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Full Length Research Paper

Screening of oleaginous yeast with xylose assimilating capacity for lipid and bio-ethanol production

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Microbial oil is a promising new biodiesel resource, which have great potential in industrial-scale production. In our preliminary study, 57 oleaginous yeast with xylose assimilating capacity were isolated from 13 soil samples, 16 strains were identified as potential lipid biomass producer. Four strains which showed higher lipid content were used for further ethanol fermentation at different conditions. Strain 9-44 belonging to *Pichia guillermondii* showed the highest ethanol production (21.91 g/l), and the theoretical ethanol yield was 85.90%. Our study will be of great significance for coupling of lipid and bio-ethanol production, and also provide a choice of cellulocis biomass utilization.

Key words: Microbial oil, oleaginous yeast, *Pichia guillermondii*, bio-ethanol, cellulocis biomass.

INTRODUCTION

A rapid expansion in biodiesel production capacity is being observed not only in developed countries but also in developing countries (Li et al., 2008). Generally, the cost of triacylglycerol such as vegetable oils, animal fats or recycled greases and other oil resources is about 70-75% of the total biodiesel production (Park, 2003). High cost of triacylglycerol lipid feedstock is the major barrier to commercial production of biodiesel (Chen et al., 2009). Developing of cheap triacylglycerol lipid will release the pressure on the ever-increasing prices of vegetable oils in the future. Currently, different cheap lipid feedstocks from oleaginous microorganisms, such as bacteria (Mona et al., 2008), fungi (Du, 2007), yeast (Li et al., 2008) and microalgae (Chisti, 2007, 2008) have being paid more and more attention, which might become one of the potential oil sources for biodiesel production in the future (Ma, 2006; Li et al., 2008). Among all microorganisms, yeast shows advantages in terms of its fast growth rate and high lipid content (Li et al., 2008), which have great potential in industrial-scale production of biodiesel. However, the key technical barrier to this goal is the efficiency of fermentation process and robust organisms. To reduce the cost of microbial oils, exploring other cheap carbon sources (such as lignocellulose hydrolysate) instead of glucose is very important for biodiesel production. Furthermore, oleaginous micro-organisms capable of metabolizing lignocellulose derived sugars could also provide a practical option with higher economic competitiveness (Gerpen, 2004).

Alternatively, the production and utilization of bio-ethanol has also attracted more and more attention as a strategy for reducing greenhouse gas (GHG) emissions and enhancing global energy security. If biodiesel and bioethanol can be produced simultaneously from agricultural and forestry residues, the benefits can be much more significant than singly. In this work, we screened different oleaginous yeasts with xylose assimilating capacity from soils, and then used them for lipid and bio-ethanol production. This study will be of great significance in the

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Abbreviations: GHG, Greenhouse gas; **YPG,** yeast peptone glucose; **YPX,** yeast peptone xylose; **FID,** flame ionization detector; **DNS,** dinitrosalicylic acid; **PCR,** polymerase chain reaction; **YPD,** yeast peptone dextrose.

Table 1. Screening of yeast from 13 different soil samples.

Soil sample number	Source of soil sample	Odour and appearance of enriched culture	Colonial morphology	Yeast
1	Long-term cropped corn land in Douliang village	Light red flocc. suspension	Silkiness, brown or yellow colony	–
2	Plum grove in Douliang village	Yellow turbidness flocc. suspension	Silkiness, brown colony	–
3	Tea grove in Chuanzhu country	Yellow turbidness flocc. suspension	Silkiness, brown , yellow or white colony	–
4	Long-term cropped corn land in Chuanzhu village	Iivory turbidness with strong bouquet	Round glossy white colony	+
5	Long-term cropped rape land in Chuanzhu village	Ivory turbidness with bouquet	Silkiness or round glossy white colony	+
6	Rape land in Tianhui town	Ivory turbidness	Silkiness, white or light yellow colony	–
7	Common Camptotheca Fruit grove in chengdu botanical garden	Ivory flocc. suspension	Silkiness, white or light green colony	–
8	Pinery in chengdu botanical garden	Ivory turbidness	Silkiness, white colony	–
9	Sweet osmanthus grove in chengdu botanical garden	Ivory turbidness with strong bouquet	Round glossy white colony	+
10	Chinese Tupelo grove in chengdu botanical garden	Ivory turbidness with light fruit flavour	Silkiness, white or light yellow colony	–
11	Bamboo grove in chengdu botanical garden	Yellow flocc. suspension with light fruit flavour	Silkiness, white colony	–
12	Begonia grove in chengdu botanical garden	Ivory turbidness with strong bouquet	Silkiness or round glossy white colony	+
13	Pinus massoniana Lamb grove in chengdu botanical garden	Yellow flocc. suspension with light fruit flavour	Silkiness, white colony	–

coupling of lipid and bio-ethanol production, and also provide a choice of cellulocis biomass utilization.

