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Enzymatic production of 2-amino-2,3-dimethylbutyramide by cyanide-resistant nitrile hydratase

Zhi-Jian Lin · Ren-Chao Zheng · Ya-Jun Wang · Yu-Guo Zheng · Yin-Chu Shen

Abstract A novel enzymatic route for the synthesis of 2-amino-2,3-dimethylbutyramide (ADBA), important intermediate of highly potent and broad-spectrum imidazolinone herbicides, from 2-amino-2,3-dimethylbutyronitrile (ADBN) was developed. Strain *Rhodococcus boritolerans* CCTCC M 208108 harboring nitrile hydratase (NHase) towards ADBN was screened through a sophisticated colorimetric screening method and was found to be resistant to cyanide (5 mM). Resting cells of *R. boritolerans* CCTCC M 208108 also proved to be tolerant against high product concentration (40 g l^{-1}) and alkaline pH (pH 9.3). A preparative scale process for continuous production of ADBA in both aqueous and biphasic systems was developed and some key parameters of the biocatalytic process were optimized. Inhibition of NHase by cyanide dissociated from ADBN was successfully overcome by temperature control (at 10°C). The product concentration, yield and catalyst productivity were further improved to 50 g l^{-1}, 91% and 6.3 g product/g catalyst using a 30/70 (v/v) *n*-hexane/water biphasic system. Furthermore, cells of *R. boritolerans* CCTCC M 208108 could be reused for at lease twice by stopping the continuous reaction before cyanide concentration rose to 2 mM, with the catalyst productivity increasing to 12.3 g product/g catalyst. These results demonstrated that enzymatic synthesis of ADBA using whole cells of *R. boritolerans* CCTCC M 208108 showed potential for industrial application.

Introduction

Imidazolinone herbicides, such as imazethapyr, imazaquin, and imazapyr are widely used around the world for broad-spectrum control of broadleaf weeds and grasses in rice and leguminous crops [1, 33]. They are also important tools for weeds management in forests, railways, freeways, and plantation crops [27, 34]. 2-Amino-2,3-dimethylbutyramide (ADBA) is a key intermediate of these highly potent and broad-spectrum herbicides [11, 28, 31]. In conventional synthesis of ADBA, 2-amino-2,3-dimethylbutyronitrile (ADBN) was hydrated by excess concentrated sulfuric acid with concentrated ammonia at 100°C [11, 28] or considerable hydrogen peroxide solution with concentrated ammonia and palladium-on-carbon [3], resulting in large amounts of salt wastewater and by-products. In addition, ADBA produced by these methods required time-consuming and expensive isolation procedures involving extraction with five equivalent volumes of dichloromethane [11, 28]. Chemical methods had drawbacks such as low product yield and high waste production, rendering them both energy-intensive and eco-harming.

Nitrile hydratase (NHase, EC 4.2.1.84) catalyzes hydration of a wide variety of nitrile compounds into higher-value amides under mild conditions [2, 18, 32]. It has been successfully applied to the industrial production of acrylamide

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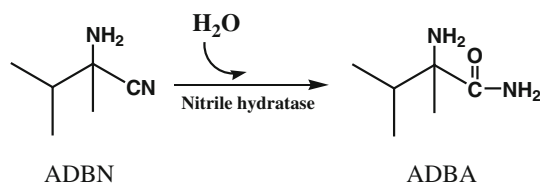
Scheme 1 Nitrile hydratase-catalyzed preparation of ADBA, a key intermediate for imidazolinone herbicides [28, 31]

[2, 20, 21], nicotinamide [25, 29], and 3-cyanovaleramide [21]. NHase provides a more efficient and greener alternative approach for the synthesis of ADBA. To the best of our knowledge, there have been few studies on biocatalytic preparation of ADBA reported up to now [17].

The main challenge in developing biocatalytic process of ADBA is the high sensitivity of NHase to low concentration of cyanide dissociated from ADBN (α-aminonitrile). It is well known that α-aminonitriles are unstable in water, and decompose spontaneously to form aldehydes or ketones accompanied with hydrogen cyanide [4, 5]. These by-products, mainly cyanide, are known to be strong inhibitors of NHase [5, 10, 15, 22]. As previously reported, in the presence of 0.01 and 1 mM cyanide, NHases from *R. rhodochrous* J1 and *Pseudomonas putida* NRRL-18668 lost more than 62 and 70% of their activity, respectively [10, 22], while whole cells of *R. erythropolis* 870-AN019 lost half of its NHase activity after incubation in 25 μM KCN [5]. What's more, inhibition of NHase caused by cyanide was irreversible [30]. As a result, reduction of NHase inhibition by cyanide or screening of NHase tolerant to cyanide is crucial for the industrial bioproduction of α-aminoamides. Another approach is to use water-immiscible organic solvent to establish a biphasic system, which can minimize inhibition of substrate and/or product [7, 13]. Although biphasic system has been well established in many biocatalytic processes, its application in NHase-mediated bioconversion is rarely reported.

