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Acetonitrile Biotransformation into Less Toxic Compounds by a Bioprocess Based on the Nitrile Hydratase/Amidase Sequential Enzymatic Reactions

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The nitrile hydratase/amidase *in-situ* sequential enzymes of *Microbacterium imperiale* CBS 498-74 were used, as biocatalysts, for the biotransformation of acetonitrile streams into less toxic compounds: the corresponding acetamide and acetic acid.
This preliminary study aimed to investigate the possible use of resting cells of this strain for pollutant removal.
The kinetic parameters, K_M and V_{max}, evaluated in batch reactor, at 20 °C, and in 50 mM sodium phosphate buffer, at pH 5.0 and 7.0, were at pH 5.0, 60.4 mM (or at pH 7.0, 74.96 mM) and 0.44 μmol min^{-1} mg_{DCW}^{-1} (or at pH 7.0, 0.46 μmol min^{-1} mg_{DCW}^{-1}), respectively. The reactions were unaffected in the whole range of pH, 3.5 till 8.5.
Acetonitrile biotransformation, up to 300 mM, into the corresponding acetamide and acetic acid was driven to completeness, at steady state, in Continuous Stirred UF-Membrane Reactor (CSMR). The importance of stirring was also assessed

Introduction

Both industries and laboratories make a large use of acetonitrile, as solvent, in the extraction of products or in the manufacturing of cosmetics, pharmaceutical and agricultural products, in the preparation of plastic materials and of lithium batteries, or as a mobile phase in HPLC analyses (Ahangar et al., 2014). Thus, generating huge amount of acetonitrile waste streams, classified as toxic compound, that if released in the environment and not correctly disposed, represent a severe risk for the human health (Feng and Lee, 2009). Acetonitrile can generate hydrogen cyanide and acetaldehyde in living cells.
So far, incineration has been largely used to reduce the amount of acetonitrile wastes, and detoxification has been also suggested (Gilomen et al., 1995).
Recently, great attention has been paid to detoxification methods by means of microbial degradation. Numerous papers appeared in the literature, investigating strains such as *Pseudomonas putida* (Nawaz et al., 1989), *Chromobacterium* sp., *Pseudomonas aeruginosa* (Nawaz and Chapatwala, 1991), *Geotrichum sp. JR1* (Rezende et al., 2003), *Rhodococcus sp.* RHA1 (Li et al., 2013), and *Mesorhizibium* sp. F28 (Feng and Lee, 2009). Many of these strains contain the nitrile hydratase (NHase, EC 4.2.1.84)/amidase (AMase, EC 3.5.1.4) enzyme system.
Two microbial pathways are able to degrade nitrile compounds. Nitrilases catalyse the hydrolysis of nitriles to the corresponding acids and ammonia, in one step reaction. Whereas the two-step reactions involve NHase, catalysing the hydration of nitriles to the corresponding amides, followed by AMase, hydrolysing the amides to the corresponding acids and ammonia (Martínková and Křen, 2010).
This study focuses on the use of *Microbacterium imperiale* CBS 498-74 resting cells as a potential candidate for acetonitrile waste stream biodisposal, following the reaction depicted in Figure 1. This strain expresses

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constitutively the two-step pathway (catalysed by NHase and AMase) that has been successfully adopted in previous studies (Cantarella et al., 2011, 2013; Pasquarelli et al., 2015) for the hydration of a large number of diverse nitriles to their corresponding acids and ammonia, *via* an amide as an intermediate. This paper investigates in a batch reactor configuration the enzyme kinetics, and in a Continuous Stirred UF-Membrane Reactor (CSMR) the pH dependence, the effect of stirring and the biodegradation of acetonitrile up to 2 M.

