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Isolation of the Oleaginous Yeasts from the Soil and Studies of Their Lipid-Producing Capacities

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Summary

D-xylose is one of the most abundant pentose sugars in nature. To isolate oleaginous yeasts that can utilize xylose from diverse soil samples, soils from a litchi orchard, longan orchard, carambola orchard, and woods were collected, yeasts were isolated by the glycerol enrichment and their xylose-assimilating capacities were measured. A subset of these isolates was grown in nitrogen-limited media and then screened for potential oleaginous yeasts by the Sudan Black B staining, after which their lipid-producing capacities were studied. There were 13 strains of oleaginous yeasts identified, and a rapid microbiological assay was provided to exploit microbial lipids that may one day be used as biodiesels or cocoa butter substitute.

Key words: isolation and identification of oleaginous yeast, xylose, glycerol, biodiesel, cocoa butter substitute

Introduction

Because of the environmental problems and limited petroleum reserve on Earth, it is more and more difficult to exploit fossil fuel resources. In this century, these problems have become more severe, and researchers are called to search for the renewable biofuels.

Biodiesel is a useful alternative energy resource and may be used as a substitute for petroleum-based diesel. The conventional method to produce biodiesel is to transesterify plant oils with methanol (*1,2*). However, the cost of biodiesel produced with this method is currently more expensive than that of conventional diesel due to high cost (70–85 %) of the raw material. Increasing interest is generated to explore ways to reduce the high cost of biodiesel, especially the cost of the raw materials (*3*). With the increasing focus on regenerative energy sources, researchers turn their attention to the cheap carbohydrate source, lignocellulosic materials (*4*).

Lignocellulosics are widely distributed in nature. They contain sugars that are polymerized to cellulose and hemicellulose, and need to be liberated by gasification or hydrolysis for subsequent conversion to biofuels. A great amount of these materials are burned, causing environmental problems. If biodiesel could be produced from lignocellulosic materials, both environment and economy would benefit.

One way of exploiting natural carbohydrates as energy sources is to use carbohydrate-based microbial oils, because they consist of fatty acids similar to those of the plant oils (*5*). There is a small number of microorganisms in nature that can convert carbohydrates into oils and store these energy sources under appropriate conditions. Lipid-producing (oleaginous) microorganisms have been known for many years, and their potential as alternative sources of plant oils has been periodically assessed (*6,7*). The majority of those lipids in these organisms are triacylglycerols (TAG), containing long-chain

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fatty acids that are comparable to conventional plant oils (*8*). It would be important to develop new oil resources by using microbes, which offers many advantages compared with traditional methods using animal fat and plant oils. A large amount of raw materials and high-value products can be produced in relatively short time at low cost and without the seasonal weather effects. This approach may offer an alternative way to obtain biofuels (*9*).

Recent reports in the international publications indicate problems concerning cocoa butter production due to the failure of cocoa plant protection measures against harmful insects and infections. This problem can result in a significant increase in the prices of cocoa butter. However, the oleaginous microorganisms have noticeable application in production of lipids which can be used as cocoa butter substitute. Some oleaginous yeasts show an unusual capacity to synthesize interesting lipid profiles with high percentages of stearic acid and non-negligible percentages of palmitic and oleic acid, with a composition resembling that of cocoa butter (*10,11*).

Xylose and glucose are the main products of the hydrolysis of lignocellulosic materials. These carbohydrates can be used by the oleaginous yeasts isolated from the soil or other natural sources to produce lipid oils. In this paper, the isolation of oleaginous yeasts utilizing xylose from the soil is reported using the glycerol enrichment approach. Also, the lipid-producing capability of oleaginous yeasts is investigated.

Materials and Methods

Soil sample collection

Ten soil samples were obtained for the isolation of yeasts. Bulk samples (5 kg) were collected from the upper 5–20 cm of a litchi orchard, longan orchard, carambola orchard, and the woods in Nanning, Guangxi Chuang Municipality, People's Republic of China, where the climate is semi-tropical.

