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Significantly improved esterase activity of *Trichosporon brassicae* cells for ketoprofen resolution by 2-propanol treatment

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Abstract

The kinetic resolution of racemic ketoprofen was carried out by enantioselective hydrolysis of ketoprofen ethyl ester using intact cells of *Trichosporon brassicae* CGMCC0574 as a biocatalyst. After the yeast cells were pretreated by 2 vol.% of 2-propanol for 10 h, the esterase activity on the (*S*)-ketoprofen ester increased dramatically, by a factor of ca. 310% without reducing the enantioselectivity of enzymatic resolution.

Keywords: Ketoprofen; Kinetic resolution; Esterase; *Trichosporon brassicae*; Cell treatment

1. Introduction

Ketoprofen (2-(3-benzoylphenyl)propionic acid), is widely used to reduce inflammation and relieve pain resulted from arthritis, sunburn, menstrual flow, and fever. Previous studies showed that its anti-inflammatory activity lies mainly in its (*S*)-enantiomer, while its (*R*)-enantiomer acts as an analgesic for neuropathic pain and as an antipyretic [1–3]. Therefore, it is of great interest to prepare optically pure (*S*)- and/or (*R*)-ketoprofen through optical resolution of their racemic mixture.

Biocatalysis becomes increasingly attractive in optical resolution of chiral drugs due to the simplicity of process and the high enantioselectivity of biocatalysts [4]. Over the past several years, the application of commercial enzymes to obtain chiral chemicals has received considerable attention [1,5–8]. In many cases, however, different strategies such as optimization of reaction conditions [7,8], modification of substrate and/or enzyme [9,10], purification of commercial enzyme preparations and introduction of additives [11] are indispensable to obtain satisfactory enantioseletivity and activity. These cumbersome processes have increased operational difficulty and process cost. In recent years, many researchers turned to seeking for more cost-effective methods such as microbial resolution [12] or inversion [13] for production of optically pure profens.

In a previous study [14], we reported the optical resolution of ketoprofen by a newly isolated yeast strain, *Trichosporon brassicae* CGMCC0574 (Scheme 1). However, just like many other whole-cell biocatalysts, the catalytic activity of the CGMCC0574 cells in resolution was not sufficiently exhibited owing to the permeability barrier of the cell membrane against the transfer of substrates and products. Although there is no work reported previously to alleviate this problem in optical resolution of profens by microor-

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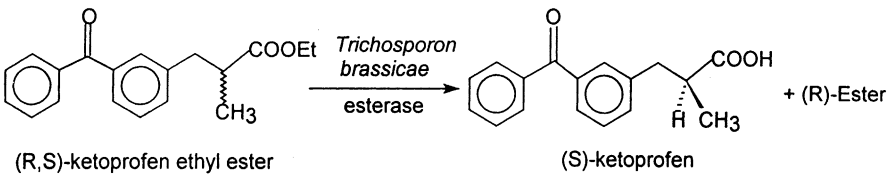

Scheme 1. Biocatalytic resolution of ketoprofen using *T. brassicae* CGMCC0574.

ganisms, a number of permeabilizing procedures for various organisms have been described to partially or totally overcome this transmembrane barrier, by using chemical reagents such as detergents [15–17] and alcohols [18,19] or by physical methods such as osmotic shock [20], ultrasonification [21], drying [22] and temperature shock [23].

In this paper, we developed an efficient cell-pretreatment procedure for the preparation of whole-cell biocatalyst with significantly improved activity. After treated by 2 vol.% of 2-propanol, the activity of resting cells of *T. brassicae* CGMCC0574 toward ethyl ester of ketoprofen was markedly increased up to 310%.

2. Materials and methods

2.1. Chemicals

Racemic ketoprofen was provided by Xi'nan Pharmaceutical Factory, Chongqing, China. Racemic ethyl ester of ketoprofen was prepared following the procedure described in [24]. All the other chemicals used were obtained commercially and of analytical grade.

