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Efficient microbial elimination of methanol inhibition for Naproxen resolution by a lipase

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Abstract

To eliminate methanol inhibition of the activity of a lipase, methanotrophic bacteria, which can convert methanol into water and CO_2, were introduced to the reaction of enantioselective hydrolysis of Naproxen methyl ester catalysed by lipase from *Candida rugosa*. Both the activity and stability of lipase were improved by the removal of methanol by the bacteria.

Introduction

(*S*)-(+)-2-(6-Methoxy-2-naphthyl)propionic acid (Naproxen) is a non-steroidal, anti-inflammatory drug, which belongs to the family of 2-aryl propionic acid derivatives. As the physiological activity of the *S*-Naproxen is 28-fold higher than that of the *R*-form (Pandey *et al.* 1999), the production of *S*-Naproxen has been intensively explored. Among the methods used, enzymatic resolution of Naproxen is efficient and can be performed by hydrolysis (Battistel *et al.* 1991, Chang *et al.* 1999) or esterification (Tsai *et al.* 1996, Duan *et al.* 1997). For enzymatic hydrolysis, the resolution of Naproxen can be performed in aqueous system or in aqueous-organic biphasic system. In the former system, the relatively low solubility of substrate causes a low reaction rate (Akita *et al.* 1989), and the separation of substrate and product is also difficult. In the latter system, the substrate can be dissolved in an organic phase while the emulsion in the aqueous-organic biphasic system may cause complicated performance and decreased stability of lipase (Rakel *et al.* 1994).

To overcome the aforementioned problem, an improved trapped, aqueous-organic biphasic system was developed recently (Halling 1987, Xin *et al.* 2000a), which avoids the emulsification of water and organic solvent and increases the activity of lipase. However, the inhibition of lipase by methanol due to the low water content in a trapped aqueous-organic biphasic system becomes significant. In other words, methanol, at a relatively low conversion in a trapped aqueous phase, will severely inhibit lipase activity. Several methods, including photocatalysis and continuous extraction of methanol, have been proposed to eliminate methanol (Battistel *et al.* 1991, Xin *et al.* 2000a,b). However, photocatalysis decreases the stability of lipase due to the formation of intermediates, such as formaldehyde, formic acid, etc. while the continuous extraction method complicates the reaction and consumes more energy.

Here we present a new and simple method for the elimination of methanol in a batch reaction by introducing methanotrophic bacteria to the reaction system. Methanotrophic bacteria, containing methane monooxygenase, alcohol dehydrogenase and other enzymes, can convert methane into methanol, and then methanol is converted into water and CO_2 subsequently under mild condition. The introduction of this kind of bacteria to the resolution process is then beneficial for the elimination of methanol. In addition,

because it is biocompatible it should be harmless to the activity of lipase.

Materials and methods

Preparation of cells

Two methanotrophic bacteria, *Methylosinus trichosporium* IMV 3011 (a kind gift from Russian Academy of Sciences) and *Methylomonas* sp. GYJ3 (isolated in our laboratory), were used. The bacteria were cultivated in a shaking flask (1000 ml) with methane/air (1:1, v/v) at 32 °C for 3–4 days. After cultivation, the cells were harvested by centrifugation at 9000 × *g* and washed several times with 0.1 M sodium phosphate buffer (pH 7.5). The washed cells were resuspended in the above phosphate buffer at about 4 mg dry cell per ml.

Degradation of methanol in aqueous solution

The methanol was added to a 250 ml conical flask containing 10 ml suspension of bacteria (both the cell dry wt of strain GYJ3 and 3011 were $\sim$ 4 mg ml^{-1}). The initial concentration of methanol for strain IMV 3011 was 25 mM. For strain GYJ3, it was 25 mM and 2 mM. The reaction was started with shaking the flask at 30 °C. At intervals, samples (0.5 ml) were centrifuged and the supernatants were analyzed using GC to determine the concentration of methanol. GC analysis was performed using a 25 m × 0.25 mm SE-54 capillary column and an FID detector.

Immobilization of lipase

The mesoporous molecular sieve support, MCM-41 (pore diam. 2.78 nm and a surface area of 1000 m^2 g^{-1}), which was kindly provided by Dr Xin Jing in our laboratory, 0.5 g, was suspended in 25 ml isooctane in a 250 ml conical flask. *Candida rogusa* lipase (free form, 780 U mg^{-1}, from Sigma), 100 mg, was dissolved in 0.75 ml sodium phosphate buffer (0.1 M, pH 7.5) and then added into the conical flask with gentle stirring. After adsorption (30 min at room temperature), the isooctane was decanted and the molecular sieve-immobilized lipase was ready for the resolution of Naproxen.

