Screening and Production Optimization of a New Organic Solvent-Tolerance Fungal Lipase

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Keywords: Lipase, Screening, Organic solvents tolerance, Fermentation.

Abstract. A novel fungus which produced lipase extracellularly was isolated from soil. The strain was named after *Candida tropicalis* SD7 based on morphological features and ITS1-5.8S–ITS4 region sequences of ribosomal RNA. The fermentation conditions were optimized in order to improve lipase production of the strain. The maximum lipase activity was achieved at 72h. The optimum lipase production was observed in the medium with glycerol as carbon source and peptone as nitrogen source through submerged fermentation at 30 ℃ and initial pH 7.0. The lipase showed stability in the presence of hydrophobic organic solvents such as methylbenzene, petroleum ether, isooctane and n-hexane. It was moderately stable in polar solvents, including DMSO, acetone, acetonitrile and methanol.

Introduction

Lipases (E.C.3.1.1.3) constitute a versatile group of enzymes which catalyze different sorts of reactions such as hydrolysis, esterification, transesterification, aminolysis, acidlysis and alcoholysis [1]. The multifaceted features have enabled the use of lipase in medicine, food, detergent, leather, paper industries and waste treatment [2]. These extensive applications of lipase have placed greater stress on researchers to search for more efficient lipase production. More recently, organic solvents tolerance lipases have captured increasing attention due to altered regiospecificity and steroselectivity, higher solubility of substrate and ease of products recovery and ability to shift the equilibrium toward synthetic reaction, etc. [3]. Organic solvents tolerance lipases are produced by a slice of microorganisms such as *Pseudomonas aeruginosa* S5 [4], *Pseudomonas aeruginosa* LST-03 [5], *Pseudomonas aeruginosa* CS2 [6], *Bacillus sphaericus* 205y [7], *Serratia marcescens* ECU1010 [8], *Arthrobacter sp.*SD5 [9], *Staphylococcus saprophyticus* M36 [10], etc. However, less information is available on the organic solvents tolerance lipase originated from fungi [11].

In the present work, a novel fungi producing lipase with organic solvents stability was isolated and identified. The fermentation conditions of the strain were optimized for lipase production. The stability of the crude lipase in the presence of organic solvents was also investigated.

Materials and Methods

Materials and Reagents

p-NPP was purchased from Fluka Co.Ltd. Fungal genome extraction kit and gel extraction kit were from Sanboyuanzhi Ltd. All other chemicals were obtained from various commercial sources and were of analysis grade or higher grade.

Isolation and Screening of Lipase Producing Fungi

Different samples were collected from soil contaminated with oil in Nanchang city, China and stored in closed containers at 4 ℃ prior to use. Ten grams of soil was suspended in 90 ml sterilized saline water and 200μl of suspension was transferred into 25 ml enrichment culture medium containing peptone 1%, olive oil 2.3%, K_2HPO_4 0.35%, KH_2PO4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.25%, rose bengal 0.0033%, streptomycin 0.003% and then was incubated at 30 ℃ on a rotary shaker at 160rpm for 4 days. The culture were diluted 100 times and spread on the solid plate (tri-*n*-butyrin 2.6%, KH_2PO_4 0.35%, K_2HPO_4 0.1%, $MgSO_4$ 0.25%, peptone1%, agar 3%, rose bengal 0.0033%, streptomycin 0.003%). The plates were incubated at 30 ℃ for 72 h. Lipases secreted by microorganisms catalyzed the hydrolysis of tri-*n*-butyrin to form clear halo. Lipase production was approximately evaluated by measuring the H/C ratios (diameter of hydrolysis halo/ diameter of cell colony). According to H/C ratios, the strains with high H/C ratio value were selected for the further screening. The selected strains were inoculated in fermentation medium containing peptone1%, olive oil 2.3%, K_2HPO_4 0.35%, KH_2PO4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.25% and incubated at 30 ℃ on a rotary shaker with 160 rpm for 3d. The culture was centrifuged at 4 ℃ (10,000 rpm) for 2 min. The resultant supernatant was subject to the assay of lipase activity by spectrophotometric method.

