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# Operational stability of *Brevibacterium imperialis* CBS 489-74 nitrile hydratase

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**Abstract**

*Brevibacterium imperialis* CBS 489-74 was grown in broths prepared with yeast and malt extract, bacteriological peptone and 2% glucose or differently modified with the addition of Na-phosphate buffer, $FeSO_4$, $MgSO_4$ and $CoCl_2$. The peak production of nitrile hydratase (NHase) did not change significantly. At the stationary growth phase, the units per milliliter of broth (60 units $ml^{-1}$) were more important than those at the exponential growth phase.

The NHase operational stability of whole resting cells was monitored following the bioconversion of acrylonitrile to acrylamide in continuous and stirred UF-membrane reactors. The rate of inactivation was independent on buffer molarity from 25 to 75 mM and on pH from 5.8 to 7.4. Enzyme stability and activity remained unchanged in distilled water. The initial reaction rate increased from 12.8 to 23.8 g acrylamide/g dry cell/h, but NHase half-life dropped from 33 to roughly 7 h when temperature was varied from 4°C to 10°C. The addition of butyric acid up to 20 mM did not improve enzyme operational stability, and largely reduced (94%) enzyme activity. Acrylonitrile caused an irreversible damage to NHase activity. High acrylonitrile conversion (86%) was attained using 0.23 mg cells/ml in a continuously operating reactor.


*Keywords:* *Brevibacterium imperialis*; Nitrile hydratase; Operational stability; UF-membrane reactor

## 1. Introduction

Nitrile-hydrolysing enzymes produced by bacteria and fungi catalyse the conversion of a large number of chemically diverse nitriles into amides and acids [1–3]. Microorganisms expressing NHase have already been used for the industrial production of acrylamide and nicotinamide [4–6]. They can also be used efficiently in environmental remediation for the conversion of nitrile wastes to less toxic amides [7,8].

Acrylamide, a commodity chemical widely used to produce polymers for application in sewage treatment, petroleum recovery, papermaking and textile sizing industrial processes, is manufactured from acrylonitrile mainly by chemical processes (more than 200,000 tons/year in the world). Acrylonitrile biotransformation to acrylamide has been also developed up to the industrial scale [5,9]. Nitto Chemical Industries in Japan has operated an industrial plant

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for several years [10], and, at present, it produces more than 30,000 tons of acrylamide/year [11]. A similar plant is in operation in Russia [12]. Both processes use resting cells from *Rhodococcus* species. The bioproduction of acrylamide under moderate reaction conditions limits the uncontrolled polymerisation favoured by the presence of double bonds in the molecule.

Several university and industrial laboratories are still interested in the optimization of the bioprocess [13–17] through the improvement of NHase-producing microorganisms such as *Brevibacterium imperialis*, *Pseudomonas chlororaphis*, *Corynebacterium*, *Arthrobacter*, *Norcadia* and *Rhodococcus rhodochrous*. Bioreactor configurations and downstream processing [18,19] are also under study since acrylamide needs to be of high quality, and any additives such as buffer salts, metal ions and impurities from cells are undesirable in the product stream.

This paper presents the results of experiments carried out with *B. imperialis* CBS 489-74 resting cells. This strain is particularly interesting for acrylamide production since the hydratase/amidase system exhibits high NHase activity and almost nil amidase activity. Therefore, very little amount of the acrylamide produced is transformed into undesired acrylic acid in the course of the reaction; and the downstream processing is also minimised. Whole cells were used as sources of enzyme because in continuous processes: (i) they can be easily separated from the reaction mixture by simple centrifugation or filtration; (ii) the isolation of the intracellular enzyme is generally both expensive and laborious, and preliminary experiments also showed that recovery of NHase activity was poor.

The research was oriented to expand on our previous investigations on the optimisation of *B. imperialis* fermentation [16,17,20,21] and to characterise kinetically the NHase in the resting cells. Biomass growth and NHase activity in the cells were monitored at several fermentation periods varying the composition of the broth. The operational stability of the NHase in cells, harvested at peak production of enzyme, was determined in experiments performed at different substrate and cell concentrations, varying buffer molarity, pH, temperature and adding butyric acid. Continuous ultrafiltration membrane reactors were used since this configuration is powerful at the laboratory scale for monitoring the activity and the stability of biocatalysts in systems operating at well-controlled substrate and product concentrations.

