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Screening and fermentation optimization of microbial lipid-producing molds from forest soils

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Sixty-one isolates including molds, yeasts and bacteria from the forest soils collected from Hunan Province, China were tested for their potentiality to accumulate microbial lipid as alternative sources for biodiesel production. The results showed that sixteen mold isolates were potential oleaginous microorganisms, among which, strain SCIM 3.009 was the best lipid producers, which may accumulate up to 50.4% of lipids in dry biomass. Further study showed that the components profile of the lipid from strain SCIM 3.009 had the similar characters to that of vegetable oil, abundant in low degree unsaturated long chain fatty acid (C18:1) and saturated long chain fatty acids (C16:0), suggesting the lipid is a potential source for biodiesel production. Based on the morphology and a commercial identification system, the strain SCIM 3.009 was found to be *Thamnidium ctenidium*. To enhance the lipid production by the strain, the fermentation parameters were optimized, the optimium conditions were as follows: glucose as carbon source with initial concentration 60 g/L, NH_4NO_3 as nitrogen source at 3.0 g/L, culture temperature was 30°C, initial pH = 6.5, culture volume was 50 mL in a 250 mL flask, agitation speed was 220 rpm. Results on verification of the optimium conditions in a 5 L stirred-tank bioreactor reveal that the strain accumulate up to 66.02% of lipids in dry biomass and the lipid yield significantly enhanced from 6.4 ± 0.39 to 13.6 ± 0.37 g/L, while biomass enhanced from 12.7 ± 0.72 to 20.6 ± 0.52 g/L.

Key words: Biodiesel, microbial lipid, oleaginous microorganism, strains screening, submerged fermentation.

INTRODUCTION

Biodiesel (fatty acid methyl esters, FAME) has attracted much attention recently because it is made from renewable resources and it has remarkable environmental benefits (Krawczyk, 1996; Li et al., 2008; Ye et al., 2010). It was firstly prepared with transesterification of renewable biological sources, such as vegetable oils and animal fats (Shay, 1993; Ye et al., 2010). Although, examples of such species, vegetable oils, have been used as diesel fuels in emergency situation as early as in 1930 and 1940s, it has recently been recommended as

alternative for fossil fuel due to its renewable, easily available, easily biodegradable characters and low emission profiles and friendship to environment (Shay, 1993) . With the resources shortage of fossil, the price of crude oil increasing sharply and more problems from environment, research on biodiesel fuels from vegetable oil and animal fat have received much attention (Krawczyk, 1996; Sivasamy et al., 2009).

Compared to fossil fuel, the main hurdle for the commercialization of biodiesel is the higher price, which is determined by resources of vegetable oil or animal fat. The total produce cost is mainly depended on the resources used (Su et al., 2004; Gao et al., 2004; Sivasamy et al., 2009). Using waste cooking oil or extending resource ranges is strategies for reducing the

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cost. However, we have to add equipment investment heavily if using waste cooking oil (Zhang et al., 2003a; 2003b). Considering the total cost, there is no advantage. Therefore, extending the resource ranges may be a potential means to reduce the produce cost.

Some microorganism can transform such carbon sources as carbohydrate or hydrocarbon to lipid that stored in the body of it at certain condition, if the lipid content surpasses 20%, this microorganism may be called as oleaginous microorganism. There are strains that can produce lipid among bacteria, yeast, mycetes etc, especially yeast and mycetes (Peralta-Yahya and Keasling, 2010; Kohse-Höinghaus et al., 2010) . Microorg-nism resources are abundant and can grow at various culture conditions, having large potential in industrial scale production and development. Furthermore, as the profile of fatty acid components of some oleaginous microorganism is similar to that of vegetable oils such as rapeseed oil, palm oil and soybean oil, abundant in saturated and low degree unsaturated long chain fatty acid (Calvin, 1985; Goering et al., 1983; Ma et al., 2009). Therefore, the lipid obtained from oleaginous microorga-nisms is potential resource for biodiesel production.

