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Heavy oils, principally long-chain *n*-alkanes secreted by *Aureobasidium pullulans* var. *melanogenum* strain P5 isolated from mangrove system

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Abstract In this study, the yeast strain P5 isolated from a mangrove system was identified to be a strain of *Aureobasidium pullulans* var. *melanogenum* and was found to be able to secrete a large amount of heavy oil into medium. After optimization of the medium for heavy oil production and cell growth by the yeast strain P5, it was found that 120.0 g/l of glucose and 0.1 % corn steep liquor were the most suitable for heavy oil production. During 10-l fermentation, the yeast strain P5 produced 32.5 g/l of heavy oil and cell mass was 23.0 g/l within 168 h. The secreted heavy oils contained 66.15 % of the long-chain *n*-alkanes and 26.4 % of the fatty acids, whereas the compositions of the fatty acids in the yeast cells were only $C_{16:0}$ (21.2 %), $C_{16:1}$ (2.8 %), $C_{18:0}$ (2.9 %), $C_{18:1}$ (39.8 %), and $C_{18:2}$ (33.3 %). We think that the secreted heavy oils may be used as a new source of petroleum in marine environments. This is the first report of yeast cells which can secrete the long-chain *n*-alkanes.

Keywords Heavy oils · *Aureobasidium pullulans* var. *melanogenum* · Long-chain *n*-alkanes · Secretion · Mangrove system

Introduction

Recently, hydrocarbon production by microorganisms has received increasing attention because they may play a role in petroleum formation and genesis of marine sediments.

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They may also be used for the production of renewable fuel and valuable hydrocarbon compounds, such as the rubber-like polymers [11]. It has been known that bacteria, fungi and algae can produce intracellular and extracellular hydrocarbons. Generally, intracellular hydrocarbons include mainly long-chain C_{25}–C_{35} *n*-alkanes (50–90 %), while extracellular fractions are represented by hydrocarbons with the lower chain lengths. For example, hydrocarbons C_{19}–C_{21} and C_{16}–C_{18} are produced by *Clostridium pasteurianum* and *Desulfovibrio desulfuricans*, respectively [11]. Many fungi are known to produce octane, 1-octene and lower-molecular-mass hydrocarbons [25]. Metzger and Largeau [15] reported that some algae produced essentially *n*-alkadiene and triene hydrocarbons, odd-carbon-numbered from C_{23} to C_{33}, some algae produced triterpenoid hydrocarbons, C_{30}–C_{37} botryococcenes and C_{31}–C_{34} methylated squalenes and other algae produced a single tetraterpenoid hydrocarbon and lycopadiene. So far, a biosynthesis pathway (elongation–decarboxylation) for straight-chain hydrocarbons in cyanobacteria has been proposed and the genes encoding the fatty acyl-CoA or fatty acyl-ACP reductase and fatty aldehyde decarboxylase have been cloned and characterized in cyanobacteria [7, 21]. The "elongation–decarboxylation" pathway envisages de novo synthesis of long-chain fatty acids (e.g. C_{16} and C_{18}), which are elongated by the continuous addition of a C_2 unit derived from malonyl-CoA with the subsequent decarboxylation. In addition to the "elongation–decarboxylation", the other widely considered pathway for the alkane biosynthesis involves the head-to-head condensation of two fatty acids (or suitable derivatives) with the subsequent decarboxylation of one of them. This route was investigated in detail in bacterium *Sarcina lutea* [11]. The physiological roles of hydrocarbons in microorganisms include that hydrocarbons arranged at the wall surfaces

of fungal spores appear to fulfill protective function, the straight-chain hydrocarbons produced by sulfate-reducing bacteria and clostridia seem to be associated with the formation of capsules on the cell surface, which protect bacteria from high concentrations of excreted acids [2] and extracellular saturated C_{21}–C_{33} hydrocarbons produced by bacterium *Psudomonas fluorescens* are involved in the autoregulation of the cell adhesion to a glass surface and promote the cell aggregation [17].

Aureobasidium spp. are cosmopolitan yeast-like fungi and popularly known as black yeasts due to their melanin production. They have been well known because of production of different kinds of extracellular enzymes, single cell protein, single cell oil, siderophore, aureobasidin A, pullulan, aubasidan, polymalate and heavy oils [13]. For example, Manitchotpisit et al. [14] reported that in more than 50 diverse strains of *Aureobasidium pullulans*, 21 strains produced extracellular heavy oils. Their partial structure of the heavy oils is 3,5-dihydroxydecanoyl esters of mannitol. The liamocins produced by *A. pullulans* strain NRRL 50380 were the heavy oils composed of a single mannitol headgroup partially *O*-acylated with three or four 3,5-dihydroxydecanoic ester groups [18]. In this study, the yeast strain P5 isolated from the mangrove system was found to be able to produce heavy oils with mainly long-chain *n*-alkanes. Therefore, the yeast strain P5 was identified and characterized and the medium for production of the heavy oil was optimized.

