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Kinetic resolution of ketoprofen ester catalyzed by lipase from a mutant of CBS 5791

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Abstract A biotransformation process was developed for the production of (*S*)-ketoprofen by enantioseletive hydrolysis of racemic ketoprofen ester using the mutant *Trichosporon laibacchii* strain CBS 5791. A satisfactory result was obtained, in which the E was 82.5, with an ee of 0.94 and a conversion of 0.47 under the optimum hydrolysis conditions [E is enantiomeric ratio, $E = \ln[1-X(1+ee)]/\ln[1-X(1-ee)]$; ee is enantiomeric excess, $ee = (C_S-C_R)/(C_S+C_R)$: temperature of hydrolysis was 23°C]. The medium used in biotransformation was a mixture of growth broth and biotransformation broth at a ratio of 1:9, the concentration of Tween 80 was 15 g/l, the time of hydrolysis, 72 h. These results are promising for further scale-up. Tween 80 significantly improved lipase enantioselectivity and activity at the optimum concentration.

Keywords Microbial hydrolysis · Biotransformation · Lipase · Enantioselective resolution · Ketoprofen

Introduction

As optically pure single enantiomers are often more specific targets and have fewer side effects than their corresponding racemates [4], there is a real driving force today for the production of new drugs and agrochemicals with high optical purity and for the enantioenrichment of previously exisitng racemic products [3]. Ketoprofen, [(*R*, *S*)-2-(3-benzoylphenyl) propionic acid] is an inhibitor of prostaglandin synthesis, an analgesic and an anti-inflammatory agent (4). The anti-inflammatory activity of ketoprofen is mainly attributed to the (*S*)-enantiomer. However, ketoprofen is mainly produced by chemical synthesis and sold as a mixture of stereoisomers.

Several methods to obtain the (*S*)-enantiomer, free of contamination, are known; these include asymmetric chemical synthesis, enzymatic resolution and chemical resolution, such as the stoichiometric crystallization of diastereomeric salts formed with various chiral amines. Tsai et al. [10] reported the enantioselective synthesis of 2-arylpropionic acid, related to ketoprofen; Liu et al. [7] reported significant enhancement of lipase enantioselectivity towards (*S*)-ketoprofen ester at pH 2. Efficient preparation of optically active ketoprofen by *Mucor javanicus* lipase immobilized on an inorganic support was reported by Kato et al. [5]. Enantioselective esterification of racemic ketoprofen in non-aqueous solvent under reduced pressure was carried out by De Crescenzo et al. [2]. Kim et al. [6] reported an improved enantioselectivity of *Candida rugosa* lipase towards ketoprofen ethyl ester by a simple two-step treatment. Nicola et al. [9] reported the large-scale preparation of enantiopure (*S*)-ketoprofen by biocatalyzed kinetic resolution using immobilized lipase from *Candida antarctica* (Novozym 435).

An alternative approach has been employed in our work. This method involves biocatalytic hydrolysis of a racemic ketoprofen ester, to yield a biotransformation broth containing ketoprofen acid substantially enriched with (*S*)-enantiomer and ketoprofen ester substantially enriched with (*R*)-enantiomer. The strain used in our work was the *Trichosporon laibacchii* mutant strain CBS 5791(Centralbureau Voor Schimmelcultures (CBS) in Netherlands). The acid product of the biotransformation was sufficiently enriched with the desired (*S*)-enantiomer. The residual ketoprofen ester from the biotransformation can be readily purified, chemically racemized and recycled for use in further biotransformations to minimize raw-material costs.

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Materials and analysis

Strain

The mutant strain *T. laibacchii* CBS 5791 was used for the biotransformation. The bacterium has been identified as being extremely useful in carrying out the required reaction. The strain was obtained by mutagenesis with two kinds of mutagens, such as $C_4H_{10}O_4S$ and ultraviolet. The mutant is stable, and its activity is higher than that of the wild-type. Characterization of the mutant has not yet been accomplished.

