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Evaluation of single cell oil from *Aureobasidium pullulans* var. *melanogenum* P10 isolated from mangrove ecosystems for biodiesel production

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ABSTRACT

In this study, the yeast strain P10 which was identified to be a member of *Aureobasidium pullulans* var. *melanogenum* isolated from the mangrove ecosystems was found to be able to accumulate high content of oil in its cells. After optimization of the medium for lipid production and cell growth by the yeast strain P10, it was found that 8.0 g of glucose per 100 ml, 0.02 g of yeast extract per 100 ml, 0.02 g of ammonium sulfate per 100 ml, pH 6.0 in the medium were the most suitable for lipid production. During 10-l fermentation, a titer was 66.3 g oil per 100 g of cell dry weight, cell mass was 1.3 g per 100 ml, a yield was 0.11 g of oil per g of consumed sugar and a productivity was 0.0009 g of oil per g of consumed sugar per h within 120 h. At the same time, only 0.07 g of reducing sugar per 100 ml was left in the fermented medium. The compositions of the fatty acids produced were $C_{16:0}$ (26.7%), $C_{16:1}$(1.7%), $C_{18:0}$ (6.1%), $C_{18:1}$ (44.5%), and $C_{18:2}$ (21.0%). The biodiesel produced from the extracted lipid could be burnt well.

1. Introduction

In recent years, more and more researchers have attempted to use single cell oil (SCO), whose lipid composition is similar to that of traditional vegetable oils, as alternatives for biodiesel production because of the current energy crisis [1]. Biodiesel can be produced by transesterification of triacylglycerols from SCO, yielding monoalkyl esters of long-chain fatty acids with glycerol. The biodiesel produced from natural lipids has many merits. For example, it is biodegradable, can be used directly in the existing engines, and causes less contaminated gas emissions such as sulfur oxide than the conventional fuel [2]. Biodiesel also reduces net carbon dioxide emissions by 78 per cent, particulate matter emissions by 66.7 per cent and unburned hydrocarbons by 45.2 per cent compared to the conventional diesel fuel [2]. Traditionally, microorganisms that can accumulate lipids to more than 20 per cent of their dry weight are considered as oleaginous microorganisms [3]. Although many oleaginous microorganisms, such as bacteria, yeast, fungi and microalgae have been used as the producers of SCO, yeast and microalgae have been used more frequently than fungi and bacteria for lipid and biodiesel production. *Rhodotorula glutinis* [4], *Rhorosporidium toruloides* [5], *Trichosporon fermentans* [4], *Lipomyces starkeyi* [6], *Yarrowia lipolytica* [7], *Pichia guilliermondii* [8], *Rhodotorula mucilaginosa* [9], *Pichia kudriavzevii* [10], *Trichosporon capitatum*, *Apiotrichum curvatum*, *Candida curvata*, and *Cryptococcus curvatus* have the potential to produce biodiesel from SCO via the transesterification process mentioned above.

Furthermore, screening for optimal oleaginous microorganisms became a key mission to many scientists in the field of SCO. *Aureobasidium* spp. are cosmopolitan yeast-like fungi and popularly known as black yeasts due to their melanin production [11]. So far, the genus *Aureobasidium* spp. have been divided into four species, *A. pullulans*, *A. leucospermi*, *A. proteae* and *A. thailandense* [12] and it has been found that *A. pullulans* has five varieties: *A. pullulans* var. *pullulans*, *A. pullulans* var. *melanogenum*, *A. pullulans* var. *subplaciale* and *A. pullulans* var. *namibiae* [13,14]. From an ecological point of view, *Aureobasidium* spp. are ubiquitous species found mainly in soil, including Antarctic soils, water, the phylloplane, wood, and many other plant materials, rocks, monuments, and limestone [15]. *Aureobasidium* spp. have also been reported as slime producing contaminants of paper mills and can colonize optical lenses [16]. In recent years, they also have been found to be widely distributed in hypersaline habitats, coastal water, deep sea, marine sediments of Antarctica and mangrove ecosystems [5,17–20]. Recently, it has been found that some strains of *Aureobasidium* spp. can produce heavy oil [21]. Therefore, the ability to produce single cell oil by *Aureobasidium* spp. isolated from mangrove ecosystems was

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examined in this study. It was found that the strain P10 among them can produce over 50 g of oil per 100 g of cell dry weight and optimization of the medium for SCO production by it was carried out. This is the first time to report that *Aureobasidium* sp. can be a candidate for SCO production.

