

Nicotinic acid bioproduction in UF-membrane reactor *via* nitrile hydratase-amidase catalyzed reactions

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In this study *Microbacterium imperiale* CBS 498-74 resting cells are used as catalyst for the bioconversion of 3-cyanopyridine into nicotinic acid. Nitrile bioconversion into the corresponding acid is performed by this strain *via* a two step cascade reaction catalysed by nitrile hydratase and amidase with an amide as intermediate. Both enzymes that operate under mild conditions suitable for the synthesis of labile organic molecules have been characterized independently for activity and stability in a UF-membrane bioreactor. The reactor was fed with buffered solutions of appropriate substrate, either 3-cyanopyridine or nicotinamide. The effluent containing unreacted substrate, products and buffer were collected and analysed for product determination. The operational conditions (temperature, residence time) that strongly influence continuous UF-membrane bioreactor performances (conversion yield and selectivity) are presented.

1. Introduction

Nowadays there is a widespread use of nicotinic acid (niacin, a form of soluble vitamin B3) in the treatment of schizophrenia, diabetes, auto-immune diseases and cholesterol-related diseases and in cosmetic skin care. However, both nicotinamide and nicotinic acid, that are building blocks for NADH and NADPH co-enzymes, have to be supplied to the human body through food. Niacin is also exploited in animal feed supplementation, for vitamin enrichment of cereal products, as meat additive, as a biostimulator for the formation of activated sludge and as deodorant for air and waste gases in pollution control.

Recently much industrial and academic interest has been focused on the search of a bioproduction route alternative to the chemical one. The chemical production of nicotinic acid from 3-cyanopyridine or picoline is an uneconomical process (low yield and high cost) that calls for harsh conditions such as strong base, high temperature, catalyst (Anderson and Bovine, 1985). Thus, the conversion of 3-cyanopyridine using nitrile hydrolyzing enzymes is attractive owing to the mild conditions and high conversion (Nagasawa et al., 1988) due to the absolute selectivity of the bioconversion. At industrial level Nitto, BASF and Lonza companies developed a biotechnological

process to convert 3-cyanopyridine into niacinamide making use of enzymes from *Rhodococcus rhodochrous* (Chuck, 2005).

In most cases the microbial degradation of nitrile proceeds through two different pathways as shown in Figure 1: nitrile hydratase (NHase) catalyzes the hydration of a nitrile to the corresponding amide which is further converted into the acid; this second reaction being catalyzed by amidase (AMase), while in the second pathway nitrilase catalyses the direct transformation of the nitrile into the corresponding acid (Kobayashi and Shimizu, 1994; Martínková and Křen, 2002; Martínková et al., 2008; Stolz et al., 1998; Sugai et al., 1997).

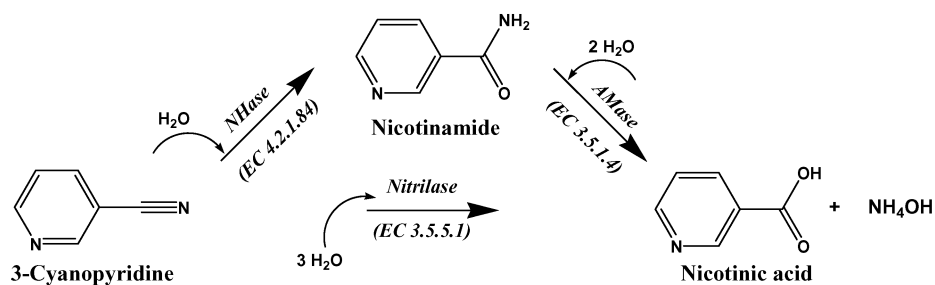


Figure 1: Alternative enzymatic pathways in microbial degradation of nitrile

This study makes use of resting cells of *Microbacterium imperiale* CBS 498-74 as catalyst for the bioconversion of 3-cyanopyridine into nicotinic acid. The strain follows the two step cascade reactions catalysed by NHase and AMase in nitrile bioconversion into the corresponding acid *via* an amide as intermediate. Both enzymes operate under mild conditions suitable for the synthesis of organic molecules (Cantarella et al., 1998; Cantarella et al., 2006). Though, the activity loss due to operational conditions strongly affects conversion yield and selectivity of the investigated process, NHase activity being rather labile (Alfani et al., 2001; Cantarella et al., 2004).

The scale up of 3-cyanopyridine bioconversion calls for the characterization of each enzyme of the cascade system. To this end, NHase and AMase have been characterized independently for activity and stability using a UF-membrane bioreactor, a useful reactor configuration (Cantarella et al., 2006, 2008a, 2008b) fed with buffered solutions of appropriate substrate, 3-cyanopyridine or nicotinamide. The effects of different operational conditions (temperature, residence time) on continuous UF-membrane bioreactor performances are discussed and a process layout with reactors in series is examined.