## 2. Materials and methods

### 2.1. Microorganism, culture and growth medium

The strain *B. imperialis* CBS 489-74 was utilised in all the experiments. Stock cultures were routinely maintained on yeast extract, malt extract, bacteriological peptone, α-D-glucose and agar slant (YMPG-agar) at 4°C and transferred monthly. YMPG-agar plates contained the following components from Oxoid, England (g $l^{-1}$): yeast extract, 3; malt extract, 3; bacteriological-peptone, 5; agar, 20; and from Serva, Germany: glucose 2% (w/w). Sufficient Erlenmeyer flasks (500 ml) (to allow triplicate samples to be taken), containing 100 ml of nutrient broth, were inoculated with one colony picked off the YMPG-agar plate. The growth was carried out, at 28°C, on a rotary shaker (220 rpm). Cell transfers were made in an aseptic manner. Two culture broths were mainly used unless otherwise specified. The first one, referred to as the minimal culture medium or as YMPG broth, contained the above-mentioned constituents dissolved in distilled water and adjusted to pH 7.0 with 1 N NaOH. The second culture medium, in the following referred to as modified YMPG broth, contained the same constituents in 50 mM $Na_2HPO_4/NaH_2PO_4$ buffer, pH 7.0. Metal salts, $FeSO_4 \cdot 7H_2O$ (0.01 g $l^{-1}$) and $MgSO_4 \cdot 7H_2O$ (0.88 g $l^{-1}$) were added as suggested by Bernet et al. [22]. Both media were sterilised by autoclaving at 121°C for 20 min. Glucose, 50% (w/w) solution, separately sterilised, was added to the broth after sterilisation. All other chemicals were commercially available reagents of analytical grade.

### 2.2. Resting cell preparation

NHase is an intracellular enzyme as confirmed by the absence of activity in the supernatant culture medium at all fermentation conditions. The cells

were harvested at different fermentation times. The broth was centrifuged, at 11,400 rpm for 15 min at 4°C, and the cells washed three times with 100 ml/wash of 50 mM $Na_2HPO_4/NaH_2PO_4$ buffer, pH 7.0. The cell paste was then diluted in the same buffer and stored in the refrigerator (−18°C) until it is used as a source of biocatalyst in reactions.

### 2.3. Cell concentration evaluation

The amount of cells present in the fermentation broth or in the cell suspension was rapidly estimated by optical density at 610 nm wavelength. The solutions were diluted before spectrophotometric readings to give an absorbance below 1.0. Four dilution ratios were usually adopted: 1:150, 1:60, 1:30 and 1:15. The relationship between absorbance and cell dry weight was determined through experimental data correlating the absorbance vs. dry-cell weights obtained by drying solutions with known optical density to constant weight. From this reference curve, every unit of optical density (at 610 nm) of *B. imperialis* suspension corresponds to 0.26 mg $ml^{-1}$. The absorbance readings were multiplied by the dilution and the average was taken giving an effective absorbance. Zero absorbance was set using water.

### 2.4. Analysis of product and substrate

The amount of acrylamide and acrylonitrile in the reaction mixture was determined by high-performance liquid chromatography (HPLC). The HPLC analysis was performed with a Perkin Elmer Series 2 system equipped with an UV detector and a MERCK C18 column (reversed-phase column, 25 cm × 4 mm) operating at 30°C and at 0.5 ml $min^{-1}$ of mobile phase (pure acetonitrile and $KH_2PO_4/H_3PO_4$ buffer (10 mM, pH 2.8) at a volumetric rate from 1 to 10). The retention time of acrylonitrile and acrylamide being quite different, the $A_{220\ nm}$ allowed the determination of both concentrations. Acrylamide concentration was also measured spectrophotometrically (Perkin Elmer, Spectrophotometer mod. Lambda 2) at 235 nm, a wavelength at which neither the acrylonitrile nor the buffer interfere with the readings.

### 2.5. Enzyme activity assay

The NHase activity was assayed by the following procedure: reaction mixture was 2 ml containing acrylonitrile (Aldrich, Germany), 50 mM, in 50 mM $Na_2HPO_4/NaH_2PO_4$ buffer, pH 7.0, and an adequate amount of cells (0.1–0.2 mg dry weight) depending on the specific activity. The reaction was carried out at 20°C for 15 min with continuous stirring at 250 rpm. Adding 1 ml of 0.5 M HCl stopped the reaction. A centrifugation step at 11,400 rpm for 10 min at room temperature was adopted for separating the cells from the reaction medium. The acrylamide concentration in the supernatant was measured spectrophotometrically or by HPLC. One unit (U) of NHase activity was defined as the amount of resting cells that catalysed the formation of 1 μmol acrylamide/min under the adopted conditions. The specific activity was expressed as units per milligram of dry cells. Reactions were always run at least in duplicate to ensure reliability.

### 2.6. Operational stability studies in UF-membrane reactors

An ultrafiltration cell (UF-cell), Amicon mod. 72 (Grace, USA), equipped with FS81PP membrane (molecular weight cut-off 6000) from DDS (Denmark) was used as the membrane bioreactor. A previous study [21] showed that no retention of substrate and product occurs in the UF-cell and these organic chemicals do not damage the membrane. The volumetric flow rate was controlled using a peristaltic pump (Gilson Miniplus, France). Standard flow rate was 12 ml $h^{-1}$ and acrylonitrile concentration was 100 mM in 50 mM $Na_2HPO_4/NaH_2PO_4$ buffer, pH 7.0. Resting cells (2 mg dry weight) were added to the reactor to start the reaction. The system was continuously stirred at 250 rpm to avoid cell deposition onto the membrane due to concentration polarisation. All the runs were carried out under these conditions unless otherwise stated. The effluent stream was collected in a fraction collector and the samples were analysed every hour to determine the acrylamide concentration. Reaction rate (μmol $min^{-1}$) and specific reaction rate (μmol $min^{-1}$

$mg^{-1}_{cells}$) were calculated from product concentration determinations ($\mu$mol $ml^{-1}$), mass of dry cells ($mg_{cells}$) and volumetric flow rate (ml $min^{-1}$).

