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Screening of Oleaginous Yeasts and Optimization for Lipid Production Using Crude Glycerol as a Carbon Source

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Abstract

Eight hundreds and eighty nine yeast strains were isolated from soils and wastes of palm oil mill and biodiesel plant in southern region of Thailand using glucose or glycerol as carbon source and at acidic condition (pH 4.0) or neutral condition (pH 6.0) with 0.0001% chloramphenicol. By applying Sudan Black B tests, 23 strains were identified as potential lipid producer or oleaginous yeast. The lipid contents of these 23 strains were compared in crude glycerol based medium. It was found that BY4-523 accumulated highest lipid content up to 53.28% while JU4-57 grew fastest and gave comparable high lipid content 41.50%. They were identified as *Kodamaea ohmeri* and *Trichosporonoides spathulata*, respectively. Among organic nitrogen sources tested, a mixture of yeast extract and peptone (1:1) gave the best biomass (17.05 g/L for *T. spathulata* and 11.1 g/L for *K. ohmeri*) and the maximum lipid production (10.43 g/L for *T. spathulata* and 4.53 g/L for *K. ohmeri*). In the view point of economic strategy, the cheaper inorganic nitrogen sources were also tested. Among inorganic nitrogen sources tested, ammonium sulfate was selected as a suitable nitrogen source. It gave the best biomass (9.17 g/L for *T. spathulata* and 10.45 g/L for *K. ohmeri*) and the maximum lipid production (3.85 g/L for *T. spathulata* and 3.17 g/L for *K. ohmeri*). The results showed that the newly isolated yeasts could grow and accumulate high lipid content in crude glycerol based medium supplemented with only ammonium sulfate. The optimal medium composition for both strains was 0.5% ammonium sulfate and 10% crude glycerol (C/N ratio of 17). Under this condition, the maximum biomass of 10.40 g/L and lipid production of 4.45 g/L were achieved for *T. spathulata*. Similarly, *K. ohmeri* also reached the maximum biomass of 10.50 g/L and lipid production of 3.22 g/L.

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Keywords: Biodiesel; Crude glycerol; Lipid; Oleaginous yeast; Screening

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1. Introduction

Biodiesel has become more attractive recently because of its environmental benefits, and the fact that it is made from renewable resources. It contributes no net carbon dioxide or sulfur to the atmosphere and emits less gaseous pollutants than normal diesel. Generally speaking, biodiesel is prepared through transesterification of vegetable oils, animal fats and waste oils (Meng *et al*., 2009) with short chain alcohols. If plant oil was used for biodiesel production, the cost of source has account to 70–85% of the whole production cost. Therefore, taking into account of these inhibition factors, exploring ways to reduce the high cost of biodiesel is of much interest in recent research, especially for those methods concentrating on lowering the cost of oil raw material. Moreover, the lack of oil feedstocks also limits the large-scale development of biodiesel to some extent. Recently, much attention has been paid to the development of microbial oils and it has been found that many microorganisms, such as algae, yeast, bacteria, and fungi, have the ability to accumulate oils under some special cultivation conditions. Microorganisms that can accumulate oils in lipid form more than 20% of their biomass are defined as oleaginous species. Some yeast strains, such as *Rhodosporidium* sp., *Rhodotorula* sp. and *Lipomyces* sp. can accumulate intracellular lipids as high as 70% of their biomass dry weight. The yeast oils are now believed as a promising potential feedstock for biodiesel production due to their similar composition of fatty acids to that of vegetable oils. It has been reported that such yeast oils can be used as oil feedstocks for biodiesel production with the catalysis either by lipase or chemical catalyst (Li *et al*., 2008). Compared to other plant oils, microbial oils have many advantages, such as short life cycle, less labor required, less affection by venue, season and climate, and easier to scale up. In addition, oleaginous yeasts can not only accumulate lipids within a short period of time but grow well on a variety of substrates, even inexpensive material, such as nutritional residues from agriculture and industry, thus lowering the cost of oils (Zhu *et al*., 2008). With the rapid expansion of biodiesel, microbial oils might become one of potential oil feedstocks for biodiesel production in the future, though there are many works associated with microorganisms producing oils need to be carried out further.