In this study, sixty- one isolates from the forest soils collected from hunan Province, China were tested for their potentiality to produce microbial lipid as alternative sources for biodiesel production. Sixteen potential mold strains were potential and were further screened, and the strain SCIM 3.009 identified as *Thamnidium ctenidium* was the highest yield lipid-producing strain and after optimization of fermentation parameters, the strain accumulate up to 66.02% of lipids in dry biomass and the lipid yield significantly enhanced from 6.4 ± 0.39 to 13.6 ± 0.37 g/L.

MATERIALS AND METHODS

Microorganisms and screening

Sixty-one strains including molds, yeasts and bacteria from forest soils in Hunan province in China were isolated and collected in strain collection of industrial microorganisms (SCIM), Central South University of Forestry and Technology (Changsha, China). The strains were preliminary screened through Sudan Black B staining method (monitoring lipid production by microscopic observation) (Thakur et al., 1988), then screened according to their capacity in producing lipid and their fatty acid profile in shake flask culture. The strain that showed the most potential ability in producing lipid and had the similar fatty acid profile to plant oils (abundant in low degree unsaturated long chain fatty acid (C18: 1) and saturated long chain fatty acids (C16: 0) was selected for further investigation. The final selected mold was preliminary identified by morphology and further identified with a commercial identification system (SHERLOCK® Microbial Identification System, MIDI Corporation, America).

Maintenance and culture of the strains

The strains were maintained on YPD (yeast, peptone and dextrin) slant. The slant was inoculated and incubated at 30°C for 2 - 3

days. The seed cultures were incubated in a rotary shaker-incubator at 180 rpm at 30°C for 2 - 3 days. The seed medium and the medium for screening of potential strains contained of 30 g glucose, 5 g peptone and 5 g yeast extract and water to One liter (pH 5.0). Ten percent of the seed broth was used as inoculum for the production medium (basal medium, BM) consisting: 50 g glucose, 3 g $(NH_4)_2SO_4$, 0.8 g KH_2PO_4, 0.2 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$ and water to 1 L. The pH was adjusted to 5.5. The 250 ml culture flasks each containing 50 mL of production medium were incubated in a rotary shaker-incubator at 180 rpm at 30°C for 4 days.

Bioreactor fermentation

The fermentation medium was inoculated with 10% (v/v) of the liquid seed culture and then cultivated in a 5 L stirred-tank fermenter. Fermentations were performed under the optimized conditions: work volume: 3.5 L, stirring rate: 220 rpm, culture tem-perature, 30°C, initial pH, 6.5, aeration rate: 1.5 vvm and culture time, 100 h.

Determination of mycelial biomass

Biomass was obtained by centrifuging the mycelium at 10 000 rpm for 15 min, washing the precipitated cells for three times with distilled water and drying at 60°C for sufficient time to a constant weight.

Lipid extraction and determination

The lipids were extracted with chloroform/methanol/water as described by Bligh and Dyer 1959 (Bligh and Dyer, 1959), then freeze-dried after removal of solvents by evaporation.

Analysis of fatty acids

For analysis of fatty acids of microbial lipids, FAME was prepared by 15 min incubation at 95°C in boron trifluoride/methanol using the method reported by Morisson and Smith (Morison and Smith, 1964). FAME were extracted with hexane and analyzed by GC-MS immediately.

Measurement of residual sugar

Residual sugar content was determined as 3.5-dinitro-salicylic acid method (Miller, 1959).

Statistical analysis

Incubations were performed at triplicate and data were analyzed using SAS 8.1 version. The results were expressed as the mean ± SD.