MATERIALS AND METHODS

Soil samples and culture media

Thirteen soil samples were collected from Ermei (Sichuan, China) or Chengdu in July 2009, as summarized in Table 1. All soil sample were collected from underlayer soil without plant residual body, plant root system or soil animals (such as earthworm), and stored in plastic bags at -20 ℃ until further screening. The culture media used in this study is summarized in Table 2.

Screening of oleaginous yeast tolerant heat

One gram of each soil sample was dissloved with 10 ml of sterilized H_2O in a 25 ml disposable plastic centrifuge tube, and then enriched in 50 ml enrichment medium for 72 h at 35℃ with shaking at 180 rpm. The enriched culture was identified firstly by an integrated approach which include odour, appearance and colonial morphology. The enriched culture with bouquet was diluted in a series of 10-fold dilutions in order to achieve a countable number of cells in 0.1 ml. Then 0.1 ml diluted culture was inoculated onto solid isolation medium using spread-plate technique for 72 h at 35℃. A total of 83 yeast strains were isolated from all soil samples (Table 1). The isolated yeast strains were stored at -80℃ and then sub-cultured on yeast peptone dextrose (YPD) agar slopes for further screening with a rapid and sensitive technique.

Secondly, the cells were stained with Sudan Black B so that the absorbance measured at 580 nm gave lipid concentration in the fermentation broth using unstained cells as control (Thakur, 1989).

Assay of xylose-utilization

Xylose plate assay was performed to determine the selected strain that gained xylose assimilating capacity. Five to ten microliter of the cell was spotted on the xylose plate (Table 2), then cultured for 72 h at 35℃ to observe and record the development of colonies.

Further screening of oleaginous yeast

After Sudan III tests and assay of xylose-utilization, further screening of oleaginous yeast was performed to determine which strain showed the highest lipid content. Selected organism was subcultured to fresh YPD (Table 2) for 48 h at 30℃ before being inoculated into the fermentation medium (YPD). After 96 h culture,

Table 2. Medium composition used in this study.

Component	Enrichment medium	Isolation medium	Lipid production medium	Inoculum medium	YPD	Xylose plate	YPG	YPX	YPGX
Glucose (g/l)	50.0	50.0	100.0	40.0	50.0	-	50.0	-	25.0
Xylose (g/l)	-	-	-	-	-	50.0	-	50.0	25.0
Yeast extract (g/l)	5.0	5.0	8.0	15.0	50.0	5.0	50.0	50.0	50.0
Peptone (g/l)	-	-	3.0	5.0	50.0	-	50.0	50.0	50.0
$(NH_4)_2SO_4$ (%)	0.1	0.1	-	-	-	0.1	0.1	0.1	0.1
KH_2PO_4 (%)	0.25	0.25	-	-	-	0.25	0.25	0.25	0.25
K_2HPO_4 (%)	0.05	0.05	-	-	-	0.05	0.05	0.05	0.05
$MgSO_4$ (%)	0.1	0.1	-	-	-	0.1	0.1	0.1	0.1
$Fe_2(SO_4)_3$ (%)	0.01	0.01	-	-	-	0.01	0.01	0.01	0.01
Amp (%)	-	0.003	-	-	-	-	-	-	-
Agar (g/l)	-	20.0	-	-	20.0	20.0	-	-	-
pH value	5.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0

the total lipid and biomass concentration of the selected strains were determined.

Ethanol production by selected oleaginous yeast strain

The first fermentation assay was carried out in 100 ml shaken flasks, containing 50 ml fermentation medium (YPD) and 10.0% (v/v) inoculum were inoculated for 96 h at 35°C with shaking at 180 rpm. The strains that showed higher lipid content and xylose assimilating capacity were selected for ethanol fermentation experiments. Organism was subcultured to fresh YPD for 48 h at 35°C before being inoculated into the fermentation medium. After 96 h culture, ethanol concentration was determined.

The second assay was carried out according to the first assay which yielded higher concentration of ethanol at different conditions. Sets of six 100 ml shaken flasks, containing 50 ml fermentation medium (YPG, YPX and YPGX) and 10.0% (v/v) inoculum were inoculated for 96 h at 35°C with different conditions (Tables 2 and 5). Sample aliquots of 1 ml were taken and centrifuged, which will be used for monitoring biomass concentration and ethanol.

