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Optimization of trace elements in purified glycerol for microbial lipid and citric acid production by Yarrowia lipolytica SKY7

--Manuscript Draft--

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Full Title:	Optimization of trace elements in purified glycerol for microbial lipid and citric acid production by Yarrowia lipolytica SKY7
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Abstract:	Purified glycerol obtained after acid treatment of crude glycerol solution was used as the carbon source for lipid and citric acid production using Y. lipolytica SKY7. Although purified glycerol was high in phosphorus, it was important to investigate the impact of fortification of trace elements in the medium on cell growth, lipid and citric acid (CA) production. When all the trace elements (including phosphates and sulphates) required for growth and lipid production were added to the purified glycerol medium, high biomass (51.67 g/L) and lipid concentration (19.47 g/L) were observed at 96 h of fed-batch fermentation with low CA concentration of 5.42 g/L. The purified glycerol medium without additional trace elements gave low biomass (27.67 g/L), lipid concentration (9.35 g/L) at 80 h of fed-batch fermentation, but gave high CA concentration (24.51 g/L). When purified glycerol was provided with only sulphates or all elements except KH 2 PO 4 , low biomass (32.59 g/L & 38.52 g/L) and citric acid concentration (1 g/L & 2.42 g/L) were obtained at 96 h.
Corresponding Author:	Rajeshwar Tyagi INRS Eau Terre et Environnement CANADA
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	INRS Eau Terre et Environnement
Corresponding Author's Secondary Institution:	
First Author:	Lalit R Kumar, M. Tech
First Author Secondary Information:	
Order of Authors:	Lalit R Kumar, M. Tech Sravan K Yellapu, PhD Rajeshwar Tyagi Patrick Drogui, PhD
Order of Authors Secondary Information:	
Author Comments:	
Response to Reviewers:	The reviewer's comments have been responded with answer and the changes in the revised manuscript have been highlighted in yellow. Reviewer #1: Crude glycerol solution is cheap and easily acquired bioresource. Pretreated crude glycerol, namely purified glycerol, can be used as substrate in fermentation to produce chemicals. As this substrate has complex composition, it is important to investigate the impact of fortification of trace elements in the medium on cell growth and chemicals production. The authors therefore performed shaking flask and fermenter cultivations of Y. lipolytica SKY7 using purified glycerol with different trace elements to find suitable conditions for the production of microbial lipid and citric acid. The work is interesting

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and provides novel insights on the utilization of industrial wastes. For the most part, this work is based on sound designs and experiments. However, the authors should make sure the manuscript flows in a logical way.

Major comments:
1. Also, the authors present tons of results within the manuscript, the main line of the study is not clear. If the authors intend to optimize the media for fermentation, systematically design is needed. On the other hand, although different combinations of purified glycerol and trace element had divergent effect on chemical production, the readers could be hard to find which in the substrate is the key positive factor or which one is negative specifically. I suggest the authors could focus more on purified glycerol itself and clearly illustrate the characteristic of its content as a fermentation medium. This will provide reference value for the readers.
Answer: The main objective of the study is highlighted in lines 41-48 in the introduction section of the revised manuscript.
The systematic design of experiments has been provided in Table 2.
The important finding of the study is highlighted in the abstract of the revised manuscript. Pl see lines 11-17 of the revised manuscript. The detailed metabolism of YL for cell growth, lipid and citric acid synthesis is discussed in section 3.1.
The characteristics of crude and purified glycerol are presented in Table 1. Purified glycerol as a growth medium has been tabulated in Table 5 and discussion is provided in lines 218-221 of the revised manuscript.

Minor comments
1. P3 L46-47 Please rephrase this long sentence.
Answer: The sentence has been rephrased. Lines 64-66 of the revised manuscript.

2. P3 2.2 strain used only introduces the products of SKY7, other aspects should be included from cell physiology point of view, such as temperature, pH, substrate spectrum….
Answer: Other physiological details about the strain used are provided in lines 67-69 of the revised manuscript.

3. P4 L24-34 I don't know why there are some words underlined. It would be better to list the ingredient of different flasks in a table.
Answer: The underlined words have been removed. The ingredients used in different flask medium are provided in Table 2.

4. P6 L12-27 Are they repetition of flask media?
Answer: Yes, they are repetition of flask media in fermenter. This was necessary as in shake-flask studies, there is no-control of pH due to which, pH of the medium decreases with time due to production of organic acids by microbes. However, pH-control is important for lipid and citric acid production. Controlled- pH conditions are obtained in fermenter. This argument is provided in lines 206-209 of the revised manuscript.

5. P7 L4 What is the solid content of the culture? Will it affect the biomass assay?
Answer: There is no solid content in the culture medium. The solid content was only in the form of biomass generated during growth. Biomass concentration was estimated by standard method of weighing the washed and dried centrifuged biomass pellet of 15 mL sample volume.

6. P8 L43 KH2PO4 also contains phosphorus, which is an important substrate for cell growth. How would you evaluate it’s effect on the results?
Answer: The effect of phosphorus has been incorporated in the revised manuscript. Pl check lines 221-224 of the revised manuscript for effect of phosphorus.

7. Please correct all "KH2PO4" in the figures.
Answer: KH2PO4 has been written as “monopotassium phosphate” in all figure legends.

Reviewer #2:
1) Write the collage address in English.

Answer: The college name is in French and cannot be translated in English. Pl check Line 4 of the revised manuscript.

2) Give the full company name of 'BIOLIQ-INC'.
Answer: BIO-LIQ is the full name of the company.

3) As the YPD contains '20 g/L glucose, 20 g/L peptone and 10 g/L yeast extract' (in page 4), what does 'fortified with 50 g/L YPD' mean in line 12, page 5?
Answer: The statement has been modified. Line number 91-92 of the revised manuscript.

4) In table 1, not good to keep only 1 significant digit for concentration of Cr, Mg and Zn in crude glycerol, and for pH, concentration of Ni and Pb in purified glycerol.
Answer: The suggestion has been incorporated in table 1 and highlighted in the revised manuscript.

5) Provide details of the methods of LC-MS-MS for organic acids analysis and ICP-MS for elemental concentration determination in page 7.
Answer: The column and equipment details for LC-MS and ICP-MS have been provided in lines 137-141 of the revised manuscript.

6) All the tables need to be changed to three-line form.
Answer: All the tables have been changed to 3-line format.

7) In figure 1, the biomass is still growing dramatically in exponential phase and the glycerol concentration is still decreasing linearly, it seems the fermentation can be continued for better results, why stopped the fermentation at 96 h?
Answer: The fermentations could have been conducted for a longer time. But the main objective of the study was to compare the effect of different trace elements in purified glycerol on biomass, lipid and citric acid, which is clearly visible in 96 h run.

8) In table 2, why the initial glycerol added in the sample of 'purified glycerol with all the trace elements' is 25 g/L instead of 20 g/L like other samples? It makes this medium different from the other three samples and may influence the results.
Answer: The high initial glycerol concentration in ‘purified glycerol with all trace elements’ is due to manipulation error during glycerol addition. Yes, the difference in initial glycerol concentration might influence the results. But considering very high glycerol consumption rates (Table 8) in Fermenter 4 (purified glycerol with all trace elements), it can be concluded that the ‘presence of all trace elements including KH_2PO_4’ is major reason for enhanced biomass concentration and glycerol consumption in Fermenter 4. Pl check lines 332-335 of the modified manuscript.

9) How to decide the fermentation ending time? Either the same fermentation time or the same feeding times are acceptable, but this experiment ended in different time with different feeding strategy.
Answer: The feeding time is dependent on residual glycerol concentration in the fermenter. Pl see Figure (3a) of the revised manuscript. When glycerol concentration went below 5 g/L, glycerol feed was imparted. Even though fermentation ending time are different, the results can be compared based on productivities. Biomass, lipid and citric acid productivities are discussed in section 3.2.5.

10) In discussion of '3.2.7 comparison of yield coefficients' page 16, it is reasonable that the decrease of lipid yield was caused 'by the shift of metabolism from cell membrane lipid production (cell growth phase) to cytosolic lipid production (lipid production phase)', but the time points can't be explained so well. For 'purified glycerol with sulphates and expect KH_2PO_4, a dip in lipid yield was after 32 h' in figure 6a, but the change of cell growth phase was earlier than 24 h in figure 2.
Answer: Although change of cell growth phase was earlier than 24 h in figure 2. But please see figure 4a and table 8 of the modified manuscript where in fermenter 3 (all except KH_2PO_4), change in lipid productivity was observed from 32 h. The sentence (L357-359) has been written considering this fact.

11) Write details of all the instruments, its model number, country of manufacturer.
Answer: All the instruments have been written with manufacturer and the country

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(highlighted).

12) English needs to be checked carefully. For instance, the word in abstract 'high biomass (51.67 g/L) and lipid concentration (19.47 g/L) WAS observed' needs to be changed to WERE.
Answer: English corrections have been rectified in the revised manuscript.
The sentence has been changed in the revised manuscript. Pl check lines 13-14 of the revised manuscript.