Identification of the Isolated Strain

The isolated strain was identified and classified according to ITS1-5.8S–ITS4 region sequences of ribosomal RNA and morphological features [12]. The genomic DNA was extracted using fungal genome extraction kit. The partial sequences of ribosomal RNA of the isolate were amplified using ITS1 primer (5′- TCCGTAGGT GAACCTGCGG-3′) and ITS4 primer (5′-TCCTCCGCTTATTGATATGC-3′). The amplification was carried out by initial denaturation at 94 ℃ for 5 min followed by 35 cycles of 94 ℃ for 45 s, 52 ℃ for 45 s, 72 ℃ for 1.5 min and final extension at 72 ℃ for 10 min. PCR product was purified by PCR Clean-Up Kit. The purified DNA fragment was sequenced using an automated sequencer. The sequence was deposited in NCBI GenBank database under the accession number of KT692959. Multiple sequence alignment was performed between the contig obtained and sequences homologous to stain SD7, using ClustalX. A neighbor-joining phylogenetic tree was constructed by using MEGA 5.05 software. The morphological features of the isolated strain were observed and described.

Time Course of Growth and Lipase Production of Candida Tropicalis SD7

The seed culture of *Candida tropicalis* SD7 was inoculated to 50ml of fermentation medium (peptone1%, olive oil 2.3%, K_2HPO_4 0.35%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.25%, Tween-80 0.21%) in a 250mL Erlenmeyer flask. The strain was incubated at 160rpm in an orbital shaker incubator at 30 ℃. Samples were harvested at certain intervals for determination of growth and lipase activity of *Candida tropicalis SD7*. The growth of *Candida tropicalis SD7* was determined by the counting of yeast cells.

Optimization of Fermentation Conditions for Lipase Production

Optimization of fermentation conditions for lipase production was performed by only changing one factor, while maintaining other factors constant. In order to study the effects of culture temperature, different culture temperatures (25 ℃, 28 ℃, 30 ℃, 35 ℃, 37 ℃ and 40 ℃) were used. In order to study the effects of initial pH value, different initial pH values (4, 5, 6, 7, 8, 9 and 10) were adjusted in the fermentation

medium, respectively. In order to study the effects of diverse nitrogen sources on lipase production, peptone in the fermentation medium (peptone1%, olive oil 2.3%, K_2HPO_4 0.35%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.25%, Tween-80 0.21%) was replaced with urea, potassium nitrate, yeast extract and ammonium sulfate. In order to study the effects of diverse carbon sources on lipase production, olive oil in the fermentation medium was replaced with glycerol, maltose, glucose and sucrose. After inoculation, the submerged fermentation was carried out at 160rpm for 72 h. Samples were harvested for lipase activity assay.

Influence of Different Organic Solvents on the Activity of Crude Lipase

Crude lipase was harvested by centrifugation at 4 ℃ (12,000rpm) for 15 min. Effect of different organic solvents on the activity of crude lipase was investigated. 1 ml of crude lipase was mixed with 0.25ml of different organic solvents (DMSO, methanol, acetone, acetonitrile, chloroform, methylbenzene, n-hexane, petroleum ether, isooctane). The mixture was incubated for 0.5h at 30 ℃ under shaking condition (160 rpm). The residual activity was assayed after incubation. The relative activity with organic solvents was determined by regarding the control (no organic solvents in the mixture) as 100%.

Assay of Lipase Activity with a Spectrophotometric Method

Lipase activity was measured according to Peng *et al.* [6] First, p-NPP was dissolved in 2-propanol (20 mM) as a substrate, and then the substrate (75 μl) was mixed with 3 ml Tris–HCl buffer (50 mM, pH 8), followed by pre-incubating them at 50 ℃ for 5 min. The mixture was incubated for 10 min at 50 ℃ after adding 50 μl appropriately diluted lipase. The reaction was terminated by adding 1 ml SDS (0.05% w/v). The absorbance of liberated p-nitrophenol was measured at 410 nm. One unit of lipase activity was defined as the amount of enzyme to produce 1 nmol p-nitrophenol in 1 min under the described conditions.

Statistical Analysis

A statistical package (SPSS version 11.5) was used for the data analysis. Experimental data were presented as mean±SD.

