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Screening of Yeast Strains in producing biodiesel

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Abstract. Microbial oil production has many advantages such as rapid proliferation of microbial cells and short production cycle. The raw materials needed for growth are abundant and inexpensive such as starch, sugar, especially the waste which can be utilized in food or paper industry. 170 oil-producing strains were screened by Sudan black staining observation using fat grains as screening index, among which, 6 high lipid yield yeast strains were identified by residual sugar as indirect index. By comparing the oil-producing ability of this strain, it was determined that the first strain was a good strain for oil production by fermentation. The biomass, oil content and oil yield of the strain were 11.58 g/L, 36.68%, 4.25 g/L, respectively. Through analysis on 26S rDNA sequence and observation on morphological characteristics, culture characteristics and physiological and biochemical characteristics, this strain belonged to *Cryptococcus* spp.

1. Introduction

Oil is not only one of the basic substances that make up and maintain life, but also an important industrial raw material. It has wide application value. Traditional oil resources mainly come from animals and plants, while energy oil mainly comes from mineral resources. Population growth and social progress have intensified the contradiction between oil demand and serious shortage of natural resources. In addition, environmental pollution has become more and more serious, forcing us to seek to develop new oil resources and clean energy sources. With the rapid development of biotechnology, the research on microbial lipids has been deepening. Hence, oil production by microbial fermentation provides a new way for the development of oil resources [1].

At present, the research of microbial grease mainly focuses on two aspects: microbial production of special functional grease and microbial development of biodiesel. Microbial oil production not only has the advantages of high oil content, abundant source of raw materials, low production cost and short production cycle, but also can use cell fusion, cell mutagenesis and other methods to make microorganisms produce high nutritive oil or special oil composed of some specific fatty acids, such as cocoa butter, cocoa butter and so on [2]. Moreover, microbial cells proliferate rapidly, the raw materials needed for the growth of microorganisms with short production cycle are abundant and the price is cheap. For example, starch, sugar, especially the waste of food industry and paper industry can be utilized. At the same time, they can be continuously produced on a large scale without seasonal and climate change restrictions. The production cost is low, and high-tech methods such as cell fusion and cell mutagenesis can be used to make microorganisms produce. Highly nutritious oils or oils that are better suited to people's needs than animal or vegetable oils [3]. Therefore, development and utilization of microbial oils can not only alleviate the shortage of vegetable oils, but also produce functional oils. In addition,



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microbial oils can be used to produce biodiesel instead of vegetable oils to alleviate the global energy crisis. Nowadays, many kinds of microbial lipids have been studied and some progress has been made. However, further research is still needed in the aspects of strain selection, extraction technology and large-scale industrial production [4-6]. Therefore, screening strains with high oil production has far-reaching significance for accelerating the development of microbial oil.

2. Material and Methods

2.1. Experimental materials

2.1.1. Soil sample. Soil samples were taken from alpine regions and orchards cultivated for many years.

2.1.2. Culture medium. Enrichment medium contains 50 g/L glucose, 1 g/L urea, 1 g/L ammonium sulphate, 2.5 g/L KH_2PO_4 , 0.5 g/L Na_2HPO_4 , 1 g/L MgSO_4 , 0.1 g/L FeSO_4 , 0.5 g/L yeast extract, 0.03 g/L Bengal red. Isolation medium contains 50 g/L glucose, 1 g/L urea, 1 g/L ammonium sulphate, 2.5 g/L KH_2PO_4 , 0.5 g/L Na_2HPO_4 , 1 g/L MgSO_4 , 0.1 g/L FeSO_4 , 0.5 g/L yeast extract, 0.03 g/L penicillin, 0.03 g/L streptomycin, 0.03 g/L tetracycline, 20 g/L agar.