Materials and methods

Yeast strains

Aureobasidium spp. strains P3 (collection number 2E01390 at the Marine Microorganisms Culture Collection of China), P4 (collection number 2E01391 at MCCC), P5 (collection number 2E01391 at MCCC), P7 (collection number 2E01393 at MCCC), P10 (collection number 2E01394 at MCCC), P12 (collection number 2E01298 at MCCC)and P39 (collection number 2E012306 at MCCC) isolated from the mangrove systems 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 heavy oil producers in this study.

Media

The yeast strains were kept at 4 °C on YPD agar slant. The medium for screening the yeast strains secreting heavy oil contained 12.0 % glucose, 0.05 % yeast extract, 0.1 % NH_4NO_3, 0.01 % KH_2PO_4, 0.05 % KCl and 0.02 %

$MgSO_4·7H_2O$. The medium used for heavy oil production by the yeast strains contained 12.0 % glucose, 0.1 % corn steep liquor, 0.1 % NH_4NO_3, 0.01 % KH_2PO_4, 0.05 % KCl, 0.02 % $MgSO_4·7H_2O$. The medium for seed culture was YPD medium [18].

Sampling

The roots, stems, branches, leaves, barks, fruits, and flowers (35 °C, summer of 2012) from 12 species of the mangrove plants at 6 different places in Hainan Province of China were used as the sources for yeast isolation in this study [28].

Yeast isolation

Yeast isolation was done according to the methods described by Wang et al. [28].

Screening of the yeast strains secreting heavy oils

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 168 h. Fifty milliliters of the cultures were centrifuged at $12,000\times g$ and 4 °C. The pellets including the heavy oils obtained were mixed thoroughly with 10 ml of methanol and chloroform (1:1). The mixtures were centrifuged at $12,000\times g$ and 4 °C for 10 min. The organic phase was transferred into a round bottom flask. The solvent was removed on a vacuum rotary evaporator at 80 °C. The residues (the crude heavy oils) were mixed thoroughly again with 10 ml of the solvent. The mixture was centrifuged at $12,000\times g$ and 4 °C for 10 min. The organic phase was transferred into a new round bottom flask. The solvent was removed on a vacuum rotary evaporator at 80 °C. The same procedure for purification of the heavy oils was repeated three times. After drying, the residues (heavy oils) were weighed and mg of the residues was calculated. The yeast strains without secreting heavy oils were also selected.

Observation of the secreted heavy oils in the culture

The yeast cells with secreted heavy oils and without secreted heavy oils were observed under blue light with Olympus U-LH100HG fluorescent microscope with $100\times$ oil immersion objective. Images were recorded using the cellSens Standard software.

Identification of the yeast

Routine identification of the yeast strain P5 was performed using the methods described by Kurtzman and Fell [10].

DNA extraction and PCR

The total genomic DNA of the yeast strain P5 was isolated and purified by using the methods as described by Sambrook et al. [20]. Amplification and sequencing of ITS sequence from this yeast strain were performed according to the methods described by Chi et al. [5] and the common primers for amplification of ITS in yeasts were used; the forward primer was IT-5 (5′-TCCGTAG-GTGAACCTGCGG-3′) and the reverse primer was IT-6 (5′-TCCTCCGCTTATTGATATGC-3′).

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 2007.10.18). 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].

Heavy oil production at flask level

The effects of different carbon sources on heavy oil production and cell growth by the yeast strain P5 were performed by incubating the culture in the oil production medium containing different carbon sources (glucose, inulin, sucrose, fructose and glycerol), different concentrations of glucose from 100.0 to 180 g/l and different concentrations of corn steep liquor from 0.1 to 0.5 %. The cells of the yeast strain P5 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/ml) was transferred to 50 ml of the oil production medium and cultivated at the shaking speed of 180 rpm and 28 °C for 120 h. The heavy oil extraction and total heavy oil determination from the culture were performed as described above.

Ten-liter batch fermentation

The seed culture of the yeast strain P5 was prepared as described above. 450 ml of the seed culture ($OD_{600nm} = 18.0$) was transferred to 6,000 ml of the heavy oil production medium with initial 120.0 g/l and 0.1 % CSL 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, the aeration rate of 500 l/h, the temperature of 28 °C and the fermentation period of 168 h. Only 50.0 ml of the culture

was collected in the interval of 12 h and was centrifuged at $5,000 \times g$ and 4 °C for 5 min and heavy 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 heavy oil extraction and total heavy oil determination from the culture were performed as described above.