RESULTS

Strain screening

Sixty-one isolates of molds, yeasts and bacteria were obtained from forest soils collected in Hunan province in China. They were preliminary screened through Sudan

Strains	Biomass (g/L)	Lipid yield (g/L)
SCIM 3.001	11.8 ± 0.70	4.3 ± 0.21
SCIM 3.002	9.1 ± 0.48	4.0 ± 0.23
SCIM 3.003	3.6 ± 0.18	1.1 ± 0.11
SCIM 3.004	8.6 ± 0.43	2.2 ± 0.17
SCIM 3.005	7.2 ± 0.31	1.7 ± 0.15
SCIM 3.006	3.6 ± 0.18	1.1 ± 0.10
SCIM 3.007	5.3 ± 0.21	2.1 ± 0.17
SCIM 3.008	9.2 ± 0.44	2.7 ± 0.13
SCIM 3.009	12.7 ± 0.72	6.4 ± 0.39
SCIM 3.010	7.8 ± 0.38	2.1 ± 0.15
SCIM 3.011	8.7 ± 0.43	3.1 ± 0.19
SCIM 3.012	8.5 ± 0.41	3.1 ± 0.13
SCIM 3.013	7.8 ± 0.38	2.6 ± 0.15
SCIM 3.014	3.8 ± 0.18	1.8 ± 0.12
SCIM 3.015	8.2 ± 0.43	3.1 ± 0.16
SCIM 3.016	7.3 ± 0.31	2.6 ± 0.11

Black B staining method. The results showed that sixteen isolates of molds (26.2% of the total isolates) could be clearly seen having lipid bodies in their hyphae when examined with optical microscopy. Therefore, these isolates were further tested for ability in producing lipid and their fatty acid profile in shake flask culture. Table 1 shows biomass and lipid yield of the tested sixteen isolates. Strain SCIM 3.009 displayed the most potential ability in accumulating microbial lipid, the lipid content reached 50.4% of dry cell weights and the lipid yield was 6.4 ± 0.39 g/L.

Table 2 shows the profiles of fatty acids of lipids from the sixteen isolates of molds. Besides strain SCIM 3.005, SCIM 3.008, SCIM 3.011, SCIM 3.013 and SCIM 3.015, the fatty acid content were similar to each other and the profile of fatty acids of these microbial lipids were also similar to that of vegetable oils, abundant in low degree unsaturated long chain fatty acid (C18: 1) and saturated long chain fatty acids (C16: 0), suggesting these lipids may be suitable for biodiesel production (Calvin, 1985; Goering et al., 1983; Ma et al., 2009). As shown in Tables 1 and 2, strain SCIM 3.009 produced microbial lipid with the highest yield and the components profile of the lipid was suitable for biodiesel production. Therefore, strain SCIM 3.009 was selected for further research.

Identification of strain SCIM 3.009

Newly isolated mycelium of SCIM 3.009 grew well and dense on PDA. Colonies with a regular margin attained 35 – 45 mm in diameter after incubation on PDA at 30°C for 4 days and became umber in PDA plate, sporran-giferous hyphae profuse, sporangiophores slender, sporangium densely packed with sporangiophores which

are smooth, globese. These morphological characte-ristics allowed the identification of the fungus as *T. ctenidium*, which was reinforced by a commercial identify-cation system (SHERLOCK® Microbial Identification System, MIDI Corporation, America).

Effects of carbon sources on biomass and lipid production

To study the effects of carbon sources on lipid production and biomass, various available substrates were used instead of glucose in BM (Figure 1) . The results show that glucose, sucrose, lactose and maltose were the suitable carbon sources for the growth and lipid accumu-lation of the strain, among which glucose is the most suitable, the lipid production was 7.3 ± 0.19 g/L, followed by lactose with a yield of 6.2 ± 0.29 g/L. Therefore, glucose was chosen as carbon source in following experiments. Figure 2 shows the effect of initial glucose concentrations on lipid production and biomass. Both the biomass and lipid yield enhanced with an increase in initial glucose concentration at a range of 40 - 60 g/L. Higher levels of glucose concentration could not increase the lipid and biomass production.

Effects of nitrogen sources on biomass and lipid production

The effect of nitrogen sources on lipid production and biomass was shown in Table 3. Compared with organic nitrogen sources (peptone, yeast extract, corn powder, soybean powder, wheat bran), when inorganic ones (NH_4NO_3, $NaNO_3$, KNO_3 and $(NH_4)_2SO_4$) were used, the cells grew more quickly and reached a higher lipid yield. Among the inorganic nitrogen sources, NH_4NO_3 was the most suitable and the lipid yield and biomass was 9.1± 0.43 and 17.5 ± 0.71 g/L, respectively. It is reasonable that $(NH_4)NO_3$ was chosen as nitrogen source in following experiments.