Reagents

Taq polymerase was obtained from Takara (Shiga, Japan). EZNA Plasmid MiniprepI and a Gel Extraction Kit were obtained from Omega Bio-Tek (Doraville, GA, USA). All other reference substances and chemicals were purchased from Generay Biotech Co., Ltd. (Shanghai, PR China), and were of analytical grade unless otherwise specified.

Yeast growth from soils

A mass of 1 g of soil was added into 50 mL of glycerol-enriched medium containing (in g/L): glycerol 100, $(NH_4)_2SO_4$ 1, KH_2PO_4 1, $MgSO_4{\cdot}7H_2O$ 0.5, and yeast extract 0.2 in a 250-mL Erlenmeyer flask, and incubated in an incubator shaker at 28 °C for 24 h with shaking at 180 rpm, so that the targeted yeasts from the soil would be enriched to a greater number.

Yeast isolation

A volume of 1 mL of the above pre-cultured yeasts was added to 9 mL of distilled water and 10-fold serial dilutions were made. Portions of 0.1 mL from each dilution ranging from 10^{-1} to 10^{-5} were spread onto plates made with 2 % xylose, 0.5 % $(NH_4)_2SO_4$, 0.1 % KH_2PO_4, 0.05 % $MgSO_4{\cdot}7H_2O$, 0.05 % yeast extract, 2 % agar, 20 mL of 2 % fresh sterilized sodium deoxycholate ($C_{24}H_{39}O_4Na$) and 3.3 mL of streptomycin solution (10 000 U/mL). The plates were incubated at 28 °C for 2 to 4 days, and those containing isolated colonies with the morphology typical of yeasts were used for further study.

Screening for oleaginous yeasts

The isolated colonies of yeasts were further screened for their lipid-producing abilities by qualitative analysis with the Sudan Black B staining technique (*12*). The potential oleaginous yeast colonies were maintained on YEPD slant containing (in g/L): glucose 20, yeast extract 20, and peptone 20 at 4 °C and transferred once every 2 months. Stock cultures were incubated for 2 days, and then stored in a refrigerator before use.

Yeast inoculation and flask culture

The oleaginous yeast colonies were initially streaked onto YEPD slant and grown for 2 days. After that they were transferred to 250-mL Erlenmeyer flasks containing 50 mL of inoculation medium containing (in g/L): xylose 20, $(NH_4)_2SO_4$ 5, KH_2PO_4 1, $MgSO_4{\cdot}7H_2O$ 0.5, and yeast extract 0.5 and grown at 28 °C on a rotary shaker at 180 rpm for 2 days. In flask cultures, 5 mL of these cultures were transferred to 45 mL of nitrogen-limited fermentation medium containing (in g/L): xylose 40, $(NH_4)_2SO_4$ 2, KH_2PO_4 7, NaH_2PO_4 2, $MgSO_4{\cdot}7H_2O$ 1.5 and yeast extract 1 in 250-mL Erlenmeyer flask, and incubated in a rotary shaker at 180 rpm and 28 °C for 5 days. Duplicate samples were analyzed for dry mass, percentages of lipids, residual xylose and nitrogen amount.

Determination of yeast dry mass

Portions of 2-mL cultures were harvested by centrifugation at 5000×*g* for 5 min. The supernatants were analyzed for xylose and ammonium concentrations as described below. Harvested biomass was washed twice with 5 mL of distilled water and then dried at 60 °C to constant mass (usually after 24 h). The biomass was determined gravimetrically.