Results and Discussion

Isolation, Screening and Identification of lipase Producer

Lipases occur widely in animals, plants and microorganisms. Isolation and screening of suitable organisms are essential for the production of lipase. Microbial lipases are superior to lipases from animals and plants owing to their catalytic versatility, high yield and ease of genetic engineering. To date, about 65 genera from microorganism have been reported to produce lipase [2]. Some simple methods were applied to isolate microorganisms which secret lipase. Wang *et al.* used modified rhodamine B agar plates to obtain a thermostable alkaline lipase from *bacillus* strain A30-1 (ATCC 53841) [13]. In the present study, olive oil in the enrichment culture and tri-*n*-butyrin in the solid plate was used to isolate fungi which produced lipase. Rose bengal and streptomycin in the medium was intended to suppress growth of actinomyces and bacteria, respectively. On the basis of screening results, the strain SD7 achieved the highest lipase activity of 464.1 U/ml among all of lipase producers. The strain SD7 was ellipse-shaped and the middle of the round colony had a creamy white bulging, surrounded by arachnoid tomentum . The ITS1- 5.8S- ITS4 region of ribosomal RNA

of Strain SD7 was sequenced and is available in NCBI GenBank database under the accession number of KT692959. The phylogenetic analysis of the ITS1- 5.8S- ITS4 region sequence indicated that strain SD7 was a clade of *Candida tropicalis* (Fig. 1) . Accordingly, strain SD7 was tentatively named as *Candida tropicalis* SD7. *Candida tropicalis* have reported to produce xylitol [14], long chain dicarboxylic acid [15] and carbonyl reductase [16] etc, but few data concerning lipase production by *Candida tropicalis* were appeared in the literature. In the present work, *Candida tropicalis* SD7 was a novel and promising fungus used in the production of lipase.

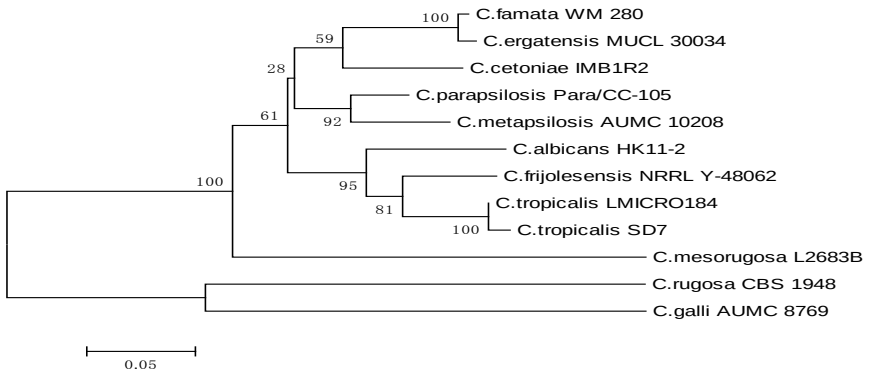

Figure 1.Neighbor-joining phylogenetic tree constructed on the basis of ITS sequence

Time Course Studies of Growth and Lipase Production of Candida Tropicalis SD7

The growth and lipase production of *Candida tropicalis* SD7 with regard to time were indicated in Figure 2. The maximum growth was achieved after 24h of fermentation. Lipases are detected during the lagging phase of growth, but optimum production was achieved at 72h. This suggests the production of lipase from *Candida tropicalis* SD7 did not synchronize with the yeast growth. Generally, the production of hydrolase such as lipase and protease tended to occur during the logarithmic phase of growth [17], whereas the obvious production of lipase from *Candida tropicalis* SD7 appeared after 3h of fermentation and the lipase production sharply decreased after 72h of incubation. Degradation by protease in the medium and low culture pH may account for diminishing lipase activity after prolonged fermentation.