Analysis of heavy oils by GC–MS

For the fatty acid methyl esters, 100 mg of the extracted heavy oils 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. The GC/MS analysis of the treated sample was performed on an Agilent 7890A/5975C gas chromatograph interfaced with an Agilent 5975C mass-selective detector configured in an electron impact (EI) mode and equipped with a Hewlett-Packard (Santa Clara, CA) 7683 series autoinjector. Chromatography was achieved on an Agilent HP-INNOWax Polyethylene Glyco (30 m × 250 µm × 0.25 µm film thickness) using helium as the carrier gas. The oven temperature was ramped over a linear gradient from 100 to 240 °C at 15 °C per min within 10 min. GC–MS of each sample was run for 19.333 min. Mass spectra were recorded in positive-ion mode over the m/z range of 60–550. Injector and detector/interface temperatures were 275 and 300 °C, respectively. The GC–MS measurements were made triple with comparable results. The data from each peak were acquired and processed using the Agilent Masshunter version Nist08 software.

Determination of the compositions of fatty acids in the yeast cells

The cells in the cultures were collected and washed three times with sterile saline water by centrifugation at $5,000 \times g$ and 4 °C. 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. [6]. The fatty acid methyl esters were prepared as described above. 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 °C 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 [12].

Determination of reducing sugar in the fermented media

Reducing sugar in the initial media and fermented media was determined by the Nelson–Somogyi method [24].

Measurement of cell dry weight

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 5,000×g for 5 min. The washed cells were dried at 80 °C until the cell dry weight was constant [4].

Results and discussion

Screening of heavy oil producers of the yeast strains isolated from the mangrove systems

It has been shown that many strains of *Aureobasidium* spp. isolated from terrestrial environments can produce heavy oils [9, 14, 18]. Recently, it has also been found that *Aureobasidium* spp. are widely distributed in mangrove ecosystems [13]. In order to know if some stains of *Aureobasidium* spp. isolated from mangrove ecosystems can produce heavy oils, a total of 200 yeast strains were isolated from the samples of the mangrove systems and purified as described in "Materials and methods" (data not shown). It was found that the morphologies of many yeast strains were similar to those of *Aureobasidium* spp. [10]. After determination of the amount of the secreted heavy oil of seven yeast strains of the possible *Aureobasidium* spp. isolated from the mangrove system, the results in Fig. 1 indicated that the yeast strain P5 secreted the highest amount of the heavy oil (22.5 g/l), while the yeast strain P10 did not produce any heavy oils. However, cell mass of the different yeast strains was also different (Fig. 1). After calculation of the amount of heavy oil per gram of cell dry weight, the yeast strain P5 produced the highest amount of heavy oil while the yeast strain P10 produced no heavy oil (Fig. 1). Therefore, the yeast strains P5 and P10 were used in the subsequent investigations.

After the yeast strains P5 and P10 were grown in the heavy oil production medium, the cultures were observed under blue light with Olympus U-LH100HG fluorescent microscope. It can be seen from the results in Fig. 2a that the culture of the yeast strain P5 grown in the heavy oil production medium contained a large amount of the secreted
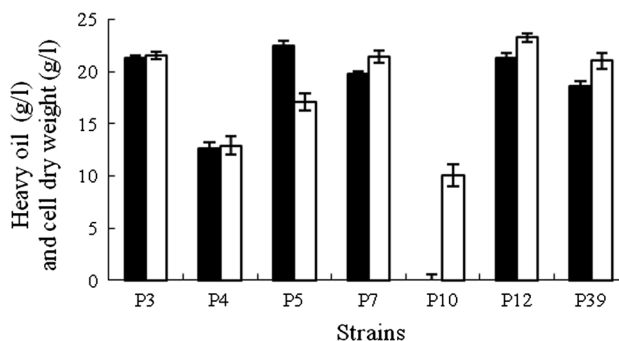

Fig. 1 The amount of the heavy oil (*black*) produced by different strains of *Aureobasidium* spp. and their cell mass (*white*). Data are given as mean ± SD, $n = 3$

oil. Even, after the yeast was grown on the plate containing the heavy oil production medium, the secreted yellow oil could be observed (Fig. 2b). However, Fig. 2c shows that the culture of the yeast strain P10 grown in the same heavy oil production medium had no secreted oil. Even, after the yeast was grown on the plate containing the heavy oil production medium, the secreted yellow oil could not be observed (Fig. 2d). This confirmed that the yeast strain P5 indeed could produce high amount of the secreted heavy oil. It has been reported that oil colors produced by 21 strains of *Aureobasidium pullulans* range from bright yellow to malachite [14].

