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Full Length Research Paper

Optimization of lipid production in the oleaginous bacterium *Rhodococcus erythropolis* growing on glycerol as the sole carbon source

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The growth of an oleaginous bacteria strain *Rhodococcus erythropolis* on glycerol was studied. Lipid accumulation was influenced by the glycerol concentration, nitrogen source, nitrogen concentration, pH medium, incubation time and aeration rate. Under the best optimized flask culture medium; 30 g/L glycerol plus 0.75 g/L urea, incubation temperature at 30°C, agitation rate of 150 rpm and 96 h of cultivation time, 3.93 g/L of dry biomass, 1.84 g/L cellular lipid accumulation and 45.8% accumulated lipid of total dry biomass were produced. The scaling up of *R. erythropolis* in bioreactors yielded the biomass and lipid content of 11.74 g/L and 14.1% of total dry biomass, respectively. The lipid composition of oleaginous microorganisms contained a high proportion of C16 and C18 fatty acids. The extracted lipids were mainly 16.48% C14:1, 16.69% C16:1, 20.16% C16:0, 18.90% C18:1. *R. erythropolis* could be directed to using raw glycerol obtained from biodiesel by-product as substrate, in order to accumulate lipids for biodiesel production.

Key words: *Rhodococcus eryhropolis*, glycerol, lipid accumulation, biodiesel, fatty acid composition.

INTRODUCTION

Biodiesel (fatty acid methyl esters, FAME) fuel originating from vegetable oils and animal fats mainly by trans-esterification of triacylglycerols (TAGs) has drawn attention as an alternative, renewable, biodegradable and nontoxic fuel (Easterling et al., 2009; Papanikolaou et al., 2008). Because of these environmental advantages, biodiesel fuel is expected as a substitute for conventional fossil fuel and has been industrially produced from vegetable oil in North America and Europe (Demirbas, 2006), and from waste edible oils in Japan (Watanabe et al., 2000). The high cost of the virgin vegetable oils is a major obstacle for biodiesel production, although the usage of waste edible oils can reduce the costs, the process requires additional cheap raw materials. In such a situation, the oleaginous microorganisms may be suitable alternatives, as they have the capacity to convert a number of raw materials into the series of value added products, such as oils and fats and their structure and fatty acid composition instead of vegetable oils (Li et al., 2008).

Microbial lipophilic compounds, called microbial oils or single cell oils (SCO), produced by oleaginous microorganisms involving bacteria, yeasts, fungi and algae (Li et al., 2007; Zhu et al., 2008). They can accumulate lipids at more than 20% of their cell dry weight (Li et al., 2007). Bacteria belonging to the actinomycetes group such as *Streptomyces, Nocardia, Rhodococcus, Mycobacterium, Dietzia* or *Gordonia* are able to accumulate lipids and TAGs under nitrogen-

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limiting conditions (Alvarez and Steinbüchel, 2002). Gram-positive, aerobic bacteria belonging to the genus *Rhodococcus* are of considerable interest to bio-technologists due to their ability to catalyze the biotransformation and degradation of various substances (Larkin et al., 2005). Gouda et al. (2008) reported that *Rhodococcus opacus* strain PD630 grown on sugar cane molasses medium is capable of accumulating intracellular lipids to as high as 93% of their cell dry weight and lipid bodies were mainly composed of TAGs (50%). Interestingly, not only *R. opacus* but also many other species of this genus such as *R. ruber*, *R. erythropolis* and *R. fascians* are capable of synthesizing TAGs as storage compounds for carbon and energy (Alvarez et al., 2000).

Glycerol is the principal by-product of biodiesel production which is 10% of the product output or 1lb of glycerol for each gallon of biodiesel fuel. Crude glycerol derived from biodiesel production possesses very low value because of the impurities (Easterling et al., 2009; Dasari, 2007; Papanikolaou and Aggelis, 2002). Therefore, using glycerol for the production of various chemicals and biofuel is advantageous both economically and environmentally (Moon et al., 2010). There has been studied in the lipid production of microbial oil from oleaginous yeasts by using and glycerol crude glycerol obtained from biodiesel process (Papanikolaou et al., 2008). The aim of the present study was to determine optimal conditions for lipid production by oleaginous bacteria strain *R. erythropolis* using glycerol as substrate.

