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Production of (2*R*,3*S*)-3-(4-Methoxyphenyl) Glycidic Acid Methyl Ester Using an Emulsion Bioreactor Containing Lipase from *Serratia marcescens*

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ABSTRACT

An emulsion bioreactor for production of (2*R*,3*S*)-3-(4-methoxyphenyl) glycidic acid methyl ester ([−]MPGM) from a racemic mixture ([±]MPGM) using the lipase from *Serratia marcescens* has been proposed. Kinetics of hydrolyzing reaction and purification of (−)MPGM from the reaction mixture were investigated to provide a basis for industrial application of this bioreactor. The hydrolyzing reaction in the bioreactor proceeded at a rate that was first order in substrate concentration. The reaction rate was affected by a stirring speed and the ratio of the aqueous phase containing lipase to the toluene phase containing substrate. Phase separation after the enzymatic reaction was accomplished by addition of surfactant to the reaction mixture, and crystalline (−)MPGM with a chemical purity of 100% and optical purity of 100% enantiomeric excess was obtained in a high yield of 40–43% by concentration of the toluene solution.

Index Entries: 3-Phenylglycidic acid ester; lipase; *Serratia marcescens;* emulsion bioreactor; biphasic reaction.

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INTRODUCTION

An optically active (2*R*, 3*S*)-3-(4-methoxyphenyl) glycidic acid methyl ester ([−]MPGM) is an important intermediate for the production of diltiazem hydrochloride that is useful as a coronary vasodilator and produced enzymatically by asymmetric hydrolysis of (±)MPGM with the lipase from *Serratia marcescens* Sr 41 8000 (*1*). We previously reported that the hydrolyzing reaction proceeds efficiently in an organic-aqueous biphasic system (2). However, the biphasic system was unfavorable for industrial purpose, because the destruction of emulsion after the enzymatic reaction and subsequent phase separation are difficult and the desired product cannot be recovered in high yields. From this viewpoint, it was of interest to develop an effective emulsion bioreactor for industrial production of (−)MPGM. We investigated the demulsification and phase separation after the enzymatic reaction by various procedures, and found that the addition of surfactant to reaction mixture was effective. This article describes the kinetics of enzymatic hydrolysis and the phase separation for industrial application of the emulsion bioreactor.

MATERIALS AND METHODS

Chemicals

(±)MPGM was purchased from Juzen Chemical Co. (Toyama), meast from Asahi Brewery Co. (Tokyo), Tween-20 from Katayama Chemical Industries Co. (Osaka), and Colorine 102 from Sanyo Chemical Industries Co. (Kyoto). L-Proline was a product of Tanabe Seiyaku Co. (Osaka) and all other chemicals were of reagent grade.

Preparation of Lipase

Lipase was the metabolite from *S. marcescens* Sr 41 8000. *S. marcescens* was cultivated in a 30-L jar fermentor (B. E. Marubishi Co., Tokyo) with a working volume of 18 L under aerobic conditions for 28 h at 25°C. The media (pH 7.0) containing meast (360 g), KH_2PO_4 (36 g), $(NH_4)_2SO_4$ (36 g), $MgSO_4 \cdot 7H_2O$(9 g), $FeSO_4 \cdot 7H_2O$ (0.18 g), sorbitan oleate (Span85, 270 g), colorine 102 (36 g), and dextrine (180 g) was used.

During the culture, 1 L of a 1.5% L-proline solution was added continuously at a flow rate of 67 mL/h to increase lipase activity. The resulting culture broth was separated into a supernatant and cells by means of centrifugation. The supernatant containing lipase was passed into a MF membrane to obtain a cell free filtrate, and then concentrated about 10–20-fold by the use of UF membrane.

