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ORIGINAL PAPER

Development of nitrilase-mediated process for phenylacetic acid production from phenylacetonitrile

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Abstract This study aimed at developing an efficient biotransformation process for phenylacetic acid production from phenylacetonitrile by using recombinant *Escherichia coli* M15 harboring a double mutant MG nitrilase (I113M/Y199G) from *Burkholderia cenocepacia* J2315. A yield of 2310 U/mL nitrilase was obtained by fermentation after the optimization of cultivation conditions, with a specific activity of 64 U/mg dcw. The MG nitrilase showed high substrate tolerance and completely hydrolyzed 100 mM phenylacetonitrile in 30 min under optimal conditions. To alleviate substrate inhibition, periodic or continuous batch-feeding of substrate was used during the biotransformation. Up to 164 g/L substrate was completely hydrolyzed in 9 h with continuous batch-feeding using resting cells, corresponding to 400 U/mL of nitrilase activity, and leading to production of 163.4 g/L phenylacetic acid. The hydrolysis process has potential application for phenylacetic acid production on a large scale.

Keywords Biotransformation · Fed-batch reaction · Fermentation · Nitrilase · Phenylacetic acid

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Introduction

Phenylacetic acid is an important intermediate with a high demand in the pharmaceutical and perfume industries, and the major application of phenylacetic acid is as a key precursor for the synthesis of penicillin G (Zhu et al. 2011). Several methods have been developed to produce phenylacetic acid. The classic industrial process is the phenylacetonitrile hydrolysis method, which requires the presence of mineral acids at temperatures of 80–250 °C (Kagawa et al. 1980).

Alternative biotechnology methods for phenylacetic acid production have been reported. Koma et al. (2012) reported a metabolically engineered *Escherichia coli* strain with an expanded shikimate pathway for production of phenylacetic acid. Another potential route for phenylacetic acid production was by a fermentation process mediated by *Pseudomonas* or *Comamonas* using phenylalanine as starting material (Farbood et al. 1995). Bertokova et al. (2015) and Mihal et al. (2016, 2017) used whole-cell *Gluconobacter oxydans* as a biocatalyst for the production of phenylacetic acid through 2-phenylethanol oxidation. However, these methods had limited applications in industry due to low production efficiency.

Nitriles constitute a versatile class of intermediates for the production of various pharmaceuticals and fine chemicals (Banerjee et al. 2002; de Oliveira et al. 2013; Kielbasinski et al. 2008). Compared to chemical transformation methods, nitrilases allow nitrile hydrolysis to occur under mild conditions of temperature and pH (Vergne-Vaxelaire et al. 2013; Xue et al. 2016; Zhu et al. 2008). In recent years, nitrile hydrolysis by nitrilase has been successfully applied for the commercial production of carboxylic acids (Wang 2015). Therefore, the biotransformation of

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phenylacetonitrile by nitrilase could be a promising method for production of phenylacetic acid.

In our previous work, a novel nitrilase from *Burkholderia cenocepacia* J2315 (BCJ2315) was discovered, and a double mutant MG (I113M/Y199G) that had higher activity for the hydrolysis of arylacetonitriles was identified (Wang et al. 2013, 2015). In the present work, for biotransformation of phenylacetonitrile to phenylacetic acid using resting cells of *E. coli* expressing MG as a biocatalyst, optimal reaction conditions were determined, including the co-solvents, concentrations of substrate and resting cells, pH, and temperature. Then, to alleviate substrate inhibition, a fed-batch reaction system with a periodic or continuous substrate feeding in a mono aqueous phase system was developed.

Experimental

Materials

Phenylacetonitrile and phenylacetic acid were purchased from Aladdin Co., Ltd. (Shanghai, China). Tryptone and yeast extract powder were purchased from Oxoid Ltd. (Cambridge, UK). All the other chemicals used in this study were of analytical grade.

Optimization of culture media and conditions

A medium named FM designed based on Terrific Broth (TB) was used for cultivation of the recombinant *E. coli*/MG cells. First, the cultivation temperature after induction and the IPTG concentration were optimized. Then FM medium (12 g/L tryptone, 16 g/L yeast extract, 24.75 g/L K_2HPO_4, 3.47 g/L KH_2PO_4, 7 mM glycerol, 10 mM $MgCl_2$) was used as the fermentation medium for high-density cultivation of the *E. coli*/MG in a bioreactor. Different glycerol concentrations in FM medium were used for optimization. Ampicillin (0.1 mM) and kanamycin (0.05 mM) were added to all media to maintain selection pressure during cultivation of *E. coli*/MG.

