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Screening Intracellular Lipase-Producing Microbial with Selective Synthesis of 1,3-diolein

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The main purpose of the work was to screen intracellular lipase-producing microbial with high selective synthesis of 1,3-diolein, a healthy lipid. The screening model is a combination of Rhodamine B-olive oil agar plat, para-nitrophenyl palmitate (ρ-NPP) for ester synthetase activity and HPLC analysis of products of glycerolysis of triolein. The results show that the strain GZUF36 (CCTCC No. M2012538) can be used to catalyze the selective synthesis of 1,3-diolein. 1,3-Diolein yield is 11.12% (w/w) by whole-cell of GZUF36 catalyzed glycerolysis and it is accounted for 72% of total diolein (including 1,3- and 1,2-isomer). Then, GZUF36 was identified as *Aspergillus niger* by morphology and 18S rDNA sequence analysis. It is also suggested that the model can be applied to screen other lipases with high ester synthetase activities.

Key words: *Aspergillus niger*, 1,3-diolein, Identification, Intracellular lipase, Screening.

Obesity is a growing threat to human health. A person is susceptible to cardiovascular diseases owing to obesity. Then, what is the cause of obesity? One of the major reasons is the accumulation of triglyceride in the body. 1,3-Diglyceride (not 1,2-isomer) can protect against diet-induced obesity since it may reduce body-fat accumulation[1]. So 1,3-diglyceride is a sort of health lipid. What's more, it can be utilized as starting materials for drug formulation, such as 1,3-diglyceride conjugated chlorambucil for treatment of lymphoma[2]. So it makes some sense to prepare 1,3-diglyceride. Large-scale preparation of highly pure 1,3-diglyceride still remains a problem with traditional chemical methods. And it is unfriendly to the preparation of 1,3-diglyceride with chemical methods. Enzymatic approach has advantages of mild conditions, the high regioselectivity and friend to the environment.

There are some reports on preparation of 1,3-diglyceride catalyzed by lipase[3, 4]. It is difficult to control lipase-catalyzed hydrolysis to the preparation of 1,3- diglyceride. And it is common to the preparation of 1,3-diglyceride by ester synthesis[4] or transesterification[3]. The lipases used are mainly commercial enzymes[3, 4], which is unfavorable to its industrial applications. So it makes differences to screen lipase-producing microbial with high selective synthesis of 1,3-diglyceride. Moerover, it is better if the lipase produced is an intracellular enzyme. The enzyme-

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producing cell can be as the catalyst without purification of enzymes. There are few reports on preparation of 1,3-diglyceride by whole-cell lipase.

Therefore, the main purpose of the present study was to screen intracellular lipase-producing microbial with high selective synthesis of 1,3-diglyceride. It is crucial to build up a screening model. The model would be the primary screening with Rhodamine B-olive oil agar plat, the first rescreening with ρ-NPP for lipase synthetic activity (a quick and economic spectrophotometry method) and the second rescreening by HPLC analysis of products of glycerolysis of triolein.

MATERIALS AND METHODS

Soil samples for the separation of lipase-producing microorganisms were taken from soil rich in lipid. 1-Monoolein, 2-monoolein, 1,3-diolein, 1,2-diolein and triolein (purity > 99%) as standards were purchased from Sigma. Acetonitrile and hexne were chromatographically pure. All other chemicals used in this work were of analytical grade and commercially available.

Enrichment medium contained (g/L): agar 20.0, $(NH_4)_2SO_4$ 1.5, K_2HPO_4 0.1, $MgSO_4 \cdot 7H_2O$ 0.5, NH_4NO_3 0.5, NaCl 1.5, olive oil 1.0. It was adjusted to pH 7.0.

Screening medium contained (g/L): glucose 2.0, soluble starch 5.0, peptone 5.0, yeast extracts 5.0, K_2HPO_4 1.0, $MgSO_4 \cdot 7H_2O$ 0.2, $NaNO_3$ 2.0, agar 20.0. It was adjusted to pH 6.8.

Rescreening fermentation medium contained (g/L): soybean 20; corn syrup 20; K_2HPO_4 0.5; $NaNO_3$ 0.5. Its pH was adjusted to 7.0.

Enrichment culture was carried out as follows: Addition of soil sample 5.0 g to 50 ml sterilized physiological saline in a 250-ml erlenmeyer flask was mixed well at 30 °C and 180 r/min for 30 min. Then, adding one ml sample diluent to 50 ml enrichment medium was incubated at 30 °C and 180 r/min for two days.

