Organic Process Research & Development

Article

pubs.acs.org/OPRD

Efficient Production of (*R*)-*o*-Chloromandelic Acid by Recombinant *Escherichia coli* Cells Harboring Nitrilase from *Burkholderia cenocepacia* J2315

Hualei Wang, Huihui Sun, Wenyuan Gao, and Dongzhi Wei*

State Key Laboratory of Bioreactor Engineering, New World Institute of Biotechnology, East China University of Science and Technology, Shanghai 200237, People's Republic of China

ABSTRACT: A solvent engineering approach and an extended fed-batch reaction mode were introduced to increase the activity and enantioselectivity and alleviate the substrate inhibition of nitrilase BCJ2315 from *Burkholderia cenocepacia* J2315 toward *o*-chloromandelonitrile. Among the seven water-miscible organic solvents tested, ethanol (30%, v/v) demonstrated the highest reaction conversion (55.7%) and enantioselectivity (enantiomeric excess, 98.2% ee) compared with those of the control [which did not contain any organic solvent (13% and 89.2%, respectively)] and was thus chosen as the suitable cosolvent. In the extended fed-batch reaction mode, *o*-chloromandelonitrile (solubilized in ethanol, 5 M) was continuously fed into the reaction mixture containing ethanol as cosolvent (20%, v/v) to ensure an optimal reaction rate by adjusting the feeding rate and simultaneously increasing the enantioselectivity due to the increased concentration of ethanol. Finally, a maximum of 415 mM of product was produced with an enantiomeric excess value of 97.6% ee. The hydrolysis process was easily scaled up to 2 L, demonstrating that the described biocatalytic process was rationally designed and could be applied further on an industrial scale.

INTRODUCTION

Optically pure 2-hydroxycarboxylic acids, such as (*R*)-mandelic acid and its derivatives, are important intermediates with broad use in the pharmaceutical and the fine chemical industries.[1] (*R*)-*o*-chloromandelic acid, one of the most important representatives, is a key intermediate for the preparation of clopidogrel,[2] a highly market-occupied platelet aggregation inhibitor. Considering the great importance of (*R*)-*o*-chloromandelic acid, numerous biological methods for the synthesis of (*R*)-*o*-chloromandelic acid and its derivatives have been reported.[1a,2] Among these described methods, the nitrilase-mediated pathway has been proven as a simple and practical approach for the commercial production of (*R*)-*o*-chloromandelic acid and its derivatives due to the non-involvement of a cofactor, the cheap starting material in the form of *o*-chloromandelonitrile, and the 100% theoretical manufacture of the target product (Scheme 1).

Although the nitrilase-mediated pathway exhibits great potential in the production of optically pure (*R*)-*o*-chloromandelic acid, the technical usage of nitrilase has still remained limited.[3] The steric hindrance effect and the insufficient solubility of *o*-chloromandelonitrile impede the substrate affinity to nitrilase, resulting in low specificity and enantioselectivity. Thus, only very few truly enantioselective nitrilases that could hydrolyze *o*-chloromandelintrile to optically pure (*R*)-*o*-chloromandelic acid are available.[1b,4] Furthermore, the high toxicity of *o*-chloromandelonitrile also prevents the efficient production of (*R*)-*o*-chloromandelic acid through the hydrolysis of *o*-chloromandelonitrile in high concentrations (>50 mM).[2] In recent years, solvent engineering has been successfully used to enhance the performance of biocatalysts, such as activity and enantioselectivity.[5] Moreover, the fed-batch mode and the biphasic system have been successfully used to alleviate the inhibition of the highly toxic substrate.[4a,6] Therefore, a combination of these methods would be a potential solution to achieve the production of (*R*)-*o*-chloromandelic acid in high concentration and high enantioselectivity.

In our previous work, a novel nitrilase BCJ2315 from *Burkholderia cenocepacia* J2315 has been discovered using phylogeny-based enzymatic substrate-specificity prediction.[7] This nitrilase is an arylacetonitrilase that could efficiently hydrolyze mandelonitrile in high concentrations to produce optically pure (*R*)-(−)-mandelic acid with high enantioselectivity (98.7% ee). However, BCJ2315 exhibits a relatively low enantiomeric excess (ee) value of 89.2% toward *o*-chloromandelonitrile due to the steric hindrance effect. In the present study, a solvent engineering approach and an extended fed-batch mode are introduced to improve the enantioselectivity and activity of BCJ2315, and to achieve the efficient production of optically pure (*R*)-*o*-chloromandelic acid in high concentration and high enantioselectivity.

RESULTS AND DISCUSSION

Effects of Temperature and pH on Activity and Enantioselectivity of *Escherichia coli* M15/BCJ2315. The effects of temperature and pH on nitrilase activity and enantioselectivity were investigated in the present study, and the results are shown in Figure 1.