Characterization of the yeast strain P5

In our previous study [28], the yeast strain P10 mentioned above has been assigned to a strain of *Aureobasidium pullulans* var. *melanogenum*. As the yeast strain P5 used in this study could synthesize and secrete a large amount of heavy oil (Figs. 1, 2), it was identified as described in "Materials and methods". It could be observed that the colony and cell morphology of the yeast strain P5 were close to those of *A. pullulans* (Fig. 2a, b). Based on the fermentation and carbon source assimilation profiles and characteristics of the yeast type strain [10], it was found that the yeast strain P5 was also closely related to *A. pullulans* (data not shown). ITS sequence (accession number was KF891885) of the yeast strain was determined and aligned and its phylogenetic tree was constructed as described in "Materials and methods". Figure 3 reveals that many phylogenetically related yeast species were similar to the yeast strain P5 obtained in this study. The topology of the phylogenetic tree (Fig. 3) confirmed that the yeast strain P5 was also assigned to a 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 % and the similarity between ITS of the yeast strain P5 and that of the yeast strain P10 was 100 %

Fig. 2 The heavy oils (*yellow*) secreted by the yeast cells in the culture of the yeast strain P5 (**a**) and grown on the plate (**b**) while no heavy oil was secreted by the yeast cells in the culture of the yeast strain P10 (**c**) and grown on the plate (**d**); *Arrow* 1 shows the yeast cell; *Arrow* 2 shows heavy oil
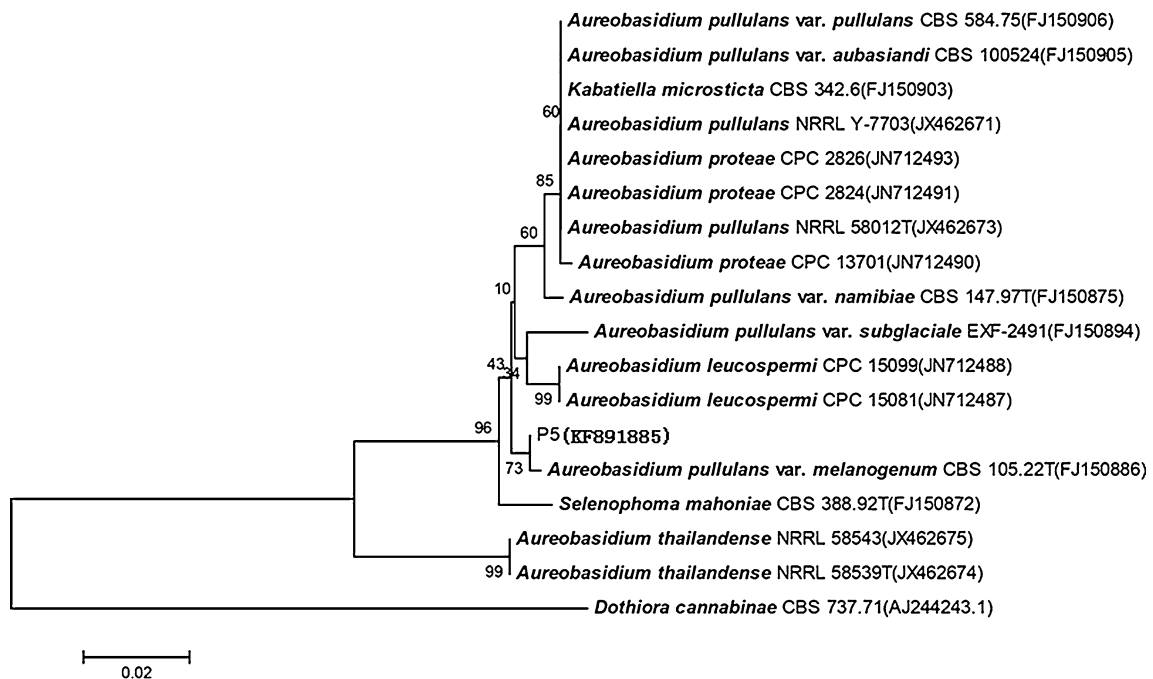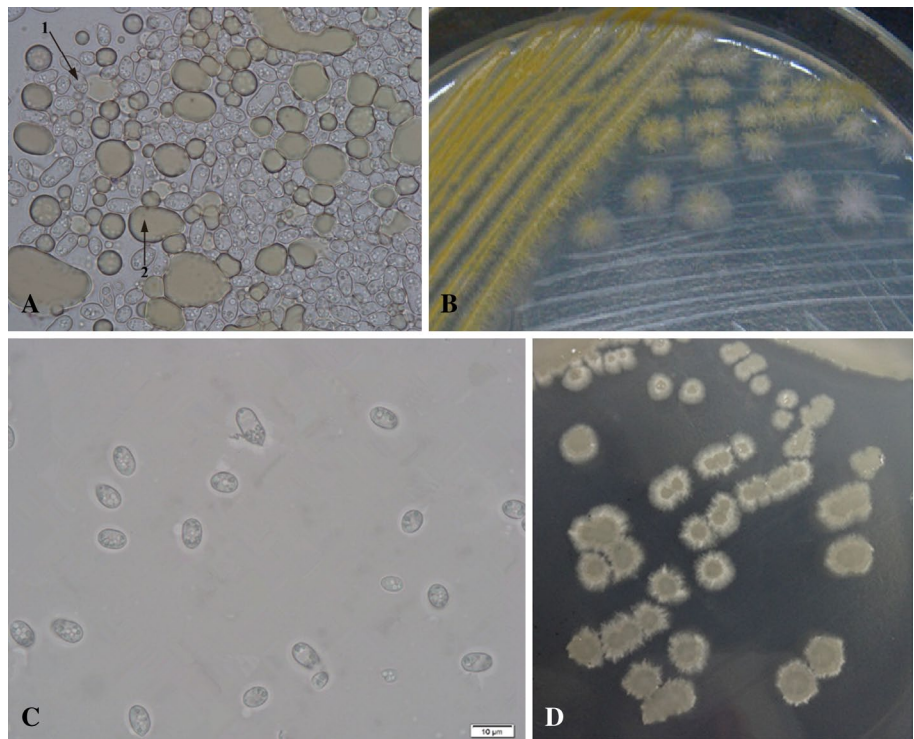