Extraction of methanol

To demonstrate that the methanol can be extracted from the trapped aqueous phase into the silicone tubing, an experiment without the lipase and substrate was performed: 0.5 g MCM-41 was added to a 250 ml conical flask containing 20 ml isooctane, then 2 ml sodium phosphate buffer (0.1 M, pH 7) containing 0.25 mmol methanol was added to the conical flask with gentle stirring. Thirty min later, a silicone tubing (50 cm) with 0.7 mm wall thickness and 6 mm inner diameter (purchased from Rubber Medical Materials Factory in Shandong Province, China) containing 10 ml sodium phosphate buffer (0.1 M, pH 7) was coiled on the bottom of the conical flask to make contact with the isooctane. To avoid the leakage of suspension of bacteria, the two ends of the silicone tubing were kept upward and near the top of the conical flask. The flask was shaken at 30 °C. At intervals, samples (0.5 ml) were centrifuged and the supernatants were analyzed using GC.

Enzymatic resolution of Naproxen

In a 250 ml conical flask, 0.6 g immobilized lipase (0.5 g support and 0.1 g *Candida rogusa* lipase) containing 0.75 ml sodium phosphate buffer (0.1 M, pH 7.5) was added to 20 ml isooctane that contained 0.68 mmol Naproxen methyl ester. Silicone tubing (50 cm) containing 10 ml suspension of *Methylosinus trichosporium* IMV 3011 (4 mg dry wt ml^{-1}) was coiled on the bottom of the conical flask and the two ends of the silicone tubing were kept upward and near the top of the conical flask. The resolution reaction was started by shaking the flask at 30 °C. The substrate solution and suspension of bacteria were replaced with fresh substrate solution and suspension of bacteria every 72 h. The concentration of Naproxen methyl ester was quantitatively measured spectrophotometrically at 270 nm.

Results and discussion

Selection of suitable methanotrophic bacteria from different sources

Our previous experiments showed that adding various amounts of methanol at the beginning of a hydrolysis reaction decreased the enzyme activity (Xin *et al.* 2000a). The elimination of methanol is therefore desirable. In our present experiments, two types of

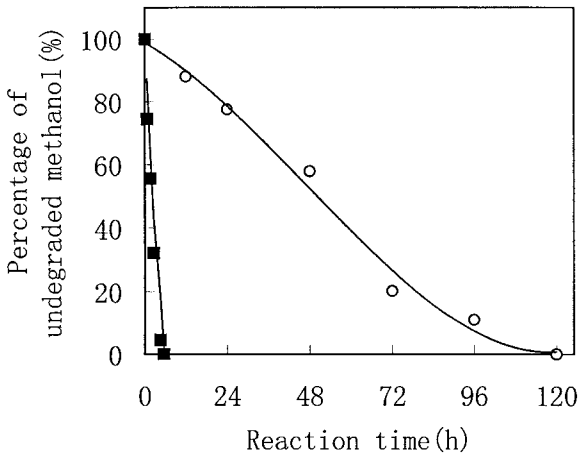

Fig. 1. Time-course of methanol degradation by *Methylosinus trichosporium* IMV 3011 (■) and *Methylomonas* sp. GYJ3 (○). The methanol was added to a 250-ml conical flask containing 10 ml suspension of bacteria (both the cell dry wt of strain GYJ3 and 3011 were ~4 mg ml^{-1}). The initial concentration of methanol for strain IMV 3011 was 25 mM. For strain GYJ3, it was 2 mM. The reaction was started with shaking the flask at 30 °C. In intervals, samples (0.5 ml) were centrifuged and the supernatants were analyzed using GC.

Table 1. Conversion of Naproxen methyl ester as a function of time in the trapped aqueous-organic biphasic system with/without bacteria from *Methylosinus trichosporium* IMV 3011 for the elimination of methanol.