2.2. Microorganism and culture

The isolation of *T. brassicae* CGMCC0574 has been described elsewhere [14]. The strain was identified by Institute of Microbiology, The Chinese Academy of Sciences (Beijing, China) and currently deposited in China General Microbiological Culture Collection Center (Beijing). The strain was grown on a medium containing 5 g/l peptone, 5 g/l yeast extract and 10 g/l glucose. Cultivation was carried out in a 5 l fermenter at 30 °C with agitation of 600 rpm and aeration rate of 0.4 vvm.

2.3. Treatment of cells with organic solvents

Resting cells of CGMCC0574 were permeabilized by pretreatment with various organic solvents. The cells were harvested from a 15 h culture by centrifugation at 5000 rpm for 10 min and washed once with 0.85% NaCl solution. The washed cells were then treated by re-suspending at a cell concentration corresponding to 0.3 g dry weight/10 ml in potassium phosphate buffer (50 mM, pH 7.0) with a certain organic solvent. After incubated on a shaker at 120 rpm and 30 °C for a desired period of time, the suspension was centrifugated at 5000 rpm for 10 min. The cells separated were washed once again with 0.85% NaCl solution before used for enzyme assay.

2.4. Enzyme assay

Resting cells or treated cells of CGMCC0574 were re-suspended at 0.3 g dry weight/10 ml in potassium phosphate buffer (pH 7.0, 50 mM) containing 10 mM substrate and 0.5% (w/v) Tween 80. The mixture was incubated at 30 °C and 180 rpm for 1 h, then the residual substrate and its hydrolytic product were extracted by the same volume of ethyl acetate with phenanthrene as an internal standard. The extract was directly subjected to HPLC analysis. One unit of hydrolytic activity was defined as the amount of the enzyme that catalyzes the hydrolysis of ketoprofen ethyl ester to release 1 mmol of ketoprofen/min under above conditions.

2.5. Analytical methods

Concentrations of ketoprofen and its ethyl ester were determined by HPLC with an UV detector at 254 nm, using a reverse phase column, Lichrosorb RP-18 (200 mm × ϕ5.0 mm, Merck, Germany). The mobile phase was composed of methanol and water

(85/15, v/v) and its flow rate was 0.8 ml/min. The retention time of ketoprofen and its ethyl ester were 2.8 and 9.0 min, respectively.

The enantiomers of ketoprofen were analyzed using a chiral column (Chiralcel OJ, 25 cm × ϕ0.46 cm, Daicel Co., Japan) with hexane/2-propanol/acetic acid (90/10/0.1, v/v) as mobile phase (1.0 ml/min) and detected at 254 nm. The retention time of (*S*)- and (*R*)-ketoprofen ethyl ester and (*R*)- and (*S*)-ketoprofen were 9.5, 10.0, 13.0 and 17.4 min, respectively.

The structure of product was confirmed to be (*S*)-ketoprofen by ^{1}H NMR (500 MHz, MeOH-d_4), δ_{ppm} 7.77–7.48 (m, 9 H, Ar**H**), 3.81 (q, 1 H, J = 7.16 Hz, –C**H**(CH$_3$)COOH), 1.48 (d, 3 H, J = 7.17 Hz, –C**H**$_3$) and mp 74–78 °C, $[\alpha]_D^{20} + 45^\circ$ (c = 1.0, MeOH).

3. Results and discussion

3.1. Selection of treating agent

While the catalytic activity of whole cells of *T. brassicae* CGMCC0574 in the enantioselective hydrolysis of ketoprofen ester (Scheme 1) was not satisfactory, a significant increase in activity was observed after incubating the cells in an alcoholic buffer for several hours (unpublished results). This suggested the possible existence of a permeability barrier of the cell membrane against the transfer of substrates and/or products. Thus, the cell permeabilization was considered to be favorable for the enzymatic resolution of ketoprofen.

The choice of a suitable permeabilizing agent depends on the organism and the composition of the cell membrane. Many investigators employed toluene as a permeabilizing agent [20]. However, in our study, a 30% decrease in the cell activity after toluene treatment was observed. Whereas, among several other reagents tested (Table 1), acetone and 2-propanol contributed to a significant increase in the cell activity, up to 161 and 235% respectively of the original activity, indicating that acetone and 2-propanol were very effective for treatment of the yeast cells.