Fig. 3 The phylogenetic tree of the strain P5 and other yeasts and fungal species relatives based on a neighbor-joining analysis of ITS sequences. Bootstrap values (1,000 pseudoreplications) were ≥43 %

(data not shown). Therefore, both the yeast strains P5 and P10 belonged to the same variety of *A. pullulans*. However, it is still unknown why *A. pullulans* var. *melanogenum* strain P5 can secrete a large amount of heavy oil, but

A. pullulans var. *melanogenum* strain P10 can not. It has been reported that *A. pullulans* var. *melanogenum* can produce melanin and the center of its colonies is olive brown to black [29]. However, during the heavy oils production,
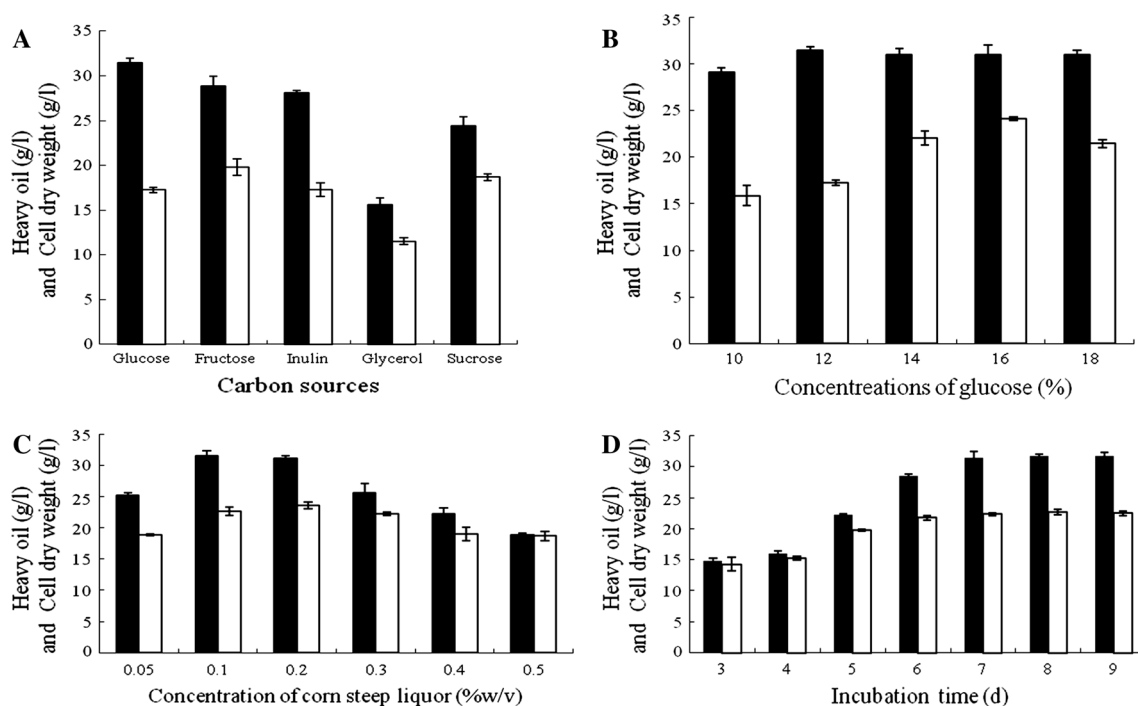
Fig. 4 Effects of different carbon sources (**a**), different concentrations of glucose (**b**), different concentrations of CSL (**c**) and different time (**d**) on heavy oil production (*black*) and cell growth (*white*). Data are given as mean ± SD, $n = 3$