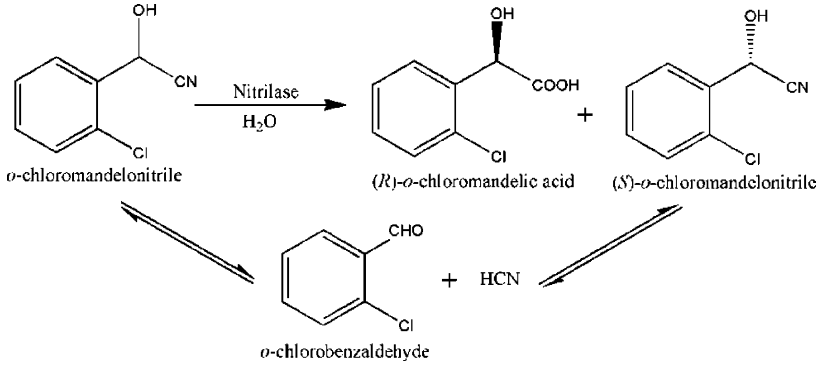
The effect of temperature, ranging from 20 to 70 °C, on nitrilase activity and enantioselectivity were investigated (Figure 1a). Nitrilase exhibited its highest activity at 50 °C. When the temperature was raised to 70 °C, nitrilase fully lost its function

Special Issue: Biocatalysis 14

Received: June 30, 2013

dx.doi.org/10.1021/op400174a | *Org. Process Res. Dev.* XXXX, XXX, XXX−XXX

Scheme 1. Nitrilase-mediated pathway for producing (*R*)-*o*-chloromandelic acid through the hydrolysis of *o*-chloromandelonitrile



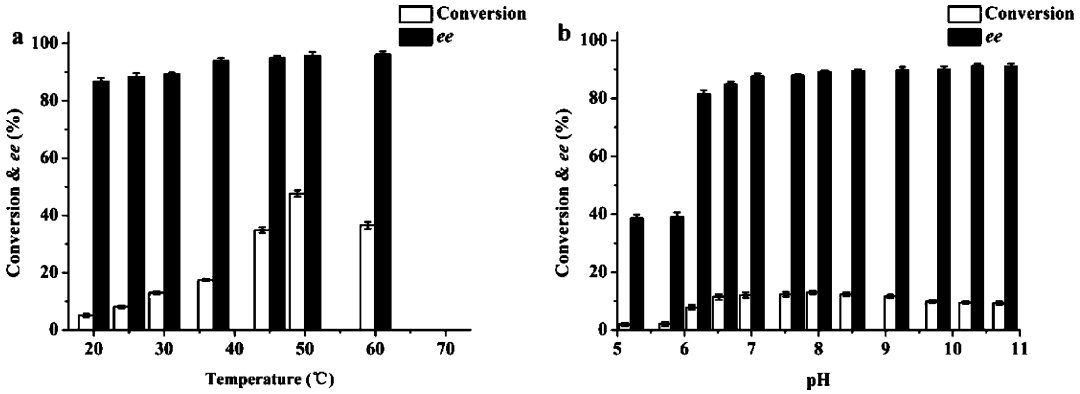


Figure 1. Effects of temperature (a) and pH (b) on the activity and enantioselectivity of *E. coli* M15/BCJ2315 toward *o*-chloromandelonitrile.

due to protein denaturation. In the case of enantioselectivity, temperature exhibited dramatic influence on the enantioselectivity of the reaction. The ee value of the product increased rapidly with the increase of temperature and reached the maximum of 96.2% at 60 °C from a starting value of 86.8% at 20 °C. In higher temperature, both the racemization of unreacted (*S*)-*o*-chloromandelonitrile and hydrolysis rate were accelerated. When the racemization was more rapid than the hydrolysis, redundant (*R*)-*o*-chloromandelonitrile was supplied to the enzyme, and thus the enantioselectivity was increased. Enthalpy and entropy were shown to play an important role in temperature dependence of enzyme enantioselectivity.[8] The enantioselectivity is the result of differences in activation enthalpy and entropy between the enantiomers. The contributions of differential activation enthalpy and entropy to enantioselectivity can be determined by determination of the temperature dependence of the enantiomeric ratio. Ottosson et al.[8a] have carefully studied the temperature dependence of the enantioselectivity of *Candida antarctica* lipase B towards secondary alcohols through a new experimental and molecular modeling study, giving a better understanding of the role of enthalpy and entropy on a molecular level.

Nitrilase exhibited its highest activity at pH 8.0 and demonstrated a wide pH tolerance between pH 6.6 and pH 9.2 (Figure 1b). The activity of the nitrilase sharply dropped when the pH was below 6.2, reflecting either significantly low enzyme stability at those pH levels or a critical change in the ionization status of the active site of the substrate-binding residues. The pH of the reaction also influences the enantioselectivity of the reaction. In contrast with the temperature value, the ee value of the product increased slowly with the increase in pH at neutral to alkaline pH values. The results can also be explained by the higher bioavailability of (*R*)-*o*-chloromandelonitrile due to the faster racemization of the unreacted (*S*)-*o*-chloromandelonitrile at high pH levels in combination with significant residual enzyme activities at the said pH levels. When the reaction was carried out at either pH 5.2 or pH 5.8, the ee value of the product was only 38.7% or 39.1%, respectively. In acidic conditions, the spontaneous racemization of the enantiomers of *o*-chloromandelonitrile was significantly inhibited,[9] and the hydrolysis process was also inhibited. Both of the steps may affect the enantioselectivity of the reaction.