## 3. Results and discussion

### *3.1. Time course of enzyme production in YMPG and in modified YMPG broths*

The relationship between *B. imperialis* growth and NHase production was determined in shake-flask experiments up to roughly 160 h. Samples were withdrawn regularly from the flasks under aseptic conditions and analysed for biomass content and NHase activity.

Figs. 1 and 2 show the growth curves and the time course of enzyme production in the YMPG broth and in the modified YMPG broth. The biomass concentration ($mg_{cells}$ $ml^{-1}$) is reported on the left-hand axis. The right-hand axis shows the specific activity of NHase (U $mg^{-1}_{cells}$) and the total units of NHase activity per milliliter of broth. A lag phase of 8–14 h followed by the exponential cell growth was typically observed. The growth rate slowed significantly 20 h after inoculation and the stationary phase was usually reached within 90–100 h. The cells expressed more peaks of NHase activity during this fermentation period. In the YMPG broth the first peak occurred at 21 h, associated with the exponential phase, and had a specific activity of 9 U $mg^{-1}_{cells}$.

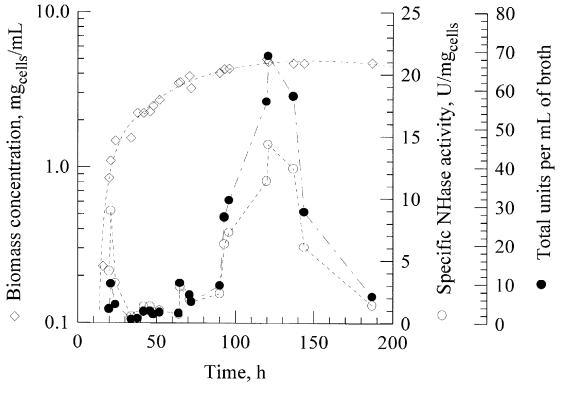


Fig. 1. Growth curve of *B. imperialis* in YMPG broth and NHase activity of resting cells grown in shake flasks (500 ml) at 28°C, 220 rpm.

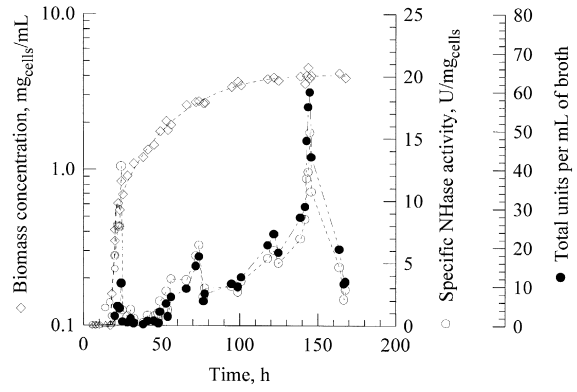


Fig. 2. Growth curve of *B. imperialis* in modified YMPG broth and NHase activity of resting cells grown in shake flasks (500 ml) at 28°C, 220 rpm.

The most significant peak occurred at 120 h with a specific activity of 14.3 U $mg^{-1}_{cells}$. The total activity at this point of the fermentation was much more significant because of the greater cell concentration (4.8 $mg_{cells}$ $ml^{-1}$) in comparison with the value at 21 h (1.1 $mg_{cells}$ $ml^{-1}$). The medium pH was not controlled continuously and its value dropped to 6.8 after 24 h and to 5.7 after 120 h of fermentation.

Further experiments were made adjusting the pH at the beginning of the fermentation with $Na_2HPO_4/NaH_2PO_4$ buffer 50 mM, pH 7.0 and adding $FeSO_4$ and $MgSO_4$. A higher level of enzyme activity was expected since phosphate is a chelating agent that could capture $Ca^{2+}$ and reduce protease synthesis during cell growth. The addition of both salts should increase the NHase activity as already reported in the literature [22,23].

The fermentation in the modified YMPG broth presented a different time course of enzyme production as plotted in Fig. 2. The first peak of enzyme activity appeared at 24 h with a specific activity of 12.7 U $mg^{-1}_{cells}$. A small peak occurred after 74 h (6.4 U $mg^{-1}_{cells}$) and the third one was observed after 144 h with a specific activity of 15.5 U $mg^{-1}_{cells}$. The latter was the most important since at this point of the fermentation the bacterial growth was in the stationary phase and the cell concentration reached the highest level. This gives a high total activity (60 U $ml^{-1}$). After this fermentation time, the NHase activity fell rapidly. During the fermentation, there was only a slight pH change, the final value being 6.6.