13) In line 21, page 2, 'involve not only problem' needs to change order to put 'INVOLVE' behind 'NOT ONLY'.
Answer: The mistake has been rectified in the revised manuscript. Pl check lines 30-31 of the revised manuscript.

14) In line 39, page2, 'catalyst' needs to be in plural form 'catalysts'.
Answer: The mistake has been rectified in the revised manuscript. Pl check line 37 of the revised manuscript.

15) The second sentence in '2.1 crude glycerol purification', page 3, is incorrect. It only contains the subject 'BIOLIQ' and a subordinate clause. In line 31 the same page, 'protocols given in (Chen et al. 2017)' needs to be changed to 'protocols given by Chen et al. (2017)'.
Answer: The correction has been incorporated in the revised manuscript. Lines 53 and 59-60 of the revised manuscript.

16) In line 10, page 4, '(or PC1)' can omit the word 'OR'.
Answer: The suggestion has been incorporated in line 89 of the revised manuscript.

17) In line 5, page 7, 'reported by Chen et al. (2017);(Zhang et al. 2017)', change the sentence.
Answer: The suggested change has been made in lines 134-135 of the revised manuscript.

18) In line 16, page 8, 'Specific growth rate (μ, h-1)' needs to be 'Specific growth rate (μ/h)' to consist with other parts of the article.
Answer: The suggestion has been incorporated in line 160 of the revised manuscript.

19) In line 25, page 11, the sentence does not have a verb.
Answer: The sentence has ben corrected, pl check lines 233-234 of the revised manuscript.

20) In line 33, page 18, 'when compared to compared to pure glycerol' needs to delete the extra 'compared to'.
There are so many grammar errors in this article.
Answer: The error has been fixed, pl check line 399 of the revised manuscript.
Grammar errors in the article has been rectified.

Reviewer #3: This is an experimental report on the effect of trace metals on the growth and product formation by a Yarrowia lipolytica strain. It is essentially a preliminary medium optimization study and unfortunately in its current form the paper lacks the scientific significance to justify publication in SMAB. The result is well-expected, although the exact numbers should to be dependent on the glycerol purification protocol and the composition of the crude glycerol. In terms of experimental methods, the trace element concentrations (Page 4) appear to be arbitrarily determined. If the authors are willing to, a detailed design of experiment is expected to gain deeper understanding in the roles of the metal ions involved.

Answer: The detailed design of experiment is provided in table 2. The trace element concentration might seem to be arbitrarily determined, but it is an effective way to conclude the effect of particular trace element on cell metabolism of YL for cell growth, lipid or citric acid synthesis.

"Investigating the" in the title does not serve much purpose.

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	Answer: The title of the manuscript has been changed to ‘Optimization of trace elements in purified glycerol for microbial lipid and citric acid production by Yarrowia lipolytica SKY7’

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Optimization of trace elements in purified glycerol for microbial lipid and citric acid production by *Yarrowia lipolytica* SKY7

Lalit R Kumar[a], Sravan K Yellapu[a], RD Tyagi[a*], Patrick Drogui[a]

[a]*INRS-ETE, 490, Rue de la Couronne, Québec, Canada, G1K 9A9*

**Corresponding author: Tel: 1-(418) 654-2617; Fax: 1-(418) 654-2600. Email:Rd.tyagi@ete.inrs.ca*

ABSTRACT

Purified glycerol obtained after acid treatment of crude glycerol solution was used as the carbon source for lipid and citric acid production using *Y. lipolytica* SKY7. Although purified glycerol was high in phosphorus, it was important to investigate the impact of fortification of trace elements in the medium on cell growth, lipid and citric acid (CA) production. When all the trace elements (including phosphates and sulphates) required for growth and lipid production were added to the purified glycerol medium, high biomass (51.67 g/L) and lipid concentration (19.47 g/L) were observed at 96 h of fed-batch fermentation with low CA concentration of 5.42 g/L. The purified glycerol medium without additional trace elements gave low biomass (27.67 g/L), lipid concentration (9.35 g/L) at 80 h of fed-batch fermentation, but gave high CA concentration (24.51 g/L). When purified glycerol was provided with only sulphates or all elements except KH_2PO_4, low biomass (32.59 g/L & 38.52 g/L) and citric acid concentration (1 g/L & 2.42 g/L) were obtained at 96 h.

Keywords: *Yarrowia lipolytica* SKY7, Purified glycerol, Trace elements, Microbial lipid, Citric acid

1. Introduction

Current biodiesel feedstock like vegetable oil, fossils, and wood cause environmental concerns (e.g., global warming, greenhouse gas emissions) and lead to depletion of natural resources including deforestation. Animal slaughtering for biodiesel production will lead to fuel vs food competition. Microbial oil or microbial lipid can be quickly synthesized by bacteria, yeast and fungi and its fatty-acid composition is highly similar to that of vegetable oil (Wang et al. 2015; Marjakangas et al. 2015). Since commercial substrates like glucose not only involves problem of food versus fuel competition, but also are expensive for microbial lipid production and increases the final biodiesel production cost. Therefore, alternative substrates need to be explored. Crude glycerol has attracted researchers' attention in past few years because it is a by-product of the biodiesel industry and application of crude glycerol as a substrate for lipid production will help to boost biodiesel industry economy by maintaining the circular economy (Kumar et al. 2019).

However, crude glycerol contains several impurities, which emanate from the trans-esterification process due to the use of different catalysts such as methanol, soap, free fatty acids, metals and salts. These impurities may inhibit cell growth and lipid production depending on the stain and concentration of the impurity (Kumar et al. 2019). Acidification is an effective method to remove soap, metals and salts from the crude glycerol (Nanda et al. 2014; Chen et al. 2018; Kumar et al. 2019). However, purified glycerol obtained after acid treatment may contain several trace elements required for microbial growth, but they may not be present in sufficient concentration needed for lipid and citric acid production or they may not be consumed by microbe. If they are not present in sufficient quantity, what extra elements would be needed to be supplied for high lipid and citric acid production? Therefore, the objective of this study was to investigate whether purified glycerol had sufficient trace elements for high lipid and citric acid production by

Yarrowia lipolytica SKY7 (YL). Also, optimum trace element concentration was found out to obtain high cell biomass and lipid concentration by YL.

2. Methodology

2.1 Crude glycerol purification

The crude glycerol was obtained from Canadian biodiesel producing company BIO-LIQ INC. BIO-LIQ crude glycerol had high potassium concentration because potassium methoxide was used as a catalyst during trans-esterification process step. The high potassium concentration in BIO-LIQ crude glycerol was treated with phosphoric acid (by adjusting pH to 2) followed by centrifugation at 6000 rpm for 10 minutes. During the purification process of crude glycerol using phosphoric acid, precipitates of KH_2PO_4 are formed as indicated by the following equation:

$$RCOOK + H_3PO_4 \rightarrow RCOOH + KH_2PO_4\ (precipitate) \qquad (1)$$

The characterization for crude and purified glycerol was performed using protocols given in Chen et al. (2017). The composition of crude glycerol solution and that of purified glycerol is mentioned in table 1.

2.2 Strain Used

Y. lipolytica SKY7 (YL) isolated in INRS laboratory was used in this study. YL is a wild strain and can accumulate lipids up to 50% of cell dry weight (w/w). The lipids produced by the strain closely resemble with vegetable oil and could serve as a feedstock for biodiesel production. The optimum pH and temperature for growth of YL are 6.5 and 28^{o}C. The strain is capable of

assimilating a wide variety of substrates like glycerol, Tween 80, D-glucose, N-acetyl-D-glucosamine, inulin, L-proline, L-glutamic acid and succinic acid (Kuttiraja et al. 2015). The strain also produces citric acid, which could be recovered and used by the food and/or chemical industry since the organism is safe to use at the industrial level (Kuttiraja et al. 2015).

2.3 Shake-flask studies

The dormant pure culture of *Y. lipolytica* (4ºC) was revived by cultivating in 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 a shaking incubator with agitation 180 rpm and temperature of 28ºC. PC1 volume of 6.25% v/v was used for inoculation of experimental flasks. Experiments were carried out in duplicate in 1 L Erlenmeyer flask with a working volume of 300 mL. The shake-flasks experiments were started with purified glycerol (20 g/L) while 4 g/L peptone was added at 0 h to provide nitrogen source. Initial pH was 6.5 but the pH was not controlled in shake-flasks. No additional trace elements were added in shake flask 1 at 0 h. The media composition of shake flask 2, 3 and 4 is tabulated in table 2. The flasks were incubated at 180 rpm and 28ºC for 96 h. The samples were withdrawn every 24 h for biomass and glycerol estimation while lipid and citric acid were estimated for 96 h sample.