In this paper, a NHase-producing strain *R. boritolerans* CCTCC M 208108 with high activity towards ADBN and resistance against cyanide was isolated. We developed a biocatalytic method for continuous production of ADBA in both aqueous and biphasic systems using *R. boritolerans* CCTCC M 208108 (Scheme 1). Bioconversion parameters, reuse of biocatalysts, isolation and purification of ADBA were investigated.

Materials and methods

Materials and strains

ADBN and ADBA were purchased from Jintan Dengguan Chemical Co., Ltd. (Jiangsu, China). All other chemicals were of analytical purity and commercially available. Soil samples used to isolate bacteria harboring NHase were collected from nitrile-contaminated areas around chemical plants in Zhejiang and Fujian Province, China.

Potential NHase-producing strains including *R. boritolerans* CCTCC M 208108, *Serratia marcescens* CCTCC M 208231, *Alcaligenes faecalis* CCTCC M 208168, *Bacillus subtilis* CCTCC M 206038, *R. ruber* CCTCC M 206040, *Rhodococcus* sp. G20, and *Rhodococcus* sp. N595 were previously isolated from soil and preserved in our laboratory [14, 16]. *Rhodococcus* sp. G20 and *Rhodococcus* sp. N595 producing NHases towards other nitriles were previously reported by us [14, 16]. Strain *R. boritolerans* CCTCC M 208108 was deposited in the China Center for Type Culture Collection (CCTCC, Wuhan, China) and identified as *Rhodococcus boritolerans* with regard to its morphology, physiological tests and 16S rRNA sequence. The sequence was deposited in the GenBank database under accession number FJ597543.

Media

The enrichment medium consisted of (g l^{-1}) glucose (10.0), KH$_2$PO$_4$ (3.3), K$_2$HPO$_4$ (0.8), MgSO$_4$·7H$_2$O (0.2), NaCl (1.0), FeSO$_4$·7H$_2$O (0.01), CoCl$_2$ (0.01), CaCl$_2$ (0.015) and ADBN (2.0), pH 7.0.

The rich medium consisted of (g l^{-1}) glucose (10.0), yeast extract (5.0), ε-caprolactam (1.0), KH$_2$PO$_4$ (1.0), K$_2$HPO$_4$ (1.0), MgSO$_4$·7H$_2$O (0.2), NaCl (1.0), FeSO$_4$·7H$_2$O (0.01), CoCl$_2$ (0.01) and CaCl$_2$ (0.015), pH 7.0.

The nutrient broth for *R. boritolerans* CCTCC M 208108 was obtained by optimization of culture conditions. It contains (g l^{-1}) sucrose (7.0), sodium citrate (3.0), beef extract (5.13), yeast extract (5.0), ε-caprolactam (1.5), NaCl (1.0), KH$_2$PO$_4$ (1.0), K$_2$HPO$_4$ (1.0), FeSO$_4$·7H$_2$O (0.005), CoCl$_2$ (0.005) and MnSO$_4$ (0.005), pH 6.5.

Isolation and screening of microorganisms

Soil samples and potential NHase-producing strains in our laboratory were collected. Isolation of bacteria hydrating ADBN was performed by an enrichment procedure using ADBN as the sole nitrogen source. One loop of strains (from slants) or soil sample (1.0 g) was added to 10 ml physiological saline and allowed to stand for 5 min. The suspension (2 ml) was used to inoculate 38 ml enrichment medium. Shake flasks containing media were placed on a rotary shaker operated at 30°C, 150 rpm. The culture broth (2 ml) was taken after 3–7 days depending on the developing turbidity (indicating microbial growth) and transferred into 38 ml of fresh media. This procedure was performed four times, and then the resulting cultures were diluted and plated onto agar plates for cultivation. Isolated pure

colonies were grown aerobically at 30°C for 48 h in rich medium. Cells were separated by centrifugation (12,000 × g, 5 min) and stored at 4°C for further use.

Biotransformation was performed at 30°C, 150 rpm, in Erlenmeyer flasks (50 ml) with a screw cap unless noted otherwise. Cell pellet was suspended in 5 ml of distilled water (4.0 g CDW l^{-1}). After pretreating for 5 min, reactions were started by adding 50 μl ADBN. Samples (0.5 ml) were withdrawn at regular intervals and then centrifuged. The isolated strains capable of hydrating ADBN to ADBA were rapidly screened through a high-throughput colorimetric method reported by us [17]. Formation of ADBA was detected by addition of ferrous and ferric ion solutions successively, which resulted in yellow precipitate. If ADBN was not converted, the final color was deep blue. Samples with positive reaction were marked for further confirmation by gas chromatography (GC). Consequently, seven strains (including *R. boritolerans* CCTCC M 208108) were selected from culture collections in our laboratory and two strains (ZA0707 and P4) were screened from soil samples. Strains ZA0707 and P4 were later identified as *Rhodococcus* sp.