The semilogarithmic plot of biomass against time indicates that the cells follow the normal exponential-type growth pattern. As far as enzyme production is concerned, in both media under study, NHase activity is expressed either during the exponential growth (20–24 h) or in the late stationary phase, when the culture becomes substrate limited. The biological origins, probably due to variations induced by still unknown factors linked to batch fermentation, should be investigated further. Due to higher biomass concentration, as well as higher specific activity, the total amount of enzymatic units produced per milliliter of broth is by far the most interesting when the cells are harvested, in both fermentation broths, from stationary phase cultures. In the modified YMPG broth, the maximum in enzyme production was delayed 24 h in comparison with that in the YMPG broth, and this determines a lower specific productivity. This lowered from 0.56 U $ml^{-1}$ $h^{-1}$ in YMPG to 0.41 U $ml^{-1}$ $h^{-1}$ in the modified YMPG broth. The productivities at the exponential growth phase are comparable, 0.45 U $ml^{-1}$ $h^{-1}$ in modified YMPG and 0.47 U $ml^{-1}$ $h^{-1}$ in YMPG. The cells harvested at exponential growth (24 h in modified YMPG broth) are roughly 1.4 times more active than those cultivated 21 h in the YMPG broth. These findings agree with those of Bernet et al. [22] who found that addition of $FeSO_4$ and $MgSO_4$ promotes NHase production. In contrast, when the two peaks of highest enzyme production, attained at the stationary phase culture, are compared no significant differences in NHase activity were realised by the addition of these chemicals to buffered media.

Further attempts to enhance biomass growth and NHase activity have been pursued with the addition of acrylonitrile and methacrylamide, reported as being NHase inducers [10,24]. Their concentrations in the modified YMPG broth were 5, 10, 20 and 50 mM. Biomass growth, together with NHase specific activity, were evaluated at different fermentation times corresponding to peak production. The addition of either acrylonitrile or methacrylamide at any concentration did not determine favourable effects either on the bacterial growth or on the NHase induction. The specific activity was always lower than that in their absence. The total units of NHase produced at the highest concentration (50 mM) of both chemicals dropped to only 14% of the level attained without their addition.

Another series of fermentations was performed aiming to verify the effect of Cobalt ions on both biomass growth and NHase production. Cobalt-induced NHase is reported in the literature [25]. Biomass and enzyme activity were measured after 24, 120 and 144 h of fermentation in the YMPG broth and in the modified YMPG broth. The presence of $CoCl_2$ at the explored concentration (0.01, 0.015, 0.02 mg $ml^{-1}$) did not promote enzyme formation markedly although it did not inhibit cell growth.

### *3.2. Dependence of kinetic parameters and operational NHase stability on fermentation conditions*

The higher activity and the larger NHase production attained at the maximum activity peak may not justify the cost of a longer fermentation time in the industry, unless operational parameters might be more interesting for a continuous process. Cells with significant NHase activity could be collected from both exponential and stationary phase cultures and from both YMPG and modified YMPG broths. A previous kinetic characterisation of the cells from the two broths proved that the Michaelis constant ($K_m$), the activation energy and the optimum pH of the enzyme are slightly affected by fermentation conditions and the values are in fairly good agreement with those reported in the literature [17,23]. The $K_m$ was 13.0 mM for NHase in cells harvested from the minimal broth and 10.1 mM for that from the modified broth. The activation energy for the acrylamide production catalysed by both cultured cells was 57.53 kJ $mol^{-1}$ in the range from 5°C to 20°C. In contrast, the $V_{max}$ ($\mu$mol $min^{-1}$ $mg_{cells}^{-1}$) was strongly dependent on culture conditions, being different for the NHase units per dry-cell weight. These findings suggested the hypothesis that the same form of NHase was always present and has the same kinetic behaviour of the one isolated and characterised by Nagasawa et al. [10,23]. This hypothesis could be strengthened by the data on NHase operational stability of cells grown for different fermentation times. Further on, the stability investigation could also indicate if the release of metabolites or proteases, which

denature the enzyme and limit the process use of *B. imperialis* cells, occurs.

NHase operational stability was monitored in UF-membrane reactors. This reactor, regarded as a useful tool for laboratory-scale investigation [26], could also be a suitable configuration for continuous industrial processes using soluble biocatalysts. It can operate at both differential and integral conditions depending on the dilution rate, and it allows monitoring of biocatalyst behaviour at well-controlled operational conditions, useful for the simulations of full-scale bioreactors. Performances of reactors operating at 4°C with cells from peak production are illustrated in Fig. 3. The system was stirred to prevent cell deposition on the membrane and was fed (12 ml $h^{-1}$) with acrylonitrile (100 mM) in 50 mM $Na_2HPO_4/NaH_2PO_4$ buffer, pH 7.0. Cell mass (dry weight) in the reactor was 2 mg to prevent an excess of biocatalyst concentration masking the deactivation kinetics. Acrylamide concentration in the effluent stream was measured, and the reaction rate was calculated from product concentration and volumetric flow rate.