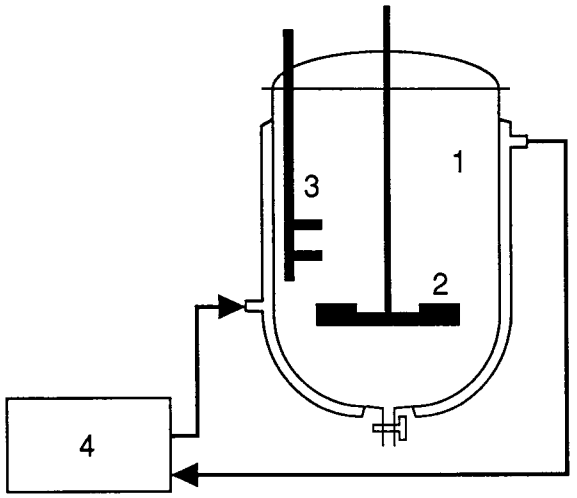


Fig. 1. Schematic diagram of emulsion reactor. (1) Vessel (working volume: 1500 mL; 120 × 230 mm); (2) impeller (3-paddles; 86 mm diameter); (3) buffle; (4) thermostat.

Standard Assay

Lipase activity of the concentrated enzyme preparation was determined according to the procedure previously described (*1*).

Reaction Experiments

A schematic diagram of the emulsion reactor designed for this study is given in Fig. 1. The reactor (working volume: 1500 mL), made of glass, was equipped with a stainless steel impeller (3-paddles; 86 mm diameter), a glass finger baffle, and jacket to control the temperature. The operation of the reactor was carried out as follows. (±)MPGM (208.2g) and toluene (1000 mL) were placed in the reactor and mixed at a stirring speed of 600 rpm at 30°C. After confirmation of the complete dissolution of (±)MPGM, a potassium phosphate buffer (330 mL; pH 8.5) preheated at 30°C containing the concentrated enzyme preparation (7.8 mL; 4000 U/mL), and $CaCl_2 \cdot 2H_2O$ (90 mg) as an activator was added and the reaction was started. After a predetermined time, the concentration of (−)MPGM in the organic phase was determined. The concentration of (+)MPGM was determined at the same time. The experiments were done with various stirring speeds and phase ratios with similar conditions.

Assay and Data Analysis

(−) and (+)MPGMs were determined by HPLC (Simadzu Corp., Kyoto, LC-6A) with the following conditions: column, Chiralcel OB (Daicel Chemical Corp., Osaka, 0.4 diameter × 25 cm); mobile phase, *n*-hexane/

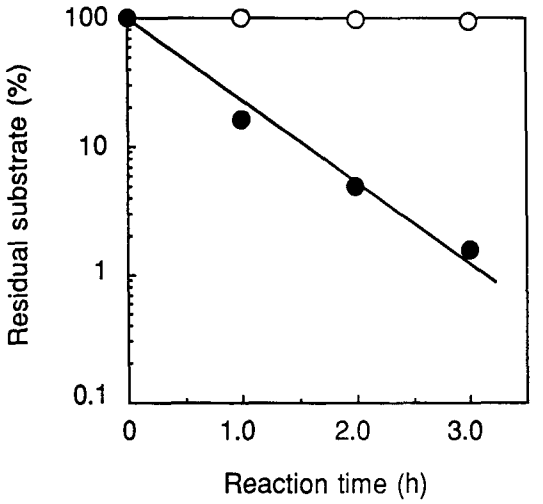


Fig. 2. Typical time course for hydrolysis of (±)MPGM by lipase from *S. marcescens*. The reaction was done at the conditions as shown in Materials and Methods. Symbols: ●, (+)MPGM; ○, (−)MPGM.

isopropanol = 10/1; detection, 236 nm; temperature, 40°C; flow rate, 1.0 mL/min. The reaction rates were calculated from the rate of decrease of (−) and (+)MPGMs.

RESULTS AND DISCUSSION

Reaction Rate

In order to provide a basis of reactor design, we investigated the reaction rate. Figure 2 shows the plots of the relative value of residual substrates ([−] and [+]MPGMs) vs reaction time. The relative residual concentration of (+)MPGM was decreased with reaction time and a good linear relationship was found between them. As the similar behavior was also found for (−)MPGM, it was concluded that this hydrolyzing rate with the lipase from *S. marcescens* obeys the following first order reaction kinetics:

$$r_S = -dC_S / dt = k_S C_S \quad (1)$$

$$r_R = -dC_R / dt = k_R C_R \quad (2)$$

where r_S and r_R are reaction rates for (±) and (−)MPGMs (M/h); C_S and C_R, (+) and (−)MPGM concentrations (M); k_S and k_R, reaction rate constant (h^{-1}); t, reaction time (h).