MATERIALS AND METHODS

Microorganism and medium

R. erythropolis IGTS8 was purchased from the American type Culture Collection (ATCC) and maintained at 4°C on LB agar plates. The composition of minimal salt medium used in this study was as follow (g/L): KH_2PO_4 2; K_2HPO_4 7; $ZnCl_2$ 0.01; $MgCl_2.6H_2O$ 0.2; $FeCl_3.6\ H_2O$ 0.01; $MnCl_2.4\ H_2O$ 0.01; $CaCl_2.2\ H_2O$ 0.01; Na_2SO_4 0.2; NH_4NO_3 1 and yeast extract 0.005 (Nekodzuka et al., 1997) and this was for all growth experiments. In many experiments this medium was modified with respect to the nitrogen source and concentration in order to promote the accumulation of lipid. Glycerol (95% wt/wt) was used as sole carbon source.

Preparation of seed culture

A loopful of cells from single colony on LB agar plate was aseptically transferred to 50 mL of culture medium containing 10 g/L glycerol as carbon source in a 125-mL Erlenmeyer flask. The cultures were incubated at 30°C shaking at 150 rpm for 48 h.

Culture conditions

Shake flask experiments were carried out in 250-ml Erlenmeyer flask containing 50 mL culture medium and inoculated with 1 mL of a 48-h preculture (2.0×10^7 - 3.0×10^7 cells/mL). All cultures were incubated in an orbital shaker at 150 rpm for 96 h. Reactor cultures was performed in a 2-L stirred tank bioreactor (New Brunswick Bioflo*110 Fermenter/Bioreactor) with an active volume of 1.5-L, inoculated with 0.05 L of a 48-h preculture at two dissolved oxygen (DO) levels, corresponding to oxygen saturation of 50% (5 SLPM) and 75% (6 SLPM), respectively. Cultivations were done at initial medium pH of 7.4 and incubated at 30°C with an agitation at 100 rpm for 96 h.

Analytical methods

For cell dry weight determination, 10 ml of the culture were centrifuged at 6,000 x *g* for 10 min. The pellet was washed three times with deionized water and dried at 80°C until constant weight (typically 24 h). The dry cell weight was determined gravimetrically.

Total lipids were extracted from whole yeast cells by the method of Folch et al. (1957) with some modifications. Briefly, 30 ml of the cell culture were centrifuged at 6000 × *g* for 15 min to collect yeast pellet. This pellet was washed twice with deionized water and the supernatant was discarded. To an aliquot of 100 to 1000-mg (wet weight) of the pellet was added 3.75 ml of chloroform/methanol solution (2:1, v/v). The mixture was vortexed for 15 min at 22 to 24°C. To this was added 1.25 ml of chloroform. The mixture was vortexed for 1 min followed by addition of 1.25 ml of 1 M NaCl to the mixture and vortexed again for 1 min. The mixture was centrifuged at 3000 x *g* for 15 min to separate the aqueous and organic phases. The lower organic phase containing lipids was recovered with a Pasteur pipette and transferred to another glass tube. The organic solvent was evaporated under the stream of N_2 gas and the lipid weight was determined gravimetrically. The amount of total dry cell lipid was expressed as a percentage of total dry cell weight.

The total lipids were saponified and methyl esterified to yield methyl esters of fatty acid which were analyzed using an Agilent 6890N/5973 GC/MSD fitted with a capillary column (30.0 m × 0.32 m × 0.25 µm) under the following conditions: Initial oven temperature at 45°C ramped to 260°C at the rate of 2°C/min.; injector temperature held at 250°C; detector temperature at 280°C and carrier gas (helium) flow rate of 2 ml/min.

To determine the amount of residual glycerol, 2 ml of the liquid culture were centrifuged at 10,000 x *g* for 10 min and passed through 2 µm nylon filters to remove the cell mass and other debris. The glycerol content in the supernatant was analyzed by Agilent 2695 HPLC equipped with a Hypersil APS-2 column (250 × 4.6 mm.) and Refractive Index (RI) detection operated at 30°C. The mobile phase was 75% acetonitrile with a flow rate of 1.0 ml/min. Glucose served as the internal standard.

Statistical analysis

Statistical analysis was done by one-way analysis of variance (ANOVA) using SPSS software ver 11.5. Means were compared using Tukey's test at 95% confidence interval ($p < 0.05$).

RESULTS

The effect of several culture conditions on growth and lipid production of *R. erythropolis* on glycerol is given below.

Batch flask experiments

In order to select the optimum concentration of glycerol as carbon source, four concentrations were used as 30.0, 50.0, 70.0 and 100.0 g/L. Substantial growth was observed at 30.0 and 50.0 g/L glycerol (biomass = 3.17 and 2.96 g/L, respectively) while cellular lipid accumulation was favored at 30.0 g/L glycerol (1.51 g/L, lipid content = 47.72% of total dry biomass). At 70.0 and 100.0 g/L glycerol, biomass and cellular lipid accumulation gradually decreased (Figure 1A). An initial glycerol concentration of 30.0 g/L was chosen for all following experiments. NH_4Cl and urea were individually used as nitrogen sources to replaced NH_4NO_3, the nitrogen source in the cultivation medium as the control. As shown in Figure 1-B the highest biomass yield was found with urea (3.77 g/L) whereas the highest cellular lipid accumulation was observed in media containing NH_4Cl (1.89 g/L). Based on the biomass yield and cellular lipid accumulation, urea was chosen for all following experiment as nitrogen source.