According to the analysis reported in Ref. [26] specific reaction rate depends only on the instantaneous level of the active biocatalyst in the reactor. Specific reaction rate ($\mu$mol $min^{-1}$ $mg^{-1}_{cells}$) was reported in the semilogarithmic plot vs. process time; and the straight lines indicate a first-order deactivation mechanism of NHase. The initial transient period corresponds to the product concentration in the reactor reaching steady state rather than being an indication of enzyme behaviour. When this period has elapsed, regression of data allows evaluation of the specific activity at time zero ($r_0$), as *y*-axis intercept, and of the operational deactivation constant ($k_d$), as slope of the straight portion of the curve. The values depend on the temperature, mixing rate, and enzyme and substrate concentrations. In our experiments, the $r_0$ values also vary because of the different specific activity of cells and they agree well with the specific units evaluated from the cell growth cultures. The $k_d$ values are listed in Table 1 and the small differences are within the limits of data reproducibility ($\pm$10%). With the deactivation patterns of NHase in cells harvested from YMPG and modified YMPG being very similar, the indication arises that the same enzyme (at least with the same overall kinetic behaviour) was expressed during the different phases of growth. The differences in medium composition and in the time of harvesting did not affect enzyme properties; and likely neither metabolites nor proteases, which could alter NHase stability, were released from cells during the stationary phase.

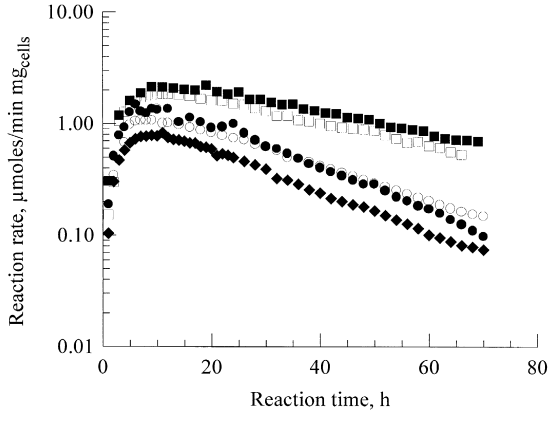


Fig. 3. NHase operational stability in UF-membrane reactor of cells collected at different fermentation times, 4°C, 100 mM acrylonitrile in 50 mM $NaH_2PO_4/Na_2HPO_4$ buffer pH 7.0 and 2 mg of dry cells. Modified YMPG broth: (●) 24 h; (♦) 72 h; (■) 144 h. YMPG broth: (○) 21 h; (□) 120 h.

Table 1
Effect of fermentation conditions on activity and stability of NHase in UF-membrane reactor

| Fermentation broth | Fermentation time, h | $k_d$, $h^{-1}$ | $r_0$, $\mu$mol $min^{-1}$ $mg^{-1}_{cells}$ |
|---|---|---|---|
| YMPG | 21 | 0.035 | 1.68 |
| YMPG | 120 | 0.023 | 2.51 |
| Modified-YMPG | 24 | 0.044 | 2.33 |
| Modified-YMPG | 72 | 0.042 | 1.30 |
| Modified-YMPG | 144 | 0.033 | 3.01 |

### *3.3. Effect of buffer molarity, pH and temperature on enzyme operational stability*

A previous study showed that the change in NHase activity over the pH range from 6 to 8 was almost negligible [17]. The investigation was here enlarged to monitor the operational stability of NHase activity as a function of pH, since enzyme stability too can greatly depend on the pH of the reaction medium.

Runs were performed with 2 mg of cells, collected from the modified YMPG broth at stationary phase, and the UF-membrane reactor was fed with 100 mM acrylonitrile in 50 mM $Na_2HPO_4/NaH_2PO_4$ buffer whose pH ranged from 5.8 to 7.4. Stirring (250 rpm) and temperature (4°C) were selected to minimise deactivation caused by shear stress and temperature. The semilogarithmic plots of reaction rate vs. process time are reported in Fig. 4. The curves are superimposable showing that the deactivation kinetics, as well as the activity are unaffected by pH over this range. This result also clarifies that pH variations during the fermentation periods lead to only negligible differences in cell growth and NHase units between the two broths and at the peak productions. These experiments and those of Ref. [20] performed at constant pH 7.0 and in Na-phosphate buffer at 25, 50 and 75 mM clearly prove that cells can be used in a broad range of pH and ionic strength. The bioconversion of acrylonitrile with NHase from *B. imperialis* could allow operation of an industrial process that has pH fluctuations without loss of performance. This is rather important since exact pH control is harder to achieve than at laboratory scale and more costly are the equipment needed to keep the pH within a narrow range.

