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Enhanced biotransformation of (*R*,*S*)-mandelonitrile to (*R*)-(−)-mandelic acid with *in situ* production removal by addition of resin

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ABSTRACT

Bioconversion of (*R*,*S*)-mandelonitrile (*R*,*S*-MN) to prepare (*R*)-(−)-mandelic acid (*R*-MA) with nitrilase is an attractive method in industrial application. However, during this bioconversion by whole cells of *Alcaligenes faecalis* CCTCC M 208168, *R*-MA was found to inhibit its own production. To improve *R*-MA productivity, a new biocatalytic process of *in situ* product removal (ISPR) has been developed utilizing anion-exchange resin. To optimize the bioconversion of *R*-MA from *R*,*S*-MN, several anion-exchange resins were examined: HZ202, demonstrated several exciting features including high *R*-MA and low *R*,*S*-MN adsorbance. In batch biotransformation, ISPR by addition of HZ202 increased *R*-MA volumetric productivity to 0.285 mmol/l/min. The kinetic models for enantioselective hydrolysis of *R*,*S*-MN by *A. faecalis* CCTCC M 208168 with ISPR were established, and they were well fitted to the experimental data of the reaction kinetics. In fed-batch biotransformation with ISPR was also performed. As compared to the conventional fed-batch mode, this approach allowed *R*-MA volumetric productivity and biocatalyst productivity to be increased from 0.083 mmol/l/min and 55.1 mmol/g to 0.281 mmol/l/min and 185.5 mmol/g, respectively.

1. Introduction

Nitrilases have recently received considerable attention due to their ability of conducting nitrile hydrolysis to produce corresponding carboxylic acid at ambient conditions with precise chemo-, regio- and enantioselectivity [1–8]. They have mediated the industrial conversion of aliphatic, arylaliphatic, aromatic and heterocyclic nitriles [9–11]. Quantitatively the most important industrial application of nitrilases is the transformation of (*R*,*S*)-mandelonitrile (*R*,*S*-MN) to (*R*)-(−)-mandelic acid (*R*-MA). This reaction exhibits excellent enantioselectivity, cheap starting material and above all a possibility of carrying out a dynamic kinetic resolution of racemic substrate which provides theoretically 100% yield of the product (Fig. 1) [12]. *R*-MA is an important intermediate for the production of pharmaceuticals such as semi-synthetic penicillins, cephalosporins, antitumor agents, antiobesity agents [13–15] and is also used as chiral resolving agent for the resolution of racemic alcohols and amines [16,17].

The nitrilases from *Alcaligenes faecalis* ATCC 8750 [12,18,19], *Pseudomonas putida* MTCC 5110, *Microbacterium paraoxydans*, *M. liquefaciens* [20,21], and *Alcaligenes* sp. ECU0401 [22] have been reported to hydrolyze *R*,*S*-MN to *R*-MA. However, these nitrilases show poor activity and/or stability, lowering their viability for industrial applications. Recently, the strain *A. faecalis* CCTCC M 208168 with outstanding nitrilase activity for racemic mandelonitrile was newly isolated in our laboratory by using screening and mutagenesis methods [23]. The further research showed that the product exhibited inhibitory effect on *A. faecalis* CCTCC M 208168 nitrilase. Thus, measures to decrease this inhibition are very important for the improvement of *R*-MA productivity. The approach that can accomplish this task is the implementation of an *in situ* product removal (ISPR) technique [24–27]. The benefit of ISPR in overcoming product inhibition has been successfully demonstrated [28–32]. This includes the application of anion exchange resins for improving productivities and final product concentrations of whole-cell bioprocesses for the production of organic acids. For instance, Roddick and Britz [28] employed an anion exchange resin Amberlite IRA 400 during culture of *Megasphaera elsdenii* ATCC 25940 for ISPR to improve hexanoic acid production. The equivalent of 11 g/l of hexanoic acid was made with an estimated yield of up to 39%. Wei et al. [29] investigated the production of glycolic acid from ethylene glycol with *Gluconobacter oxydans* DSM 2003 by ISPR using anion exchange resin D315. This approach increased the yield of glycolic acid to 93.2 g/l, compared to 74.5 g/l obtained from a conventional fed-batch mode. Wang et al. [30] developed a technology

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Nomenclature

q^*	adsorption capacity, which represents the quantity of adsorbate adsorbed on 1.0 g of wet resin at adsorption equilibrium (mmol/g wet resin)
E	adsorption ratio, which means percentage of total adsorbate being adsorbed at adsorption equilibrium
C_0	initial concentration of adsorbate (mmol/l)
C^*	equilibrium concentration of adsorbate (mmol/l)
V_0	initial volume of solution added into the flask (l)
W	weight of the wet resin (g)
t	reaction time (min)
c_0	initial concentration of *R*,*S*-MN (mmol/l)
c_1	concentration of *R*,*S*-MN adsorbed on the resin (mmol/l)
$c_{R,S\text{-MN}}$	concentration of *R*,*S*-MN in solution (mmol/l)
$c_{R\text{-MA}}$	concentration of *R*-MA in solution (mmol/l)
$c'_{R\text{-MA}}$	total *R*-MA including in solution and resin phase (mmol/l)
$c_{R\text{-MN}}$	concentration of *R*-MN (mmol/l)
$c_{S\text{-MN}}$	concentration of *S*-MN (mmol/l)
v_0	initial reaction rate (mmol/l/min)
v	reaction rate (mmol/l/min)
K_m	kinetic constant (mmol/l)
V_{max}	maximum reaction rate (mmol/l/min)
K_i	product inhibition constant (mmol/l)

of citric acid fermentation with *in situ* product separation by ion-exchange resin, in which an anion exchange resin packed column was attached to a fermenter for separation of citric acid from the fermentation broth. Citric acid productivity was increased from 0.38 g/l/h to 0.543 g/l/h. Mirata et al. [31] established a method for ISPR based on anion exchange resin Amberlite IRA 410 Cl to overcome product inhibition in the process for the oxidation of limonene to perillic acid with *P. putida* DSM 12264. This led to a cumulative perillic acid concentration of 187 mmol/l after 7 days. To date, however, the application of ISPR to overcome the product inhibition for biocatalytically produced *R*-MA has not been reported.