Taking stability into account, the whole-cell system at 30 °C and pH 8.0 should be optimal for the enantioselective hydrolysis of *o*-chloromandelonitrile enantiomers. Higher stability of the enzyme at 30 °C and slightly alkaline pH provides an excellent opportunity to establish a dynamic kinetic resolution (DKR) process for the production of (*R*)-*o*-chloromandelic acid from readily available *o*-chloromandelonitrile.

Effects of Solvents on the Activity and Enantioselectivity of *E. coli* M15/BCJ2315. Recently, organic solvents have been reported to influence the enantioselectivity of biocatalysts through the regulation of enantiomer traffic at the active site.[10] Moreover, a number of remarkable achievements in the field of solvent engineering have been made to successfully improve enantioselectivity of biocatalysts.[11] Although the effect of an organic solvent cannot be reliably predicted, the addition of such solvents to aqueous medium has become a useful approach for improving enantioselectivity. Moreover, the insolubility of the *o*-chloromandelonitrile in the

dx.doi.org/10.1021/op400174a | *Org. Process Res. Dev.* XXXX, XXX, XXX−XXX

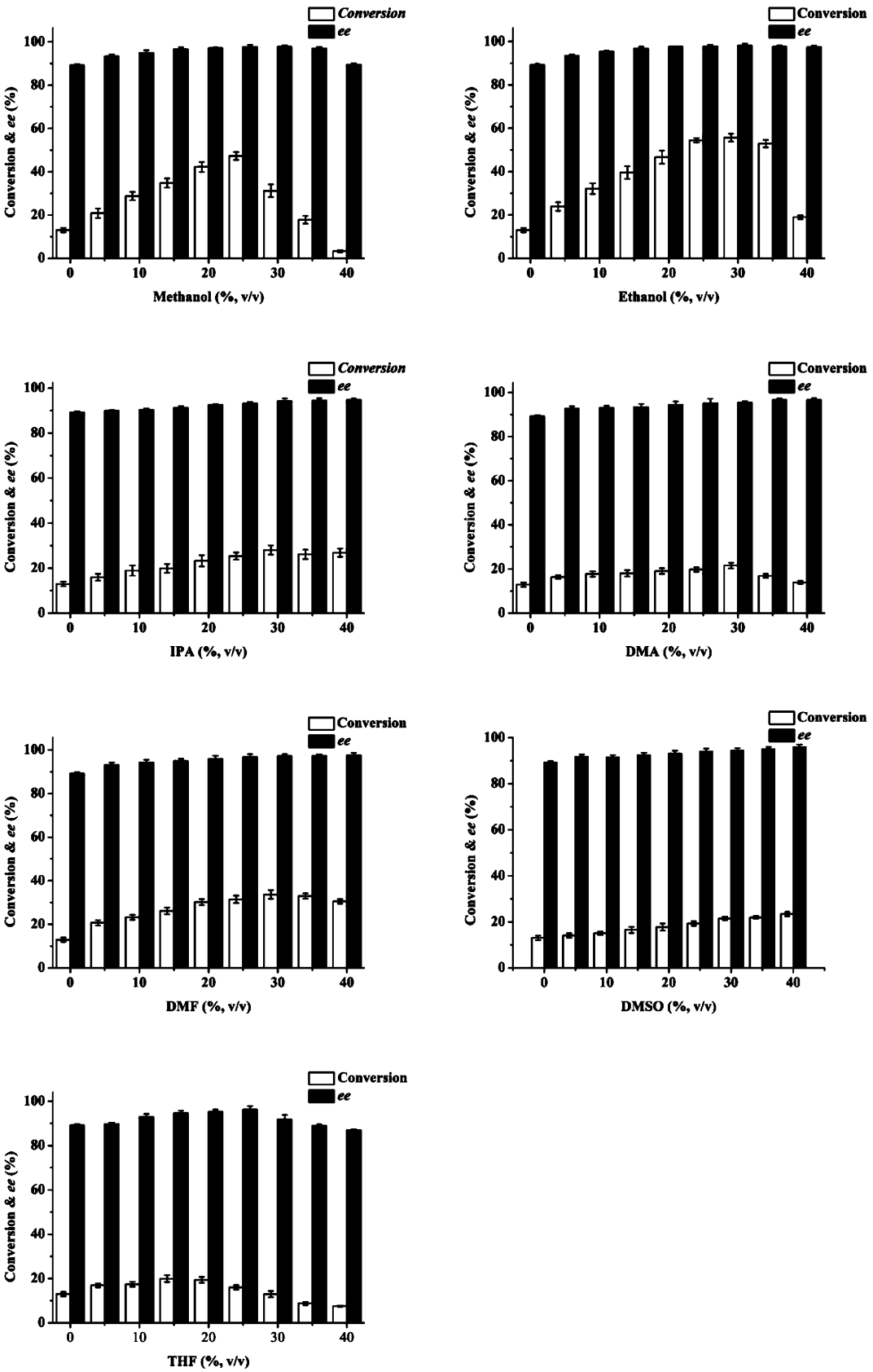


Figure 2. Effects of solvents on the activity and enantioselectivity of *E. coli* M15/BCJ2315 towards *o*-chloromandelonitrile.