2. Materials and methods

2.1. Yeast strains

Aureobasidium spp. strains P10 (collection number 2E01290 at the Marine Microorganisms Culture Collection of China), P20 (collection number 2E01299 at MCCC), P24 (collection number 2E012303 at MCCC), P25 (collection number 2E012304 at MCCC), P26 (collection number 2E012305 at MCCC), P27 (collection number 2E012306 at MCCC), P28 (collection number 2E012307 at MCCC), P29 (collection number 2E012308 at MCCC), P30 (collection number 2E012309 at MCCC), P33 (collection number 2E012312 at MCCC), P34 (collection number 2E012313 at MCCC), P35 (collection number 2E012314 at MCCC), P36 (collection number 2E012315 at MCCC), P45 (collection number 2E012324 at MCCC) and P147 P30 (collection number 2E012338 at MCCC) isolated from mangrove ecosystems at DongZaiGang, Haikou, Hainan Province (N19°53′ E110°19) were preserved at −80 °C in this laboratory. These yeast strains were used to screen the SCO producers in this study.

2.2. Media

The yeast strains were kept at 4 °C on YPD agar slant. The medium for screening the yeast strains containing higher lipid content was the medium which contained 0.7 g of KH_2PO_4 per 100 ml, 0.25 g of Na_2HPO_4 per 100 ml, 0.15 g of $MgSO_4{\cdot}7H_2O$ per 100 ml, 0.015 g of $CaCl_2$ per 100 ml, 0.015 g of $FeCl_3{\cdot}7H_2O$ per 100 ml, 0.02 g of $ZnSO_4{\cdot}7H_2O$ per 100 ml, 0.002 g of $MnSO_4{\cdot}H_2O$ per 100 ml, 0.02 g of $(NH_4)_2SO_4$ per 100 ml, 0.04 g of yeast extract per 100 ml, 6.0 g of glucose per 100 ml, and pH 6.0. The medium used for oil production by the yeast strains contained 0.7 g of KH_2PO_4 per 100 ml, 0.25 g of Na_2HPO_4 per 100 ml, 0.15 g of $MgSO_4{\cdot}7H_2O$ per 100 ml, 0.015 g of $CaCl_2$ per 100 ml, 0.015 g of $FeCl_3{\cdot}7H_2O$ per 100 ml, 0.002 g of $ZnSO_4{\cdot}7H_2O$ per 100 ml, 0.002 g of $MnSO_4{\cdot}H_2O$ per 100 ml, 0.02 g of $(NH_4)_2SO_4$ per 100 ml, 0.02 g of yeast extract per 100 ml, 8.0 g of glucose per 100 ml, pH 6.0 [22].

2.3. Sampling and yeast isolation

The roots, stems, branches, leaves, barks, fruits, and flowers (35 °C, summer of 2012) from 12 species of the mangrove plants at six different places in Hainan Province of China were used as the sources for yeast isolation in this study. Latitude and longitude of the sampling sites at DongZaiGang, Haikou, Hainan Province are N19°53′ E110°19. Two grams of the roots, stems, branches, leaves, barks, fruits, and flowers from different mangrove trees were immediately suspended in 50.0 ml of sterile YPD medium containing 2.0 g of glucose per 100 ml, 2.0 g of polypeptone per 100 ml, and 1.0 g of yeast extract per 100 ml and supplemented with 0.05 g of chloramphenicol per 100 ml in 250-ml shaking flasks after the sampling and cultivated at 28 °C for 5 days. After suitable dilution of the cell cultures, the diluted sample was plated on YPD plates with 0.05 g of chloramphenicol per 100 ml and the plates were incubated at 28 °C for 5 days. Different colonies from the plates were transferred to the YPD slants, respectively.

2.4. Screening of the yeast strains with high oil content

All the cultures were incubated in the screening medium in an orbital shaker at a shaking speed of 180 rpm and incubation temperature of 28 °C for 120 h. The lipid extraction and estimation were performed as described below. Finally, it was found that the yeast strain P10 among 15 yeast strains contained the highest amount of total lipids and the yeast strain P35 contained the lowest amount of lipid. Therefore, the yeast strains P10 and P35 were used in the subsequent investigations.

2.5. Staining and observation of lipid particles in the yeast cells

The yeast strain P10 and the yeast strain P35 were grown in the oil production medium at 28 °C for 120 h by shaking, respectively. The cells were harvested and washed by centrifugation at 4 °C and 5000 × *g* for 10 min. The washed cells were stained with Nile Red (GenMed Scientifics Inc., USA; 0.05 mg per 100 ml in DMSO) for 5 min at room temperature [8]. After stained, the cells were observed under blue light with Olympus U-LH100HG fluorescent microscope with 100× oil immersion objective. Images were recorded using the cellSens Standard software.

2.6. Identification of the yeast

Routine identification of the yeast strain P10 was performed using the methods described by Kurtzman and Fell [23].

