Isolation and Characterization of Lipid-Producing *Bacillus* sp. TC14

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**Abstract.** In order to obtain microbes producing microbial oil from environment, a strain, marked as TC14 was isolated by replica plating and Sudan Black staining on the selected media. Its colony is smooth; milk white, slightly transparent, round edge, sticky. Its cell is long-rod, G-, intracellular lipid granule. The results of physiological and biochemical research: catalase, denitrification and fluorescent pigment tests are negative; metachromatic granule staining, amylolysis, lecithinase tests are positive. According to its sequencing result of 16S rDNA, strain TC14 is classified and belonged to *Bacillus*, namely sp. TC14. The optimum inoculation temperature is 35°C and the most suitable initial pH value is 10. The intracellular lipid of strain TC14 is extracted by the way of hydrochloric acid hydrolysis and the calculated ratio of oil-production is 5.08%.

1. Introduction
Oil is not only one of the basic substances that make up and maintain our life, but is an important industrial raw material as well which has wide application value. Traditional oil resources mainly come from animals or even 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 resource. In order to solve the increasingly serious problem of energy shortage and environmental degradation, countries around the world are vigorously developing renewable liquid fuels, among which biodiesel is the most potential bioenergy product. Currently, supply of animal and vegetable oils is also very limited, and oil resources are bound to be developed in various ways. There are a few microorganisms in nature that could transform carbohydrate and hydrocarbon into oil and even store them in large quantities under suitable conditions. The highest content of oil in bacteria can exceed 70% of its dry weight. It is known that bacteria, yeasts, molds and algae all have strains which can accumulate oil, but most of them are yeast and molds. Compared with the traditional methods which rely on animals and oil plants to obtain oil, the microbial production of oil has the advantages of continuous production, short cycle, free from the influence of seasons and climate, wide source of raw materials and great potential of high value products, and moreover, has a broad industrial application prospect. Therefore, it is a good way to develop new oil resources by using microorganisms to transform carbohydrate into oil.

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At present, commercial mushroom oil has been listed in Japan, Germany, the United States and other countries. Moreover, microbial oils have been allowed to be added to baby food in Europe, Middle East, South Asia and Australia. With the increase of population, contradiction between oil demand and serious shortage of natural resources is becoming more and more serious. In this study, oil producing bacteria were isolated from riverside soil before investigating its morphology, physiological and biochemical characteristics. In addition, culture conditions were optimized, taxonomic status was determined by molecular biological identification, and intracellular oil was extracted by hydrochloric acid hydrolysis method, hence, oil production rate was calculated. Consequently, this study not only enriches the microbial resources in China, but also provides new microbial materials for oil production, and theoretical basis for future use of microbial oil in place of vegetable oil to produce biodiesel and solve the problem of human energy shortage.

2. Material and Methods

2.1. Experimental materials

2.1.1. Sample. 3 g topsoil from the river was taken before being added into 100 ml sterile water and shaked for 30 min.

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 KH2PO4, 0.5 g/L Na2HPO4, 1 g/L MgSO4, 0.1 g/L FeSO4, 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 KH2PO4, 0.5 g/L Na2HPO4, 1 g/L MgSO4, 0.1 g/L FeSO4, 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 method

0.5 ml mixed water sample was shaken for gradient dilution. 0.1 ml 10-6 gradient bacterial solution was taken and coated on the separation medium plate for culturing at 28℃ for 2-4d, separate the single colonies under the same culture conditions for more than 3 times, purify the strains, and number them respectively. Replica plating is a method used for screening oil producing strain. It is a simple and convenient method for the detection of lipid producing microorganisms. When adopting this method, the single colony on the plate which has high fat content should choose an optional dyeing method so that the dye can not only be absorbed and utilized by the cell, but also penetrate into the oil drop of the cell to indicate the accumulated concentration of oil.

For morphological observation and physiological and biochemical identification, manual for systematic identification of common bacteria and the manual for identification of Berger's bacteria was taken as reference. Agarose gel electrophoresis was used to analyze the concentration and quality of 16S PCR products. The PCR products were recovered by gel recovery kit after gel cutting. Positive clone was screened and sequenced by Shanghai Biotechnology Co., Ltd. the 16S r DNA sequence was compared with blast in GenBank database, and the phylogenetic tree was constructed by Mega 7.0 software.

3. Result and Discussion

3.1. Isolation and purification of oil strains

After dilution and coating separation, large amount of colonies were grown on three different screening media. Through observation, strains with larger colonies were selected, and the strains with higher oil production were screened and numbered by photocopy plate method. Among them, the oil in the cell formed by the strain TC14 was dark blue, so it was used as the experimental strain. The colony of strain TC14 is smooth, milky white, slightly transparent, round and easy to pick and sticky. The cells are
stained with Sudan black as shown in Figure 1. The cells are long rod-shaped, and there are blue black oil particles in the cells.