Cultivation of the recombinant *E. coli* M15/MG in a fermenter

Cultivation was performed in a volume of 2 L in a 7-L fermenter. A 100-mL seed culture was incubated in LB broth in a shake flask at 37 °C, 200 rpm for 8 h and then inoculated into the fermenter. The initial parameters were set at pH 7.2, 37 °C, a stir rate of 300 rpm, and an aeration rate of 0.5 vvm. A supplementary medium (40 g/L tryptone and 30 g/L yeast extract) was used for continuous feeding of carbon and nitrogen sources during the fermentation period. The feeding started with an initial speed of 10 mL/h when the optical density at 600 nm (OD_{600}) of the culture reached 4–4.5. Ammonia was used to maintain the pH above 7.0. The growing cells were induced with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) when the OD_{600} reached 20. The cultivation temperature was reduced to 30 °C immediately before the induction. After induction, the supplementary medium feeding rate was increased to 20 mL/h. The dry cell weight (dcw), OD_{600}, and nitrilase activity were determined by taking samples at different time points.

Nitrilase activity assay

The reaction mixture (5 mL) containing sodium phosphate buffer (100 mM, pH 8.0), ethanol (10% v/v), and an appropriate amount of resting cells was incubated at 30 °C for 10 min. The reaction was then initiated by adding 100 mM phenylacetonitrile. Samples were periodically withdrawn and quenched by the addition of 10% (v/v) diluted HCl (2 M). The concentration of phenylacetic acid was determined by high-performance liquid chromatography (HPLC). One unit of nitrilase activity is defined as the amount of nitrilase producing 1 μmol phenylacetic acid per minute under the assay conditions.

Effect of reaction temperature and pH

The determination of optimal reaction temperature and pH was carried out by incubating the *E. coli*/MG with phenylacetonitrile (100 mM) at different temperatures (20–50 °C) or in buffers with different pH values (100 mM sodium phosphate buffer for pH 6.0–8.0 and 100 mM Tris–HCl buffer for pH 8.5–8.9). The thermostability was investigated by incubation of the *E. coli*/MG at different temperatures (20–50 °C) in 100 mM sodium phosphate buffer (pH 8.0).

Effect of co-solvent

The reaction mixture (5 mL) containing sodium phosphate buffer (100 mM, pH 8.0), resting cells (100 mg), and the organic solvent (10% v/v) was incubated at 30 °C for 10 min and then the reaction was initiated by addition of phenylacetonitrile to 100 mM final concentration. The reaction was terminated after 10 min and the conversion extent was determined. In some cases, ethanol (5–25% v/v) was added as co-solvent for investigation of the effects of ethanol concentration on the conversion.

Effect of phenylacetonitrile and resting cell concentrations on phenylacetic acid production

The hydrolysis was performed with substrate concentrations ranging from 25 to 150 mM in a 5-mL reaction system which contained sodium phosphate buffer (100 mM, pH 8.0), the resting cells (400 U/mL), and ethanol (10% v/v) at 30 °C for 10 min. The concentration of product was determined by HPLC analysis. To obtain complete conversion, different concentrations of resting cells (200–600 U/mL) were used in the reaction and the conversion was determined after 30 min of hydrolysis.

Fed-batch production of phenylacetic acid

The fed-batch reaction (300-mL scale) with periodic or continuous substrate feeding mode was performed in a 500-mL three-necked round-bottom flask. The reaction temperature was maintained at 30 °C in a water bath. A pH auto-controller was used to maintain the pH value between 7.9 and 8.1 by addition of ammonia. The agitation speed was set at 300 rpm. The reaction mixture (300 mL) containing sodium phosphate buffer (100 mM, pH 8.0), resting cells (400 U/mL), and ethanol (10% v/v) was incubated at 30 °C for 20 min; then the reaction was initialized by addition of 100 mM substrate. In periodic substrate feeding mode, phenylacetonitrile (100 mM) was fed into the reaction mixture when the last batch of substrate was completely converted. In continuous substrate feeding mode, phenylacetonitrile feeding was started after 30 min with a rate of 23 mL/L h. To monitor the reaction process, samples were taken at intervals and analyzed by HPLC.