2.2. Experimental methods

1 g separation material was shaken in sterile water. The suspension was obtained by oscillation, and the supernatant was absorbed into the enriched medium. The suspension was cultured by 180 rpm for 3 days. The enriched medium was diluted appropriately, and then coated with separation plate, incubated at constant temperature for several days. The suspected yeast colonies were observed and stained with Sudan black, and the colonies with obvious intracellular fat grains were picked out and moved to the inclined plane. 30 ml shake flask fermentation medium was packed in 250 mL triangle flask, one by one flask was connected with a ring of inclined bacteria, and then cultured at 4°C and 180 rpm for 4 days. The formation of intracellular fat grains was observed by dyeing and the residual sugar in fermentation broth was determined. The strains with low residual sugar and large and large intracellular fat grains were selected for re-screening.

3. Result and Discussion

3.1. Screening of Yeast Strains with High Oil Yield

A total of 170 oil-producing yeast strains were isolated and screened from the collected soil samples by plate separation and Sudan black staining (Figure 1). The results of plate preliminary screening are shown in the Table 1.

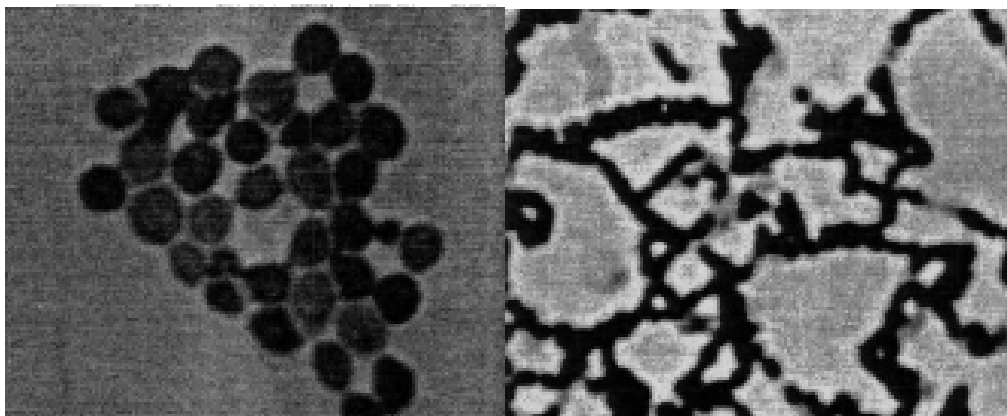


Figure 1. Results of colouring by sudan black B

Table 1. Results of plate screening.

Number	Source material	Number of yeast strains
1	Low Temperature Vineyard	7
2	Cold Pasture 1	23
3	Vineyards in High Temperature Zone	0
4	Cold Pasture 2	2
5	Low temperature forest	2
6	Cold Pasture 3	29
7	Peach Grove	18
8	Apricot forest	6
9	Apple Grove	17
10	Vegetable shed	23
11	Vegetable garden	7
12	Cultivated land	3
13	Hibiscus woods	7
14	Peach Grove	4
15	Pear orchard	3
16	Pear orchard	0
17	Potato Garden	4
18	Terek bostan	4
19	Apple orchard	4
20	Soil and water samples	7
Total	/	170

The yeast strains obtained by plate screening were screened one after another for shaking flask fermentation. At the end of fermentation, smears were used to observe the formation of intracellular fat granules and analyze the consumption of glucose in fermentation broth. After two rounds of screening, 10 strains with high oil production were obtained. The results are shown in Table 2.

Table 2. Preliminary fermentation screening results of high-yielding lipid strains.

Number	Fat granule staining	Remnant (g/L)
6-1	+++	0
6-5	++	0
6-6	+++	0
6-7	+++	0
6-14	+++	10
6-15	+++	0
6-18	++++	7.85
6-21	+++	0
7-1	++	0
9-5	+++	0

Note: "-" means that half of the pigments are stained, but it is uncertain whether all pigments are in the cell or not; "+++" means basically all coloring, but it is not sure whether it is in the cell." It means that fat grains can be observed clearly; "++++" indicates fat particles could be observed more clearly.

