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SPECIAL TOPIC: BIODETERIORATION

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Nitrile bioconversion by *Microbacterium imperiale* CBS 498-74 resting cells in batch and ultrafiltration membrane bioreactors

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Abstract The biohydration of acrylonitrile, propionitrile and benzonitrile catalysed by the NHase activity contained in resting cells of *Microbacterium imperiale* CBS 498-74 was operated at 5, 10 and 20°C in laboratory-scale batch and membrane bioreactors. The bioreactions were conducted in buffered medium (50 mM Na_2HPO_4/NaH_2PO_4, pH 7.0) in the presence of distilled water or tap-water, to simulate a possible end-pipe biotreatment process. The integral bioreactor performances were studied with a cell loading (dry cell weight; DCW) varying from 0.1 mg_{DCW} per reactor to 16 mg_{DCW} per reactor, in order to realize near 100% bioconversion of acrylonitrile, propionitrile and benzonitrile without consistent loss of NHase activity.

Keywords Bioconversion · Nitrile hydratase · Acrylonitrile · Propionitrile · Benzonitrile · Ultrafiltration membrane bioreactors

Introduction

Nitriles are organo-cyanide (R-CN) compounds which appear in the environment via either natural or industrial syntheses. Most of them are not biodegradable and are generally highly toxic, as they release cyanide. Some are mutagenic, carcinogenic and may persist in the soil or surface water for a long time. Their indiscriminate use as an agricultural herbicide and their uncontrolled release in the wastewater of chemical industries leads to their increasing distribution throughout the environment.

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Great concern about their ecological impact and treatment is raised nowadays, because industrial nitrile wastes are dilute solutions which are either disposed at sea or pumped into deep pressure wells below the water table [18]. Efficient processes are becoming urgent either for their preparation or for their disposal and degradation once released into the soil or in industrial waste waters [12, 19].

The use of conventional waste treatment processes for the biodegradation of difficult wastes presents several limits. Micro-organisms do not metabolise toxic organic chemicals when easily degradable substances are present [22]. The biodegradation of hydrophobic compounds may be restricted by their low solubility coupled with strong binding/sorption onto solids [17]. Weeks or months can be required to reach significant degradation; and the operation cost ultimately increases [10]. The effectiveness of a treatment plant may also depend on the ability of the biomass to respond to changes in the composition of the effluent stream [11]. Organic chemicals also determine anti-microbial effects and conventional degradation methods may require large-capacity plants and become time-consuming [16]. Hence, for many years difficult wastes were treated with physical and chemical processes, even though both might be impractical mainly because of the high operational costs, due to additional treatments or the release of hazardous by-products [9].

Biodegradation of difficult wastes calls for important system innovation, such as end-of-pipe technology, to bring the bioprocess to full-scale operation. The use of specialised microbial cultures or monocultures/enzymes was suggested to overcome the preferential metabolisation of easily degradable substances that usually occurs. The mild reaction conditions, the absence of additional toxic materials and the reduction of emissions and wastes make bioprocessing advantageous. Effluent streams containing toxic organic chemicals might be treated directly at their source in small-scale reactors, achieving a better reactor control and assuring higher efficiencies than in conventional sewage treatment

reactors. This technology has already been studied for phenol degradation by *Pseudomonas cepacia* G4 [22], biodegradation of quinoline by an immobilised culture of *Comamonas acidovorans* [23] and phenol bioconversion catalysed by a thermostable beta-tyrosinase [16].

Nitrile biodegradation is quite common among micro-organisms and a wide variety of plants and bacteria and some fungi possess nitrile-hydrolysing enzymes. Three different groups of enzymes are involved in the degradation. Nitrilases (EC 3.5.5.1, 3.5.5.7) catalyse the direct hydrolysis of nitriles to the corresponding carboxylic acids, forming ammonia. Nitrile hydratases (NHases, EC 4.2.1.84) and amidases (EC 3.5.1.4) sequentially hydrolyse nitriles to their acids, in a two-step reaction with the amide as intermediate product.

