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BIOCATALYSIS

Screening, cultivation, and biocatalytic performance of *Rhodococcus boritolerans* FW815 with strong 2,2-dimethylcyclopropanecarbonitrile hydratase activity

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Abstract In this work, a mild, efficient bioconversion of 2,2-dimethylcyclopropanecarbonitrile (DMCPCN) to 2,2-dimethylcyclopropanecarboxamide (DMCPCA) in distilled water system was developed. The isolate FW815 was screened using the enrichment culture technique, displaying strong DMCPCN hydratase activity, and was identified as *Rhodococcus boritolerans* based on morphological, physiological, biochemical tests and 16S rRNA gene sequencing. Cultivation outcomes indicated that *R. boritolerans* FW815 was a neutrophile, with a growth optimum of 28–32°C; its DMCPCN hydratase belonged to the Fe-type family, and was most active at 38–42°C, pH 7.0, with maximal activity of 4.51×10^4 U g^{-1} DCW. *R. boritolerans* FW815 was found to be DMCPCA amidase-negative, eliminating the contamination of dimethylcyclopropanecarboxylic acid. Moreover, it displayed high activity and acceptable reusability in the non-buffered distilled water system, comparable to those in pH 7.0 phosphate buffer (50.0 mmol l^{-1}).

Keywords 2,2-Dimethylcyclopropanecarboxamide · 2,2-Dimethylcyclopropanecarbonitrile · Nitrile hydratase · *Rhodococcus boritolerans*

Introduction

Imipenem (MK0787), *N*-formimidoyl thienamycin, exhibits extremely broad antibacterial spectrum and is always used in critically infected patients before specific culture and sensitivity information are available. Unfortunately, it is rapidly degraded by renal dehydropeptidase in human and animals [12, 18]. Therefore, imipenem is commercially supplied together with cilastatin (MK0791), a dehydropeptidase inhibitor, to block its hydrolysis and retain the potency by a probenecid-like effect. Up to now, the in vitro and in vivo activities of the combination of imipenem with cilastatin against aerobic and anaerobic bacteria have been well documented [3]. Since *S*-2,2-dimethylcyclopropanecarboxamide (*S*-DMCPCA) and *S*-2,2-dimethylcyclopropanecarboxylic acid are the chiral building blocks of cilastatin, their production is of considerable interest.

So far, it has been extensively documented that two metabolic pathways to convert nitriles to the corresponding carboxylic acids exist in organisms. Nitrilases catalyze the direct hydrolysis of nitriles into the corresponding carboxylic acids, while nitrile hydratases (NHases) catalyze nitrile hydrations to amides that are subsequently hydrolyzed into carboxylic acids by the action of amidases. Commonly, aliphatic nitriles are metabolized by a combination of NHase and amidase, while nitrilases act preferentially on aromatic nitriles [2, 4, 8, 9, 16]. It is well known that NHases are a group of bacterial metalloenzymes containing either a low spin non-heme Fe(III) or a low-spin non-corrin Co(III) ion in the catalytic center, designated as Fe-type NHase family and Co-type NHase family, respectively. As a group of industrial biocatalyst, NHases are at the center of large-scale production of a variety of valuable amides, including acrylamide and niacinamide. During the past 20 years, nitrile hydration and hydrolysis catalyzed by nitrile-converting enzymes has been regarded as a powerful tool in organic synthesis for preparation of some valuable amides and acids [1, 15, 19, 20, 25, 26]. Since biocatalysis provides an efficient, environmentally benign strategy for creating optically pure chemicals and

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operates under mild conditions [1, 4, 14], bioconversions of *S*-DMCPCA and *S*-2,2-dimethylcyclopropanecarboxylic acid are attractive.

Two decades ago, bioresolution of *S*-DMCPCA with *R*-amidase active isolates and recombinant microorganisms was firstly developed by Lonza [17, 30]. Later, Wang and Feng [19, 20] reported that *S*-2,2-dimethylcyclopropanecarboxylic acid was prepared in a 26% yield with an ee value up to 90% by using *Rhodococcus* sp. AJ270, which harbors nitrile hydratase and amidase. In recent years, we have conducted extensive studies on the bioresolution of enantiopure *S*-DMCPCA with *Delftia tsuruhatensis* ZJB-05174, *Brevibacterium epidermidis* ZJB-07021 [11, 23, 24, 27–29]. To make these enzymatic processes more competitive, economic, also inspired by our vast experiences in developing biocatalytic processes with nitrile-converting enzymes [10, 21, 22, 25, 26], an attempt to explore a two-step enzymatic process for *S*-DMCPCA by introducing 2,2-dimethylcyclopropanecarbonitrile (DMCPCN) hydration with NHase was proposed (Fig. 1).

In the present work, an isolate, strain FW815, with strong DMCPCN hydratase activity was screened using the enrichment culture technique, and identified as *Rhodococcus boritolerans* according to morphological, physiological, biochemical tests and 16S rRNA gene sequencing. Characteristics of *R. boritolerans* FW815 cultivation were examined, along with its performance evaluation in DMCPCN hydration.

