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A study in UF-membrane reactor on activity and stability of nitrile hydratase from *Microbacterium imperiale CBS 498-74* resting cells for propionamide production

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This paper is dedicated to the memory of Prof. Francesco Alfani

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Abstract

The bioconversion of propionitrile to propionamide was catalysed by nitrile hydratase (NHase) using resting cells of *Microbacterium imperiale* CBS 498-74 (formerly, *Brevibacterium imperiale*). This microorganism, cultivated in a shake flask, at 28 °C, presented a specific NHase activity of 34.4 U mg_{DCW}^{-1} (dry cell weight). The kinetic parameters, K_m and V_{max}, tested in 50 mM sodium phosphate buffer, pH 7.0, in the propionitrile bioconversion was evaluated in batch reactor at 10 °C and resulted 21.6 mM and 11.04 μmol min^{-1} mg_{DCW}^{-1}, respectively. The measured apparent activation energy, 25.54 kJ mol^{-1}, indicated a partial control by mass transport, more likely through the cell wall.

UF-membrane reactors were used for kinetic characterisation of the NHase catalysed reaction. The time dependence of enzyme deactivation on reaction temperature (from 5 to 25 °C), on substrate concentrations (from 100 to 800 mM), and on resting cell loading (from 1.5 to 200 μg_{DCW} ml^{-1}) indicated: lower diffusional control (E_a = 37.73 kJ mol^{-1}); and NHase irreversible damage caused by high substrate concentration. Finally, it is noteworthy that in an integral reactor continuously operating for 30 h, at 10 °C, 100% conversion of propionitrile (200 mM) was attained using 200 μg_{DCW} ml^{-1} of resting cells, with a maximum volumetric productivity of 0.5 g l^{-1} h^{-1}.

Keywords: *Microbacterium imperiale*; Nitrile hydratase; Propionitrile biotransformation; Enzyme kinetics; UF-membrane reactor

1. Introduction

Nitriles appear in the environment via natural or industrial syntheses; thereby the ability to degrade nitriles is quite common among microorganisms. Their microbial hydrolysis to carboxylic acids occurs either through a one-step reaction catalysed by nitrilases (EC 3.5.5.1 and 3.5.5.7); or a two-step reaction catalysed by nitrile hydratases, NHase (EC 4.2.1.84) and amidases (EC 3.5.1.4) with formation of the corresponding amide as intermediate. The current industrial production of acrylamide and nicotinamide operates with whole cells, the purified NHase being too unstable, of *Rhodococcus rhodochrous* J1 [1–5] while that of 5-cyanovaleramide, an intermediate for herbicide synthesis, uses *Pseudomonas chlororaphis* B23 [6].

The potential of nitrile-degrading enzymes has been also assessed in fine chemical and pharmaceutical syntheses [5,7–10], and waste treatments [11–13]. The past two decades have seen tremendous development of reactions catalysed by nitrile-hydrolysing enzymes in organic synthesis where nitriles and amides are widely exploited [14]. Biocatalytic steps involving nitrile hydratase and amidase catalysed reactions are already being used to obtain enantiopure pipecolic and piperazine carboxylic acids from aromatic nitrile precursors [15]. Problems associated with chemical hydrolysis of nitriles and amides (low yields, poor regio-, chemo- and stereoselectivity) can be overcome by their stereoselective hydrolysis with enzymes from different strains [9,16–20]. So far, some nitrilases, nitrile hydratases and amidases have also been purified [21]. However, the

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industrial use of nitrile-hydrolysing enzymes, which offer attractive mild reaction conditions, has been hampered by many factors, a very important one being the poor enzyme stability. Much research is focused on this subject to satisfy the demand for efficient biocatalysts and bioprocesses.

Among the numerous microorganisms investigated the wild type and different mutants of *Brevibacterium* species have received much attention [22–26].

We have been investigating acrylonitrile biotransformation using *Brevibacterium imperiale* CBS 498-74 (new classification *Microbacterium imperiale*) for a few years [27–32] and to further explore its potential we have tested other hydration reactions.

This paper reports on the propionitrile bioconversion into propionamide using resting cells of the same strain that follows the two-step degradation pathway of nitriles, and presents a high nitrile hydratase activity while amidase activity is negligible. The optimisation of microorganism growth for NHase production was already reported, and shake flask cultures (at 28 °C) with a specific NHase activity as high as 34.4 U/mg_{DCW} were obtained [28,30].

Use was made of dead-end UF-membrane bioreactor, operating in differential (low substrate conversion) or integral (high substrate conversion) mode, for kinetic characterisation of NHase. Different operating conditions allowed us to investigate enzyme thermostability and deactivation mechanism. High substrate concentrations were also tested to verify whether the observed activity loss was due to substrate inhibition or to substrate toxicity. Finally, the integral bioreactor was studied varying enzyme loading for realising 100% bioconversion without consistent activity loss.