2.4 Fermentation under controlled conditions

2.4.1 Pre-culture and inoculum preparation

The dormant pure culture of *Y. lipolytica* SKY7 (4ºC) was revived by cultivating in pre-culture 1 (PC1) synthetic media (given above) for 24 h in a 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 medium was prepared by dissolving 50 g YPD in 1L tap water and purified glycerol was added with concentration of 20 g/L. This was done to acclimatize *Y. lipolytica* in purified glycerol medium. PC2 was grown at 28ºC in an incubating shaker at 180 rpm for 36 h before being transferred to the production fermenter.

2.4.2 Fermentation Operation

Fermentations were carried out in stirred tank fermenters (SARTORIUS BIOSTAT, USA and LABFORS 3, INFORS AG, Switzerland) 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 (Mathiazhakan et al. 2016). During the fermentation, DO was maintained between 25-40% saturation by adjusting the agitation rate (250–600 rpm) and airflow rate (1–2.5 L/min). The limitation of DO favors lipid production (Yen and Zhang 2011). 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, temperature and pH were continuously monitored by means of a polarographic dissolved oxygen probe (Mettler-Toledo, USA), temperature probe (Mettler-Toledo, USA) and a pH sensor (Mettler-Toledo, USA), respectively.

2.4.3 Fed-batch strategy

Fermentations were conducted using purified glycerol as a carbon source. The fermenters were operated under fed-batch mode to control substrate limitation. The production fermenters were operated at temperature 28ºC and pH 6.5 for *Y. lipolytica* SKY7 as reported by Kuttiraja et al. (2018). No additional trace elements were added in fermenter 1 at 0 h. The fermenter 2 had only different sulphates at 0 h: 0.2 g/L $MgSO_4.7H_2O$, 0.0055 g/L $FeSO_4.7H_2O$, 0.001 g/L $ZnSO_4.7H_2O$ and 0.00076 g/L $MnSO_4.H_2O$. All trace elements except KH_2PO_4 were added at 0 h in fermenter 3: 0.95 g/L Na_2HPO_4, 0.2 g/L $MgSO_4.7H_2O$, 0.0055 g/L $FeSO_4.7H_2O$, 0.001 g/L $ZnSO_4.7H_2O$ and 0.00076 g/L $MnSO_4.H_2O$. In fourth fermenter, all the additional trace elements were added in the medium at 0 h with composition: 2.7 g/L KH_2PO_4, 0.95 g/L Na_2HPO_4, 0.2 g/L $MgSO_4.7H_2O$, 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 production fermenters were started with 8-10 g carbon /L (or 20-25 g glycerol /L) and C/N ratio (molar) of 10 at 0 h. When glycerol concentration in the medium reached below 5 g/L, a glycerol feed was added so that the glycerol concentration in the medium reaches the initial glycerol concentration (20-25 g/L). Peptone was added at 0 h only to provide nitrogen for cell growth. However, no nitrogen (peptone) and trace elements were imparted during the feed at later stages. The reason for it is that high initial nitrogen concentration or low initial C/N ratio will help to build high lipid- free biomass during early stages of fermentation and once nitrogen concentration will become limiting, the addition of glycerol feed will increase the C/N ratio in the medium, which will result in high lipid accumulation (Chen et al. 2017). Feeding details are presented in Table 3. Samples were withdrawn every 8 h during the fermentation to determine the biomass, lipid, glycerol and organic acid concentration in the fermentation medium.

2.5 Analytical Techniques

Biomass concentration and lipid concentration were determined as reported in Chen et al. (2017). Glycerol was measured according to the method by Bondioli and Della Bella (2005). Citric acid estimation was carried out according to the method by Marier and Boulet (1958). Other organic acids (keto-glutamic acid, pyruvic acid, malic acid) in the supernatant were analyzed using LC-MS (Liquid chromatography-mass spectrometry with 2x150 mm column VG-50 2D, Shodex, Japan). Elemental concentration in the samples was determined by inductively coupled plasma mass spectroscopy (ICP-MS) after acid digesting the samples (model DRE, Leeman Labs Inc, USA) (Kaur et al. 2019).

For 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, USA) equipped with flame ionization detector. Column length was 60 m (Agilent J&W, USA); the carrier gas was helium at a flow rate of 1.18 mL/min with the oven temperature at 230 °C. Trans-esterified sample (1µL) 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. Biomass, lipid and citric acid productivity will be calculated by equation 2.

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

Product yield coefficient ($Y_{P/S}$) is defined as the amount of product produced per g of substrate consumed. It 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) was determined by the equation (4):

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

Where X represents biomass concentration at particular time t

Biomass productivity and specific growth rate have been calculated with respect to cell biomass including intracellular lipid.

3. Results and discussion

3.1 Shake-flask studies

Biomass profile for different trace element media is presented in Figure 1a. At 96 h, the highest biomass concentration (9.73 g/L) was obtained in shake flask 4 where all the trace elements were present in the medium including KH_2PO_4. It indicates that presence of additional potassium in the purified glycerol medium assisted in cell growth of *Y. lipolytica* SKY7. It has been reported that potassium in the media is essential for cellular activities such as maintaining cell volume, enzyme activity, compensation of negative charges of macromolecules to electroneutrality, protein synthesis, maintenance of intracellular pH and membrane potential (Yenush 2016). At 96 h,

biomass concentration in shake flask 3 (containing sulphates and Na_2HPO_4) was higher than biomass concentration in shake flask 2 (containing only sulphates). It reveals that the added sodium salt assisted in cell growth of *Y. lipolytica* SKY7. This is in agreement with the observations of Gao et. al. (2016) who reported that increased concentration of sodium salt in the medium provided *R. toruloides* with osmoregulation and a good physiological state for growth and reproduction. At 96 h, the biomass concentration in shake flask 2 (containing sulphates) was higher than shake flask 1 (no trace elements). The above fact revealed that the added sulphate salts assisted in cell growth of *Y. lipolytica* SKY7. It has been reported that the presence of sulphates in the growth medium provided sulphur element for growth of *R. toruloides*, which is essential for the provision of acyl-SCoA and S-containing amino acids (Yang et al. 2014).

Citric acid and lipid concentration in different flasks at 96h of fermentation are presented in table 4. It shows that without the addition of any trace elements in shake flask 1 leads to citric acid production (4.05 g/L). However, the addition of sulphates in the medium (shake flask 2, 3 and 4) leads to no citric acid production. The citric acid production (citrate concentration in the cytoplasm) is one of the important factors that control the de-novo lipid accumulation (EVANS et al. 1983). 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) (Kuttiraja et al. 2018). If ACL enzyme gets inhibited, accumulated citrate in cytoplasm comes out of the cell instead of being converted into lipids. The metal ions like Mg^{2+}, Fe^{2+}, Mn^{2+} and Zn^{2+} which were supplied in the form of sulphate salts in shake flasks 2, 3 and 4 play an important role

in the functioning of ACL enzyme and lipid production. It has been reported that addition of 1.5 g/L Mg^{2+}, 0.0001 g/L Mn^{2+}, 0.1 g/L Fe^{3+}, 0.0001 g/L Cu^{2+}, 0.0001 g/L Co^{2+}, 0.1 g/L Ca^{2+}, and 0.0001 g/L Zn^{2+} in the growth medium after 48 h increased the lipid accumulation in fungi *Cunninghamella bainieri* (Shuib et al. 2014). Metals like Mg^{2+} and Fe^{2+}act as cofactor for ACL enzyme and are essential for lipid production (Ma et al. 2009). Moreover, iron is vital for cell metabolism as it impacts the transfer of electrons, DNA synthesis and nitrogen fixation (Concas et al. 2014). A beneficial effect of Mn^{2+} in the concentration range of 2‑500 mg/L on lipid production has been observed in *Mortierella* sp.(Šajbidor et al. 1992). The lipid concentration in shake flask 4 was higher due to highest biomass concentration. The reason for low lipid concentration and biomass lipid content in shake flask studies might also be due to uncontrolled pH in shake flask experiments. It has been reported that lipid and citric acid production improves under pH control (pH – 6.5) condition for *Y. lipolytica* SKY7 (Kuttiraja et al. (2018).

The residual glycerol profile for different trace elements has been highlighted in Figure 1b. At 96 h, glycerol consumption was highest (10.54 g/L) where all the trace elements were added when compared to without the addition of trace elements (8.42 g/L), addition of only sulphates (8.42 g/L), the addition of all elements except KH_2PO_4 (8.89 g/L) (Table 4). It can be concluded that chemical elements present in the purified glycerol are not sufficient to promote cell growth and lipid production in *Y. lipolytica* SKY7. The chemical elements contributed by the added purified glycerol in the medium (Shake flask 1) and concentration of complete trace elements added in the medium (shake flask 4) are compared with the actual requirement (Table 5). It can be observed from table 5 that sufficient Fe, K, Mg, Mn and Zn were not present where purified glycerol was added in the medium and no extra trace elements were added. Hence, they had to be imparted

through the addition of trace elements - sulphates and phosphates. It can also be concluded from tables 4 and 5 that although the purified glycerol medium had sufficient phosphorus in it, additional phosphorus imparted through Na_2HPO_4 and KH_2PO_4 had no detrimental effect on the biomass concentration.