Primary screen by Rhodamine B-olive oil agar plate: Screening medium was supplemented with both 2.0 % olive oil and 0.001 % Rhodamine B according to the method described by Kouker and Jaeger[5]. Culture plates were incubated at 30 °C and examined for three days. Lipolytic activity was monitored by irradiation at 365 nm.

First rescreening by ρ-NPP: The screened lipase-producing microbial by Rhodamine B-olive oil agar plate method were incubated in the rescreening fermentation medium at 30 °C and 180 $r \cdot min^{-1}$ for three days. The harvested cells were frozen dry and used as whole-cell biocatalyst. Their synthetase activities were determined by ρ-NPP[6]. One unit of ester synthetase activity (U) was deûned as the amount of dry whole-cell lipase required to produce 1.0 µmol of para-nitrophenol in one minute, under the determination conditions. Then, microbial was chosen with high synthetase activity.

Second rescreening by HPLC analysis products of glycerolysis: The glycerolysis reactions were performed in a sealed 50-ml flask containing glycerol 25 µmol, triolein 50 µmol, 0.2 g dry whole-cell lipase, 4.9 ml dry hexane and 0.1 ml dry t-butanol at 40 °C and 200 r/min for 12 h. Hexane and t-butanol were dehydrated by incubation with 4 Å activated molecular sieves, prior to their addition to the reaction system. After the reaction, the organic phase was filtered with a 0.45 ¼m microporous membrane for HPLC analysis of triolein, 1,3-diolein, 1,2-diolein, and momooleins. The analysis condition: HPLC (Agilent technologies 1260 infinity) was equipped with a Nova-Pak column (3.9 mm×150mm, Waters, USA) and a diode array detector. The mobile phase was acetonitrile and isopropanol (v/v, 60:40) from 0 to 27 min, and then acetonitrile/isopropanol was adjusted to 80:20 (v/v) from 27 min to 30 min. The flow-rate was 0.6 ml/min and the column temperature was 30 °C. The efûuent was monitored at 210 nm. Then lipase-producing microbial with high selective synthesis of 1,3-diolein was chosen.

Identification: The lipase-producing microbial with high selective synthesis of 1,3-diolein was identified by China Center for Type Culture Collection (CCTCC) through its morphological characteristics and 18S rDNA sequence analysis. A phylogenetic tree was constructed by the neighbor-joining algorithm using the maximum composite likelihood method in MEGA 5.0.

RESULTS AND DISCUSSION

The main purpose of the work was to screen intracellular lipase-producing microbial with high selective synthesis of 1,3-diglyceride. It is

critical to build up a model for screening. First, enrichment cultures of lipid-rich soil samples were carried out to make objective microbial dominant using olive oil as the sole carbon source. This makes it simple to screen. Then, the primary screening is general to use a transparent/color circle plate (For example, Rhodamine B-olive oil plate) formed by lipase-catalyzed hydrolysis. In this way, 72 strains produced lipase were chosen by Rhodamine B-olive oil agar plates for the lipase-producing microbial were indicated by the presence of orange ûuorescent halos around colonies when the plates were irradiated under UV light [5,7].

There is no direct relationship between the hydrolytic activity and synthetic activity of lipase[8] while the transesterification activity is general positive correlation to synthetic activity[9]. So, for the first rescreening, ester synthetase activity was determined by ρ-NPP, a quick and economic method. The results show that the ester synthase activity of most of them is low and there are only nine stains with higher ester synthase activity (>3 U·g^{-1}dry whole cell) than the others (Table 1). Hence, the nine strains were chosen for a subsequent glycerolysis of triolein.

For the second rescreening, it needs to analyze the selectivity of the above nine strains to the synthesis of 1,3-diolein through glycerolysis. So the nine stains were cultured and prepared as dried whole-cell lipase to catalyze the glycerolysis of triolein. The result indicates that GZUF36 is an excellent lipase-producing strain with high selective synthesis of 1,3-diglyceride (Fig. 1). 1,3-Diolein content in total diolein was up to 72.54% after glycerolysis of triolein by whole-cell lipase of GZUF36. The diolein can be as healthy lipid, for a