aqueous reaction mixture could escalate the mass-transfer problem, thereby decreasing the enzymatic reaction rate, which presents a challenge to enzymatic hydrolysis and hampers the production of (*R*)-*o*-chloromandelic acid in high concentrations. Water-miscible organic solvents in the reaction medium can enhance the availability of insoluble nitrile substrate to the

dx.doi.org/10.1021/op400174a | *Org. Process Res. Dev.* XXXX, XXX, XXX–XXX

nitrilase active site, thereby increasing the catalytic efficiency.[10,12] Accordingly, seven different water-miscible solvents [methanol, ethanol, isopropyl alcohol (IPA), dimethyl acetamide (DMA), dimethyl formamide (DMF), dimethyl sulphoxide (DMSO), and tetrahydrofuran (THF)] were used to test the effects of organic solvents on the reaction conversion and enantioselectivity in *E. coli* M15/BCJ2315-mediated hydrolysis of *o*-chloromandelonitrile by the addition of the organic solvents to the aqueous reaction at different concentrations. The obtained results are shown in Figure 2.

The addition of an organic solvent up to a critical concentration could enhance the reaction conversion by increasing the availability of the substrate. However, further increase in concentration beyond the critical concentration leads to the decrease in catalytic efficiency of the enzyme, signifying the occurrence of protein denaturation. Among the seven tested solvents, methanol and ethanol both exhibited significant enhancement of reaction conversion below the critical concentration compared with the control, which did not contain any organic solvent. The incorporation of either 25% methanol or 30% ethanol to the reaction mixture enhanced reaction conversion to 47.3% and 55.7%, respectively, compared with 13% for the control. While the presence of other organic solvents increased the activity slightly, the enhancement of reaction conversion was not that significant. Below the critical concentration (30%), ethanol demonstrated the highest reaction conversion among the seven tested solvents added in the same final concentration.

In the case of enantioselectivity, each of the seven organic solvents significantly improved the enantioselectivity of nitrilase within a certain range of concentration added to the reaction medium. Three of the solvents, namely, methanol, ethanol, and THF, had a critical concentration below which the enantioselectivity was improved with the increase of concentration. The critical concentrations were 30%, 30%, and 25% for methanol, ethanol, and THF, respectively. The enantioselectivity increased with the increase in the concentration of IPA, DMSO, DMA, and DMF. A critical concentration has not been observed in the tested concentrations for these four solvents. The highest enantioselectivity observed are 94.8% (IPA), 95.9% (DMSO), 96.7% (DMA), and 97.5% (DMF) in the highest concentration (40%) tested, compared with 89.2% in the control, which did not contain any organic solvent. Among the tested solvents, methanol and ethanol demonstrated significant enhancement of the enantioselectivity in a relatively low concentration. The highest enantioselectivity observed was 97.8% in 30% methanol and 98.2% in 30% ethanol. With 20% (v/v) concentration, the enantioselectivity was decreased to 97.1% and 97.6% for methanol and ethanol, respectively. DMF also showed relatively high influence on enantioselectivity, whereas the last four solvents exhibited relativity low enhancement of enantioselectivity compared with methanol and ethanol, especially at low concentrations (5−10%). Therefore, with the obtained results, the addition of either methanol or ethanol was very beneficial to the enhancement of enantioselecitivity. Ethanol is considered as the more suitable solvent candidate for the improvement of the enantioselectivity of the biocatalysts because it exhibited a slightly higher enhancement than methanol.

Changes in solvent type and concentration were shown to yield different effects on enzyme enantioselectivity. Various hypotheses have been proposed to rationalize this phenomenon.[13] Solvent physicochemical properties, such as polarity, could influence the enantioselectivity through altering the molecular recognition process between substrate and enzyme by modifying the enzyme conformation. In another hypothesis, solvent could bind within the enzyme active site and interfere with the association or transformation of one enantiomer more than that of the other, thus influencing the enantioselectivity. Moreover, solvent effects on enzyme enantioselectivity could also contribute to the changes of both differential activation enthalpy and entropy.[14] However, it is likely that the solvent influences enzyme enantioselectivity through more than one mechanism.

Stability is another critical factor that must be considered in choosing the optimal solvent. The stability of the biocatalysts is dependent on the extent of its exposure to the solvent. In this regard, the recombinant *E. coli* M15/BCJ2315 (5 mg of wet cells) resuspended in sodium phosphate buffer (100 mM, pH 8.0) was mixed with each if the seven different organic solvents (20%, v/v). The resulting mixtures (5 mL) were shaken at 200 rpm and 30 °C. At different time intervals (2, 4, 6, and 8 h), an aliquot of each mixture (500 μL) was withdrawn and centrifuged. The obtained cells were washed with physiological saline (NaCl solution, 0.9% w/v), diluted 10-fold, and subsequently used to assay the residual activity toward *o*-chloromandelonitrile. The results are shown in Figure 3. The

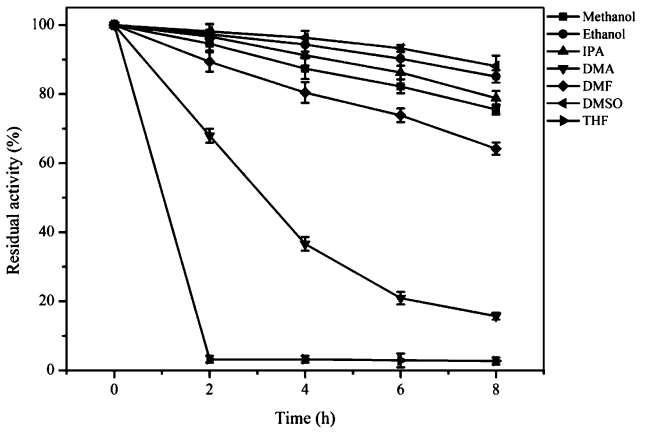


Figure 3. Organic solvent tolerance of the recombinant *E. coli* M15/BCJ2315.