2. Materials and methods

2.1. Chemicals

Acrylonitrile and propionitrile were supplied by Aldrich (Germany). All the experiments were performed with reagent-grade, commercially available, compounds.

2.2. Microorganism culture conditions

The strain *M. imperiale* CBS 498-74 was utilised throughout this study. A 100 ml nutrient broth culture was used to inoculate 500 ml flasks in a rotary shaker G25-KC from New Brunswick Scientific (USA). All fermentations were carried out at 28 °C and 220 rev min^{-1} orbital shaking. The composition of the culture medium (YMP-medium) was as follows: 3 g l^{-1} yeast extract (Oxoid, England), 3 g l^{-1} malt extract (Oxoid), 5 g l^{-1} bacteriological-peptone (Oxoid). The medium was prepared in 50 mM Na_2HPO_4/NaH_2PO_4 buffer, pH 7.0, and sterilised by autoclaving at 121 °C for 20 min. Sterile glucose at initial concentration 5 g l^{-1} was separately added in cultures.

Inoculum preparation of *M. imperiale* was performed in YMP-medium without added glucose and was started aseptically with one colony picked off an agar-nutrient plate, where the strain was routinely maintained. After 24 h incubation at 28 °C, 10 ml of inoculum were transferred into the fermentation flask. The cells were centrifuged at 11,400 rev min^{-1} for 15 min at 4 °C for the recovery from the culture broth. The harvested cells were washed three times with 50 mM Na-phosphate buffer, pH 7.0, and then suspended in the same buffer. Till their use in reactions they were kept in the refrigerator.

The amount of cells in the broth or in the cell suspension was estimated by optical density (OD) measurement at 610 nm. Dry cell weight (DCW) was obtained by drying to constant weight a solution with a known OD. An average value of 0.26 mg_{DCW} ml^{-1} for every unit of OD was determined. The starter culture was 0.97 ($\pm$0.18) mg_{DCW} ml^{-1} on average and the NHase specific activity was 25.41 ($\pm$3.0) U mg_{DCW}^{-1}. All runs were replicated at least twice and averaged values are presented in this work.

2.3. Enzyme activity assay

NHase activity was assayed for 15 min at 20 °C in a reaction medium containing an amount of whole cells from 0.1 to 0.2 mg_{DCW}, depending on the specific activity, and using 50 mM acrylonitrile as substrate. The reaction medium (2 ml volume) was buffered with 50 mM Na_2HPO_4/NaH_2PO_4 buffer, pH 7.0, and continuously stirred at 250 rev min^{-1}. The reaction was halted by adding 1 ml of 0.5 M HCl and centrifuging for 10 min at 11,400 rev min^{-1}. The acrylamide formed was determined spectrophotometrically with a Perkin-Elmer spectrophotometer mod. Lambda 2 (USA), at 235 nm, a wavelength at which neither the acrylonitrile nor the buffer interfere with the readings. A calibration curve was obtained in advance to determine the molar extinction coefficient of acrylamide, which was determined to be 1100 mol^{-1} l cm^{-1}. One unit (U) of NHase activity was defined as the amount of resting cells that catalyses the formation of 1 μmol of acrylamide per min under the adopted conditions [27–31]. Specific activity was expressed as U mg_{DCW}^{-1}.

2.4. Analytical determinations

Protein contents were measured by the Lowry–Hartree method [33] using as a standard crystalline bovine serum albumin from Aldrich (Germany). Propionamide was identified by its retention time in gas chromatography analysis and quantitative measurements were obtained by comparison with a calibration curve of known concentration of the compound. Sample of supernatant fractions of reaction mixture were filtered through Millipore membrane (USA) to remove any high molecular weight contaminants, and analysed with 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 m × 0.53 mm i.d.) (USA). A base deactivated guard column Restek Cat 10002 (5 m × 0.53 mm i.d.) was also used. Helium carrier gas flow rate was 1.6 ml min^{-1}. The column, injector and flame ionisation detector were held at 220 °C.

2.5. Biotransformations

2.5.1. In batch reactor

Kinetic studies were performed within the propionitrile concentration range 10–500 mM in buffered reaction medium with 50 mM Na-phosphate buffer, pH 7.0, at 20 °C. Reaction volume was 2 ml; resting cell concentration 100 μg ml^{-1}. The activation energy of the propionamide formation catalysed by NHase was evaluated from runs performed in the temperature range from 5 to 25 °C. The reaction was carried out in batch reactors, 50 ml volume, resting cell load 0.3 mg_{DCW} and 500 mM substrate in 50 mM Na-phosphate buffer, pH 7.0. At known time intervals a sample was withdrawn and analysed for product formation.