Meanwhile, the concentration of proteins measured in the supernatant of the treated cell suspension was very low, and no notable activities toward ethyl ester of ketoprofen were detected (data not shown). We can, therefore, conclude that the treatment of whole cells by organic solvents did not lead to the cell disruption, and that the process of treatment did not cause the leakage of the requisite enzyme from the cell.

Table 1
The activity and enantioselectivity of *T. brassicae* cells after treatment with various organic solvents

Organic solvent[a]	Relative activity (%)[b]	Enantiomeric ratio[c]
Control	92	54
Toluene	67	N.D.[d]
Ethanol	79	N.D.[d]
2-Propanol	235	56
Acetone	161	51
DMSO	111	N.D.[d]

[a] Various organic solvents (1 vol.%) in potassium phosphate buffer (50 mM, pH 7.0), 0.3 g dry cell weight in 10 ml, 30 °C, 120 rpm, 12 h.
[b] Activity of 100% corresponds to the activity of fresh cells harvested (2.13 U/g dry weight).
[c] The enantioselectivity of the esterase, calculated using the equation of Chen et al. [25]: $E = \ln[1-x(1+ee)]/\ln[1-x(1-ee)]$.
[d] N.D.: not determined.

In a study by Colton et al. [9], an improvement in the activity and enantioselectivity of commercial lipase by 2-propanol treatment of the crude enzyme was reported, but in our case the effect of 2-propanol on the enzyme itself might be ignorable as the reasons listed below: (1) as described in Section 3.2, 2-propanol itself exhibited a negative effect on the cell activity; (2) the possible positive effect of 2-propanol on the enzyme itself is limited to a great extent because in our case the enzyme is tightly bound to the cell membrane, so its structure and activity is somewhat fixed; (3) in our work, the cell activity increased up to 310% after the treatment under optimal conditions as described later, which is much greater than 136% of their work [9].

As for enantioselectivity of the cells treated by acetone or 2-propanol, it was shown that treatment with these two solvents did not cause notable changes in enantioselectivity of the cells toward ethyl ester of (*S*)-ketoprofen. Finally, 2-propanol was chosen as the best treating agent for further study owing to the much better effect than that of acetone.

3.2. Effect of treatment time

In order to determine the optimal condition for cell treatment, the effect of incubation time was investi-

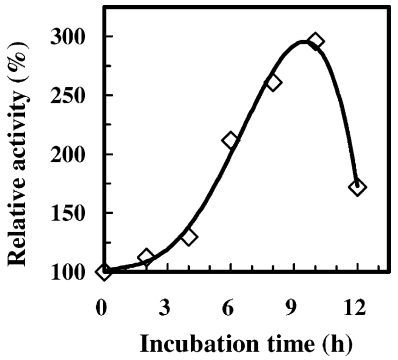

Fig. 1. Effect of incubation time on treatment of yeast cells by 2-propanol. Conditions for treatment: 30 mg dry cell weight/ml and 1 vol.% of 2-propanol in potassium phosphate buffer (50 mM, pH 7.0) 30 °C, 120 rpm. Activity of 100% corresponds to that of untreated cells (2.13 U/g dry cell weight).

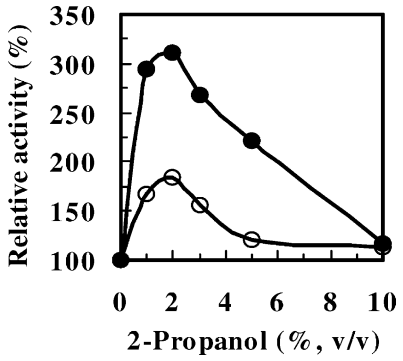

Fig. 2. Effect of 2-propanol concentration on the treatment of the yeast cells. Conditions for treatment: 30 mg dry cell weight/ml and specified concentrations of 2-propanol in potassium phosphate buffer (50 mM, pH 7.0) 30 °C, 120 rpm. Duration of treatment: (●) 10 h; (○) 5 h. Activity of 100% corresponds to that of untreated cells (2.13 U/g dry cell weight).