Chemicals

Racemic ketoprofen was purchased from Ningbo Medicine (Ningbo, Zhejiang Province, China). Ethanol, methanol, acetone, glyceryl polyether (antifoam, from Qingdao Chemicals, CHian)), sodium phosphate, potassium dihydrogen, $MgSO_4{\cdot}7H_2O$, KOH, metal Na, glucose, and Tween 80 were of analytical grade; some compounds were further purified.

Analysis

All HPLC analyses were carried out using a Waters Alliance liquid chromatography system.

Resolution of the ketoprofen enantiomers was accomplished with a HPLC system equipped with a chiral column (Chiralcel OJ (4.6×25 cm, Exton, Penn., USA) connected to a UV detector. Each sample was dissolved in the mobile phase (hexane/2-propanol/acetic acid 9/1/0.05, v/v/v). The flow rate was maintained constant at 1.0 ml/min and the column temperature at 20°C.

The compounds were separated with a HPLC system equipped with a reversed-phase column (Symmetry-Shield RP18, 5 μm, 3.9×150 column C18). Each sample was dissolved in the mobile phase (acetone/acetonitrle/1 mM acetic acid 50/50/1, v/v/v). The flow rate was maintained constant at 1.0 ml/min and the column temperature at 20°C; 20 μl of each sample was injected. The conversion of the racemic substrate, (*S*, *R*)-ketoprofen ester,was estimated, $X=(Ce_0-Ce)/Ce_0=(C_S+C_R)/Ce_0$. Agitation at 1,500 rpm was maintained for 20 min to emulsify the broth. The broth remained as an emulsion because of the intensive agitation and the presence of Tween 80.

Cell concentration and cell size in the culture broth were measured with a blood cell plate. Cell concentration is expressed as the number of cell per ml, and cell length in microns.

Experiments

Preparation of biotransformation broth

Preparation of biotransformation broth included three phases: (1) seed broth, or seed phase; (2) growth broth, or growth phase; (3) biotransformation broth, or biotransformation phase.

All media were heat-sterilized at 121°C for 15–30 min prior to inoculation. First, cells were inoculated onto YM (Difco) agar plates and incubated for 2 days. Second, a single colony was transferred into seed medium in a flask and incubated on a rotary shaker at 150 rpm with an aeration of 0.5 vvm for 20–26 h, and then this culture was immediately reincubated for the second fermentation stage, the growth broth phase. The contents of the rotary shaker flask were transferred into a fermenter containing growth medium with sufficient stirring and aeration to maintain oxic conditions for 8–15 h. Finally, the cell broth was transferred to biotransformation medium. The mixture of the cell broth and the biotransformation medium was the biotransformation broth. Before the cell broth was added to the biotransformation medium, the ketoprofen ethyl ester was sterilized at 121°C for 20–30 min. Care was taken to maintain sterile conditions during cell transfers, and culture purity was monitored by plating culture samples and examining the colonies microscopically. composition of theThe composition of the seed medium, the growth medium and the biotransformation medium are shown in Table 1 ; the trace elements are listed in Table 2.

Enzymatic hydrolysis of ketoprofen ethyl ester

Ketoprofen ethyl ester was added to the biotransformation broth to produce a final concentration of 50–70 g/l after the addition of the culture broth. The biotransformation broth was transferred into the hydrolysis vessel to start the biotransformation. During the biotransformation, intensive agitation and aeration of 0.5 vvm were maintained. Samples were periodically withdrawn and analyzed.