whole cell demonstrated high tolerance toward the majority of the tested organic solvents. Moreover, the whole cell showed the highest tolerance toward DMSO. After 8 h of incubation, the residual activity was 88.1% compared with the control. Ethanol demonstrated the second best biocompatibility toward the whole cell, with a residual activity of 85.1% after 8 h of incubation. THF and DMA were highly detrimental and resulted in the immediate deactivation of the biocatalysts. Particularly, THF resulted in the full deactivation of nitrilase after 2 h of incubation. Methanol, IPA, and DMF showed moderate biocompatibility toward the whole cell. Thus, the "nontoxicity" of ethanol was also an advantage as compared to that of methanol. Accordingly, taking enantioselectivity, activity, and stability into account, ethanol was chosen as the most suitable cosolvent to significantly enhance the properties of *E. coli* M15/BCJ2315, especially its enantioselectivity.

Effect of *o*-Chloromandelonitrile Concentration on (*R*)-*o*-Chloromandelic Acid Production. *o*-Chloromandelonitrile is highly detrimental to nitrilases, only allowing transformation to occur in low concentrations (below 50

dx.doi.org/10.1021/op400174a | *Org. Process Res. Dev.* XXXX, XXX, XXX−XXX

mM).[2] To study the tolerance of *E. coli* M15/BCJ2315 toward *o*-chloromandelonitrile, the transformation reaction was performed with the use of different concentrations of the substrate (30−200 mM) in an aqueous system containing 20% ethanol as cosolvent. The results are shown in Table 1. The

Table 1. Effect of *o*-chloromandelonitrile concentration on (*R*)-*o*-chloromandelic acid production

substrate concn (mM)	rxn time (min)	product concn (mM)	acid ee (%)
30	15	30	96.9
50	30	50	96.3
100	360	42.3	89.7
150	360	17.4	72.5
200	360	12.5	61.5

whole-cell system converted up to 50 mM *o*-chloromandelonitrile within 30 min, with 96.3% ee of the product. Increasing the concentration would result in low conversion due to the inhibition of the substrate. When 100 mM of the substrate was used, the concentration of the product reached to 42.3 mM at 6 h of hydrolysis, after which no product was produced. The ee value of the product decreased to 89.7%. Increasing the amount of the biocatalyst resulted in the complete hydrolysis of 100 mM of substrate, although with a low ee of 74.3% (data not shown). When the substrate concentrations were increased to 150 mM and 200 mM, only 17.4 mM and 12.5 mM of the product were produced in 6 h. Therefore, to obtain a substantial amount of product, the reaction mode must be considered.

Extended Fed-Batch Reaction for (*R*)-*o*-Chloromandelic Acid Production. To obtain higher yields of (*R*)-*o*-chloromandelic acid, an extended fed-batch mode was designed to alleviate the substrate inhibition. In this mode, *o*-chloromandelonitrile was continuously fed into the reaction mixture, and the feeding rate was adjusted to guarantee the reaction conversion above 90% and avoid the inhibition of the substrate at high concentrations. Ethanol (20%, v/v) was used as cosolvent to provide relatively high reaction conversion and ee value of the product. With the feeding of *o*-chloromandelonitrile solubilized in ethanol (5 M), the concentration of ethanol in the reaction increased steadily, and the enantioselectivity of the product and the reaction conversion increased simultaneously. The obtained results are shown in Figure 4.

The substrate-feeding rate was initially set as 22.6 g/L/h. Using this feeding rate, the reaction rate was kept at a high level, and a total of 180 mM (*R*)-*o*-chloromandelic acid was produced within 1.5 h. Subsequently, the feeding rate was adjusted to 11.3 g/L/h due to the decreased reaction conversion, probably mainly caused by the inhibition of the product. The concentration of the product increased to 315 mM within 4 h of reaction time. Finally, the substrate-feeding rate was decreased to 5.5 g/L/h, and substrate feeding was stopped after 7.5 h of reaction time. The reaction was allowed to proceed for another 30 min to achieve complete *o*-chloromandelonitrile conversion. Ultimately, 494 mM of *o*-chloromandelonitrile was completely hydrolyzed, and a total of 415 mM (*R*)-*o*-chloromandelic acid was produced in the final reaction mixture due to the effect of dilution caused by substrate feeding (9.34 mL) and pH control (11 mL) for 8 h. To our knowledge, this value is the highest reported concentration of (*R*)-*o*-chloromandelic acid produced by nitrilase. The ee value of the product was determined to be