2.5.2. Continuous ultrafiltration (UF) membrane bioreactor

An ultrafiltration kit (Amicon Model 52, Grace, USA), of stirred type was employed as a dead-end UF-membrane bioreactor. A fluoro-polymer membrane FS81PP produced by Dow Liquid Separations (England) with a molecular weight cut-off of 10,000 was used. The same stirring, by means of a magnetic stirrer set at 250 rpm, was provided in all the experiments to limit resting cell deposition onto the membrane due to concentration polarisation. The substrate solution was fed to the reactor using a peristaltic pump (Gilson Minipuls, France) and the filtrate was collected for analysis, using a fraction collector (LKB Instruments, Sweden). The average volumetric flow rate adopted in all experiments was 12.7 (±0.7) ml h^{-1}. Both reactor and substrate reservoir were placed in a water bath, the temperature of which was controlled within ±0.1 °C. The membrane resistance to chemicals was fair at the investigated range of propionitrile and propionamide concentrations. No rejection of solute was determined. Membranes totally retained the resting cells and no fouling was observed working under stirring conditions. Membrane compaction was responsible for flux loss during the first 3–4 h of operation.

The operational stability of NHase was investigated in experimental runs performed with propionitrile solutions, 200 mM, buffered with phosphate buffer (50 mM, pH 7.0). Cell amount and residence time in the bioreactor were selected to assure conditions of differential reactor operation. The reaction temperature was varied from 5 to 25 °C.

The effect of substrate concentration on the NHase activity was studied in different runs performed feeding the UF-membrane bioreactor with buffered propionitrile solutions at concentration from 100 to 800 mM. Membrane permeability was not affected by propionitrile concentration and solute rejection coefficient of the UF-membrane was zero (data not shown).

The effect of enzyme concentration on reaction rate was investigated in the range 0.11–14.9 mg_{DCW} of resting cells per reactor. The substrate solution, prepared in 50 mM Na-phosphate buffer, pH 7.0, was 200 mM, as this concentration showed negligible substrate inhibition.

3. Results and discussion

3.1. Kinetic parameters in batch reactors

The first part of the study was focused on the evaluation of kinetic parameters in batch reaction. Kinetic studies, performed within the substrate concentration range 10–500 mM, revealed that the Michaelis–Menten equation holds and the kinetic parameters: K_m, Michaelis constant, and V_{max}, maximum velocity, have been determined. The Hanes–Woolf plot of kinetic runs carried out at 10 °C allowed the evaluation of V_{max}^{app} (11.04 μmol min^{-1} mg_{DCW}^{-1}) and of K_m^{app} (21.6 mM). The K_m^{app} value for propionitrile substrate is roughly twice that for acrylonitrile thus indicating a higher affinity to the latter. The activation energy of the propionamide formation catalysed by NHase was evaluated from runs performed in the temperature range from 5 to 25 °C as described in Section 2. The reaction rate, r_0, was evaluated from plots of product concentration against time. The values varied with temperature according to the known Arrhenius equation:

$$r_0 = A \exp\left(-\frac{E_a}{RT}\right)$$

where A is the pre-exponential factor, E_a the activation energy, R the gas constant, and T the absolute temperature. E_a^{app} was found to be 25.54 kJ/mol a rather low value indicating the presence of diffusional resistances.

3.2. Effect of temperature on the stability and activity of NHase in resting cells

The second part of the investigation was devoted to the study of operational stability of NHase in a UF-membrane reactor. This reactor configuration is particularly suitable to perform long-term continuous reaction under controlled operating conditions such as substrate and enzyme concentration, pH, temperature, mixing speed, and residence time, all of which affect enzyme activity.

The bioreactor operated as a completely mixed system so that the enzyme, substrate and product concentration in the reactor were uniform, and the substrate and product concentration were equal to their value in the permeate. The specific reaction rate was evaluated from product concentration in the reactor effluent, C_P (μmol l^{-1}), flow rate, Q (l min^{-1})