Reaction time (h)	24	72	144	216
Conversion (%)[a]	9.3	21	20	20
Conversion (%)[b]	8.5	16	7.1	4.2

[a]In a 250-ml conical flask, 0.6 g immobilized lipase (0.5 g support and 0.1 g *Candida rogusa* lipase) containing 0.75 ml sodium phosphate buffer (0.1 M, pH 7.5) was added to 20 ml isooctane that contained 0.68 mmol Naproxen methyl ester. Then a silicone tubing (50 cm) containing 10 ml suspension of bacteria from *Methylosinus trichosporium* IMV 3011 (4 mg dry wt ml) was added to the conical flask. The reaction was started by shaking the flask at 30 °C. The substrate solution was decanted every 72 h and 20 ml fresh substrate solution was added to the conical flask. At the same time, the suspension of bacteria in the silicone tubing was replaced with 10 ml fresh suspension of bacteria. The concentration of Naproxen methyl ester was quantitatively measured by spectrophotometer HP 8453 at 270 nm.

[b] The reaction was performed in the same way as [a] except that no bacteria were used.

methanotrophic bacteria, GYJ3 and 3011, were tested for the elimination of methanol. The limiting values for the degradation of methanol by GYJ3 and 3011 were different although both of them can degrade methanol. Our present experiment indicated that GYJ3 could not degrade 25 mM methanol (data not shown) but strain IMV 3011 had a high degradation activity (Figure 1 shows that methanol is degraded within 6 h). When the initial concentration of methanol was decreased to 2 mM, GYJ3 degraded methanol but still had very little activity (Figure 1 shows that 120 h was needed to remove all methanol). *Methylosinus trichosporium* IMV 3011 was therefore used in the following experiments.

The use of hydrophobic silicone tubing

If centrifuged cells were added directly to the trapped aqueous-organic biphasic system, the degradation rate of methanol was much more slower than that in aqueous solution. At the same time, the water content in the trapped aqueous-organic biphasic system will be changed as the centrifuged cells contain a certain amount of water. According to the previous report (Xin *et al.* 2000a), water content is a sensitive factor that influences the activity of lipase in the trapped aqueous-organic biphasic system. To avoid the change of water content in the enzymatic reaction system and to preserve the degradation rate of methanol, a hydrophobic silicone tubing was used to separate the suspension of bacteria and the organic solvent, so that no additional water was introduced to the trapped aqueous-organic biphasic system. Some hydrophobic organic solvents will cause the silicone rubber to swell and will permeate through the silicone tubing (Brook & Livingston 1995) while water cannot. Figure 2 shows the schematic diagram of the elimination of methanol by methanotrophic bacteria in a trapped aqueous-organic biphasic system with the aid of a silicone tubing. Methanol produced during the reaction was extracted from the aqueous phase around the carrier into the organic phase. Then it was extracted into the silicone tubing and degraded to water and CO_2. Figure 3 shows the diffusion of methanol from the trapped aqueous-organic biphasic system to the aqueous solution inside of the silicone tubing. More than 50% methanol was extracted into the silicone tubing within 6 h. From this result, it can be speculated that if methanol produced in the reaction system was extracted into the silicone tubing and degraded by methanotrophic bacteria continuously, the concentration of methanol in the reaction system will be controlled at very low level. The inhibition of methanol on the activity of lipase will then be eliminated.