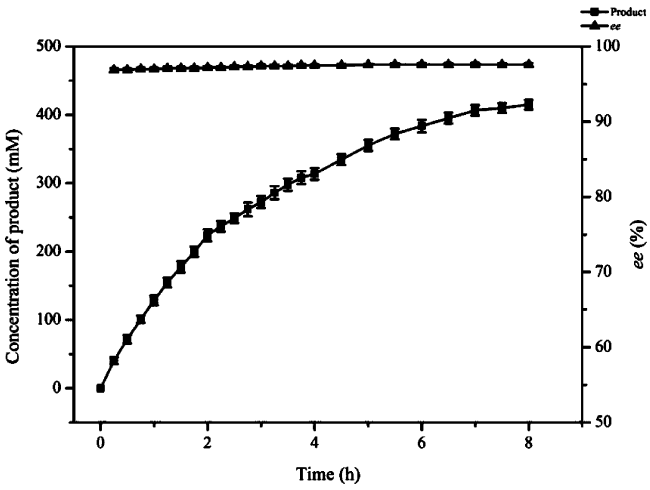


Figure 4. Concentration and ee value of (*R*)-*o*-chloromandelic acid during extended fed-batch reaction (100 mL scale).

96.9% by high performance liquid chromatography (HPLC) analysis at 30 min of reaction time. With the increase of ethanol in the reaction mixture, the enantioselectivity increased concurrently. The ee value of the final product also increased to 97.6%. After recrystallization in toluene, the optical purity of the product further improved to 99.5%. The product was further characterized as follows: $[\alpha]^{25}_{D} = -152.2$ (c = 0.5, ethanol) (literature, $[\alpha]^{25}_{D} = -154.5$; c = 0.52, ethanol);[15] ^{1}H NMR (400 MHz, D_2O) δ/(ppm): 5.65 (s, 1H), 7.39−7.43 (m, 2H), 7.45−7.49 (m, 1H), and 7.51−7.54 (m, 1H).

Through the extended fed-batch method, substrate inhibition was alleviated, and high product concentration with high enantioselectivity was obtained. Compared with the traditional fed-batch mode that fed the substrate intermittently, the extended fed-batch mode ensured optimal reaction rate and avoided substrate inhibition by adjusting the substrate concentration and enhanced the enantioselectivity and reaction conversion by increasing the concentration of ethanol through the feeding of substrate solubilized in ethanol. Ultimately, the use of the extended fed-batch method led to the production of (*R*)-*o*-chloromandelic acid in high concentration and high enantioselectivity in a short period, which is particularly important in large-scale experiments.

Scale-Up of Extended Fed-Batch Reaction to 2 L. On the basis of the observations above, the reaction was scaled up to 2 L. Figure 5 shows the concentration and ee value of the product at various time intervals. About 100% of the feeding substrate was converted into the product within 8 h. A productivity of 27.7 g of (*R*)-*o*-chloromandelic acid per day was obtained in the presence of 1 g of the biocatalyst (wet cells). The process was very stable during scale-up. The ease of the scale-up process makes the extended fed-batch method a favorite route for the industrial production of (*R*)-*o*-chloromandelic acid.

CONCLUSIONS

In summary, a combination of solvent engineering and an extended fed-batch method was successfully used to produce (*R*)-*o*-chloromandelic acid in high concentration and high enantioselectivity by deracemization of *o*-chloromandelonitrile. Ultimately, the recombinant *E. coli* M15/BCJ2315 harboring an arylacetonitrilase from *B. cenocepacia* J2315 produced a maximum of 415 mM (*R*)-*o*-chloromandelic acid in 8 h, with

dx.doi.org/10.1021/op400174a | *Org. Process Res. Dev.* XXXX, XXX, XXX−XXX

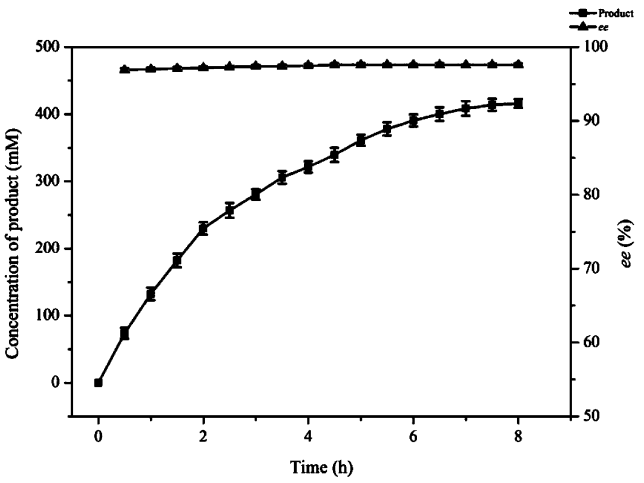


Figure 5. Concentration and ee value of (*R*)-*o*-chloromandelic acid during extended fed-batch reaction (2-L scale).

an ee value of 97.6%. The hydrolysis process was easily scaled up, indicating a great potential for the industrial production of optically pure (*R*)-*o*-chloromandelic acid in high concentrations.

EXPERIMENTAL SECTION

Materials. *B. cenocepacia* J2315 was ordered from American Type Culture Collection (ATCC). *E. coli* M15 and the plasmid pQE30 were used for the expression of nitrilase. *o*-Chloromandelonitrile was ordered from Trademax Pharmaceuticals and Chemicals Co., Ltd. (Shanghai, China). All other chemicals used were of analytical grade and purchased from standard companies.