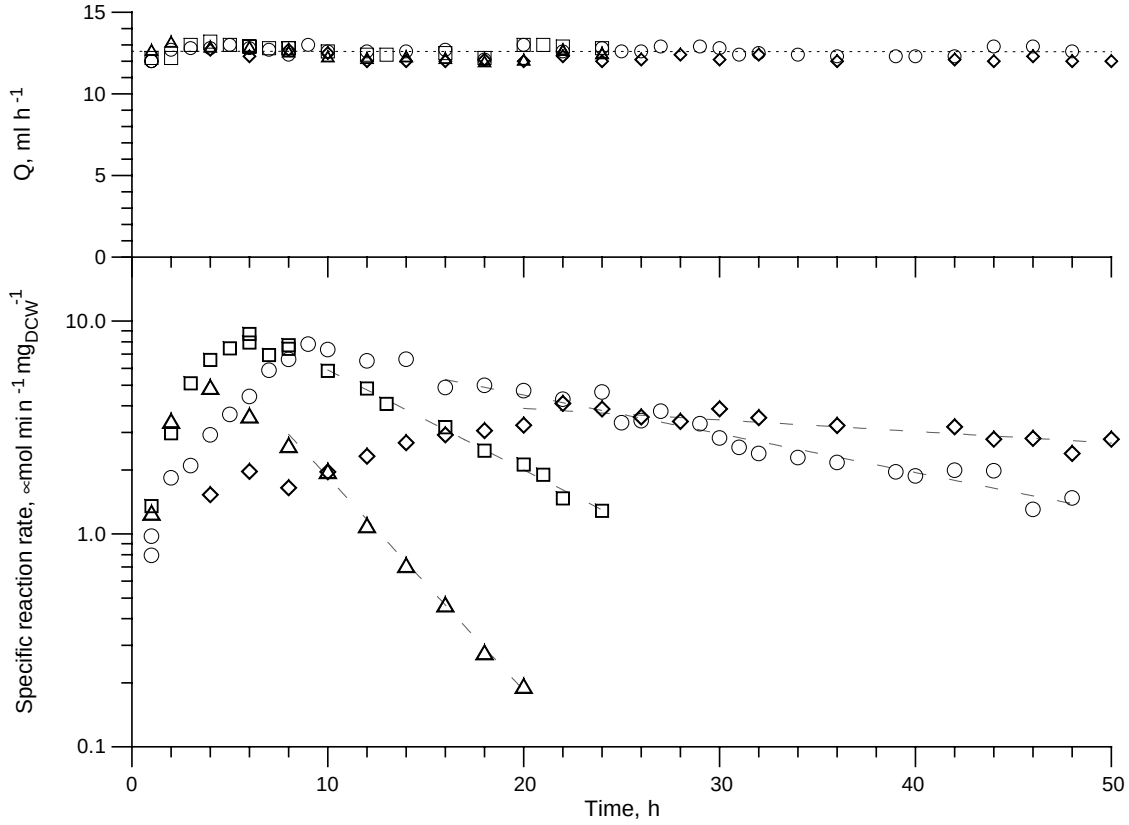

Fig. 1. Time course of NHase specific reaction rate as a function of reaction temperature in UF-membrane bioreactor. In the upper plot: time course of instantaneous flow rate in the runs. Reaction conditions—flow rate: 12.7 (±0.7) ml h^{-1}; cell load: 2.3 mg_{DCW} of *M. imperiale* resting cells; propionitrile solution: 200 mM in 50 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7.0; temperature: 5 °C (◇); 10 °C (○); 20 °C (□); 25 °C (△).

and amount of cells (mg_{DCW}) introduced in the bioreactor using the following relationship [34]:

$$r = \frac{Q \times C_P}{mg_{DCW}}$$

The specific reaction rate of propionamide production is reported in a semi-logarithmic plot as a function of process time in Fig. 1. The upper plot shows the time course of flow rate in the runs. It can be observed an initial rapid flux decay, which lasts 2–3 h, and from this time onward membrane permeability remained almost unchanged at all tested temperatures thus indicating that the adopted membrane has a very good resistance to the chemical species involved in the reaction. The lower plot shows that after a transient period, due to the initial accumulation of product in the bioreactor, the data points give straight lines with negative slope, indicating that NHase exhibited a first-order deactivation mechanism described by:

$$r = r_0\, e^{-k_d t}$$

Exponential fitting of data (linear portion of the curve) allowed the evaluation of NHase first-order deactivation constant, k_d, from the slope, while the intercept of the extrapolated straight line on *y*-axis gave the specific initial reaction rate, r_0. The values of r_0 depended on temperature, and the corresponding Arrhenius plot is shown in Fig. 2. An apparent activation energy of roughly 37.7 kJ mol^{-1} was evaluated between 5 and 25 °C. The discrepancy between the E_a^{app} values in batch and UF-bioreactor could be attributed to diffusional resistances through the cell wall, being the NHase intracellular. These two reactor configurations explore a different reaction time, 15 min for batch reactor, while in the UF-bioreactor the mean residence time of cells was roughly of 6 h. In this latter configuration. most likely the transport properties through the cell wall could be modified by such a long contact with substrate and/or permanence time in stirred conditions. In any case absence of cell lysis was ascertained. The insert of Fig. 2 shows that k_d values changed with temperature too. NHase appears to be a thermolabile enzyme that loses its activity quite rapidly at 20 and 25 °C, the half-lives being 6.3 and 3.0 h, respectively. A better operational stability is obtained at 5 and 10 °C (half-lives: 38.5 and 16.5 h, respectively).

