing due to added glycerol feed (Table 2), lipid concentration started increasing irrespective of type of glycerol.

Lipid characterization revealed that Palmitic acid (C16:0), Oleic acid (C18:1) and Linoleic acid (C18:2) were produced irrespective of glycerol medium. Oleic acid (C18:1) and linoleic acid (C18:2) are the major components in the case of SKY7 as reported in other studies [15, 17]. These lipids have the resemblance with the vegetable oil. The presence of polyunsaturated fatty acids (PUFAs) makes the isolate *Y. lipolytica* SKY7 important for biodiesel production.

3.1.4 Variation of organic acids production

Citric acid is the major acid produced by *Y. lipolytica*, which is a well-known industrial scale citric acid producer. Citric acid production in the medium with purified and crude glycerol is presented in Figure 3b. Higher concentration of citric acid (12 g/L) in the medium was attained with crude glycerol than that with purified glycerol (5.42 g/L) at 96 h. A lower concentration of

potassium in the medium with purified glycerol (1.8-2.34 g/L) and pure glycerol (1.13-1.46 g/L) resulted in lower citric acid production, which was almost similar in two cases (3.92 g/L citric acid) (Figure 3b). On the other hand, a high potassium concentration in the crude glycerol medium increased the citric acid production that resulted in a final concentration of 12g/L. It has been reported that a higher potassium concentration in the media exerted a positive effect on the citric acid production [22]. The citric acid production (citrate concentration in the cytoplasm) is one of the important factors that control the de-novo lipid accumulation [23]. Citrate is known as the acetyl donor for fatty acid biosynthesis and is transported from mitochondria to the cytoplasm. A constant supply of intracellular citrate will generate adequate amounts of acetyl-CoA in the cytoplasm by the enzyme ACL (ATP citrate lyase). Acetyl CoA is converted to malonyl-CoA (a step-in lipid synthesis) using ACC enzyme (acetyl-CoA carboxylase) [17]. High concentration of potassium (4.8-8.42 g/L) in crude glycerol media might have inhibited ATP-citrate lyase (ACL) required for breakdown of citrate. Due to inhibition of ACL, accumulated citrate in cytoplasm comes out of the cell instead of being converted into lipids.

Other organic acids produced using different types of glycerol in the medium were pyruvic acid, alpha-ketoglutaric acid, malic acid, glutamic acid and fumaric acid (Table 3). Pyruvic acid, malic acid and alpha-keto-glutaric acid are intermediates of citric acid cycle and all have commercial applications [24]. Pyruvic acid is used as a weight-loss supplement, malic acid is used as food additive while alpha-keto glutaric acid is used for immune regulation and as anti-oxidant. However, the concentration of these acids in the medium is very low and recovery may be expensive.

3.1.5 Variation of biomass, lipid and citric productivities

In order to calculate biomass productivity, the biomass curve can be divided in different sections (Table 4) and each section is represented by a straight line. The slope of each line represents biomass productivity (dX/dt) during that fermentation period. Lipid productivity (dL/dt), citric acid productivity (dC/dt) and carbon consumption rates (dS/dt) have been calculated by similar method (Table 4).

Biomass productivity was higher during the initial 24 h for all three-glycerol medium and was lower in the later period of fermentation (24-96 h) (Table 4). Higher biomass productivity during the initial 24 h is due to abundance of nitrogen and other nutrients, which resulted in a higher cell growth. Also, during the initial 24 h, carbon of peptone (contains 10% carbon) was also used for cell growth. During later phases, biomass productivity was lower due to reduction in nutrients (pure glycerol and purified glycerol) or inhibition (in the crude glycerol due to feed addition). Biomass productivity in purified glycerol was observed higher than crude glycerol and it was comparable to that of pure glycerol. This indicates no inhibition was observed in purified glycerol. At 96 h, the overall biomass productivity for purified glycerol, pure glycerol and crude glycerol medium was 0.54 g/L/h, 0.565 g/L/h and 0.21 g/L/h, respectively.

Irrespective of glycerol medium, lipid productivity was higher during initial 16-24 h period, because during this period membrane lipids develop with biomass growth [2]. While during later stages of fermentation (>24h), lipid was accumulated as intracellular lipids in cytosol. For crude glycerol, two sections are identified where membrane lipids are developed (1^{st} section, 0-16 h) and lipids accumulate in cytosol (2^{nd} section, 16-96 h) (Table 4). For purified and pure glycerol,

three sections were identified: 1st section (accumulation of membrane lipids), 2nd and 3rd section (accumulation of cytosol lipids). However, 3rd section had higher lipid productivity than 2nd section due to higher C/N ratio (Table 2). Throughout the fermentation, lipid productivity was higher in purified glycerol when compared to crude glycerol. At 96 h, the overall lipid productivities for purified glycerol, pure glycerol and crude glycerol medium were 0.2 g/L/h, 0.184 g/L/h and 0.075 g/L/h respectively. Since lipid and biomass productivities are the most important process parameters affecting the final cost [25], they are compared with the productivities reported in literature [14, 17, 26-28] (Table 5). Lipid productivity in fed-batch study (this study) was higher than those reported in batch studies conducted on *Y. lipolytica* and was comparable to that of oleaginous yeast *T. oleaginosus* grown on crude glycerol [14].