Table 1 Composition of the seed, growth, and biotransformation media (g/l)

Components	$(NH_4)_2SO_4$ g/l	KH_2PO_4 g/l	$MgSO_4{\cdot}7H_2O$ g/l	Yeast extract g/l	Trace element g/l	Glyceryl polyether ml/l	Glucose ml/l	Tween 80 g/l	Na_2HPO_4 g/l	Malt extract l/l
Seed medium	2	10	0.5	30	1	1	–	–	–	0.5
Growth medium	2	10	0.5	50	1	1	50	–	–	–
Biotransformation Medium	–	–	–	10	–	1	–	5-15	27.6	–

Table 2 Composition of trace elements

Components	$CaCl_2$	$ZnCl_2$	$CuCl_2$	$(NH_4)_6MoO_4$	$(NH_4)_2MnO_4$	$FeSO_4{\cdot}7H_2O$	H_3BO_3	$CoCl_2{\cdot}6H_2O$	HCl(ml)
Content (g/250 ml)	0.675	0.84	0.1675	1.325	0.4075	1.6	0.075	0.6	25

The acid product of the biotransformation was sufficiently enriched with the desired enantiomer. In order to minimize raw-material costs, the residual ketoprofen ester from the biotransformation can be readily purified, chemically racemized and recycled for use in further biotransformations. The progress of the biocatalytic hydrolysis was monitored by thin-layer chromatography (TLC) on GF254 plates with a mixture of acetone/petroleum ether (60–90°C) 4/5, v/v. The spots were visualized under UV light. The experiment was run 2–4 times for each test.

Growth broth was added to biotransformation medium immediately after the growth phase finished;the ratio of growth broth to biotransformation medium was 1:9, and the Tween 80 concentration was 15 g/l. Biotransformation was performed in a reactor at 18, 20, 23, 25 and 30°C, with agitation, aeration of 0.5 vvm, and a reaction time of 72 h. Samples were periodically withdrawn and analyzed by HPLC or chiral HPLC.

Separation of ketoprofen ethyl ester and ketoprofen acid

Hydrolysis broth contained cells, unreacted ketoprofen ethyl ester, (*S*)-ketoprofen acid and (*R*)-ketoprofen acid produced by hydrolysis, enzyme, and nutrient ingredients of culture medium. Unreacted ketoprofen ethyl ester, (*S*)-ketoprofen acid and (*R*)-ketoprofen acid were separated from other components. The separation procedure used was as follows: (1) the pH of the broth was adjusted to 9–10 with NaOH; (2) the resultant broth was centrifuged at 5,000 rpm for 5 min; (3) supernatant , which contained ketoprofen acid and some other components, was removed. The pellet contained ketoprofen ethyl ester, cells and some other compounds. The pH of the supernatant was adjusted to 2–3 with HCl, and ketoprofen acid was precipitated. The turbid liquor was centrifuged at 5,000 rpm for 5 min and supernatant was removed. The precipitate contained (*S*)-ketoprofen acid and (*R*)-ketoprofen acid [4] There were ketoprofen ester (liquor) and cells in the residue of the third step; the liquor was removed and the solid was washed with ethanol to recycle the ester.

The specific optical rotation of the ketoprofen acid produced by biocatalytic hydrolysis of a racemic ketoprofen ester was measured using a polarimeter to analyze the chirality.

Results and discussion

Preparation of biotransformation broth

The experiments were carried out in a flask in a rotary shaker at 150 rpm with aeration of 0.5 vvm. The optimal parameters obtained were as follows: (1) seed phase: temperature 23°C, time 23 h, ratio of malt extract to elementary solution 0.5, concentrations of $(NH_4)_2SO_4$ 2 g/l, pH 6.5, $MgSO_4$ 0.7 g/l and KH_2PO_4 20 g/l, (2) growth phase: glucose 50 g/l, $(NH_4)_2SO_4$ 2 g/l, malt extract 500 g/l, $MgSO_4$ 0.7 g/l, trace elements 1 ml/l, glyceryl polyether 1 ml/l, yeast extract 15 g/l, KH_2PO_4 20 g/l, pH 6.5, time 13 h and an aeration of 0.5 vvm . Seed broth was added to growth medium at a ratio of 1:5 or 1:9.