Effects of Temperature and pH on Activity and Enantioselectivity of *E. coli* M15/BCJ2315. The effects of temperature and pH were determined by incubating the recombinant *E. coli* M15/BCJ2315 with *o*-chloromandelonitrile (10 mM) at different temperatures (20−70 °C) or in buffers with different pH levels (pH 5.2−10.8). To determine the optimum temperature, the reaction was performed with M15/BCJ2315 (1 mg of wet cells) in 1 mL of sodium phosphate buffer (100 mM, pH 7.0) with *o*-chloromandelonitrile (10 mM) at 20−70 °C. To determine the optimum pH, sodium citrate/citric acid buffer (pH 5.2−6.6, 0.1 M), sodium phosphate buffer (pH 7.0−8.0, 0.1 M), Tris-HCl buffer (pH 8.5, 0.1 M), and glycine/sodium hydroxide buffer (pH 9.1−10.8, 0.1 M) were used in the experiment. The recombinant *E. coli* M15/BCJ2315 (1 mg of wet cells) was incubated in 1 mL of each respective buffer with *o*-chloromandelonitrile (10 mM) at 30 °C. The conversion was determined using RP-HPLC after 10 min of reaction. The enantioselectivity was determined using chiral HPLC (see Analytical Methods).

Effects of Solvents on Activity and Enantioselectivity of the *E. coli* M15/BCJ2315. The reaction was performed in a reaction mixture (5 mL) containing sodium phosphate buffer (100 mM, pH 8.0), recombinant *E. coli* M15/BCJ2315 (5 mg of wet cells), and the appropriate amount of organic solvent (0−40%, v/v). After incubation for 20 min at 30 °C, *o*-chloromandelonitrile (5 M, solubilized in organic solvent) was added to a final concentration of 10 mM to initiate the reaction. The conversion was determined using RP-HPLC after 10 min of reaction. The enantioselectivity was determined using chiral HPLC (see Analytical Methods).

Effect of *o*-Chloromandelonitrile Concentration on (*R*)-*o*-Chloromandelic Acid Production. To study the tolerance of *E. coli* M15/BCJ2315 toward *o*-chloromandelonitrile, the concentration of the substrate (30−200 mM) was varied in a 5 mL reaction mixture containing sodium phosphate buffer (100 mM, pH 8.0), ethanol (20%, v/v), and the recombinant *E. coli* M15/BCJ2315 (50 mg of wet cells). The concentration and ee value of the (*R*)-*o*-chloromandelic acid formed in the reaction were determined by HPLC (see Analytical Methods).

Extended Fed-Batch Reaction for (*R*)-*o*-Chloromandelic Acid Production (100-mL scale). To obtain a significant amount of (*R*)-*o*-chloromandelic acid with high enantioselectivity, the biotransformation of *o*-chloromandelonitrile was conducted in an extended fed-batch mode through the continuous feeding of the substrate solubilized in ethanol (5 M). The reaction was performed in a 250-mL, three-necked, round-bottom flask equipped with two bladed turbine impellers driven by a speed-variable motor in its middle neck. A glass electrode was inserted into the flask through one of the side necks to detect the pH of the reaction mixture using a pH/ORP controller. The glass electrode was secured with a rubber stopper, which also had two pipes that were respectively used to supply the substrate and to add NaOH (2 M) for pH adjustment. The left-side neck was used for obtaining samples. The reaction temperature was maintained at 30 °C by a thermostatted water bath. The pH was maintained at 7.5−8.0 by a pH/ORP controller. About 80 mL of sodium phosphate buffer (100 mM, pH 8.0) containing the recombinant *E. coli* M15/BCJ2315 (1 g of wet cells) was added to the flask and stirred for 30 min to equilibrate at 30 °C. Twenty milliliters of substrate (solubilized in ethanol, 150 mM) was then added to initiate the reaction. After 10 min of hydrolysis, *o*-chloromandelonitrile (solubilized in ethanol, 5 M) was fed into the reactor using a peristaltic pump at a feeding rate of 22.6 g/L/h. Samples were periodically withdrawn by syringe and analyzed by HPLC to determine the conversion of *o*-chloromandelonitrile and the ee value of (*R*)-*o*-chloromandelic acid. The feeding rate was adjusted on the basis of HPLC analysis to guarantee a conversion above 90%. When the feeding was stopped, the reaction was allowed to proceed for another 30 min to achieve complete *o*-chloromandelonitrile conversion. Samples were periodically withdrawn by syringe and analyzed by HPLC to determine the conversion and enantioselectivity.

Scale-Up of Extended Fed-Batch Reaction to 2 L. The conversion of *o*-chloromandelonitrile to (*R*)-*o*-chloromandelic acid was scaled up to 2 L in a 5-L double jacket glass reactor (ShenSheng, Shanghai, China) equipped with four-bladed turbine impellers driven by a speed-variable motor (200 rpm). The temperature of the jacketed glass vessel was maintained at 30 °C with the use of water circulating from a heated water bath. In the reaction mixture, 400 mL of substrate (solubilized in ethanol, 150 mM) was added to 1600 mL of sodium phosphate buffer (100 mM, pH 8.0) containing the recombinant *E. coli* M15/BCJ2315 (20 g of wet cells). After 10 min of hydrolysis, *o*-chloromandelonitrile was fed into the reactor using a peristaltic pump at the feeding rate of 22.6 g/L/h. The substrate-feeding rate was adjusted on the basis of the above result.