Cyanide-resistance of NHase in different strains

Cells suspension (2.0 g CDW l^{-1}) was treated with 5 mM KCN at 30°C, 150 rpm for 10 min, and then its residual activity was assayed by the following method. Because ADBN may release cyanide to the aqueous solution, acrylonitrile was selected as substrate. Acrylonitrile (50 μl) was added into the cells suspension (5 ml). NHase activity of the testing strains towards acrylonitrile was determined as described in the "Standard activity assay" section. Relative activity was expressed at the percentage of trials without KCN.

Product tolerance of *R. boritolerans* CCTCC M 208108

ADBA was dissolved in distilled water to concentrations of 20 and 40 g l^{-1}. Resting cells were suspended in 5 ml of each ADBA solution (6.0 g CDW l^{-1}). After incubating at 30°C, 150 rpm for 10 h, reactions were started by addition of ADBN (final concentration of 9 g l^{-1}). Control experiments without ADBA were carried out. Samples were taken at regular intervals.

Effect of water-immiscible co-solvents on *R. boritolerans* CCTCC M 208108 NHase activity

Various water-immiscible organic solvents (0.5 ml) including *n*-hexane, cyclohexane, ethyl acetate, methylene dichloride, toluene, and iso-octane were added to 5.0 ml cells suspension (2.0 g CDW l^{-1}). NHase activity of *R. boritol-erans* CCTCC M 208108 towards ADBN was determined as described in the "Standard activity assay" section. Relative activity was expressed at the percentage of control experiments without co-solvent.

Effect of temperature on ADBN stability in aqueous solution

Flasks containing 50 ml of distilled water were incubated in water baths of different temperatures (5, 10, 20, and 30°C). After incubating for 5 min, ADBN was added into each flask to an equal concentration of 50 mM. Aliquots were taken at different intervals to determine the cyanide concentration.

Optimization of *R. boritolerans* CCTCC M 208108 productivity in continuous biotransformation

The reactions were carried out in a 2-l round-bottom flask with a paddle agitator and rubber plugs. Cells of *R. boritol-erans* CCTCC M 208108 were suspended in 800 ml of distilled water (6 or 8 g CDW l^{-1}). The mixture was stirred (200 rpm) in a water bath at 5, 10, 20, or 30°C. To the cells suspension, ADBN was added dropwise by a peristaltic pump at various flow rates (1, 2, or 3 g h^{-1}). Samples were withdrawn at different intervals and the reactions were ceased when the product concentration stopped increasing. In the biphasic approach, *n*-hexane was added to 800 ml of cells suspension to give a volume ratio (*n*-hexane/water) of 10, 20, 30, or 40%.

Separation, purification, and identification of product

A 300-ml reaction mixture containing 42.5 g l^{-1} ADBA was centrifuged (12,000 × g, 10 min). The supernatant was treated by activated carbon absorption (0.5%, w/v) and then filtered. Thereafter, the solvent and small amount of unconverted ADBN was removed by evaporation under reduced pressure at 45°C, giving white solids. For further purification, the product was recrystallized from ethyl acetate at 4°C with *n*-hexane as a salting-out agent. The crystals were harvested by filtration, dried in a vacuum desiccator, and then weighed.

Molecular structure of the white crystals was determined by Fourier transform-infrared (FT-IR) spectroscopy (Nicolet 6700, Thermo Scientific, Waltham, MA, USA), nuclear magnetic resonance (NMR) (Inova 400-MHz NMR spectrometry, Varian, USA) and positive-mode mass spectra (ESI–MS, *m/z*, 50–850). Data of FT-IR spectroscopy of the product revealed the characteristic bands at 3371.5/1,602.2 (v_{N-H}), 2,972.7 (v_{C-H}) and 1,398.6 cm^{-1} (v_{C-N}), which were also discernible in that of ADBN. However, extra signals at 3,521.2, 1,675.4, and 708.1 cm^{-1} were ascribed to amino

and carbonyl of amide, and the characteristic signal of cyano at $2,220$ cm^{-1} was not visible in the spectroscopy of the product. Moreover, the product was further identified by ^{1}H NMR [δ_H(500 MHz; CDCl$_3$) 0.86 (3H, d, Me), 0.90 (3H, d, Me), 1.3 (3H, s, Me), 2.2 (1H, m, CH), 5.5 (1H, br s, NH), 7.4 (1H, br s, NH)], ^{13}C NMR [δ_C(100 MHz; CDCl$_3$) 15.9 (CH$_3$), 17.9 (CH$_3$), 26.1 (CH$_3$), 34.3 (CH), 60.7 (C), 180.8 (C=O)]. The molecular weight of the product was confirmed by ESI–MS, which revealed the following results: 283 (*m/z*) ([2 M + Na]$^+$), 261 ([2 M + H]$^+$), 131 ([M + H]$^+$), calculated value for C$_6$H$_{14}$ON$_2$, 130. All of these results were identical to the spectra obtained by standard sample of ADBA, which indicated that ADBN has been hydrated to ADBA. The IR, NMR, and MS spectra of the product are shown in the supplementary material.