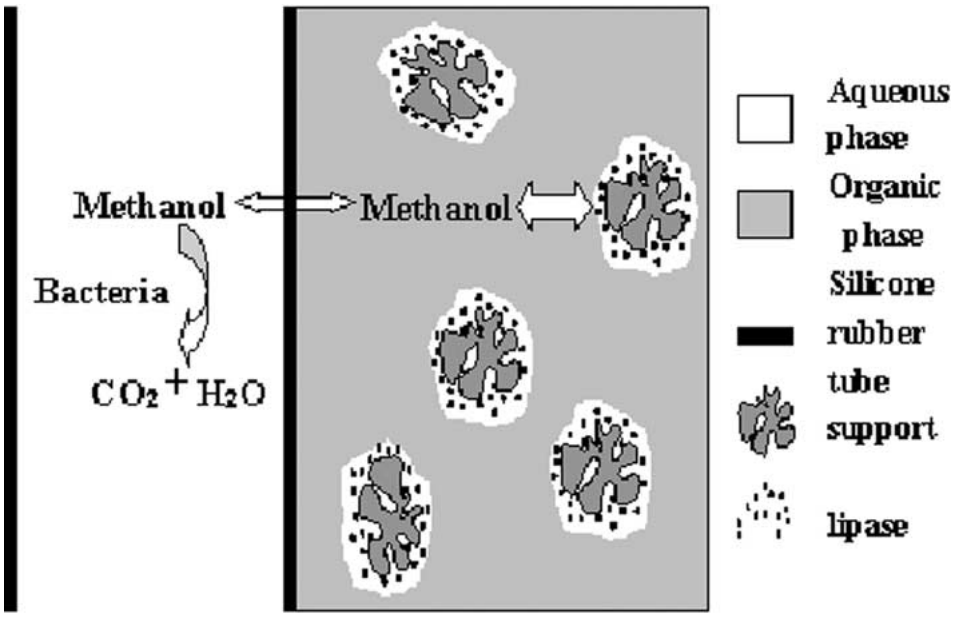

Fig. 2. Schematic diagram of the elimination of methanol by bacteria in the trapped aqueous-organic biphasic system. The methanol produced during the resolution process was extracted from the trapped aqueous phase to the organic phase, then to the suspension of cells in the silicone tubing. Subsequently, it was degraded to CO_2 and water by *Methylosinus trichosporium* IMV 3011.

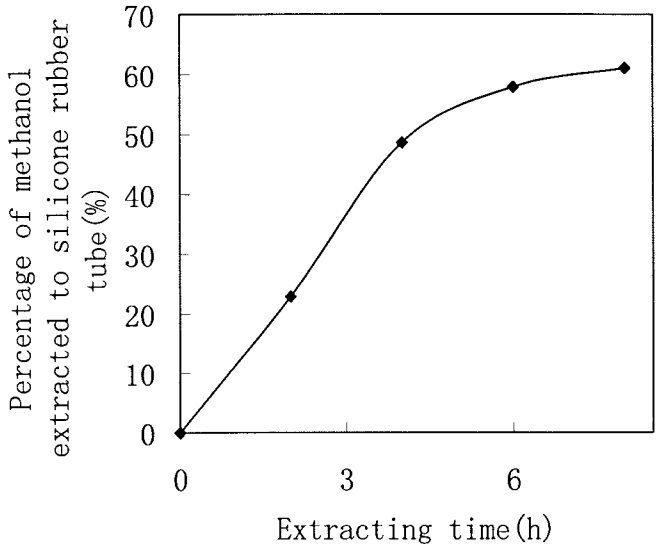

Fig. 3. Time-course of the percentage of methanol extracted into the silicone tubing (100% = 0.25 mmol methanol). 0.5 g support was added to a 250-ml conical flask containing 20 ml isooctane. Then 2 ml sodium phosphate buffer (0.1 M, pH 7) containing 0.25 mmol methanol was added to the conical flask with gentle stirring. Thirty min later, a silicone tubing (50 cm) with 0.7 mm wall thickness and 6 mm inner diameter containing 10 ml sodium phosphate buffer (0.1 M, pH 7) was added to the conical flask. The reaction was started with shaking the flask at 30 °C. In intervals, samples (0.5 ml) were centrifuged and the supernatants were analyzed using GC.

Elimination of the inhibition of methanol

On the basis of the above experiments, a novel and efficient reaction pattern was established. In the reaction system, methanol produced during the hydrolysis of Naproxen methyl ester was continuously extracted into the silicone tubing and degraded by methanotrophic bacteria quickly and completely. A control experiment without bacteria was also performed. The results shown in Table 1 indicated that the activity was increased significantly compared with the control experiment. The batch reaction was repeated for 3 times and the conversion of Naproxen methyl ester dropped slightly. However, the conversion in the control experiment decreased to 26% of the initial one after three batches. No methanol was detected in the silicone tubing during the course of reaction while in the control experiment the concentration of methanol increased. Moreover, the conversion after 24 h in the first batch of the two reaction systems was similar (9.3% and 8.5%, respectively) but the difference of conversion between the two reaction systems became larger (21% and 16%, respectively) at 72 h. This phenomenon implied that low concentration methanol inhibited the activity of lipase slightly, while high concentration methanol led to serious inhibition (Battistel *et al.* 1991).

In this paper, we presented a novel and efficient reaction pattern. With the help of silicone tubing, both

the activity and stability of lipase were improved significantly as methanol produced in the reaction system was degraded by methanotrophic bacteria in time. This method can also be used in other similar reaction system.

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