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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.