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Enzyme and Process Development for Production of Nicotinamide

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Abstract:

Through screening a library of nitrile hydratases developed in-house, NHT-120 was selected to catalyze the hydration of 3-cyanopyridine for production of nicotinamide. After reaction optimization, a fed-batch method was used to alleviate the substrate inhibition. Under this approach, 200 g/L of 3-cyanopyridine could be converted to nicotinamide in 5.5 h, and no nicotinic acid was produced. Under a substrate to enzyme ratio of 100:1 (w/w), nicotinamide in the reaction solution could reach 230 g/L.

Introduction

As one of the two principal forms of the B-complex vitamin niacin, nicotinamide has a wide range of industry applications in the medicinal and food industries.[1] Nicotinamide is an intermediate for the synthesis of pharmaceuticals and pesticides and is also used as a plant growth regulator and additive.

The most common preparation method of nicotinamide is ammonia oxidation of 3-methylpyridine followed by the inorganic acid hydrolysis of the resulting 3-cyanopyridine. It is not easy to keep this reaction in the amidation stage without hydrolysis in concentrated sulfuric acid under limited water content. Therefore, the production of nicotinamide requires harsh reaction conditions and high-standard equipment, rendering the production of nicotinamide more difficult than that of nicotinic acid, and leading to higher manufacturing costs.

As an improved method, the biotransformation of 3-cyanopyridine to nicotinamide using nitrile hydratases has attracted increasing attention owing to the enzyme's high selectivity under mild reaction conditions in water (Scheme 1). Nitrile hydratases have been used at commercial scale for the manufacture of several products including acrylamide.[2−8] Almost all of them use resting cells containing nitrile hydratases to reduce manufacturing costs. However, whole cells have their own problems. Many substrates cannot penetrate into cells efficiently, and products may not be able to diffuse out of cells readily. Moreover, cells contain many other biocatalysts, which may lead to side products. For example, in whole-cell-catalyzed production of nicotinamide from 3-cyanopyridine, unacceptable amounts of nicotinic acid and ammonia byproducts are usually generated by amidases, of which their genes typically sit downstream from the genes of nitrile hydratases. Substrate inhibition is another issue for many whole-cell-catalyzed processes. It is generally easier to solve substrate inhibition for processes catalyzed by isolated enzymes than for those catalyzed by whole cells. To overcome these problems, Lonza developed a continuous three-step cascade nicotinamide process through extensive reaction engineering. We report here an efficient nicotinamide process using isolated recombinant nitrile hydratase (NHT).

Results and Discussion

Enzyme Screening. In order to obtain the desired nitrile hydratase, using plate-based screening (1 mg of enzyme and 0.1 mg of substrate in 100 μL buffer, pH 7.0), we compared over 20 NHTs which were obtained through cloning, expression, and isolation. The results showed that more than half of them catalyzed conversion of 3-cyanopyridine to nicotinamide, but with different reaction efficiency. To identify better ones, the substrate concentration was increased to 5 g/L with a substrate to enzyme ratio of 100:1 (w/w) using water as the only solvent. From the results (Table 1), NHT-120 showed the highest specific activity and therefore was chosen for future research.

Effect of 3-Cyanopyridine Concentrations on Nicotinamide Production. As the concentration of substrate increased (2−40 g/L) in the reaction mixture, there was a rise in the NHT activity. However, at higher substrate concentrations (40−50

Scheme 1. **Enzymatic preparation of nicotinamide from 3-cyanopyridine**

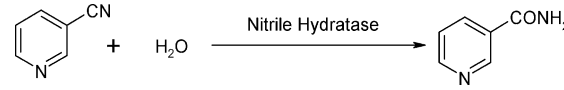

Table 1. **Screening results with 5 g/L of substrate and a substrate to enzyme ratio of 100:1 (w/w) using water as the only solvent**

	conversion (%)	
enzyme no.	1 h	3 h
NHT-105	8.9	12.1
NHT-106	4.3	8.4
NHT-107	35.4	52.9
NHT-108	4.6	7.2
NHT-112	2.3	4.9
NHT-114	3.3	5.1
NHT-120	81.7	97.5
NHT-121	1.1	1.8
NHT-124	9.0	15.5

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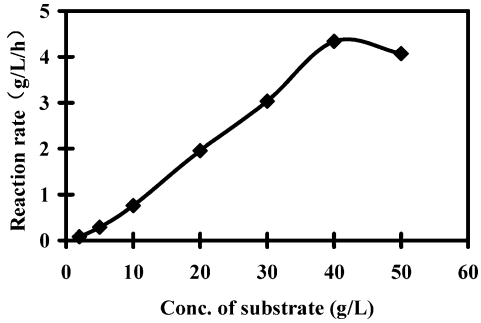