Materials and methods

Chemicals

2,2-Dimethylcyclopropanecarbonitrile and 2,2-dimethylcyclopropanecarboxamide were provided by Shangyu Huakang Chemicals Ltd (Zhejiang, China). Peptone, yeast extract, and agar were purchased from Huadong Medicine Group (Hangzhou, China). All other chemicals were of analytical reagent grade purity and obtained from commercial sources.

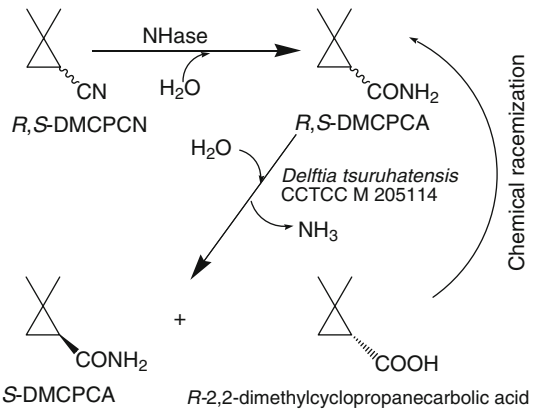

Fig. 1 A two-step enzymatic process for S-DMCPCA from racemic DMCPCN: *step 1* DMCPCN hydration to DMCPCA by the action of NHase; *step 2* bioresolution of *S*-DMCPCA by *R*-amidase of *Delftia tsuruhatensis* CCTCC M 205114

Microbial screening for DMCPCN-hydrating activity

The soil and wastewater samples used for isolation of DMCPCN hydratase active bacteria were longtime polluted with nitrile compounds, collected from local chemical plants. The soil samples were weighed and dispersed into sterile 0.95% (w/v) physiological saline solution at approximately 0.1 g ml^{-1} level. A 2.0-ml aliquot of soil suspension or wastewater sample was transferred to 50.0-ml enrichment media in a 250-ml Erlenmeyer flask that contained (g l^{-1}) 10.0 glucose, 0.2 $MgSO_4$, 1.0 K_2HPO_4, 1.0 KH_2PO4, 2.0×10^{-3} $FeSO_4{\cdot}7H_2O$, 2.0×10^{-3} $CoCl_2{\cdot}6H_2O$, 1.5×10^{-2} $CaCl_2$, and 0.34 DMCPCN acting as sole nitrogen source without pH adjustment. Enrichment cultures were incubated at 30°C with shaking at 150 rpm until the broths became turbid. Afterwards, 1.0 ml of culture was transferred into 50 ml of sterile enrichment medium in a 250-ml Erlenmeyer flask at inoculum size of 2.0% (v/v). Cultivation was carried out under the same conditions as those mentioned above until the broths became turbid. After three successive transfers and cultivation, the broths were gradually diluted with sterile 0.95% (w/v) physiological saline solution to an appropriate cell density and spread onto solid agar plates, which consisted of (g l^{-1}) 15.0 glucose, 7.5 yeast extract, 3.0 $(NH_4)_2SO_4$, 0.3 $MgSO_4$, 1.5 K_2HPO_4, 1.5 KH_2PO_4, 1.5×10^{-2} $FeSO_4{\cdot}7H_2O$, 20.0 agar. Plates were cultivated at 30°C until visible colonies emerged. Each representative single colony was streaked onto sterile agar slant, grown at 30°C for 2 days, and then stored at 4°C for further evaluation and identification. Among all the isolates, strain FW815 was the most active to hydrate DMCPCN and was chosen for further studies. For the storage purpose, strain FW815 was maintained as frozen stocks in 20% (v/v) glycerol at −70°C.

Strain identification

Physiological and biochemical tests, including growth at different temperatures and pH values, Voges-Proskauer test, methyl red, and production of H_2S and indole, were performed to characterize strain FW815, according to Bergey's *Manual of Systematic Bacteriology* [7]. Morphology of strain FW815 cells was observed using a field-emission scanning electron Microscope (FEI/Philips XL30, USA). Catalase activity was detected by the evolution of oxygen upon addition of 30 g l^{-1} H_2O_2 to the single colonies of strain FW815. For genotypic identification, the

chromosomal DNA of strain FW815 was isolated according to the method described by Frederich et al. [6] and 16S rDNA nucleotide sequence analysis was enzymatically amplified. Amplification was carried out with primers: p16S-8: 5′-aga gtt gat cct ggc tca g-3′ and p16S-1541: 5′-aag gag gtg atc cag ccg ca-3′ in a thermal cycler (PTC-200, Bio-Rad, USA) under the following conditions: 5 min at 95°C, 35 cycles of 40 s at 95°C, 60 s at 53°C, 2 min at 72°C and one final step of 10 min at 72°C. The PCR products were extracted and purified from the agarose gel using the High Pure PCR Product Purification Kit (Roche, Germany). The resulting PCR fragment was ligated with pMD18-T (Takara, Japan) according to the T/A cloning procedure [13]. The constructed vector was transferred into the competent *E. coli* JM109 [5], and then spread on the Luria-Bertani (LB) agar plates containing X-gal (40 μg ml^{-1}), isopropyl β-D-1-thiogalactopyranoside (IPTG, 24 μg ml^{-1}) and ampicillin (50 μg ml^{-1}). The positive clones grown on the LB agar plates were designated *E. coli* JM109/pMD18-T-FW815. DNA was sequenced on both strands using an Applied Biosystems Model 377 automatic DNA sequencer and a dye-labeled terminator sequencing kit (Applied Biosystems, Foster City, CA, USA). The sequences obtained were compiled and compared with sequences in the GenBank databases using the BLAST program. The sequences were aligned using multiple sequence alignment software (CLUSTAL W ver. 1.81). A phylogenetic tree was constructed with MegAlign software (DNASTAR Inc., Madison, WI, USA) based on the partial 16S rDNA sequences of 16 strains similar to strain FW815. The GenBank accession number for strain FW815 is No. GU181290. Since it had 100% sequence similarity with *Rhodococcus boritolerans*, strain FW815 was identified as *R. boritolerans*.