Effect of temperature on hydrolysis of ketoprofen ester

At the beginning of hydrolysis all of the cells were alive. However, cells died gradually during hydrolysis, and about 20% of the cells were dead by 72 h at 23 °C. The amount of dead cells increased with the rise in temperature, and the amount of dead cells was 80% greater at 30°C. Results are shown in Fig. 1. X represents the conversion of racemic substrate, (*S*, *R*)-ketoprofen ester, and the conversion is for (*S*, *R*)-ketoprofen, $X = (Ce_0 - Ce)/Ce_0 = (C_S + C_R)/Ce_0$; Ce is the concentration of racemic ester in hydrolysis broth (g/l); Ce_0, the initial concentration of racemic ester in hydrolysis broth (g/l); C_R, the concentration of (*S*)-ketoprofen in hydrolysis broth (g/l); C_S, the concentration of (*S*)-ketoprofen in hydrolysis broth (g/l). E is the enantiomeric ratio [1], $E = \ln[1 - X(1 + ee)]/\ln[1 - X(1 - ee)]$; X_S is the relative concentration of (*S*)-ketoprofen, $X_S = C_S/(C_S + C_R)$, and X_R, the relative concentration of (*R*)-ketoprofen, $X_R = C_R/(C_S + C_R)$. C_S is the concentration of (*S*)-ketoprofen in hydrolysis broth analyzed by chiral HPLC (g/l) and C_R, the concentration of (*R*)-ketoprofen; ee is the enantiomeric excess [10], expressed as $ee = (C_S - C_R)/(C_S + C_R)$. As shown in Fig. 1, ee at 18°C was the highest, but its conversion was the lowest, only 18%. At 30°C, the conversion was 20%, the second lowest rate

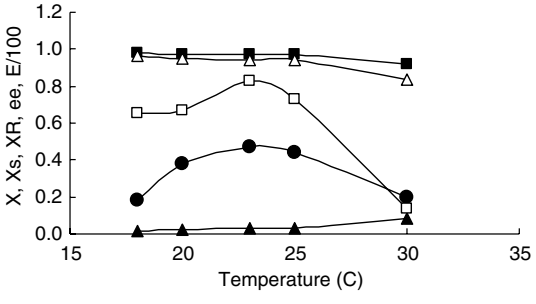

Fig. 1 Effect of temperature on hydrolysis of ketoprofen ester. *Filled square* Xs, *open triangle* ee, *open squares* E/100 *filled circles* X, *filled triangles* XR

recorded, and the ee was 88.3%, the lowest value obtained. Thus, 18 and 30°C are not suitable temperatures for hydrolysis. At 23°C the enantiomeric excess for ketoprofen (ee%) was 94%, with a conversion of 47% and an enantiomeric ratio (E) of 82.5. The E involved both ee and conversion; E is an appropriate assessment criterion of the enzyme's enantioselectivity and reaction conditions. The larger the value of E, the better the enzyme enantioselectivity and reaction conditions. Considering ee, E and conversion, the best result was obtained at 23°C . Analysis of the samples showed that 8.83–22.8 g ketoprofen/l had accumulated, with an ee of 0.96–0.95 in favor of the (*S*)-enantiomer. The lipase produced by the cells was intracellular, and living cells were needed for hydrolysis of the ester. The optimum temperature for growth of the cells was 23°C in seed broth, on YM agar plates, and in hydrolysis broth. Neither the maximum nor the minimum temperature was optimal for cell growth. Consequently, 23°C was used for further work.

Effect of Tween 80 on hydrolysis of ketoprofen ester

The hydrolysis conditions were similar to those for experiments examining the effect of temperature on hydrolysis, except for the concentration of Tween 80; the temperature was 23°C. Biotransformation was carried out in a reactor at the concentrations of 0, 5, 10, 15 and 20 g Tween 80/l, with intensive agitation and aeration of 0.5 vvm for 72 h. The result is shown in Fig.2.

