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Isolation of Lipase Producer and Its Performance in Enantioselective Hydrolysis of Glycidyl Butyrate

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Abstract

A racemic glycidyl butyrate resolving strain, preliminarily identified as a *Rhizopus* sp., had been isolated from soil. Its extracellular lipase was found to enantioselectively hydrolyze the (*S*)-enantiomer of the chiral ester, with optimal activities at pH 5.3 and 42°C. Higher enantioselectivity of the enzyme was observed at lower temperatures, while the best enantioselectivity was obtained at pH 5.5–6.0, with an *E* value (enantiomeric ratio) of 57.

Index Entries: (*R*, *S*)-Glycidyl butyrate; optical resolution; lipase; *Rhizopus* sp.; isolation.

Introduction

Nonracemic glycidols are versatile chiral synthons for the synthesis of many pharmaceuticals such as β-blockers, L-carnitine, platelet aggregation factor, and (*S*)-HMPA (anti-viral), and are also used as synthetic intermediates of a large number of biologically active compounds such as chiral phospholipids, chiral glycols, and insect pheromones. Moreover, they have found use in new materials such as ferroelectric liquid crystals and biochemical probes into the mechanism of enzyme systems. Their wide application has made them attractive goals for many companies and institutes to pursue *(1,2)*.

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So far, there are five categories of preparation methods of nonracemic glycidols: chemical syntheses, asymmetric epoxidation, lipase-catalyzed hydrolysis, enzymatic esterification in organic solvent, and microbial transformation. The earliest approach to nonracemic glycidol could be traced back to 1914 by Abderhalden and Eichwald *(3)*. By chemical methods, there were two classical routes: from D-mannitol to (*R*)-glycidol *(4)* and from L-serine to (*S*)-glycidol *(5)*, but excessive steps and expensive reagents involved prevented their commercialization. Asymmetric epoxidation of allyl alcohol is a commercialized method, and both enantiomers of glycidol can be produced in the presence of Sharpless catalyst *(6–8)*; however, the resultant enantiomeric excess (ee) of the isomer formed is not yet satisfactory. Lipase-catalyzed hydrolysis of esters of glycidol is regarded as an elegant method *(9)*. After the enzymatic resolution of (*R,S*)-glycidyl butyrate by porcine pancreatic lipase (PPL) that has a preference for (*S*)-ester, (*R*)-glycidol and (*R*)-ester were prepared. The later compound could be further converted into (*S*)-glycidol by hydrolysis *(9)* or transesterification *(10)*. Recently, this method has been improved by immobilizing PPL on a hydrophobic carrier, octyl-agarose *(11)*. The preparation via lipase-catalyzed reaction in nonaqueous medium has also been considered. Optically active glycidol or ester was produced after transesterification of racemic glycidol with carboxylates *(12–14)*, or esterification of glycidol with butyric acid in organic solvent *(15)*. However, the application of these approaches are still technically difficult due to some disadvantages of enzymatic reaction in organic solvents *(16)*. Recently, microbial methods have been reported, in which enantiomers could be obtained via enantioselective oxidation of racemic glycidol by some bacteria *(17)*, enantioselective hydrolysis of glycidyl fatty acid esters by microorganisms *(18)*, or in other ways *(19)*.

Usually, high optical purity of the products could be achieved by microbial methods owing to the high enantiospecificity of their enzymes. Because of the advantages of low cost of materials and simple downstream process, hydrolysis is liable to be performed in the preparation of nonracemic glycidol. We report here the isolation and some characteristics of a wild-type strain capable of selectively hydrolyzing the (*S*)-enantiomer of racemic glycidyl butyrate.

Materials and Methods

Chemicals

Glycidyl butyrate (substrate) was synthesized according to the literature method *(20)*, with purity ≥98%. All other chemicals used were from commercial sources and of analytical reagent grade.

Soil Samples and Some Strains

More than 200 soil samples were collected in China. *Aspergillus niger* Bc0-11, *Candida lipolylica* Bc2-01, *Mucor* sp. Bc0-01, and *Pseudomonus aeruginosa* Bc1-01 were preserved in our laboratory.