Effects of culture temperature on biomass and lipid production

In order to investigate the effects of culture temperature on biomass and lipid production, the strain SCIM 3.009 was incubated at varying temperature (20 - 35°C). The result in Figure 3 shows that both the biomass and lipid yield enhanced with an increase in culture temperature at a range of 20 - 30°C. But above 30°C, there was significant reduction in the lipid formation. Therefore, the optimum culture temperature was 30°C.

Effects of initial pH on biomass and lipid production

To study the effects of initial pH on biomass and lipid

Table 2. Contents of fatty acids of lipids from several strains.

|	Contents of fatty acids of lipids (%)					
Strains	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3
SCIM 3.001	1.35 ± 0.08	16.36 ± 0.75	5.00 ± 0.03	54.99 ± 2.78	6.37 ± 0.03	1.03 ± 0.05
SCIM 3.002	1.41 ± 0.09	18.99 ± 0.77	5.52 ± 0.02	48.90 ± 2.82	7.34 ± 0.04	1.04 ± 0.03
SCIM 3.003	1.27 ± 0.08	21.36 ± 0.65	4.91 ± 0.03	47.99 ± 2.78	5.97 ± 0.03	1.23 ± 0.04
SCIM 3.004	1.06 ± 0.06	19.39 ± 0.98	6.66 ± 0.04	55.39 ± 2.90	11.1 ± 0.05	1.21 ± 0.01
SCIM 3.005	1.11 ± 0.07	45.53 ± 0.94	9.76 ± 0.05	23.79 ± 2.69	7.7 ± 0.05	1.12 ± 0.02
SCIM 3.006	1.92 ± 0.08	19.98 ± 0.81	5.56 ± 0.03	59.32 ± 3.01	7.59 ± 0.04	0.98 ± 0.08
SCIM 3.007	1.55 ± 0.06	14.54 ± 0.77	4.49 ± 0.03	58.91 ± 2.72	7.11 ± 0.05	1.11 ± 0.07
SCIM 3.008	1.21 ± 0.06	50.49 ± 0.79	6.31 ± 0.05	21.02 ± 2.91	5.37 ± 0.04	1.02 ± 0.05
SCIM 3.009	1.18 ± 0.07	17.32 ± 1.01	5.60 ± 0.09	61.83 ± 2.85	7.75 ± 0.02	1.21 ± 0.07
SCIM 3.010	1.41 ± 0.09	15.99 ± 0.77	4.52 ± 0.02	60.30 ± 2.82	7.54 ± 0.04	1.04 ± 0.05
SCIM 3.011	1.82 ± 0.03	43.53 ± 0.94	9.76 ± 0.08	26.79 ± 2.69	7.7 ± 0.06	1.32 ± 0.04
SCIM 3.012	1.35 ± 0.08	14.36 ± 0.75	5.00 ± 0.03	54.99 ± 2.78	6.37 ± 0.03	1.03 ± 0.05
SCIM 3.013	1.33 ± 0.06	51.49 ± 0.79	8.71 ± 0.03	20.02 ± 2.91	5.47 ± 0.03	1.44 ± 0.03
SCIM 3.014	1.05 ± 0.06	22.54 ± 0.77	2.49 ± 0.03	50.91 ± 2.72	7.11 ± 0.05	1.11 ± 0.07
SCIM 3.015	1.11 ± 0.07	48.47 ± 0.71	8.76 ± 0.06	22.79 ± 2.69	5.70 ± 0.01	1.23 ± 0.03
SCIM 3.016	1.31 ± 0.04	13.91 ± 0.72	3.99 ± 0.04	57.92 ± 2.72	7.01 ± 0.04	1.22 ± 0.06
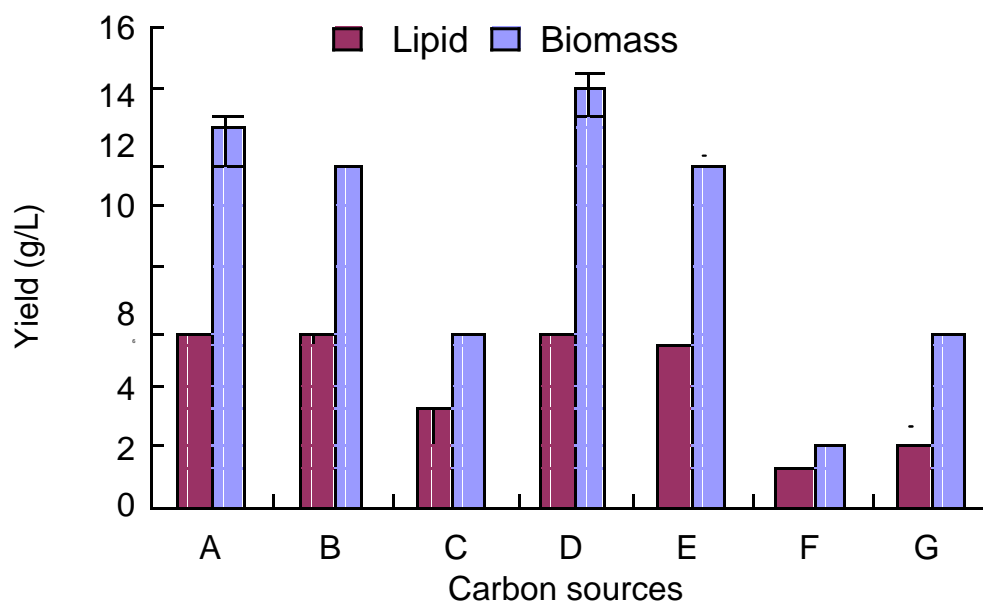
Figure 1. Effect of carbon sources on biomass and lipid production. A, Lactose; B, Sucrose; C, Fructose; D, Glucose; E, Maltose; F, Xylose; G, Starch. Cells were cultivated in a rotary shaker-incubator at 180 rpm at 30°C for 4 days.