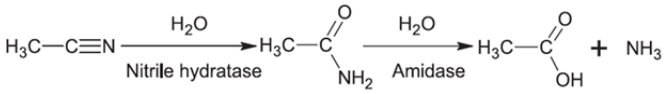

Figure 1: Acetonitrile biodegradation via a two-step catalysed pathway

Materials and methods

1.1 Microorganism

Microbacterium imperiale CBS 498-74 was maintained and cultured, as described previously (Cantarella et al., 2002), using reagents of the highest purity available and purchased from Oxoid (England).
All cultures were performed in duplicate and the relative standard deviations were < 5 %. A resting cell preparation (1.0 unit of OD_{610}) contained *approx*. 0.26 mg_{DCW} mL^{-1}, 0.76 U_{NHase} mg_{DCW}^{-1} and 0.09 U_{AMase} mg_{DCW}^{-1} (DCW means Dry Cell Weight).

1.2 Product analysis

Samples of reaction products (filtered and diluted) were analysed on an HPLC Waters Alliance 2695 separation module (Waters Corp., Milford, MA, USA) equipped with a UV detector (Waters Corp.) and using a Waters Spherisorb column, 5 □m ODS2, 4.0x250 mm, maintained at 35 °C. The flow rate of mobile phase was 1.0 mL min^{-1}, and the injection volume of samples was 20 µL. The mobile phase was an aqueous solution of 0.1 % H_3PO_4. Data analysis was performed using Software Empower Waters. The substrate and products were identified by their characteristic retention time and quantified by integrating peak areas and comparing them to calibration curves, got with external standards. All samples were analysed at least in two replicates.

1.3 Activity assay

One unit of U_{NHase} (or U_{AMase}) activity was defined as the amount of enzyme that transforms 1 µmol of acrylonitrile (or acrylamide) per min at 20 °C under stirring (250 rpm) with a proper amount of whole cells (5 or 10 □g_{DCW}) re-suspended in 2 mL of Na-phosphate buffer, 50 mM, pH 7.0, with acrylonitrile (or acrylamide) (100 mM), respectively. After 15 min incubation, the reaction was quenched by addition of HCl (1 mL, 0.5 M) and centrifuged (10 min at 10,000 rpm). The products were spectrophotometrically determined at 235 nm.

1.4 Long-term runs in CSMR

Continuous long-term experiments were performed in an Amicon stirred cell module (Model 8010, Cat. No. 5121; Amicon, USA) fitted with a 20,000 MWCO UF-membrane GR61PP (DDS Danish separation system, DK), used only once to exclude interferences linked to membrane fouling. The reactor, magnetically stirred (250 rpm) for minimizing the concentration polarization phenomenon, was filled with the appropriate amount of resting cells. A BIORAD ECONO pump (USA), set at the suitable flow-rate, fed the substrate solution to the module, fully immersed in a water bath. The temperature was controlled (± 0.1 °C) by means of a thermostat. A fraction collector, kept in cool room, collected the reactor permeates, which was HPLC analysed.

Results and discussion

1.1The effect of pH on the reaction kinetics

The products of acetonitrile biodegradation, being acetic acid and ammonia, can affect the medium pH and thus the kinetics. The reaction kinetics was explored at pH 5.0 and 7.0, using the substrate concentration range within 25-500 mM. The experiments were performed in a thermomixer that assured an orbital agitation (750 rpm). The reaction, carried out at 20 °C, in a reaction medium buffered with 50 mM Na-phosphate, at the two pHs, and with a cell loading at 5 U_{NHase} mL^{-1}, was quenched with HCl 0.5 M, within 20 minutes. The plot of initial reaction rate *versus* substrate concentration obeys the Michaelis-Menten kinetics, as illustrated in Figure 2A that shows the kinetic behavior at pH 5.0 and 7.0**.** Within 500 mM acetonitrile concentration, a moderate substrate inhibition takes place. Interestingly both pH-curves of reaction rate *vs.* acetonitrile overlap, indicating that the intracellular pH could be not affected at least in short-term experiments.