3.2 Fermenter studies

3.2.1 Biomass concentration

Impact of the trace elements in purified glycerol medium on biomass concentration is highlighted in Figure 2. The biomass results in fermenter studies follow a similar trend as that of shake flask studies. At 96 h, the highest biomass concentration (51.67 g/L) was obtained in fermenter where all trace elements were added in the medium, including KH_2PO_4, followed by purified glycerol except KH_2PO_4 (38.32 g/L), purified glycerol with only sulphates (32.59 g/L). Throughout fermentation, lowest biomass concentration was observed in purified glycerol medium without any trace elements. Thus, additional potassium, sulfur and sodium at 0 h in the medium assisted in cell growth of *Y. lipolytica* SKY7.

3.2.2 Glycerol consumption

The maximum glycerol consumption was observed when all the trace elements were imparted at 0 h in the purified glycerol medium (92.43g glycerol/L at 96 h) (Figure 3b). The presence of all trace elements in purified glycerol provided a complete growth medium for *Y. lipolytica* due to which higher glycerol consumption was observed. The maximum number of glycerol feeds (four) were also required and provided when all the trace elements were present (Fig 3a). However,

higher glycerol consumption was observed when no trace elements were provided compared to only sulphates and all trace elements except KH_2PO_4. This is due to higher citric acid production when no trace elements were provided in the purified glycerol medium (discussed later).

3.2.3 Lipid production

At 96 h, highest lipid production (19.47 g/L) occurred in purified glycerol medium where all the trace elements were present (Figure 4a). This was followed by purified glycerol medium where all trace elements were present except KH_2PO_4 (15.72 g/L at 96 h), purified glycerol medium with only sulphates (12.24 g/L at 96 h) and purified glycerol medium without trace elements (9.35 g/L at 80 h). The lipid results obtained in fermenter studies follow a similar trend as that of shake-flask studies. For lipid production, a greater concentration of biomass is needed so that empty cells can be filled with microbial lipids. Microbial lipid production takes place due to increase of the C/N ratio in the fermentation medium. Lowest biomass concentration in the absence of trace elements is the reason for lowest lipid production. The reason for the high lipid accumulation in presence of all trace elements is because of the high biomass concentration in the medium. When nitrogen concentration started decreasing the C/N ratio started increasing due to glycerol feeding (Table 3), lipid concentration started increasing in all glycerol media. Lipid characterization observed in different cases has been provided in table 6.

Lipid characterization revealed that palmitic acid (C16:0), Oleic acid (C18:1) and linoleic acid (C18:2) were produced in all glycerol media. Oleic acid (C18:1) and linoleic acid (C18:2) are the major components in the case of SKY7 as reported in other studies (Kuttiraja et al. 2015; Kuttiraja et al. 2018). These lipids have a resemblance with the vegetable oil. The presence of

polyunsaturated fatty acids (PUFAs) makes the isolate *Y. lipolytica* SKY7 important for biodiesel production. Other fatty acids like mysteric acid (C14:0), stearic acid (C18:1), arachidic (C20:0), and arachidonic acid (C20:4) were produced in minor quantities.

3.2.4 Organic acids production

Citric acid is the major acid produced by *Y. lipolytica*, which is a well-known industrial scale citric acid producer. Highest citric acid concentration (24.51 g/L) was observed at 80 h in the fermenter without additional trace elements (Fig 4b). In other fermenters, low citric acid production was obtained, 1 g/L-5.42 g/L at 96 h. The absence of additional trace elements assisted in citric acid production, as discussed in shake-flask studies. In another three fermenters, these metal ions were supplied in the form of sulphates at 0 h, due to which low citric acid and increased lipid production were detected.

Other organic acids produced during fermentation were pyruvic acid, alpha-ketoglutaric acid, malic acid, glutamic acid and fumaric acid. Other organic acids produced during fermentation at 96 h are highlighted in table 7. Pyruvic acid, malic acid, fumaric acid and alpha-keto-glutaric acid are intermediates of citric acid cycle and all have commercial applications (Vuoristo et al. 2016). Malic acid was majorly produced acid in the medium with only sulphates and trace elements except KH_2PO_4. It is used as a food additive. Alpha-keto glutaric acid was produced in all glycerol media and is used for immune regulation and as anti-oxidant. Pyruvic acid, fumaric acid and glutamic acid were produced in lower quantities. However, the concentration of these acids in the medium is very low and recovery may be expensive.

3.2.5 Variation of biomass, lipid and CA productivities

In order to calculate biomass productivity, the biomass curve can be divided into different sections (Table 8) 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 glycerol consumption rates (dS/dt) have been calculated by similar method.

Biomass productivity was higher during the initial 24 h for all four-glycerol medium and was lower in the later period of fermentation (24-96 h) (Table 8). Higher biomass productivity during initial 24 h is due to abundance of nitrogen in the medium, which resulted in cell growth. Also, during the initial 24 h, carbon of peptone (contains 10% carbon) was used for cell growth. During later phase, biomass productivity was lower due to reduction in nutrients and nitrogen concentration. Biomass productivity in the purified glycerol medium with all trace elements was observed highest among all conditions. At 96 h, the overall biomass productivities for purified glycerol without trace elements, purified glycerol with only sulphates, purified glycerol except for KH_2PO_4 and purified glycerol with all trace elements were 0.35 g/L/h, 0.34 g/L/h, 0.40 g/L/h and 0.54 g/L/h, respectively.

Irrespective of trace elements, lipid productivity was higher during initial 16-32 h period because during this period, membrane lipids develop with biomass growth (Mathiazhakan et al. 2016). While during later stages of fermentation, lipid was accumulated as intracellular lipids in cytosol. For purified glycerol medium with no trace elements and only sulphates, two sections are

identified where membrane lipids are developed (1st section, 0-32 h) and lipids accumulate in cytosol (2nd section, after 32 h) (Table 8). For purified glycerol with all trace elements and except KH_2PO_4, 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. Throughout the fermentation, lipid productivity was the highest in purified glycerol with all trace elements followed by purified glycerol except KH_2PO_4, purified glycerol with only sulphates and purified glycerol with no trace elements. At 96 h, the overall lipid productivities for purified glycerol with all trace elements, purified glycerol except KH_2PO_4, purified glycerol with only sulphates and purified glycerol with no trace elements were 0.2 g/L/h, 0.164 g/L/h, 0.128 g/L/h and 0.117 g/L/h, respectively.

For all trace element's media, citric acid productivities were lower during the initial stages of fermentation (24-48 h) and were higher during late stages of fermentation (Table 8). Citric acid productivities were higher 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 highest in purified glycerol with no trace elements due to inhibition of ATP-citrate lyase enzyme responsible for the breakdown of intracellular citrate. All other trace element media resulted in very low citric acid productivities. At 96 h, the overall citric acid productivities for purified glycerol with all trace elements, purified glycerol except KH_2PO_4, purified glycerol with only sulphates and purified glycerol with no trace elements were 0.056 g/L/h, 0.025 g/L/h, 0.01 g/L/h and 0.31 g/L/h, respectively.

The glycerol consumption rate in Fermenter 4 (all trace elements) was twice as that of other fermenters. The difference in initial glycerol concentration might influence the results. However, the presence of all trace elements, including KH_2PO_4 is a major reason for enhanced biomass and glycerol consumption in Fermenter 4.

3.2.6 Comparison of specific growth rates

For all types of glycerol medium, the specific growth rate monotonically decreased until the end of the fermentation process (Figure 5). Maximum specific growth rate (μ_{max}) for purified glycerol with all trace elements, purified glycerol except KH_2PO_4, purified glycerol with only sulphates and purified glycerol with no trace elements was 0.329, 0.188, 0.253 and 0.178 h^{-1}, respectively at 8 h (Figure 5a). Thus, trace elements are necessary for cell growth of *Y. lipolytica* because lowest μ_{max} was obtained in purified glycerol with no trace elements. The effect of specific growth rate during initial 24 h is more prominent on final biomass concentration.