Biodiesel production is a process in which 10% glycerol is separated from the triacylglycerides in fats or vegetable oils. The future supplies and usage of glycerol are expected to increase as biodiesel plants increase production, and the output will greatly outpace demand. Some alternative uses for this glycerol that have been investigated are substrates for fermentation process (Morita *et al*., 2007). Another alternative use for glycerol is as a growth substrate for the cultivation of oleaginous yeasts. Oleaginous yeasts have the ability to grow and accumulate lipids when grown on glycerol (Easterling *et al*., 2009) and have short generation times, and very minimal nutrient requirements (Meesters *et al*., 1996). The crude glycerol produced during biodiesel manufacturing contains macro elements such as calcium, potassium, magnesium, sulfur and sodium. Thus, using the crude glycerol to produce yeast oils to be used as biodiesel feedstock would provide an added bonus of offsetting costs of production.

The objective of this study is to produce oils from crude glycerol, a by-product of biodiesel plant, by using oleaginous yeast. First, the high lipid accumulating oleaginous yeast will be screened using glucose and glycerol as a carbon source. The growth and lipid production of selected oleaginous yeasts will be optimized using crude glycerol as a carbon source.

2. Materials and methods

2.1. Screening of oleaginous yeasts

The waste samples were collected from soil and waste of palm oil mill and biodiesel plant in southern region of Thailand. 5% of samples will be enriched in 25 mL YPD medium (yeast extract 10 g/L and

peptone 10 g/L) by using 40 g/L glucose or glycerol as a carbon source at acid pH (4.0) or neutral pH (6.0) with 0.0001% chloramphenicol. Then 0.1 mL diluted culture was isolated on YPD agar medium using spread-plate technique for 72 h at room temperature. Total yeast strains were stained with Sudan black B technique and observed under a phase contrast microscope on oil immersion for the presence of blue or greyish colored fat globules within the cell [7]. The yeast strains showing fat globules within the cell were selected for further quantitative analysis. The yeast strains were precultured in inoculum medium, and then 24 h old preculture were inoculated in 25 mL crude glycerol based (crude glycerol 100 g/L with corresponding glycerol concentration of 40 g/L, yeast extract 10 g/L and peptone 10 g/L, pH 6.0) at the C/N molar ratio of 7.4 and incubated for 72 h at room temperature with shaking at 140 rpm.

2.2. Identification of oleaginous yeast strain

The selected oleaginous yeast strains were identified based on its 26S rDNA sequence. The 26S rDNA was amplified by PCR using various universal primer sets and sequenced. The obtained sequences were BLAST searched against National Center for Biotechnology Information (NCBI) database.

2.3. Optimization of medium components and culture condition

The influence of organic nitrogen sources (yeast extract, peptone, urea) and inorganic nitrogen sources ($(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3), nitrogen concentrations (0, 0.5, 1 and 2% w/w) and glycerol concentration (5, 10, 15 and 20% v/v) on the biomass and lipid production were investigated. The preculture inoculums were transferred into 50 mL sterilized medium in a 250-mL Erlenmeyer flask and incubated at room temperature for 3 days on rotary shaker at 140 rpm.

2.4. Analytical methods

Biomass concentration was determined gravimetrically. Samples containing 10 mL fermentation broth withdrawn from the flasks will be centrifuged at 4,000 rpm for 10 min, the cell pellet was collected and washed twice with distilled water, and then will be dried at 60°C to constant weight [8]. The dry biomass was ground into a fine powder. The powder was blended with 1 mL chloroform: methanol (2: 1) and the mixture was sonicated for 30 min at 70 Hz and room temperature. Solvent phase was recovered by centrifugation. The process was repeated two more times. The combined solvent was removed by drying at 60°C. Lipid content was expressed as gram lipid per gram dry biomass [8].