In this study, irrespective of type of glycerol medium, citric acid productivities were lower during the initial phase of 24-32 h and increased during later stages of fermentation (Table 4). Citric acid productivity increased during later stages of fermentation due to a reduction in nitrogen concentration and an increase in the C/N ratio in the medium. Citric acid productivity was the highest in the crude glycerol due to inhibition of ATP-citrate lyase enzyme responsible for the breakdown of intracellular citrate (as discussed earlier). Pure glycerol had lowest citric acid productivities because no growth inhibitory element was present in the medium (only required trace elements were added) while in purified glycerol, the citric acid productivity was very low due to the presence of low potassium concentration (1.8-2.34 g/L) in medium. At 96 h, the overall citric acid productivity for purified glycerol, pure glycerol and crude glycerol medium was 0.056 g/L/h, 0.04 g/L/h and 0.125 g/L/h, respectively. Citric acid productivity as reported in the literature is compared in Table 6 [17, 29-31]. It can be found that citric acid productivity in this study (wild

strain) is lower than genetically engineered strain [31]. However, citric acid productivity is higher in glucose when compared to pure glycerol [30]. With glycerol as the sole carbon source, only 6.7% of its uptake was directed to phosphate pentose pathway (PPP) compared to 35% with glucose [30]. On the other hand, higher fluxes toward tri-carboxylic acid (TCA) cycle were observed with glycerol rather than glucose as substrate. Relatively lower TCA cycle and higher PPP fluxes could explain the higher citrate produced with glucose as the sole carbon source. Moreover, the higher PPP fluxes would also reduce the fluxes toward the NADP dependent isocitrate dehydrogenase, reported to be present in *Y. lipolytica*, the major citrate degrading enzyme.

3.1.6 Comparison of yield coefficients

Point yield of biomass, lipid and citric acid was calculated by dividing respective point productivity with glycerol consumption rate (calculated and presented in Table 4) at a particular point of time and presented in Figure 4. Irrespective of type of glycerol medium, biomass yield (g biomass produced/ g glycerol consumed) was higher during the initial 24 h than later period of the fermentation process (24-96 h) (Figure 4a). Higher biomass yield during the initial 24-32 h is due to an abundance of nitrogen and other nutrients available in the medium for growth. Biomass yield was observed highest for pure glycerol indicating no growth inhibition or absence of inhibitory compounds in the medium. The biomass yield was the lowest for crude glycerol indicating growth inhibition due to high potassium concentration. During 32-96 h, the biomass yield for purified glycerol and crude glycerol was 0.34 g/g and 0.234 g/g, respectively (Figure 4a). Although comparable biomass concentration was observed in pure and purified glycerol medium, however, the biomass production was a little lower and glycerol consumption rate was higher in purified

glycerol (Table 4) leading to a lower biomass yield in purified glycerol medium. Overall yield is calculated for total fermentation time, which is total product produced divided by the total glycerol consumed. At 96 h, the overall biomass yield obtained with purified glycerol, pure glycerol and crude glycerol was 0.56, 0.65 and 0.35 g biomass/g glycerol consumed, respectively.

Lipid yield (Yl/s, g of lipid produced per g of glycerol consumed) and biomass yield was observed lowest for crude glycerol because most of the carbon consumed was diverted towards citric acid synthesis resulting in lowest Yl/s value and highest citric acid yield (Figure 4c). For crude glycerol (having high potassium concentration), lipid yield was slightly increased between 24h -32h and after that stayed nearly constant throughout the fermentation. This was due to a shift in metabolism from growth phase to citric acid production phase (Figure 4c). A dip in lipid yield was observed at 24 h (irrespective of glycerol medium) due to metabolic shift from cell growth phase to either lipid production phase (Figure 4b) or citric production phase (Figure 4c). Irrespective of glycerol medium, lipid yield was higher during initial 24 h due to the formation of cell membrane lipids. For purified glycerol, an increase in C/N ratio (46.61) with glycerol feeding at 72 h (Table 2) resulted in increased lipid yield. Same phenomenon was also observed for pure glycerol. In the case of pure glycerol, C/N ratio of the same order (46.21), as in case purified glycerol, reached earlier at 64h (due to faster growth) and resulted in increased lipid yield. At 96 h, the overall lipid yield obtained with purified glycerol, pure glycerol and crude glycerol was 0.21, 0.22 and 0.124 g biomass/g glycerol consumed, respectively.

Irrespective of glycerol medium, citric acid yield was lower during initial 24-32 h and was higher during later stages of fermentation (Figure 4c). Citric acid yield was higher during later

stages of fermentation due to a decrease in nitrogen concentration and increase in the C/N ratio in the medium. After 24 h, citric acid yield was observed highest in crude glycerol medium due to inhibition of ATP-citrate lyase enzyme responsible for the breakdown of intracellular citrate (as discussed earlier). At 96 h, overall citric acid yield was the highest for crude glycerol (0.2 g/g) followed by purified glycerol (0.059 g/g) and pure glycerol (0.048 g/g).