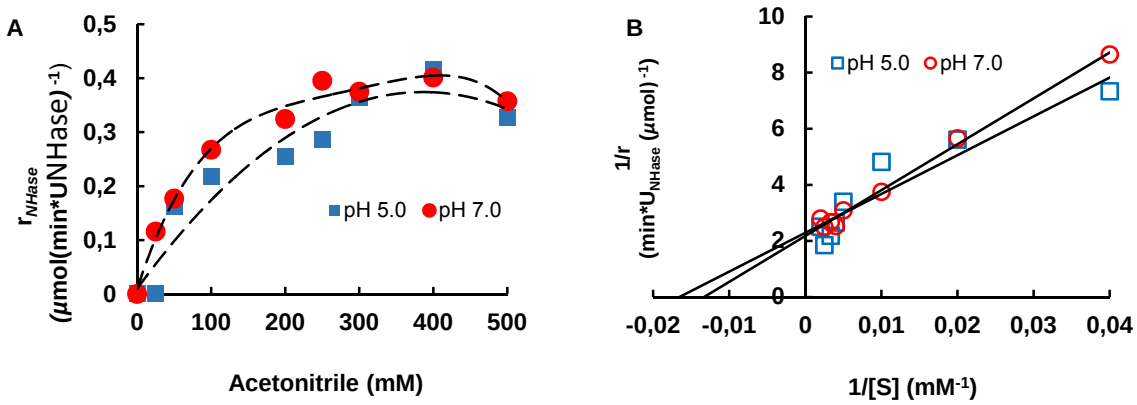

Figure 2: A) Reaction rate versus acetonitrile concentration at pH 5.0 and 7.0. B) Lineweaver Burk plot. The tests were performed in batch type reactor at 20 °C and 750 rpm (orbital shaking). The resting cell concentration was 5 U_{NHase} mL^{-1}. Standard deviations of the product evaluation were always < 5 %.

The apparent kinetic parameters, obtained from both Lineweaver-Burk (shown in Figure 2B) and Hanes-Wolf plots (not shown), are reported in Table 1. Apparently, both the maximal reaction rate (V_{max}) and the affinity for the substrate (K_M) are not affected by the medium pH, being the numerical values similar.

Table 1: Apparent kinetic parameters obtained at pH 5.0 and 7.0

	Hanes – Woolf			Lineweaver-Burk		
	K_M	V_{max}	R^2	K_M	V_{max}	R^2
pH 5.0	79.87	0.45	0.818	60.40	0.44	0.885
pH 7.0	83.86	0.49	0.991	74.96	0.46	0.992

The resting cells exhibited a similar kinetic behavior in the 3.5-8.5 pH interval, obtained using 50 mM Na-citric and Na-phosphate buffers (data not shown). Moreover, the potential of these enzymes in acetonitrile biodegradation remained similar, replacing the buffer solution by distilled water (data not shown).

1.2 The effect of speed stirring in CSMR runs

The CSMR runs were performed under stirring conditions to ensure good mixing and good solubility of the substrate feeding in the reactor and to improve the homogeneous reaction mass transfer between resting cells and substrate. Different stirring conditions were explored to verify if membrane fouling, due to protein released from damaged cells or cell deposition on the membrane surface, occurred. As known membrane fooling causes severe flux decline and may require intense chemical cleaning or membrane replacement.

In Figure 3 three long-term runs performed in CSMR, at different stirring speed, are compared, and product concentration are reported *vs.* process time. A feed stream of 500 mM acetonitrile, buffered by Na-phosphate buffer (50 mM and pH 7.0), continuously fed, at 12.0 ± 0.7 mL h^{-1}, the CSMR operated at 10 °C with a cell load of 40 U_{NHase}. Figure 4 shows how the three runs reached the same reaction rates within the first 50 h. Then the reaction rate of the CSMR agitated at lower speed rate (100 rpm) drop slowly, indicating that the concentration polarization phenomenon most likely occurred. The slope calculated from 55 h onwards, from the data in the semi-log plot, gave an estimate of the apparent inactivation that took place.