Cultivation

Rhodococcus boritolerans FW815 was streaked on agar plates containing (g l^{-1}) 15.0 glucose, 7.5 yeast extract, 3.0 $(NH_4)_2SO_4$, 0.3 $MgSO_4$, 1.5 K_2HPO_4, 1.5 KH_2PO_4, 1.5×10^{-2} $FeSO_4 \cdot 7H_2O$, and 20.0 agar. A single colony of *R. boritolerans* FW815 was used to inoculate each 50 ml liquid seed media in a 250-ml Erlenmeyer flask, which consisted of (g l^{-1}) 10.0 glucose, 3.0 yeast extract, 1.0 NaCl, 0.3 K_2HPO_4, 0.3 KH_2PO_4, 0.2 $MgSO_4$. After 24 h culture at 30°C, 150 rpm, 1.0 ml of seed culture was aseptically transferred to 50.0 ml basal fermentation media, composed of (g l^{-1}) 10.0 glucose, 5.0 yeast extract, 2.0 peptone, 1.0 NaCl, 1.0 K_2HPO_4, 1.0 KH_2PO_4, 0.2 $MgSO_4$, 3.0×10^{-2} $FeSO_4 \cdot 7H_2O$, 5.0×10^{-2} $CaCl_2$, 0.34 DMCPCN; DMCPCN acts as the inducer, and was supplemented after media were sterilized and cooled down. All media were adjusted to pH 7.0 with 1.0 M NaOH prior to sterilization by steam autoclaving at 121°C for 20 min. Unless otherwise specified, *R. boritolerans* FW815 was cultivated aerobically at 30°C until maximal NHase activity was observed.

DMCPCN biohydration by *R. boritolerans* FW815 resting cells

A 0.25-ml aliquot of *R. boritolerans* FW815 broth was centrifuged at 6,500 × *g* for 10 min. Cell paste was washed three times with 0.95% (w/v) physiological saline solution, suspended into 10 ml, pH 7.0 phosphate buffer (50 mmol l^{-1}). After 20 s of vortexing, the resultant homogenous *R. boritolerans* FW815 suspensions were preheated in a water bath to 39°C; afterwards, DMCPCN was added at a concentration of 17.1 mmol l^{-1} to initialize the hydration. The enzymatic hydration reaction was conducted at 39°C and lasted 1.0 min. An aliquot of 800 μl was withdrawn from the bioconversion mixture and NHase was quenched with 100 μl, 5.0 mol l^{-1} HCl, and then neutralized with 100 μl 5.0 mol l^{-1} NaOH. The resultant suspensions were centrifuged at 9,000 × *g* for 10 min, and the supernatants containing the formed DMCPCA and residual DMCPCN were further microfiltrated through 0.45-μm membranes. The obtained clarified filtrates were used for DMCPCA and DMCPCN determinations.

Temperature and pH dependence of *R. boritolerans* FW815 NHase

Temperature dependence of DMCPCN hydratase activity of *R. boritolerans* FW815 was examined in a temperature range within 18–48°C, at 17.1 mmol l^{-1} DMCPCN, pH 7.0. After 1.0-min conversion, reactions were quenched, and the conversion mixtures were neutralized, centrifuged, and microfiltrated as mentioned above. The resultant clarified filtrates were used to assay the DMCPCA formed. Similarly, pH dependence of DMCPCN hydratase activity of *R. boritolerans* FW815 was examined in citrate–phosphate, glycine–NaOH, and phosphate buffers with pH values covering 5.0–10.6, at 39°C, initial DMCPCN concentration of 17.1 mmol l^{-1}.

Reusability evaluation of *R. boritolerans* FW815 resting cells in phosphate buffer and non-buffered water system

Two systems including 50 mmol l^{-1}, pH 7.0 phosphate buffer and non-buffered water system were used to evaluate the reusability of *R. boritolerans* FW815 resting cells.

About 37 mg of dried cells collected from 10.0 ml of broth were suspended into each 10.0 ml of phosphate buffer (50 mmol l^{-1}, pH 7.0) or distilled water. The hydration was initialized by adding 17.1 mmol l^{-1} DMCPCN into these suspensions. After a 5-min reaction at 39°C, the mixture was centrifuged at 6,500 × *g* for 10.0 min. The resultant pellets were washed three times with each reaction medium, and suspended into 10 ml of reaction media. After vortexing for 20 s, DMCPCN was added at concentration of 17.1 mmol l^{-1} to initialize the hydration that was performed at 39°C for 5 min. In this way, *R. boritolerans* FW815 resting cells repeatedly catalyzed DMCPCN hydration in phosphate buffer (pH 7.0, 50 mmol l^{-1}) or distilled water for nine consecutive runs. In each run, DMCPCN hydratase was measured, along with DMCPCN conversion that was calculated based on its concentration alteration before and after the action by *R. boritolerans* FW815.