Phosphate ions in the product stream are undesirable since they cause during polyacrylamide production the water insolubilisation of polymers,

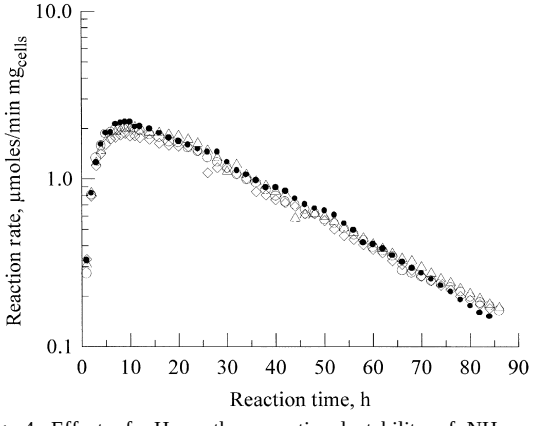


Fig. 4. Effect of pH on the operational stability of NHase in resting cells grown 144 h in modified YMPG broth. Runs in UF-membrane reactors, at 4°C, 2 mg dry cells and 100 mM acrylonitrile in 50 mM $NaH_2PO_4/Na_2HPO_4$ buffer: (△) pH = 5.8; (●) pH = 6.6; (○) pH = 7.0; (◇) pH = 7.4.

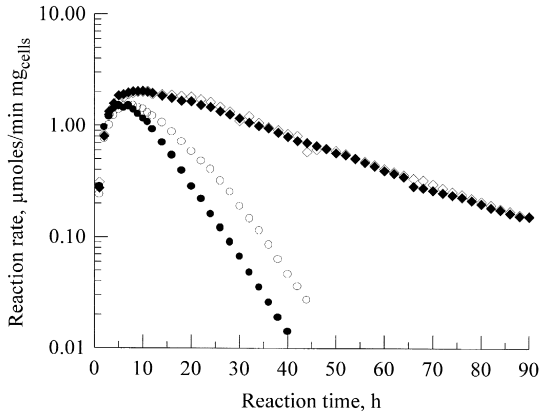


Fig. 5. Effect of temperature on the operational stability of NHase in resting cells grown 144 h in modified YMPG broth. Runs in UF-membrane reactor, 2 mg dry cells and 100 mM acrylonitrile in 50 mM $NaH_2PO_4/Na_2HPO_4$ buffer, pH 7.0 (closed symbols) and in distilled water (open symbols). (●, ○) 10°C; (■, □) 4°C.

especially those of high molecular weight [14]. Generally, a further downstream-processing step is needed to remove the ions and to get a high-quality product. Processes, such as ion-exchange chromatography, are costly and can largely annihilate the advantages of the biological reaction. However, it has been reported in the literature [14] that in unbuffered media the cells swell and the NHase deactivates rapidly. This industry requirement and the fact that NHase in *B. imperialis* is quite stable at low buffer concentration suggested to verify the possibility of replacing the buffer with distilled water. Parallel runs were carried out in distilled water or in 50 mM $Na_2HPO_4/NaH_2PO_4$ buffer, pH 7.0, at two different temperatures, 4°C and 10°C, since 10°C has previously been reported [10] as an optimum temperature for industrial operation.

The data plotted in Fig. 5 show that at 4°C, the specific activity and the deactivation kinetics were the same in distilled water and in phosphate buffer. Only minor differences were observed when operating at 10°C. NHase half-life at 10°C in distilled water (6.0 h) is only 30% the value at 4°C. However, operation at this lower temperature increases energy requirements, and although the cost could be balanced by savings in catalyst requirement and downstream processing, the optimum temperature for industrial processes cannot be settled only on the basis of these results.

### 3.4. Effect of butyric acid on enzyme stability

The addition of butyric acid to the reaction medium as stabilizer of NHase activity in *P. chlororaphis* B23 and *Brevibacterium* CH2 was reported in the literature [14,27]. The operational stability of resting cells from modified YMPG broth was investigated at 4°C in UF-membrane reactors. Butyric acid, 8 and 20 mM, was added in the standard feedstream (100 mM acrylonitrile in 50 mM $Na_2HPO_4/NaH_2PO_4$ buffer, pH 7.0). The results are shown in Fig. 6. The NHase deactivation constant was unaffected by butyric acid at 8 mM ($k_d$ = 0.020 $h^{-1}$) and was slightly lowered at 20 mM ($k_d$ = 0.015 $h^{-1}$). In contrast, enzyme activity was greatly depressed, with 70% and 94% losses of reaction rate measured in the presence of 8 and 20 mM butyric acid, respectively, in comparison with the value in buffer (3.01 μmol $h^{-1}$ $mg^{-1}_{cells}$). This result cannot be attributable to the slight pH shift of the feedstream solution. Consequently, in our process conditions, the addition of this chemical does not contribute any advantages.