All experiments were performed in two or three replicates. Analysis of variance was performed to calculate significant differences in treatment means, and the least significant difference ($p \leq 0.05$) was used to separate means, using the SPSS software.

3. Result and discussion

3.1 Screening and identification of oleaginous yeasts

Eight hundred and eighty nine yeast strains were isolated from soil and waste of palm oil mill and biodiesel plant in southern region of Thailand in YPD medium using glucose or glycerol as carbon source at acid pH (4.0) or neutral pH (6.0) with 0.0001% chloramphenicol. In Table 1, 266 and 176 yeast strains were isolated using glycerol as a carbon source at initial pH 4 and 6, respectively. 267 and 220 yeast strains were isolated using glucose as a carbon source at initial pH 4 and 6, respectively. They were

stained with Sudan black B technique and observed under a phase contrast microscope on oil immersion for the presence of blue or greyish colored fat globules within the cell. 86 and 63 yeasts strains were found positive for showing fat globules within the cell but only 5 and 3 yeast strains could show big fat globules or high positive from the 266 and 176 yeast strains which were isolated using glycerol as a carbon source at pH 4 and pH 6, respectively. While 104 and 81 yeasts strains were found positive for showing fat globules within the cell but only 16 and 2 yeast strains could show big fat globules from the 267 and 220 yeast strains which were isolated by using glucose as a carbon source at pH 4 and pH 6, respectively. These 23 yeast strains which showed big globules or high positive were selected as potential lipid biomass producer**.**

Table 1. Number of yeast strains isolated by using glucose and glycerol as a carbon source.

Initial pH	Carbon source					
	Glycerol			Glucose		
	Total isolates	+	++	Total isolates	+	++
pH 4	226	86	5	267	107	13
pH 6	176	63	3	220	81	2
Total	402	149	8	487	185	15

+: showing fat globules; ++: showing big fat globules

The isolated 23 yeast strains were precultured in inoculum medium, and then 24 h old preculture were inoculated in 125 mL flasks containing 25 mL crude glycerol based medium and incubated for 72 h at room temperature with shaking at 140 rpm. Among 23 isolates, four of them (BY4-523, BY4-571, JU4-57 and BU4-598) gave high lipid yield with lipid content higher 40% when grown in crude glycerol base medium broth. Fig. 1 shows time courses of cell growth and lipid yield of four strains. It was found that BY4-523 accumulated highest lipid content up to 53.28% (10.28 g/L of biomass and 5.48 g/L of lipid yield) while JU4-57 grew fastest and gave comparable high lipid content 41.50% (17.05 g/L of biomass and 7.48 g/L of lipid yield). It should be noted that the isolated yeast strains were not inhibited by crude glycerol. Easterling *et al*. [5] reported that oleaginous yeasts have the ability to grow and accumulate lipids when grown on glycerol and have short generation times, and very minimal nutrient requirements. It was also reported that the crude glycerol from biodiesel manufacturing contains macro elements such as calcium, potassium, magnesium, sulfur and sodium [6]. Thus, using the crude glycerol to produce yeast oils as biodiesel feedstock would provide an added bonus of offsetting costs of production. Yeast strains JU4-57 and BY4-523 which gave highest lipid yield and highest lipid content, respectively, were selected for the next study.

Strains JU4-57 and BY4-523 were grown on YPD agar with staining and observed under microscopy (10 x 100) as shown in Fig. 2. Primers used for the amplification of the D1/D2 fragment yield a fragment about 542 and 623 bp for the JU4-57 and BY4-523 strains, respectively. The sequence obtained were compared with those available in the GenBank, and the results showed high sequence similarity (99%) with the type strain of *Trichosporonoides spathulata* for JU4-57 and *Kodamaea ohmeri* for BY4-523 base on 23S rDNA. Therefore, JU4-57 and BY4-523 strains belonged to *Trichosporonoides spathulata* and *Kodamaea ohmeri*, respectively.