3.3. The effect of propionitrile substrate concentration on the activity of whole cells

Our previous studies [27–31] clearly indicated that *M. imperiale* resting cells are not tolerant to high acrylamide concentrations. Thereby, another part of the present study was devoted to ascertain whether propionitrile might induce a similar behaviour. Operational temperature was set

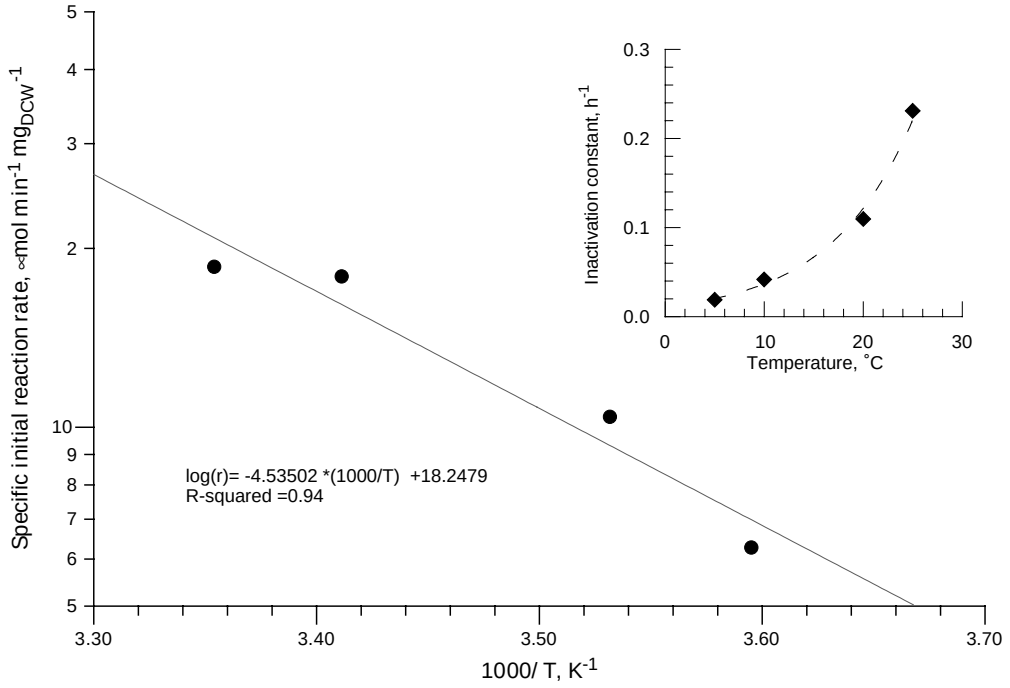

Fig. 2. Arrhenius plot of propionitrile hydratation catalysed by NHase from *M. imperiale* resting cells. Insert shows the dependence of inactivation constant on temperature.

at 10 °C, a compromise between the need to get a rapidly vanishing transient (10 h at 10 °C against 20 h at 5 °C) and a high reaction rate, while keeping the deactivation constant acceptably low for a possible real process. Fig. 3 shows semi-logarithmic plots of specific reaction rate against process time. The operating time was 15–60 h according to the rate of enzyme activity decays. Data points have been collected at least until the NHase activity lowered down to 10% of the initial activity. This figure illustrates well the dependence of NHase deactivation kinetics on substrate concentration. As in the analysis previously described, the identification of a first-order deactivation mechanism was done using the exponential fitting of data. The equation of the best-fit allowed to estimate both the specific initial reaction rate and the inactivation constant.

For each run the initial specific reaction rate, r_0, was evaluated and plotted against nominal substrate concentration (inlet one) as illustrated in Fig. 4 (left plot). It should be pointed out that the effective concentration of substrate varies in the bioreactor, due to the reaction progress. This variation could be quite important especially at lower concentration of substrate as indicated by the horizontal bar introduced in the plot. The solid curve shows an apparent maximum of specific reaction rate of 11.45 μmol min^{-1} mg$^{-1}_{DCW}$ for propionitrile 250 mM that dropped to 1.47 μmol min^{-1} mg$^{-1}_{DCW}$ for 800 mM substrate feed (vertical error bars indicate the error on r_0 evaluation).

The pattern of specific reaction rate decay appears to be quite similar to that postulated for substrate inhibition kinetics in differential reactor. To investigate this hypothesis, different predictive curves were obtained with the well-known Haldane equation using different order of magnitude of K_I ($10\times K_m$ and $100\times K_m$). The calculated curves indicate that enzyme activity depression might be due to superimposed phenomenon in addition to substrate inhibition: most likely toxic effects on cells that modify transport properties of the membrane and/or an inactivating effect on the enzyme itself. Studies devoted to ascertain these effects and to better characterise the phenomenon are in progress.

At the end of reaction, the retentate of the bioreactor was collected and centrifuged in Eppendorf to spin down the resting cells. No proteins were evaluated in the supernatant demonstrating the absence of cell lysis and cell damage during the experiments.

Interestingly, as shown by the right plot of Fig. 4, k_d values are not at all influenced up to 300 mM substrate concentration, the numerical value being (0.031 ± 0.0044 h^{-1}) quite similar to that obtained above in runs performed varying the temperature. However, when propionitrile concentrations higher than 300 mM were fed to the bioreactor, k_d values increase dramatically by a factor of 1.8, 2.5 and 5 for substrate concentrations 400, 500, and 800 mM, respectively.