2.7. DNA extraction and PCR

The total genomic DNA of the yeast strain P10 was isolated and purified by using the methods as described by Sambrook et al. [24]. Amplification and sequencing of ITS sequence from this yeast strain were performed according to the methods described by Chi et al. [25] and the common primers for amplification of ITS in yeasts were used, the forward primer was IT-5 (5′-TCCGTAGGTGAACCTGCGG-3′) and the reverse primer was IT-6 (5′-TCCTCCGCTTATTGATATGC-3′).

2.8. Phylogenetic analysis and identification of the yeast

The sequence obtained above was aligned using BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi, last accessed 18.10.07). The sequences which shared over 98% similarity with currently available sequences were considered to be the same species and multiple alignments were performed using ClustalX 1.83 and phylogenetic tree was constructed using MEGA 4.0 [26].

2.9. Lipid production at flask level

The effects of different carbon sources and different nitrogen sources on oil production and cell growth by the yeast strain P10 were performed by incubating the culture in the oil production medium containing different kinds of carbon source (glucose, maltose; inulin, sucrose, tuber meal of Jerusalem artichoke), different concentrations of glucose from 2.0 g per 100 ml to 14.0 per 100 ml and different nitrogen sources (the initial concentrations of nitrogen sources were kept at 0.04 g per 100 ml) such as yeast extract, peptone, $NaNO_3$, $(NH_4)_2SO_4$ and NH_4NO_3. The cells of the yeast strain P10 were transferred to 50 ml of YPD liquid medium and cultivated at the shaking speed of 180 rpm and 28 °C for 24 h. Five milliliters of the culture (2.5×10^6 cells per ml) were transferred to 50 ml of the oil production media and cultivated at the shaking speed of 180 rpm and 28 °C for 120 h. The cells in the culture were collected and washed three times by centrifugation at 5000 × *g* and 4 °C with sterile saline water. The washed cells were dried at 80 °C until their weight was constant. The total lipids in the cells (1.0 g) were extracted according to Folch et al. [27]. The extracted lipids were weighted and oil content per 100 g of cell dry weight was calculated.

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2.10. Ten-liter batch fermentation

The seed culture of the yeast strain P10 was prepared as described above. 450 ml of the seed culture (OD_{600nm} = 18.0) was transferred to 6000 ml of the oil production medium with initial 8.0 g of glucose per 100 ml, 0.02 g of $(NH_4)_2SO_4$ per 100 ml and 0.02 g of yeast extract per 100 ml in the 10-l fermenter [BIOQ-6005-6010B, Huihetang Bio-Engineering Equipment (Shanghai) Co. Ltd.]. The fermentation was carried out in the fermenter equipped with baffles, a stirrer, heating element, oxygen sensor, and temperature sensor. The fermentation was performed under the conditions of the agitation speed of 300 rpm, aeration rate of 500 l per h, the temperature of 28 °C and the fermentation period of 132 h. Only 120.0 ml of the culture was collected in the interval of 12 h and was centrifuged at 5000 × *g* and 4 °C for 5 min and oil content in the cultures and reducing sugar in the supernatant obtained were determined as described above and below, respectively. The cell dry weight in 10.0 ml of the culture during the 10-l fermentation was also determined as described below. The lipid extraction and total lipid determination from the cultivated cells were performed as described above.

2.11. Determination of the compositions of fatty acids in the extracted oil

For fatty acid analysis, 100 mg of the dried cells was added to 5.0 ml of 0.4 M potassium hydroxide–methanol solution, and the mixture was incubated in water bath at 50 °C for 1 h. Then, 5.0 ml of 14.0 g of boron trifluoride–methanol per 100 ml was added and mixed well and further incubated in water bath at 50 °C for 1 h. Hexane (5.0 ml) was then added to the mixture, and 2.0 ml of saturated sodium chloride was added at last. Gas chromatography analysis of the fatty acid methyl esters obtained was carried out by using 5890-II (Agilent Company, USA). The chromatography column was a fused silica AC2.0 capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness); injector temperature, 250 °C; carrier gas, N_2, 1.0 ml per min; temperature program, 50 °C, held for 2.0 min, from 150 to 200 °C at 15 °C per min, held for 2.0 min, then to 250 °C at 2 °C per min, held for 5.0 min. The fatty acid with $C_{19:0}$ was used as an internal standard [9].

2.12. Determination of reducing sugar in the fermented media and cell dry weight

Reducing sugar in the initial media and fermented media was determined by the Nelson–Somogyi method [28]. Measurement of cell dry weight was conducted according to the methods [29]. Ten milliliters of the culture during the 10-l fermentation were collected and the cells in the cultures were washed three times using sterile saline water by centrifugation at 4 °C and 5000 × *g* for 5 min. The washed cells were dried at 80 °C until the cell dry weight was constant.