Analytical method

2,2-Dimethylcyclopropanecarboxamide and 2,2-dimethylcyclopropanecarbonitrile detection was performed on a GC-14C instrument (Shimadzu, Japan), equipped with a flame ionization detector (FID) and a UA-5 capillary column (Frontier Laboratory, Japan). Nitrogen was used as the carrier gas at a flow rate of 1.0 ml min^{-1}. The column was conditioned and operated at 140°C; the detector and injector temperatures were run at 220°C. One NHase unit (U) was defined as the amount of biocatalyst required for 1 μmol DMCPCA formation per min at 30°C, pH 7.0.

Each run of culture experiments and analysis was replicated three times to ensure consistency and accuracy. Hence, data obtained for a specific experimental condition are the results of three separate batch experiments, and provided as mean ± standard deviation.

Results and discussion

Phenotypic and biochemical characteristics of strain FW815 and its identification

Strain FW815 was oval, 0.2–0.5 μm wide by 0.8–1.2 μm long (Fig. 2), non-motile, non-sporulating, and Gram-positive. It produced urease, catalase, lecithinase and β-galactopyranosidase, hydrolyzed glutin, casein, and reduced nitrate. It formed orange-yellow, circular, convex colonies on agar plates. The colony margin was smooth and the surface texture was glossy. Colonies were about 2 mm in diameter after 48 h culture at 30°C, and turned red-pink with prolonged culture time. Strain FW815 tested negative for methyl red, indol, and Voges-Proskauer reactions. It was capable of utilizing numerous carbon sources including starch. These morphological and chemotaxonomic characteristics of strain FW815 were consistent with the diagnostic properties of the genus *Rhodococcus*. The partial 16S rDNA sequence of FW815 was further determined and a phylogenetic tree was constructed based on the 16S rDNA sequence (Fig. 3). The 16S rDNA sequence was deposited in the GenBank database with accession No. GU181290. Comparative ribosomal DNA gene sequence analysis supported a strong relationship between strain FW815 and members of the genus *Rhodococcus*. Particularly, strain FW815 had 100% sequence similarity with *R. boritolerans* (GenBank accession No. AB288061). Based on physiological and biochemical characteristics and 16S rDNA sequence comparison, the strain was identified as a strain of *R. boritolerans*, and named *R. boritolerans* FW815. It was deposited at the China Center for Type Culture Collection (CCTCC) under accession number of CCTCC M208108. Moreover, *R. boritolerans* FW815 is able to hydrate DMCPCN but without enantioselectivity; it was amidase negative to DMCPCA at ambient temperatures.

Cofactor determination for *R. boritolerans* FW815 NHase

Commonly, NHases are divided into two classes, namely Fe-type NHase family and Co-type NHase family, dependent on the metal ions located in the active site. To judge its NHase type, *R. boritolerans* FW815 was grown in a mineral medium in the presence or absence of ferrous and cobalt ions at 30°C; the chemically defined mineral medium consisted of (g l^{-1}) 10.0 glucose, 1.3 NH_4NO_3, 1.0 NaCl, 1.0 K_2HPO_4, 1.0 KH_2PO_4, 0.2 $MgSO_4$, 0.05 $CaCl_2$, 0.34 DMCPCN. A trial included one mineral medium group as the control and three