Determination of lipid content

To determine the lipid content in yeast cells, lipids were extracted, dried and weighed, based on the method of Bligh and Dyer (*13*) with modifications. This is a fast procedure allowing complete lipid extraction. Briefly, a 50-mL sample was centrifuged at 5000×*g* for 5 min, after which the yeast was washed twice with 50 mL of distilled water, then added into 10 mL of 4 M HCl, and incubated at 60 °C for 1 to 2 h. Then the acid-hydrolyzed mass was stirred with 20 mL of chloroform/methanol mixture (1:1) at room temperature for 2 to 3 h, followed by centrifugation at 2000×*g* for 5 min at room temperature to separate the aqueous upper phase and organic lower phases. After that the lower phase containing lipids was recovered with a Pasteur pipette, and evaporated under reduced pressure for 10 min. The dry lipids were weighed.

Determination of xylose and ammonium concentrations

The xylose concentration in the culture medium was estimated with the dinitrosalicylic acid (DNS) method. The ammonium concentration in the culture medium was estimated by the method of Chaney and Marbach (*14*).

Molecular analyses of the 26S D1/D2 rDNA

The genome DNA of the strains for the PCR was gained by using the phenol/chloroform method. The sequences of primes for 26S D1/D2 rDNA were 5′-GCATATCAAAAGCGGAGGAAAAG-3′and 5′-GGTCCGTGTTTCAAGACGG-3′. The PCR product was ligated with the vector of pMD18-T and sequenced by Generay Biotech Co., Ltd. (Shanghai, PR China). Comparisons of sequences with those in the databases were made with BlastN (BLAST, basic local alignment search tool) at NCBI.

Statistical analysis

Experiments were performed in triplicates and the data were analyzed using one way analysis of variance (ANOVA). Differences with $p<0.05$ were considered statistically significant.

Results

The isolation of yeast colonies from soil samples

In this preliminary study, 40 colonies with the morphology typical of yeast were isolated from ten soil samples that can utilize xylose or glycerol as the sole carbon source.

Screening and characterization of oleaginous yeast colonies

The lipid accumulation process requires the exhaustion of a nutrient, usually nitrogen, to allow excess carbon to be incorporated into lipids. With the nitrogen-limited growth, Sudan Black B staining identified 20 colonies as potential lipid biomass producers (Table 1). Although this technique did not allow precise insight into cellular lipid content, it did give, at least partially, information on the lipid accumulation ability of the tested yeast colonies.

These colonies were then further characterized for a series of parameters as listed in Table 2 including their biomass, lipid yield, lipid content and lipid coefficient. The ammonium concentration was close to zero (data ellipsis).

Table 1. Qualitative analysis by Sudan Black B staining

Colony serial number	Fat droplet number	Colony serial number	Fat droplet number
H2-1	+ + +	J2-3	++ + + + +
H2-2	+ +	J2-4	+ + + + +
H2-3	+ +	J3-1	+ + + + +
H2-4	+ +	J3-2	+ + + + +
H3-1	+	J3-3	+ + + + +
H3-2	+	Q2-1	+ + + + +
H3-3	+	Q2-2	++
H3-4	+ +	Q2-3	+ + + + +
J2-1	+	Q2-4	++
J2-2	+	J3-4	+ + + + +

Cells containing different amounts of fat droplets were indicated by + signs: + for one, + + for two, + + + for three or more, + + + + + for many fat droplets in pseudohyphae

Table 2. Characterization of 20 oleaginous yeast colonies

Colony serial number	γ(biomass)	γ(lipid yield)	w(lipid)	Lipid coefficient	γ(xylose utilized)
	g/L	g/L	%	g lipid/100 g xylose	g/L
H2-1	12.5	4.867	38.94	14.56	33.43
H2-2	10.5	2.490	23.71	6.45	38.60
H2-3	9.5	2.020	21.26	6.80	29.71
H2-4	14.00	3.060	21.86	8.46	36.17
H3-1	18.50	3.420	18.49	9.29	36.81
H3-2	8.10	1.994	24.62	7.39	26.98
H3-3	7.00	2.240	32.00	6.34	35.33
H3-4	8.50	3.070	36.12	7.78	39.46
J2-1	10.50	2.670	25.43	6.70	39.85
J2-2	16.32	3.788	23.21	10.73	35.30
J2-3	5.60	0.970	17.32	12.13	8.00
J2-4	6.50	1.950	30.00	4.88	39.96
J3-1	20.50	5.100	24.88	13.00	39.23
J3-2	20.90	4.900	23.44	12.75	38.43
J3-3	10.40	2.548	24.50	8.37	30.44
J3-4	4.90	0.960	19.59	4.42	21.72
Q2-1	22.30	5.680	25.47	15.38	36.93
Q2-2	21.50	4.490	20.88	12.13	37.02
Q2-3	18.50	3.660	19.78	9.57	38.24
Q2-4	14.80	4.780	32.30	11.98	39.90