2.13. Preparation of biodiesel

The H_2SO_4 solution (0.1 M) (in methanol) was prepared by dissolving 1.0 g of H_2SO_4 in 100.0 ml of methanol anhydrous. 50.0 ml of the total lipid extracted from the biomass according to Folch et al. [27] was mixed with 500 ml of the H_2SO_4 solution (in methanol). The mixture was heated at 70 °C on a magnetic stirrer with mild mixing for 20 min. The mixture was cooled to the room temperature. After separation, the upper phase was removed and lower organic phase obtained was incubated at 80 °C for 1.5 h. The residual organic compounds were used as biodiesel. Finally, the burning of the biodiesel was tested.

3. Results and discussion

3.1. Screening of SCO producers of the yeast strains isolated from the mangrove ecosystems

After isolation and purification of the yeast strains from the samples of the mangrove ecosystems, a total of 200 yeast strains were obtained (data not shown). It was found that the morphologies of only 15 yeast strains were similar to those of *Aureobasidium* spp. [24]. In order to know if the 15 yeast strains are the oleaginous yeasts, oil contents of the 15 yeast strains of the possible *Aureobasidium* spp. isolated from the mangrove ecosystem were determined as described in Materials and methods. The results in Fig. 1 indicated that the yeast strain P10 contained the highest oil content (54.2 g per 100 g of cell dry weight) and the yeast strain P35 contained the lowest amount of lipid (33.8 g per 100 g of cell dry weight). However, cell mass of the different yeast strains was almost the similar (Fig. 1). Therefore, the yeast strains P10 and P35 were used in the subsequent investigations.

After the yeast strains P10 and P35 were grown in the oil production medium, the yeast cells were stained with Nile Red and observed under blue light with Olympus U-LH100HG fluorescent microscope. The results in Fig. 2 showed that each cell of the yeast strain P10 grown in the oil production medium contained big lipid bodies, even the whole cell of some of them was stained by Nile Red while each cell of the yeast strain P35 grown under the same conditions contained only very small lipid particles. This meant that the yeast strain P10 indeed could produce higher amount of oil and more lipid bodies in its cells than any other yeast strains tested in this study. It has been reported that in the oleaginous yeast cells, as neutral lipids such as triacylglycerols (TAGs) and steryl esters (SEs) synthesized are unable to be integrated into phospholipid bilayers, they are stored in the hydrophobic core of lipid bodies which can be stained by Nile Red and TAG is the main component of the lipid body [30,31]. However, it is still completely unknown why the yeast strain P10 could produce higher amount of oil and more lipid bodies than any other yeast strains tested in this study.

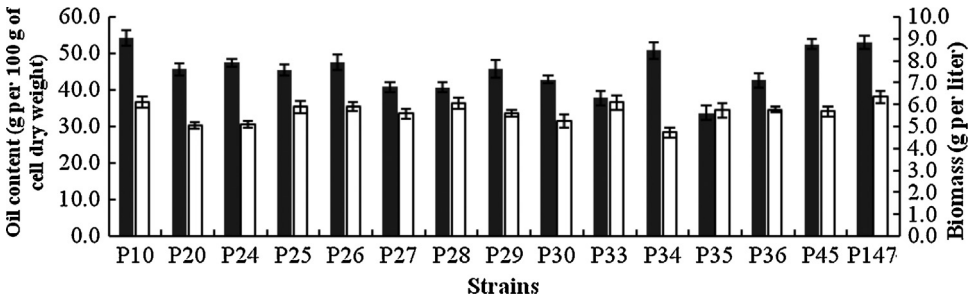

Fig. 1. Oil contents (black) and biomass (white) of the different yeast strains isolated from the mangrove ecosystems. Data are given as means ± SD, n = 3.

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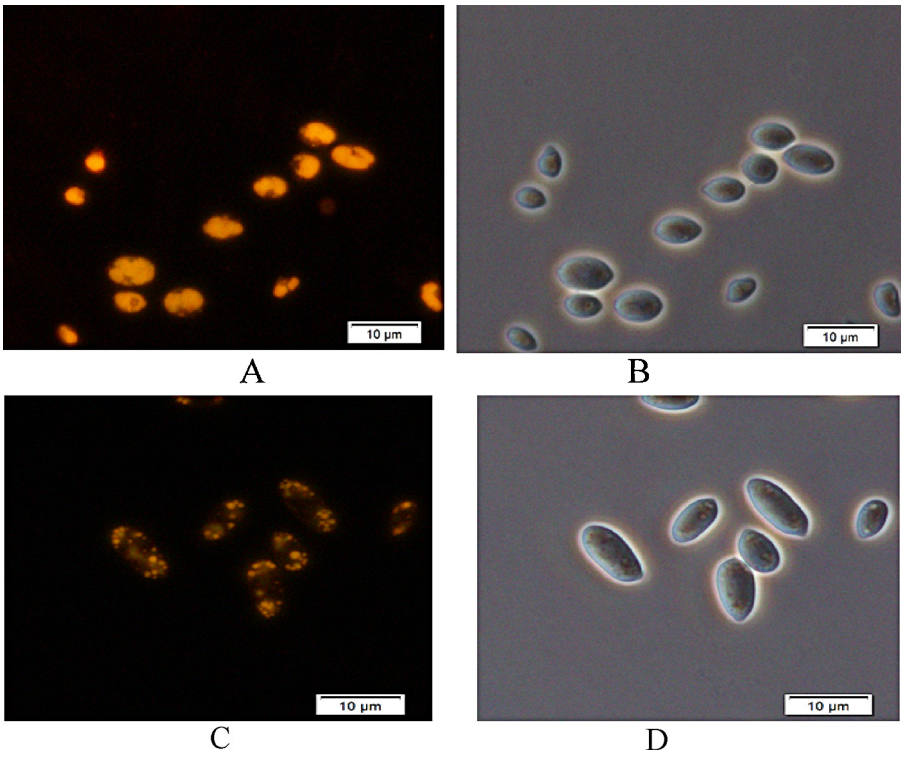