It is worth mentioning that NHases are useful biocatalysts currently applied in the industrial production of acrylamide [27], nicotinamide and interesting intermediate compounds [14]. The literature reports on the optimisation of amide production but rather neglects the disposal of nitrile wastes, even though it is known that acrylamide can be converted further into acrylic acid by amidase. This latter is in turn degraded to L-lactate through several reaction steps catalysed by constitutive enzymes of micro-organisms within conventional treatment plants and enters the intermediary metabolism. Accordingly, the biotechnological relevance of NHases for bioremediation and their ability to transform highly toxic nitriles into more-friendly compounds for the environment is beyond argument [13, 21]. NHase activities have been identified in bacterial sources such as *Arthrobacter*, *Bacillus*, *Brevibacterium*, *Comamonas*, *Corynebacterium*, *Nocardia*, *Rhodococcus*, *Pseudonocardia* and *Pseudomonas* [12, 15, 20, 24]. These micro-organisms can be used in environmental bioremediation and for end-of-pipe conversion of nitrile wastes to amides [3, 25, 26]; and some of them have been used in the treatment of organocyanide-polluted industrial effluents [4].

In the present work, use was made of resting cells of *Microbacterium imperiale* CBS 498-74, a strain that allows the bioconversion of nitrile via a two-step degradation pathway, catalysed by NHase and amidase. The proper choice of operational conditions in acrylonitrile bioconversion makes the amidase activity negligible as compared with NHase activity and near 100% conversion yields into amide are possible [2, 5–8].

This study investigates the possibility to adopt an end-pipe biotreatment process that would reduce the environmental impact of nitrile waste streams. To this end, simulated streams of acrylonitrile, propionitrile and benzonitrile were biodegraded in continuous and batch laboratory-scale bioreactors. High conversion yields were obtained in appropriately buffered media and in the presence of distilled or tap-water.

Materials and methods

NHase and amidase reactions

M. imperiale CBS 498-74 is able to use a NHase/amidase system to catalyse the reactions indicated in Fig. 1.

Chemicals

Substrates and products were obtained from Sigma–Aldrich (Munich, Germany) and used without further purification. Reagent grade, commercially available chemicals were used in all experiments.

Shake-flask cultures of *M. imperiale* CBS 498-74

M. imperiale CBS 498-74 was grown at the optimum initial glucose concentration for NHase production (5 g L^{-1}) in Erlenmeyer flasks (500 mL) at 28°C for 24 h of incubation. All shake-flask cultures were carried out in a rotary shaker (G25-KC; New Brunswick Scientific, Edison, N.J.) with orbital shaking (220 rpm), using 100 mL of culture medium whose composition was (per litre): 3 g yeast extract, 3 g malt extract and 5 g bacteriological peptone (all from Oxoid, Basingstoke, UK). The medium was prepared in 50 mM Na_2HPO_4/NaH_2PO_4 buffer, pH 7.0, sterilized by autoclaving at 121°C for 20 min and then sterile glucose was added [8].

The organism was routinely maintained on nutrient agar plates using the above-described medium in 2% agar. Sub-culturing was performed monthly and plates were stored at 4°C. A single colony from nutrient agar plate stock cultures was used to inoculate aseptically 100-mL volumes of culture medium (without glucose). The growth of inocula was carried out at 28°C with orbital shaking (220 rpm) for 24 h; and subsequently 10-mL volumes (1.1 mg biomass mL^{-1}, calculated as dry cell weight; DCW) were used to inoculate shake-flask cultures.