Identifying oleaginous yeast colonies

By sequencing their 26S rDNA, 20 colonies were identified as 13 different strains. Results from the sequencing, as listed in Table 3, confirm their genetic differences, thus supporting their identification. Because Q2-4 could not be identified, it was named *Rhodotorula* sp., which might be a new strain.

The ability of several typical oleaginous yeasts to assimilate glycerol and xylose

Oleaginous yeasts are organisms capable of synthesizing and accumulating oil, wherein oil accumulation ranges from at least about 20 up to about 80 % of the cellular dry mass. Yeast genera that have been identified as oleaginous yeasts, but are not limited to only this property are: *Yarrowia, Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon* and *Lipomyces.* More specifically, illustrative oil-synthesizing yeasts include: *Rhodosporidium toruloides, Lipomyces starkey, L. lipoferus, Candida revkaufi, C. pulcherrima, C. tropicalis, C. utilis, Rhodotorula minuta, Trichosporon pullans, T. cutaneum, Cryptococcus curvatus, Rhodotorula glutinis, R. graminis and Yarrowia lipolytica* (formerly classified as *Candida lipolytica*).

The ability of these typical oleaginous yeasts to assimilate glycerol and xylose was also studied, as listed in Table 4, which shows that most oleaginous yeasts can assimilate glycerol and xylose. Consequently, glycerol enrichment could effectively isolate oleaginous yeasts from the samples. *Yarrowia lipolytica* yeast could not be obtained for this study by our isolation method, because it does not utilize xylose.

Discussion

The basic physiology of lipid accumulation in microorganisms has been well studied. It is known that lipid production requires a medium with an excess of sugars or similar components such as glycerol and polysaccharides, but with little other nutrients, usually nitrogen (*15*). When nitrogen is low in the medium, the activity of nicotinamide adenine dinucleotide isocitrate dehydrogenase (NAD-IDH) decreases or even disappears from the mitochondria of the oleaginous yeasts. Then tricarboxylic acid cycle (TCA) is repressed, metabolism pathway altered, protein synthesis stopped and lipid accumulation activated (*16–18*). Cupp *et al.* (*19,20*) discovered that yeast strains lacking the activity of NAD-IDH could not utilize acetic acid as a carbon source, but utilized glycerol or lactic acid. Glycerol as a lipid analogue could play a positive role in the lipid accumulation. Papanikolaou *et al.* (*21*) reported that the increase of glycerol concentration in the growth medium somehow increased the cellular unsaturated fatty acid content of the lipids. The utilization of technical glycerol and stearin as co-substrates resulted in higher lipid synthesis and increased

Table 3. Result of the identification of 20 oleaginous yeast colonies

Colony serial number	Name	Colony serial number	Name
H2-1	*Rhodosporidium toruloides*	J2-2	*Lipomyces starkey*
		J2-3	*Trichosporon cutaneum*
H2-2	*Rhodotorula glutinis*	J2-4	*Candida pulcherrima*
H2-3	*Rhodotorula graminis*	J3-1	*Candida tropicalis*
H2-4	*Rhodotorula minuta*	J3-2	*Candida utilis*
		J3-3	*Candida utilis*
H3-1	*Rhodosporidium toruloides*	J3-4	*Trichosporon cutaneum*
H3-2	*Rhodotorula minuta*	Q2-1	*Cryptococcus curvatus*
H3-3	*Rhodotorula glutinis*	Q2-2	*Lipomyces starkey*
H3-4	*Rhodotorula mucilaginosa*	Q2-3	*Rhodosporidium toruloides*
J2-1	*Lipomyces lipoferus*	Q2-4	*Rhodotorula* sp.