From the slopes of individual straight lines, k_S and k_R were calculated to be 1.40/h and 0.017/h, respectively. The ratio of k_R to k_S was a very small value, indicating that the lipase from *S. marcescens* had a very high enantioselectivity (E = 138) on hydrolysis of (+)MPGM (3).

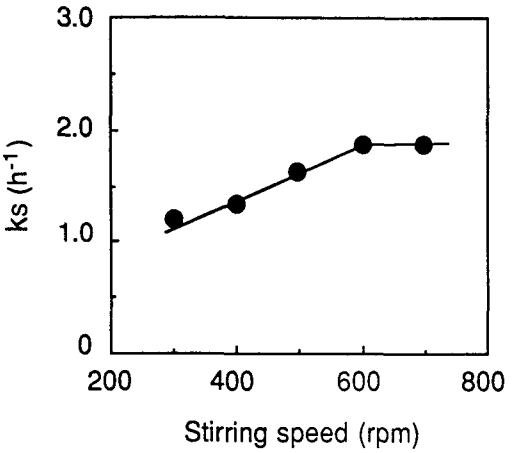


Fig. 3. Effect of stirring speed on reaction rate. k_s value calculated from Eq. 1 was plotted on stirring speed.

Factors Governing Reaction Rate

Stirring Speed

In comparison experiments, the hydrolyzing reaction proceeds faster with a high stirring speed than with a low speed. This higher reaction rate is assumed to be caused by the influences of outer diffusion and contact area. To confirm this assumption, the reaction rate was investigated in various stirring speeds. Figure 3 shows the plots of k_s vs stirring speed. As the k_s increased linearly with increasing stirring speed and reached a constant value at 600 rpm, we concluded that the aforementioned assumption is reasonable. Similar behavior is reported on the hydrolysis of esters in biphasic systems (4). They measured the emulsion pore size, and the average diameters of the suitable particles was decreased with increasing the stirring speed.

Phase Ratio

To design a more practical emulsion bioreactor for industrial production of (−)MPGM, the influence of phase ratio on the reaction rate should be ascertained. We investigated the reaction rate in various ratios of aqueous phase to organic phase. As shown in Fig. 4, the hydrolyzing reaction proceeded more rapidly in a high-phase ratio than in a low one. This is considered to be attributable to the difference of interfacial area between aqueous and organic phases.

Demulsification

The demulsification after the enzymatic reaction and subsequent phase separation are a serious problem to accomplish the industrial application of emulsion bioreactor. Several means involving an electrical method with supersonic irradiation or coalescer; a physical method with filtration,

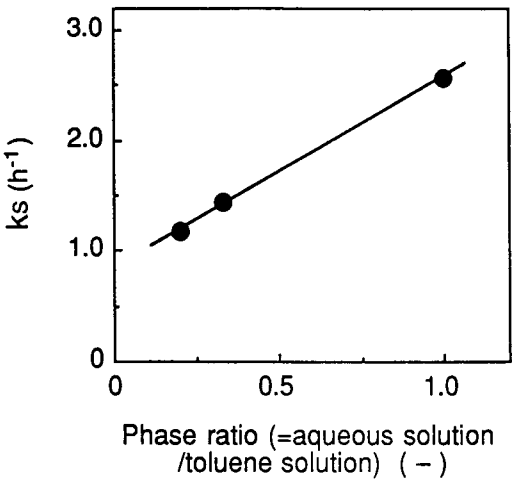


Fig. 4. Effect of phase ratio on reaction rate.

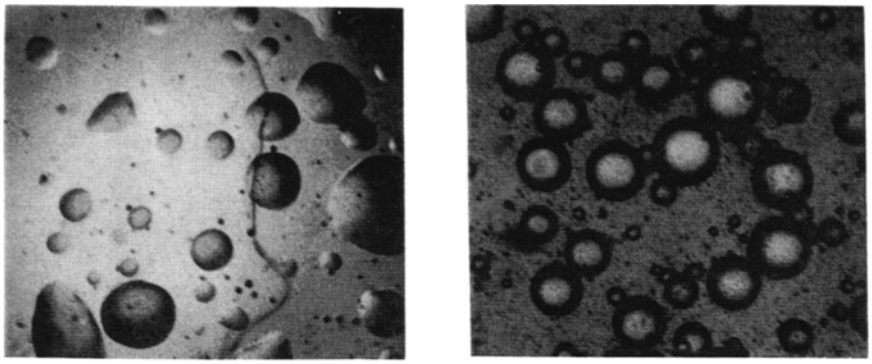
Table 1
Amount of Emulsion After Enzyme Reaction