Fig. 2. The lipid particles of the yeast strain P10 (A and B) and the yeast strain P35 (C and D) grown in the lipid production medium. The cells were stained using Nile Red and observed under microscope. A and C were taken under phase microscope and B and D were taken under fluorescent microscope.

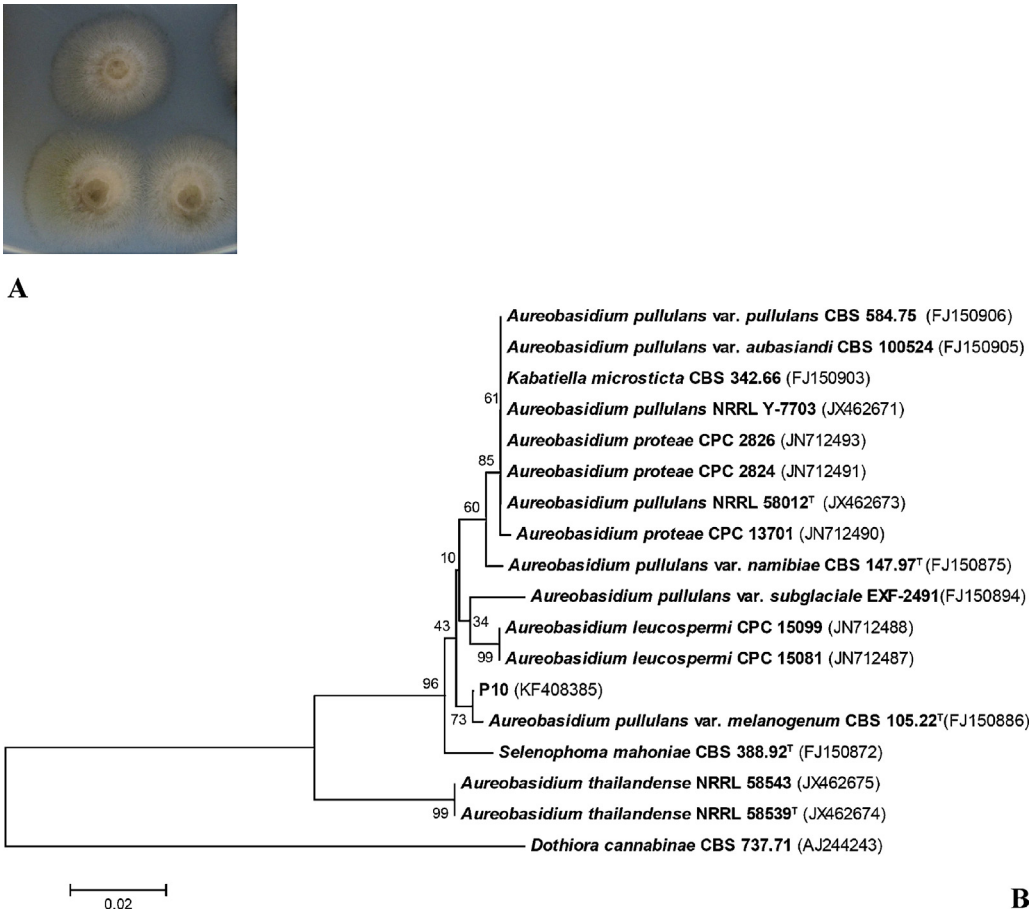

Fig. 3. The morphology of the colonies (A) of the strain P10 and the phylogenetic tree (B) of the strain P10 and other yeasts and fungal species relatives based on a neighbor-joining analysis of ITS sequences. Bootstrap values (1000 pseudoreplications) were 42%.