Standard activity assay

An appropriate amount of wet cells and organic solvent (needed in the biphasic approach only) were suspended in 5 ml of distilled water and kept in a water bath for 5 min. Then ADBN or acrylonitrile (50 μl) was added to initiate the biotransformations. Reactions were performed at 30°C, 150 rpm, on a reciprocating shaker and allowed to proceed for 3 min after which samples (500 μl) were withdrawn. The samples were mixed with 20 μl HCl (6 M) to terminate the reaction and then centrifuged. Supernatant was directly assayed by GC. One unit of NHase activity was defined as the formation of 1 μmol ADBA (acrylamide) from ADBN (acrylonitrile) per minute.

Analytical procedures

ADBA and acrylamide were quantified by GC. A 6890 N instrument (Agilent, USA), equipped with an AT.FFAP column (Lanzhou Institute of Chemical Physics, China) was employed. Nitrogen was used as a carrier gas at a flow rate of 2.0 ml min^{-1}. Temperatures of the inlet, column, and detector were 250, 180, and 250°C, respectively. Under these conditions, the retention times of ADBA and acrylamide were 3.5 and 2.3 min, respectively.

Concentration of cyanide was determined using the Merck Spectroquant® cyanide test kit for the determination of free and readily liberated cyanide in water according to the manual of the manufacturer.

Results

Screening of bacterial strains

Various bacterial strains were isolated from soil samples and our culture collections through enrichment culture.

Candidates capable of hydrating ADBN were screened quickly by a sophisticated colorimetric screening method [17]. Microbes with positive reactions were selected out and further confirmed by GC. Through these methods, seven microorganisms from our culture collections and two strains from the soil were screened out of 418 isolates (Table 1). Since NHase with stronger tolerance against cyanide is more favorable in the hydration of α-aminonitrile, the cyanide-resistant capacity of these strains was further studied. As shown in Table 1, strain *R. boritolerans* CCTCC M 208108 exhibited the highest NHase activity and strongest cyanide tolerance among the nine microbes. *R. boritolerans* CCTCC M 208108 NHase activity was reasonably preserved (>80%) after incubation in 5 mM KCN for 10 min. While NHases of *A. faecalis* CCTCC M 208168 and *B. subtilis* CCTCC M 206038 were very sensitive to cyanide, in which more than 82% of their activity were inhibited. Therefore, *R. boritolerans* CCTCC M 208108 was selected as the best biocatalyst for the subsequent study.

Table 1 Hydration activity and cyanide-resistance of nine screened NHase-producing strains

Entry	Catalyst	Yield (%)	Relative activity in 5 mM cyanide (%)
1	*Rhodococcus* sp. ZA0707	83 ± 3.2	75 ± 0.9
2	*Rhodococcus* sp. P4	49 ± 2.2	41 ± 0.3
3	*Serratia marcescens* CCTCC M 208231	27 ± 2.0	36 ± 0.3
4	*Alcaligenes faecalis* CCTCC M 208168[a]	43 ± 1.6	18 ± 0.2
5	*Bacillus subtilis* CCTCC M 206038	35 ± 1.0	16 ± 0.9
6	*Rhodococcus* sp. G20	80 ± 3.2	34 ± 0.8
7	*R. ruber* CCTCC M 206040	52 ± 2.2	31 ± 1.1
8	*Rhodococcus* sp. N595	63 ± 3.0	30 ± 1.0
9	*R. boritolerans* CCTCC M 208108	93 ± 3.3	81 ± 0.7
10	Control[b]	No activity	No data

Reaction conditions of hydration activity: cells suspension in distilled water (4.0 g CDW l^{-1}), 50 μl ADBN, 30°C, 150 rpm, 60 min, reaction mixture volume: 5 ml. All the values were the mean of three independent experiments, and the values after the "±" represent standard deviation, similarly hereafter

Reaction conditions of cyanide-resistance: cells suspension (2.0 g CDW l^{-1}) was treated with 5 mM KCN at 30°C, 150 rpm for 10 min, and then its residual NHase activity was assayed using acrylonitrile (50 μl) as substrate. Reaction mixture volume: 5 ml. Relative activity was obtained at the percentage of experiments without KCN

[a] NHase was induced by addition of ε-caprolactam (5.0 g l^{-1}) to the culture medium