2. Materials and Methods

2.1 Materials

3-Cyanopyridine, nicotinamide and nicotinic acid were of analytical grade and purchased from Aldrich USA.

2.2 Methods

2.2.1 Culture conditions and preparation of resting cells

The cultivation conditions for *Microbacterium imperiale* CBS 498-74 for the production of nitrile hydratase were optimized previously (Cantarella et al., 2002). The subculture

was carried out in 100 mL of sterile YMP medium, prepared in 50 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 7.0, consisting of (in g/L) yeast extract 3.0, malt extract 3.0, bacteriological peptone 5.0. After 24 h, 10 ml of subculture were used to inoculate 90 mL of YMPG medium (YMP added with 5g/L glucose). Both cultures were carried out at 28°C for 24 h in an orbital shaker (220 rpm). The cells obtained were washed three times with buffer, pH 7.0, centrifuged at 11,400 rpm, and then suspended in buffer. This cell suspension was kept at -18 °C till its use in the resting-cell reaction.

2.2.2 Activity assays

NHase activity (EC 4.2.1.84) was assayed for the production of nicotinamide using 50 mM 3-cyanopyridine, as substrate. AMase activity (EC 3.5.1.4) was assayed with nicotinamide (50 mM) as substrate. Each reaction was carried out, for 20 min. under stirring conditions (250 rpm) in a standard reaction mixture (2 ml) containing 50 mM Na-phosphate buffer (pH 7.0) and 1 and 2 mg_{DCW} of cell suspension. The reaction was stopped by adding 1mL of 0.5 M HCl, and centrifugating at 10,000 rpm for 10 min. One unit of NHase or of AMase activity was defined as the amount of enzyme (cells) required to release 1 $\mu\text{mol min}^{-1}$ of relative product.

2.2.3 Analytical high-pressure liquid chromatography (HPLC)

The amounts of residual substrate and products in the reaction mixture were assayed by HPLC performed with a Perkin-Elmer Series 2 HPLC system (USA) equipped with a Merck (Germany) LiChro-CART 250-4 LiChrospher 100 reverse-phase-C18 column (5 μm) and a Merck (Germany) LiChro-CART 4-4 guard column. The mobile phase was: acetonitrile (12% v/v) -10 mM $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$ buffer (pH 7.0 at a flow rate of 1.0 ml/min at 30°C. The samples adequately diluted with mobile phase were placed in an LC Auto-sampler LS 3200 SGE (Australia). The absorbance of products and substrates was measured at 230 nm with a UV detector from Perkin-Elmer LC 290 (USA), associate to JASCO Borwin Chromatography software for area integration.

2.2.4 Bioconversion in continuous UF-membrane bioreactor

Amicon stirred cell (Mod 8050, Grace, USA) equipped with a fluoro-polymer membrane (DDS, Denmark) (NMWCO, 20 kDa) were used in continuous runs as fully described elsewhere (Cantarella et al., 2008).

3. Results and Discussion

3.1 Effect of temperature on NHase-AMase activities

The effect of temperature was tested on the NHase/AMase system in a UF-membrane reactor. The NHase activity was characterized feeding the reactor with 3-cyanopyridine, 10 mM as substrate; the operational conditions are those detailed in caption of Figure 2 that displays the results.

As part of the nicotinamide produced by the NHase activity is rapidly transformed by the AMase activity, as shown in the reaction scheme illustrated in Figure 1, the nicotinamide concentration, produced by NHase activity, is therefore calculated as the sum of the nicotinamide plus the nicotinic acid concentration evaluated in the sample. At 10°C the activity and stability of NHase are acceptable but the AMase activity at that temperature is almost negligible, the amount of nicotinic acid produced being rather low. Different attempts have been carried out to drive the reaction to completeness. At 30°C, all the nicotinamide produced by NHase is transformed by the successive step, catalyzed by AMase, but NHase activity drops rapidly, arresting the reaction. Higher amount of resting cells in the reactor, apparently mitigates the inactivation process, and

assures higher process time. Such limitations could be to some extent overcome with a series arranged bioreactors. To this end the dependence of AMase activity on temperature was investigated in a previous paper (Cantarella et al., 2006) where UF-membrane reactors were fed with nicotinamide as substrate. AMase displayed its activity in a larger temperature range and resulted quite stable up to 50 °C, its thermal inactivation constant, k_d , being equal to 0.0034 h⁻¹ as a first-order deactivation process holds (Cantarella et al., 2008).