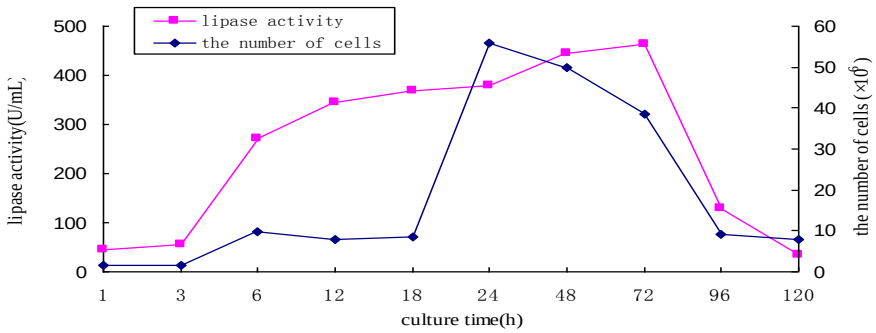

Figure 2. Time course of growth and lipase production of Candida tropicalis SD7

Optimization of fermentation conditions for lipase production

The yield of lipase was dependent on growth factors such as pH and temperature and nutrient requirements. Thus it is essential to obtain suitable cultural parameters and medium components to enhance the production level. The effect of temperature on lipase production is closely related to the growth of the organism. In general, lipases are produced in the temperature ranged from 20 ℃ to 45 ℃ [18]. Although *Candida*

tropicalis SD7 can grow at higher temperature, the best temperature for lipase production was 30 ℃. Further increase in the temperature gave diminished lipase production. Kantak *et al.* reported a similar result that maximum lipase production from a mesophilic *Rhizopus* JK-1 was seen at 30 ℃ [12]. The pH profile for lipase from *Candida tropicalis* SD7 was determined in various pH values ranging from 4 to 10. Optimum lipase activity was observed at 7. Further increase or decrease in pH gave rise to obvious reduction of lipase production. Generally, bacteria prefer to neutral pH for lipase production [18]. In the case of *Pseudomonas areuginosa* EF2 [19] and *Bacillus licheniformis* [20] alkalinity is beneficial, whereas acidic pH is optimum for lipase production by *R. homothallicus* [21]. In addition to physiological parameter, various components in the medium also play an important role in the production of lipase by all sorts of microorganisms. Nitrogenous sources in the medium are important in the synthesis of lipase. The influence of nitrogen source on lipase production was shown in Fig.3. Peptone was determined to be the best nitrogen source for lipase yield. Rahman *et al.* reported peptone was also superior to other nitrogen source for producing S5 lipase [17]. The major factor for the expression of lipase has always been carbon source [22]. In our study, the most suitable carbon source was glycerol among all carbon source studied, while the lowest lipase activity was obtained with maltose (Fig. 4).

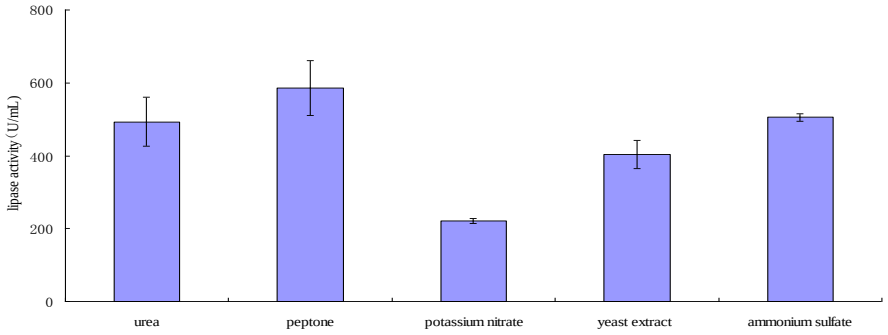

Figure 3. The effects of different nitrogen source on the production of lipase

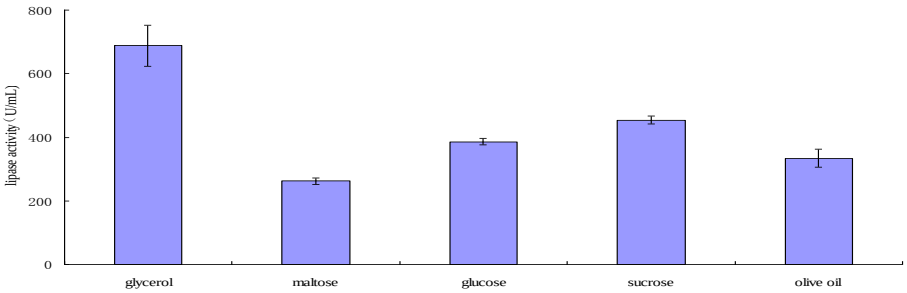

Figure 4. The effects of different carbon source on the production of lipase

Effects of Different Organic Solvents on the Activity of the Lipase from Candida Tropicalis SD7