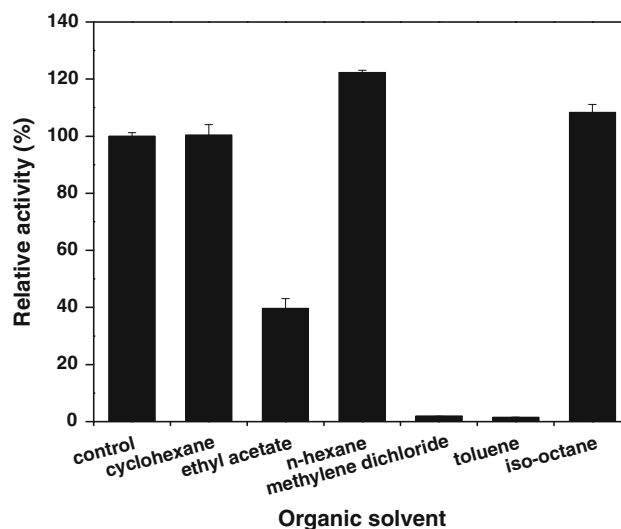[b] Control experiments without cells
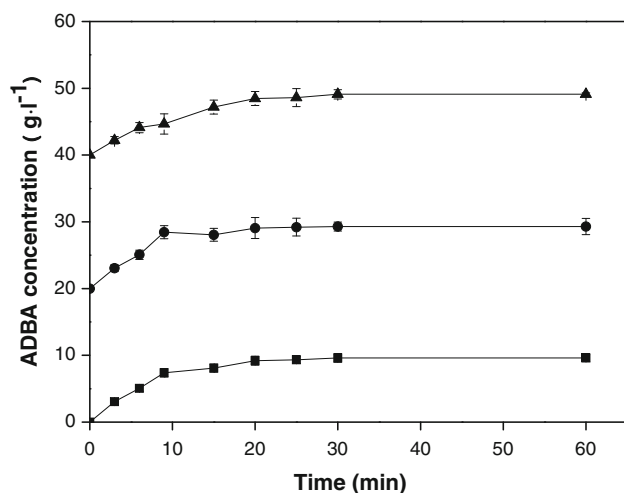
Fig. 1 Time course of ADBA formation catalyzed by resting cells of *R. boritolerans* CCTCC M 208108 at different initial ADBA concentrations. Reaction conditions: cells suspension in distilled water (6.0 g CDW l^{-1}), different concentrations of ADBA solution, 9 g l^{-1} ADBN, 30°C, 150 rpm, reaction mixture volume: 5 ml. Samples were taken at regular intervals shown in the figure. Symbols for initial ADBA concentration: *filled square* 0 g l^{-1}; *filled circle* 20 g l^{-1}; *filled triangle* 40 g l^{-1}. Standard deviations for three independent experiments were represented by *error bars*, similarly hereafter


Fig. 2 Effect of water-immiscible co-solvents on *R. boritolerans* CCTCC M 208108 NHase activity. Reaction conditions: 5 ml cells suspension in distilled water (2.0 g CDW l^{-1}), 0.5 ml different organic solvents, 50 μl ADBN, 30°C, 150 rpm, 3 min. Relative activity was obtained at the percentage of control experiments without co-solvent

However, after the first batch biotransformation, the product concentration no longer increased when additional substrate was fed. It indicated that *R. boritolerans* CCTCC M 208108 NHase was completely deactivated after the first batch reaction (30 min, at 30°C). The reasons probably lay in three aspects, high concentration of product, high pH environment of the reaction mixture owing to the product or cyanide released from ADBN solution.

Tolerance of *R. boritolerans* CCTCC M 208108 against high product concentration

In order to study the reasons of NHase inactivation in the above-mentioned fed-batch reactions, the effect of ADBA pretreatment on *R. boritolerans* CCTCC M 208108 NHase activity was investigated (Fig. 1). In the presence of 0, 20, and 40 g l^{-1} ADBA, the product increments were 9.6, 9.3, and 9.1 g l^{-1}, respectively. These results suggested that *R. boritolerans* CCTCC M 208108 NHase activity was not notably inhibited by a long treatment (10 h) in high product concentration (40 g l^{-1}). Furthermore, taking into account the pH value of 40 g l^{-1} ADBA was up to 9.3, *R. boritolerans* CCTCC M 208108 not only tolerated high product concentration but also exhibited good stability at relatively high pH. Consequently, we supposed that inactivation of *R. boritolerans* CCTCC M 208108 in the fed-batch experiments was mainly caused by the cyanide dissociated from ADBN.

Selection of water-immiscible co-solvents

To further improve the productivity, a biphasic approach was introduced. Among the solvents tested (Fig. 2), *n*-hexane proved to be the most appropriate, leading to the highest relative activity (122.3%). Moreover, ADBN was soluble in *n*-hexane, while ADBA was practically insoluble (data not shown). In the bioconversion process, the formed ADBA was found exclusively in the aqueous phase, whereas the substrate ADBN was partly stored in *n*-hexane and gradually released to the aqueous phase as the reaction proceeded. On the other hand, addition of ethyl acetate, methylene dichloride or toluene to the reaction system gave poor results. This was probably due to the low solubility of substrate in these solvents or damage of NHase activity caused by them [21]. Thus, *n*-hexane was considered as a suitable co-solvent in the biphasic biotransformation of ADBN.