3.1.7 Comparison of specific growth rates

Irrespective type of glycerol in the medium, the specific growth rate decreased until the end of fermentation process (Figure 5). Such type of decreasing trend of sp. growth rate of YL was reported in the literature [17, 32]. Maximum specific growth rate (μ_{max}) 0.329, 0.273 and 0.083 h^{-1} for purified glycerol, pure glycerol and crude glycerol respectively was observed at 8 h (Figure 5a). This indicates that growth inhibition was significantly reduced in purified glycerol medium and it gave comparable performance to pure glycerol medium. The effect of specific growth rate during initial 24 h is more prominent on final biomass concentration.

In first 16 h, sp. growth rate of YL on pure and purified glycerol decreased rapidly and became equal to sp. growth rate on crude glycerol at 24 h (Figure 5a). This was due to the fact that initially all nutrients were available in the medium. Therefore, in the absence of inhibition in pure and purified glycerol medium YL grew rapidly. But due to their rapid growth, nutrients in the medium depleted faster. As a result, sp. growth rate also decreased rapidly and at 24h became equal to sp. growth rate of YL in crude glycerol. After 24 h, due to nutrients depletion/limitation, the sp. growth rate of YL in purified and pure glycerol medium became lower than that of crude glycerol (Figure 5b). On the other hand, due to the presence of inhibitor (high potassium

concentration), YL grew slowly and rate of decrease of sp. growth rate was lower and consequently nutrients did not deplete at a faster rate.

3.1.8 Computation of half velocity constant

According to Monod model, the half velocity constant is the substrate concentration at which specific growth rate is half of the maximum specific growth rate (μ_{max}) value [33]. Accordingly, the calculated values of the half-velocity constant for purified, pure and crude glycerol medium were 23.5 g/L, 22.5 g/L and 4 g/L, respectively. Lower value of half velocity constant for crude glycerol indicates lower affinity of *Y. lipolytica* for crude glycerol because it contains high potassium concentration, which is inhibitory for cell growth. On the other hand, pure and purified glycerol exhibited comparable half velocity constant values, which were higher than that of crude glycerol due to very low potassium concentration in the medium.

3.2 Mass Balance for purification of 1 L crude glycerol

During purification of crude glycerol using phosphoric acid, a precipiate of KH_2PO_4 is formed through following equation:

$$RCOOK + H_3PO_4 \rightarrow RCOOH + KH_2PO_4\ (precipitate) \qquad (5)$$

The chemicals requirement and KH_2PO_4 generated during the crude glycerol purification process and their corresponding cost is presented in Table 7. Although precipitated KH_2PO_4 is not commerical grade but can be rinsed with isopropyl alcohol (IPA) follwed by heat treatment (90^{o}C for 60 min) to obtain high purity ($\geq$ 97%) phosphate salts [33]. KH_2PO_4 has applications as food additive, buffering agent and fungicide [33]. Considering the cost of phosphoric acid and

potassium dihydrogen phosphate, the glycerol purification strategy is economical because of the following facts: i) purified glycerol can be used as substarte for lipid production using *Y. lipolytica* and ii) the precipitated potassium salts have commercial applications.

Although potassium salts are produced as by-product during glycerol purification, however, after acidification the purified glycerol has pH 2 and needs to be brought to 6.5 for YL cultivation. For fermentation with 3 L working volume, 10 mL of 4 M NaOH (1.6 g NaOH flakes) was utilized to bring pH to 6.5. Considering NaOH bulk price of 0.5 $/kg, cost of pH adjustment is 8×10^{-4} $ for 3 L (0.267$/m^3) working volume. Although pH adjustment cost seems negligible in lab scale reactors, the cost will increase with working volume of the reactor.

4. Conclusion

In this study, crude glycerol with high potassium concentration was purified by precipitating excess phosphorus as KH_2PO_4 with phosphoric acid. The purified glycerol thus obtained was employed as carbon source for lipid production using *Y. lipolytica* SKY7. At 96 h of fed-batch fermentation, the purified glycerol gave higher biomass (51.67 g/L) and lipid concentration (19.47 g/L) than crude glycerol (20.36 g/L and 7.21 g/L, respectively). The process performance of purified glycerol medium was comparable to that of pure glycerol medium in terms of biomass concentration, lipid yield and half-velocity constant. Precipiatated KH_2PO_4, obtained during glycerol purification, improves the process economics as it has commercial applications.

Lalit R Kumar: Conceptualization, Methodology, Formal Analysis, Writing – Original Draft, Data curation

Sravan K Yellapu: Investigation, Resources, Validation

RD Tyagi: Supervision, Funding Acquisition, Project Administrator, Writing – Review & Editing

