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Methods for Increasing Nitrile Biotransformation into Amides Using *Mesorhizobium* sp.[1]

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Abstract—Nitriles are potential soil pollutants from industrial wastewater. There has been increased demand for an efficient process for the nitrile degradation process. Nitrile hydratase (NHase) has been extensively used in the production of acrylamide and treatment of organocyanide-contaminated industrial effluents. The NHase of *Mesorhizobium* sp., isolated from polyacrylonitrile (PAN) activated sludge from fiber manufacturing wastewater treatment systems was studied in the whole bacterial cells. Different chemicals were added to observe the variation in the percentage of acrylonitrile converted into acrylamide. The result indicated that cobalt ions were the NHase cofactor and could increase the NHase activity. The addition of propionaldehyde, or butyraldehyde, could enhance the acrylonitrile conversion rate. Therefore, acrylamide could be accumulated effectively and the percentage of acrylonitrile converted into acrylamide increased. Propionaldehyde was the most effective NHase activator. The percentage of acrylonitrile converted into acrylamide was nearly 100% at 3.8 h when propionaldehyde was added at about 207.4 mg/l. The addition of benzaldehyde was unable to increase the percentage of acrylonitrile converted into acrylamide. EDTA and acrylamide showed no effect on NHase activity. However, 0.1 mg/l of Ag_2SO_4 would slightly inhibit NHase activity, producing an acrylonitrile conversion rate of 492.9 mg/l with 54.9% converted at 29.1 h. The ability of the acrylonitrile biotransformation was completely inhibited if the Ag_2SO_4 concentration was above 0.5 mg/l.

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Nitrile compounds are organocyanides (R–CN) existing widespread in nature [1–3]. Synthetic nitrile compounds are widely used in benzonitrile herbicides [4] and as chemical solvents, extractants, and recrystallizing agents in a number of industrial operations [5]. Consequently, using these toxic compounds in industry has led to their increased distribution throughout the environment. The potential of nitrile hydratase/amidase systems for converting toxic nitriles to high-value amide has already been demonstrated [6–8]. Nitrile hydratase (NHase, EC 4.2.1.84) is a microbial enzyme that catalyzes nitrile compounds hydration into corresponding amides and could be used as catalysts for industrial production of important commodity chemicals, acrylamide [9]. NHase is generally characterized by the presence of a coordinated metal, iron or cobalt [10, 11] or, recently, both [12]. The NHase production in some microbial systems has been reported as constitutive [13], while in most of the nitrile degrading organisms, it is generally induced or inactive by amides [14–17]. Several researchers have investigated that NHase could be inactivated using different chemicals, for instance, Ag_2SO_4 [18, 19], EDTA, or hydrogen peroxide [20]. In this paper, *Mesorhizobium* sp. containing NHase was studied in a resting cell with different chemicals added to observe variation in the percentage of acrylonitrile converted into acrylamide. Through increasing the NHase activity of *Mesorhizobium* sp., poisonous acrylonitrile could be effectively converted into high-value acrylamide.

MATERIALS AND METHODS

Organisms, media, and culture conditions. *Mesorhizobium* sp., isolated from polyacrylonitrile (PAN) activated sludge from fiber manufacturing wastewater treatment systems, containing both NHase and amidase enzymes, could convert acrylonitrile into acrylamide [21]. The treatment bacteria was cultivated on R2A medium [22].

All microbial experiments were performed in phosphate buffered medium (PBM) [23, 24]. The PBM compositions for these experiments contained the following (g/l): $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.02; K_2HPO_4, 1.0; KH_2PO_4, 1.0. The PBM trace element solution was 10 ml/l. The trace element solution composition was as follows (mg/l): $FeSO_4 \cdot 7H_2O$, 300; $MgCl_2 \cdot 4H_2O$, 180; $CoCl_2 \cdot 6H_2O$, 106; $Na_2MoO_4 \cdot 2H_2O$, 34; $ZnSO_4 \cdot 7H_2O$, 40. The final pH value was 7.5 at PBM. The pH of the phosphate buffered medium was adjusted to 7.5 with NaOH/HCl.

[1] The text was submitted in English.