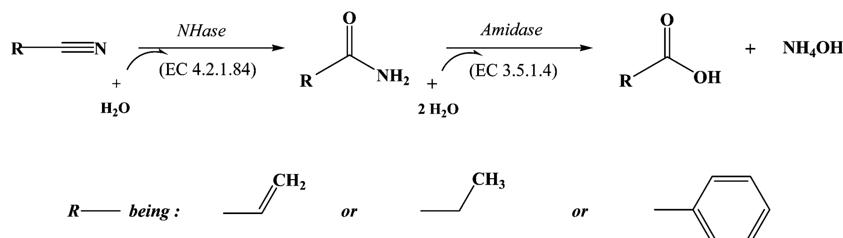

Fig. 1 NHase- and amidase-catalysed reactions investigated in this study

Cell harvesting

The bacteria were harvested from shake-flask cultures at the mid-exponential phase of growth (3–3.5 mg_{DCW} mL^{-1}) by centrifugation (11,400 rpm, 15 min, 4°C). The bacteria were washed three times with 50 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 7.0) and re-suspended in the same buffer. This cell suspension was either used directly (resting cells) for the biocatalytic transformations or stored at 18 °C until used.

Dry weight determination

The dry weight estimation was carried out with a resting cell suspension previously washed three times with 100 mL of distilled water per wash to eliminate the spent growth medium. The suspension was then dried (24 h, 80°C) until a constant weight was achieved.

Growth measurement

Growth of *M. imperiale* was monitored by taking pairs of aliquots at regular time intervals and measuring the optical density at 610 nm (OD_{610}, Lambda 2 spectrophotometer; Perkin–Elmer, Boston, Mass.). For the measurements, the cell suspensions were diluted with distilled water in order to keep the OD_{610} below 1.0. A mean value was calculated from the two aliquot measurements. The $OD_{610}$of 0 was set using water. An OD_{610} of 1.0 corresponded to 0.26 mg bacterial DCW mL^{-1} culture.

Enzyme assay

NHase activity was measured using resting cell suspension in 2-mL assays containing 25–50 μL of a suspension of 3 mg_{DCW} mL^{-1}, 50 mM acrylonitrile and Na_2HPO_4/NaH_2PO_4 buffer (50 mM, pH 7.0), continuously stirred with a magnetic stirring bar at 250 rpm . The mixture was incubated at 20°C for 15 min and the reaction stopped by adding 1 mL of 0.5 M HCl and centrifuging for 10 min at 11,400 rpm. The supernatant was used for the determination of product and residual substrate. One unit of NHase activity was defined as the amount of resting cells that catalysed the formation of 1 μmol min^{-1} acrylamide under the adopted conditions. Specific activities in the cell were found to be roughly 18–20 units mg_{DCW}^{-1} . All runs were replicated at least twice and averaged values are reported.

Hydration of substrates in batch bioreactor

Nitriles were added to phosphate-buffered (50 mM, pH 7.0) reaction media, or the described media, containing resting cell suspension. Poorly soluble substrates were added from a stock solution in methanol (5%) to give a final concentration of 10 mM in the suspension medium. Biotransformations were carried out at 10°C (unless otherwise stated) in reactor vessel of 100 mL volume. Stirring was assured by a magnetic bar and the temperature was maintained by circulating water through the jacket from the constant-temperature water bath. Samples (1 mL) of the reaction mixture were removed at intervals; and the sample reaction was halted by the addition of 0.5 M HCl and centrifugation at 11,400 *g* for 5 min. The supernatant was removed (neutralised) and an appropriate dilution assayed for product determination. The rate of hydrolysis was expressed as micromoles of product min^{-1} mg_{DCW}^{-1}. All experiments were carried out at least in duplicate and the mean activity calculated.

Ultrafiltration membrane bioreactor

An ultrafiltration (UF) kit (Amicon 52; Amicon–Grace Co., Danvers, Mass.) of the stirred type was employed as a membrane bioreactor. A fluoro-polymer membrane (FS81PP; Dow Liquid Separations, Edegem, Belgium) with a molecular weight cut-off of 10,000 was used. Stirring, by means of a magnetic stirrer set at 250 rpm, was provided in all the runs to limit cell deposition onto the membrane due to concentration polarisation. The buffered substrate solutions were fed to the reactor using a peristaltic pump (Gilson Minipuls, Villiers-le-Bel, France), assuring a constant flow rate of 12.0 ± 0.7 mL h^{-1}. The filtrates were collected using a fraction collector (LKB RediRac; Pharmacia, Golden Valley, Minn.) and diluted aliquots were analysed for product determination. The reaction temperature was controlled within ± 0.1°C. In bioreactor experiments, membranes totally retained the resting cells and no fouling was detected, under stirred conditions, within the explored cell concentrations. The membrane resistance to chemicals was fair and no rejection of solutes was determined.