Four concentrations of urea (0.375 to 1.5 g/L) were individually replaced in the cultivation medium to select the optimum concentration required for lipid production as shown in Figure 1-C. Noticeable growth and lipid accumulation were observed in all concentrations, except for the lowest concentration with 0.375 g/L urea. The highest amount of cellular lipid accumulation was observed in media with urea of 0.75 g/L (1.87 g/L, lipid content = 46.97% of total dry biomass). Therefore 0.75 g/L urea was chosen and used as nitrogen source concentration for further experiments.

In order to examine the effect of initial pH value of the cultivation medium, 5 different pH were used at pH 4.0, 6.0, 7.4, 8.0 and 10.0 (medium at pH 7.4 as control). Expected difference between the initial and final pH values were noticed as shown in Figure 1-D. Slight changes in the final pH value was found among different treatments. Growth was observed in all cases. At pH 6.0, the biomass accumulated to 4.49 g/L containing cellular lipid 1.39 g/L while pH 7.4 recorded the highest cellular lipid accumulation of 1.83 g/L. For further experiments pH 7.4 was chosen.

The behavior of *R. erythropolis* exhibited different activities with different incubation time as shown in Figure 1-E. Incubation periods were carried out at 0, 24, 48, 72 and 96 h. After 48 h, the amounts of biomass and cellular lipid accumulation increased from 1.33 to 3.77 g/L and cellular lipid accumulation from 0.19 to 1.81 g/L. While the amounts of glycerol gradually reduced from 29.88 to 15.22 g/L. The highest values of biomass and lipid production were found at 96 h.

Batch reactor experiments

At an oxygen saturation of 50%, remarkable growth was observed (biomass = 5.86 g/L) and the lipid accumulation increased to 25% of total dry biomass in *R. erythropolis* cells. After 96 h of incubation, the unconsumed glycerol was 17.76 g/L. In the highly aerated culture condition (oxygen saturation of 75%), further increase in growth was observed (biomass = 11.74 g/L) with significant increase in lipid accumulation. At the end of 96h, 12.21 g/L of glycerol remained unconsumed in the spent medium (12.21 g/L) (Figure 2).

Cellular lipid accumulation

Fatty acid analysis of 75% dO_2 batch fermentation and reserve fat at 96 h showed that C13 to C18 were predominant fatty acid while the amounts of storage lipid were mainly composed of unsaturated fatty acids (70 to 79% of total fatty acids) such as C13:1, C14:1, C15:1, C16:1, C16:2, C17:1 and C18:1. C16:0 was found at 22% of total fatty acids in storage lipid following C18:1, C14:1 and C16:1, respectively (Figure 3).

DISCUSSION

The well known oleaginous bacteria *R. opacus* PD630 produced significant amounts of lipid and TAGs in the cells when the cells were cultivated on various carbon sources such as sodium gluconate, glucose, fructose, acetate, citrate, succinate, propionate, valerate, phenylacetate, olive oil, phenyldecane, n-alkanes (Avalez and Steinbüchel, 2002) while *R. erythropolis* could grow on various carbon sources such as gluconate, pentadecane, hexadecane, valerate and produced 21% TAGs when grown on sodium gluconate (Avalez and Steinbüchel, 2002). Fermentative metabolism of glycerol has been studied in great detail in several bacterial genera such as *Citrobacter*, *Klebsiella*, *Clostridium*, *Lactobacillus*, *Bacillus*, *Escherichia coli Propionibacterium* and *Anaerobiospirillum* and several attempts have been made to achieve efficient production of fuels in micro-organisms that ferment glycerol in anaerobic conditions (Yazdani and Gonzalez, 2007). The present study is concerned with the analysis of the optimization and quantity of lipid accumulation *R. erythropolis*, which have not been studied in detail on glycerol cultivation of fuels previously.