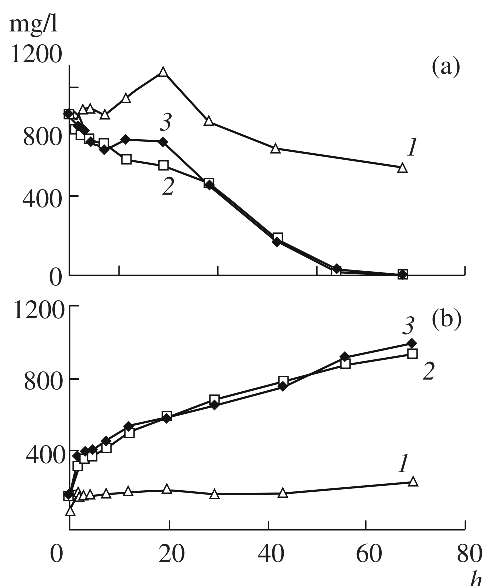

Fig. 1. Effect of metal ions on *Mesorhizobium* sp. converting 975.6 mg/l acrylonitrile(a) and the variation of acrylamide concentration (b). The phosphate buffers containing iron ions (*1*), cobalt ions (*2*), and both iron and cobalt ions (*3*) were used to investigate the NHase cofactor of *Mesorhizobium* sp.

Batch reactor. A midexponential culture of *Mesorhizobium* sp. was centrifuged at 6000 *g* for 12 min at 4°C and washed three times with phosphate buffers. A series of batch experiments were conducted in 120-ml serum bottles, each serum bottle containing cells suspended in 40 ml phosphate buffer to a cell concentration of 10^7 cfu ml^{-1} (resting cell). Three kinds of phosphate buffers containing metal ions (Co, Fe, Co/Fe) were prepared to determine the cofactor of NHase from *Mesorhizobium* sp., and the appropriate media would be used in subsequent experiments. Acrylamide, aldehyde (propionaldehyde, butyraldehyde, and benzaldehyde), and an inhibitor (EDTA and Ag_2SO_4) were respectively added to the phosphate buffer to investigate the effect of different chemicals on the bacteria to convert acrylonitrile into acrylamide. After sealing with teflon/silicon stoppers, the reactors were shaken at 120 rpm in the dark at 30°C. The acrylonitrile, acrylamide, acrylic acid, and ammonia concentration changes, and the pH and OD_{600} values were analyzed at regular intervals.

Table 1. The NHase cofactor of *Mesorhizobium* sp.

Cofacter	Time, h	The conversion rate of acrylonitrile, %*	The percentage of acrylonitrile converted into acrylamide, %**
Fe/Co	69.5	100	72.1
Co	69.5	100	76.3
Fe	69.5	100	19.9

Notes: * The conversion rate of acrylonitrile = (initial acrylonitrile concentration – final acrylonitrile concentration)/(initial acrylonitrile concentration) × 100%.
** The percentage of acrylonitrile converted into acrylamide = (acrylamide accumulatiion molar concentration/acrylonitrile conversion molar concentration) × 100%.

Analytical methods. Samples were collected directly from the reactors using a syringe. After membrane filtration, the ammonia concentration was measured using the indophenol blue method [25]. The acrylonitrile, acrylamide, and acrylic acid concentrations were measured using high performance liquid chromatography (HPLC) with an ultraviolet detector. HPLC was performed with a Hitachi system equipped with a Merck Lichrospher 100 PR–18 endcapped (5 m) column at a flow rate of 0.6 ml/min. The solvent system consisted of 300 ml acetonitrile and 700 ml water and the UV detector absorbency wavelength was fixed at 210 nm.

The pH and optimum density (OD) were measured using a pH meter and Spectrophotometer at 600 nm, respectively.

RESULTS AND DISCUSSION

The NHase cofactor. Two kinds of metal ions, Fe and Co, were used to determine the NHase cofactor. Fig. 1 shows the variation in acrylonitrile and acrylamide concentration using three kinds of phosphate buffers containing iron ions, cobalt ions, and both iron and cobalt ions when converting 975.6 mg/l acrylonitrile.

The result shows that the acrylonitrile conversion rates when using phosphate buffer containing both iron and cobalt ions and phosphate buffers containing just cobalt ions were similar, with a 97.0% and 98.7% conversion rate at 55.8 h, respectively. Under both conditions, acrylonitrile was completely converted at 69.5 h and the acrylamide concentration was accumulated gradually during the conversion process. The acrylonitrile conversion rate for a phosphate buffer containing only iron ions was substantially lower, reaching only 32.6% at 69.4 h. The accumulated acrylamide concentration was distinctly lower than that from a phosphate buffer containing both iron and cobalt ions or only cobalt ions. Table 1 shows the experimental data when using phosphate buffers containing iron ions, cobalt ions, and both iron and cobalt ions. During the course of these experiments, with the acrylonitrile conversion, the acrylic acid concentration also appeared to increase, reaching values of 336.3, 378.6, and 313.4 mg/l, respectively. Under all three circumstances, the pH value decreased, while the OD value underwent no substantial change (data not shown).