Here we report the use of ISPR method to eliminate the product inhibition and enhance the biotransformation of *R*,*S*-MN to *R*-MA by whole cells of *A. faecalis* CCTCC M 208168.

2. Materials and methods

2.1. Chemicals

R,*S*-MN, *R*-MA and *S*-MA were purchased from J&K Chemical Co., Ltd. (Shanghai, China). Six anion-exchange resins including

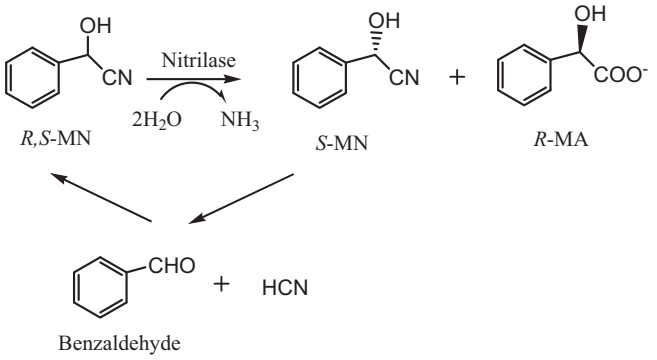

Fig. 1. Scheme of enantioselective hydrolysis of *R*,*S*-MN for production of *R*-MA with nitrilase, which was modified from Fig. 1 in reference [12]. The residual *S*-MN which is unreactive to the nitrilase is spontaneously racemized under alkaline pH and then used as the substrate.

D202, D315, D293, HZ202, 335 and 717 were provided by Shanghai Huazhen Sci. & Tech. Co., Ltd. (Shanghai, China). Their relevant properties are given in Table 1. All the other chemicals were of reagent grade and obtained from commercial sources.

2.2. Microorganism

A. faecalis CCTCC M 208168, which was screened by our lab and previously deposited at the China Center for Type Culture Collection (CCTCC, Wuhan, China), was used in this work. This strain was maintained at 4 °C on agar slants which contained 10 g/l glucose, 5 g/l yeast extract, 5 g/l peptone, 5 g/l K_2HPO_4, 0.2 g/l $MgSO_4$, 0.03 g/l $FeSO_4$, 1 g/l NaCl, and 20 g/l agar (pH 7.0). It was transferred every three months.

2.3. Culture medium and culture condition

Seed medium contained: 10 g/l ammonium acetate, 6 g/l yeast extract; 5 g/l K_2HPO_4, 0.2 g/l $MgSO_4$, and 1.0 g/l NaCl (pH 7.2). The optimized fermentation medium contained: 12.14 g/l ammonium acetate, 7.79 g/l yeast extract, 5 g/l K_2HPO_4, 0.2 g/l $MgSO_4$, and 1.0 g/l NaCl (pH 7.5). All media were prepared with tap water and sterilized at 115 °C for 20 min.

The inoculum was prepared by transferring a loopful of colony from 2-day-old slant into a 50-ml seed medium in a 250-ml flask and being cultivated at 30 °C for 24 h on a rotary shaker (150 rpm). After a subsequent preculturing in flasks with larger volume, the cultures were used as an inoculum and inoculate the optimized fermentation media at a level of 3% (v/v). Then, *n*-butyronitrile (3.29 g/l) was added to induce the nitrilase activity. Incubation was carried out on a rotary shaker at 150 rpm and 30 °C. The cells were harvested by centrifugation at 4 °C, 9000 × *g* for 15 min, and stored at 4 °C until further use.

Table 1
The resins examined and their properties.

Resins	Polymer structure	Functional group	Water content (%)	Particle size (mm)	Bulk density (g/ml)	Total capacity [mmol/g (dry resin)]
D202	Macroporous polystyrene crosslinked with divinylbenzene	Quaternary ammonium	47.0–57.0	0.3–1.2	0.65–0.75	≥3.5
D315	Macroporous polyacrylic acid crosslinked with divinylbenzene	Tertiary amine	50.0–62.0	0.3–1.2	0.68–0.8	≥6.5
D293	Macroporous polystyrene crosslinked with divinylbenzene	Quaternary ammonium	50.0–60.0	0.3–1.2	0.65–0.75	≥3.3
HZ202	Gel polystyrene crosslinked with divinylbenzene	Quaternary ammonium	65.0–75.0	0.3–1.2	0.50–0.70	≥3.8
335	Porous polyethylene polyamine crosslinked with epichlorohydrin	Tertiary amine	65.0–75.0	0.3–2.0	0.65–0.8	≥9.5
717	Gel polystyrene crosslinked with divinylbenzene	Quaternary ammonium	42.0–52.0	0.3–1.2	0.65–0.75	≥3.4