Table 4. The ability of typical oleaginous yeast strains to assimilate xylose and glycerol

Oleaginous yeast	Xylose	Glycerol	Oleaginous yeast	Xylose	Glycerol
Rhodosporidium toruloides	+	+	*Trichosporon pullans*	u.	u.
Lipomyces starkey	+	+	*T. cutaneum*	+	+
L. lipoferus	u.	u.	*Cryptococcus*	+	+
Candida revkaufi	u.	u.	*curvatus*		
C. pulcherrima	+	+	*Rhodotorula glutinis*	+	+
C. tropicalis	+	+	*R. graminis*	+	+
C. utilis	+	+	*Yarrowia lipolytica*	–	+
Rhodotorula minuta	+	+			

Yeast culture media were collected and the xylose and glycerol were detected as described in Materials and Methods. + indicates the positive results, – indicates the negative results, u. indicates unknown results due to the unavailability of the yeast strain

citric acid production than the combination of glucose and stearin. Briefly, this suggested that glycerol as a sole carbon source in the growth medium may have positive effect on the enrichment of oleaginous yeasts.

Oleaginous yeasts are often considered for the production of single cell oil (SCO). The economics of these bioprocesses has become more favourable when zero or negative value waste substrates are utilized as carbon or nitrogen sources (*11*,*22*–*24*). As a primary component of hydrolysis of lignocellulosic materials, xylose utilization has become a key problem in the production of single cell oil. Oleaginous yeasts have bright prospects in industrial applications since they can utilize xylose and glucose at the same time.

After 5 days of incubation, the analysis of residual xylose is performed. The result shows that xylose is not completely utilized by the yeasts. Before the depletion of the carbon source in the culture medium, oleaginous microorganisms are not able to consume their own lipid reserves (*25*–*27*). In this case, the accumulated lipids are not used for lipid-free biomass synthesis.

Lipid coefficient is expressed as the amount of lipid produced in each 100 g of xylose in the substrate consumed. The higher the lipid coefficient, the higher the efficiency of substrate utilization. Li *et al*. (*28*) investigated the lipid coefficients of 10 oleaginous yeasts utilizing xylose and showed that *Rhodosporidium toruloides* AS 2.1389 had the maximum conversion rate of 10.6 g of lipid per 100 g of consumed xylose. In this study, 8 strains of oleaginous yeasts were obtained whose lipid coefficients were more than 10. Lipid coefficients of these oleaginous yeasts were higher than those of previously reported strains (*28*,*29*).

In order to further increase the lipid production from xylose, we believe that future research efforts should be focused on the following three aspects: (*i*) fermentation conditions for lipid production by oleaginous yeasts should be optimized, (*ii*) oleaginous yeasts should be subjected to mutagenesis to select better strains with the highest lipid productions, and (*iii*) xylose assimilation should be further improved by the genetic engineering methods.

Our results suggest that components from the lignocellulosic biomass may be utilized to produce microbial oil, which could be an alternative biofuel and a promising energy source. The 13 yeast strains we isolated have the potential to be used for the industrial production of biofuels or cocoa butter substitute, because they assimilate a wide range of sugars with xylose as the main component.

Conclusion

In this study, a simple method was used to isolate oleaginous yeasts utilizing xylose from the soil. There were 13 strains identified and it was shown that several strains produced lipids in high yields when fed with carbohydrates. This is an efficient way of producing lipids from carbohydrate. Therefore, potentially it could be used to produce a large amount of lipids for biofuels or cocoa butter substitute.

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