3.4. The effect of NHase concentration on the specific reaction rate

Most of this study was performed operating in differential reactor conditions, that is, at low conversion, in order to quantify the role of parameters such as temperature and

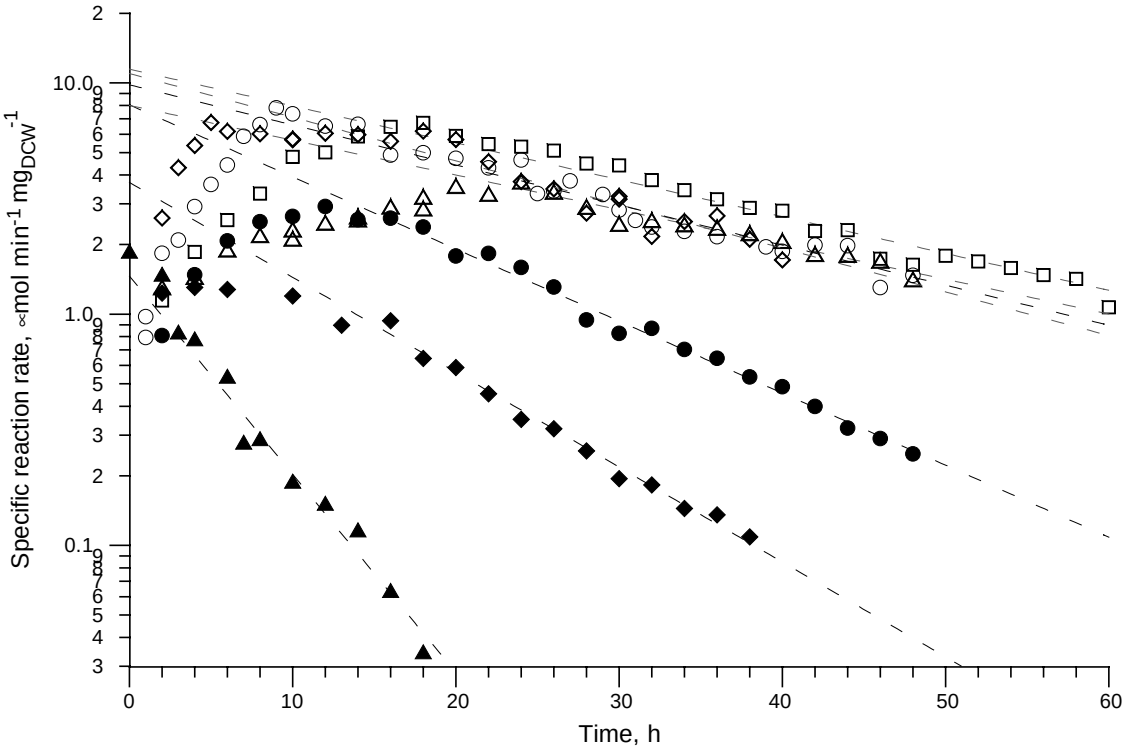

Fig. 3. Time course of NHase specific reaction rate as a function of substrate concentration solution prepared in 50 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7.0: (◇) 100 mM; (○) 200 mM; (□) 250 mM; (△) 300 mM; (●) 400 mM; (◆) 500 mM; (▲) 800 mM. Reaction conditions—flow rate: 12.7 (±0.7) ml h^{-1}; reaction temperature: 10 °C; cell load: 2.3 mg_{DCW} of *M. imperiale* resting cells.