3.2.7 Comparison of yield coefficients

Point yield coefficients have been calculated by diving point productivity by the glycerol consumption rate (calculated from Table 8) at a particular point of time. Overall yield is calculated for a total fermentation time, total product produced divided by total glycerol consumed. Point lipid yield and point citric yields are depicted in Figure 6. Lipid yield (Yl/s, g of lipid produced per g of glycerol consumed) was observed lowest for purified glycerol without any trace elements because of highest citric aid yield (Figure 6b). In a purified glycerol medium with all trace elements, lipid yield was comparatively higher during initial 16 h due to the formation of cell membrane lipids and after 72 h, as C/N ratio increased with glycerol feeding at 72 h (Table 3)

which resulted in lipid accumulation. A dip in lipid yield was observed at 24 h for purified glycerol with all trace elements because of the shift of metabolism from cell membrane lipid production (cell growth phase) to cytosolic lipid production (lipid production phase) (Figure 6a). For purified glycerol with sulphates and expect KH_2PO_4, a dip in lipid yield was after 32 h where there was a shift in metabolism from growth phase to lipid production phase (Figure 6a). The point of dip in yield is delayed for purified glycerol with sulphates and except KH_2PO_4 due to prolonged cell growth phase because of lack of sufficient trace elements in the medium. At 96 h, the overall lipid yield obtained in purified glycerol medium without any trace elements, purified glycerol medium with sulphates, purified glycerol except KH_2PO_4 and purified glycerol medium with all trace elements were 0.15 g/g glycerol, 0.25 g/g glycerol, 0.28 g/g glycerol and 0.21 g/g glycerol respectively. Table 9 indicates lipid productivity and lipid yield obtained in this study and that reported in the literature. The lipid yield and productivity observed in purified glycerol was comparable to crude glycerol for *Y. lipolytica* SKY7 (Mathiazhakan et al. 2016). Lipid productivity recorded in this study was comparable to that of *T. oleaginous* grown on crude glycerol (with high soap content) (Chen et al. 2017) and was higher than genetically engineered *Y. lipolytica* grown on glucose (Tai and Stephanopoulos 2013), *C. curvatus* and *L. starkeyi* grown on crude glycerol (Liang et al. 2010) (Signori et al. 2016). Microorganisms like *T. oleaginosus*, *C. curvatus* and *L. starkeyi* are strictly lipid producers and do not produce extracellular organic acids while *Y. lipolytica* also produces organic acids besides intracellular lipids.

In this study, irrespective of glycerol medium, citric acid yield was lower during the initial stages of fermentation and was higher during later stages of fermentation (Figure 6b). Citric acid yield was higher during later stages of fermentation due to a decrease in nitrogen concentration

and an increase in the C/N ratio in the medium. Throughout fermentation, citric acid yield was observed highest in purified glycerol medium without trace elements due to inhibition of ATP-citrate lyase enzyme responsible for the breakdown of intracellular citrate, as stated above. At 96 h, overall citric acid yield was the highest for purified glycerol (0.4 g/g) without any trace elements followed by purified glycerol with all trace elements (0.059 g/g), purified glycerol except KH_2PO_4 (0.042 g/g) and purified glycerol with sulphates (0.02 g/g). Table 10 indicates citric acid yield and productivity of studies reported in literature. Citric acid productivity for *Y. lipolytica* SKY7 in purified glycerol (this study) was higher than reported on crude glycerol (Kuttiraja et al. 2018).

The citric acid productivity and yield in this study is lower when compared to genetically engineered strain grown on glucose (Table 10). High citric acid production in genetically modified strain was due to cloning and over-expression of pyruvate carboxylase gene, *PYC1* gene (obtained from marine fungus) in citric acid producing wild strain of *Y. lipolytica* SWJ- 1b (Fu et al. 2016). In *Y. lipolytica* SWJ- 1b, pyruvate carboxylase catalyzes the carboxylation of one mole of pyruvic acid to oxaloacetic acid by fixing 1 mol of CO_2. The oxaloacetic acid formed is reduced to malate, and the malate enters into mitochondria where the malate is oxidized to oxaloacetic acid. At the same time, another mole of pyruvic acid is converted into acetyl-CoA under the catalysis of mitochondrion pyruvate dehydrogenase (mPDH) by releasing 1 mol of CO_2 in the mitochondria. Then, oxaloacetic acid and acetyl-CoA are condensed to form CA in mitochondria. Therefore, due to overexpression of pyruvate carboxylase, more oxaloacetic acid exists in the mitochondria and hence higher CA is formed. It can also be noted (Table 10) that citric acid yield and productivity were higher on glucose when compared to pure glycerol (Sabra et al. 2017). With glycerol as the sole carbon source, only 6.7% of its uptake rate is directed to phosphate pentose pathway (PPP)

compared to 35% with glucose. On the other hand, higher fluxes toward the tri-carboxylic acid (TCA) cycle are observed with glycerol rather than glucose as substrate. The 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. Although citric acid yield with respect to glucose is higher than from glycerol, glucose is an expensive substrate (1 $/kg) for fermentation and in this study, during crude glycerol purification, KH_2PO_4 is produced as by-product, which has application in the food industry, as buffering agent and fungicide (Javani et al. 2012).

4. Conclusion

In this study, the purified glycerol was investigated as carbon and trace element source for lipid and citric acid production using *Y. lipolytica* SKY7. It was revealed that in purified glycerol, sufficient Fe, K, Mg, Mn and Zn were not present for cell growth and lipid production. Hence, low biomass and lipid concentration were obtained in the absence of trace elements. Hence, these metal ions had to be imparted through external trace elements (in the form of sulphates and phosphates) to promote cell growth and lipid production. However, high CA concentration was obtained when no additional trace elements were added in purified glycerol.

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Conflict of Interest:

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

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Table 1: Characterization of crude and purified glycerol

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 ± 1	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	5.0×10^{-3}	3.88×10^{-3}

Table 2: Media composition used in different set of shake flasks experiments

Medium components	**Shake flask 1 (without trace elements)**	**Shake flask 2 (with sulphates only)**	**Shake flask 3 (all trace elements without KH_2PO_4)**	**Shake flask 4 (with all trace elements)**
Purified glycerol (g/L)	20	20	20	20
Peptone (g/L)	4	4	4	4
$MgSO_4.7H_2O$ (g/L)	-	0.2	0.2	0.2
FeSO4.7H2O (g/L)	-	0.0055	0.0055	0.0055
$ZnSO_4.7H_2O$ (g/L)	-	0.001	0.001	0.001
$MnSO_4.H_2O$ (g/L)	-	0.00076	0.00076	0.00076
Na_2HPO_4 (g/L)	-	-	0.95	0.95
KH_2PO_4 (g/L)	-	-	-	2.7

Table 3: Feeding strategy and intervals for fed-batch fermentation

Feeding time and component	Fermenter 1 (without trace elements)				Fermenter 2 (with sulphates only)			Fermenter 3 (all trace elements without KH_2PO_4)			Fermenter 4 (with all trace elements)				
Time	**0 h**	**24 h**	**40 h**	**56 h**	**0 h**	**24 h**	**48 h**	**0 h**	**24 h**	**48 h**	**0 h**	**20 h**	**32 h**	**48 h**	**72 h**
Glycerol added (g/L)	20	16	16	15.83	20	15.7	16	20	18.7	19	25	20	20	18.5	19.5
Carbon added (g/L)	8	6.4	6.4	6.33	8	6.28	6.34	8	7.48	7.54	10	8	8	7.4	7.8
Peptone added (g/L)	8	-	-	-	8	-	-	8	-	-	9.5	-	-	-	-
Nitrogen added (g/L)	0.96	-	-	-	0.96	-	-	0.96	-	-	1.1	-	-	-	-
C/N (molar) in the medium	10.08	16.63	17.20	23.75	10.21	15.63	21.32	10.11	16.87	24.21	10.23	20.47	33.33	40.23	46.67
Microbial Lipid concentration (g/L)	0	4.47	6.03	7.43	0	4.68	7.05	0	4.76	7.99	0	5.21	6.59	8.49	12.21

Table 4: Fermentation data obtained in shake flask studies at 96 h of fermentation.

Parameter	No trace elements (Shake flask 1)	Only sulphates (Shake flask 2)	All except KH_2PO_4 (Shake flask 3)	All trace elements (Shake flask4)
Citric acid (g/L)	4.05 ± 0.2	0	0	0
Lipid (g/L)	0.79 ± 0.04	0.99 ± 0.05	1.08 ± 0.02	1.13 ± 0.02
Glycerol consumed (g/L)	8.42 ± 0.4	8.42 ± 0.4	8.89 ± 0.45	10.54 ± 0.5
Biomass Lipid content (%)	9.38	11.76	12.15	10.72

Table 5: Different elements composition in shake flask medium with and without addition of trace elements.