High performance liquid chromatography

Quantitative analysis of reference compounds and products was carried out using a Perkin–Elmer series 2 high performance liquid chromatography system (Perkin–Elmer, Boston, Mass.) equipped with UV detector and a Merck LiChroCART 250-4 with LiChrospher 100 reverse phase 18 (5 micron; Merck & Co., New Jersey, N.J.), fitted with a Merck LiChroCART 4-4 guard column. The auto-sampler was a LS 3200 SGE auto-sampler (LC, Ringwood, Australia). The operating temperature and flow rate were 30°C and 0.8 mL min^{-1}, respectively. The mobile phase (acetonitrile with KH_2PO_4/H_3PO_4 buffer, 10 mM, pH 2.8; volumetric ratio of 1:10 for acrylonitrile determination, ratio of 1:2 for benzonitrile determination) was degassed by helium for 15 min. Injections of 20 μl of the supernatant appropriately diluted were used. The concentrations of

acrylonitrile, benzonitrile and their amides were detected at 220 nm and 230 nm, respectively. Since the nitrile and amide retention times were quite different, these absorbance measurements allowed their quantitative determination. In rapid analysis, the acrylamide concentration was also measured spectrophotometrically at 235 nm (ϵ_{235} = 1,106 cm^{-1} M^{-1}), a wavelength at which neither the acrylonitrile nor the buffer interfered with the readings.

Gas chromatography

Propionamide was identified by its retention time in a Hewlett Packard 5890 series II gas chromatograph, equipped with a HP 3396 series II integrator and a RT-QPLOT-restek Cat 19716 capillary column (30 mm long, 0.53 mm i.d.; Hewlett Packard, Ramsey, Minn.). A base-deactivated Restek 10002 guard column (5 m long, 0.53 mm i.d.) was also used. Helium carrier gas flow rate was 1.6 mL min^{-1}. Injection volume was 1 μl, in splitless mode. The column, injector and flame ionization detector were held at 220°C.

Results and discussion

Biodegradation of acrylonitrile

In our previous investigations on acrylamide production using resting cells from the same strain, much attention was paid to the determination, in buffered medium, of kinetic parameters and operational conditions such as temperature, substrate and biocatalyst concentration, in order to reach a high acrylonitrile conversion [2, 5–8]. The body of the experimental results achieved suggested the opportunity to investigate the process for environmental applications as well.

To this end in a first series of experiments, a dilute stream of acrylonitrile was biodegraded in a continuously operating stirred UF-membrane reactor. This reactor proved to be powerful at the laboratory scale for monitoring the activity and stability of a biocatalyst in systems operating at well controlled substrate and product concentrations [1]. Furthermore, this reactor can operate at both differential and integral conditions, depending on the dilution rate and the biocatalyst amount. Since this reactor configuration is suitable for continuous industrial processes, the derived operational parameters can be valuable to predict full-scale bioreactor performances.

The biocatalyst (as resting cells) concentration-effect on the conversion yield was investigated and time-course experiments are shown in Fig. 2. The system was stirred to prevent cell deposition on the membrane and was fed with 100 mM acrylonitrile in 50 mM Na_2HPO_4/NaH_2PO_4 buffer, pH 7.0 (for details, see Materials and methods). The bioreactor was operated at 10°C, a temperature which is optimal for acrylamide industrial production and represents a good compromise between a sufficiently high reaction rate and an acceptable thermal inactivation of NHase. In the presence of small amounts of resting cells (0.5 mg_{DCW}, 1.3 mg_{DCW} per reactor), the bioreactor is unable to operate as an integral one and, at stationary conditions, barely 15% of acrylonitrile is converted into the less toxic acrylamide. A higher cell concentration (16 mg_{DCW} per reactor) allows the bioreactor to convert 85% of the substrate, leaving 0.8 g l^{-1} acrylonitrile in the effluent stream.