Stability of ADBN at different temperatures

It was previously reported that a higher temperature led to a faster spontaneous degradation of α-aminonitriles in aqueous solution [5]. In order to reduce the inhibition of NHase by cyanide, thermal stability of ADBN solution (50 mM) was studied. At 30°C, cyanide concentration showed a sharp increase to 8.80 mM within 10 h, indicating that ADBN was unstable at this temperature (Fig. 3). In contrast, nitrile decomposition was less at lower temperatures.
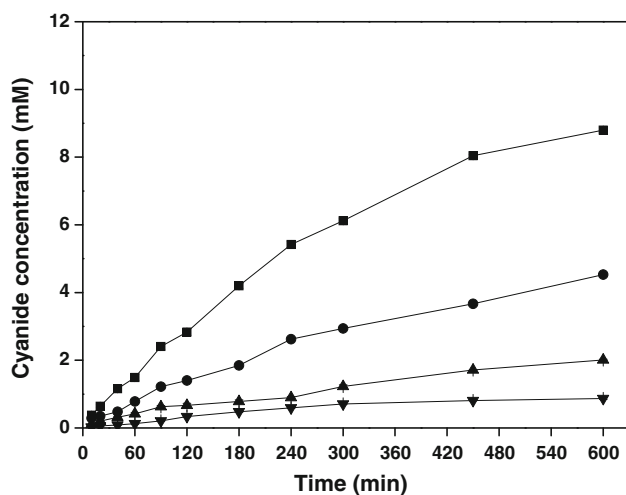
Fig. 3 Effect of temperature on ADBN stability in aqueous solution. Reaction conditions: 50 mM ADBN, distilled water, different temperatures, reaction mixture volume: 50 ml. Aliquots were taken at regular intervals to determine the cyanide concentration. Symbols: *filled square* 30°C; *filled circle* 20°C; *filled triangle* 10°C; *filled inverted triangle* 5°C

Especially at 10 and 5°C, cyanide accumulated very slowly in the initial 10 h, giving concentrations of 2.01 and 0.87 mM, respectively. Therefore, low temperature could markedly suppress the decomposition of ADBN, which was beneficial to the stability of NHase.

Continuous production of ADBA in aqueous and biphasic systems

Reaction parameters including temperature, substrate feeding rate and cells loading in an aqueous system were optimized at 800-ml scale (Table 2). So as to achieve a high yield, the initial cell concentration and substrate flow rate were set at 8 g CDW l^{-1} and 2 g h^{-1}. Because decomposition of ADBN can be readily reduced by lowering temperature, the reaction temperature was firstly studied. As the reaction temperature decreased from 30 to 10°C, the final product concentration rose (Table 2, entry 1–3). While further lowering the temperature (5°C) gave poor results. At 10°C (entry 3), with less inhibitor (mainly cyanide) the biotransformation proceeded smoothly, giving a high product concentration (40 g l^{-1}) and yield (91%). The catalyst productivity at 10°C (5.0 g product/g catalyst) was almost twice as that at 30°C (2.7 g product/g catalyst). These results prompted us to accelerate the rate of substrate addition (entry 5) or reduce cells loading (entry 7). However, the product concentration and yield decreased in both cases. On the other hand, if the substrate flow rate was turned down to 1 g h^{-1} (entry 6), the product concentration was increased to 43 g l^{-1}. Nevertheless, the space–time yield (1.35 g l^{-1} h^{-1}) was much lower than that of entry 3 (2.64 g l^{-1} h^{-1}).

Based on the optimum conditions of entry 3, *n*-hexane was introduced as co-solvent to establish a biphasic biotransformation procedure (entry 8–11). Addition of *n*-hexane notably improved both product accumulation and yield. Further experiments were conducted to explore the possibility to increase the productivity by variation of the *n*-hexane/water ratio. Satisfactorily, at 30% the conversion proceeded efficiently and the product concentration came up to 50 g l^{-1} with a good yield (91%) (entry 10). As a result, cells of *R. boritolerans* CCTCC M 208108 displayed a much higher operational stability than in batch reactions at 30°C without co-solvent (Fig. 4), highlighting the considerable efficiency of this methodology.

Table 2 Continuous biotransformation of ADBN to ADBA using resting cells of *R. boritolerans* CCTCC M 208108

| Entry	ADBN (g h^{-1})	Cells loading (g CDW l^{-1})	T (°C)	*n*-hexane/water (%, v/v)	Time (h)	Product (g l^{-1})	Yield (%)	Catalyst productivity (g product/g catalyst)
1	2	8	30	0	9	21 ± 1.0	82 ± 3.7	2.7 ± 0.12
2	2	8	20	0	12	31 ± 0.6	88 ± 1.8	3.8 ± 0.08
3	2	8	10	0	15	40 ± 1.4	91 ± 3.2	5.0 ± 0.17
4	2	8	5	0	14	35 ± 1.0	86 ± 2.4	4.4 ± 0.12
5	3	8	10	0	10	34 ± 0.5	78 ± 1.2	4.3 ± 0.07
6	1	8	10	0	32	43 ± 1.0	93 ± 1.9	5.4 ± 0.11
7	2	6	10	0	13	32 ± 0.8	86 ± 2.3	5.4 ± 0.15
8	2	8	10	10	17	46 ± 1.4	94 ± 2.7	5.8 ± 0.17
9	2	8	10	20	18	48 ± 0.6	93 ± 1.6	6.1 ± 0.08
10	2	8	10	30	19	50 ± 0.7	91 ± 1.3	6.3 ± 0.09
11	2	8	10	40	19	49 ± 0.6	90 ± 1.1	6.2 ± 0.08