Patrick Drogui: Supervision

Declaration of interests

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

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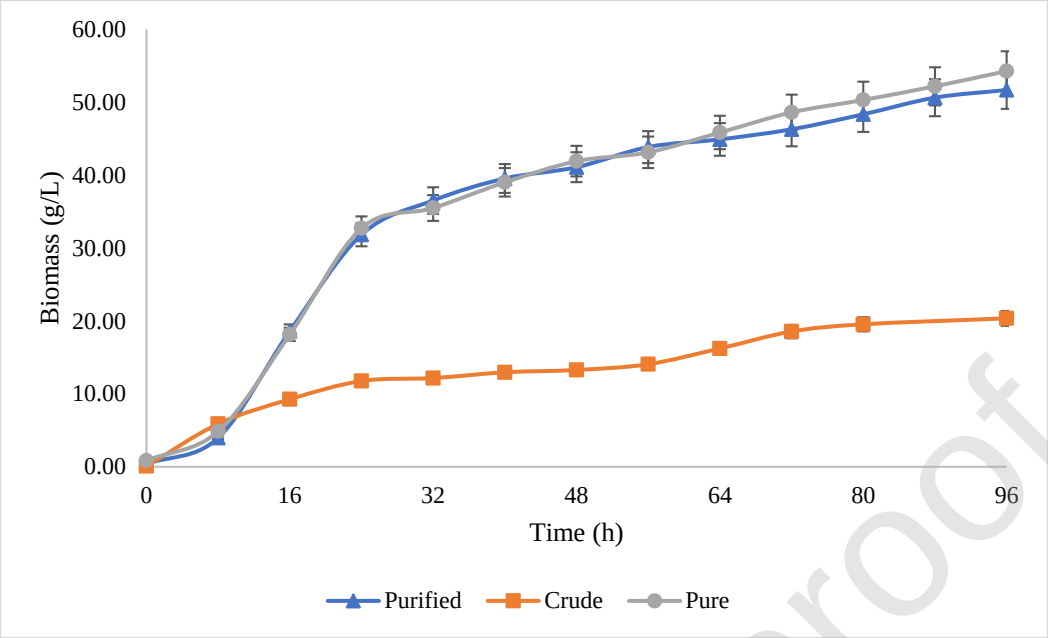
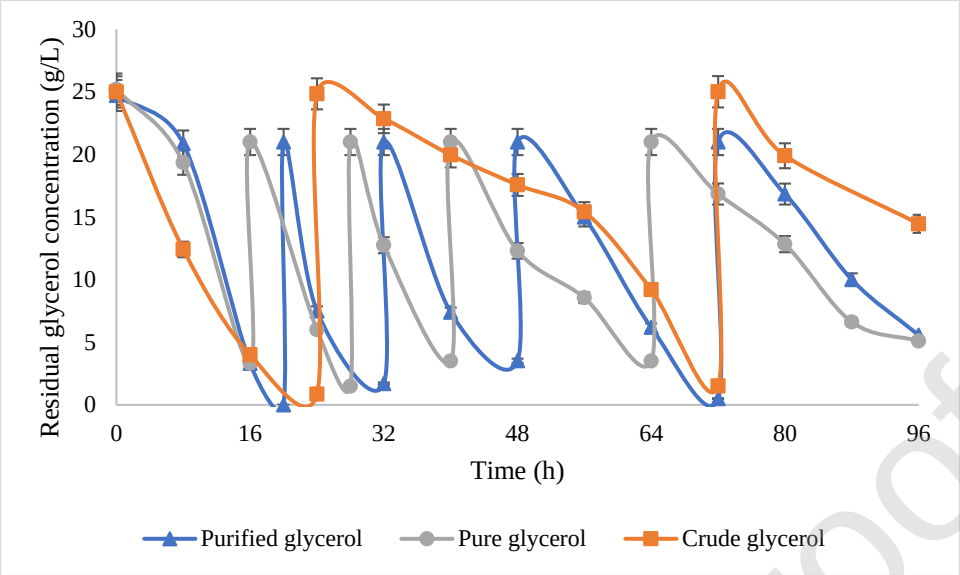


Figure 1: Growth profile in three types of glycerol media. Growth of YL in pure, crude and purified glycerol media at 28ºC and pH 6.5 using fed-batch strategy.



a)