An end-pipe treatment of industrial nitrile streams would be of great potential interest if bioconversion into less toxic amides or carboxylic acids could be performed in non-buffered media. Following this reasoning, acrylonitrile bioconversion was studied in a second series of runs performed in batch reactors in either distilled or tap-water. The reaction mixture contained 10 mg_{DCW} of cells and a lower concentration of acrylonitrile, since the economic balance of the main process calls for a high substrate conversion, which implies a very low concentration in the waste streams. Interestingly, in both media bioconversion reaches 100%, within roughly 30 min (data not shown). A longer contact period in distilled water led to acrylonitrile increase in the stream, but this could be due to the fact that residual acrylonitrile was calculated from a mass-balance between the amount of acrylonitrile introduced into the reactor and that transformed into acrylamide. Most likely the amidase activity, which is inhibited by high acrylonitrile concentration, was activated and part of amide converted into acid. This reaction was also conducted at 10, 15 and 20°C (data not shown), and 20–30 min were necessary to accomplish the total conversion of acrylonitrile in the presence of distilled water. Apparently 10°C and 15°C could be chosen too for performing the bioreaction, although the reaction rate would be lower.

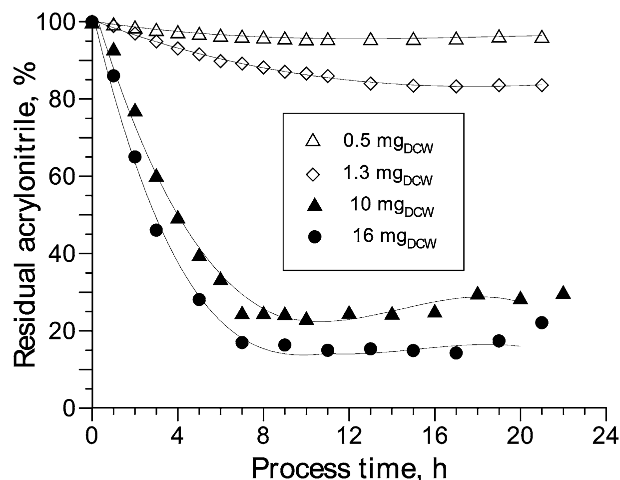

Fig. 2 Time-course experiments of continuous acrylonitrile conversion in UF-membrane bioreactors as a function of resting cell concentration. Reactor feed: 100 mM acrylonitrile in 50 mM Na-phosphate buffer, pH 7.0. Operating temperature 10°C, magnetic stirring 250 rpm, flow rate 12.0 ± 0.7 mL h^{-1}. Lines are added for ease of following data points

Finally, the bioreaction in distilled water was performed in a continuous UF-membrane reactor operating in differential mode (2 mg_{DCW} of resting cells) at 4°C and 10°C. The time-course is reported in Fig. 3. The exponential equation derived from the best fit of the data (once the system reached a steady state) allowed an estimation of the initial reaction rate at 4°C and 10°C: 1.17 μmol min^{-1} mg_{DCW}^{-1} and 1.72 μmol min^{-1} mg_{DCW}^{-1} respectively. The inactivation constants (k_d) was obtained from the slope of the straight lines. The NHase activity decay rate, when compared with that obtained in a parallel experiment carried out in buffered medium, was roughly the same at 4°C (k_d = 0.034 h^{-1}, half-life 40 h), while at 10°C the half-life in distilled water was slightly higher (k_d = 0.148 h^{-1}, 15 h) than that in buffered medium (k_d = 0.116 h^{-1}, 12 h). In the continuous process, the temperature choice appears more crucial, since the lower temperature increases energy requirements and part of the cost could be balanced by savings in catalyst. The optimum temperature cannot be settled only on the basis of these results, although they do confirm the industrial-level possibility of carrying out the biodegradation of effluent streams in unbuffered media.