Elements	**Shake flask 1 (without addition of trace elements)**	**Shake flask 4 (with all trace elements)**	**Actual Requirement** (Kuttiraja et al. 2018)
Fe (mg/ L)	0.178	1.178	1
K (g/L)	0.447	1.221	0.774
Mg (mg/L)	0.389	20.089	19.7
Mn (mg/ L)	0.008	0.258	0.25
Na (g/ L)	0.540	0.694	0.154
P (g/ L)	1.002	1.824	0.207
S (g/L)	0.140	0.166	0.026
Zn (mg/ L)	0.164	0.394	0.23

Table 6: Lipid profile of *Y. lipolytica* SKY7 obtained in different trace element media at end of fermentation

Fatty acid	Fermenter 1 (Without trace elements)	Fermenter 2 (With only sulphates)	Fermenter 3 (Except KH_2PO_4)	Fermenter 4 (With all trace elements)
C14:0 (Mysteric acid)	16.01%	8.46%	4.15%	3.17%
C16:0 (Palmitic acid)	24.59%	11.67%	5.78%	4.64%
C18:0 (Stearic acid)	2.6%	3.97%	1.82%	1.51%
C18:1 (Oleic acid)	18.76%	15.91%	27.46%	31.7%
C18:2 (Linoleic acid)	34.99%	55.05%	60.79%	37.23%
Others	3.05%	4.94%	-	21.74%

Table 7: Organic acid production in different trace element media at the end of fermentation

Medium	Pyruvic acid (mg/L)	Glutamic acid (mg/L)	α-ketoglutaric acid (mg/L)	Malic acid (mg/L)	Fumaric acid (mg/L)
Fermenter 1 (No trace elements)	200	200	250	104	9.2
Fermenter 2 (With only sulphates)	34	14	200	2000	120
Fermenter 3 (Except KH_2PO_4)	290	4.0	460	1500	100
Fermenter 4 (All trace elements)	7.9	15.0	150	12	1.9

Table 8: Interpretation of biomass, lipid, citric acid production curves and glycerol consumption curve (fermenter studies)

Fermenter 1 (no trace elements)	**Fermenter 2 (only sulphates)**	**Fermenter 3 (except KH_2PO_4)**	**Fermenter 4 (all trace elements)**
	Biomass productivity (g/L/h)		
0-24 h dX/dt = 0.69 R^2 = 0.95	0-24 h dX/dt = 0.77 R^2 = 0.94	0-24 h dX/dt = 0.85 R^2 = 0.98	0-24 h dX/dt = 1.31 R^2 = 0.95
24-80 h dX/dt = 0.20 R^2 = 0.97	24-96 h dX/dt = 0.195 R^2 = 0.98	24-96 h dX/dt = 0.24 R^2 = 0.99	24-96 h dX/dt = 0.26 R^2 = 0.97
	Lipid productivity (g/L/h)		
0-32 h dL/dt = 0.18 R^2 = 0.98	0-32 h dL/dt = 0.19 R^2 = 0.98	0-32 h dL/dt = 0.20 R^2 = 0.98	0-16 h dL/dt = 0.42 R^2 = 0.99
32-80 h dL/dt = 0.08 R^2 = 0.99	32-96 h dL/dt = 0.1 R^2 = 0.99	32-72 h dL/dt = 0.1 R^2 = 0.99	16-72 h dL/dt = 0.129 R^2 = 0.99
		72-96 h dL/dt = 0.225 R^2 = 0.99	72-96 h dL/dt = 0.31 R^2 = 0.95
	Citric acid productivity (g/L/h)		
0-24 h dC/dt = 0.074 R^2 = 0.98	0-48 h dC/dt = 0 R^2 = 1	0-48 h dC/dt = 0 R^2 = 1	0-32 h dC/dt = 0 R^2 = 1
24-80 h dC/dt = 0.42 R^2 = 0.99	48-96 h dC/dt = 0.021 R^2 = 0.97	48-96 h dC/dt = 0.044 R^2 = 0.9	32-96 h dC/dt = 0.075 R^2 = 0.95
	Glycerol consumption rate (g/L/h)		
0-80 h dS/dt = 0.8 R^2 = 0.99	0-48 h dS/dt = 0.67 R^2 = 0.99	0-48 h dS/dt = 0.80 R^2 = 0.99	0-24 h dS/dt = 1.59 R^2 = 0.94
	48-96 h dS/dt = 0.36 R^2 = 0.99	48-96 h dS/dt = 0.41 R^2 = 0.98	24-96 h dS/dt = 0.77 R^2 = 0.99

Table 9: Comparison of results on lipid production obtained in this fermenter study with those reported in the literature

Micro-organism	Substrate	Cultivation Mode	Time (h)	Lipid productivity (g/L/h)	Yield (g/ g substrate)	Reference
Y. lipolytica (Engineered)	Glucose	Batch	120	0.143	0.195	(Tai & Stephanopoulos, 2013)
Y. lipolytica SKY7	Crude glycerol	Batch	60	0.2	0.179	(Mathiazhakan et al., 2016)
Y. lipolytica SKY7	Purified glycerol (with all trace elements)	Fed-batch	96	0.2	0.21	This study
T. oleaginosus	Crude glycerol (high soap content)	Batch	56	0.22	-	(Chen et al., 2017)
C. curvatus	Crude glycerol	Fed-batch	288	0.06	0.21	(Liang et al., 2010)
L. starkeyi	Crude glycerol	Fed-batch	112	0.13	0.124	(Signori et al., 2016)

Table 10: Comparison of results on citric acid production obtained in this fermenter study with those reported in the literature

Micro-organism	Substrate	Cultivation Mode	Time (h)	Citric acid productivity (g/L/h)	Yield (g/ g substrate)	Reference
Y. lipolytica SKY7	Purified glycerol (without trace elements)	Fed-batch	80	0.31	0.4	This study
Y. lipolytica SKY7	Crude glycerol	Batch	120	0.093	-	(Kuttiraja et al., 2018)
Y. lipolytica	Waste cooking Oil	Batch	336	0.09	0.4	(Liu et al., 2015)
Y. lipolytica	Pure Glycerol	Batch	90	0.2	0.27	(Sabra et al., 2017)
Y. lipolytica	Glucose	Batch	90	0.6	0.58	(Sabra et al., 2017)
Y. lipolytica (Engineered)	Glucose	Fed-batch	240	0.46	0.93	(Fu et al., 2016)

Figure

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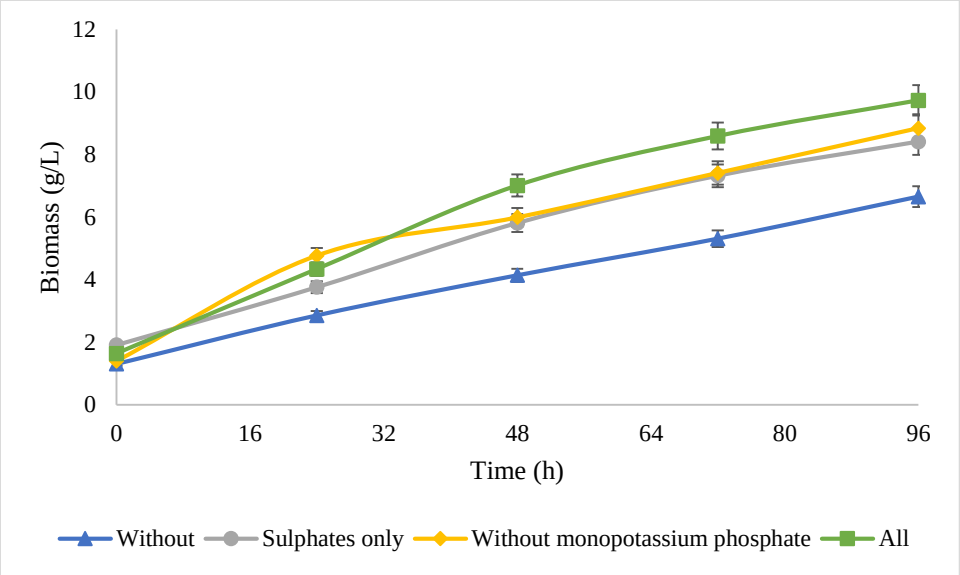
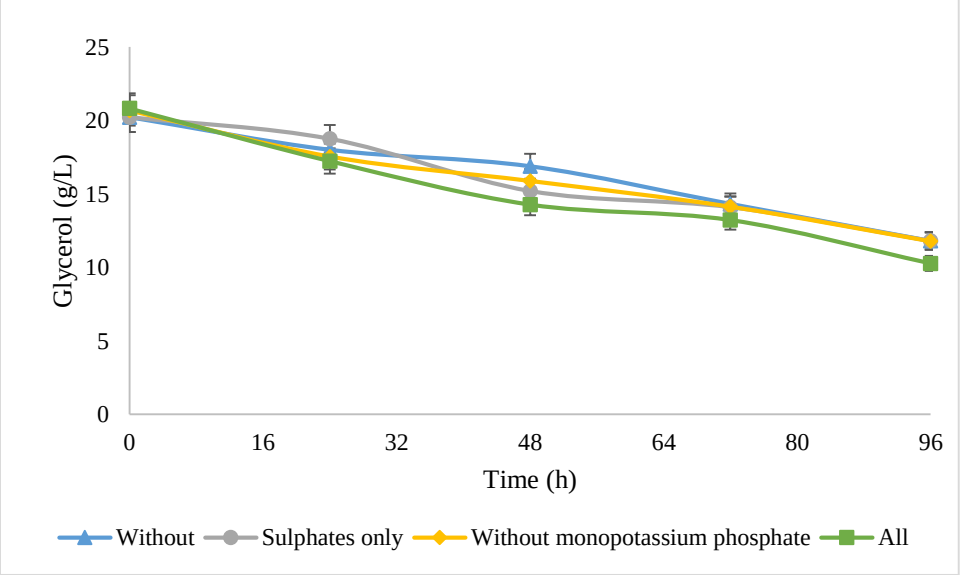


a)



b)