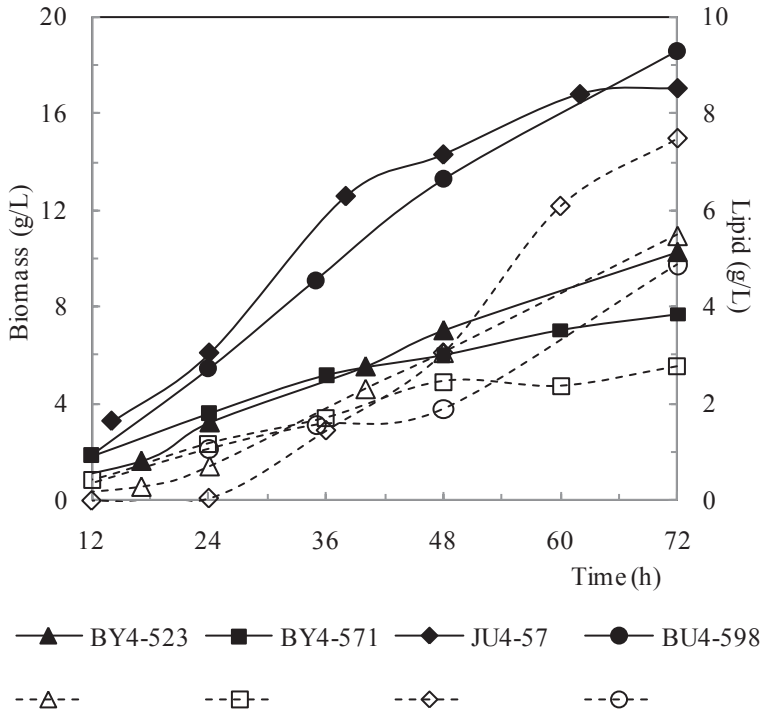

Fig. 1. Time course of cell growth (close symbol) and lipid accumulation (open symbol) with oleaginous yeast strains. 10% crude glycerol (0.13 M of carbon) was used as a carbon source and 1% yeast extract and 1% peptone were used as nitrogen source with the C/N molar ratio of 7.4.

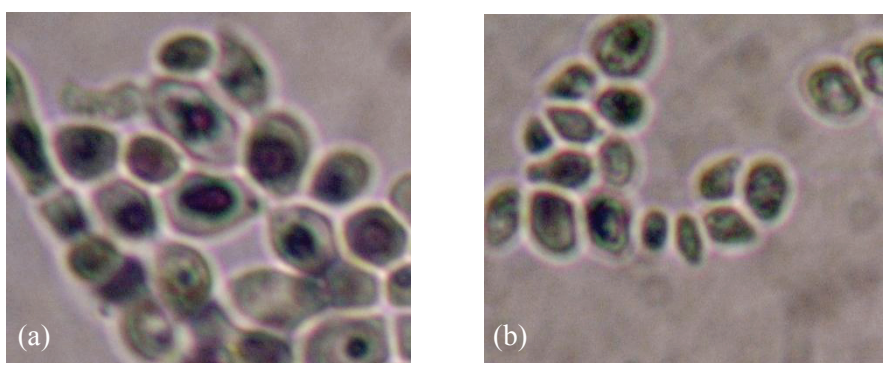

Fig. 2. Microscopic view of *T. spathulata* (a) and *K. ohmeri* (b) on YPD agar with staining.

3.2. Optimization of medium components and culture condition

3.2.1 Effect of nitrogen source on growth and lipid accumulation

Many factors including medium components, such as carbon source, nitrogen source and C/N molar ratio etc. as well as culture conditions (temperature and pH) have significant influences on cell growth and lipid accumulation of oleaginous microorganism [9]. Effects of nitrogen source on biomass and lipid production of *T. spathulata* and *K. ohmeri* strains were presented in Fig. 3. Among the nitrogen sources tested for *T. spathulata* strain (Fig. 3a), a mixture of yeast extract and peptone (1:1) gave the maximum biomass (17.05 g/L) and lipid yield (10.43 g/L), followed by yeast extract (16.02 g/L biomass and 7.12 g/L lipid yield), peptone (10.88 g/L biomass and 5.43 lipid yield) and $(NH_4)_2SO_4$ (9.17 g/L biomass and 3.85 g/L lipid yield).