Biodegradation of propionitrile

The investigation into the effect of resting cell concentration on the conversion of propionitrile was carried out in the stirred UF-membrane reactor. A feed stream of 200 mM propionitrile was continuously biodegraded, as shown in Fig. 4a. Small amounts of cells (from 0.1 mg_{DCW} to 1.1 mg_{DCW} per reactor) prevented the bioreactor from operating as an integral one and the bioconversion was very low. Total biodegradation was realized with 14.9 mg_{DCW} per reactor and proceeded once a steady state was attained for at least 30 h of continuous performance. However, after 30 h of operation, enzyme inactivation became evident (data not shown) and propionitrile appeared in the effluent stream. The effect of enzyme inactivation is clearly shown in the experiment carried out with 5.7 mg_{DCW} per reactor. In the first 14 h of operation, the residual propionitrile in the effluent stream decreased with time; and the biodegradation reaction rate was higher than the enzyme inactivation rate. From 14 h of operation onwards, the activity decay increased and the residual propionitrile in the effluent increased considerably. Of course, in order to keep the reaction going on a 100% conversion basis, an adequate policy for refreshing the cell population should be pursued [5].

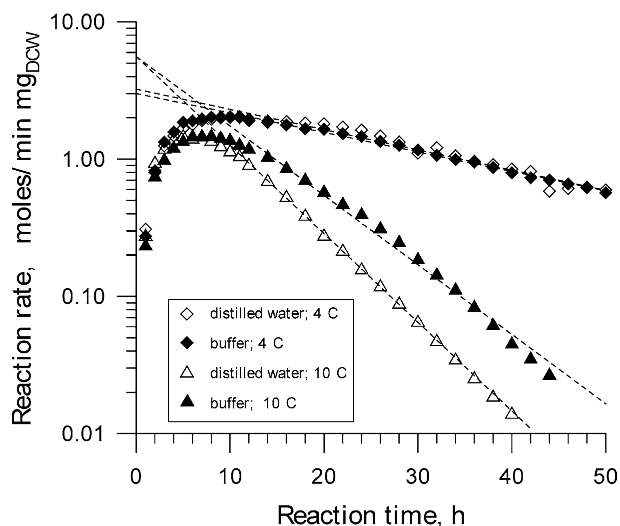

Fig. 3 Time-course of continuous acrylonitrile conversion in UF-membrane bioreactors. Reactor feed: 100 mM acrylonitrile in distilled water or buffered medium (50 mM Na-phosphate buffer, pH 7.0), with 2 mg_{DCW} of resting cells. Operating temperature 4°C or 10°C, magnetic stirring 250 rpm). Flow rate 12.0 ± 0.7 mL h^{-1}. Regression lines added