melanin and such morphology of the colonies of the yeast strain P5 were not observed (Fig. 2b). Figure 3 shows that *A. pullulans* var. *melanogenum* was also distributed in the mangrove systems (Fig. 3). It has been evidenced that many strains of *A. pullulans* can synthesize and secrete heavy oil into medium [9, 14, 18], but this is the first report that *A. pullulans* var. *melanogenum* can be a candidate for heavy oil production.

Optimization of the medium for heavy oil biosynthesis and secretion

After effects of different carbon sources on heavy oil production and cell growth by the yeast strain P5 were examined, Fig. 4a indicates that when the heavy oil production medium contained glucose, the secreted heavy oil reached the highest (31.4 g/l) and cell dry weight was 17.3 g/l. After effects of different concentrations of glucose on heavy oil production and cell growth by the yeast strain P5 were tested, Fig. 4b shows that when the oil production medium contained 120.0 g/l of glucose, the secreted heavy oil of the yeast cells was the highest (31.4 g/l) and cell dry weight was 17.3 g/l. After effects of different concentrations of corn steep liquor (CSL) on heavy oil production and cell growth by the yeast strain P5 were examined, the results in Fig. 4c indicated that when the heavy oil production medium contained 0.1 % CSL, the amount of the secreted

heavy oil in the yeast culture reached 31.5 g/l and cell dry weight was 22.7 g/l. It can be seen from data in Fig. 4d that under the optimal conditions, the yeast cell could secrete 31.8 g/l of heavy oil and cell dry weight reached 22.7 g/l within 8 days of the cultivation at flask level. When the medium contained 5.0 % sucrose, oil yields produced by 21 strains of *A. pullulans* ranged from 0.5 to 6 g/l of oil [14]. Many isolated strains of *Aureobasidium* sp. were found to produce extracellular lipids as heavy oils in culture media containing no $CaCO_3$ as a neutralizing agent. The lipids (35 g) were recovered from the culture broth (1 l) of a light-colored mutant of *Aureobasidium* sp. A-2 and proved to be a mixture of fatty acid esters of arabitol and mannitol [9]. CSL has been shown to stimulate pullulans and L-malate production by *A. pullulans* and *Penicillium viticola* [8, 23]. It has been reported that CSL would provide sufficient biotin or at least make quite a significant contribution to biotin requirement [16].

Heavy oil secretion by batch fermentation

To scale up for heavy oil production from glucose, 10-l fermentation was carried out. The results in Fig. 5 revealed that 32.5 g/l of heavy oil, 23.0 g/l of dry cell mass, 0.275 g of oil per gram of consumed sugar and 0.195 g of biomass per gram of consumed sugar were obtained in the culture of the yeast strain P5 after 168 h of the batch fermentation.
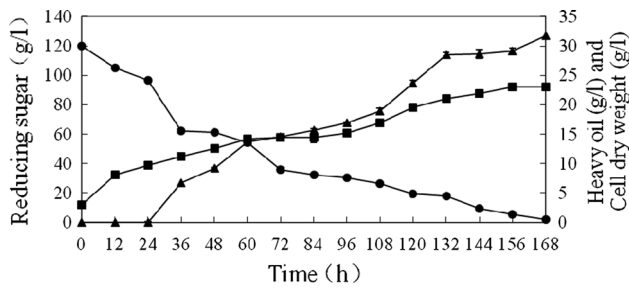
Fig. 5 The time course of heavy oil secretion (*filled triangle*), cell growth (*filled square*) and the change in reducing sugar (*filled circle*) during 10-l fermentation. Data are given as mean ± SD, $n = 3$

At the end of the batch fermentation, only 0.2 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 cell mass, heavy oil and CO_2. This demonstrated that the yeast strain P5 used in this study also could secrete very high concentration of heavy oil into the medium during the 10-l batch fermentation.