Among the nitrogen sources tested for *K. ohmeri* strain (Fig. 3b), there was no significant difference in biomass between the mixture of yeast extract and peptone (1:1) (11.1 g/L) and yeast extract alone (11.2 g/L), followed by $(NH_4)_2SO_4$ (10.45 g/L). For the lipid production, the mixture of yeast extract and peptone gave the maximum lipid yield (4.53 g/L), followed by yeast extract and $(NH_4)_2SO_4$ (3.17 g/L and 3.03 g/L, respectively). Disappointingly, urea and NH_4NO_3 gave both poor biomass and lipid yield by *T.spathulata* and *K. ohmeri* strains, indicating that organic nitrogen sources are more beneficial to lipid production of oleaginous yeast than inorganic nitrogen sources. On the contract, Zhu *et al*. [3] reported that urea supported the maximum biomass of *Trichosporon fermentans* (23.1 g/L), followed by peptone (19.7 g/L).

Although $(NH_4)_2SO_4$ gave lower biomass and lipid production of *T. spathulata* and *K. ohmeri* compared with organic nitrogen source, it was selected as a suitable nitrogen source because of its cheaper cost and inorganic nitrogen is more economical choice.

3.2.2 Effect of ammonium sulphate concentration on growth and lipid accumulation

As shown in Fig. 4a, the biomass of *T. spathulata* increased when ammonium sulfate was added. It reached the maximum value of 10.15 g/L at 0.5% ammonium sulfate. At this concentration, the maximum lipid yield was also obtained (4.13 g/L). Further rise in ammonium sulfate concentration beyond 0.5% resulted in a drop of biomass, lipid yield and lipid content. In Fig. 4b, biomass of *K. ohmeri* increased gradually with the increase of ammonium sulfate concentration and reached the maximum biomass of 10.45 g/L at 0.5% ammonium sulfate. Lipid yield showed a sharp increase when ammonium sulfate concentration increased from 0.25% to 0.5%, and reached the maximum lipid yield of 3.17 g/L at 0.5%. From Fig. 4, it can be seen that at lower concentration of nitrogen source, the lipid content was higher. This result indicated that the exhaustion of ammonium-N apparently greatly promoted lipid accumulation. The similar results were also observed in lipid content of *Rhodosporidium toruloides* Y4, *Rhodotorula glutinis* and *Trichosporon fermentans* [11].

3.2.3 Effect of glycerol concentration on growth and lipid accumulation

The biomass of *T. spathulata* increased gradually with an increase of glycerol concentration and reached the maximum value of 10.40 g/L at 10% crude glycerol (Fig. 5a) in 72 h. Lipid yield also increased when glycerol concentration increased to 10%, and reached the maximum lipid of 4.45 g/L for

72 h. The biomass and lipid yield of *K. Ohmeri* also increased gradually with an increase of glycerol concentration and reached the maximum values at 10% crude glycerol (Fig. 5b).