Figure 1: Profiles for different trace elements added in shake flask studies: a) Biomass profile and b) glycerol profile

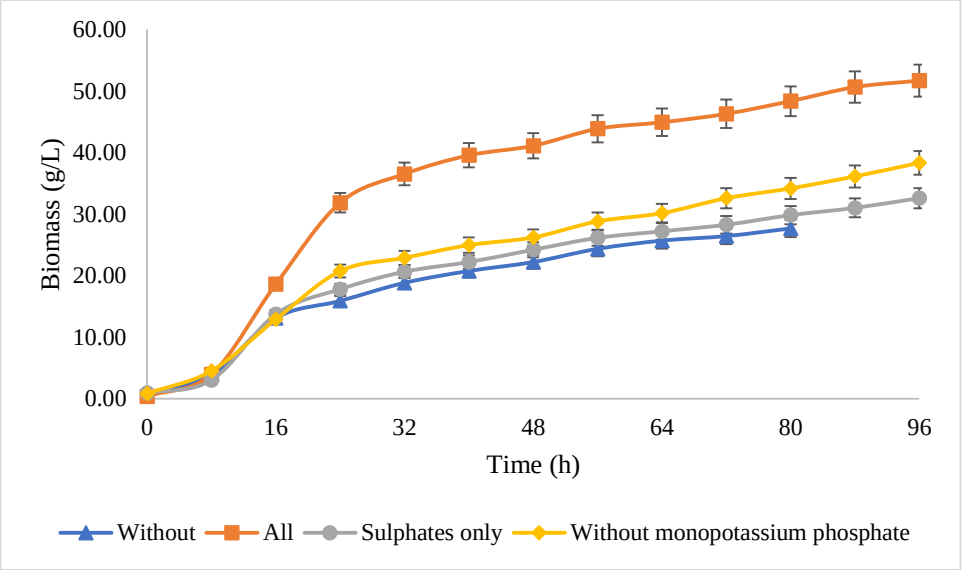
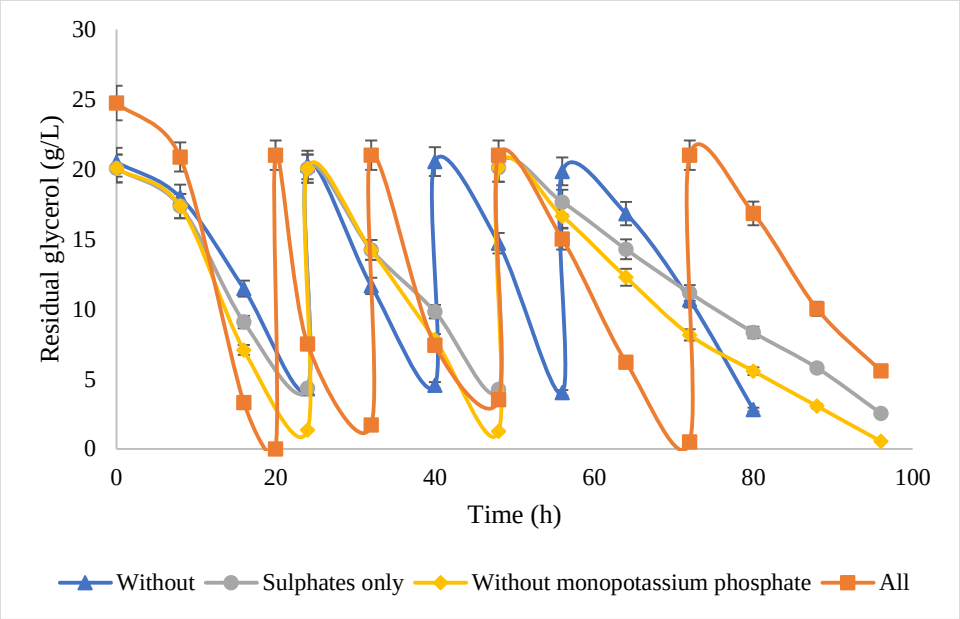
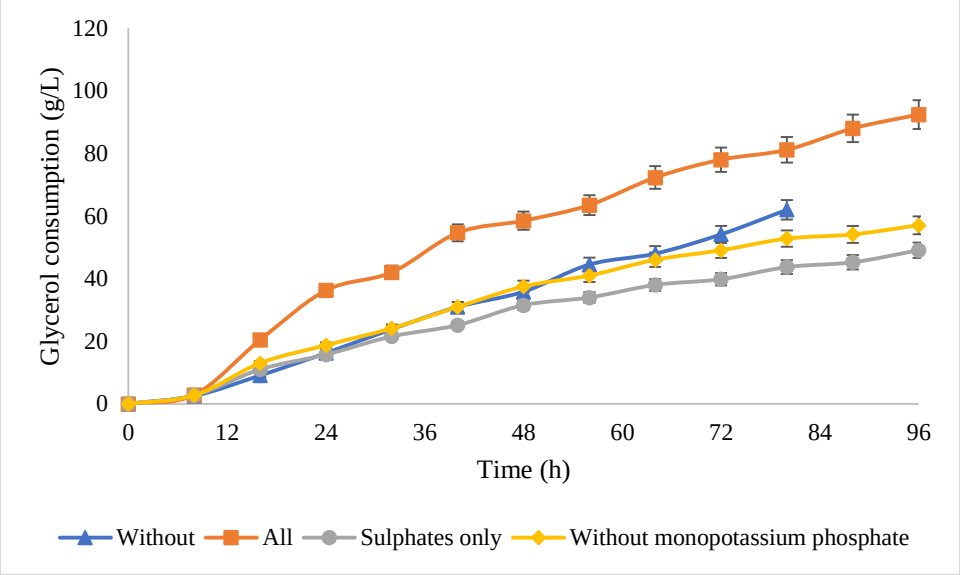


Figure 2: Biomass profile obtained during fermenter studies with different trace elements

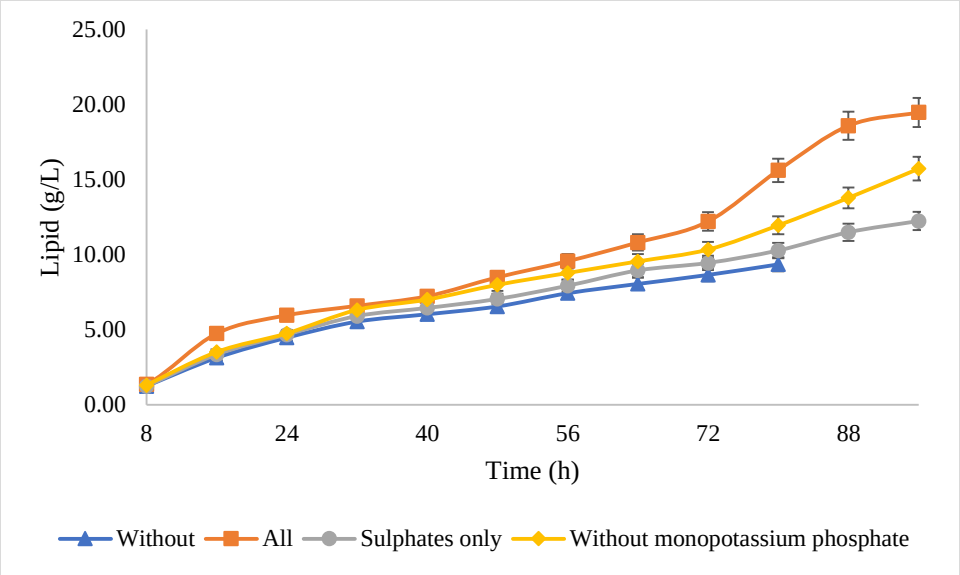


a)

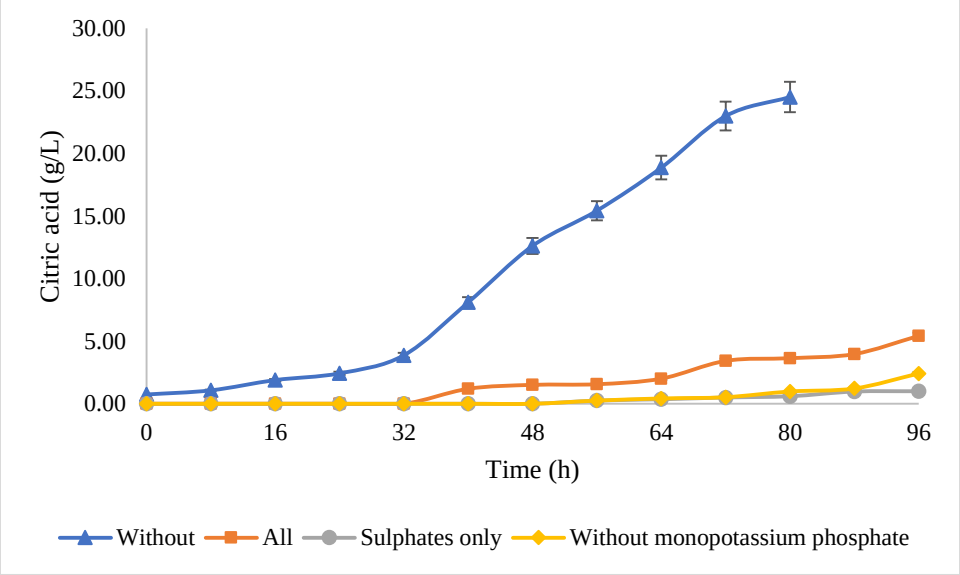


b)

Figure 3: Variation of glycerol concentration and consumption during fermenter studies for different trace elements: a) Residual glycerol concentration and b) glycerol consumed.