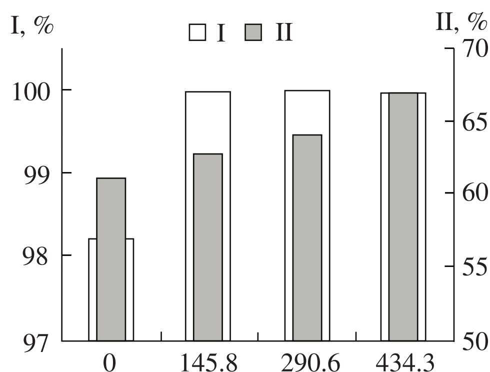

Fig. 2. The acrylamide effect on the NHase of *Mesorhizobium* sp. Reactions were carried with 970.0 mg/l acrylonitrile and acrylamide at 145.8, 290.6, and 434.3 mg/l. The conversion rate of acrylonitrile (%) after 45 h of reaction (I). The percentage of acrylonitrile converted into acrylamide (%) after 45 h of reaction (II).

From the above experiment, a phosphate buffer containing cobalt ions was the best suited for converting acrylonitrile into acrylamide. This meant that the NHase of *Mesorhizobium* sp. was more active with cobalt ions as a cofactor. A phosphate buffer containing cobalt ions was used to conduct further experiments.

The acrylamide effects on the NHase of *Mesorhizobium* sp. Some researches have reported that NHase activity in some organisms might be inhibited by acrylamide, and this would affect bacteria to convert acrylonitrile into acrylamide consecutively. Raj et al. have reported that the NHase activity of R. rhodochrous PA–34 would not be detected when 0.2% (w/v) acrylamide existed in the medium [16]. The NHase activity of *Rhodococcus* sp. gt1 was 50 U in the absence of nitriles and amides, but significantly decreased to 4.2 U after addition of acrylamide to the medium [17]. In this experiment, acrylamide was added to observe the effect on the NHase of *Mesorhizobium* sp. Fig. 2 showed the results on the addition of acrylamide. From the results, it could demonstrate that acrylamide had no obvious influence on the acrylonitrile conversion rate or the percentage of acrylonitrile converted into acrylamide. Thus, *Mesorhizobium* sp. could be used to efficiently convert acrylonitrile into acrylamide and the accumulation of acrylamide would not inhibit the NHase activity.

The aldehyde effects on the NHase of *Mesorhizobium* sp. Fig. 3 shows the results from converting 493.8 mg/l acrylonitrile into acrylamide by the resting cell of *Mesorhizobium* sp. when the phosphate buffer media were supplemented with 207.4 mg/l propionaldehyde, 210.0 mg/l butyraldehyde, and 210.0 mg/l benzaldehyde. As adding propionaldehyde and butyraldehyde, the acrylonitrile conversion rates were both nearly 100% at 3.7 h and 3.6 h, and the percentages of acrylonitrile converted into acrylamide were 100% and 88.2%, respectively. To further examine acrylic acid concentration, it was exhibited that acrylic acid concen-

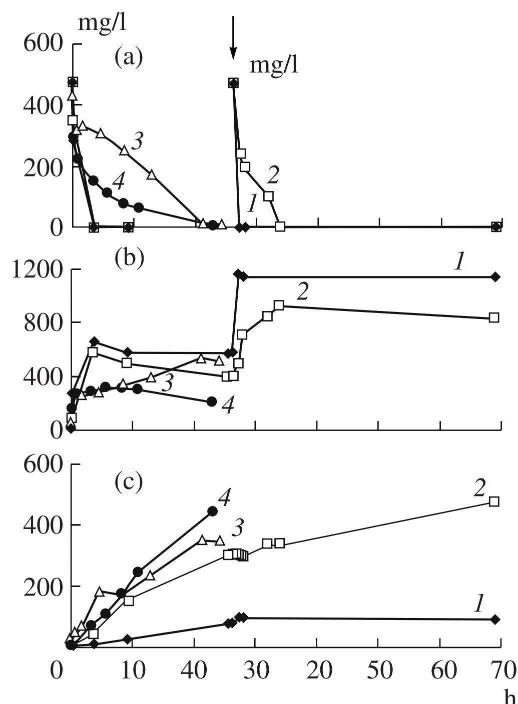

Fig. 3. The variation of acrylonitrile (a), acrylamide (b), and acrylic acid concentration (c) as adding 207.4 mg/l propionaldehyde (*1*), 210.0 mg/l butyraldehyde (*2*), and 210.0 mg/l benzaldehyde (*3*), control (*4*). Reactions were carried with 493.8 mg/l acrylonitrile. 487.8 mg/l acrylonitrile was supplemented at 26.3 h (the arrow shows) to confirm the acrylonitrile conversion.