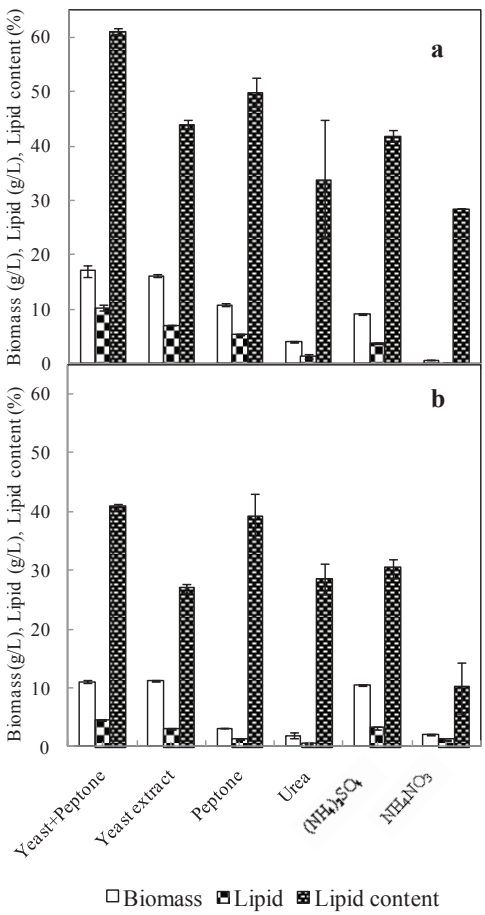

Fig. 3. Effect of nitrogen sources on cell growth and lipid accumulation of *T. spathulata* (a) and *K. ohmeri* (b). 10% crude glycerol (0.13 M of carbon) was used as a sole carbon source and the nitrogen source was added to obtain the C/N molar ratio of 7.4.

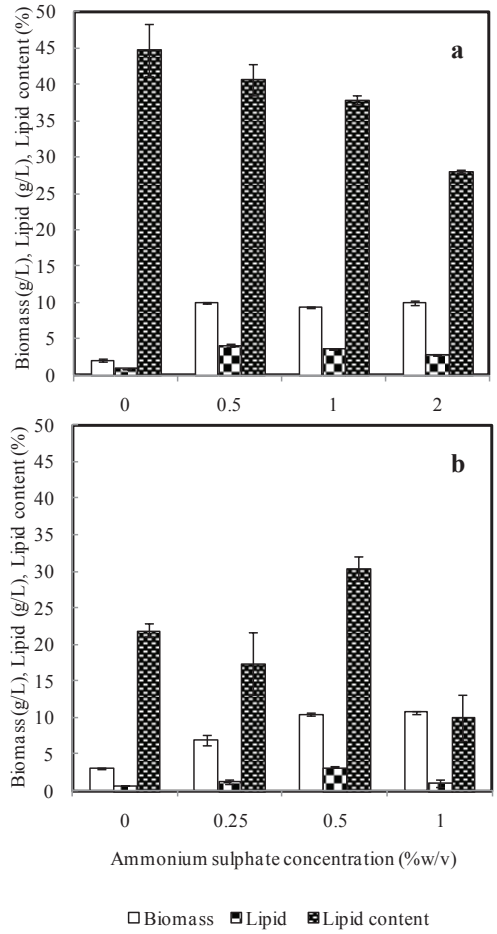

Fig. 4. Effect of ammonium concentration on cell growth and lipid accumulation of *T. spathulata* (a) and *K. Ohmeri* (b). 10% crude glycerol (0.13 M of carbon) was used as a sole carbon source.

The time courses of biomass and lipid accumulation of *T. spathulata* and *K. Ohmeri* using 10% crude glycerol and 0.5% ammonium sulphate as a carbon source and a nitrogen source, respectively, are shown in Fig. 6. *T. spathulata* gave the higest biomass of 10.4 g/L and the maximum lipid production of 4.45 g/L at 72 h (Fig. 6a). *K. Ohmeri* also gave the higest biomass of 10.5 g/L and the maximum lipid production of 3.22 g/L at 72 h (Fig. 6b). It should be noted that the lipid content of *T. spathulata* reached the maximum value at 24 h and gradually decreased. The similar changes were also observed in lipid content of *Yarrowia lipolytica, Cunninghamella echinulata* and *Mortierella isabellina* after exhaustion of the carbon source in the growth environment [12-14]. The use of lipid for cell proliferation owing to the lack

of glycerol could account for this phenomenon. It has been reported that the breakdown of lipid could be repressed in multiple-limited media [13-14].