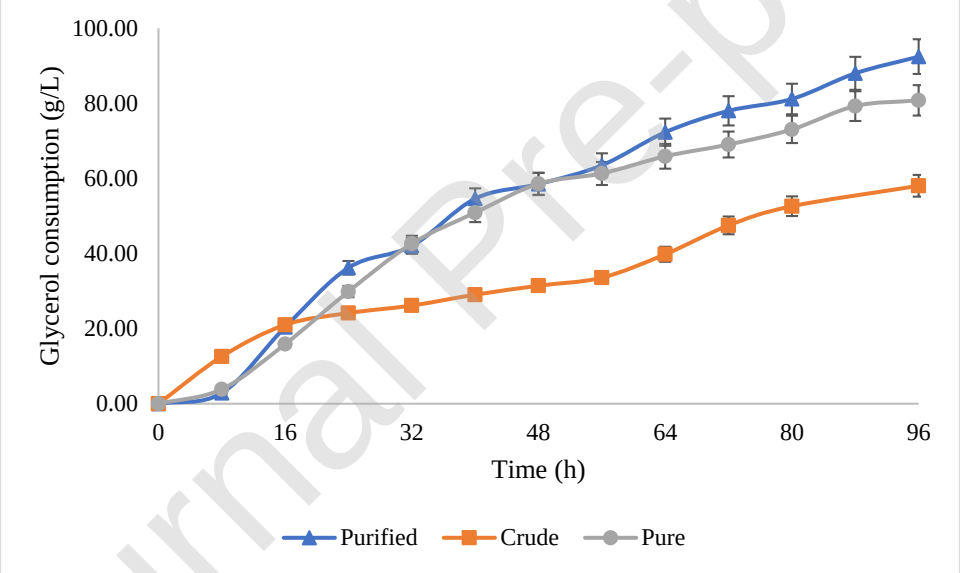


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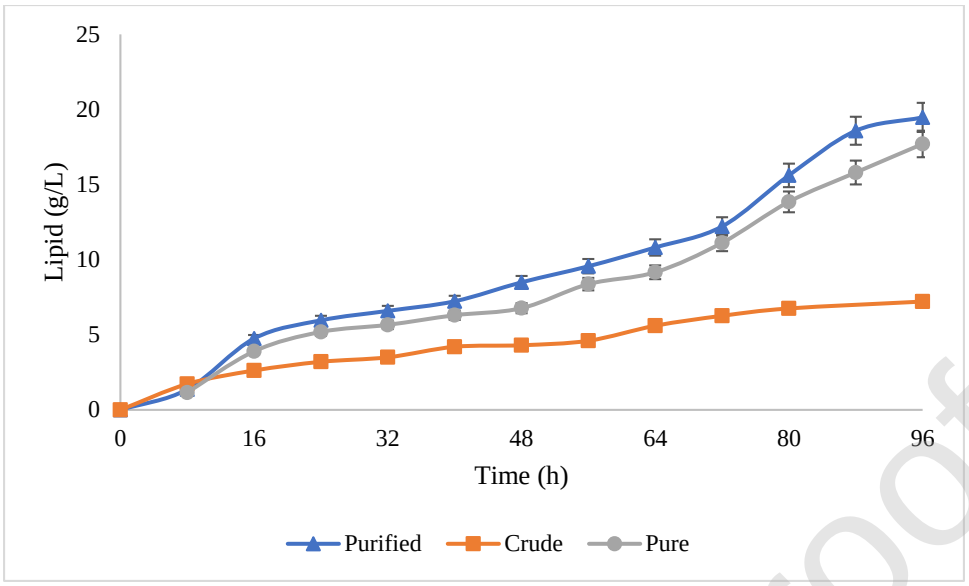
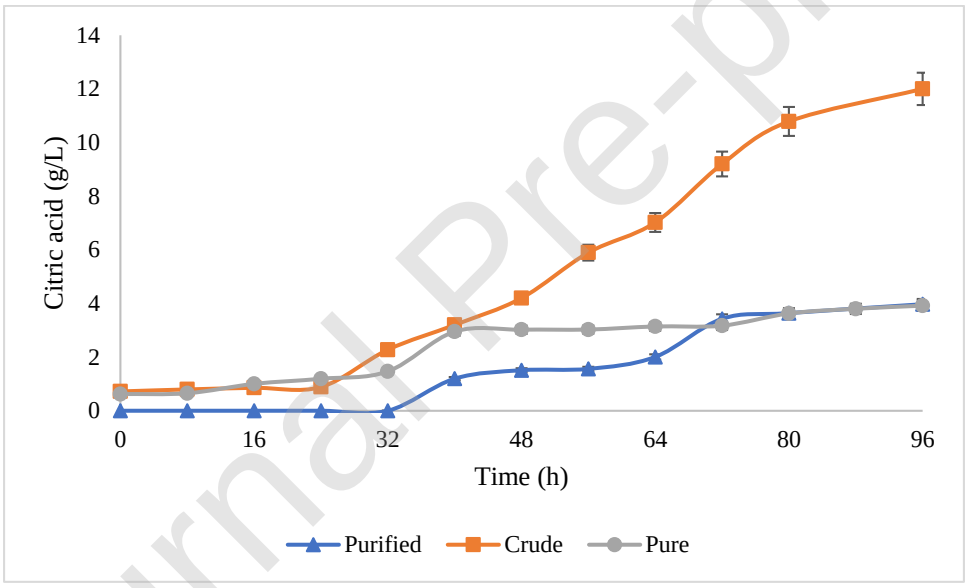
Figure 2: Variation of glycerol concentration and consumption in three different types of glycerol media: a) Residual glycerol concentration and b) glycerol consumed.



a)



b)

Figure 3: Lipid and citric acid production by YL in three different types of glycerol media: a) Lipid concentration and b) Citric acid concentration.