According to Fig.2, when the concentration of Tween 80 was 15 g/l, E was maximum (82.5), with a conversion of 0.47 and an ee of 0.95. This result yielded the best profile and is promising for further scale-up. The hydrolysis reaction was poor when no Tween 80 was added to the hydrolysis broth; the conversion was only 0.02, with an E of 10.2 and an ee of 0.82. The low concentration of ketoprofen ester in water may have been the main reason for the poor conversion. At this low concentration, the reaction surface of lipase with ketoprofen ester is very small, and conversion is poor. When Tween 80 was added to hydrolysis broth, both E and ee increased considerably. By increasing the amount of Tween 80 from 0 to 20 g/l, ee increased from 0.82 to 0.94, and E, from 10.2 to 82.5. This result indicated that Tween 80 may significantly improve lipase enantioselectivity toward (*S*)-ketoprofen ester.

Liu et al. [8] reported that some surfactants, such as Tween 80 and nonyl phenol polyleneoxy ether, can significantly improve lipase enantioselectivity toward (*S*)-ketoprofen ester. Since Tween 80 is a non-ionic surfactant, there are only hydrogen bonds and hydrophobic action between lipase and surfactant, which might change the lipase selectivety toward (*S*)-ketoprofen ester in the reaction. At higher concentrations of Tween 80, conversion rose significantly, from 0.12 to 0.49 until the concentration reached 15 g Tween 80/l. Tween 80 efficiently emulsifies ketoprofen, thus the reaction surface of hydrolysis increases greatly, as might penetrance of the cell membrane. Tween 80 does not inhibit lipase activity; however, with an increase of Tween 80 from 15 to 20 g/l, conversion did not increase further. The might be due to the fact that at above concentrations of 15 g/l the critical micellar concentration (CMC) is reached; therefore, emulsification did not improve. In addition, the higher the concentration of Tween 80 , the more difficult downstream processing of hydrolysis broth . Therefore, the concentration of 15 g/l is suitable for the biotransformation.

Effect of the ratio of growth broth on hydrolysis

Hydrolysis conditions were similar to those used for experiments studying the effect of Tween 80 concentration on hydrolysis, except for the ratio of growth broth to biotransformation medium. The ester was hydrolyzed at 23°C in a reactor, with a ratio of growth broth to biotransformation broth of 1:2, 1:5, 1:9, and 1:10. The results are shown in Fig.3. When the ratio of growth broth to hydrolysis broth was 1:2, 1:5, 1:10, E was 10.2, 39.2 and 64.6 Compared with the E of 82.5 at the ratio of 1:9, none of these values was acceptable. When the ratio was 1:2, the concentration of lipase was higher than that obtained at a ratio of 1:9. More ketoprofen ester was hydrolyzed, including some of the (*R*)-ester, which resulted in a small E of 10.2. At the ratio of 1:10,

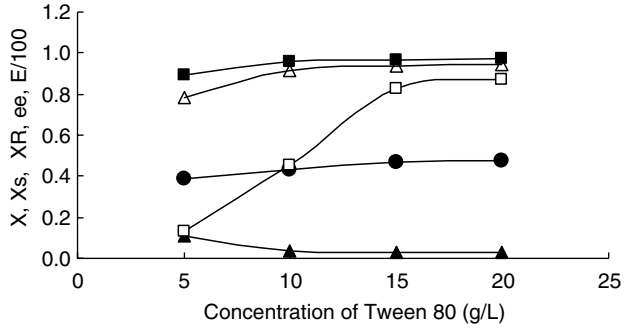

Fig. 2 Effect of Tween 80 on hydrolysis of ketoprofen ester (Ce0 is 50 g/l). *Filled square* Xs, *open triangles* ee, *open squares* E/100, *filled circles* X, *filled triangles* XR

