Screening and identification of Lipase Producing Bacterium

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Abstract. 55 samples from different regions were selected and screened by Rhodamine B flat transparent circle method to observe lipase producing effect, among which, LHY-1, identified as Serratia sp. has the characteristics of fast growth, high enzyme production and stable ability. The colony of this strain is white, the edge is smooth and tidy, the surface is moist, the cell is straight, rod-shaped, gram negative, 0.1-0.2 μm in diameter and, length 0.3-0.5 μm in length.

1. Introduction
Lipase (Lipase, EC 3.1.1.3) is a special kind of triglyceride hydrolase in the oil-water interface, which could not only catalyze the hydrolysis of triglyceride hydrolysis into glycerol and fatty acids, but promote the hydrolysis of esters, trans esterification and esterification as well. Lipase has been widely used in the fields of drug synthesis, food processing, detergent and cosmetics because of its specific biotransformation capability. Lipase has become one of the leading biological catalyst due to its great potential in the development of the lack of billions of dollars in lipid biology industrial technology market, and it also has been applied in natural and non natural lipid metabolism in many aspects. In the beginning of 70s, the number of available lipase increases, this is because the cloning and expression of the lipase from microorganism has made great achievements and continue to have the value and the temperature anomaly and the specific nature of the need for agent to increase in specificity, stability, pH. A variety of lipase producing organisms are present in the animal kingdom, plant kingdom and microbial community. Because of its stability, selectivity and extensive substrate specificity, lipases from microorganisms have received special attention in industry. Lipases are ubiquitous in some plants, animals, and microorganisms. Many bacteria, yeasts, and fungi are called producers of extracellular lipase. Lipase produced by microorganisms is the most widely used and important enzyme in the fields of biological conversion, catalysis and biochemistry. More recently (from 2004 to the present), the potential microorganisms producing lipase on liquid and solid fermentation are shown in Table 1.
Most of the lipase is extracellular lipase, hence, lipase production is based on physicochemical factors (temperature, pH and dissolved oxygen). As lipase is an inducible enzyme, the most important nutrient for lipase activity is carbon source such as oils or other inducers (three acyl glycerol, fatty acids, hydrolyzable esters, Twain, bile salts, and glycerol). The synthesis of biodiesel is mainly lipase transesterification, namely oil and short chain alcohols (usually methanol and ethanol) by esterification, among which, lipase mainly plays the significant role in catalyst. Three glycerol esters were hydrolyzed into fatty acids, fatty acids and short chain alcohol esterification of fatty acid. That is to say, lipases have catalytic ability of oil especially in low temperature and thermal stability, which is widely applied in food, washing, pharmaceutical, lipids, and low temperature bio-remediation industry.

2. Materials and Methods

Esterase is an enzyme that catalyzes the hydrolysis of esters. Lipase is an enzyme that catalyzes the hydrolysis of fats into glycerol and fatty acids. The substrate has different carbon chains: esterase preferentially hydrolyze short chain fatty acids (C3-C8), and lipase preferentially hydrolyze long-chain fatty acids (C14 or more). The physical state of the substrate is different: esterase hydrolysis of water soluble substrate, lipase hydrolysis of oil-water interface substrate. The lipase was screened according to the hydrolysis of the carbon chain. This chapter will use Rhodamine B tablet were screened from farmland, petroleum, water and soil pollution and oil pollution in soil samples, isolated strains, lipase producing strains, and then combined with the strain to strain morphological, physiological and biochemical and 16S rDNA sequence analysis and preliminary identification of a series of experiments.

2.1. Enrichment, screening and purification of Lipase Producing Bacteria

5.0g samples were added to 50mL sterile water, and the 30min was diluted at 25 and 150r/min for 10 0.5-1h, 100 times and even 1000 times. Draw 50 L dilution coating on the surface of the culture medium in detection of lipase, incubation 16h culture at 30 °C, according to the halo around the colony sooner or later and the size of single colonies producing halo larger and faster enzyme production has been purified to pure strains. Purified lipase producing bacteria were transferred to LB liquid aseptic medium from LB solid culture before shaking at 180 rpm at 30 °C for 12 h. In a 1.8 mL sterileD tube, glycerol was added to the suspension to a final concentration of 20%. After mixing, the bacteria tube was stored at -72°C in the refrigerator for further use.