Table 2: NHase inactivation induced by stirring speed at 10 °C and pH 7.0 in CSMR runs

Agitation rpm	U_{NHase}	*Acetonitrile mM*	r_o (µmol (min*U_{NHase})$^{-1}$	k_d (25-50h) (h^{-1})	k_d (50-100h) (h^{-1})
100	40	500	0.20	0.3 (R^2=0.96)	0.5 ((R^2=0.92)
250	40	500	0.20	0.3 (R^2=0.93)	0.3 (R^2=0.93)
350	40	500	0.24	0.3 (R^2=0.93)	0.3 (R^2=0.93)

Table 2 reports the r_0-and k_d-values, calculated with the following Eq(1), together with the R^2 (linear regression coefficient).

$$ln\, r_t = ln\, r_0 + k_d t \qquad (1)$$

The concentration polarization phenomenon that occurs, at 100 rpm, reduces the activity by an order of magnitude.

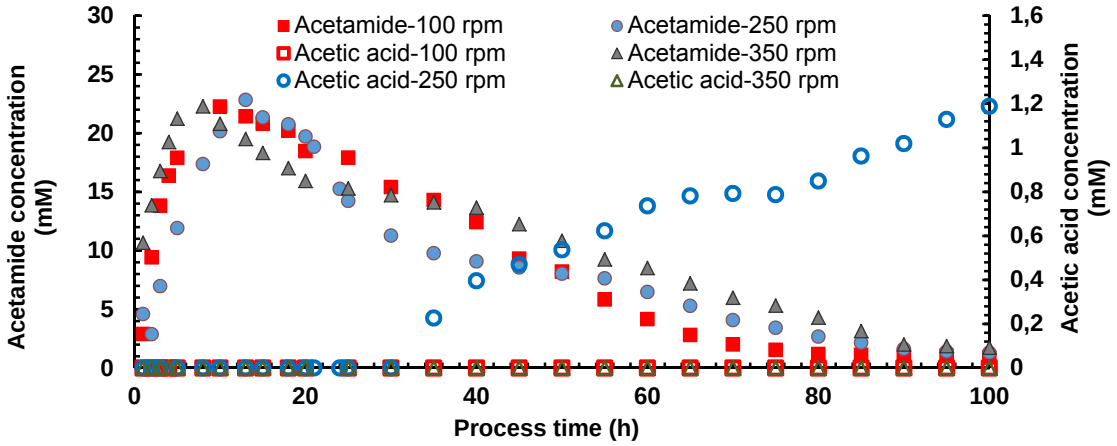

Figure 3: Time course of product concentration in the long-term experiments performed in CSMR, and operated at different stirring speed and at 10 °C, with 40 U_{NHase}. Substrate feed 0.5 M in Na-phosphate buffer (50 mM and pH 7.0). Standard deviations of the product evaluation were always < 5 %.

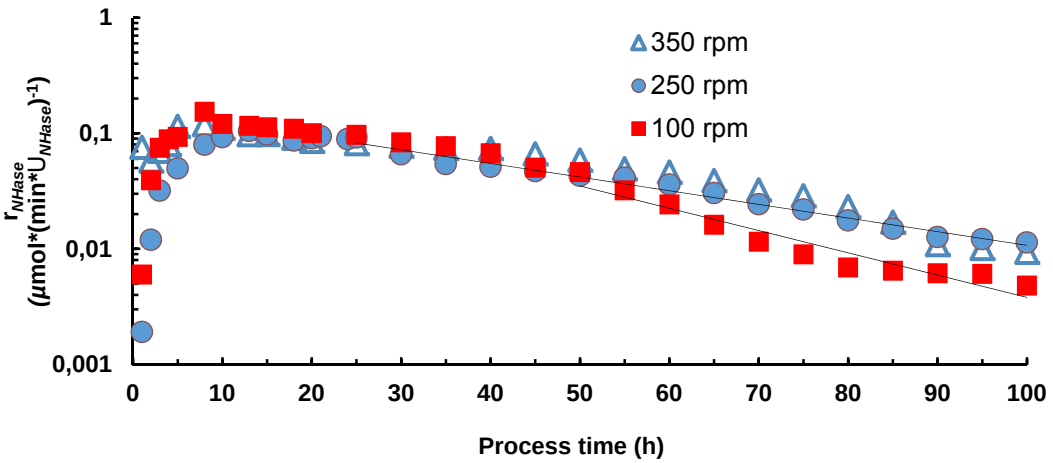

Figure 4: Time course of NHase reaction rate in the long-term experiments performed in CSMR, and operated at different stirring speed and at 10 °C, with 2,880 U_{NHase}. Substrate feed 0.5 M in Na-phosphate buffer (50 mM and pH 7.0). Standard deviations of the product evaluation were always < 5 %.