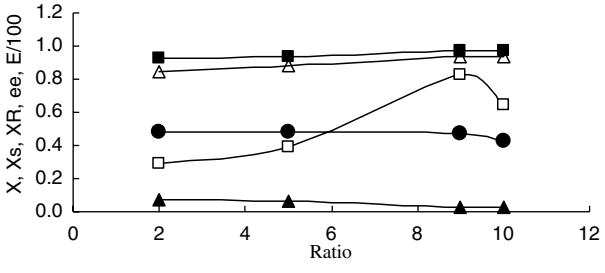

Fig. 3 Effect of ratio of growth broth to biotransformation broth on hydrolysis. *Filled squares* Xs, *open triangles* ee, *open squares* E/100, *filled circles* X, *filled triangles* XR

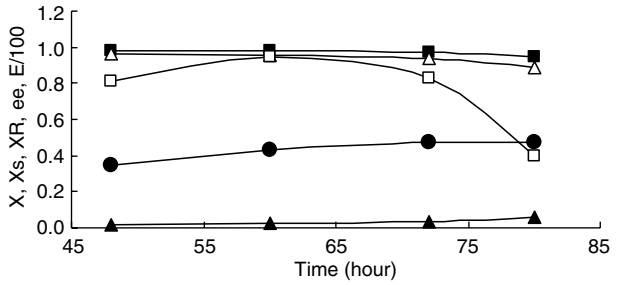

Fig. 4 Effect of time of on hydrolysis. *Filled squares* Xs, *open triangles* ee, *open squares* E/100, *filled circles* X, *filled triangles* XR

lipase activity was less than at a ratio of 1:9, and less ketoprofen ester was hydrolyzed, which resulted in a small E (64.6). Thus, the ratio of 1:9 was found to be optimal for hydrolysis of the ester, yielding good E, conversion and ee values.

Effect of time on hydrolysis

The medium used in biotransformation was a mixture of growth broth and biotransformation broth at a ratio of 1:9. Hydrolysis conditions were similar to those used in the experiments for effect of the ratio of growth broth on hydrolysis except for the time of hydrolysis. The ester was hydrolyzed at 23°C in a reactor for 48, 60, 72 and 80 h. Figure 4 shows the effect of length of hydrolysis on hydrolysis of ketoprofen ester. When hydrolysis was allowed to proceed for 48 h, E was 81.3, with a conversion of 0.35 and an ee of 0.96. The poor E was attributed to the lower conversion. With a hydrolysis time of 80 h, the E of 40 and the conversion 0.473; the reason for these low values was that some of (*R*)-ketoprofen was produced during the long reaction time. In general, the longer the reactiontime, the more (*R*)-ketoprofen is produced by hydrolysis, which results in a poor E. The E of 82.55, measured at 72 h, was excellent ; the ee was 0.94 and the conversion 0.47. Therefore 72 h is considered as the optimal time of hydrolysis.

Conclusions

A biotransformation process was developed for production of (*S*)-ketoprofen by enantioseletive hydrolysis of racemic ketoprofen ester using mutant *Trichosporon laibacchii* strain CBS 5791. A satisfactory result was obtained, in which the E was 82.5, with an ee of 0.94 and a conversion of 0.47 under optimum hydrolysis conditions. The hydrolysis temperature was 23°C, the ratio of growth broth to biotransformation medium, 1:9; the concentration of Tween 80, 15 g/l; the time of hydrolysis, 72 h. These results were found to be promising for further scale-up of the reaction. E was an appropriate criterion for the enantioselectivity as well as the enzyme activity and the reaction conditions. The experimental results showed that Tween 80 significantly improved lipase enantioselectivity and activity at the optimum concentration. Tween 80 efficiently emulsified ketoprofen thereby greatly increasing the reaction surface of the hydrolysis, and, most likely, penetrance of cell membrane. Consequently, conversion rose significantly.

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