Table 1. Ester synthase activity (U·g^{-1} dry whole cell) of isolated strains

Ester synthase activity	<1	1-2	2-3	>3
Strains (number)	47	9	7	9

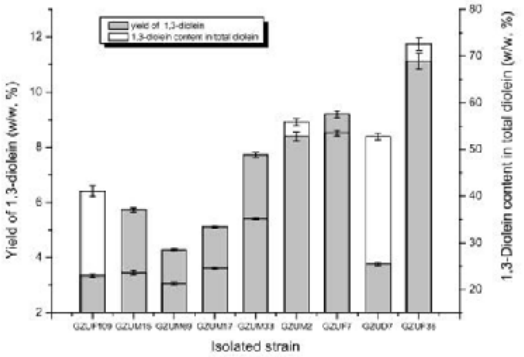

Fig. 1. Whole-cell lipase-catalyzed glycerolysis of triolein. Data are means of three determinations

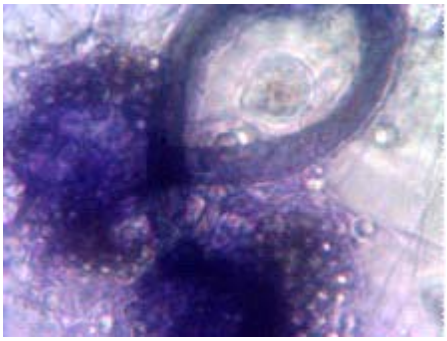

Fig. 2. A microscope (400X) photos of the strain

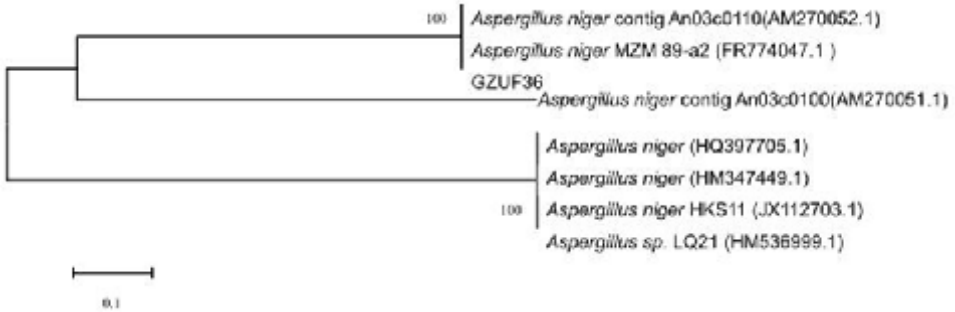

Fig. 3. Neighbor joining phylogenetic tree derived from partial 18S rDNA sequences. The evolutionary distances were computed using the maximum composite likelihood method in MEGA 5.0. The numbers at the nodes represent percentage bootstrap values based on 1000 replicates. The horizontal scale bar indicates a distance of 0.1

type of cooking oil of diglyceride is consisting of about 70% 1,3- and 30% 1,2-iso-mers, which is claimed to be clinically beneûcial [2].

The results also prove that it is an economic and rapid model to screen intracellular lipase-producing microbial with selective synthesis of 1,3-diolein through Rhodamine B-olive oil agar plate, ρ-NPP for ester synthetase activity and an accurate method for measure of 1,3-(1,2-)diolein content. Similarly, the model can be applied to screen other lipases for ester synthesis or transesterification.

Next, the strain GZUF36 needs to be identified by traditional morphology and modern molecular biology. The colony of the strain GZUF36 growth on potato dextrose agar medium is dark-brown and powder-like. The vesicle of conidia of the strain is flask-shaped, covered by a single layer of small metula (Fig. 2). Then, phylogenetic analysis based on 18S rDNA sequence indicates that the newly isolated strain GZUF36 is closely related to *Aspergillus niger* contig An03c0110 (AM270052.1) and *Aspergillus niger* MZM 89-a2 (FR774047.1) (Fig. 3).

So, GZUF36 is identified as *Aspergillus niger* by a combination of colony morphology, cell morphology, sequencing of 18S rDNA and phylogenetic analysis. It is now collected in CCTCC, numbered as CCTCC No. M2012538.

CONCLUSIONS

GZUF36 with high selective synthesis of 1,3-diolein was screened from soil rich in lipid by building up a simple, economic and rapid model. The model is a combination of quick measurement methods including Rhodamine B-olive oil agar plate and ρ-NPP for ester synthetase activity and an accurate method to measure of yield of product by lipase-catalyzed ester synthesis. 1,3-Diolein content in total diolein is 72.54%, yield 11.12% by GZUF36-catalyzed glycerolysis. Then, GZUF36 is further identified as Aspergillus niger by colony morphology, cell morphology and 18S rDNA analysis.

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