The compositions of the heavy oils

After the compositions in the heavy oils were separated on the column in GC machine as described in "Materials and methods", the data from each peak were acquired and processed using the Agilent Masshunter version Nist08 software. The results showed that the percentage of the long-chain *n*-alkanes was 66.15 % while that of the fatty acids was 26.38 % (Fig. 6; Table 1). Two biochemical pathways in microorganisms have been known for the production of straight-chained hydrocarbons. These are the 'elongation–decarboxylation' and the 'head-to-head condensation'

pathways as discussed by Ladygina et al. [11]. The latter pathway seems most likely to be involved in the formation of long-chain hydrocarbons such as tetradecane, heptacosane, hexacosane, octacosane and tetratetracontane by the yeast strain P5 used in this study (Table 1). This pathway would necessarily involve such precursors as acetyl-CoA and malonyl-CoA condensing to increase chain length, and undergoing other reactions such as decarboxylation and decarbonylation, to yield even-numbered hydrocarbons (Table 1). This is the first report that yeast cells can secrete such long-chain *n*-alkanes. It also can be seen from the data in Table 1 that hydroxyl-fatty acids such as 3-hydroxy octanoic acid, 2-hydroxyl-tetradecane (formed after decarboxylation of the hydroxyl-fatty acid) and 8-hydroxyl-pentadecane (formed after decarboxylation of the hydroxyl-fatty acid) were produced by the yeast strain P5. According to the pathway for hydroxyl-fatty acids biosynthesis in oleaginous microorganisms [19, 22], hydroxydecanoic acid may be synthesized and regulated via fungal polyketide synthase (PKS) [13]. However, the two main components of the lipophilic moiety of the heavy oils secreted by *Aureobasidium* sp. A-2 proved to be 3, 5-dihydroxydecanoic and 5-hydroxy-2-decenoic acids [9]. The mannitol oils secreted by *A. pullulans* strain NRRL 50380 are composed of only a single mannitol headgroup partially *O*-acylated with three or four 3,5-dihydroxydecanoic ester groups [18]. The common fatty acids in plasma membrane of yeast cells such as hexadecanoic acid ($C_{16:0}$), octadecanoic acid ($C_{18:0}$) and octadecenoic acid ($C_{18:1}$) also existed in the heavy oils secreted by the yeast strain P5 (Table 1). It has been well known that lipids in most of the yeast cells contain mainly $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ fatty acids [3]. This implied that the heavy oils secreted by the yeast strain P5 also contained the common fatty acids in plasma
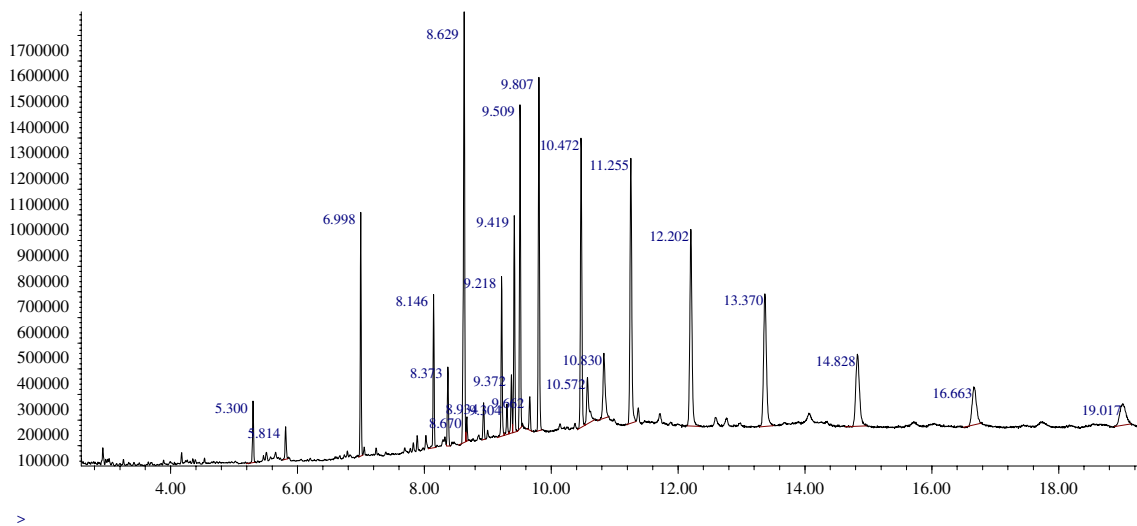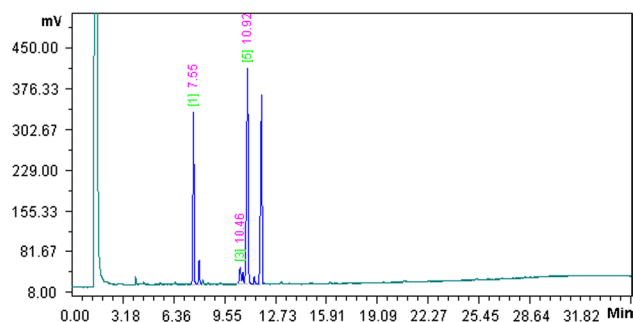