Studies on *R. erythropoilis* grown under increased glycerol concentrations presented that growth gradually decreased with increasing glycerol concentration due to osmotic stress. Papanikolaou and Aggelis (2002) referred to the several studies that the differentiations on glycerol tolerance have been equally observed during growth of various bacteria in high concentrations of this substrate such as increasing of glycerol concentration from 20 to 100 g/L resulted in a decrease of biomass values for the

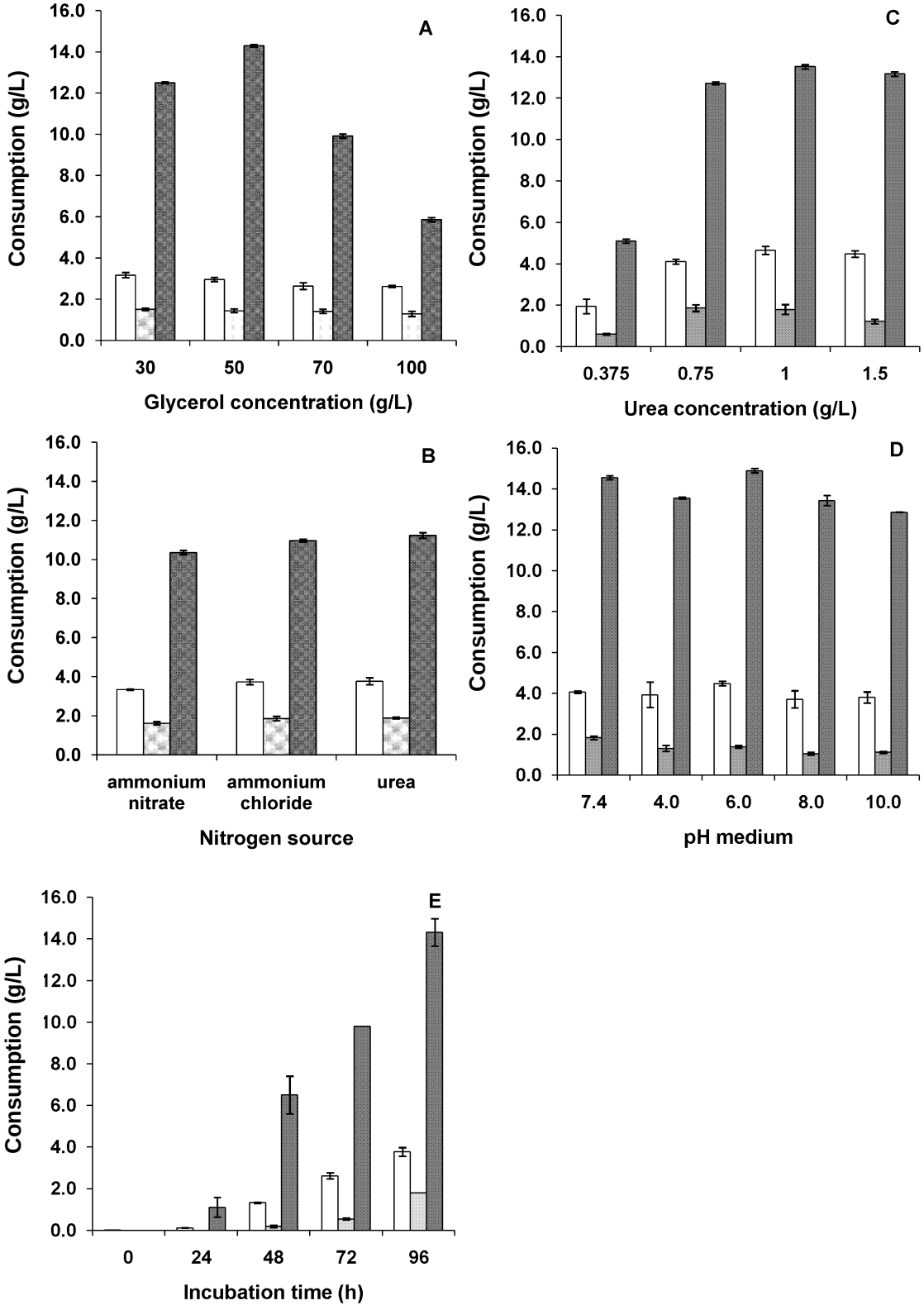

Figure 1. Dry cell weight, cellular lipid and glycerol consumption of R. erythropolis in glycerol medium. (A) glycerol concentrations; (B) nitrogen sources; (C) nitrogen concentrations; (D) pH medium and (E) incubation time. Symbols: (≤) dry cell weight g/L; cellular lipid g/L; glycerol consumption g/L.