Media

Enrichment medium for bacteria: 2.0 g glucose, 0.5 g peptone, 0.5 g yeast extract, 0.05 g $MgCl_2 \cdot 6H_2O$, 0.6 g substrate, 100 mL tap water, at pH 8.0.

Isolation medium for bacteria was the same as the enrichment medium except for the exclusion of substrate (solid medium contained 2% agar).

Enrichment medium for fungi: 1.0 g glucose, 0.5 g peptone, 0.1 g KH_2PO_4, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.3 mL 1% chloramphenicol, 0.6 mL substrate, 100 mL tap water, at pH 6.5.

Isolation medium for fungi was the same as the enrichment medium except the exclusion of substrate and chloramphenicol (solid medium contained 2% agar).

Fermentation medium: 1.0 g glucose, 0.5 g peptone, 0.3 g yeast extract, 0.3 mL malt extract, 0.1 g KH_2PO_4, 0.05 g $MgSO_4 \cdot 7H_2O$, 100 mL tap water, at pH 7.0.

All media were heat-sterilized at 121°C for 20 min before use.

Enrichment Cultivation

Soil sample (about 0.5 g) was added to a 15 × 150 mm test tube containing 3.5 mL enrichment medium, and the tubes were set inclined (to maintain aerobic condition) on a ratory shaker (180 rpm) at 28°C for 4–5 d. Inocula were diluted serially to an appropriate density, and then were spread on plates with solid isolation media and incubated at 28°C for several days. Colonies appearing on the plates were picked up and stored in a refrigerator for further isolation.

Isolation of Microorganisms

Screening was accomplished in three stages. Primary isolation was done as follows: each colony of bacterium or fungus was inoculated into a test tube containing 3 mL isolation medium and incubated inclined on a shaker (180 rpm) at 28°C for 2 or 3 d, then 20 µL of substrate was added with a pipet. One day later, 1 mL ethyl acetate was added to the mixture and shaken for 5 min, then to the separated organic layer was added 0.1 mL color developing reagent for fatty acid (aqueous solution of 4% cupric acetate, with pH adjusted to 6.1 by pyridine). The colonies with color developing reaction were picked out. Secondly, each colony was cultivated in two 250 mL Erlenmeyer flasks (each containing 30 mL medium) and into one inoculum was injected 0.2 mL of substrate, while the other one without substrate was used as a blank for color test. The mixtures were extracted with 6 mL ethyl acetate and 0.3 mL color reagent was added to each 4 mL of extracts. The concentration of butyric acid produced was expressed by the OD_{710} value measured with a spectrophotometer (Shanghai Analytic Instrument Factory, 7230-G type). The colonies having larger OD_{710} than the blank sample were picked out. Third, each colony was cultivated in several 500 mL Erlenmeyer flasks containing 60 mL of medium, and 0.5 mL

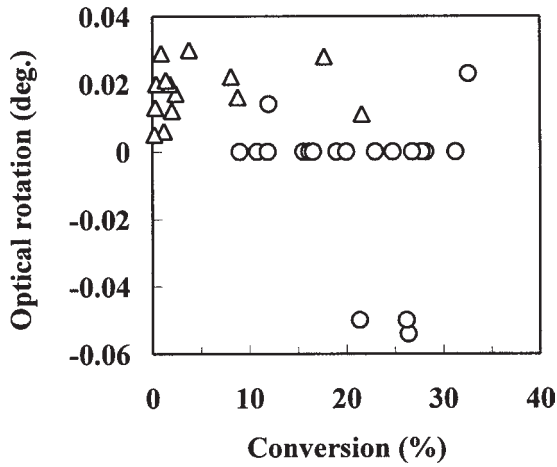

Fig. 1. Representative results of part of the isolated strains in the third screening. Symbols: △ bacteria; ○ fungi. The experiments were performed according to the method described in *Materials and Methods*.

of substrate was injected into each flask. The residual substrate (*R*-ester) in the bioconversion mixture taken at regular intervals was extracted by *n*-hexane and the optical rotation was determined with an automatic digital polarimeter (Shanghai Physico-Optical Instrument Factory, WZZ-1S type). The colonies capable of generating optical rotation were picked out (Fig. 1).