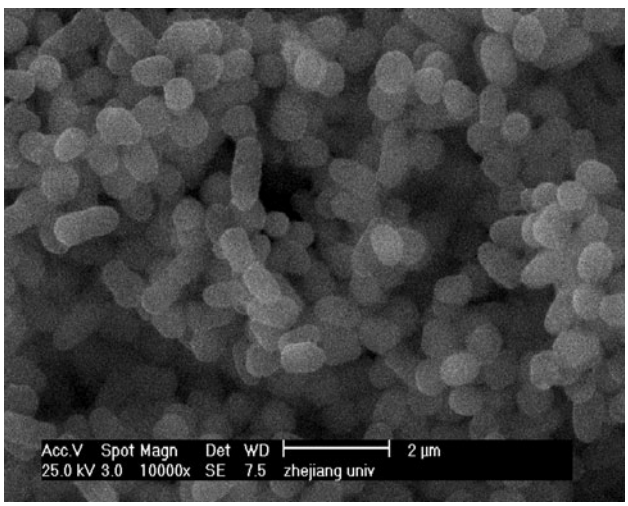

Fig. 2 Scanning electron micrography of strain FW815. *Scale* represents 2 μm

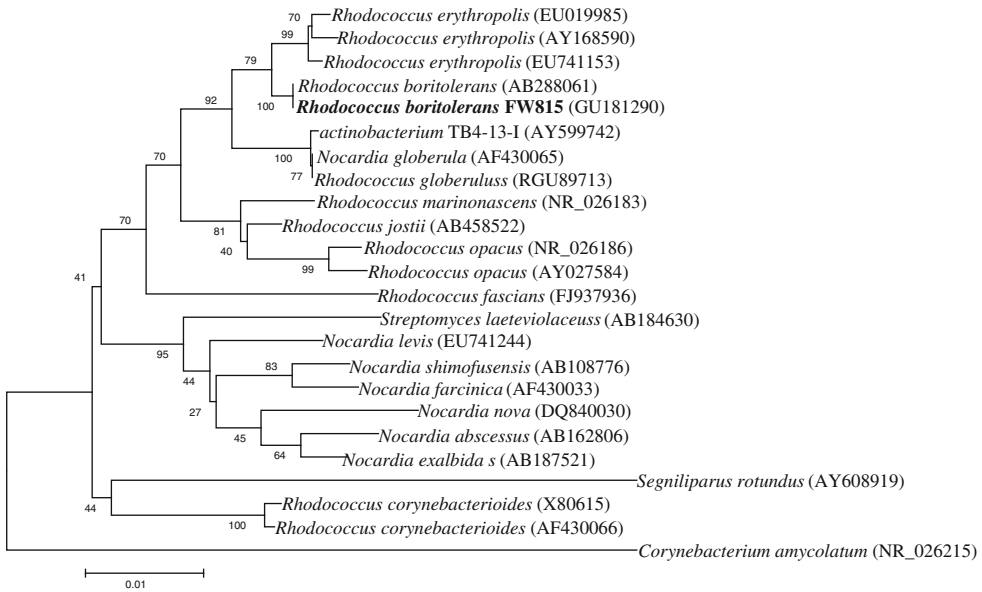

Fig. 3 Phylogenetic dendrogram for strain FW815 and related strains based on the 16S rDNA sequence. *Numbers in parentheses* are accession numbers of published sequences. Bootstrap values were based on 1,000 iterations

experimental groups, which were prepared by supplementing the control mineral media with 1.0×10^{-4} mol l^{-1} $FeSO_4 \cdot 7H_2O$, 1.0×10^{-4} mol l^{-1} $CoCl_2$, 1.0×10^{-4} mol l^{-1} $FeSO_4 \cdot 7H_2O$ plus 1.0×10^{-4} mol l^{-1} $CoCl_2$, respectively. In this section, deionized water rather than distilled water was used to prepare all the media to exclude ion interference. After 2 days cultivation at 30°C, almost no NHase activity was detectable in the control and Co^{2+} groups; in contrast, broths of Fe^{2+} group, Fe^{2+} plus Co^{2+} group exhibited significant NHase activity, approaching 30.0 U ml^{-1}, demonstrating *R. boritolerans* FW815 NHase is ferrous-dependent and cobalt-independent. Hence, we concluded that *R. boritolerans* FW815 NHase belongs to Fe-type NHase family.

Optimization of fermentation parameters for DMCPCN hydratase synthesis with *R. boritolerans* FW815

Culture temperature

The effect of culture temperature on *R. boritolerans* FW815 performance was evaluated in a range between 23 and 34°C, illustrated in Fig. 4. NHase volumetric activity increased with temperature within 23–30°C, and peaked at 30°C. A heavy loss in NHase activity was observed at temperatures exceeding 30°C. *R. boritolerans* FW815 exhibited a growth optimum at 28–32°C, relating to its wild-type nature. Hence, 30°C was the preferential culture temperature in the consideration of NHase activity and microbial growth.

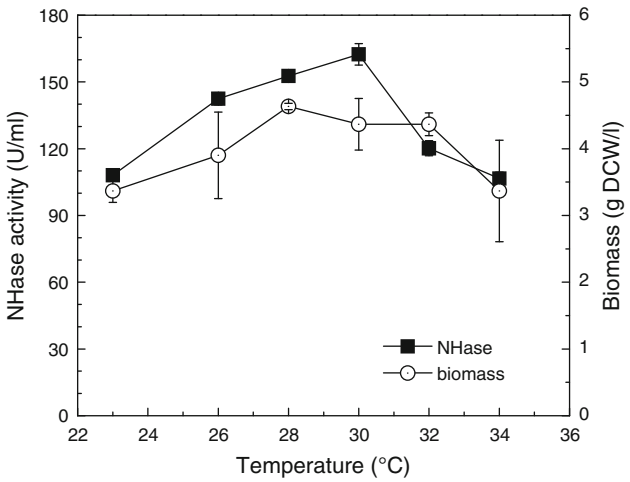

Fig. 4 Effect of culture temperature on *R. boritolerans* FW815 proliferation (*open circle*) and DMCPCN hydratase production (*filled square*). Cultivation conditions: initial medium pH 7.0, 150 rpm, cultivation time 48 h

Medium pH

Influence of initial medium pH on *R. boritolerans* FW815 growth and NHase production was examined at pH within 4.0–10.0, shown in Fig. 5. Obviously, neutral condition was supportive for microbial growth and NHase synthesis. Both volumetric NHase activity and biomass density peaked at pH 7.0, approaching 156.4 U ml^{-1} and 3.7 g DCW l^{-1}, respectively. Taken together, these results indicate pH 7.0, 28–32°C were supportive to the growth of *R. boritolerans* FW815. However, due to the constraints of