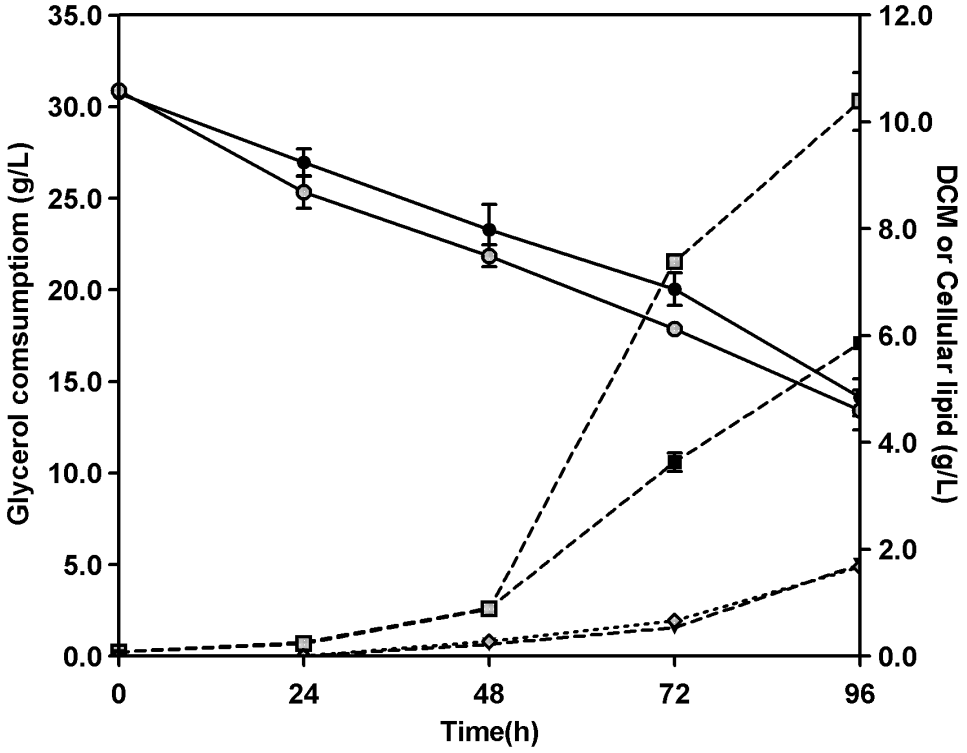

Figure 2. Biomass, cellular lipid and glycerol consumption of *R. erythropolis* during growth in bioreactor. Culture conditions: glycerol concentration 30 g/L, urea concentration 0.75 g/L, pH medium 7.4 and incubation temperature 30°C. Symbols: (■) dry cell weight g/L at aeration rate 50% (5 SLPM); (≤) dry cell weight g/L at aeration rate 75% (6 SLPM); (◆) cellular lipid g/L at aeration rate 50% (5 SLPM); (◇) cellular lipid g/L at aeration rate 75% (6 SLPM); (●) glycerol consumption g/L at aeration rate 50% (5 SLPM) and (○)glycerol consumption g/L at aeration rate 75% (6 SLPM).

case of *Krebsiella pneumoniae* DSM 2026. Increment of glycerol concentration in medium also resulted in significant decrease in growth and lipid accumulation in yeast *Cryptococcus curvatus* ATCC 20509 when glycerol concentration was increased from 32 to 128 g/L (Meesters et al., 1996). At optimal glycerol concentration (30 g/L glycerol) *R. erythropolis* produced cellular lipid 47.72% of their dry cell weight whereas *R. opacus* PD630 and *Gordonia* sp. produced cellular lipid around 20 and 30% of their dry cell weight, respectively when grown on olive mill (Gouda et al., 2008).

The influence of different nitrogen sources on lipid production using glycerol *R. erythropolis* was also evaluated. Urea was found to be the best nitrogen source for lipid accumulation. Zhu et al. (2008) reported that among the nitrogen sources tested, ammonium sulfate, ammonium chloride, ammonium nitrate, urea and peptone on growth of *Trichosporon fermentans* and lipid production, urea stimulated maximum biomass accumulation, followed by peptone. With respect to lipid content, peptone was the best with the maximum lipid yield.

The biosynthesis and accumulation of lipid were mostly influenced by an imbalance of nutrients and the C/N molar ratio. Increased amounts of lipid were detected after cultivation under N-limited conditions and the presence of a carbon surplus. *R. erythropolis* were grown on glycerol with different urea concentrations. The C/N molar ratio was 54.8, 32.8, 25.8 and 17.7. Biomass of all oleaginous microbes increased gradually with the increase of C/N molar ratio. Lipid content decreased when C/N molar ratio decreased. Papanikolaou et al. (2004) also reported such repression of reserve lipid turnover in *Cunninghamella echimulata* and *Mortierella isabellina* cultivated in multiple-limited media. When medium C/N ratio was increased from 83.5 to 133.5, lipid content in *C. echimulata* increased from 36 to 47%, and lipid content in *M. isabellina* increased from 50 to 56%. In addition, Zhu et al. (2008) also found that biomass of *T. fermentans* increased gradually with the increase of C/N molar ratio and reached the maximum of 24.0 g/L at 163. Lipid content was quite low at the C/N molar ratio of 108, then showed a sharp increase when C/N molar ratio increased from 108 to 140, and reached the maximum