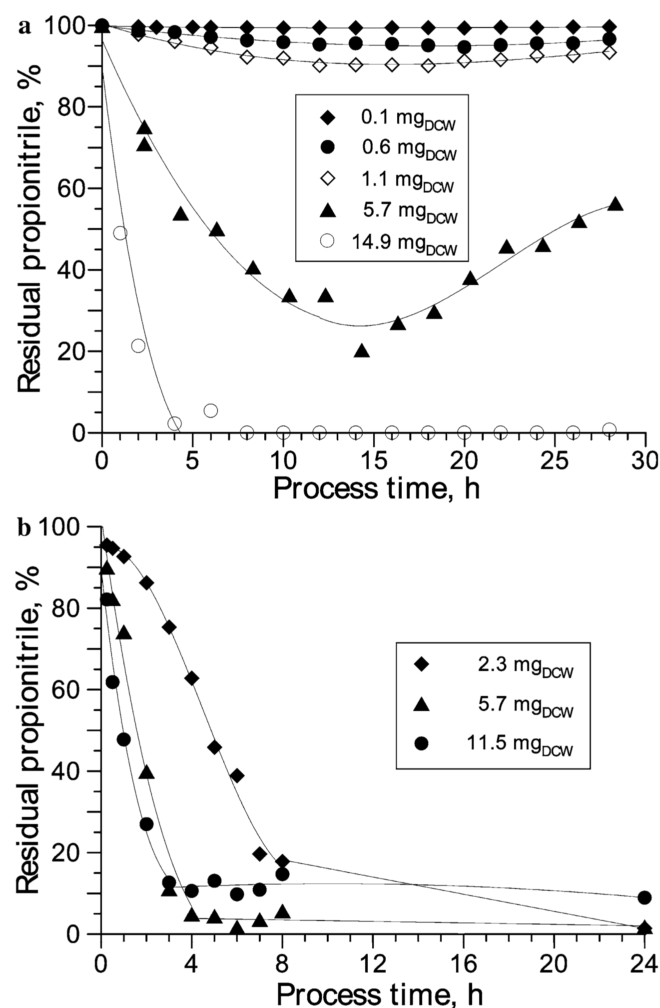

Fig. 4 a Continuous propionitrile conversion in UF-membrane bioreactors as a function of biocatalyst concentration. Propionitrile supplied at 200 mM in Na-phosphate buffer, pH 7.0. Temperature 10°C; magnetic stirring: 250 rpm, flow-rate 12.0 ± 0.7 mL h^{-1}. Lines are added for ease of following data points. **b** Time-course of propionamide bioproduction in batch reactor. Reaction volume 70 mL, 200 mM propionitrile supplied in Na-phosphate buffer, pH 7.0. Temperature 10°C, magnetic stirring 250 rpm. Lines are added for ease of following data points

In a second series of experiments, the investigation intoo the influence of different amounts of resting cells on propionitrile bioconversion was also performed in batch reactors. The results in buffered media are shown in Fig. 4b (see caption for details). The curves indicate that, within the first 8 h of operation, a conversion yield of 77.0, 88.5 and 85.5% is reached, respectively, when the reactor is operated with 2.3 mg_{DCW}, 5.7 mg_{DCW} and 11.5 mg_{DCW}. To attain 90–93% conversion requires 24 h, while total conversion is obtained after 28 h of operation. However, when 11.5 mg of cells are used in the bioreactor, the second reaction is activated and part of the formed propionamide is transformed into propionic acid.

Biodegradation of benzonitrile

Finally, a third bioconversion, reported in Fig. 5a, was tested in batch reactors using as substrate benzonitrile (10 mM). The other reaction conditions in buffered and in distilled water media are detailed in the caption. The total bioconversion of benzonitrile (5 mM) was realized after 90 min, giving benzamide (88%) and benzoic acid (data not shown). It is also quite evident from the data that increasing the process time takes the reaction through the second step (catalysed by amidase) and part of the benzamide is further converted into benzoic acid.

This result was confirmed by experiments carried out in UF-membrane reactors at 10°C and 20°C in buffered media. The substrate feed, resting cells concentration and flow rate were kept constant in order to verify the temperature effect on both enzyme activities. As shown in Fig. 5b, continuous operation at 20°C achieves the total biodegradation of benzonitrile; and roughly 60% of the benzoamide produced during the first reaction step, catalysed by NHase, is further transformed by amidase action into benzoic acid. As a result, the effluent stream is composed by benzamide and benzoic acid.

At 10°C, the response of the run is quite different, since the total benzonitrile biodegradation is not attained, presumably because of the lower amidase activity which allows the accumulation of intermediate product or benzamide. This result is quite interesting, since it could be advantageously exploited for the continuous transformation of benzonitrile streams into either almost pure benzamide or benzoic acid, by selecting the appropriate reactor operational conditions (residence time, enzyme amount, temperature). The possible environmental relevance and potential of the enzymatic system present in *M. imperiale* CBS 498-74 are clear.