Figure 1. Results of colurating by sudan black B

3.2. Physiological and biochemical characteristics of strain TC14

Some physiological and biochemical identification results of strain TC14 are shown in Table 1.

| Test item                      | Results |
|--------------------------------|---------|
| Carbohydrate fermentation      |         |
| Glucose                        | -       |
| Malt dust                      | -       |
| Fructose                       | -       |
| Lactose                        | -       |
| Fiber two pond                 | -       |
| Melezitose                     | -       |
| Soluble starch                 | -       |
| Carbohydrate fermentation      |         |
| Lactose                        | ++      |
| Sucrose                        | ++      |
| Cellose                        | +++     |
| D-sorbitol                     | +       |
| Carbon 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 negative while "++" means positive result.

The specific bands of about 1.5 kb were amplified by universal primers. According to sequencing results, homology of the strains was compared in GenBank database. It was found that the strain was in 99% similarity with Bacillus. Using GenBank database to compare the homologous sequences and construct the phylogenetic tree, the strain was identified as Bacillus sp. TC14.
3.3. Optimization of growth conditions of strain TC14

3.3.1. Effect of initial pH value on strain growth. Strain was inoculated on the medium with the initial pH value of 2, 4, 6 and 8, absorbance on 540 nm was measured every 4 hours. The results are shown in Figure 1. Strain TC14 could not grow at the initial pH value of 4. At the initial pH value of 6, 8 and 10, the growth of strain od540nm increased with the increase of pH value. When the initial pH value was 10, the growth of strain TC14 was the highest, which indicated that the growth of strain TC14 was better when the initial pH value was 10.

![Figure 2. Effect of initial pH value on strain growth](image)

3.3.2. Effect of culture temperature on strain growth. Strain TC14 was inoculated on the medium with the initial pH value of 10 and cultured at 20°C, 28°C, 35°C and 42°C. Absorption at 540nm was measured every 4 h. The results are shown in Figure 2. With the increase of temperature, absorption increased in accompany with strain growth. With the increase of temperature, the growth of strain TC14 decreased at 42°C. It can be seen that the suitable growth temperature of strain TC14 is 35°C.

![Figure 3. Effect of temperature on strain growth](image)
3.3.3. Growth curve of strain TC14. Strain TC14 was inoculated on the medium with initial pH value of 10 and cultured at 35°C. OD540 was measured every 4 hours to draw growth curve. It can be seen that 0-8 hs in the lag period, 8-16 is in the logarithmic growth period, 24h reaches the maximum growth amount with the absorption of 1.25 followed by stable period. The growth amount and growth rate of strain TC14 are relatively high.

![Figure 4. Growth curve of TC14.](image)

3.3.4. Endolipid yield of TC14. Strain TC14 was cultured for 28 h under suitable conditions. The oil in the cell was extracted by hydrochloric acid hydrolysis method. After weighing, 0.12 g was obtained. As dry cell is 2.36 g, yield of oil extracted by this method was about 5.08%.

4. Conclusion

In this experiment, TC14, a strain producing oil, was isolated from the surface soil near the river by the methods of screen plate and Sudan black staining. The results showed that contact enzyme, denitrification and fluorescent pigment tests were negative, heterochromatin staining, starch hydrolysis and lecithin tests were positive. The 16S rDNA identified the strain as *Bacillus* sp. TC14.

As one of the fermentation technologies that affect the metabolism and synthesis of oil producing strains, the temperature and pH value control in the microbial growth environment is very important. By improving the fermentation conditions, the oil production is greatly improved. The optimum culture temperature of strain TC14 was 35°C, the initial pH value was 10, and the yield of oil was 5.08% by hydrochloric acid hydrolysis. This is consistent with the fermentation temperature of oleoresins studied by most scholars. The reason may be that temperature and pH affect the synthesis and composition of microbial oil, and appropriate temperature can promote the effect of oil producing microorganisms on oil. In a word, the conversion of biomass resources into fatty acid metabolites, including oil, has become an important direction of bioenergy research, and has been paid more and more attention. Energy microbial oil technology can provide raw materials for biodiesel industry, which has made great progress. However, large-scale production of microbial oil, just like other biofuel products, is still facing difficulties. With the continuous development of modern biotechnology and synthetic biology, under the guidance of the concept of bio refining and green chemistry, the research of energy microbial oil adopts modern molecular biology technology, such as gene engineering, hybrid breeding and protoplast fusion, in order to greatly improve the oil and fat production; the research results are enlarged to improve the fermentation conditions of oil and fat at the level of fermentation tank The kinetic model of oil fermentation was established by optimization, and the pathway of oil biosynthesis in vivo was studied to further improve oil yield. It will continue to improve the comprehensive technical economy of the process and ultimately provide reliable technology for biofuel production.
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