gated. When the yeast cells were incubated in the presence of 1 vol.% 2-propanol, a steady increase in the cell activity with time was observed (Fig. 1). After 10 h of incubation with 2-propanol, the maximal activity on ethyl ester of ketoprofen, which was about 300% of the activity of untreated cells, was reached. Although the protein concentration and esterase activity in the supernatant of the cell suspension did not display any notable change after treatment for more than 10 h, cell activity dropped significantly thereafter. In a comparative experiment, a dramatic decrease in the activity of untreated whole cells was observed when 1 vol.% of 2-propanol was added directly to the reaction mixture (unpublished results), indicating that the organic solvent itself inhibit the enzyme activity. Thus, certain adverse change in the structure of the enzyme or in the cell membrane that support the enzyme after exposing for a longer time to the solution of organic solvent was a likely reason for the remarkable decrease in cell activity during prolonged treatment time, which might counteract the positive effect of treatment to some extent.

3.3. Effect of 2-propanol concentration

The effect of different 2-propanol concentrations in cell treatment on the activity of treated cells on ketoprofen ester was also studied. Fig. 2 indicates that the cell activity increased significantly by a factor of >200% after 10 h of treatment with 1–5 vol.% of 2-propanol. The maximum activity, approximately 310% higher than that of the control, was reached at 2 vol.% of 2-propanol, beyond which a steady downsizing in the activity increase was observed. The reason for this downsizing was likely the same as that for remarkable decrease in cell activity during prolonged incubation time.

Treatment in shorter incubation time was also investigated. The result (Fig. 2) showed that the curves of activity versus 2-propanol concentration were similar between 5 and 10 h of treatment, both of the two activity curves reached their maximums at 2 vol.% of 2-propanol. Therefore, it seems unfavorable to shorten the duration of treatment by raising the concentration of 2-propanol. This result is somewhat different from a recent report by Liu et al. [19], in which 2-propanol was used in a very high concentration of 40%.

3.4. Influence of cell concentration on treatment

Some previous studies showed that the amount of cells influences the effect of permeabilization [15,17]. Therefore, the effect of cell concentration on the cell activity was examined by treating different concentrations of the yeast cells with 2 vol.% of 2-propanol for 10 h. As shown in Fig. 3, the best effect of treatment was observed at a cell concentration of 30 mg

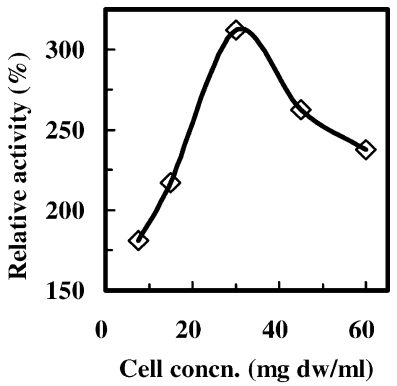

Fig. 3. Influence of cell concentration on the treatment. Conditions for treatment: specified amount of cells and 2 vol.% of 2-propanol in potassium phosphate buffer (50 mM, pH 7.0), 30 °C, 120 rpm, 10 h. Activity of 100% corresponds to that of untreated cells (2.13 U/g dry cell weight).

dry weight/ml. When the cell concentration is lower or higher than this optimum, the increase in the cell activity was somewhat depressed. Thus, we can infer that the enhancement of activity by treatment was not only influenced by 2-propanol concentration and the duration the treatment, but also affected by the relative amount of cells to the treating agent. The optimal cell concentration is possibly due to the effect of mixing intensity: a greater mixing intensity in lower cell concentration. When violently mixed, certain amount of 2-propanol will penetrate into the cell, which will impose negative effect on the activity as we described in Section 3.2. However, when the cell concentration is too high to be mixed sufficiently, the effect of 2-propanol treatment would be partly concealed.