Lipase activity was assayed by transparent plate ans spectrophotometric method. Enzyme unit was defined as the amount of lipase required for lipase hydrolysis of substrate to Nitrophenol (4-Nitrophenyl) 1nmol butyrate per minute under certain conditions.
2.2. Lipase activity assay

2.2.1. Flat transparent circle method. Various substrates hydrolyzed by lipase such as fatty acid glycerides, fatty acids, and fatty acid sodium could be combined with Rhodamine B, which is activated by UV light to produce orange fluorescence. According to orange fluorescence, depth of color and the size of the hydrolysis circle, lipase production and amount of lipase produced could judge preliminary.

2.2.2. Spectrophotometry. Substrate solution-1.5% Gum Arabic, 2% Triton X-100, 0.3 mg/ mL p-nitrobenzobutyrate was dissolved in 20 mmol Tris-HCl at pH 7.0. 1.9 mL of substrate solution was taken at 40 °C in constant temperature water bath for 5 min, 100μL enzyme solution was fully mixed and maintained at 40 °C for 10 min before adding 2 mL 95% ethanol. The final solution was measured by ultraviolet spectrophotometer at a wavelength of 410 nm.

2.3. Lipase activity assay

2.3.1. Morphological characteristics of colony. The target strain was screened out from a refrigerator at -72 °C and the bacterium to isolate a single colony for colony morphology (shape, size, color, etc.) observation, gram staining, and spore staining.

2.3.2. Physiological and Biochemical Experiments of Lipase Producing Strain. The target strain was subjected to physiological and biochemical experiments with API20E test strips in physiological and biochemical identification tubes. Each of resulting data was compared to others in Bergey's Manual of Determinative Bacteriology.

2.3.3. 16S rDNA Analysis of Lipase Production. 16S rDNA gene fragment was amplified according to the PCR amplification system in Table 2. The 16S rDNA sequence was sequenced by PCR amplification of the sample biochemical engineering (Shanghai) Co., Ltd. in the NCBI (http://blast.ncbi.nlm.nih.gov/blast.cgi) library. Hence, 16S rDNA sequence homology was compared and the phylogenetic tree was constructed.

| Table 2. The PCR amplification system. |
|--------------------------------------|
| Reaction system | Volume (μL) |
| 10×buffer | 5 |
| 25mM MgCl₂ | 3 |
| dNTP Mixture (2.5mM MgCl₂) | 1.5 |
| *Pfu* polymerase (2.5 U / μL) | 1 |
| Template DNA | 1 |
| Primer 1 (50 μM) | 0.5 |
| Primer 2 (50 μM) | 0.5 |
| Double distilled water | 37.5 |
| General system | 50 |

3. Results and discussion

3.1. Screening and identification of Lipase Producing Strain
The target strains LHY-1 were removed from the -72°C refrigerator, and the bacteria were coated on the LB solid medium, and single colonies were isolated. Then the colony morphology (shape, size, color, etc.) was observed. The colony is white, the edge is smooth and tidy, the surface is wet, and gram negative (Fig. 1).
In addition, target strain with higher activity were identified by API, 20E and physiological and biochemical identification tubes, and the result of each item was in accordance with Serratia nematodiphila in comparison with Bergey's Manual of Determinative Bacteriology, which was shown in Table 3.