Phase ratio, –	Amount of emulsion, %	Type of emulsion
0.2	5	I
0.3	5	I
1.0	70	II

The reaction was done for 3 h at various phase ratios and the resulting reaction mixture was permitted to stand for 1 h. Then a volume of emulsion was measured. The amount of emulsion was expressed by the percentage by volume of emulsion to reaction mixture.

centrifugation, or heating; and a chemical method with addition of surfactant or solvent have already been proposed to overcome this problem (*5–9*). Among them, the chemical method using surfactants would be most favorable for industrial purposes because of ease of operation. From this viewpoint, the demulsification by the use of surfactants was investigated.

We investigated the amount of emulsion after the enzyme reaction commencing with the demulsification study. As shown in Table 1, the amount of emulsion was dependent on a ratio of aqueous phase to toluene phase. For a low-phase ratio, the amount of emulsion was little and an aqueous solution containing enzyme and toluene solution containing product separated successfully. For a high-phase ratio, on the contrary, the emulsion was high and inseparable. This difference in demulsification was considered to be attributable to the type of emulsion. To confirm the finding, the photograph study using a water-soluble dye as a marker was made on these two emulsions (I and II). Figure 5 shows a microscope photograph for emulsions I and II. The results showed that the emulsion



[I] [II]

Fig. 5. Microscope photograph of emulsion. To emulsions I and II, a water soluble dye (Alizarin Red S) was added and a microscope photograph of 20 magnifications was prepared for individual emulsions. [I] W/O emulsion with colored water droplets as a dispersed phase. [II] O/W emulsion with a darkly colored water as a continuous phase.

Table 2
Effect of Surfactant on Demulsification

Surfactant	Span 85	Tween-20	CTAB[a]			SDS[b]		
Concentration[c]	0.01	0.01	0.001	0.01	0.1	0.001	0.01	0.1
Amount of emulsion, %	5–30	5–30	5–10	3–5	3–5	1–3	1	1–3

The reaction was done for 3 h at the conditions as shown in Materials and Methods. Various kinds of surfactants were added to the resulting reaction mixture and mixed for 5 min at a stirring speed of 600 rpm. After a 1-h stand, the volume of emulsion was measured.

[a]Cetyl trimethyl ammonium bromide.

[b]Sodium dodecyl sulfate.

[c]Percentage by weight of surfactant to volume of toluene solution.

I was a W/O emulsion with colored-water droplets dispersed in oil phase and the emulsion II was a O/W emulsion with an uniform distribution of a darkly colored water phase.

As mentioned earlier in this article, the extent of emulsion after the enzyme reaction could be controlled by the phase ratio but a complete demulsification was not obtained. To separate the aqueous and organic solutions more completely, we investigated the demulsification with surfactant. In this experiment, four kinds of surfactants involving Span 85, Tween 20, CTAB, and SDS were tested. Among surfactants tested, SDS, which is an anionic surfactant, showed a considerable effect on the demulsification as shown in Table 2. A near complete demulsification was obtained when a 0.01 w/v% SDS was added to the reaction mixture.