Figure 1. **NHT activity at different concentration of 3-cyanopyridine (2−50 g/L).**

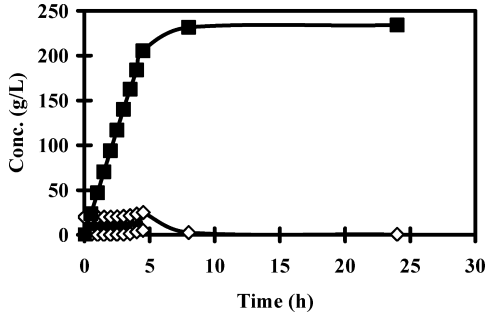

Figure 2. **Concentration of nicotinamide and 3-cyanopyridine during the fed-batch reaction (2.5 mL reaction): (□) nicotinamide; (◇) 3-cyanopyridine.**

g/L), a decrease in activity was observed (Figure 1). The rise in activity was due to the increase in the availability of the substrate for hydration. A further increase in the substrate concentration led to a decrease in the enzyme activity as a result of substrate inhibition.

Fed-Batch Reaction for Nicotinamide Production. By the fed-batch method, the inhibition of the substrate can be alleviated, and the actual ratio of the substrate to an enzyme can be improved. In order to obtain higher yields of nicotinamide, the reaction was carried out in a fed-batch mode, and the number of feeds and feeding interval were studied at a volume of 2.5 mL. The concentration of nicotinamide and 3-cyanopyridine during the fed-batch reaction is shown in Figure 2. There was complete conversion of 3-cyanopyridine to nicotinamide up to four feedings, and after that, the rate of conversion started to decrease slowly probably due to product inhibition. Upon incubation for 24 h, the substrate was completely converted into nicotinamide. In this study, 200 g/L of substrate was added by 10 feedings to the reaction mixture, and 230 g/L of nicotinamide was accumulated, leading to an actual ratio of substrate and enzyme to 100:1 (w/w).

Scale-Up of Fed-Batch Reaction to 1.5 L. On the basis of the observation above, the reaction was scaled up to 1.5 L under 240 rpm. Figure 3 shows the concentration of the product and substrate at various intervals of time. It was observed that 100% of the added substrate was converted into product within 5.5 h. This higher rate of substrate conversion on scale up is probably due to better mixing of substrate and enzyme under mechanical agitation as compared to shaking. A productivity of 502 g of nicotinamide per day was obtained in the presence of 1 g of the enzyme.

To isolate the nicotinamide, the reaction mixture was evaporated in vacuum. The solid obtained was dissolved in hot

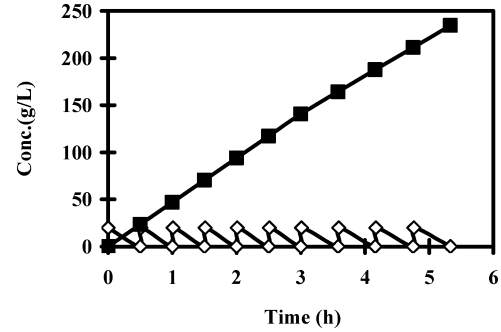

Figure 3. **Concentration of nicotinamide and 3-cyanopyridine during the fed-batch reaction (1.5 L reaction): (□) nicotinamide; (◇) 3-cyanopyridine.**

ethanol and then filtered to remove enzyme. The filtrate was concentrated under vacuum to get a white powdery solid (350 g) in 99% yield.

The product was confirmed as nicotinamide through ^{1}HNMR and mass spectra: ESI-MS *m/z*: 123 $[M + H]^+$ giving a molecular weight of 122; ^{1}H NMR (400 MHz, d^6-DMSO): 7.4, 7.5, 8.1, 8.2, 9.0 ppm.

Conclusion

According to these results, preparation of nicotinamide by isolated enzymes has the following advantages: first, high conversion of 3-cyanopyridine can be accomplished under mild conditions without the need of special equipment and harsh reagents; second, no formation of nicotinic acid was detected in the reaction mixture, which reduces the cost and time for purification. It should also be noted that the nitrile hydratase can be readily expressed in *E. coli*. with high target protein content. Further process optimization including immobilization should make this process promising for greener manufacturing of nicotinamide.

Experimental Section

Chemicals. 3-Cyanopyridine (Acros Organics, U.S.A.; Nantong Acetic Acid Chemical Co., Ltd., China) and nicotinic acid and nicotinamide (Sinopharm Chemical Reagent Co., Ltd., China) were used in this study. All of the other reagents were of analytical grade.