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3.2. Characterization of the yeast strain P10

As shown above, the yeast strain P10 could synthesize a large amount of lipid (Fig. 1) and its cells contained many large lipid particles (Fig. 2). Therefore, the yeast strain P10 was identified as described in Materials and methods. It was shown that the colony and cell morphology of the yeast strain P10 were close to those of *A. pullulans* (Figs. 2B and 3A). Based on the fermentation and carbon source assimilation spectra and characteristics of the yeast type strain [24], it was found that the yeast strain P10 was also closely related to *A. pullulans* (data not shown). ITS sequence (the accession number was KF408385) of the yeast strain was determined and aligned and phylogenetic tree was constructed as described in Section 2. The search for similarities between ITS of the isolate and those in the NCBI database showed that many phylogenetically related yeast species were similar to the yeast strain P10 obtained in this study (Fig. 3B). The topology of the phylograms in Fig. 3B confirmed that the yeast strain P10 was assigned to one strain of *Aureobasidium pullulans* var. *melanogenum* and the similarity between ITS of the isolate and that of the type strain *A. pullulans* var. *melanogenum* CBS105.22 was 99 per cent. It has been reported that *A. pullulans* var. *melanogenum* can produce melanin and the center of its colonies is olive brown to black [13]. Therefore, the results in Fig. 3 showed that it was also distributed in the mangrove ecosystems. It has been evidenced that *Y. lipolytica*, *R. toruloides*, *L. starkeyi*, *T. fermentans*, *R. mucilaginosa*, *T. capitatum*, *A. curvatum*, *C. curvata*, *P. guilliermondii* and *C. curvatus* can accumulate over 36 g of lipids per 100 g of cell dry weight in their cells [8,32]. Therefore, this is the first time to report that *A. pullulans* var. *melanogenum* can be a candidate for single cell oil production.

3.3. Optimization of the medium for single cell oil production

It has been well documented that a high initial C/N ratio in the medium is required to boost lipid accumulation in the oleaginous yeast cells [9,32]. Therefore, it is very important to optimize the C/N ratio in the oil production medium. Recently, it has been shown that the cheap materials such as inulin and tuber meal of Jerusalem artichoke can be used to produce SCO by oleaginous yeasts [3] and the yeast strains used in this study can produce low inulinase activity (data not shown), So effects of different carbon sources (glucose, inulin, sucrose, tuber meal of Jerusalem artichoke and maltose) (the initial concentrations of carbon sources were kept at 6.0 g per 100 ml) on lipid production and cell growth by the yeast strain P10 were examined. The results in Fig. 4A indicated that when the oil production medium contained glucose, lipid content in the yeast cells reached the highest (54.9 g per 100 g of cell dry weight) and cell dry weight was 6.9 g per liter. After effects of different concentrations of glucose on lipid production and cell growth by the yeast strain P10 were tested, our results showed that when the oil production medium contained 8.0 g of glucose per 100 ml, lipid content of the yeast cells was the highest (56.6 g per 100 g of cell dry weight) and cell dry weight was 10.8 g per liter (data not shown). After effects of different nitrogen sources (the initial concentrations of nitrogen sources were kept at 0.04 g per 100 ml) on lipid production and cell growth by the yeast strain P10 were examined, the results in Fig. 4B indicated that when the oil production medium contained yeast extract, lipid content in the yeast cells reached 57.0 g per 100 g of cell dry weight and cell dry weight was 11.04 g per liter. When the oil production medium contained ammonium sulfate, lipid content in the yeast cells reached 53.0 g per 100 g of cell dry weight, cell dry weight was 10.03 g per liter. It was also indicated that the