Several lipases have been reported to be stable in organic solvents [23]. To our knowledge, there were no reports on lipase with organic solvents tolerance from *Candida tropicalis*. The effects of different organic solvents on the activity of lipase from *Candida tropicalis* SD7 were investigated. On the whole, as compared to water-miscible organic solvents (DMSO, methanol, acetone, acetonitrile), lipase from *Candida tropicalis* SD7 was little inactivated by water-immiscible organic solvents including chloroform, methylbenzene, n-hexane, petroleum ether and isooctane (Fig.

5). Similarly, a thermostable lipase from *Pseudomonas fluorescens* P21 was found to keep a residual activity of 91.4%, 94.1% and 83.5% after a 2-h incubation in non-polar solvents such as heptane, hexane and styrene, respectively [24]. Generally, organic solvents with a high log P are less influence on lipase activity than organic solvents with a low log P due to their different effects of stripping the essential water from lipase molecules. The lipase from *Candida tropicalis* SD7 was moderately stable after a 30 min incubation in polar solvents, including DMSO, acetone, acetonitrile and methanol with a remaining activity of 81.2%, 78.8%, 73.4% and 63.3%, respectively. Whereas 73%, 36% and 0% of residual lipase activity from *Serratia marcescens* ECU1010 were observed after incubation for 12 h at 10% (v/v), 25% (v/v) and 50% (v/v) of ethanol [8]. Thus, *Candida tropicalis* SD7 produced an organic solvent tolerant lipase that was stable in both polar and non-polar organic solvents, suggesting the potential in biosynthetic applications.

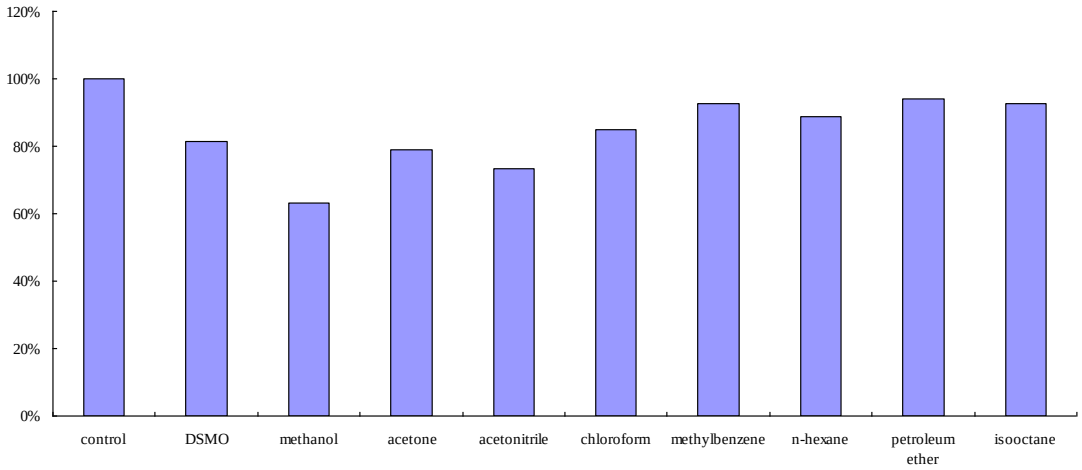

Figure 5. Effect of various organic solvents on activity of the lipase from Candida tropicalis SD7

Conclusion

A novel fungus named *Candida tropicalis* SD7 producing lipase extracellularly, which was stable in both polar and non-polar organic solvents, was isolated from soil. The optimum lipase production was observed in the medium with glycerol as carbon source and peptone as nitrogen source through submerged fermentation at 30℃ and initial pH 7.0.

Acknowledgements

The financial support from the National Natural Science Foundation of China (31360216) and Jiangxi provincial science and technology Pillar Program is thankfully acknowledged.

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