Lipase Assay

Lipase activity was measured by titrating fatty acid liberated from olive oil with 0.010 *M* NaOH. One unit (u) of lipase activity was defined as the amount of enzyme that liberates 1 µmol fatty acid per minute at 40°C and pH 7.5. An olive oil emulsion was prepared as follows: 150 mL of 4% polyvinyl alcohol aqueous solution and 50 mL olive oil were homogenized for 6 min. Then, 4 mL of olive oil emulsion and 5 mL of 0.025 *M* phosphate buffer (pH 7.5) were placed in a reciprocal bath shaker (120 strokes/min, 40°C). The mixture was preincubated for 5 min and 1 mL of fermentation broth was added. Fifteen minutes later, 15 mL ethanol was added to stop the reaction, and lipase activity was then measured by titration.

Measurements of Initial Rate and Conversion Ratio

Seventy milliliters of fermentation broth were placed in a thermostated reaction vessel equipped with a pH electrode and magnetically stirred at 200 rpm. After preincubation, the reaction was started by adding 0.72 mL (5 mmol) of substrate and the pH was kept constant by automatically adding 1 *M* NaOH with an auto-titrator. The conversion ratio was determined according to the amount of exhausted base neutralizing the butyric acid

from the substrate. The average reaction velocity in a range of 0–5% conversion was determined as the initial rate.

*Enantiomeric Excess of (*R*)-Ester*

To 70 mL of fermentation broth buffered with 0.2 *M* citrate-phosphate was added 0.72 mL substrate (5 mmol) and the mixture was incubated on a shaker (160 rpm). After reaction was completed, residual substrate was extracted by 18 mL toluene and dried over anhydrous $MgSO_4$. The optical rotation (α) and concentration (c, w/v) of the extract were determined with polarimeter and gas chromatography (Simadazu 14B, Japan), respectively. Then specific rotation [α] ($= \alpha/cl$, $l = 1$ dm) and ee ($= -[\alpha]/37.2°$) as well as enantiomeric ratio (E value) *(21)* were calculated, respectively. (Note: the reported theoretic value of $[\alpha]_D$ is –28.4° in $CHCl_3$ *(5)* and was tested to be –37.2° in toluene in our work.)

Results and Discussion

Screening of Glycidyl Butyrate Asymmetrically Hydrolyzing Strains

More than 1000 colonies (partially duplicate) had been examined after enriched culture. The purpose of adding racemate in the enrichment media was to obtain glycidyl butyrate–inducible and –tolerable hydrolase producers. It was found that no colony was obtained in some soil samples, which might be caused by the substrate inhibition presumably due to its epoxy group.

Thirty colonies of bacteria and 116 colonies of fungi (several duplicate) were obtained after the primary isolation. It was observed that many strains secreted pigments of various colors, and it was also known that many microbes produced organic fatty acids accompanying the growth. Therefore, contrast test (with control sample) was done to eliminate the interference. Twenty-five strains including 19 fungi and 6 bacteria were found to have higher lipase production. In the third isolation, a mold designated as My9 was proved capable of asymmetrically hydrolyzing the racemate and preferential to (*S*)-enantiomer; *Aspergillus niger* Bc0-11 had the similar function, but its specificity was lower than My9; a yeast (Jy19) displayed the reverse but fairly low enantioselectivity. Figure 2 shows the time-course of substrate hydrolysis by My9.

Morphological Characteristics of My9

My9 was identified as a *Rhizopus* sp. based on some typical morphological characteristics of genus *Rhizopus (22–24)* such as rhizoids, nonsepta hyphae, and ellipsoid sporangiospores, and was thus named as *Rhizopus* sp. Bc0-09 in our work. When it was inoculated onto potato dextrose agar (PDA) medium and incubated at 30°C, the strain grew rapidly and rampantly; it was white in the early stage and turned brownish gray after maturity, with enormous black sporangia appeared. The colony was