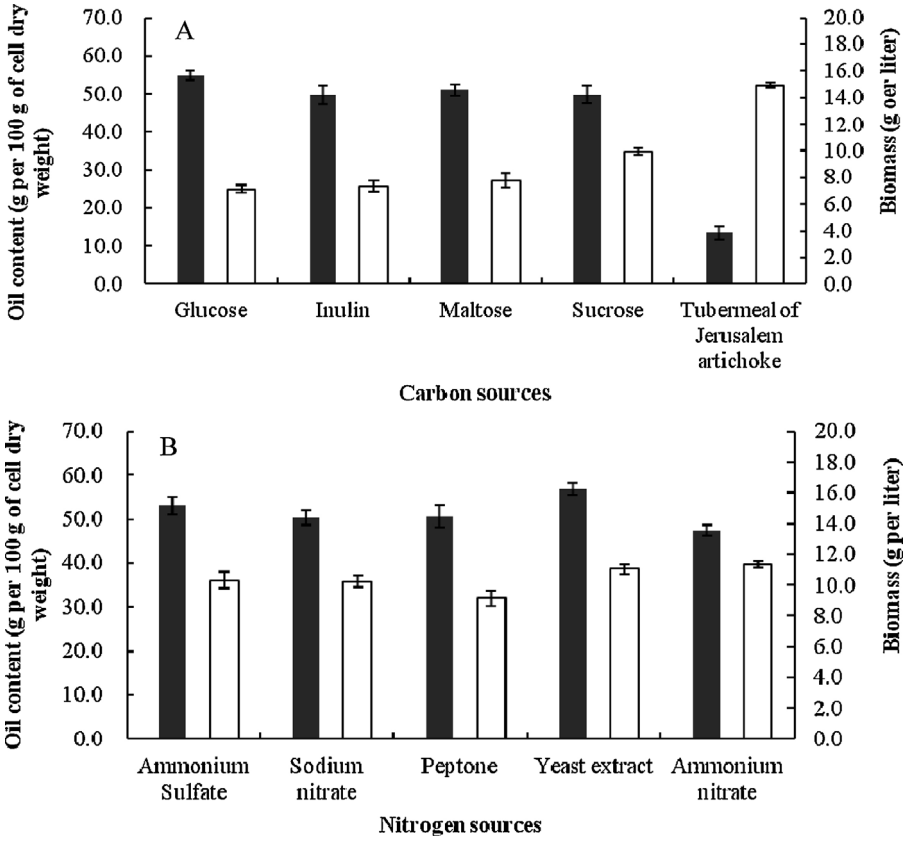

Fig. 4. Effects of different carbon sources (A) and nitrogen sources (B) on lipid accumulation (black) and cell growth (white) by the yeast strain P10. Data are given as means ± SD, $n = 3$.

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Table 1
The compositions of the fatty acids (%) of the yeast strain P10 grown in the oil production medium containing glucose.

Fatty acids	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$
Retention time (min)	7.57	7.89	10.46	10.95	11.77
Percentage	26.7% ± 0.2	1.7% ± 1.0	6.1%± 0.4	44.5% ± 4.0	21.0% ± 0.3

Data are given as means ± SD, $n = 3$.

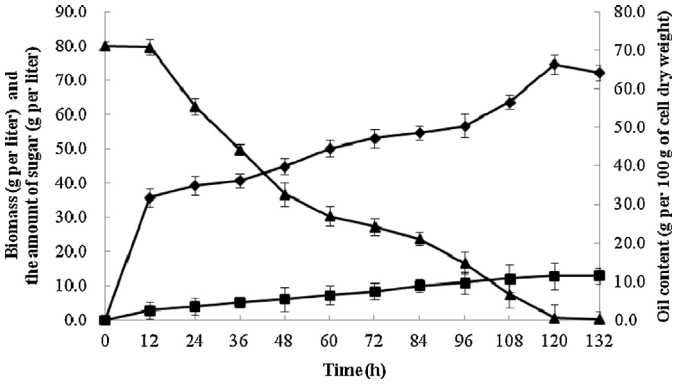

Fig. 5. Time-course of lipid content in the cells (♦), cell growth (■) and changes in reducing sugar (▲) in the yeast culture during 10-l fermentation. Data are given as means ± SD, $n = 3$.

optimal pH and temperature for lipid accumulation of the yeast strain were 6.0 and 28 °C (data not shown). Finally, in order to reduce the cost of the medium, it was determined that the carbon source in the lipid production medium was 8.0 g of glucose per 100 ml and nitrogen sources were 0.02 g of yeast extract per 100 ml and 0.02 g of ammonium sulfate per 100 ml. Under the conditions of the C/N ratio of 600, the lipid content in the yeast cells reached the highest within 120 h of cultivation (data not shown). It was reported that after optimization of the medium and cultivation conditions for SCO production, the transformant of *Y. lipolytica* could accumulate 46.3 g of oil per 100 g of cell dry weight from inulin in its cells and cell dry weight was 11.6 g per liter within 78 h at the flask level [33]. *R. mucilaginosa* TJY15a was found to be able to accumulate 48.8 g of oil per 100 g of cell dry weight from hydrolysate of inulin and its cell dry weight reached 14.8 g per liter during the batch cultivation [34]. When the immobilized inulinase-producing yeast cells were co-cultivated with the free cells of *R. mucilaginosa* TJY15a, *R. mucilaginosa* TJY15a could accumulate 53.2 g of oil per 100 g of cell dry weight from inulin in its cells and cell dry weight reached 12.2 g per liter [34]. This meant that the yeast strain P10 used in this study could accumulate very high content of lipid from glucose in its cells. Recently, sugars such as glucose, fructose, lactose, sucrose, inulin, whey, glucose-enriched wastes, molasses, cellulose, starch, glycerol and others also have been used as the substrates for SCO production [8,9,32].