Table 2. Effect of aldehyde on acrylonitrile bioconversion with *Mesorhizobium* sp.

Aldehyde	Time, h	The convertion rate of acrylonitrile, %	The percentage of acrylonitrile converted into acrylamide, %*
Control	10.8	86.7	52.8
Propionaldehyde	3.7	100	100
Butyraldehyde	3.6	100	88.2
Benzaldehyde	21.3	97.6	83.7

* The percentage of acrylonitrile converted into acrylamide = (acrylamide accumulatiion molar concentration/acrylonitrile conversion molar concentration) × 100%.

trations were just 5.8 mg/l at 3.7 h and 42.1 mg/l at 3.6 h. Compared with the result without aldehyde addition, the conversion rate of acrylonitrile and the percentage of acrylonitrile converted into acrylamide were 86.7 and 64.1% at 10.8 h. It could be demonstrated that propionaldehyde and butyraldehyde would raise the acrylonitrile conversion rate and decrease the conversion rate of acrylamide into acrylic acid, and then acrylamide would continuously accumulate. On the other hand, the acrylonitrile conversion rate and the percentages of acrylonitrile converted into acrylamide were respectively 97.3 and 83.7% at 21.3 h with benzaldehyde. It needed more time for *Mesorhizobium* sp. to completely convert acrylonitrile into acrylamide, and it was useless to increase the acrylamide accumulation. The results from the above studies were tabulated in Table 2.

The NHase inhibitor of *Mesorhizobium* sp. To determine the effect of EDTA or Ag_2SO_4 on the NHase activity of *Mesorhizobium* sp., different concentrations of EDTA or Ag_2SO_4 were added to the phosphate buffer media. Adding three kinds of 182.9 mg/l EDTA (EDTA-Mg, EDTA-Fe, and EDTA-Na), the conversion rates of 488.1 mg/l acrylonitrile were all 98.9% at 5.2 h. The conversion rate of 488.1 mg/l acrylonitrile was also 98.9% at 5.9 h when no EDTA was added (data not shown). Therefore, EDTA did not affect the NHase activity. Maier-Greiner et al. reported that the cyanamide hydratase activity is sensitive to chelating agents such as EDTA or o-phenanthroline, pointing to a functional role of zinc in the protein [26]. Chelating agents had varied effects on the B. pallidus Dac521 NHase activity. 1 mM EGTA inhibited the enzyme activity, while EDTA at concentrations of 1 mM and 5 mM had no effect [20]. Accordingly, EDTA had a distinct effect on NHase from different bacteria. This might depend upon the binding situation or strength of the NHase cofactor.

As Ag_2SO_4 was added to the phosphate buffer media, the conversion rate of acrylonitrile decreased obviously and the accumulated acrylamide concentration was low. The conversion rate of 492.9 mg/l acrylonitrile was 54.9% at 29.1 h when adding 0.1 mg/l Ag_2SO_4. The acrylonitrile was not completely converted into acrylamide or acrylic acid as the additional concentration of Ag_2SO_4 was increased to 0.5 mg/l and 1.0 mg/l (data not shown). This indicated that Ag_2SO_4 might inhibit NHase activity or be poisonous to *Mesorhizobium* sp. Therefore, *Mesorhizobium* sp. could not convert acrylonitrile effectively

The aim of this paper was to investigate the factors that affect NHase of *Mesorhizobium* sp. to convert acrylonitrile into acrylamide. This research demonstrated that the Co ions could be the cofactor of the NHase of *Mesorhizobium* sp., and propionaldehyde and butyraldehyde would increase the acrylonitrile conversion rate. To biodegrade wastewater containing acrylonitrile using *Mesorhizobium* sp., Co ions should be added to the culture medium as the NHase cofactor, and the toxic acrylonitrile could therefore be efficiently converted into acrylamide. For industrial production of high-value acrylamide with *Mesorhizobium* sp., propionaldehyde, which particularly raised the percentage of acrylonitrile converted into acrylamide, might be a useful supplement to enhance the accumulation of acrylamide.

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