Analytical methods

The formation of phenylacetic acid was analyzed by HPLC using a Zorbax SB-Aq column (4.6 mm × 250 mm, 5 μm) (Agilent Technologies, Ltd., USA) eluted with methanol/water/phosphoric acid (40:60:0.1 v/v/v) with a flow rate of 0.8 mL/min. Peaks were detected with a UV detector at 210 nm.

Results and discussion

Effect of temperature in cultivation

To determine the optimal temperature for MG expression, the effects of cultivating the recombinant cells at 20, 30, and 37 °C were investigated (Table 1). The minimum nitrilase production was obtained at 37 °C after 28 h of cultivation. SDS-PAGE analysis of the resting cells confirmed that most MG nitrilase was expressed as inclusion bodies (data was not shown). To enhance recovery of the soluble form of MG nitrilase, cultivation of the *E. coli*/MG was performed at lower temperatures. Compared to cultivation at 20 °C, the maximum cell mass and nitrilase production were both obtained at 30 °C. Therefore, 30 °C was chosen as the optimal cultivation temperature.

Effect of IPTG concentration in induction

To determine the optimal concentration of IPTG for induction during the cultivation period, varying concentrations of IPTG (0.025, 0.05, 0.1, 0.2, and 0.4 mM) were used for induction (Table 2). First, in the IPTG concentration range of 0.025–0.1 mM, nitrilase production increased with increasing IPTG concentration, indicating that the IPTG concentration for culture induction was not saturated. In the IPTG concentration range of 0.1–0.4 mM, a reduction of nitrilase production was observed, which was probably caused by IPTG inhibition of cell growth. Maximum nitrilase production and specific activity were obtained at 0.1 mM IPTG. Therefore, 0.1 mM IPTG was used as the optimal induction concentration.

Optimization of medium composition

In order to obtain a large quantity of the protein of interest, a modified fermentation medium (FM) was designed based on TB broth. The effect of glycerol concentration on nitrilase production was investigated (Table 3). Increasing the glycerol concentration led to an apparent enhancement of cell mass. However, excess glycerol (15 mM) resulted in reduced nitrilase production. Maximum nitrilase production was obtained with addition of 7 mM glycerol. Comparing addition of 7 mM glycerol with no addition, although the specific activity was reduced to approximately 86%, more than twofold greater cell mass was obtained. Taking into account the effects on nitrilase production, specific activity, and cell mass, addition of 7 mM glycerol to the FM medium was chosen for high cell density cultivation.

Fermentation in a bioreactor

Based on the optimization results in shake flasks, 2-L fed-batch fermentation was performed in a 7-L fermenter to further enhance nitrilase production and cell mass. The growth and nitrilase production profiles of *E. coli*/MG are shown in Fig. 1. The maximum nitrilase production was obtained after 28 h of fermentation, and then a decrease of nitrilase production was observed. Finally, cell mass of 35.9 mg/mL dcw and 2310 U/mL nitrilase production were obtained. Compared to the results obtained with shake

Table 1 Effect of temperature on the growth and nitrilase production of recombinant *E. coli*

Temperature (°C)	OD_{600}	Cell mass (mg dcw/mL)	Specific activity (U/mg dcw)	Production (U/mL)
20	4.43	5.11	77.4	395.3
30	5.12	5.39	92.4	498.0
37	4.62	4.23	35.7	151.0

E. coli were cultivated in FM medium in a flask at 37 °C before induction by IPTG at the indicated temperature

Table 2 Effect of IPTG concentration on the growth and nitrilase production of recombinant *E. coli*

IPTG concentration (mM)	OD_{600}	Cell mass (mg dcw/mL)	Specific activity (U/mg dcw)	Production (U/mL)
0.025	6.72	6.41	60.3	396.7
0.05	6.14	5.65	77.7	438.8
0.1	5.12	5.39	92.4	498.0
0.2	5.62	5.45	69.7	379.7
0.4	5.60	5.42	70.0	379.5

E. coli were cultivated in FM medium in a flask for 28 h at 30 °C

Table 3 Effect of medium components on the growth and nitrilase production of recombinant *E. coli*