Fig. 6 GC-MS spectrometric analysis of the extracted heavy oils from the yeast strain P5 grown in the heavy oil production medium

Table 1 The compositions of the heavy oils secreted by the yeast strain P5

Time (min)	Relative area	Percentage (%)	Possible compound	Mol. mass (Da)
5.3	2,733,545	1.32	2,4-decadienoic acid	155.21
5.8	1,578,130	0.76	3-hydroxyl-octanoic acid	160.21
6.998	10,177,714	4.93	1-acetyl-2,2-dimethyl-cyclopentane	213.29
8.146	6,809,328	3.3	Hexadecanoic acid	256.42
8.373	3,874,623	1.9	5,6-dihydro-6-pentyl-2H-pyran-2-one	168.23
8.629	23,602,735	11.4	Tetradecane	198.39
8.670	1,043,104	0.51	2,4-bis(1,1-dimethylethyl)-phenol	206.32
8.934	1,734,987	0.84	6-hydroxyl-7,7-dime-thyl-oct-3-enedithioic acid	214.38
9.216	7,650,036	3.71	Tetracosane	338.65
9.304	1,519,055	0.74	Pentadecane	212.42
9.372	2,859,883	1.39	Octadecanoic acid	284.48
9.416	10,790,645	5.23	6-ethoxy-6-methyl-2-cyclohexanone	154.21
9.509	15,352,856	7.44	Octadecenoic acid	282.47
9.662	1,734,131	0.84	Methyl-8-oxooctanoate	172.22
9.807	18,083,380	8.76	Heptacosane	380.73
10.472	17,859,329	8.65	Hexacosane	366.71
11.255	20,230,923	9.8	Octacosane	394.76
12.202	18,745,381	9.1	Octacosane	394.76
13.37	16,212,637	7.9	Octacosane	394.76
14.828	11,370,996	5.51	Octacosane	394.76
16.663	146,046	3.36	Tetratetracontane	619.19
19.017	83,374	2.71	Tetratetracontane	619.19

All the compounds in the table had mass spectra that were most closely matched with the appropriate compound in the Agilent database

membrane of the yeast cells. It has been observed that many yeast cells contain lipid particles (LPs) [27] and the LPs in yeast cells originate from the ER. The enzymes catalyzing the formation of triacylglycerols (TAGs) and steryl esters (SEs) involved in neutral lipid metabolism accumulate in certain regions of the ER. Because newly formed neutral lipids are unable to integrate into bilayer membranes, they cluster and accumulate in the hydrophobic region between the two leaflets of the ER membrane. During ongoing synthesis of TAGs and SEs the droplet grows and forms a bud. After reaching a certain size, the LP buds off the ER and is released into the cytosol [1]. Therefore, the heavy oils synthesized by the yeast strain P5 may cluster and accumulate in the hydrophobic region between the two leaflets of the plasma membrane. During ongoing synthesis of heavy oil, the droplet grew and formed a bud. After reaching a certain

Fig. 7 GC spectrometric analysis of the fatty acid esters from the membranes in the yeast strain P5 grown in the heavy oil production medium

size, the droplet buds off the plasma membrane and was released into the medium (Fig. 2). That was why the heavy oil contained the common fatty acids from plasma membrane (Table 1). However, during the 10-l fermentation, such droplet was not observed. Therefore, it is under investigation how the heavy oils are synthesized and secreted in the yeast cells used in this laboratory.

The compositions of the fatty acids of the whole yeast cells

After the fatty acids in the extracted lipids from the yeast cells were analyzed by Gas Chromatography, the results in Fig. 7 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 ($C_{18:2}$) were 21.2, 2.84, 2.88, 39.8 and 33.3 %, respectively, and the lipids in the yeast cells did not contain any other components of the heavy oils (Table 1). As discussed above, the cellular fatty acids synthesized by yeasts are usually 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 ($C_{18:3}$) [3]. This meant that all the long-chain *n*-alkanes and hydroxyl-fatty acids synthesized by the yeast strain P5 were secreted into medium and the yeast cells only contained the common fatty acids from cellular lipids. Price et al. [18] considered that only after fatty acids were hydroxylated, they could be secreted from the yeast cells.

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