3.4. Oil accumulation by batch fermentation

To scale up for lipid production from glucose, 10-l fermentation was carried out. During the fermentation, lipid content in the yeast cells, cell growth and changes in reducing sugar concentration were monitored. The results in Fig. 5 revealed that a titer of 66.3 g of lipid based on 100 g of cell dry weight, 12.8 g of dry cell mass per liter of medium, yields of 0.11 g of oil per g of consumed sugar and 0.16 g of biomass per g of consumed sugar, and a productivity was 0.0009 g of oil per g of consumed sugar per h were obtained in the culture of the yeast strain P10 after 120 h of the batch fermentation. At the end of the batch fermentation, only 0.07 g of reducing sugar per 100 ml of medium was left in the fermented medium (Fig. 5), indicating that most of the added glucose was transformed into cells mass and

Fig. 6. Burning of the biodiesel produced in this study.

SCO. A dry biomass and cellular lipid content of 151.5 g per liter of medium and 48.0 g per 100 g of cell dry weight can be reached in flask fed-batch cultures of *R. toruloides* Y4 for 25 days, respectively [5]. During the 2-l fermentation, the transformant of *Y. lipolytica* could accumulate 48.3 g of oil per 100 g of cell dry weight from inulin in its cells and cell dry weight was 13.3 g per liter of medium within 78 h [33]. *R. mucilaginosa* TJY15a could accumulate 52.2 g of oil per 100 g of cell weight from hydrolysate of extract of Jerusalem artichoke tubers and its cell dry weight reached 19.5 g per liter of medium during the fed-batch cultivations, respectively [34]. When the co-cultures were grown in 2-l fermentor, *R. mucilaginosa* TJY15a could accumulate 56.6 g of oil per 100 g of cell dry weight from the extract of Jerusalem artichoke tubers in its cells and cell dry weight reached 19.6 g per liter of medium within 48 h [35]. This meant that the yeast strain P10 used in this study could accumulate very high concentration of oil from glucose in its cells during the 10-l batch fermentation.

3.5. The compositions of the fatty acids

After fatty acids in the extracted lipids from the cells of the yeast strain P10 grown in the oil production medium were transmethylated and analyzed by Gas Chromatography, the results showed that the percentages of palmitic acid ($C_{16:0}$), palmitoleic acid ($C_{16:1}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$), and linoleic acid ($C_{18:2}$) from the extracted lipids were 26.7 per cent, 1.7 per cent, 6.1 per cent, 44.5 per cent and 21.0 per cent, respectively and over 92.2 per cent of the fatty acids from the yeast strain P10 was $C_{16:0}$, $C_{18:1}$ and $C_{18:2}$

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(Table 1). As the fatty acids compositions were similar to those from vegetable oils (Table 1) [36] and the oil feedstocks for biodiesel production require C_{16}–C_{18} fatty acids [37], the lipids from the yeast strain P10 were also a good oil feedstock for biodiesel production. The lipids from *R. toruloides* Y4 also contain mainly long-chain fatty acids with 16 and 18 carbon atoms [5]. Over 90.0 per cent of the fatty acids from the yeast strain TJY15a grown in the extract of Jerusalem artichoke tubers was $C_{16:0}$, $C_{18:1}$ and $C_{18:2}$, especially $C_{18:1}$ (50.6 per cent) [9]. It is true that the main fatty acids produced by oleaginous yeasts are myristic ($C_{14:0}$), palmitic ($C_{16:0}$), palmitoleic ($C_{16:1}$), stearic ($C_{18:0}$), oleic ($C_{18:1}$), linoleic ($C_{18:2}$), and linolenic acid ($C_{18:3}$) and such yeast oils could be used as oil feedstocks for biodiesel production [37].

3.6. Biodiesel production

After the lipids obtained from the cells of the yeast strain P10 grown in the oil production medium were transformed into biodiesel as described in Section 2, it could be clearly observed from the data in Fig. 6 that the biodiesel was burned well. It was found that over 73.7 per cent of the lipids could be transformed into biodiesel under the conditions used in this study (data not shown). It has been reported that in addition to SCO as starting material of 2nd generation biodiesel, the yeast lipids can also be used as substitutes of high added value exotic fats (e.g., cocoa butter) [32].

4. Conclusions

The yeast *A. pullulans* var. *melanogenum* strain P10 isolated from the mangrove ecosystems was found to be able to accumulate high content of oil in its cells. During 10-l fermentation, the yeast strain P10 produced 66.3 g of oil per 100 g of cell weight in its cells and cell mass was 12.8 g per liter of medium within 120 h. The compositions of the fatty acids in the produced lipid were $C_{16:0}$ (26.7 per cent), $C_{16:1}$(1.7 per cent), $C_{18:0}$ (6.1 per cent), $C_{18:1}$ (44.5 per cent), and $C_{18:2}$ (21.0 per cent). The biodiesel produced from the extracted lipid could be burnt well.

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