Medium	OD_{600}	Cell mass (mg dcw/mL)	Specific activity (U/mg dcw)	Production (U/mL)
FM	5.12	5.39	92.4	498.0
FM + 7 mM glycerol	7.04	12.09	79.5	961.2
FM + 15 mM glycerol	8.16	13.87	61.2	848.6
FM + 30 mM glycerol	9.86	16.69	52.0	867.9

E. coli were cultivated at 30 °C for 28 h. FM medium used here represents the components without glycerol

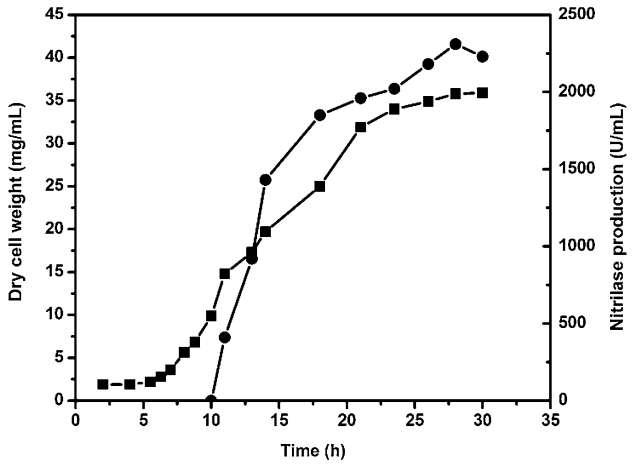


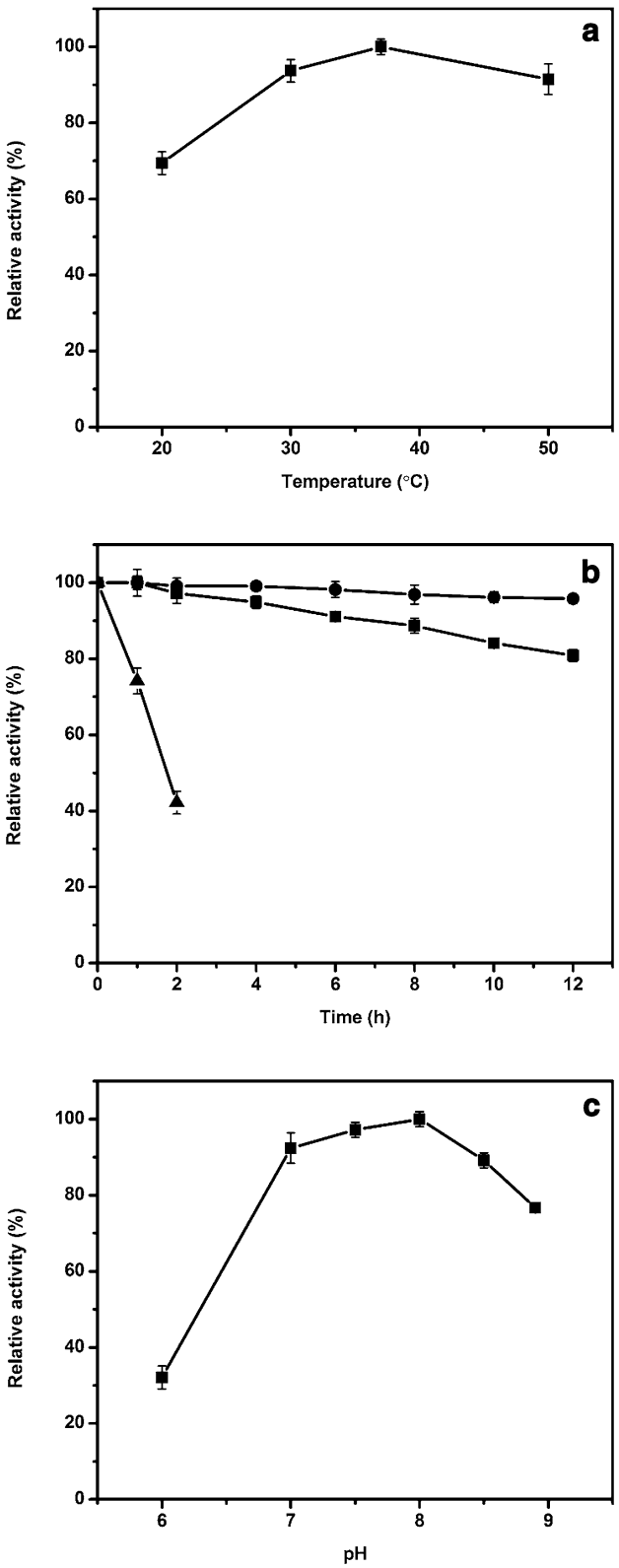
Fig. 1 *Escherichia coli*/MG fermentation in 7-L fermenter. The symbols represent: *squares* dcw; and *circles* nitrilase production. Maximum dcw and nitrilase production were obtained at 28 h as 35.0 mg/mL and 2310 U/mL, respectively