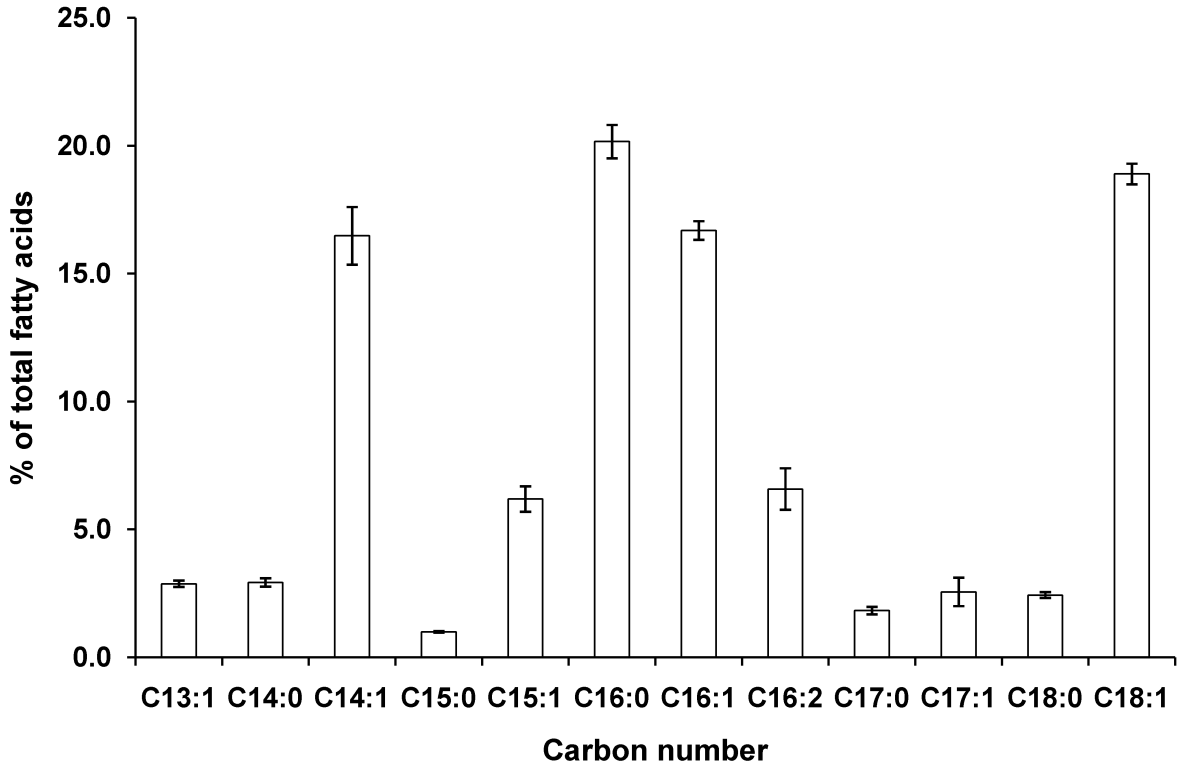

Figure 3. Distribution of fatty acids of the accumulated in *R. erythropolis* grown on glycerol medium.

of 63.1% at 140. Further rise in C/N molar ratio beyond 140 resulted in a slight drop in lipid content but a continuous increase in biomass up to 163 and the highest lipid yield of 14.8 g/L was achieved at 163.

The transesterification of oils in biodiesel process requires heat and a strong base or acid catalyst, such as sodium hydroxide or potassium hydroxide and sulfuric acid (Van Gerpen, 2005). Glycerol produced as a byproduct may be more acidic or alkaline; therefore the effect of medium pH on lipid production of *R. erythropolis* was investigated and found that the change in medium pH in flask culture. Changes in pH values usually had effect on cellular lipid content in *R. erythropolis* as observed in the fungal *Mucor circinelloides* (Xia et al., 2011). El-Fadaly et al. (2009) also observed that total lipid content in *C. curvatus* NRRL Y-1511 cells drastically decreased at pH 8.0 and at pH 4.0.

The accumulated lipid of *R. erythropolis* IGTS8 was obtained after cultured for 48 h. Increase in accumulated lipid depended on cultivation time and reached the maximum value at the end of cultivation (96 h). During the beginning of cultivation, the cells utilized the carbon and nitrogen sources not only for the synthesis of essential components for cell proliferation, but also for the biosynthesis and accumulation of TAG. When the cells had consumed the ammonium completely, they had entered the stationary growth phase (Avalrez et al., 2000).