production, the strain SCIM 3.009 was incubated at varying initial pH (4.5 - 8.5). The result in Figure 4 reveals that the lipid yield increased with the initial pH value changed from 4.5 to 6.5 and when the initial pH was over 6.5, the lipid yield was no longer enhanced and decreased to the minimal level at 8.5. The result suggests that initial pH had a significant effect on the lipid accumulation and the optimal initial pH value was 6.5.

Effects of culture volume on biomass and lipid production

The effects of culture volume on lipid production and biomass were shown in Figure 5. Both the biomass and lipid yield decreased with an increase in culture volume. This suggests that the strain might be an aerobic strain because the less culture volume the better ventilating
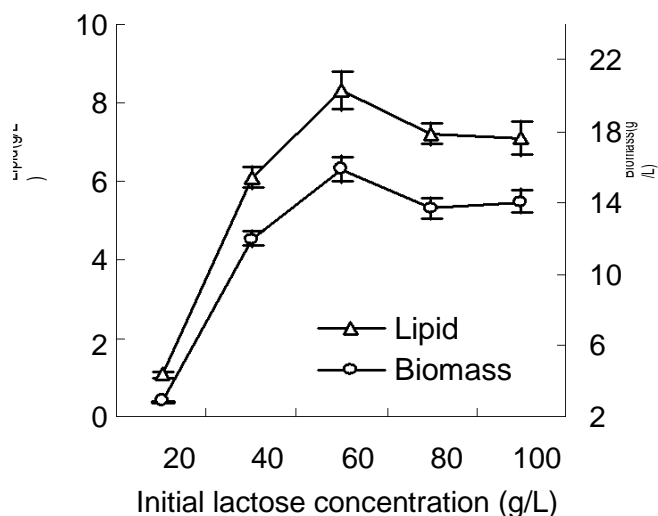
Figure 2. Effect of initial glucose concentration on biomass and lipid production. Cells were cultivated in a rotary shaker-incubator at 180 rpm at 30°C for 4 days.
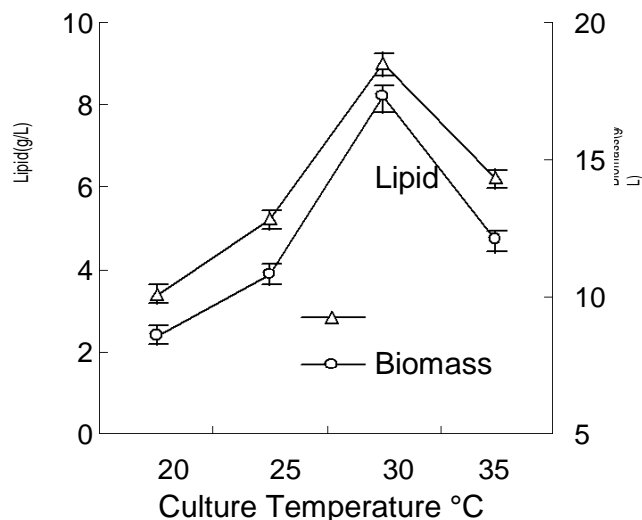