Thus the experimental investigation in CSMR was performed at 250 rpm, which appeared to be a good compromise for minimizing, at the same time, enzyme inactivation and membrane fouling.

1.3 Effect of acetonitrile concentration in long term experiments

As the concentration of acetonitrile in real waste stream is relatively high, the explored acetonitrile concentrations in long term experiments have been 0.3 and 2 M. Figure 5 reports CSMR runs, performed at 15 °C, 250 rpm, in Na-phosphate buffer (50 mM and pH 7.0.). The flow rate applied was 4 ± 0.5 mL h^{-1}, thus assuring 18 h as mean residence time. The possibility to completely convert the substrate in less toxic products was studied charging a huge amount of resting cells (4,000 U_{NHase}) into the bioreactor in order to operate the reactor as an integral one.

As shown 0.3 M acetonitrile was completely transformed for process time up to 100 h and the molar ratio of acetamide/acetic acid was 2/1. On the other hand, the increase of acetonitrile concentration, at 2 M, inactivated or inhibited rapidly the NHase activity and a steady state was reached after 40 h process time with 500 mM formed acetamide. The molar ratio of acetamide/acetic acid was roughly 5/1. Interestingly, at low

acetonitrile concentration the operational conditions adopted are ideal for the total conversion of acetonitrile into acetamide.
So far these conditions are ineffective for the complete transformation of 2 M acetonitrile concentration.
Interestingly, the concentration of the acetic acid formed reaches the steady state after 40 h of process time and remained unchanged, with a similar concentration in the two runs, as long as the run lasted. This indicates that amidase, the second enzyme of the sequential reaction is working at its maximal velocity in both cases. These preliminary results are encouraging as in other bioprocesses catalysed by the same strain the amidase activity was proved to be stable at higher temperature, driving the reaction to completeness (Cantarella et al., 2011).

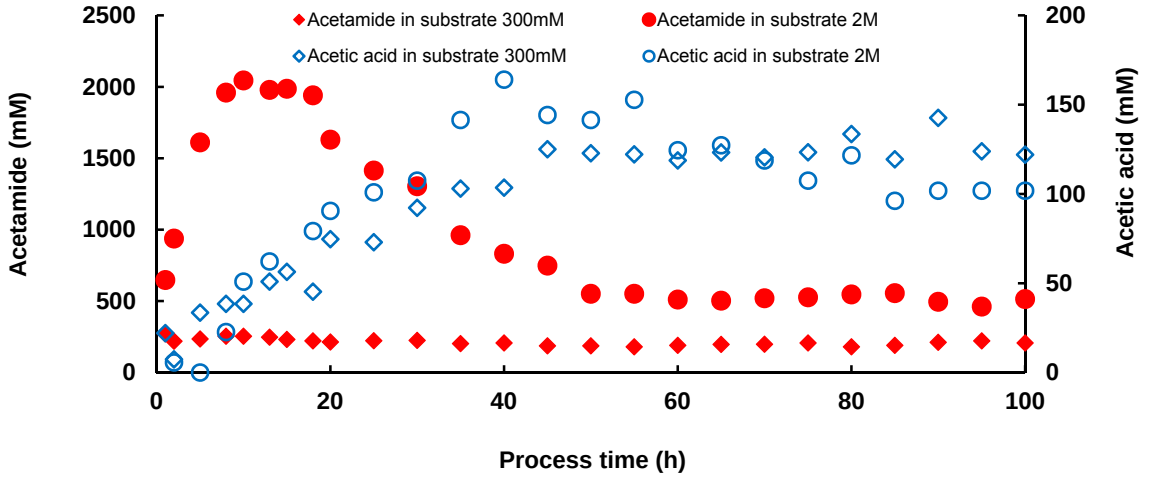

Figure 5: Time course of product concentration in long-term experiments performed in CSMR, and operated at 15 °C, 250 rpm, 4,000 U_{NHase}. Substrate feed 0.5 M and 2 M in Na-phosphate buffer (50 mM and pH 7.0). Left-hand axis acetamide produced; right-hand axis acetic acid concentration. Standard deviations of the product evaluation were always < 5 %.