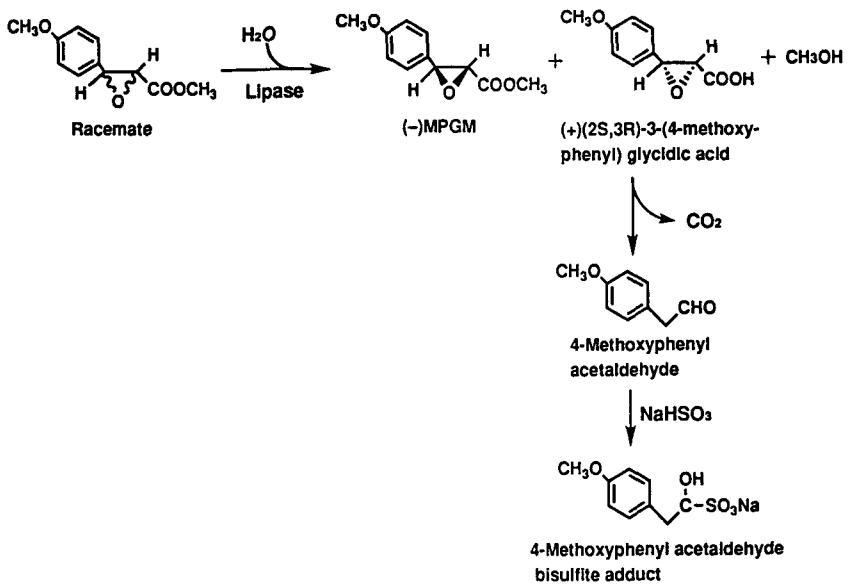


Fig. 6. Scheme of hydrolyzing reaction of (±)MPGM.

Elimination of 4-Methoxyphenylacetaldehyde from Toluene Solution

In the hydrolysis of (±)MPGM with a lipase from *S. marcescens*, 4-methoxyphenylacetaldehyde, which is a spontaneously generated derivative from (+)2*S*,3*R*-3-(4-methoxyphenyl) glycidic acid, was accumulated in the toluene solution with the desired product as shown in Fig. 6. This aldehyde compound suppressed crystallization of (−)MPGM. To isolate (−)MPGM efficiently, it was necessary to remove aldehyde from the toluene solution. It has been well known that the aldehyde compounds generally form a water soluble adduct through the reaction with bisulfite anion (*10*). So we investigated the elimination of aldehyde compound by the use of sodium bisulfite solution. As shown in Table 3, the extent of elimination of the aldehyde depended on the molar ratio of sodium bisulfite and a near complete elimination was obtained at a 1.1 molar ratio. It was considered that the extent of elimination is influenced by pH of the sodium bisulfite solution since the adduct was produced efficiently in the pH 5–9 region. We prepared sodium bisulfite solution with various pHs and used them in the experiments for removing aldehyde compounds. As shown in Table 4, the elimination rate was decreased with increasing pH. We concluded that the aldehyde compound in toluene solution can be removed successfully by using a sodium bisulfite solution with pH value around 6.

Table 3
Effect on Sodium Bisulfite on Elimination of 4-Methoxyphenylacetaldehyde from Toluene Solution

Molar ratio of sodium bisulfite to (−)MPGM	Extent of elimination,[a] %
0.5	67.5
0.9	81.6
1.0	88.8
1.05	91.7

The reaction mixture treated with surfactant (SDS) was separated into a toluene solution and an aqueous solution. The toluene solution was contacted with a sodium bisulfite solution (pH 6.3) for 5 min at 30°C and then residual aldehyde concentration in the toluene solution was measured by HPLC.

[a]Calculated on the basis of the initial concentration of aldehyde compound.

Table 4
Effect of pH of Sodium Bisulfite Solution of Elimination of 4-Methoxyphenylacetaldehyde from Toluene Solution

pH of sodium bisulfite solution		Extent of elimination, %
Initial	Final	
6.0	6.4	100
6.2	7.2	96
6.5	9.1	80
7.0	9.9	65

The experiment was done at similar conditions as described in Table 3, except that the sodium bisulfite solutions with various pHs were used as a substance to extract aldehyde compound.

Isolation of (−)MPGM

The crystallization of (−)MPGM was investigated for a toluene solution treated with sodium bisulfite solution. One liter of the toluene solution was contacted with $NaHCO_3$ solution to neutralize, washed with water, dried over anhydrous Na_2SO_4, and used as a preparation for crystallization.

When the preparation was concentrated to one-tenth of an ordinary volume, crystalline (−)MPGM was obtained with a high yield of 40–43%. Both the chemical and optical purities of (−)MPGM were 100%. As mentioned in the beginning of this article, a weak point of the emulsion bioreactor for its industrial use was that the demulsification and subsequent phase separation are difficult. However, these disadvantages could be

overcome by the use of an anionic surfactant such as SDS. This method has been applied for production of (−)MPGM using a pilot-scale emulsion bioreactor.

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