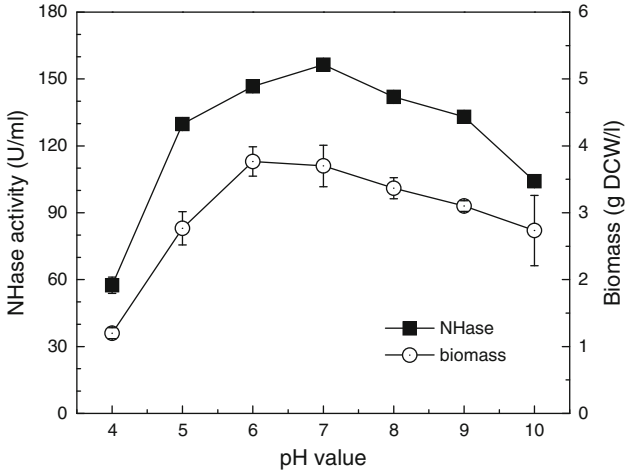

Fig. 5 Effect of initial medium pH on *R. boritolerans* FW815 proliferation (*open circle*) and DMCPCN hydratase production (*filled square*). Cultivation conditions: 30°C, 150 rpm, cultivation time 48 h

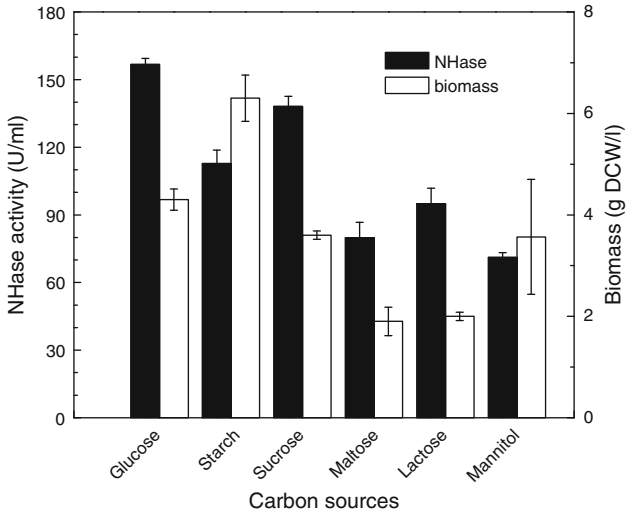

Fig. 6 Effect of carbon source on *R. boritolerans* FW815 proliferation (*open column*) and DMCPCN hydratase production (*filled column*). Cultivation conditions: 30°C, initial medium pH 7.0, 150 rpm, cultivation time 48 h. Carbon sources were added at a final carbon molar concentration of 0.33 mol l^{-1}

the one-factor-at-a-time experimentation, the reported pH and temperature values, although likely to approach the optima, cannot be conclusively taken as the optimum values.

Carbon source

To evaluate bioavailability of various carbon sources, starch, glucose, sucrose, maltose, lactose, and mannitol were supplemented into the media at the same carbon molar concentration of 0.33 mol l^{-1}. Figure 6 shows that glucose, sucrose, and mannitol yielded high DMCPCN hydratase activity, and starch supported *R. boritolerans* FW815 growth. Obviously, maltose and lactose were relatively inefficient for *R. boritolerans* FW815 in terms of NHase activity and biomass density. Glucose usage was further examined over a range of 6.0–16.0 g l^{-1}. Both NHase activity and biomass peaked at 10 g l^{-1} of glucose, with volumetric activity approaching 156.7 U ml^{-1}.

Nitrogen source

In order to evaluate bioavailability of various nitrogen sources, yeast extract, beef extract, peptone, NH_4NO_3, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, and corn steep liquor were supplemented into fermentation media at the same nitrogen molar content of 12.5 mmol l^{-1}. As shown in Fig. 7, organic nitrogen sources, particularly yeast extract, had higher bioavailability than the inorganic nitrogen sources tested. Further optimization of yeast extract usage indicated that NHase activity peaked at 164.1 U per ml broth when yeast extract was added at 8.0 g l^{-1}.

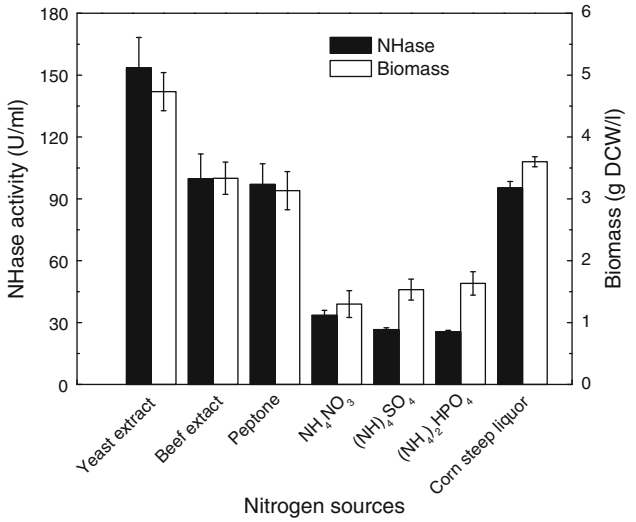

Fig. 7 Effect of nitrogen source on *R. boritolerans* FW815 proliferation (*open column*) and DMCPCN hydratase production (*filled column*). Cultivation conditions: 30°C, initial medium pH 7.0, 150 rpm, cultivation time 48 h. Nitrogen sources were added at a final nitrogen molar concentration of 12.5 mmol l^{-1}