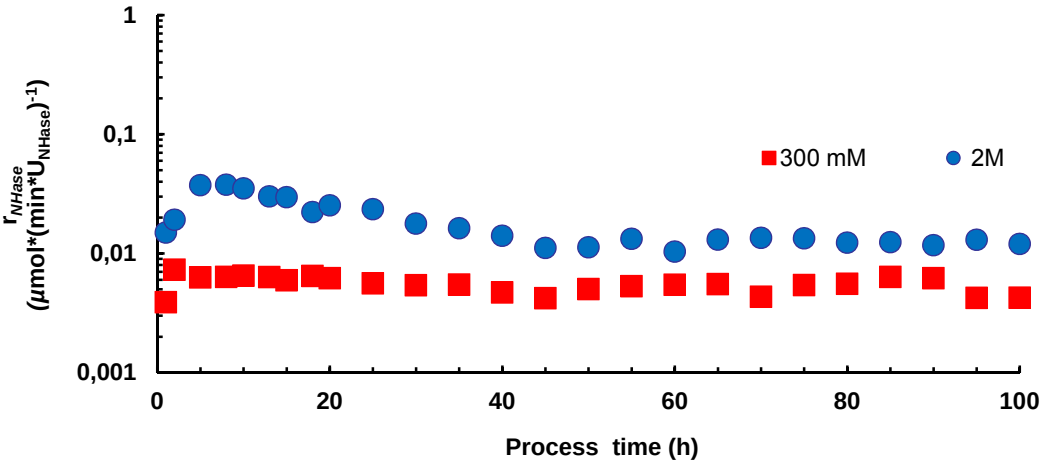

Figure 6: Time course of NHase reaction rate in the long-term experiments performed in CSMR, and operated at 15 °C, 250 rpm, 4,000 U_{NHase}. Substrate feed 0.5 M and 2 M in Na-phosphate buffer (50 mM and pH 7.0). Standard deviations of the product evaluation were always < 5 %.

Figure 6 compares the two NHase reaction rates reached by the reactors during acetonitrile biotransformation. When fed with 0.3 M the reactor operated at constant NHase activity (roughly 0.005 µmole (min U_{NHase})$^{-1}$). On the contrary, the reactor fed with 2 M started at a higher reaction rate that decreased with time and attained at steady state roughly (0.012 µmole (min U_{NHase})$^{-1}$) with a 66 % activity loss. Most likely the enzyme could be either inactivated or inhibited either by the higher substrate concentration or by the product formed.

Conclusions

The *in-situ* sequential enzymes, nitrile hydratase and amidase, of *Microbacterium imperiale* CBS 498-74, are able to convert acetonitrile into acetic acid *via* acetamide as intermediate. The independence of reaction rate from pH medium, suggests the possibility to operate the process in a non-buffered media. Agitation speed appears crucial to control both membrane fouling and enzyme inactivation. At the adopted conditions, this strain efficiently converted 300 mM acetonitrile in a continuous stirred UF-membrane bioreactor, while, the 2 M substrate reached only 32 % conversion. These results encourage a more in depth investigation oriented to ascertain the possibility to drive the biodegradation of high acetonitrile concentration to completeness (100 % transformation). This could be reached by increasing the catalyst loading or by adopting, in a real plant, a correct policy of catalyst addition. The series-arranged reactors, differently operated, which was proved to be a successful strategy in other nitrile bioconversion (Cantarella et al., 2011) could be another possibility.

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