### 3.5. Effect of substrate concentration on enzyme inactivation and bioconversion yield

A previous investigation on enzyme inactivation showed that NHase stability depends on acrylonitrile concentration being in the range from 100 to 500 mM [21]. The higher the substrate concentration in the feedstream, the higher the rate of activity loss. The present study aimed to assess the reversibility of inactivation by the substrate. Two runs were performed in UF-reactors at standard operational conditions but with 16.05 mg of cells. Acrylamide productivity is reported in Fig. 7.

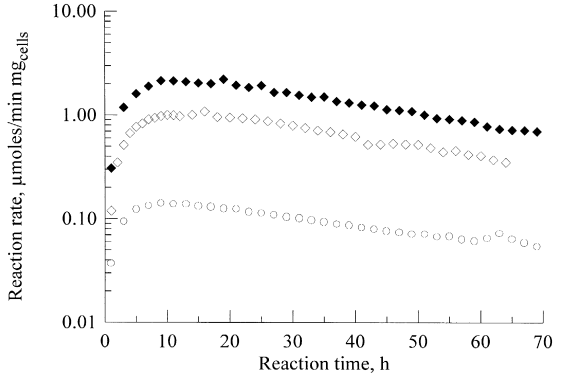


Fig. 6. Effect of butyric acid on the operational stability of NHase in resting cells grown 144 h in modified YMPG broth. Runs in UF-membrane reactors, at 4°C, 2 mg dry cells and 100 mM acrylonitrile in 50 mM $NaH_2PO_4/Na_2HPO_4$ buffer, pH 7.0 and butyric acid: (◆) = 0 mM; (◇) = 8 mM; (○) = 20 mM.

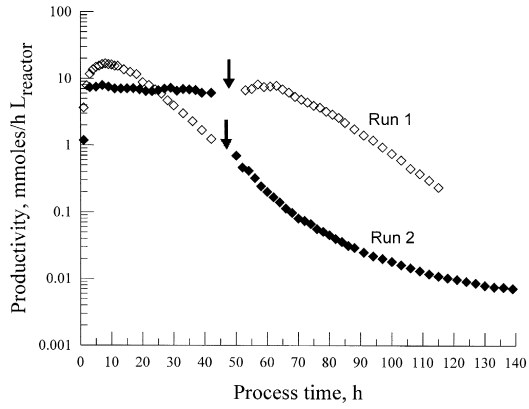


Fig. 7. Irreversible inactivation by acrylonitrile concentration of NHase from resting cells grown 144 h in modified YMPG broth. Runs in UF-membrane reactors, at 4°C and 16.05 $mg_{cells}$. Arrows indicate the change of acrylonitrile concentration in 50 mM $NaH_2PO_4/Na_2HPO_4$ buffer, pH 7.0 (◆) 100 mM; (◇) 800 mM.

In the first experiment, the reactor was continuously fed with acrylonitrile 100 mM for 45 h. After the transient period, a stationary state at 90% conversion was recorded. The reactor feedstream was successively replaced (arrow in the figure) with a solution at higher substrate concentration (800 mM). A consistent reduction in productivity occurred almost instantaneously. The second experiment was run inverting the sequence of the feed operation. Firstly, the reactor was fed with 800 mM acrylonitrile solution for 45 h and then with the 100 mM substrate solution. The reactor response was completely different. The higher substrate concentration allowed reaching a higher level of productivity at a short process time, but a faster inactivation started immediately and the specific activity was reduced. The expected new steady state condition, even at a lower value of productivity, was not attained when the lower substrate concentration feed was introduced. In contrast, the activity continued to decay. However, at this process time (45 h), roughly 95% of the cells initially charged in the reactor were already inacti-

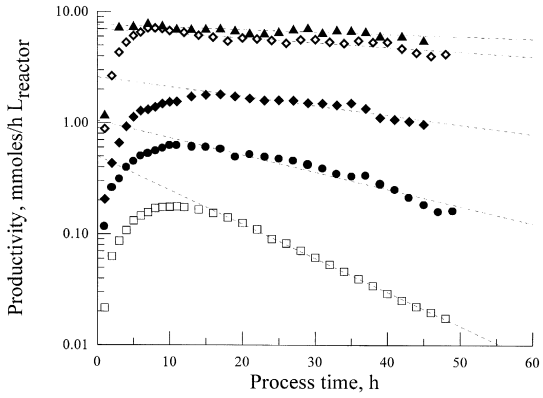


Fig. 8. Effect of resting cells (grown 144 h in modified YMPG broth) concentration on the productivity. Runs in UF-membrane reactors, at 4°C, and 100 mM acrylonitrile in 50 mM $NaH_2PO_4$ /$Na_2HPO_4$ buffer, pH 7.0. The amount of dry cells: (□) 0.27 $mg_{cells}$; (●) 0.53 $mg_{cells}$; (♦) 1.30 $mg_{cells}$; (◇) 10.00 $mg_{cells}$; (▲) 16.05 $mg_{cells}$.

vated. The second part of run 2 occurred at an effective biocatalyst concentration much lower than the first part of run 1, and this could explain the loss of apparent NHase stability. Nevertheless, the observation that the change of substrate concentration from 800 to 100 mM does not determine any recovery of the initial activity is also a clear indication that the cells are irreversibly damaged.