The above 10 strains of high yield strains were screened by shaking flask fermentation. After fermentation, centrifuge was used to collect the bacteria. After drying, Soxhlet extraction method was used to determine the fat content and calculate the oil yield. Results are shown in Table 3. The results showed that 6 strains of bacteria whose oil content exceeded the dry weight of their own cells could be defined as oil-producing microorganisms. Strain 18 has the highest oil content and rich aroma, which may contain rare fatty acid components, and has the value of further research. Therefore, it is necessary to identify the strain further.

Table 3. Secondary fermentation screening results of high-yielding lipid strains.

Number	Remnant (g/L)	Biomass (g/L)	fat content (%)	Oil yield (%)
6-5	0	12.92	22.85	2.96
6-7	8.09	11.73	23.49	2.76
6-15	0	13.47	32.98	4.44
6-18	8.33	11.58	36.68	4.25
7-1	0	12.54	31.59	3.96
9-5	0	12.83	29.5	3.78

3.2. Identification of Strain 6-18

3.2.1. Morphological observation. After the first day of culture on the isolation medium, the bacterial colonies on the plate were smaller. After the culture day, the bacterial colonies were larger, round, protuberant, neat edge, slightly yellow, smooth, opaque and lustrous (Figure 2).

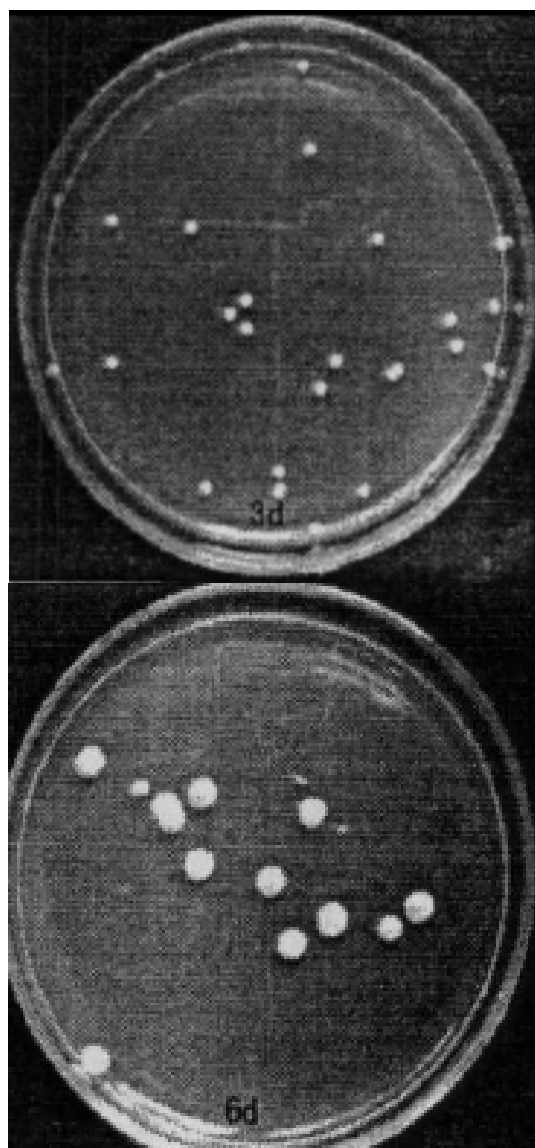


Figure 2. Colony morphology of strain 6-18 on isolation plate.

Strain 6-18 was cultured on isolation medium for 3 days, and the colony on the plate was smaller. After 6 days, the colony was larger, round, protuberant, neat edge, slightly yellow, smooth, opaque and lustrous.

3.2.2. Physiological and Biochemical Characteristics. The strain was positive in starch formation test, did not ferment glucose and maltose, and could assimilate inositol. Referring to Du Lianxiang and Wei Jingchao in Fungi Identification Manual, it was classified as *Cryptococcus* by its characteristics.

Table 4. Physiology and biochemistry characteristics of isolated strain.