### Table 3. The biochemical characteristics of the strain LHY-1

| Physiology and Biochemistry | LHY-1 | Serratia nematodiphila | Serratia marcescens | Physiology and Biochemistry | LHY-1 | Serratia nematodiphila | Serratia marcescens |
|-----------------------------|-------|------------------------|---------------------|-----------------------------|-------|------------------------|---------------------|
| Ornithine decarboxylase     | +     | -                      | +                   | maltose                      | +     | +                      | ND                  |
| Lysine decarboxylase        | +     | -                      | +                   | lactose                      | -     | -                      | -                   |
| oxidase                     | -     | -                      | -                   | Galactose                    | +     | +                      | -                   |
| V-P experiment              | +     | +                      | +                   | mannose                      | -     | +                      | ND                  |
| Hydrogen sulfide            | -     | -                      | -                   | arabinose                    | -     | -                      | -                   |
| ONPG                        | +     | +                      | +                   | turanose                     | -     | +                      | -                   |
| indole                      | -     | -                      | -                   | xylitol                      | +     | +                      | +                   |
| Glucose                     | +     | +                      | +                   | Arabia alcohol               | -     | -                      | -                   |
| xylose                      | +     | -                      | +                   | Adonis alcohol               | -     | +                      | +                   |
| fructose                    | +     | +                      | +                   | sorbitol                     | +     | -                      | +                   |
| Sorbitol                    | -     | -                      | ND                  | mannitol                     | +     | +                      | ND                  |
| Rhamnose                    | -     | -                      | -                   | inositol                     | +     | +                      | +                   |
| sucrose                     | +     | +                      | +                   | urea                         | -     | -                      | -                   |
| Ribose                      | +     | +                      | ND                  | malonate                     | -     | -                      | -                   |
| melibiose                   | -     | -                      | -                   | Amygdalin                    | +     | -                      | ND                  |

Note: "+" positive reaction; "-" negative reaction; "ND" means not detected

### 3.2. Sequence analysis of strains LHY-1, 16S and rDNA

PCR amplification was performed from the total DNA of the target strain LHY-1 using 16S rDNA universal primers. The result of agarose electrophoresis of strain LHY-1 is shown in Figure 2. PCR amplification products were sent to biological engineering (Shanghai) Co., Ltd. for 16S rDNA sequencing, and the results obtained is about 1488bp 16S rDNA fragments. The 16S rDNA fragments obtained was in comparison in GENBANK on NCBI, showing similarity of 99%, 99%, 98%, 98% to Serratia nematodiphila, Serratia marcescens, Serratia rubidaea, and Serratia ficaria according to the the phylogenetic tree constructed (Figure 3).
LHY-16S rDNA sequence is as follows:

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GACCGGCGCGCGGGCTTACCCTACCATGCAAGTGACGGGAGACAGCCATCTGCCTCC
CTGGTGACGGCAGCCAGGCGGAAGTGATCGTAATGCTGTGGAACTGCGCTCTGAGAGTG
GAGGTTCCACCCGCGAGTGGAAATTGGTGAGCAATCCGCGCGCTCAGCTGGGAACTCCT
GACGGTAAAGCCGCACGCAGCCGCTTGAAGTGGGCTCAATCGCCCTGGCCGGGGACGC
GGAAAGGCTGGTATAGTGCAGCGGTAGCTCTGCTAGCTCGCTTGCTGTCGTCAGCTG
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**Figure 2.** 16S rDNA PCR electrophoresis figure of LHY-1.

**Figure 3.** Phylogenetic tree of strain LHY-1.
CCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTA
GGGCTACACACGTGCATAATGGCCTTACGACATCAGCCACGGCTACACACGTGCTACAATGGCGTATACAAAGAGAAGCGACCTCGCGAGAGCAAGCG
GACCTCATAAAAGTACGTCTCAGTCGAGTGGAGTCTGCAACTCGACTCCATGAAGTCGAAGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCGGCGCTTGTACACA
CCGCCCGTCACACACCATGGGAGGTGGGTGCAAAAAAGAATAGGGTAGCTAACCTTACCCTTCGGGAGGGCGCTTACCACTTTATATTAAGCG

4. Conclusion

55 strains producing lipase and different bacteria were screened by Rhodamine B flat halo method in farmland, petroleum, contaminated water, polluted soil and oil pollution in soil samples, among which, 17 strains were stable with rapid growth and enzyme production, including Serratia sp., Pseudomonas aeruginosa, Aeromonas sp., Burkholderia sp., and Staphylococcus aureus. Observed from the 17 strains producing lipase strains, LHY-1 was rapidest in growth and enzyme production. The colony of strain LHY-1 were round, smooth surface and opaque, neat edge with white color. Gram staining and spore staining of strain LHY-1 revealed that gram staining was negative and no spore was produced. The 16S rDNA sequence indicated LHY-1 was in similarity with Serratia nematodiphila, Serratia marcescens, Serratia rubidaea and Serratia ficaria to 99%, 99%, 98% and 98% according to phylogenetic tree constructed and physiological, biochemical properties as well. Hence, LHY-1 was identified as Serratia (Serratia sp.)

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