Reaction conditions: cells suspension (concentration indicated above), ADBN (feeding rate indicated above), 800 ml of distilled water in a 2-l flask, different reaction temperatures (indicated above), agitation at 200 rpm. In the biphasic approach, *n*-hexane was added to the reaction mixture as a co-solvent (proportion indicated above)
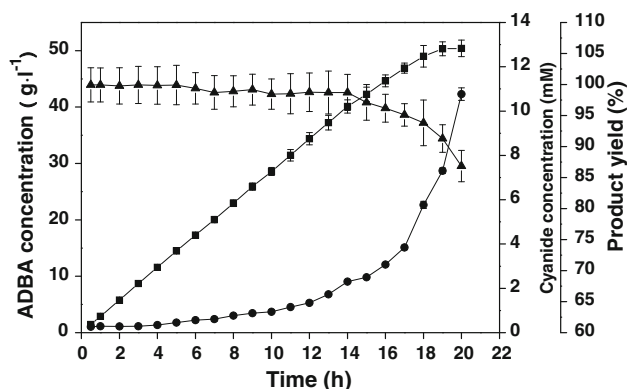
Fig. 4 Time course of continuous production of ADBA by whole cells of *R. boritolerans* CCTCC M 208108. The reaction conditions were shown in Table 2, entry 10. Symbols: *filled square* ADBA concentration; *filled circle* Cyanide concentration; *filled triangle* Product yield

Reuse of resting cells of *R. boritolerans* CCTCC M 208108

As depicted in Fig. 4, the biotransformation proceeded smoothly in the first 14 h with high yield (98%). However, it took a sharp decline as the reaction went on, while cyanide concentration notably increased from 2.3 mM (14 h) to 10.8 mM (19 h). This phenomenon suggested that long time treatment in low cyanide concentration could cause inactivation of *R. boritolerans* CCTCC M 208108 NHase. Reusability of the biocatalysts was examined by ceasing the reaction as soon as cyanide concentration increased to 2 mM. Based on this method, the biocatalyst could be reused at least twice with good yield. The first and third product concentrations of this series of recycle reactions were 39 and 26 g l^{-1}, respectively (Fig. 5). As a result, the catalyst productivity increased from 6.3 g product/g catalyst to 12.3 g product/g catalyst, which could obviously reduce the overall cost of the process. In the fourth use, about 30% of the initial enzyme activity
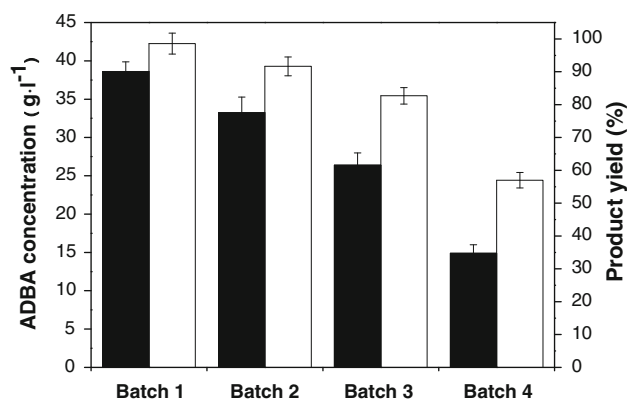
Fig. 5 Reuse of resting cells of *R. boritolerans* CCTCC M 208108. The reaction conditions were shown in Table 2, entry 10. Batch reaction was ceased as soon as the cyanide concentration reached 2 mM. Symbols: *filled square* ADBA concentration; *open square* Product yield

retained, resulting in a low product concentration (15 g l^{-1}) with an unacceptable yield (57%).

Separation and purification of product

The product could be readily separated from reaction mixture (42.5 g l^{-1} ADBA, 300 ml) and purified in a new and simple way. By centrifugation and evaporation under reduced pressure, solvent and unconverted ADBN were removed. Consequently, ADBA was isolated as white solids (13.4 g, purity 93.8%). Further purification by recrystallization from ethyl acetate afforded ADBA crystals (12.0 g, purity 98.5%) with a final recovery rate of 92.8%.