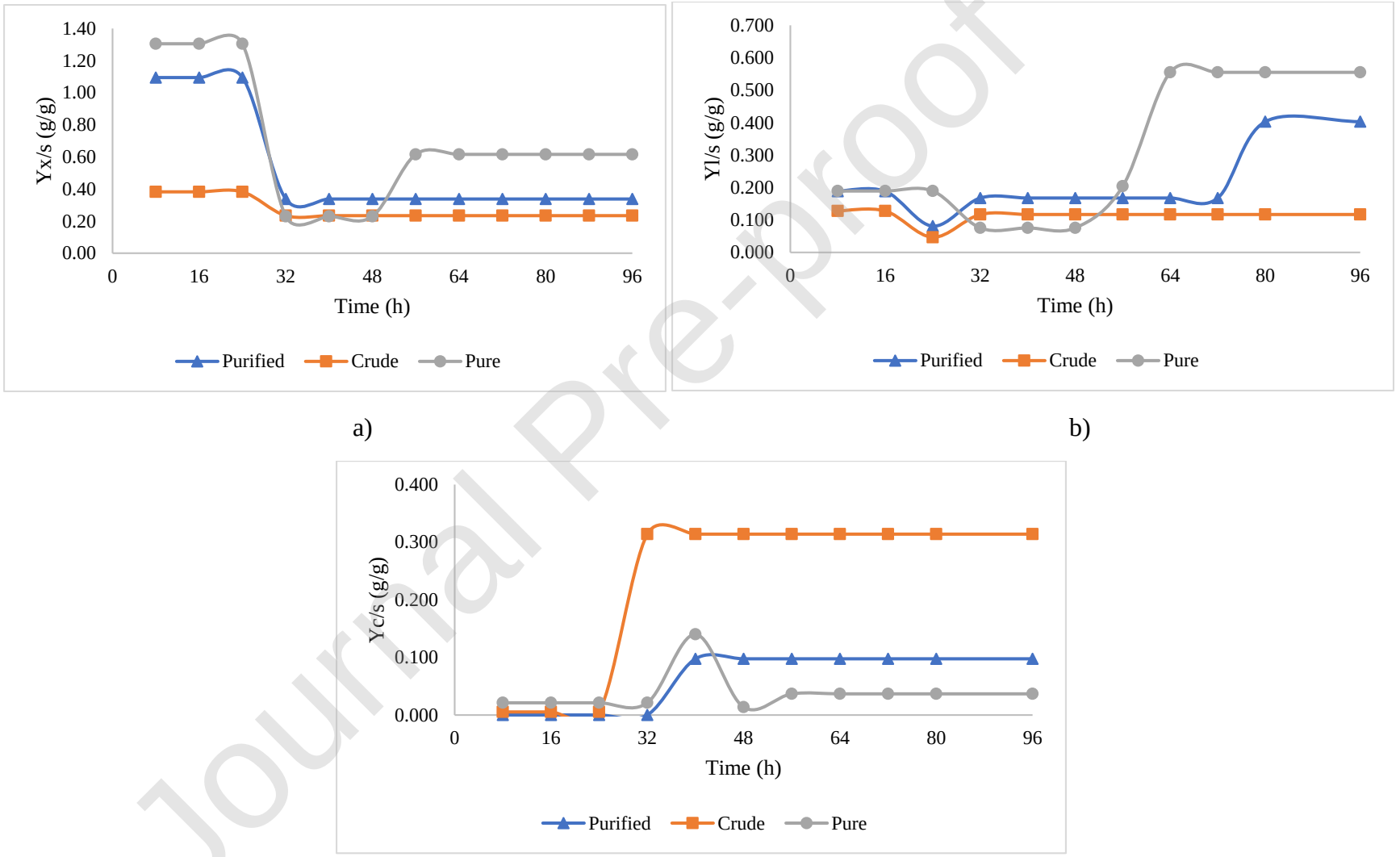
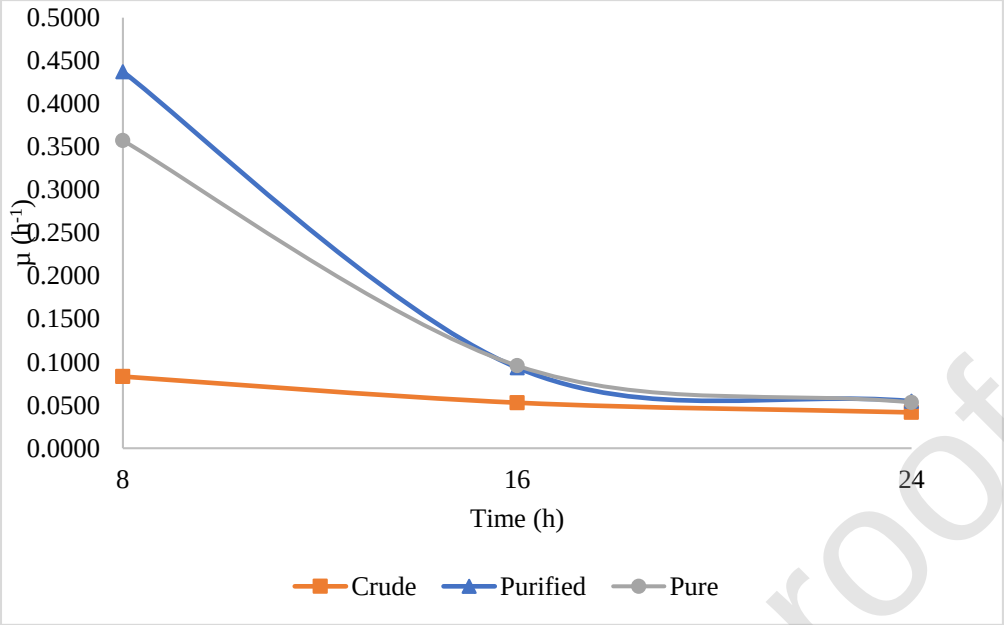
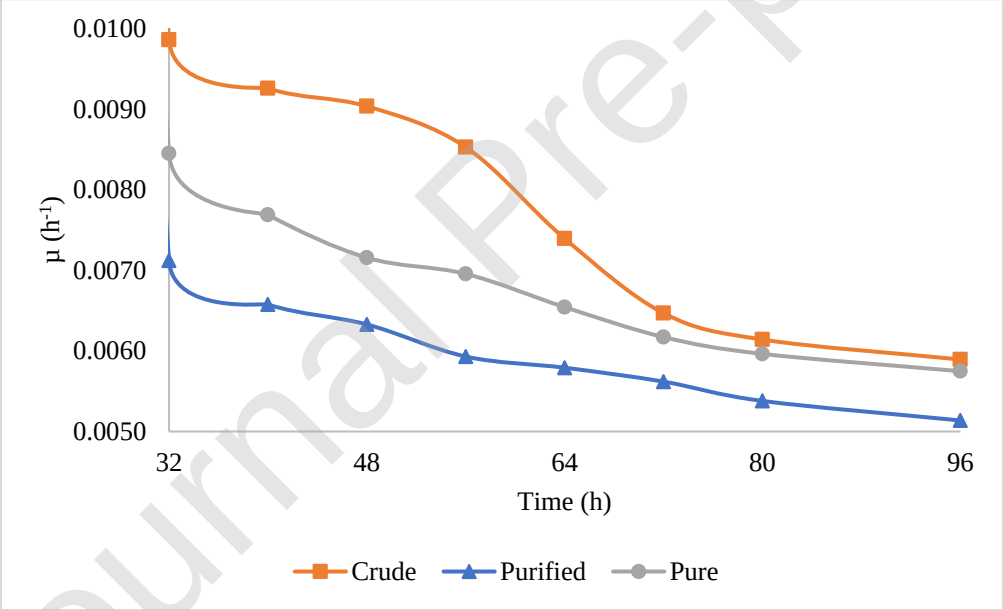