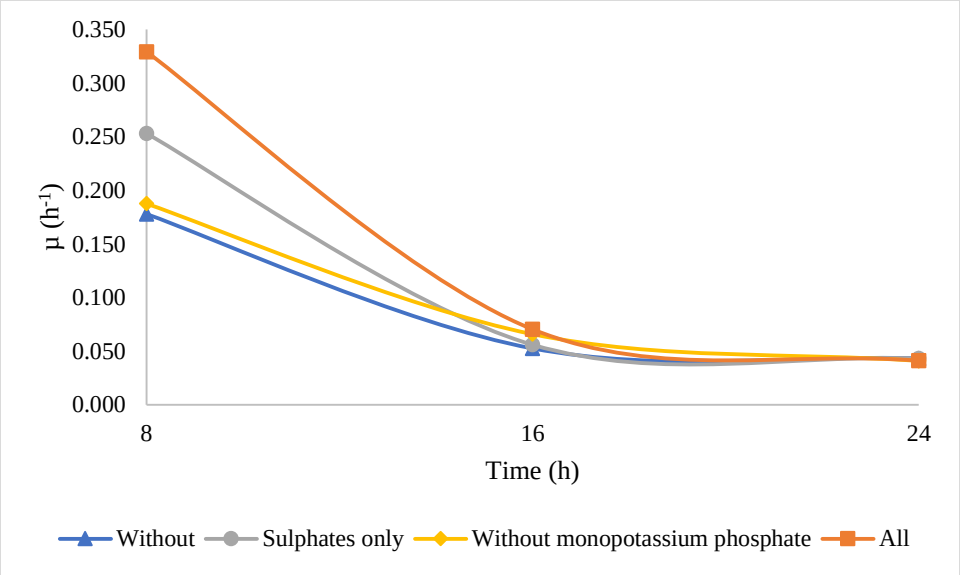


a)



b)

Figure 4: Lipid and citric production during fermentation with addition of different trace elements: a) Lipid and b) citric acid.



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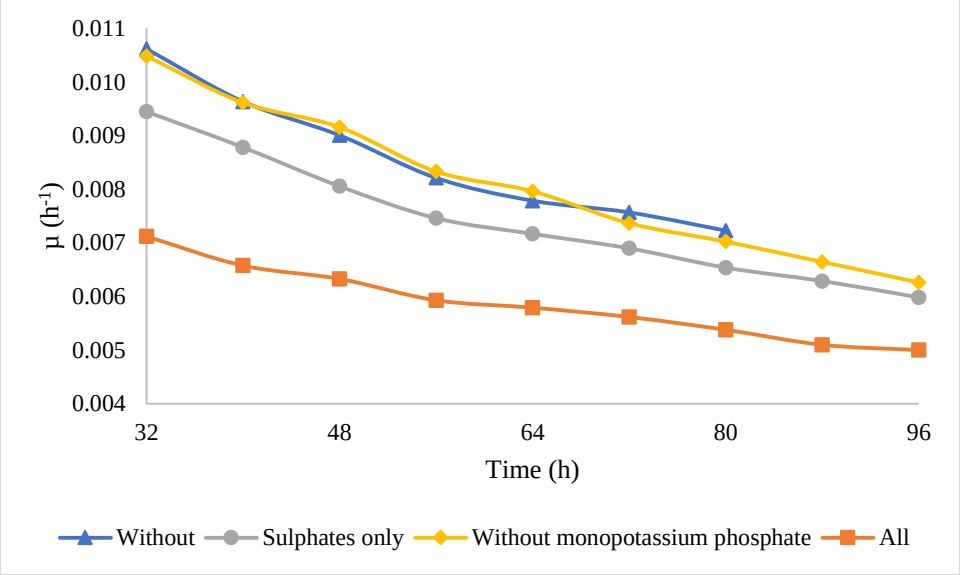
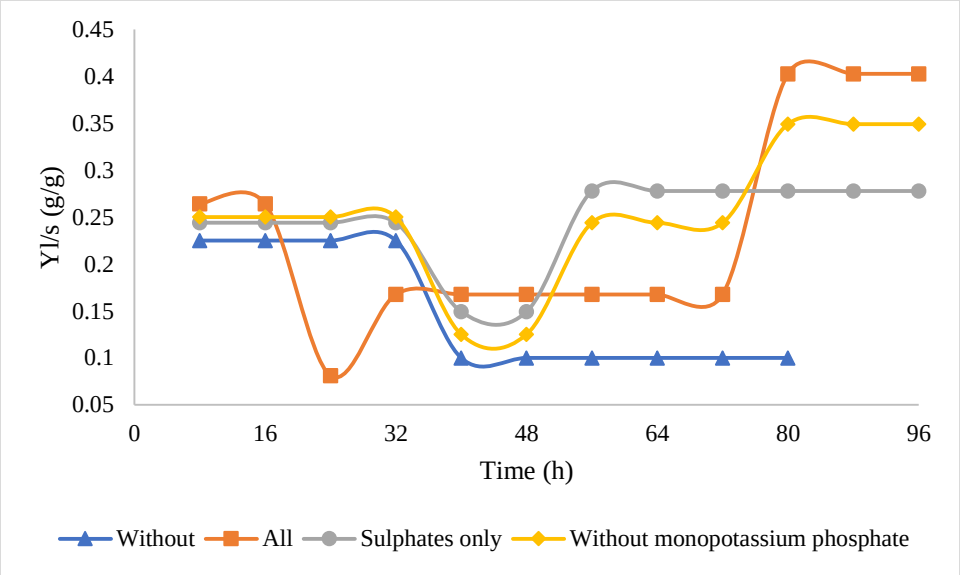
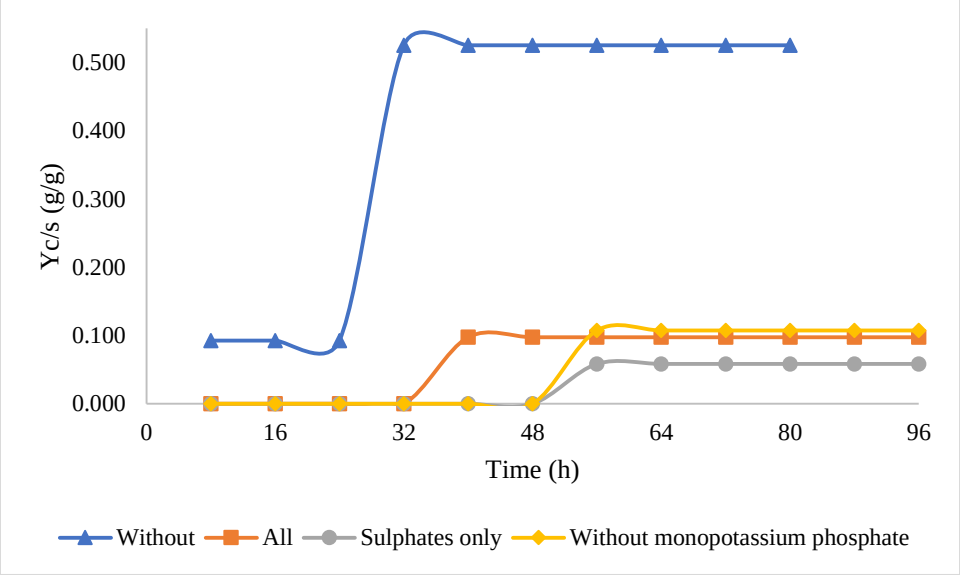


b)

Figure 5: Effect of trace elements on specific growth rate during: a) initial 24 h and b) 32-96 h of fermentation.



a)



b)

Figure 6: Effect of trace elements on product yield in fermenter study: a) Lipid yield (g/g glycerol) and b) Citric yield (g/g glycerol)