Discussion

NHase is a powerful biocatalyst and has been successfully used in the industrial production of various amides [2, 21, 25]. However, there are few reports on NHase-catalyzed production of α-aminoamides. This study was aimed at developing a biocatalytic process for continuous production of ADBA (α-aminoamide) from ADBN by NHase. The main challenge of this bioprocess was the sensitivity of NHase to cyanide dissociated from ADBN. Firstly, a bacterium, *R. boritolerans* CCTCC M 208108, which exhibited high NHase activity towards ADBN and cyanide resistance, was screened. Its cyanide-resistant capacity (5 mM) was much better than those of the other strains tested in this study (Table 1) and that of *R. erythropolis* 870-AN019 (25 μM) [5], but poorer than those of *Pseudomonas marginales* MA32, *Pseudomonas putida* MA113 (50 mM) [12], and *Nitriliruptor alkaliphilus* (11 mM) [30]. Moreover, product tolerance of an enzyme is also a parameter of great importance for high product accumulation. It was previously reported that NHase was sensitive to low concentration of α-aminoamide (*tert*-leucine amide, 3.25 g l^{-1}) [5]. *R. boritolerans* CCTCC M 208108 NHase activity was considerably preserved even after incubating in high ADBA concentration up to 40 g l^{-1} for 10 h. Considering the high pH value (9.3) of 40 g l^{-1} ADBA, *R. boritolerans* CCTCC M 208108 NHase also showed excellent pH tolerance. These characteristics demonstrated that *R. boritolerans* CCTCC M 208108 was a useful biocatalyst for practical production of ADBA. However, like most of the reported NHases [23], NHase of *R. boritolerans* CCTCC M 208108 was non-enantioselective towards ADBN.

Attempts to improve efficiency of the bioprocess were conducted by reducing NHase inhibition caused by cyanide. The bioconversions were performed at 800-ml scale and the substrate was added dropwise and slowly so as to maintain a relatively low nitrile concentration. Inhibition of NHase was notably reduced by controlling reaction temperature at

10°C, giving catalyst productivity (5.0 g product/g catalyst) nearly twice as much as that at 30°C. This is most probably because the substrate was more stable at lower temperature and released less cyanide to the reaction mixture. Similar results were found when industrial scale production of acrylamide by microbial NHase was carried out at 4–10°C in order to improve enzyme stability and catalyst productivity [6, 19, 21, 26]. On the other hand, it was reported that α-aminonitriles were more stable at lower pH circumstance [5, 8], however, *R. boritolerans* CCTCC M 208108 NHase activity was quite low in weakly acidic environment (for example, pH 6.0) in different buffers. Besides, introducing buffer to control the pH may result in a more tedious product isolation procedure and it will certainly raise the production cost.

A biphasic approach using water-immiscible organic solvent has been extensively employed in the biotransformation, for the sake of minimizing the inhibition of substrate and/or product [7, 13]. In this study, *n*-hexane was found to be favorable for the NHase-catalyzed hydration of ADBN and selected as co-solvent to set up a biphasic system. *n*-hexane was reported to be benefit for bioconversion of naproxen nitrile by NHase of *Rhodococcus* sp. C3II [9], but harmful for hydration of the same substrate using NHase from *R. equi* A4 [24]. These results suggested that effect of co-solvent on NHase activity was likely to be dependent on the source of NHase. By adding 30% (v/v) of *n*-hexane, the product concentration, yield and catalyst productivity were further improved to 50 g l^{-1}, 91% and 6.3 g product/g catalyst, respectively. We speculated that the water-immiscible solvent acted as an internal substrate reservoir and also offered the advantage in reduction of ADBN decomposition, therefore reducing the inhibition of NHase. To date, successful use of biphasic system using water-immiscible organic solvent in NHase-catalyzed biotransformation remained scarce. *n*-hexane and isooctane were applied to the biocatalytic hydration of aromatic nitrile (naproxen nitrile) [9, 24]. This study was the first report regarding biphasic methodology used in NHase-mediated hydration of aliphatic nitrile. Furthermore, whole cells of *R. boritolerans* CCTCC M 208108 could be reused at least twice by ceasing the continuous reaction before cyanide concentration reached 2 mM. As a result, the catalyst productivity was further improved to 12.3 g product/g catalyst.

ADBA formed in the biocatalytic process could be easily separated and purified with good purity (98.5%) and recovery rate (92.8%). Harsh reaction conditions, such as high temperature (100°C), concentrated sulfuric acid, ammonia, hydrogen peroxide and palladium reagent involved in the chemical processes can be obviated. For example, compared to US 4683324 [11] and EP 0123830 (A2) [28], approximate 9,000 l waste water containing concentrated ammonium sulfate and 46,000 l dichloromethane can be eliminated for pro-

duction of 1,000 kg of ADBA. In contrast to EP 0231546 (A1) [3], about 10,000 l concentrated ammonia and 20.7 kg palladium-on-carbon per 1,000 kg ADBA can be saved. Therefore, this enzymatic methodology for the production of ADBA is greener and more atom-efficient.

Conclusions

In summary, a new method for continuous production of ADBA using resting cells of *R. boritolerans* CCTCC M 208108, a cyanide-resistant bacterium harboring NHase, was developed. The data demonstrated that resting cells of *R. boritolerans* CCTCC M 208108 exhibited high NHase activity, cyanide resistance, product and pH tolerance. In addition, the enzymatic process was efficient, cost effective and environmentally benign compared to the existing chemical processes. Therefore, this study laid a solid foundation for bioproduction of ADBA on industrial scale.

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