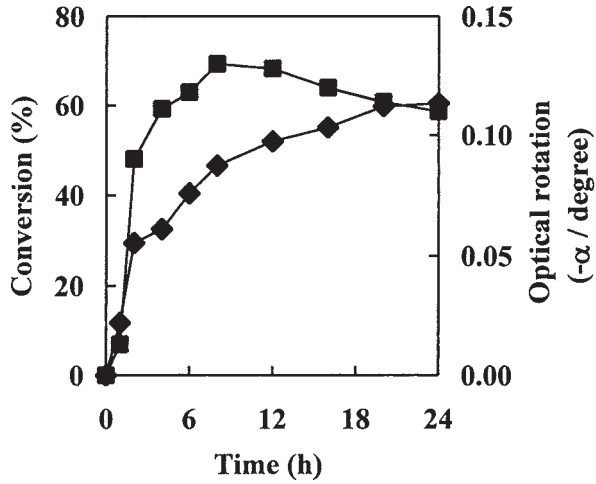

Fig. 2. Conversion ratio (◆) and optical rotation of hexane extract (■) as functions of reaction time. My9 was cultivated in several flasks with 60 mL medium for 3 d and then 0.6 mL substrate was added for conversion. At intervals, one of the flasks was withdrawn for determination of conversion ratio by titration and the residual substrate was subjected to determination of optical rotation after extraction into 18 mL *n*-hexane.

Table 1
Influences of Initial pH of Medium
and Cultivation Temperature on the Broth Lipase Activity

pH[a]	4.7	5.0	5.5	6.0	6.5	7.0	7.5
Activity (u/mL)	1.67	1.68	1.63	1.78	2.46	2.82	1.90
Temperature[b] (°C)	28		30	32		35	37
Activity (u/mL)	2.39		2.49	2.11		2.45	2.20

[a]The strain was cultivated in Erlenmeyer flasks containing 60 mL medium of different initial pH on a shaker (180 rpm) at 30°C for 48 h.

[b]The strain was cultivated with the initial pH of medium at 7.0 on a shaker (180 rpm) at different temperatures for 60 h.

cottony, dense, up to 1–2 cm thick, and there were no liquid drops or crystals on the colony. Hyphae had no septa, tube-form, 14–24 μm diameter, 2–3 erect sporangiophores arose in group opposite a tuft of rhizoids on the stolon, up to 150–270 μm long, 13–15 μm diameter. Rhizoids finger-shaped, small, 2–6 in one cluster. Sporangia globose, borne on light yellow sporangiophores, 80–100 μm diameter; apophyses funnel-shaped; spores mostly ellipsoid, 3–3.5 × 2–2.4 μm, striate.

Effects of pH and Temperature on Cultivation

Various factors such as carbon, nitrogen sources and their proportion, trace elements, pH, temperature, and aeration affect the yield of metabolites. The effects of medium pH and cultivation temperature were exam-

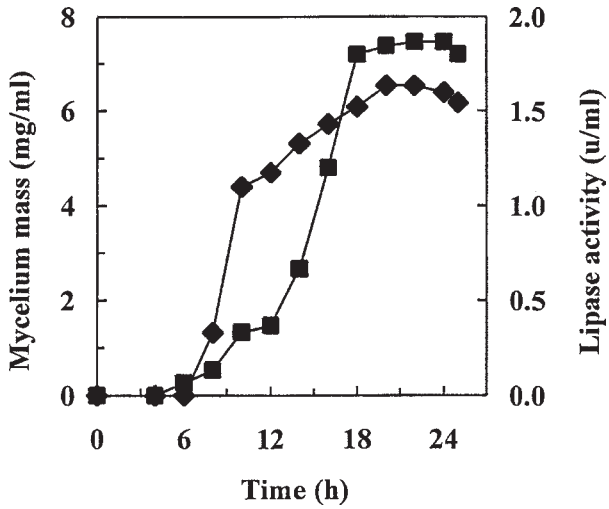

Fig. 3. The relationship between lipase activity (■) and mycelium mass (◆, dry weight) in fermentation. *Rhizopus* sp. Bc0-09 was grown in a 5-L jar fermentor (Biostat MD, B.Braun Melsungen AG, Germany) at 32°C, agitated at 700 rpm and with air of 3 L/min. The initial pH of medium was 7.0.

ined preliminarily. The pH of medium was adjusted from 4.7 to 7.5 before autoclaving, then *Rhizopus* sp. Bc0-09 was inoculated and cultivated in Erlenmeyer flasks on a shaker (180 rpm) at 30°C for 48 h. The result showed that lipase activity was affected by the initial pH of medium, especially near neutrality (Table 1). Lipase activity was not sensitive to the cultivation temperature in a range of 28–35°C (Table 1).