Identification of the selected oleaginous yeast strain

The oleaginous yeast strain was determined by sequencing of D1/D2 domain of 26S rRNA encoding gene (Botes et al., 2006; Lopandic et al., 2006). All DNA manipulations, including plasmid preparation from *Escherichia coli* JM109, ligation, *E. coli* transformation and agarose gel electrophoresis were performed according to standard protocols (Sambrook et al., 1989). Genomic DNA of yeast was extracted from overnight culture according to the manufacturer's instructions (China). Polymerase chain reaction (PCR) were carried out using Ex-Taq polymerase (TaKaRa, Dalian, China), and performed with forward primer 26SD1D2-F (5'-GCAT ATCAATAAGCGGAGGAAAAG-3') and reverse primer 26SD1D2-R (5'-GGTCCGTGTTTCAAGACGG-3') from the genomic DNA of oleaginous yeast as a template. The amplified fragment was cloned into pMD19-T, followed by sequencing (Invitrogen, Shanghai, China) for homology analysis.

Analytical methods

The total lipid concentration of the selected 16 strains was determined by soxhlet extraction method (Dai et al., 2005). Biomass concentration (g/l) was determined by dry cell weight per liter of culture. Reducing sugar was determined by the dinitrosalicylic acid (DNS) method (Miller, 1959). Ethanol was assayed using GC103 with a glass column (0.26 × 200 cm) filled with Porapak Type QS (80 – 100 mesh, Waters, Milford, MA) at 150°C and a flame ionization detector (FID) at 80°C. N_2 is the carrier gas (30 ml/min).

RESULTS AND DISCUSSION

Screening of oleaginous yeast strains

In our preliminary study, 83 yeast strains were isolated from 13 soil samples. Assay of xylose-utilization showed that 57 yeast strains have xylose assimilating capacity (data not shown). By applying Sudan III tests, 16 strains were identified as potential lipid biomass producer and 12 strains have xylose assimilating capacity (Table 3). Although Sudan III tests did not show precise insight on cellular lipid content, it did give, at least partial information on the lipid accumulation ability of the yeast strains tested. In order to screen out the best strain, lipid biomass produce by the 16 selected yeast strains were further measured with soxhlet extraction method (Dai et al., 2005). A strain 5-1 was found to be able to accumulate the highest lipid content (51.43%) (Table 3). However, it could not ferment xylose, which will be limitedly utilized in the future. Another strain, 9-44 is regarded as the best candidate for oil production than the other strain for its high lipid content (45.59%; Table 3), higher biomass (13.6 g/l; Table 3), xylose assimilating capacity and lower reducing sugar which remained the same (2.04

Table 3. Analysis of lipid content, cell biomass and xylose assimilating capacity of different selected strains.

Strain number	Dry cell weight (g)[a]	Weight of lipid (g)	Lipid content (%)	Biomass (g/l)	Xylose-utilization
4-8	0.416	0.037	8.89	16.64	+
4-11	0.2930	0.032	10.92	11.72	+
4-12	0.461	0.098	21.26	18.44	+
4-14	0.273	0.112	41.03	10.92	+
4-17	0.392	0.038	9.69	15.68	+
5-1	0.28	0.144	51.43	11.2	-
9-2	0.298	0.074	24.83	2.96	+
9-8	0.323	0.069	18.55	12.92	+
9-9	0.372	0.08	21.51	14.88	+
9-14	0.262	0.095	36.26	10.48	+
9-24	0.346	0.075	21.68	13.84	+
9-30	0.311	0.11	35.37	12.44	+
9-39	0.314	0.122	38.85	12.56	-
9-44	0.34	0.155	45.59	13.6	+
9-48	0.329	0.15	45.59	13.16	-
12-5	0.676	0.135	20.00	27.04	-

[a] Dry cell weight from 25.0 ml culture.

Table 4. Analysis of ethanol production by selected strains in 96 h culture[a].

Strain	Ethanol concentration (g/l)	Average ethanol concentration (g/l)[b]	Ethanol yield (%)	Reducing sugar remained (g/l)
4-14	12.01 13.49	12.75	50.0	2.51
5-1	10.48 15.96	13.22	51.84	2.44
9-44	22.71 21.10	21.91	85.90	2.04
9-48	14.09 19.74	16.92	66.34	2.56

[a]Fermentation was performed at 35°C with shaking at 180 rpm; [b]results were determined after 96 h fermentation by GC analysis.

g/l, Table 4).