Figure 3. Effect of culture temperature on biomass and lipid production. Cells were cultivated in a rotary shaker-incubator at 180 rpm at 30°C for 4 days.

Table 3. Effect of nitrogen source at 3.0 g/L on biomass and lipid production.

Nitrogen sources	Lipid yield (g/L)	Biomass (g/L)
NH_4NO_3	9.1 ± 0.43	17.5 ± 0.71
$NaNO_3$	7.3 ± 0.50	13.5 ± 0.32
KNO_3	7.0 ± 0.21	13.6 ± 0.67
$(NH_4)_2SO_4$	7.2 ± 0.17	13.9 ± 0.24
Yeast extract	4.1 ± 0.06	9.7 ± 0.13
Peptone	3.9 ± 0.05	8.6 ± 0.34
Corn powder	3.3 ± 0.10	7.2 ± 0.41
Soybean powder	2.9 ± 0.07	5.9 ± 0.24
Wheat bran	2.7 ± 0.09	5.4 ± 0.20

Figure 4. Effect of initial pH on biomass and lipid production. Cells were cultivated in a rotary shaker-incubator at 180 rpm at 30°C for 4 days.

status provided in the flask. When culture volume was 30 and 50 mL/250 mL, the lipid yield was 11.3 ± 0.34 and 10.4 ± 0.29 g/L, respectively. Considering economic benefit, too low culture volume is not advantageous in practice. Therefore, 50 mL (in a 250 mL flask) was selected as the suitable culture volume.

Effects of agitation speed on biomass and lipid production

To investigate the effects of agitation speed on biomass and lipid production, the strain SCIM 3.009 was incubated at varying agitation speed (140 - 220 rpm). The results were shown in Figure 6. A gradual and signi-ficant increase in both biomass and lipid content was observed with increase in speed of agitation of the medium during fermentation up to 220 rpm. At 220 rpm, the biomass yield and the lipid yield were 11.9 ± 0.41 and
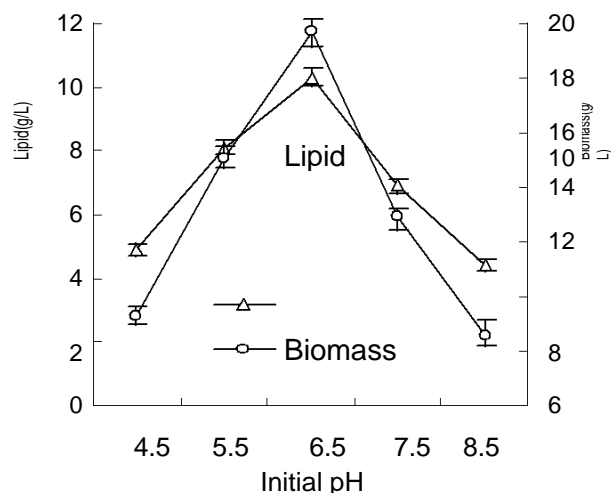
22.6 ± 0.57 g/L, respectively. In general, higher agitation speed was advantageous to oxygen supply, resulting in the higher lipid yield; therefore, the result was consistent with the results shown in Figure 5.