flasks, approximate increases of threefold in cell mass and 2.4-fold in nitrilase production were observed with a 7-L fermenter.

Effect of reaction temperature and pH

The nitrilase showed relatively high activity with phenylacetonitrile as substrate between 30 and 50 °C, and the maximum activity was observed at 37 °C (Fig. 2a). Furthermore, the thermostability of the nitrilase was determined over a period of 12 h (Fig. 2b). Although 37 °C was the optimal temperature for the MG activity, the activity was not as stable at 37 °C compared with 30 °C. About 19 and 4% of nitrilase activity were lost during 12 h incubation at 37 and 30 °C, respectively. Therefore, considering the importance of the stability of nitrilase activity for the bioconversion of phenylacetonitrile over an extended period, 30 °C was chosen as the optimal temperature for the fed-batch reaction experiments.

High activity was maintained over the pH range from 7.0 to 8.5, with the minimum relative activity observed (89.2%) at pH 8.5 (Fig. 2c). Furthermore, the MG activity showed good pH stability over the pH range of 7.0–8.5 (data not shown). Therefore, for phenylacetonitrile bioconversion, the pH of the reaction mixture was maintained at approximately 8.0.



◀**Fig. 2** **a** Effect of temperature on the conversion of phenylacetonitrile to phenylacetic acid. The activity at 37 °C was taken to be 100% (64.3 U/mg). **b** Thermostability of the nitrilase. *Circles* 30 °C, *squares* 37 °C, *triangles* 50 °C. The activity at initial time was taken as 100% (60.2, 64.3, 58.8 U/mg for 30, 37 and 50 °C, respectively). **c** Effect of pH on the conversion of phenylacetonitrile to phenylacetic acid. pH 6.0–8.0, 100 mM sodium phosphate buffer; pH 8.5–8.9, 100 mM Tris–HCl buffer. The activity at pH 8.0 was taken to be 100% (64.3 U/mg)

Effect of co-solvent

In order to enhance the catalytic efficiency, six water-miscible solvents, methanol, ethanol, isopropanol, acetone, dimethyl sulfoxide (DMSO), and tetrahydrofuran (THF), were used for the testing the effects of co-solvents on the conversion of phenylacetonitrile by increasing the solubility of the substrate (Fig. 3a). Alcohols showed the best compatibility with the nitrilase, especially as 100% conversion was achieved in the presence of methanol and ethanol. Because of environmental and toxicology considerations, ethanol was chosen as the co-solvent for the biotransformation. The optimal ethanol concentration in the reaction mixture was determined (Fig. 3b). The phenylacetonitrile could be hydrolyzed completely at ethanol concentrations up to 15% (v/v). Further increase in the ethanol concentration present in the reaction led to decreased conversion efficiency.

Effects of phenylacetonitrile and resting cell concentration

To determine the tolerance of the resting cells to phenylacetonitrile, the biotransformation reaction was performed with varying substrate concentrations in an aqueous system containing 10% (v/v) ethanol. Up to a phenylacetonitrile concentration of 100 mM, complete hydrolysis was achieved in 30 min (Fig. 4a). Further increase in the substrate concentration resulted in inhibition of conversion. Hydrolysis of 100 mM phenylacetonitrile was measured at nitrilase concentrations of 200–600 U/mL to determine a suitable catalyst concentration. Complete conversion of the substrate was achieved with 400 U/mL nitrilase in 30 min (Fig. 4b). 300 U/mL of nitrilase addition resulted in 97.7% conversion of the substrate. Therefore, 100 mM substrate with 400 U/mL of nitrilase was chosen for the fed-batch reaction experiments.