Inducer

Nitrile hydratases are prone to be induced during cultivation, typically executed by substrates, products, or their structural analogues. In order to evaluate their induction efficiencies, a variety of compounds containing cyano or amide groups involving monosodium glutamine, ε-caprolactam, urea,

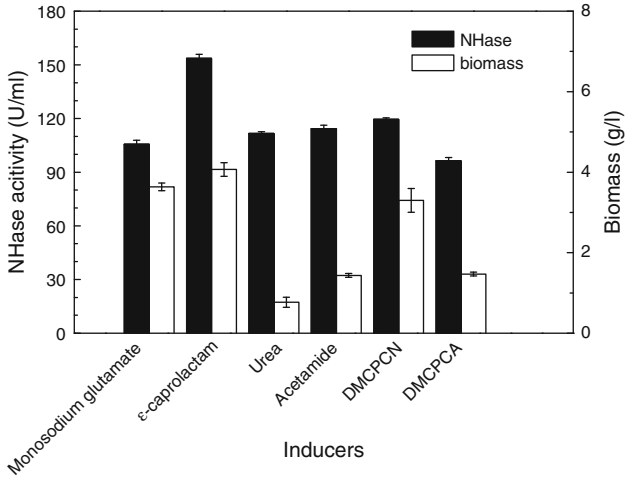

Fig. 8 Effect of inducer on *R. boritolerans* FW815 proliferation (*open column*) and DMCPCN hydratase production (*filled column*). Cultivation conditions: 30°C, initial medium pH 7.0, 150 rpm, cultivation time 48 h, inducers added at 1.0 g l^{-1} level

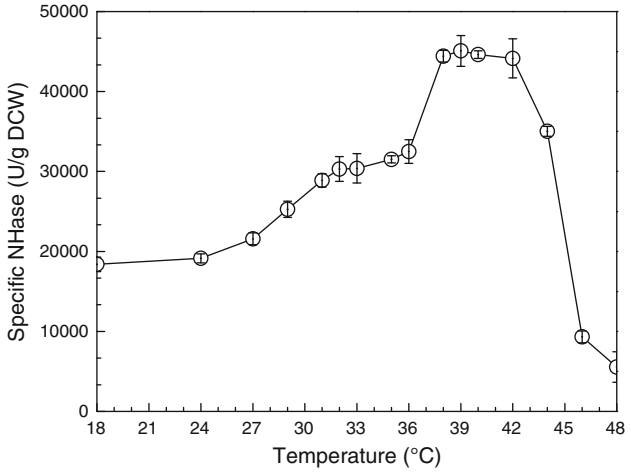

Fig. 9 Temperature dependence of specific NHase of *R. boritolerans* FW815. Reaction conditions: pH 7.0, initial DMCPCN concentration 17.1 mmol l^{-1}, conversion time 1.0 min

acetamide, DMCPCA and DMCPCN were added into the media at 1.0 g l^{-1} level, which was composed of (g l^{-1}): 10.0 glucose, 8.0 yeast extract, 1.0 KH_2PO_4, 1.0 NaCl, 1.0 K_2HPO_4, 0.2 $MgSO_4$, 1.0×10^{-2} $FeSO_4$, 5.0×10^{-2} $CaCl_2$. As shown in Fig. 8, ε-caprolactam was found to be the most effective inducer. After 2 days culture under the optimized conditions, *R. boritolerans* FW815 produced NHase activity of 166.8 U per ml broth with biomass density of 3.7 g DCW l^{-1}, corresponding to 4.51×10^4 U g^{-1} DCW.

Temperature and pH dependence of *R. boritolerans* FW815 NHase

As shown in Fig. 9, specific NHase of *R. boritolerans* FW815 increased with temperature within 18–38°C. *R. boritolerans* FW815 showed strong specific NHase at 38–42°C; afterwards, a sharp reduction of NHase was observed at temperatures exceeding 42°C. Moreover, *R. boritolerans* FW815 was sensitive to environmental pH (Fig. 10). Both acidic and alkaline conditions were adverse to DMCPCN hydratase, and neutral pH environments were preferred. Therefore, 38–42°C, pH 7.0 were preferred for *R. boritolerans* FW815 NHase activity. As mentioned above, due to the constraints of the one-factor-at-a-time experimentation, the reported pH and temperature values, although likely to be close to the optima, cannot be conclusively taken as the optimum values. So far, enzymatic hydration of DMCPCN and its derivatives has been conducted with *Rhodococcus* sp. AJ270, which harbors both NHase and *R*-amidase [19, 20]. In contrast, *R. boritolerans* FW815 displayed strong NHase activity towards DMCPCN but no detectable amidase activity towards DMCPCA with the tested conditions, suggesting it differs from *Rhodococcus* sp. AJ270 in nitrile-metabolizing enzyme system.