In large scale batch fermentation, *R. erythropolis* ITGS8 exhibited impressive cell growth when glycerol was used as carbon source. The biomass and lipid yield were critically influenced by aeration rate, biomass yield increased (at 50% dO_2 was 5.86 g/L, at 75% dO_2 was 10.83 g/L) whereas increased aeration decreased the total lipid yield (at 50% dO_2 was 25% of cell dry mass, at 75% dO_2 was 14% of cell dry mass). These findings are in agreement with the study of Papanikoloau et al. (2002). Lipid synthesis was favored in low aerated media, but in highly aerated media noticeable synthesis of fat-free material occurred and lipid production was low. Similarly, *Candida lipolytica* 1094 growing on corn oil accumulated 55% (w/w) of lipids at 0 to 5% saturation. An at low oxygen saturation (5 to 15% dO_2), lipid accumulation in *Yarrowia lipolytica* cells grown on animal fats was 44% of cell dry mass whereas at the high oxygen saturation (60 to 70% dO_2), remarkable biomass and growth were observed but lipid synthesis was insignificant (Papanikoloau et al., 2002). Conversely, oxygen concentration had no effect on lipid metabolism in the yeasts *Y. lipolytica* N 1 (Finogenova et al., 2002).

In the lipids of *R. erythropolis* obtained from nitrogen-limited batch fermentations, the proportion of C16 and

C18 were found to be the predominant one. Kurosawa et al. (2010) demonstrated that *R. opacus* PD630 grown in batch-culture with a high concentration of glucose, the accumulated fatty acids consisted primarily of palmitic acid (27.7±0.9 0%) and oleic acid (24.7 ± 0.70%). The composition of fatty acid of *Rhodotorula mucilaginosa* grown on molasses medium was high methyl ester yields with 16 and 18 carbon atom (C16:0 = 21.6% and C18:1 = 22.3%). Ahmed et al. (2008) reported the fatty acid profile of *Mucor* sp. RRL001 cultivated in bioreactor using glucose as carbon source mainly composed of palmitic acid (29 to 39.1%) and oleic acid (34 to 47%) and the fatty acid profile of fungal cell was not directly associated with dissolved oxygen. The large amounts of unsaturated fatty acids were detected, indicating that the strain might show desaturase activity under high oxygen conditions. The importance of obtained microbial oil that is close to canola oil (Abou El-Hawa et al., 2004), palm oil or sunflower oil (Moser, 2008). Li et al. (2007) has reported that bacterial oils can be used as oil feedstocks for biodiesel production.

Conclusions

A lipid-producing microorganism, *R. erythropolis* was optimized in the culture medium for lipid production from glycerol. Our studies showed that medium condition such as glycerol concentration, nitrogen source, nitrogen concentration, pH medium, cultivation time and aeration rate could influence *R. erythropolis* growth and lipid production. The properties of extracted lipid from *R. erythropolis* were comparable to conventional diesel fuel due to high amount of C14 to C18 fatty acid.

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REFERENCES

Abou El-Hawa SH, Ragab WS, El-Dengawy RA, Ali FF (2004). Composition of Canola seed oil. Yemeni J. Sci. 6(1):25-35.

Ahmed SU, Singh SK, Pandey A, Kanjilal S, Prasad RBN (2008). Fatty acid profiling during microbial lipid production under varying *p*O2 and impeller tip speeds. Appl. Biochem. Biotechnol. 151(2-3):599-609.

Alvarez HM, Kalscheuer R, Steinbüchel A (2000). Accumulation and mobilization of storage lipids by *Rhodococcus* opacus PD630 and *Rhodococcus ruber* NCIMB 40126. Appl. Microbiol. Biotechnol. 54(2):218-223.

Alvarez HM, Kalscheuer R, Steinbüchel A (2000). Accumulation and mobilization of storage lipids by *Rhodococcus opacus* PD630 and *Rhodococcus ruber* NCIMB 40126. Appl. Microbiol. Biotechnol. 54(2):218-223.

Alvarez HM, Steinbüchel A (2002). Triacylglycerols in prokaryotic microorganisms. Appl. Environ. Microbiol. 60(4):367-376.

Dasari M (2007). Crude glycerol potential described. Feedstuffs 15:1-3.

Demirbas A (2006). Biodiesel production via non-catalytic SCF method and biodiesel fuel characteristics. Energy Convers. Manag. 47(15-16):2271-2282.

Easterling ER, Todd FW, Hernandez R, Licha M (2009). The effect of glycerol as a sole and secondary substrate on the growth and fatty acid composition of *Rhodotorula glutinis*. Bioresour. Technol. 100(1):356-361.