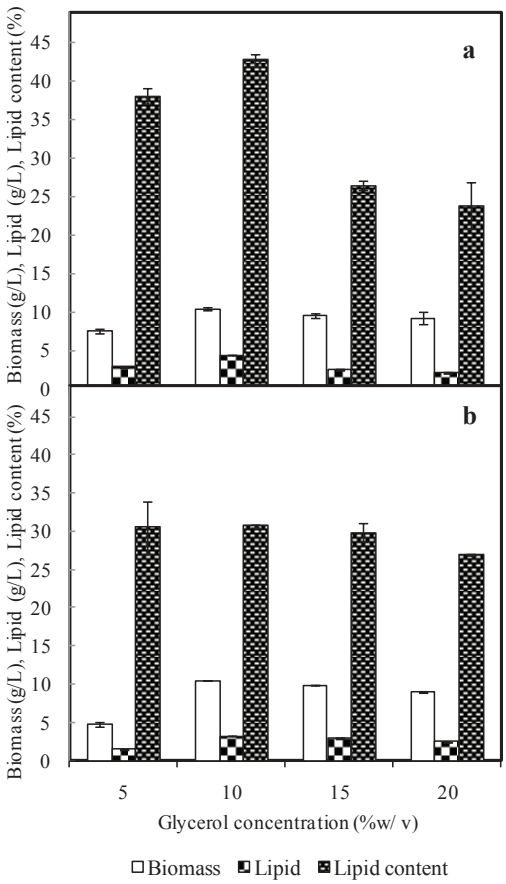

Fig. 5. Effect of glycerol concentration on cell growth and lipid accumulation of *T. spathulata* (a) and *K. ohmeri* (b). The ammonium sulphate concentration was fixed at 0.5% (w/v).

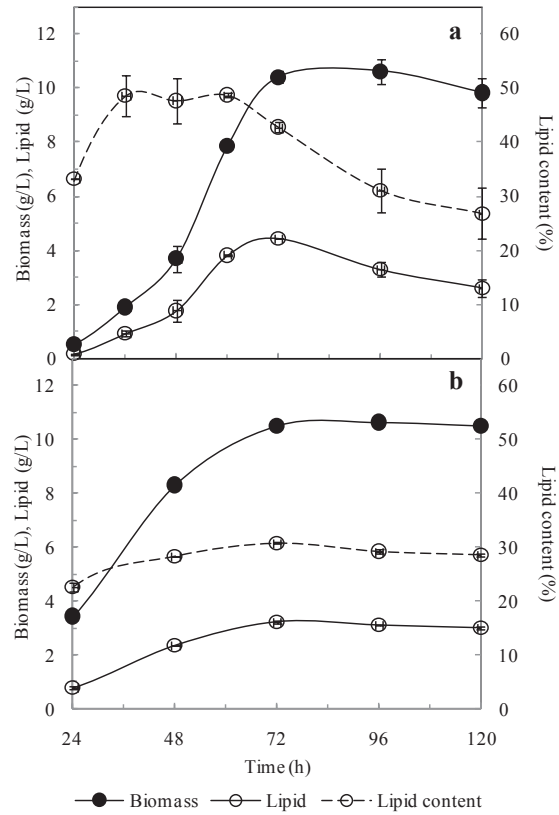

Fig. 6. Time courses of biomass (close symbol), lipid production (open symbol) and lipid content (open symbol and dash line) of *T. spathulata* (a) and *K. ohmeri* (b). 10% crude glycerol (0.13 M of carbon) was used as a sole carbon and 0.5% (w/v) ammonium sulphate was used as a nitrogen source.

4. Conclusions

The newly isolates yeasts, *T. spathulata* and *K. ohmeri* could grow and accumulate lipid in the crude glycerol supplemented with ammonium sulfate only. The optimal condition for cell growth and lipid production was determined. This study has shown that the bioconversion of crude glycerol to oils by oleaginous yeast can be a promising method to produce energy source for our limited crude oil, and may even benefit the effort for reduction of accumulated carbon in atmosphere.

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