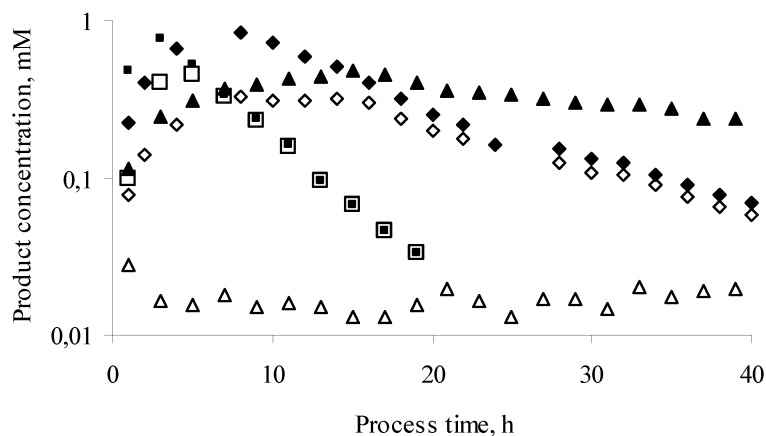


Figure 2: UF-membrane reactor operated at different temperatures, at constant flow-rate, 12,5 ml/h, loaded with 2.5 mg_{DCW} of resting cells and continuously fed with 10 mM 3-cyanopyridine buffered solution. 10°C - Δ , \blacktriangle ; 20°C - \diamond , \blacklozenge ; 30°C \square , \blacksquare . Full symbols nicotinamide produced by NHase activity; empty symbols nicotinic acid.

Several trials to reach higher yield and total conversion of substrate were made and parameters such as substrate and biocatalyst concentration, and residence time were investigated. The most satisfactory result, 80-88% conversion, was attained operating the reactor at 50°C with a mean residence time of roughly 47 h (flow-rate 1.5 ml/h) and a nicotinamide concentration of 100 mM the AMase catalyzed reaction being affected by substrate inhibition at higher concentration.

3.2 Effect of two reactors operating in series

Preliminary results of two reactors working in series are illustrated in Figure 3. The first reactor, containing 20 mg_{DCW} of resting cells, was kept at 50°C and fed at constant flow-rate of 6.5 ml/h with 100 mM nicotinamide buffered solution. The effluent was collected in a fraction collector and, once analyzed for product determination, all the fractions were pooled together and continuously fed to the second reactor, containing the same cell concentration as the first one. Nicotinic acid concentration in the effluent of the first run was about 40 mM, while after the residence in the second reactor the concentration was roughly 68-70 mM. The reaction conversion in the first and in the second reactor being 40% and 47-50%, respectively, the overall conversion reached about 70%. These first indications appear rather encouraging and a more in-depth investigation is in

progress aiming to define the optimal temperature and flow rate conditions for each reactor.

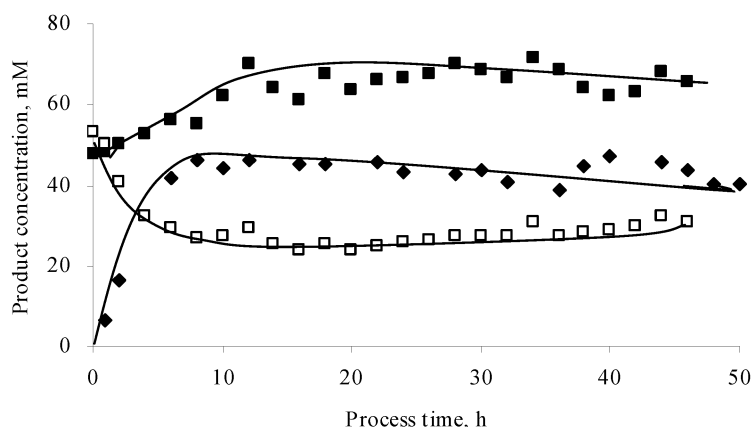


Figure 3: Two reactors operating in series at 50°C the first one was fed (flow-rate 6.5 mL/h) with buffered solution with 100 mM Nicotinamide. Once analyzed, the fractions were pooled together to feed (flow-rate 5.5 mL/h) the second reactor. ◆ - nicotinic acid in the effluent of the first reactor, ■ - nicotinic acid and □ - nicotinamide in the effluent of the second reactor .

4. Conclusions

Microbacterium imperiale CBS 498-74 resting cells were used as catalyst for 3-cyanopyridine biotransformation in a continuous dead-end UF-membrane bioreactor. A two-step cascade reaction occurs: nicotinamide, produced as intermediate in the NHase catalyzed reaction, is further transformed by AMase activity. The investigation in a continuous UF-bioreactor enlightened the difficulties to drive reaction to completeness also because of the thermal stability of the enzymes. Preliminary results in a series arranged UF-membrane reactors, indicate the possibility to increase reaction conversion.

Acknowledgments

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