### *3.6. Effect of the amount of resting cells on the reactor productivity*

Most of the study reported was carried out at low cell amount (2 mg) in order to operate in a differential reactor that allows to well clarify the role of operational parameters on NHase stability. In contrast, an industrial bioreactor needs to operate at integral conditions to increase volumetric productivity and for a prolonged process time. These conditions require a high concentration of biocatalyst in the system.

The effect of cell concentration on the bioproduction of acrylamide was investigated at 4°C; and the acrylamide productivity (mmol $h^{-1}$ $l^{-1}_{reactor}$) is illustrated in Fig. 8 as a function of process time. The stirred membrane bioreactor operated with 100 mM acrylonitrile in 50 mM Na-phosphate buffer, pH 7.0. A peristaltic pump assured a constant volumetric flow rate (12 ml $h^{-1}$).

Regression of the data allows the evaluation of the productivity at time zero ($P_0$), as *y*-axis intercept, and the operational deactivation constant ($k_d$). The results are summarised in Table 2 and clearly show that the selected residence time in the bioreactor and the biocatalyst concentration determine conditions of either differential or integral reactor. In the lower range of enzyme concentration, the productivity increases linearly with the amount of resting cells. A further increase determines a less important improvement of the productivity because the amount of resting cells is by far higher than that really involved in the reaction. At high enzyme concentration (16.05 mg), the bioconversion yield is as high as 86% and remains constant during the process. The stabilisation of the NHase activity is observed operating with 10.00 and 16.05 mg of *B. imperialis* cells in the reactor. This improved stability is partially effective since the continuous stirred reactor operated at low acrylonitrile concentration (14–25 mM), because of the higher conversion than in the runs at low cell loading (95–99 mM acrylonitrile). However, the stabilisation is also partially apparent since,

Table 2
Effect of resting cell concentration on acrylonitrile bioconversion

| Resting cells (dry weight), mg | $k_d$, $h^{-1}$ | $P_0$, mmol $h^{-1}$ $l^{-1}_{reactor}$ | Acrylamide concentration, mM | Operational substrate concentration, mM |
|---|---|---|---|---|
| 0.27 | 0.070 | 0.50 | 1–2 | 98–99 |
| 0.53 | 0.036 | 1.05 | 3–4 | 96–99 |
| 1.30 | 0.020 | 2.59 | 1–5 | 95–99 |
| 10.00 | 0.010 | 7.23 | 75–80 | 20–25 |
| 16.05 | 0.005 | 7.65 | 78–86 | 14–22 |

as already pointed out, the excess of cells in the reactor determines a reservoir of biocatalyst.

## 4. Conclusions

*B. imperialis* CBS 489-74 is a good strain for the culture of cells with important NHase activity. The production of enzyme (U $mg^{-1}_{cells}$) was high either at the exponential phase growth or at the stationary phase independently of the use of YMPG or modified YMPG broth. However, because of lower biomass concentrations within a short time, the volumetric production (U $ml^{-1}$) in the shake flask was much higher at the stationary phase. Furthermore, the units of NHase per hour and liter of fermentation broth attained either during the exponential growth phase or at the stationary phase were comparable. Both NHase activity and stability were not affected by fermentation time and broth composition. Therefore, the choice of a longer batch fermentation period (120–144 h) in comparison with the exponential growth (21–24 h) does not depend on differences of biocatalyst properties and should be made only on the basis of the optimisation of culture conditions for the saving of the chemicals used for broth preparation, for the cost of sterilisation and for the reduction of dead times in discontinuous operations.

The overall kinetics did not vary when acrylonitrile solutions were prepared with phosphate buffer at different molarities from 25 to 75 mM, at different pH from 5.8 to 7.4, or with distilled water. The choice of bioconversion temperature determines an optimisation problem. With both rates of acrylamide production and of NHase deactivation increasing with the temperature, the amount of the converted acrylonitrile varies. The weight of acrylamide produced per unit weight of dry cells, calculated on the assumption that the biocatalyst was replaced when its activity reached 10% of the initial one, is 350 g $g^{-1}_{cells}$ during 89 h at 4°C and 162 g during 18 h at 10°C. This comparison should suggest to operate at 4°C with 2 mg of cells. However, because of the different process time needed to reach the above indicated productivity, operation at 10°C should become more profitable (801 g $g^{-1}_{cells}$) at equal total time if sufficient cells are available to be replaced more often. Alternately, higher cell amounts can be initially charged in the reactor and the acrylamide production can be attained in integral conditions for a prolonged time.

Finally, it is important to define the convenient substrate concentration in the feedstream since acrylonitrile causes an irreversible damage of the NHase activity. Operation at high cell concentration could contribute to limit this problem, as the effective substrate concentration in the continuous bioreactor would be lowered.

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