4. Conclusion

In this work, we established a simple but efficient method to significantly enhance the whole cell activity of *T. brassicae* CGMCC0574. After treated by 2 vol.% of 2-propanol for 10 h, no cell disruption was observed while the cell activity on ethyl ester of ketoprofen was dramatically increased up to 310% without notable change in the enantioselectivity of resolution. Both higher 2-propanol concentration and longer treatment time will lead to remarkable decrease in the effect of treatment, which is probably due to certain adverse changes in the structure of enzyme or that of cell membrane that supports the enzyme caused by the solvent. Further study on the mechanism of this procedure is proceeding.

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References

[1] J.P. Famaey, H.E. Paulus, Therapeutic Applications of NSAIDs, Marcel Dekker, New York, 1992.
[2] W.J. Wechter, J. Clin. Pharmacol. 38 (1998) 1S.
[3] D.L. Heefner, M. Zepp, PCT Int. Appl. WO9420635A1 (1994).
[4] A.L. Margolin, Enzyme Microb. Technol. 15 (1993) 266.
[5] S.-H. Wu, Z.-W. Guo, C.-J. Sih, J. Am. Chem. Soc. 112 (1990) 1990.
[6] A. Mustranta, Appl. Microbiol. Biotechnol. 38 (1992) 61.
[7] Y.-Y. Liu, J.-H. Xu, Q.-G. Xu, Y. Hu, Biotechnol. Lett. 21 (1999) 143.
[8] H.-Y. Wu, J.-H. Xu, Y.-Y. Liu, Synth. Commun. 31 (2001) 3491.
[9] I.J. Colton, S.N. Ahmed, R.J. Kazlauskas, J. Org. Chem. 60 (1995) 212.
[10] J. Lalonde, C. Govardhan, N. Khalaf, A.G. Martinez, K. Visuri, A.L. Margolin, J. Am. Chem. Soc. 117 (1995) 6845.
[11] Y.-Y. Liu, J.-H. Xu, Y. Hu, J. Mol. Catal. B: Enzym. 10 (2000) 523.
[12] C.T. Evans, R. McCague, S.J.C. Taylor, G. Caarganico, PCT Int. Appl. WO9304190A1 (1993).
[13] W. Rhys-Williams, F. McCarthy, J. Baker, Y.-F. Hung, M.J. Thomason, A.W. Lloyd, G.W. Hanlon, Enzyme Microb. Technol. 22 (1998) 281.
[14] D. Shen, J.-H. Xu, P.-F. Gong, H.-Y. Wu, Y.-Y. Liu, Can. J. Microbiol. 47 (2001) 1101.
[15] L.R. Gowda, N. Bachhawat, S.G. Bhat, Enzyme Microb. Technol. 13 (1991) 154.
[16] S. Sekhar, N. Bhat, S.G. Bhat, Proc. Biochem. 34 (1999) 349.
[17] M.J. van der Werf, S. Hartmans, W.J.J. van den Tweel, Appl. Microbiol. Biotechnol. 43 (1995) 590–594.
[18] Y.-Y. Liu, H. Hama, Y. Fujita, A. Kondo, Y. Inoue, A. Kimura, H. Fukuda, Biotechnol. Bioeng. 64 (1999) 54–60.
[19] Y.-Y. Liu, Y. Fujita, A. Kondo, H. Fukuda, J. Biosci. Bioeng. 89 (2000) 554.

[20] E. Boye, S. Alver, K. Skarstad, J. Bacteriol. 145 (1981) 1413.
[21] G. Lin, H.-C. Liu, Tetrahedron Lett. 36 (1995) 6067.
[22] D.B. Mowshowitz, Anal. Biochem. 70 (1976) 94.
[23] D.E. Oliveira, A.L.C. Santos Neto, A.D. Panek, Anal. Biochem. 113 (1981) 188.
[24] M.J. Hernaiz, J.M. Sanchez-Montero, J.V. Sinisterra, J. Mol. Catal. 96 (1995) 317.
[25] C.S. Chen, Y. Fujmito, G. Girdaukas, C.-J. Sih, J. Am. Chem. Soc. 104 (1982) 7294.