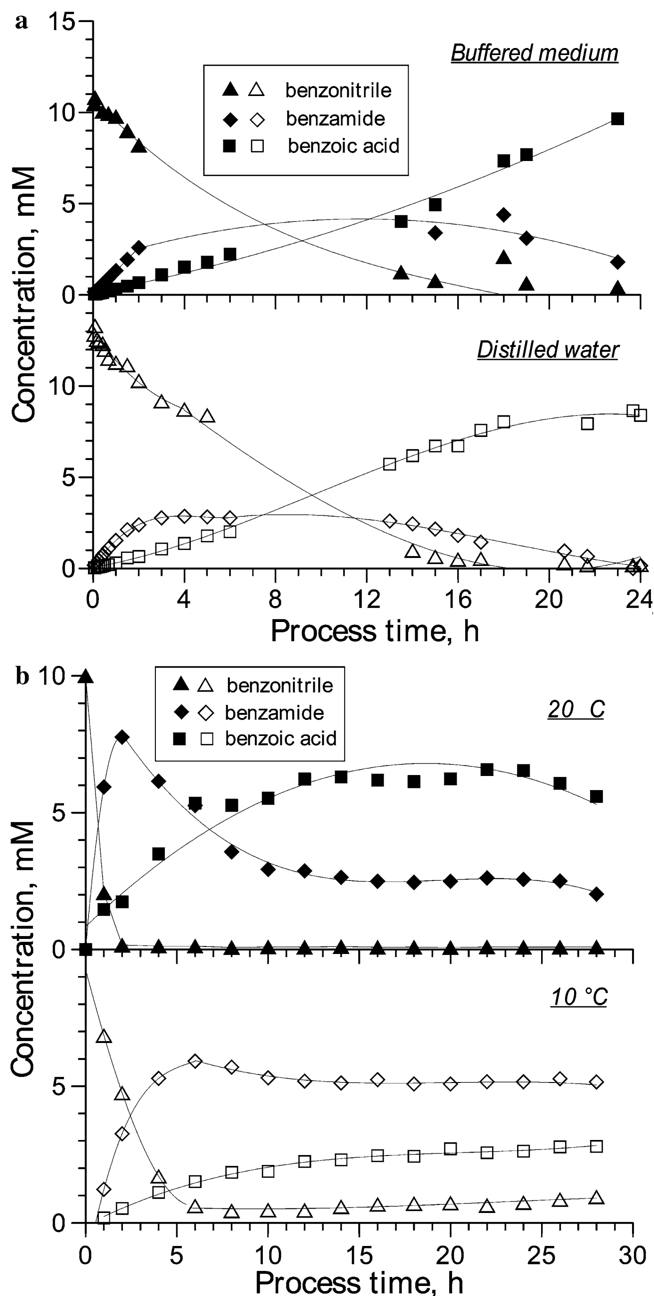

Fig. 5 a Time-course of benzonitrile bioconversion in batch reactors operating in buffered medium (*upper plot*) and in distilled water (*lower plot*). Reaction volume 50 mL, with 10 mM substrate in distilled water and 2 mg_{DCW} of resting cells. Temperature 20°C, magnetic stirring 250 rpm. Lines are added for ease of following data points. **b** Time-course of continuous benzonitrile biodegradation in UF-membrane bioreactor at 10°C and 20°C. Reactor feed 10 mM benzonitrile in 50 mM Na-phosphate buffer, pH 7.0. Resting cell concentration 22.4 mg_{DCW} per reactor, magnetic stirring 250 rpm, flow rate 12.0 ± 0.7 mL h^{-1}. Lines are added for ease of following data points

Conclusions

The results of this study allow us to conclude that:

1. The NHase of *M. imperiale* is able to catalyse the hydration of both aliphatic and aromatic nitriles.
2. Amidase activity, which is inhibited in the presence of acrylonitrile and propionitrile is quite important in the presence of benzonitrile.
3. Bioconversion is almost total for all the considered substrates, independent of whether the reaction

media contains buffer, distilled water, or tap-water. This strongly suggests the possibility of applying this strain in end-pipe biotreatments.

4. A high conversion yield can be achieved in both batch and continuous bioreactors.
5. The hydration of benzonitrile in UF-membrane bioreactors may be directed towards the intermediate amide or the final acid by the appropriate choice of operational conditions.

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