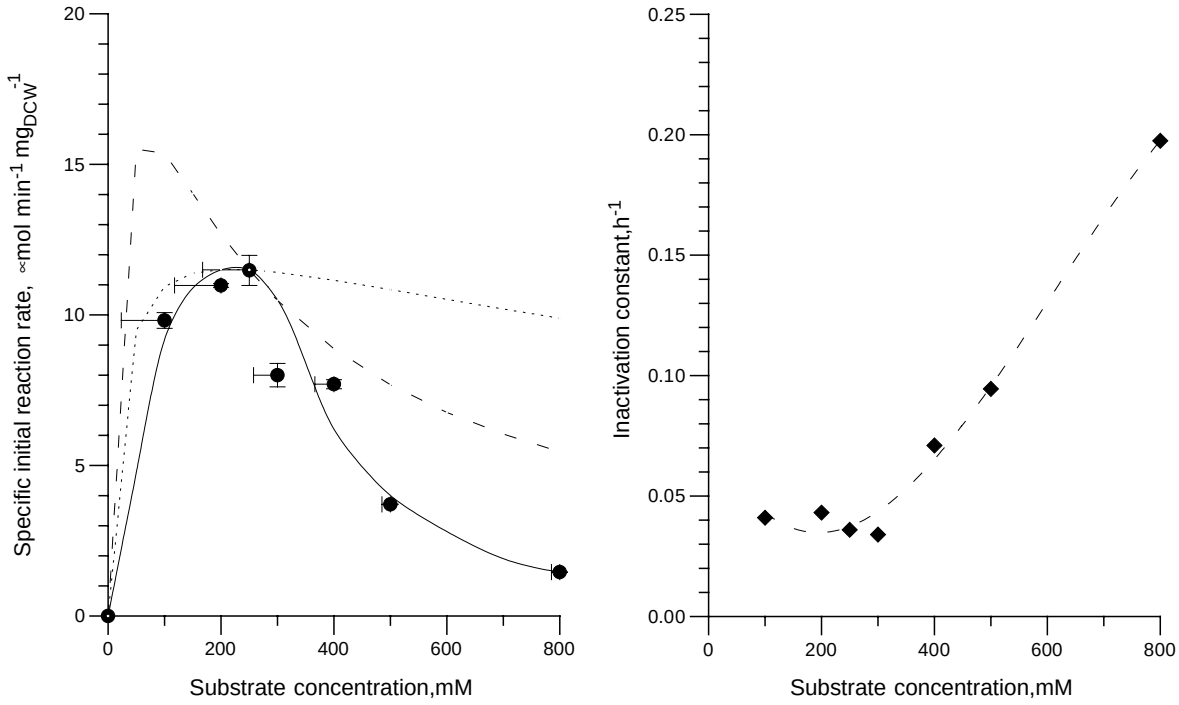

Fig. 4. Left: specific initial reaction rate as function of substrate concentration, horizontal bar indicates the range of substrate concentration due to reaction progress; vertical error bar for specific initial reaction rate evaluation. Haldane equation prediction of substrate-inhibited kinetics (--- $K_I = 216$; ··· $K_I = 2160$). Right: inactivation constant versus substrate concentration.