Figure 4: Variation of yield coefficients in three different types of glycerol media: a) Biomass yield (g/g glycerol); b) Lipid yield (g/g glycerol) and c) Citric acid yield (g/g glycerol).



a)



b)

Figure 5: Variation of specific growth rate in three different types of glycerol media: a) during initial 24 h and b) during 32-96 h of fermentation.

Table 1: Characterization of crude glycerol and purified glycerol. Crude glycerol was purified using acid treatment.

Parameter (g/L)	Crude glycerol*	Purified glycerol
Density	1385 ± 10	1242 ± 10
Glycerol concentration	453 ± 5	473 ± 5
Water	132.96 ± 5	232.25 ± 5
Methanol	-	-
pH	13.5 ± 0.1	2.0 ± 0.05
Al	2.58×10^{-3}	2.83×10^{-3}
Ca	1.3×10^{-2}	2.08×10^{-2}
Cr	1.0×10^{-4}	1.8×10^{-4}
Cu	1.72×10^{-2}	1.34×10^{-2}
Fe	8.1×10^{-3}	4.2×10^{-3}
K	73.04 ± 2.2	10.56 ± 0.5
Mg	7.0×10^{-3}	9.2×10^{-3}
Mn	1.7×10^{-4}	1.8×10^{-4}
Na	0.38 ± 0.01	0.38 ± 0.01
Ni	-	2.0×10^{-4}
P	0.18 ± 0.005	23.7 ± 1
Pb	0.8 ± 0.01	1.0 ± 0.05
S	1.2×10^{-2}	2.1×10^{-2}
Sn	3.76×10^{-2}	2.62×10^{-2}
Zn	5×10^{-3}	3.88×10^{-3}

*Samples were characterized from three different batches of same industry for crude and purified glycerol.

Table 2: Feeding strategy during fed-batch fermentation and variation of different parameters due to feed added. Glycerol was fed based on consumption in three different types of glycerol media.

Feeding time and component	Pure glycerol					Crude glycerol			Purified glycerol				
Time (h)	**0**	**16**	**28**	**45**	**64**	**0**	**24**	**72**	**0**	**20**	**32**	**48**	**72**
Glycerol added (g/L)	25 ±1	18±0.8	19.5±0.8	18±0.8	18±0.8	25±1	24±1	23.5±1	25±1	20±1	20±1	18.5±0.8	19.5±0.8
Carbon added (g/L)	10 ±0.5	7.2±0.35	7.8±0.35	7.2±0.35	7.2±0.35	10±0.5	9.6±0.4	9.4±0.4	10±0.5	8±0.4	8±0.4	7.4±0.35	7.8±0.35
Peptone added (g/L)	9.5±0.4	-	-	-	-	9.5±0.4	-	-	9.5±0.4	-	-	-	-
Nitrogen added (g/L)	1.14±0.05	-	-	-	-	1.14±0.05	-	-	1.14±0.05	-	-	-	-
C/N (molar) in the medium	10.32	13.41	32.41	38.89	46.21	10.18	25.70	45.50	10.61	20.47	33.33	40.23	46.67
Microbial Lipid Production (g/L)	-	3.9	5.36	6.54	9.16	-	3.2	6.26	-	5.21	6.59	8.49	12.21
K (g/L)	1.43	1.46	1.22	1.24	1.13	4.8	6.32	8.42	1.8	1.8	2.2	1.82	2.34
P (g/L)	0.99	0.88	0.68	0.66	0.58	0.98	0.87	0.7	1.19	1.04	1.13	1.24	1.75

Table 3: Organic acid profile. Different organic acids produced in three types of glycerol media at 96 h of fermentation.