Time Course of Lipase Production

Fermentation of *Rhizopus* sp. Bc0-09 was performed with spore inoculation in a 5-L jar fermentor. Temperature was controlled at 32°C, stirring rate was 500–800 rpm and sufficient air was supplied. Figure 3 demonstrated the progress curve of lipase generation. The lag phase of growth lasted for about 4 h, then the medium became gradually viscous and lipase activity was able to be detected. Enzyme activity rose fast at the end of exponential growth phase and reached the maximum during the stationary phase. Twenty-four hours later, the activity tended to decrease with the cell-lysis in the death phase. This graph also indicates that there was a close relationship between the broth lipase activity and the cell mass, and a measurement indicates that the cells in death phase contained at least 40% of the total lipase.

Influences of pH and Temperature on the Hydrolysis

In the optical resolution of glycidyl butyrate, it was found that the effects of pH and temperature were projecting. At 30°C, the relationships of initial rate and lipase stereospecificity versus pH were given in Fig. 4.

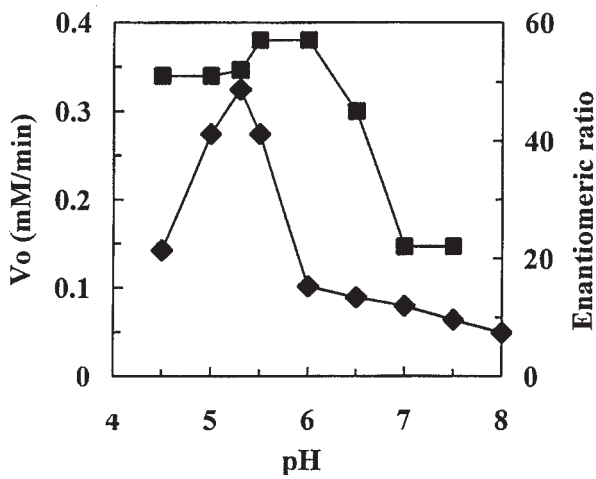

Fig. 4. Initial rate (◆) and enantioselectivity (■) as functions of system pH. Autotitration was adopted to measure the initial rate at 30°C with 5 mmol substrate in 70 mL broth (2.8 u/mL). In the test of lipase enantioselectivity, 5 mmol substrate and 70 mL buffered broth (2.8 u/mL) with 0.2 *M* citrate-phosphate were mixed and incubated at 160 rpm, 30°C for 12 h, then the ee value and the conversion were measured to give the *E* value.

The curve reveals that initial rate was remarkably sensitive to pH around 5.3 and reached the highest at pH 5.3. The enantioselectivity was higher in a range of pH 5.5–6.0. Though the peaks of two curves did not coincide, pH 5.5 was normally adopted to achieve high optical purity of (*R*)-ester.

The investigation of temperature illustrates that the initial rate increased with the rising temperature, as normal reactions rule, and reached the highest at 42°C, then there appeared a slightly decreasing tendency, which suggests that lipase would seriously denature after 42°C (Fig. 5). The profile curve of *E* value versus temperature also reveals that the specificity had a corresponding improvement with the decrease of reaction temperature, but not apparent (Fig. 5).

The actions of pH and temperature might lie in that their changes usually result in apparent variation of reaction intrinsic constants such as k_{cat}, K_m, etc., and directly affect the reaction rate, *E* value as well as enzyme stability.