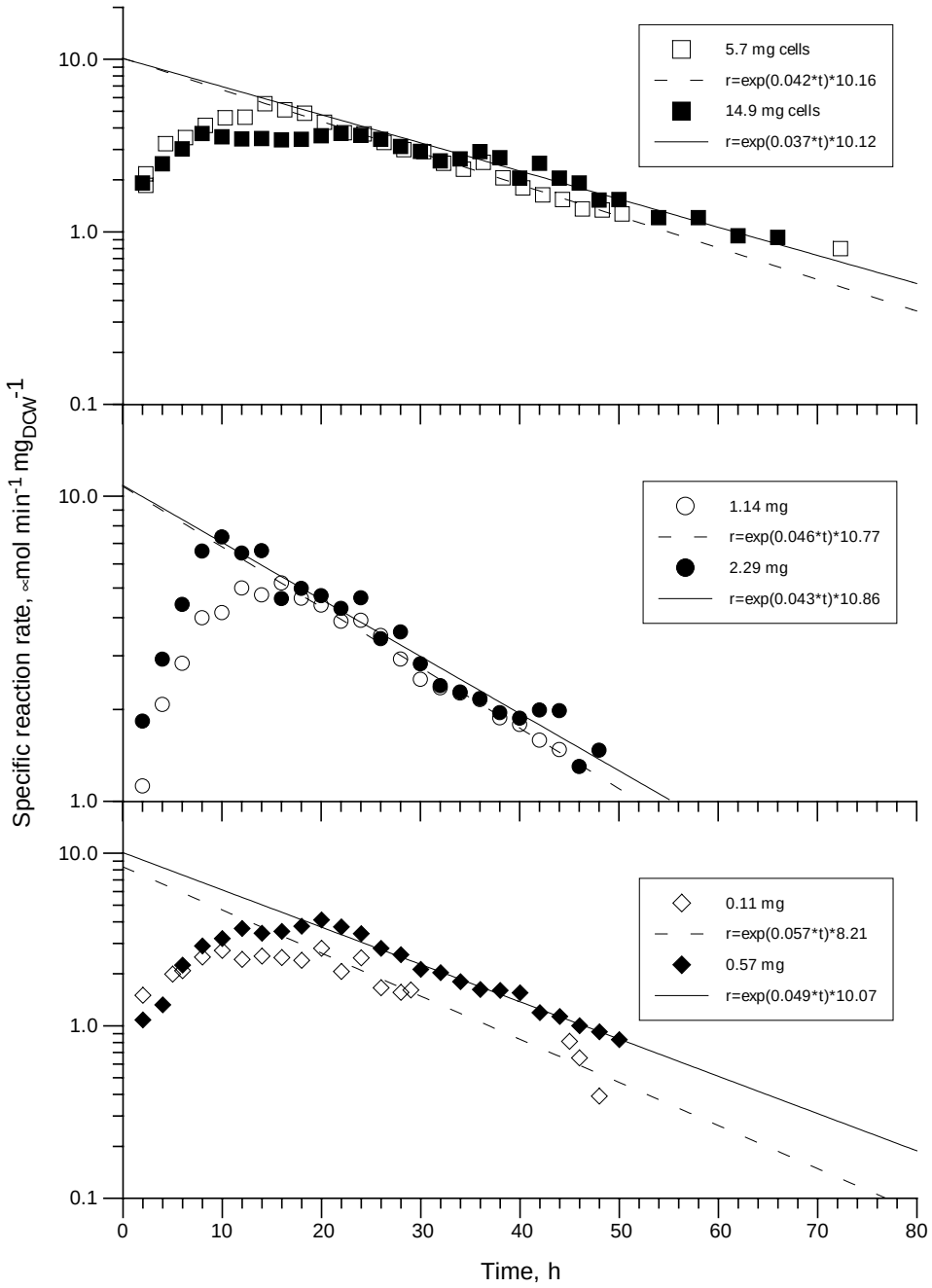

Fig. 5. Time course of NHase specific reaction rate as a function of cell load in UF-membrane bioreactor. Reaction conditions—flow rate: 12.7 (±0.7) ml h^{-1}; propionitrile solution prepared in 50 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7: 200 mM; reaction temperature was 10 °C. *M. imperiale* resting cell load: (◇) 0.1 mg_{DCW}; (◆) 0.6 mg_{DCW}; (○) 1.1 mg_{DCW}; (●) 2.3 mg_{DCW}; (□) 5.7 mg_{DCW}; (■) 14.9 mg_{DCW}.

substrate concentration on NHase activity. In a possible real scale process the bioreactor need operating at integral conditions to reach high substrate conversion. Besides, using NHase, which suffers from thermodeactivation and substrate inhibition and/or deactivation, in an industrial bioprocess a policy should be pursued for maintaining the enzyme activity in the bioreactor as high as possible, and for a period long enough, to be interesting from an industrial standpoint. To this end, several runs were performed at 10 °C (deactivation constant quite low) in the presence of different enzyme amounts as reported in Section 2. The time courses of specific reaction rate versus time for all runs are shown in Fig. 5. Table 1 reports the inactivation constant, the highest conversion attained before NHase inactivation starts, the global conversion as the integral value of the first 30 h operation, and the volumetric productivity. It is noteworthy that increasing the amount of enzyme the maximum conversion in the bioreactor changes from 0.6% (in the differential operating one) to 100% (in the integral operating one). The amount of cell charged in this last experiment (14.9 mg_{DCW})

Table 1
Effect of cell loading on bioreactor performances at 10 °C

Cell load (mg_{DCW})	Inactivation constant (h^{-1})	Highest conversion reached[a] (%)	Substrate conversion[b] (%)	Volumetric productivity ($mg\,l^{-1}\,h^{-1}$)
0.1	0.057	0.6	0.5	2.4
0.6	0.049	5.3	3.9	19.0
1.1	0.046	9.9	7.5	36.6
2.3	0.043	41.2	23.7	115.5
5.7	0.042	79.8	67.1	326.8
14.9	0.037	100.0	100.0	497.1

[a] Before NHase inactivation starts.
[b] As the integral value of the first 30 h operation.

is high enough to ensure, for 30 h, 100% conversion. During this process time, of course the enzyme activity is curtailed by inactivation but apparently no activity decay occurs since the enzyme is in excess, and only part of it, is involved in the reaction. When all the active enzyme (part being irreversibly deactivated) is involved in the catalytic reaction, the activity loss obeys the first-order deactivation kinetics previously determined. Indeed, in all experiments the relation $r = r_0 \exp(-k_d t)$ holds with k_d value $= 0.046 \pm 0.011\,h^{-1}$ (see legend inside Fig. 5 for details). To keep reaction going on a 100% conversion basis, fresh resting cells should be added to the bioreactor after the first 30 h of process.

4. Conclusions

M. imperiale CBS 498-74 resting cells are able to catalyse the propionitrile bioconversion to propionamide with a substrate affinity which is two-fold lower when acrylonitrile is used as substrate. The apparent activation energy evaluated in the temperature range from 5 to 25 °C indicated the presence of substrate mass transport effects that can be partly overcome in a UF-membrane reactor. This reactor configuration, used as a differential reactor, was proved to be a useful tool to identify a first-order deactivation mechanism. This latter was evidenced when varying parameters such as temperature and substrate concentration. Kinetic runs, performed at different substrate concentrations showed, that substrate inhibition alone cannot take into account for the enzyme activity loss at high substrate concentration but other phenomena such as substrate deactivation or toxicity should be envisaged. Finally, increasing the enzyme loading (up to 0.2 g/l of resting cells) inside the bioreactor a 100% conversion is possible for 30 h of continuous process without apparent activity decay.

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