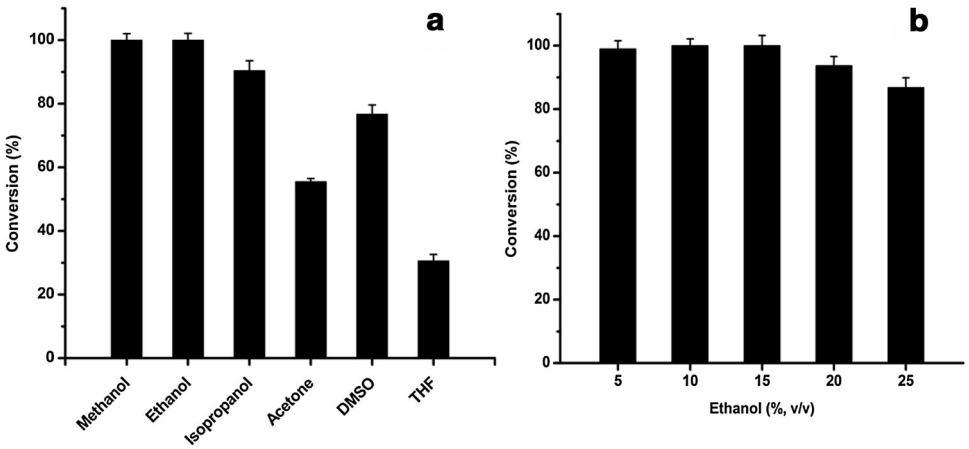


Fig. 3 Effect of co-solvents on the conversion of phenylacetonitrile to phenylacetic acid. **a** Effect of different co-solvents. **b** Effect of ethanol concentration. The 100% conversion was defined as 100 mM phenylacetonitrile completely hydrolyzed in 10 min under the described nitrilase assay conditions

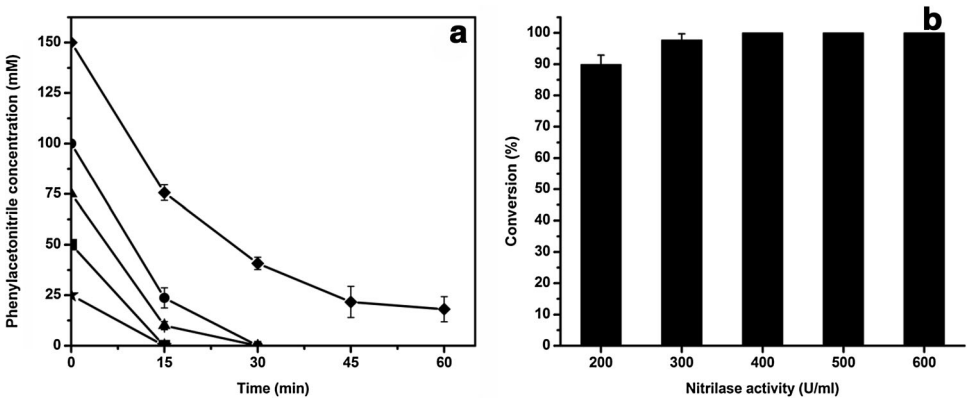


Fig. 4 **a** Effect of phenylacetonitrile concentration on phenylacetic acid production. *Asterisks* 25 mM, *squares* 50 mM, *triangles* 75 mM, *circles* 100 mM, *diamonds* 150 mM. **b** Effect of nitrilase activity dosage on phenylacetonitrile conversion. The 100% conversion was defined as 100 mM phenylacetonitrile completely hydrolyzed in 10 min under the described nitrilase assay conditions

Fed-batch production of phenylacetic acid

The catalysis efficiency of the MG nitrilase showed a reduction if the concentration of the phenylacetonitrile in the reaction mixture was higher than 100 mM. Hence, a period fed-batch mode was designed to maintain hydrolysis performance at a low substrate concentration. In this mode, 100 mM phenylacetonitrile was fed into the reaction mixture when the last substrate was completely converted. Using this mode, ten batch additions were performed in 8 h and 117.1 g/L of phenylacetonitrile was hydrolyzed (Fig. 5a). Reaction volume expansion caused by the substrate and ammonia addition resulted in the production of 0.9 M (122.5 g/L) of phenylacetic acid. During the first eight batches, the hydrolysis rate barely decreased. Due to the decrease in nitrilase activity during the reaction period, the reaction time was extended to ensure complete substrate hydrolysis for the ninth and tenth batches. Considering the biotransformation efficiency, the substrate feeding was terminated after the tenth batch.