Reusability of *R. boritolerans* FW815 resting cells in non-buffered water system

Due to its wild-type nature and neutral pH preference, reusability of *R. boritolerans* FW815 resting cells in non-buffered water system was evaluated. In distilled water, *R. boritolerans* FW815 displayed DMCPCN-hydrating activity of 4.23×10^4 U g^{-1} DCW, approximately 93.8% of that in pH 7.0 phosphate buffer (50.0 mmol l^{-1}). Its reusability in distilled water system was comparable to that in pH 7.0 phosphate buffer (Fig. 11), and in both cases, DMCPCA at a final concentration of 17.0 mmol l^{-1} was obtained in the first three runs, with DMCPCN conversion of almost 100% and space–time yield of 581 kg m^{-3} day^{-1}. In terms of the final DMCPCN concentration, *R. boritolerans* FW815 is approximately 17 times more efficient than *Rhodococcus* sp. AJ270 [19]. Interestingly, after five consecutive runs, over 90% activity remained in each reaction medium and a sharp reduction in NHase was observed from the sixth batch, demonstrating a good reusability of *R. boritolerans* FW815 resting cells. From the plots of the negative logarithm of relative remaining activity (%) versus storage time, the half-lives of *R. boritolerans* FW815 in 4°C distilled water system and 4°C, pH 7.0 phosphate buffer (50.0 mmol l^{-1}) were determined to be 74.5 and 86.6 h. Although the catalytic performance and stability in non-buffered distilled water system were a little bit lower than those in phosphate buffer (pH 7.0, 50.0 mmol l^{-1}), water system is acceptable for consideration of facilitating product recovery and eliminating desalination, effluent handling problems.

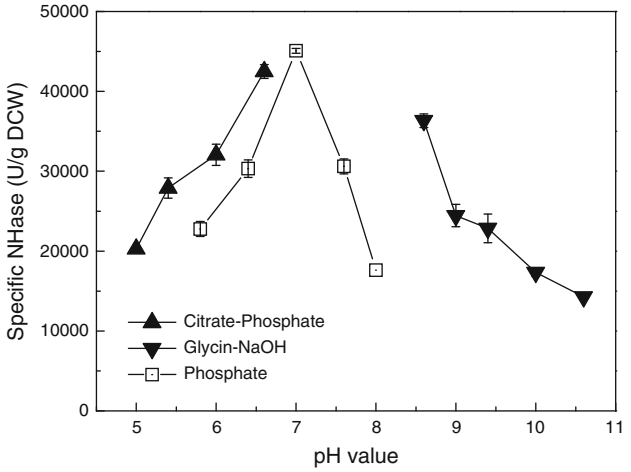

Fig. 10 pH dependence of specific NHase of *R. boritolerans* FW815. Reaction conditions: 39°C, initial DMCPCN concentration 17.1 mmol l^{-1}, conversion time 1.0 min; buffers: 50.0 mmol l^{-1} citrate–phosphate buffers (*filled triangle*) with pH covering pH 5.0–6.6, 50.0 mmol l^{-1} phosphate buffers (*open square*) with pH 5.8–8.0, 50.0 mmol l^{-1} glycine–NaOH buffers (*inverted triangle*) with pH 8.6–10.6

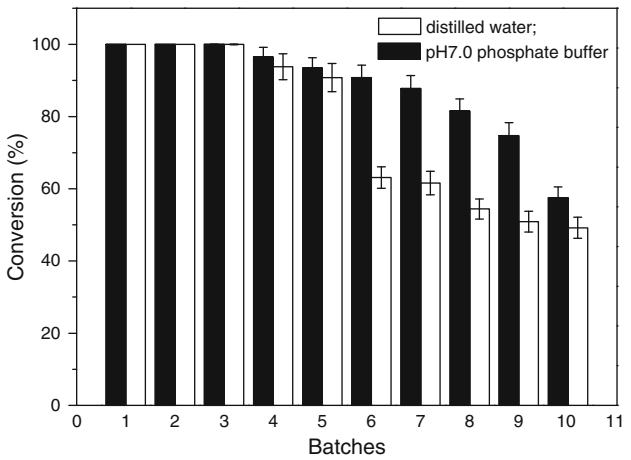

Fig. 11 Reusability of *R. boritolerans* FW815 resting cells in pH 7.0, 50.0 mmol l^{-1} phosphate buffer (*filled column*) and non-buffered water system (*open column*). Operating conditions: 39°C, initial DMCPCN concentration 17.1 mmol l^{-1}, conversion time 5.0 min

Conclusions

In this work, a mild, environmentally friendly and efficient bioconversion for DMCPCA preparation in distilled water system was developed with *R. boritolerans* FW815, which harbors excellent DMCPCN hydratase activity. Because *R. boritolerans* FW815 is DMCPCA amidase-negative, it produces high-quality DMCPCA without contamination of 2,2-dimethylcyclopropanecarboxylic acid. In this way, a promising platform technology based on the combination of active NHase and enantioselective amidase is constructed, enabling efficiently converting inexpensive racemic nitriles to valuable single enantiopure amides and acids.

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