Test item	Results	
	Strain 6-18	<i>C. aerius</i>
Carbohydrate fermentation	Glucose	-
	Malt dust	-
	fructose	-
	lactose	-
	Fiber two pond	-
	Melezitose	-
	Soluble starch	-
Carbon assimilation	lactose	++
	Sucrose	++
	Cellose	+++
	D-sorbitol	++
nitrogen assimilation	Ammonium sulphate	+
	potassium nitrate	+
	ammonium nitrate	+
	Sodium nitrate	+
High temperature resistance	10% glucose	+
	20% glucose	+
	30% glucose	+
Biochemical test	Starch-like production	+
	Acid production	-
	Ester production	+
	Urea decomposition	+
	Vitamin-free culture	-
	Reproduction mode	Multipolar budding
	Invisible pseudohyphae	-
	ascospores	-

Note: "+" means that it can ferment or grow or decompose; "-" means that it can't ferment or produce or decompose; "++" and "+++" means that it has better growth and good growth.

3.2.3. 26S sequence analysis and phylogenetic tree construction. The results showed that the sequence of the strain was 5558 bp in 26 S rDNA. By comparing with the middle sequence, the strain was in highest homology with *Cryptococcus aerius* FK5, *C. aerius* CBS4192, *C. aerius* IGC5259. Therefore, strain 6-18 could be identified as *Cryptococcus*.

Oil-producing strains can be quickly selected from the separated samples by Sudanese black fat granule staining, which reduces the workload of strain screening. However, from the results of plate screening and shaking flask screening, although the size and color of fat grains after Sudan black staining have a certain relationship with the amount of oil produced by the strain, they are not proportional. Because Sudan black dyeing is affected by test operation, systematic error is unavoidable. Sudan black dyeing can not truly and accurately reflect the oil production capacity of strains. Sudan black fat granule staining method is suitable for qualitative judgment of oil-producing strains, but it can not be used as a direct judgment criterion of oil-producing ability of strains.

Breeding low-temperature growth strains, the selected plate temperature culture, observation and selection of new doubts. Yeast-like colonies were picked out and the colonies with obvious fat granules

were moved to the inclined plane. Temperature can regulate the fatty acid composition of microbial lipids, which is caused by an adaptive response of cells to changes in external temperature. Usually, the melting point of unsaturated fatty acids is lower than that of saturated fatty acids, and the short-chain fatty acids are lower than that of long-chain fatty acids. Cell membrane is an important surface structure of microbial cells. It is composed of lipids and proteins. The higher the content of unsaturated fatty acids in lipid molecules, the greater the fluidity of cell membrane under low temperature, that is, the lower the temperature of microbial growth. Therefore, when strains grow from high temperature to low temperature, the contents of unsaturated fatty acids and short-chain fatty acids in cell membranes increase, mainly palm oleic acid or oleic acid, while the average chain length increases with the increase of temperature. These changes are designed to ensure the normal fluidity and permeability of cell membranes.

The accumulation of microbial lipids increases with the growth of bacteria. Both the growth of bacteria and the accumulation of oil need to consume glucose, so the content of reducing sugar in fermentation broth can indirectly react with the amount of oil during fermentation. The less the amount of reducing sugar, and Sudan black stained fat grains are obvious, which indicates that the strain has a higher ability to produce oil. The method of Sudan black staining and reducing sugar determination can objectively reflect the oil-producing ability of the strain. This method can significantly improve the screening efficiency of strains and greatly reduce the workload.

4. Conclusion

170 oil-producing strains were screened by Sudan black staining. Six oil-producing yeast strains were screened with residual sugar as indirect index and fat granule observation. By comparing the oil-producing ability of these six strains, it was determined that strain 6-18 was an excellent strain for oil production by fermentation. The strain 6-18 was identified as *Cryptococcus* by 26S sequence analysis combined with morphological characteristics, culture characteristics and physiological and biochemical characteristics.

Acknowledgments

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