Enzyme Development. Microorganisms were used as the DNA donors and the sources for development of nitrile hydratases. *Escherichia coli* DH5α and BL21 (DE3) were used as host cells for gene cloning and overexpression. pET system was used for in vitro protein recombination and expression. A standard procedure was used for gene cloning and in vitro expression.[9]

E. coli cells harboring the recombinant plasmids were cultured at 37 °C in Luria−Bertani (LB) medium supplemented with ampicillin (100 μg/mL) and/or kanamycin (30 μg/mL). Isopropyl-β-D-thiogalactopyranoside (IPTG, 0.1 mM) was added when the OD_{600} reached 0.6−0.8, followed by further growth overnight at 20 or 30 °C.

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The cells were harvested by centrifugation, resuspended in 10 mM sodium phosphate buffer (pH 7.0) at a concentration of 500 g/L, and disrupted with a JY 92-II DN ultrasonic cell crusher (NingBo Scientz Biotechnology, China) at 4 °C. Cell debris was removed by centrifugation (14,000 rpm, 15 min), and the supernatant was lyophilized. The powder was stored at 4 °C and used for enzyme screening.

Enzyme Screening. To 10 μL of each enzyme in a screening kit (preprepared 96-well plate with different nitrile hydratases at 100 g/L) was added 0.1 mg of the substrate dissolved in 10 μL of organic solvent (acetonitrile, acetone, or DMSO) and 80 μL of 0.1 M phosphate buffer (pH 7.5) with final reaction conditions in each well consisting of 1 g/L of substrate, 10 g/L of enzyme, and 10% organic solvent. The mixture was incubated with agitation at 30 °C for 18 h, and then an equal volume of acetonitrile was added to each mixture and the conversion monitored by LC/MS.

General Procedure for Biotransformation. The standard reaction mixture consisted of 0.5 mL of deionized water, 0.025 mg of lyophilized crude enzyme, and 2.5 mg of 3-cyanopyridine, giving a final substrate concentration of 5 g/L and a substrate to enzyme ratio of 100:1 (w/w). After incubation at 30 °C and 160 rpm in a shaker, 0.5 mL of acetonitrile was added to the reaction mixture. After centrifugation, the supernatant was directly subjected to HPLC analysis (see below) to determine the quantity of nicotinamide.

Effect of 3-Cyanopyridine Concentrations on Nicotinamide Production. To study the effect of substrate concentration on production of nicotinamide, the concentration of 3-cyanopyridine (2−50 g/L) was varied in a 0.5 mL reaction mixture at a substrate to enzyme ratio of 100:1 (w/w), and the concentration of nicotinamide formed was determined by HPLC.

Fed-Batch Reaction for Nicotinamide Production. In order to obtain high amount of nicotinamide, the biotransformation of 3-cyanopyridine was conducted in a fed-batch mode in a 10-mL Erlenmeyer flask using 2.5 mL of reaction mixture at 30 °C under 160 rpm. To the reaction mixture, 20 g/L of 3-cyanopyridine was added at an interval of 30 min, and the status of the conversion of substrate to product was analyzed by HPLC. After 10 feedings, the reaction was allowed to proceed for 19.5 h of incubation.

Scale-Up of Fed-Batch Reaction to 1.5 L. The conversion of 3-cyanopyridine to nicotinamide was carried out at 1.5-L scale in a 3-L flask at 30 °C under mechanical agitation of 240 rpm. To the reaction mixture, 20 g/L of 3-cyanopyridine was added in each feed at an interval of 20 or 30 min. After the 10th feed, the reaction was allowed to proceed for next 0.5 h.

Analytical Methods. 3-Cyanopyridine, nicotinamide, and nicotinic acid in the reaction mixture were quantitatively analyzed by HPLC system (SHIMADZU LC-2010A, Japan) equipped with C18 reverse phase column (Ø 4.6 mm $\times$150 mm, 5 μ), with detection at 254 nm. The mobile phase was a gradient of 10 to 90% acetonitrile/water (0.1% trifluoroacetic acid in both) from 0 to 3.5 min and 90% acetonitrile/water (0.1% trifluoroacetic acid in both) for 0.5 min at a flow rate of 1 mL/min. The retention time of 3-cyanopyridine, nicotinamide and nicotinic acid was 3.6 min, 2.1 and 2.2 min respectively.

The ^{1}H nuclear magnetic resonance (NMR) and mass spectra were recorded with FT-NMR (Varian, U.S.A.) and LCMS-2010 (SHIMADZU, Japan) respectively.

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