Verification of the optimum conditions for biomass and lipid production

Based on the optimum conditions obtained from the above, we further investigated the strain SCIM 3.009 grown at the optimum conditions in a 5 L stirred-tank
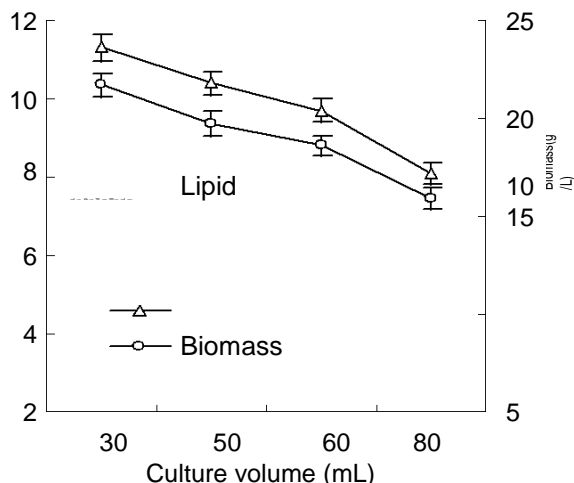
Figure 5. Effect of culture volume on biomass and lipid production. Cells were cultivated in a rotary shaker-incubator at 180 rpm at 30°C for 4 days.
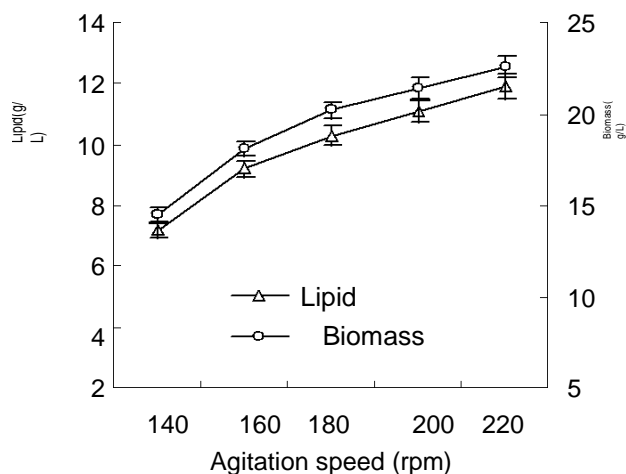

Figure 6. Effect of agitation speed on biomass and lipid production. Cells were cultivated in a rotary shaker-incubator at 180 rpm at 30°C for 4 days.

bioreactor. The time courses of cell growth, lipid production and substrate consumption under the optimized culture conditions are shown in Figure 7. The concentration of residual sugar in broth decreased to 12.1 g/L at 50 h, and 5.2 g/L at 80 h. The time for lipid production and cell growth was out of step. The amount of lipid kept rising from 20 h to 90 h and reached the maximum production (13.6 ± 0.37 g/L) at 90 h. While the biomass kept rising from 10 h to 80 h and reached the maximum weight (20.6 ± 0.52 g/L) at 80 h, and at 90 h, the biomass yield reached 20.1 ± 0.12 g/L. It was obvious that the time for lipid accumulation lags behind that for cell growth and mainly accumulated during the stationary growth phase.

DISCUSSION

Oleaginous microorganisms, such as microalgae, yeasts, fungi and bacteria can accumulate high levels of lipids and do not require arable land, so that they do not compete with food production (Gouda et al., 2008; Vicente et al., 2009). More particularly, photosynthetic microalgae have attracted attention and investment because they capture carbon dioxide in lipids using sunlight. However, their growth in bioreactor systems is problematic because of the light supply requirement (Rittmann, 2008). Oleaginous yeasts and fungi have also been considered as potential oil sources for biodiesel production (Li et al., 2008).