Ethanol production by selected oleaginous yeast strain

After further screening of oleaginous yeast, strain 4-14, 5-1, 9-44 and 9-48 showed 41.03, 51.43, 45.59 and 45.59% lipid content, respectively (Table 3). These strains were used for further ethanol fermentation at different conditions. After 96 h fermentation, the quantity of ethanol obtained from four strains is summarized in Table 4. Strain 9-44 showed the highest ethanol production (21.91 g/l), and the theoretical ethanol yield was 85.90%.

For further study of 9-44, another ethanol fermentation was carried out at different conditions. As shown in Table 5, higher ethanol concentration was obtained from yeast peptone glucose (YPG) and yeast peptone xylose (YPX) medium at shaking or stationary condition after 96 h fermentation as expected. In detail, in the case of glucose as sole carbon source, higher ethanol concentration was obtained when incubated with shaking conditions (18.84 versus 16.97 g/l; Table 5). Lower ethanol concentration was obtained when incubated at both conditions in the case of xylose as sole carbon source, and there was a satisfactory value in comparison with the data from stationary condition (1.20 versus 1.06 g/l; Table 5). However, when cultured with glucose-xylose mixture (YPGX), higher ethanol concentration was obtained from stationary condition than shaking (11.51 versus 8.61 g/l; Table 5).

Previous study has shown that the efficient and

Table 5. Analysis of ethanol production by strain 9-44 at different conditions[a].

Assay	Fermentation medium	Culture condition	Ethanol concentration (g/l)[b]	Ethanol yield (%)
1	YPG	Shaking at 180 rpm	18.84	73.88
2	YPX		1.20	4.70
3	YPGX		8.61	33.76
4	YPG	Stationary	16.97	66.54
5	YPX		1.06	4.16
6	YPGX		11.51	45.14

[a]Fermentation was performed at 35°C; [b]results were determined after 96 h fermentation by GC analysis.

simultaneous conversion of pentoses and hexoses is a significant hurdle to the economic utilization of biomass hydrolysates for the generation of any fermentation product (Eiteman et al., 2008). In our study, lower ethanol concentration was obtained when incubated with YPX or glucose-xylose mixture as carbon source. It indicated that another xylose utilization pathway may exist in this strain. On the other hand, strain 9-44 may have an exceptional capacity for rapid anaerobic growth and fermentation of hexose sugars to carbon dioxide and ethanol, and also exhibits only negligible metabolism of xylose even under aerobic conditions as *Saccharomyces cerevisiae* (Attfield and Bell, 2006). The interest in production of fuel ethanol from renewable plant material, often rich in pentose sugars such as xylose, has encouraged extensive metabolic engineering of *S. cerevisiae* for xylose metabolism. Different recombinant xylose-utilising *S. cerevisiae* strains have been constructed by introducing xylose reductase and xylitol dehydrogenase encoding genes from the xylose-fermenting yeast, *Pichia stipitis* and additionally, overexpressing the endogenous xylulokinase encoding gene (*XKS1*) (Salusjärvi et al., 2008; He et al., 2009). Strain 9-44 may also be regarded as gene source of xylose-utilising pathway for constructing xylose-fermenting yeast. Furthermore, biodiesel is an ideal substitute of the conventional vehicles diesel for its biodegradability, non-toxicity and typically produces about 60% less net carbon dioxide emissions than petroleum-based diesel. Currently, different cheap lipid feedstocks from oleaginous micro-organisms have been givenmore attention due to their advantages (such as renewability, fast growth rate, and not taking arable land) (Ma, 2006; Li et al., 2008). Lignocellulose, the most abundant renewable biomass produced from photosynthesis, has a yearly supply of approximately 200 billion metric tons worldwide (Ragauskas, 2006). It contains sugars that are polymerized to cellulose and hemicellulose and they can be liberated by hydrolysis and subsequently converted to some valuable by-products. Also, lipid and ethanol are still undergoing production simultaneously with 9-44 strain from cellulocic biomass (such as corn stover and foresty residues).

Identification of 9-44 yeast strain

Primers used for the amplification of the D1/D2 fragment yielded a fragment of about 615 bp for the 9-44 strain. The sequence obtained was compared with those available in the GenBank, and the results showed 100.0% of D1/D2 sequence similarity with the type strain of *Pichia guillermondii* L3.1 (GenBank: EU833236.1, URL: http://www.ncbi.nlm.nih.gov/nuccore/194368379). Therefore, 9-44 strain belonged to *P. guillermondii.* Thus, in our study, 57 oleaginous yeast with xylose assimilating capacity were isolated from 13 soil samples; 16 strains were identified as potential lipid biomass producer. Four strains which showed higher lipid content were used for further ethanol fermentation. A strain (9-44) belonged to *P. guillermondii* which showed the highest ethanol production, which would be used for further study.

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