Analytical Methods. The formation of *o*-chloromandelic acid was analyzed by HPLC using a Zorbax SB-Aq column (4.6 mm × 250 mm, 5 μm) (Agilent Technologies, Ltd., U.S.A.) at a

dx.doi.org/10.1021/op400174a | Org. Process Res. Dev. XXXX, XXX, XXX−XXX

flow rate of 1 mL/min with an eluting solvent system of phosphoric acid (0.1%, v/v) and methanol (40:60, v/v). The retention times for *o*-chloromandelic acid and *o*-chloromandelonitrile were 8.9 and 12.3 min, respectively. Absorbance was measured at 210 nm.

The optical purity of *o*-chloromandelic acid was determined through the analysis of the enantiomers on a CHIRALEL-OZ-RH column (4.6 mm × 150 mm, 5 μm) (Daicel Corporation, Japan) at a flow rate of 0.5 mL/min with an eluting solvent system of trifluoroacetic acid (0.05%) and isopropanol (10:90, v/v). The retention times for the (*S*)-(+)-isomer and the (*R*)-(−)-isomer were 21.5 and 25.3 min, respectively. Absorbance was measured at 210 nm.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: dzhwei@ecust.edu.cn. Telephone: +86-21-64252078. Fax: +86-21-64250068.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the National Major Science and Technology Projects of China (2012ZX09304009) and the Fundamental Research Funds for the Central Universities (WF1013008).

■ REFERENCES

(1) (a) Groger, H. *Adv. Synth. Catal.* **2001**, *343*, 547−558. (b) DeSantis, G.; Zhu, Z.; Greenberg, W. A.; Wong, K.; Chaplin, J.; Hanson, S. R.; Farwell, B.; Nicholson, L. W.; Rand, C. L.; Weiner, D. P.; Robertson, D. E.; Burk, M. J. *J. Am. Chem. Soc.* **2002**, *124*, 9024−9025. (c) Osprian, I.; Fechter, M. H.; Griengl, H. *J. Mol. Catal. B: Enzym.* **2003**, *24*−*5*, 89−98.

(2) Zhang, C. S.; Zhang, Z. J.; Li, C. X.; Yu, H. L.; Zheng, G. W.; Xu, J. H. *Appl. Microbiol. Biotechnol.* **2012**, *95*, 91−99.

(3) Martinkova, L.; Kren, V. *Curr. Opin. Chem. Biol.* **2010**, *14*, 130−137.

(4) (a) Zhang, Z. J.; Xu, J. H.; He, Y. C.; Ouyang, L. M.; Liu, Y. Y.; Imanaka, T. *Process Biochem.* **2010**, *45*, 887−891. (b) Schreiner, U.; Hecher, B.; Obrowsky, S.; Waich, K.; Klempier, N.; Steinkellner, G.; Gruber, K.; Rozzell, J. D.; Glieder, A.; Winkler, M. *Enzyme Microb. Technol.* **2010**, *47*, 140−146.

(5) (a) Olofsson, L.; Soderberg, P.; Nicholls, I. A. *Biotechnol. Lett.* **2006**, *28*, 929−935. (b) Klibanov, A. M. *Nature* **2001**, *409*, 241−246.

(6) Zhang, Z. J.; Pan, J. A.; Liu, J. F.; Xu, J. H.; He, Y. C.; Liu, Y. Y. *J. Biotechnol.* **2011**, *152*, 24−29.

(7) Wang, H.; Sun, H.; Wei, D. *BMC Biotechnol.* **2013**, *13*, 14.

(8) (a) Ottosson, J.; Fransson, L.; Hult, K. *Protein Sci.* **2002**, *11*, 1462−1471. (b) Phillips, R. S. *Enzyme Microb. Technol.* **1992**, *14*, 417−419.

(9) Rustler, S.; Muller, A.; Windeisen, V.; Chmura, A.; Fernandes, B. C. M.; Kiziak, C.; Stolz, A. *Enzyme Microb. Technol.* **2007**, *40*, 598−606.

(10) Kaul, P.; Banerjee, U. C. *J. Ind. Microbiol. Biotechnol.* **2008**, *35*, 713−720.

(11) Bornscheuer, U. T. *Curr. Opin. Biotechnol.* **2002**, *13*, 543−547.

(12) (a) Heinemann, U.; Engels, D.; Burger, S.; Kiziak, C.; Mattes, R.; Stolz, A. *Appl. Environ. Microbiol.* **2003**, *69*, 4359−4366. (b) Banerjee, A.; Kaul, P.; Banerjee, U. C. *Arch. Microbiol.* **2006**, *184*, 407−418.

(13) Carrea, G.; Riva, S. *Angew. Chem., Int. Ed.* **2000**, *39*, 2226−2254.

(14) Ottosson, J.; Fransson, L.; King, J. W; Hult, K. *Biochim. Biophys. Acta, Protein Struct.* **2002**, *1594*, 325−334.

(15) Kurono, N.; Yoshikawa, T.; Yamasaki, M.; Ohkuma, T. *Org. Lett.* **2011**, *13*, 1254−1257.