In the present study, we attempted to screen oleaginous microorganisms for production of microbial lipid as alternative sources for biodiesel production. Our results showed that sixteen mold strains were potential oleaginous microorganisms and the mold SCIM 3.009 displayed the most potential ability, and the components profile of its lipid had the similar characters to that of vegetable oils, suggesting the lipid from strain SCIM 3.009 may be suitable for biodiesel production in future. The mold SCIM 3.009 was further identified as *T. ctenidium.*

Oleaginous fungi have been considered as potential oil sources for biodiesel production because they accumulate large amounts of lipids, especially *Mortierella isabelina* and *Cunninghamella echinulata* , which may accumulate up to 86 and 57% of lipids in dry biomass, respectively (Papanikolaou et al., 2004; Fakas et al., 2008). For genera *Thamnidium*, studies have shown that *Thamnidium elegans* is a potential oil source and can be used for polyunsaturated fatty acids (PUFAs) or lipid production and it may accumulate up to 70% fat in dry microbial mass (Papanikolaou et al., 2010). However, there is no datum on the lipid production by *T. ctenidium.* Our study reveal that a newly screened *T. ctenidium* SCIM 3.009 had the ability of accumulating oil in the cell at 66.02% of the dry cell weight cultured in a 5 L stirred-tank bioreactor.

In conclusion, we have screened an oleaginous mold, *T. ctenidium* SCIM 3.009 that can accumulate microbial lipid with a higher yield. It will be attractive to use it to produce microbial lipid as alternative sources for bio-diesel production in future. Further studies are required to investigate its physiology characters and to further optimize the lipid production process.
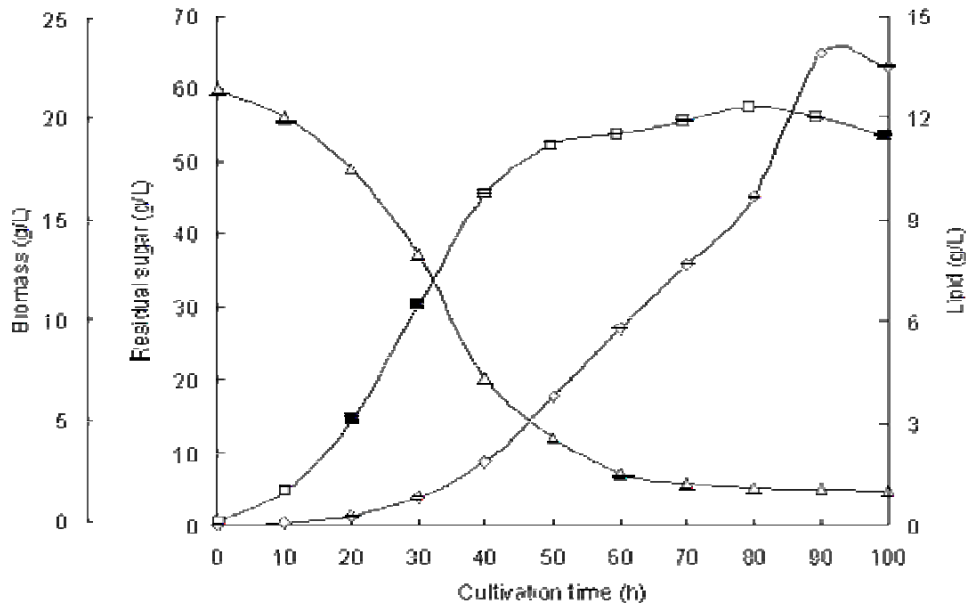
Figure 7. Time profile of cell growth (Υ), lipid (∩) and sugar consumption (≥) by strain SCIM 3.009 growth in a 5 L stirred-stank bioreactor under the optimized culture condition. Work volume: 3.5 L, stirring rate: 220 rpm, aeration rate: 1.5 vvm, culture temperature: 30°C, initial pH, 6.5; culture time: 100 h.

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