Stability of Lipase

Broth (3 u/mL) was adjusted to pH 4.5, 5.0, 5.5, or 6.7 (initial pH), stored at 20, 30, 40, or 50°C for a certain time, and then the remaining lipase activities were checked periodically. It was surprising that the half-life ($t_{1/2}$) was much shorter than that of ordinary dry enzymes (usually many days) (Fig. 6). The reason might be that free lipase could not maintain a stable conformation like that which existed in vivo or a rigid structure like crys-

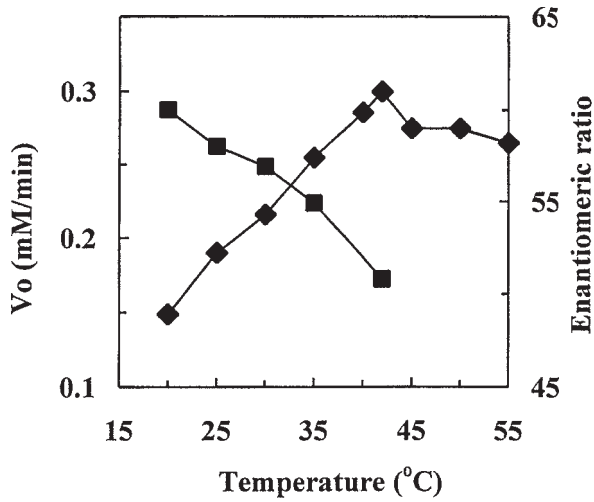

Fig. 5. Initial rate (◆) and lipase enantioselectivity (■) as functions of temperature. Auto-titration was adopted for measuring the initial rate at pH 5.5 with 5 mmol substrate and 70 mL broth (1.8 u/mL). In the test of lipase enantioselectivity, 5 mmol substrate and 70 mL buffered broth (2.8 u/mL, pH 5.5) with 0.2 *M* citrate-phosphate were mixed and incubated at 160 rpm for 12 h at different temperatures, then the ee value and the conversion were measured to give the *E* value.

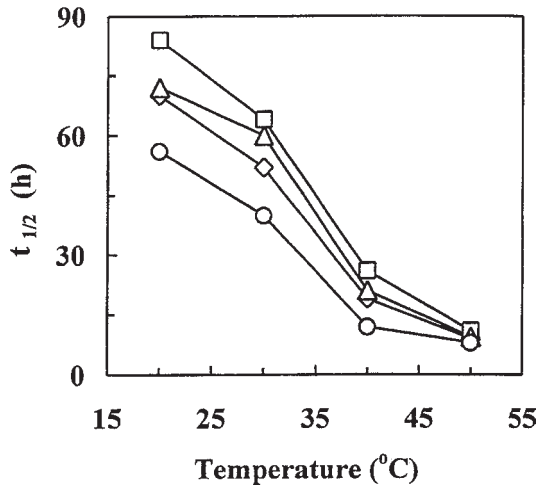

Fig. 6. Influences of pH and temperature on the lipase stability. The broth (3.0 u/mL) was adjusted to different pH and incubated at different temperatures, lipase activities were measured at regular intervals. Symbols: pH 4.5 (□), pH 5.0 (△), pH 5.5 (◇), pH 6.7 (○).

tallized enzyme. It was found that pH 5.0 was optimum for the maintenance of activity, when the environment was above 40°C, temperature would be the main factor speeding up the denaturation.

Our work focused on a convenient route of preparing (*S*)-glycidol or its precursor of highly optical purity. A *Rhizopus* sp. had been isolated from soil, which was capable of secreting extracellular lipase to hydrolyze the (*S*)-isomer of racemic glycidyl butyrate at high stereospecificity. Some properties in the optical resolution were preliminarily investigated. Under optimal conditions (pH 5.5, 30°C), the enantiomeric excess of (*R*)-ester could reach 97% at a conversion of 53%. Compared with the PPL-catalyzed hydrolysis *(2)* in which the *E* value was calculated to be only 13 according to the reported results, the higher enantioselectivity of the lipase from *Rhizopus* sp. Bc0-09 means that (*R*)-glycidyl butyrate of the same optical purity could be achieved at a smaller consumption of substrate when using *Rhizopus* sp. Bc0-09 as catalyst.

In general, high enantioselectivity is a predominant advantage of enzymatic reaction in the manufacture of chiral compounds. The mechanisms of various factors affecting enzymatic catalysis are very complicated, because pH, temperature, and other factors controlling the protonation equilibrium of acidic or basic groups of amino acids can change the polarity and geometry of the active sites of enzyme *(25)*. In order to study the mechanism of optical resolution of (*R, S*)-glycidyl butyrate, the purification of lipase from *Rhizopus* sp. Bc0-09 is essential, and this work is currently in progress.

Acknowledgment

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