Type of medium	Pyruvic acid (mg/L)	Glutamic acid (mg/L)	alpha-ketoglutaric (mg/L)	Malic acid (mg/L)	Fumaric acid (mg /L)
Crude glycerol	330 ± 15	<1	580 ± 20	210 ± 10	86 ± 4
Purified glycerol	7.9 ± 0.1	15.0 ± 0.5	150 ± 5	12 ± 0.5	1.9 ± 0.05
Pure glycerol	5.6 ± 0.1	9.9 ± 0.4	105 ± 5	10 ± 0.5	1.4 ± 0.05

Table 4: Variation of productivities. Variation of biomass, lipid and citric acid productivities and glycerol consumption rate during fed-batch fermentation process employing three types of glycerol media.

Pure glycerol	Crude glycerol	Purified glycerol
Biomass productivity (g/L/h)		
8-24 h	0-24 h	8-24 h
dX/dt = 1.74	dX/dt = 0.49	dX/dt = 1.74
R^2 = 0.99	R^2 = 0.94	R^2 = 0.99
24-96 h	24-96 h	24-96 h
dX/dt = 0.3	dX/dt = 0.12	dX/dt = 0.26
R^2 = 0.97	R^2 = 0.94	R^2 = 0.97
Lipid productivity (g/L/h)		
0-24 h	0-16 h	0-16 h
dL/dt = 0.25	dL/dt = 0.164	dL/dt = 0.3
R^2 = 0.96	R^2 = 0.97	R^2 = 0.99
24-64 h	16-96 h	16-72 h
dL/dt = 0.1	dL/dt = 0.06	dL/dt = 0.129
R^2 = 0.99	R^2 = 0.98	R^2 = 0.99
64-96 h		72-96 h
dL/dt = 0.27		dL/dt = 0.31
R^2 = 0.99		R^2 = 0.95
Citric acid productivity (g/L/h)		
0-32 h	0-24 h	0-32 h
dC/dt = 0.019	dC/dt = 0.007	dC/dt = 0
R^2 = 0.96	R^2=0.97	R^2 = 1
40-96 h	24-96 h	32-96 h
dC/dt = 0.028	dC/dt = 0.161	dC/dt = 0.075
R^2 = 0.9	R^2 = 0.987	R^2 = 0.95
Glycerol consumption rate (g/L/h)		
0-48 h	0-24 h	0-24 h
dS/dt = 1.3	dS/dt = 1.28	dS/dt = 1.59
R^2 = 0.98	R^2 = 0.98	R^2 = 0.94
48-96 h	24-96 h	24-96 h
dS/dt = 0.49	dS/dt = 0.51	dS/dt = 0.77
R^2 = 0.99	R^2 = 0.94	R^2 = 0.99

Table 5: Comparison of results on lipid production observed in this study with those reported in the literature.

Micro-organism	Substrate	Cultivation Mode	Time (h)	Biomass productivity (g/L/h)	Lipid productivity (g/L/h)	Reference
Y. lipolytica (Engineered)	Glucose	Batch	120	0.24	0.143	[27]
Y. lipolytica SKY7	Crude glycerol	Batch	60	0.3	0.13	[17]
Y. lipolytica SKY7	Purified glycerol	Fed-batch	96	0.54	0.2	This study
T. oleaginosus	Crude glycerol (high soap content)	Batch	56	0.44	0.22	[14]
C. curvatus	Crude glycerol (after soap removal)	Fed-batch	288	0.11	0.06	[28]
L. starkeyi	Crude glycerol	Fed-batch	112	0.29	0.13	[29]

Table 6: Comparison of results on citric acid production observed in this study with those reported in the literature.

Micro-organism	Substrate	Cultivation Mode	Time (h)	Citric acid productivity (g/L/h)	Reference
Y. lipolytica SKY7	Crude glycerol (high potassium content)	Fed-batch	96	0.125	This study
Y. lipolytica SKY7	Crude glycerol	Batch	120	0.093	[17]
Y. lipolytica	Waste cooking Oil	Batch	336	0.09	[30]
Y. lipolytica	Pure Glycerol	Batch	90	0.2	[31]
Y. lipolytica	Glucose	Batch	90	0.6	[31]
Y. lipolytica (Engineered)	Glucose	Fed-batch	240	0.46	[32]

Table 7: Cost of chemicals required for glycerol purification and net revenue generated. KH_2PO_4* produced as a by-product during glycerol purification was accounted as revenue.

Input		**Output**	
Chemical	**Value**	**Chemical**	**Value**
Crude glycerol	1385.2 g	Purified glycerol	869.26 g
Phosphoric Acid	304.5 g	*KH_2PO_4	820.44 g
Unit Price of phosphoric acid	55.53 $/kg	Unit Price of KH_2PO_4	119 $/kg
Cost of purification	**16.88 $**	**Revenue**	**97.63 $**