In the continuous fed-batch mode, the phenylacetonitrile feeding rate was set at 200 mM/h to keep the concentration of substrate in the reaction system below 100 mM, which should alleviate the nitrilase inhibition observed at high substrate concentrations. A total of 164 g/L phenylacetonitrile was completely hydrolyzed in 9 h, and a measured concentration of 1.2 M (163.4 g/L) phenylacetic acid was obtained because of the reaction volume expansion caused by substrate and ammonia addition (Fig. 5b). During the first 6 h, the nitrilase activity barely decreased, and a high rate of phenylacetonitrile hydrolysis was maintained. After 6 h of substrate feeding, the hydrolysis rate clearly decreased. Thus, substrate feeding was stopped after 8 h, and an additional hydrolysis period (1 h) was continued to guarantee complete hydrolysis.

At a comparable feeding rate, higher product concentration was obtained by the continuous fed-batch mode than that obtained by the periodic fed-batch mode. In addition, the continuous fed-batch reaction mode was more

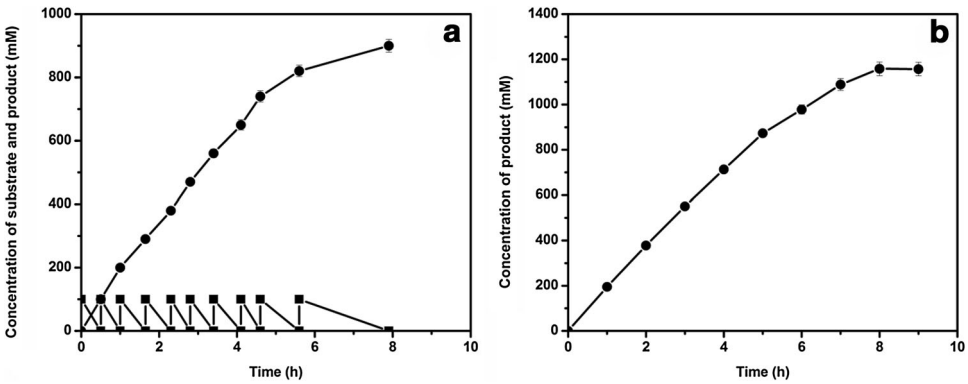


Fig. 5 Time course of phenylacetonitrile hydrolysis with fed-batch mode (300-mL scale). **a** Periodic substrate feeding mode. Phenylacetic acid and phenylacetonitrile concentrations in the reaction are denoted by *circles* and *squares*, respectively. **b** Continuous substrate feeding mode

efficient than the periodic fed-batch reaction mode (18.2 g/L h compared to 15.3 g/L h, respectively) and more convenient to operate. Using the continuous fed-batch reaction mode with *E. coli*/MG, high-volume productivity (163.4 g/L) of phenylacetic acid was obtained. Compared with the nitrilase-mediated route, there are several cases for biocatalytic production of phenylacetic acid. However, these reported processes were quite different, such as double enzymatic cascade reaction process (Mihal et al. 2016), 2-phenylethanol oxidation catalysis by whole-cell *G. oxydans* (Bertokova et al. 2015; Mihal et al. 2017), and metabolically process (Koma et al. 2012). Among them, the highest phenylacetic acid concentration (25 g/L) was obtained by whole-cell *G. oxydans* mediated 2-phenylethanol oxidation process in 7 days. Compared to these mentioned process, the nitrilase-mediated process demonstrated great potential for industrial production of phenylacetic acid.

Conclusions

In summary, optimized culture conditions were developed for the fermentation of the recombinant *E. coli* which harbored the nitrilase MG. Using the whole-cell *E. coli*/MG as catalyst, a bioprocess was developed to completely hydrolyze as high as 164 g/L phenylacetonitrile, leading to production of 163.4 g/L phenylacetic acid. The bioprocess could be easily scaled up and showed high potential for industrial application.

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