El-Fadaly HA, El-Naggar N El-Ahmady, Marwan El-Sayed M (2009). Oleaginous yeast strains in a low cost cultivation medium. Res. J. Microbiol. 4(8):301-313.

Finogenova TV, Kamzolova SV, Dedyukhina EG, Shishkanova NV, Il'chenko AP, Morgunov IG, Chernyavskaya OG, Sokolov AP (2002). Biosynthesis of citric and isocitric acids from ethanol by mutant Yarrowia lipolytica N 1 under continuous cultivation. Appl. Microbiol. Biotechnol. 59(4-5):493-500.

Folch J, Lees M, Sloane Stanley GH (1957). A simple method for the isolation and purification of total lipid from animal tissues. J. Biol. Chem. 226:497-509.

Gouda MK, Omar SH, Aouad LM (2008). Single cell oil production by *Gordonia* sp. DG using agro-industrial wastes. World J. Microb. Biot. 24(9):1703-1711.

Kurosawa K, Boccazzi1 P, Almeida NM de, Sinskey AJ (2010). High-cell-density batch fermentation of *Rhodococcus opacus* PD630 using a high glucose concentration for triacylglycerol production. J. Appl. Bacteriol. 147(3-4):212-218.

Meesters PAEP, Huijberts GNM, Eggink G (1996). High-cell-density cultivation of the lipid accumulating yeast *Cryptococcus curvatus* using glycerol as a carbon source. Appl. Microbiol. Biotechnol. 45(5):575-579.

Moon C, Ahn J, Kim SW, Sang B, Um Y (2010). Effect of biodiesel-derived raw glycerol on 1,3-propanediol production by different microorganisms. Appl. Biochem. Biotechnol. 161(1-8):502-510.

Moser BR (2008). Influence of blending canola, palm, soybean and sunflower oil methy esters on fuel properties of biodiesel. Energ. Fuel. 22(6):4301-4306.

Nekodzuka S, Kambe TN, Nomura N, Lu J, Nakahara T (1997). Specific desulfurization of dibenzothiophene by *Mycobacterium* sp. strain G3. Biocatal. Biotransfor. 15(1):17-27.

Larkin MJ, Kulakov LA, Allen CC (2005). Biodegrdation and Rhodococcus - masters of catabolic versatility. Curr. Opin. Biotechnol. 16(3):282-290.

Li Q, Du W, Lui D (2008). Perspectives of microbial oils for biodiesel production. Appl. Microbiol. Biotechnol. 80(5):749-756.

Li YH, Zhao ZB, Bai FW (2007). High-density cultivation of oleaginous yeast *Rhodosporidium toruloides* Y4 in fed-batch culture. Enzyme Microb. Tech. 41(3):312-317.

Papanikolaou S, Chevalot I, Komaitis M, Marc I, Aggelis G (2002). Single cell oil production by *Yarrowia lipolytica* growing on an industrial derivative of animal fat in batch cultures. Appl. Microbiol. Biotechnol. 58(3): 308-312.

Papanikolaou S, Aggelis G (2002). Lipid production by *Yarrowia ipolytica* growing on industrial glycerol in a single-stage continuous culture. Bioresour. Technol. 82(1):43-49.

Papanikolaou S, Fakasb S, Ficka M, Chevalota I, Galiotou-Panayotoub M, Komaitisb M, Marca I, Aggelis G (2008). Biotechnological valorisation of raw glycerol discharged after bio-diesel (fatty acid methyl esters) manufacturing process: Production of 1,3-propanediol, citric acid and single cell oil. Biomass Bioenergy 32(1):60-71.

Papanikolaou S, Sarantou S, Komaitis M, Aggelis G (2004). Repression of reserve lipid turnover in *Cunninghamella echinulata* and *Mortierella isabellina* cultivated in multiple-limited media. J. Appl. Microb. 97(4):867-875.

Watanabe Y, Shimada Y, Sugihara A, Noda H, Fukuda H, Tominaga Y (2000). Continuous production of biodiesel fuel from vegetable oil using immobilized *Candida antarctic*. JAOCS. 77(4):355-360.
Van Gerpen J (2005). Biodiesel processing and production. Fuel Process. Technol. 86(10):1097-1107.
Xia C, Zhang J, Zhang W, Hu B (2011). A new cultivation method for microbial oil production: cell pelletization and lipid accumulation by Mucor circinelloides. Biotechnol. Biofuels 4(1):15.
Yazdani SS, Gonzalez R (2007). Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry. Curr. Opin. Biotech. 18(3):231-219.
Zhu LY, Zong MH, Wu H (2008). Efficient lipid production with *Trichosporon fermentans*and its use for biodiesel preparation. Bioresour. Technol. 99(16):7881-7885.