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Influence of initial glucose concentration on nitrile hydratase production in *Brevibacterium imperialis* CBS 498-74

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We wish to dedicated this paper to the memory of Prof. Francesco Alfani, our Friend and Maestro.

Abstract

The growth of *Brevibacterium imperialis* CBS 498-74 (new classification, *Microbacterium imperiale*), with glucose, acrylonitrile, acrylamide and methacrylamide as the C-source, was studied in a shake flask at 28 °C for culture periods of up to 150 h. The optimum initial glucose concentration for nitrile hydratase (NHase) production (131 U ml^{-1} broth) was 5 g l^{-1}. Higher concentrations were found to depress the volumetric enzyme production. Acrylonitrile, acrylamide and methacrylamide cannot be used as the sole C-source. At all tested growth conditions, the highest NHase productivity (U ml^{-1} broth h^{-1}) was reached after 24 h of incubation. Specific activities (U mg^{-1} DWC) in the cell were found to be: 31 with 5 g l^{-1} glucose, 43 with 5 g l^{-1} glucose plus 20 mM acrylonitrile, 47 with 5 g l^{-1} glucose plus 20 mM acrylamide. The addition of methacrylamide was found to be detrimental under all tested concentrations. Yield coefficient increased progressively with initial glucose concentration until 3.5 g l^{-1} and then decreased. Maintenance energy requirement was continuously increasing function of the initial glucose concentration. NHase activity in the whole cell suspension was tested following the biotransformation of acrylonitrile (50 mM) into acrylamide at 20 °C in 50 mM sodium phosphate buffer, pH 7.0. The differently induced NHase had very close K_M (from 9.35 to 9.80 mM). The enzyme in cells grown using glucose as the sole C-source had a V_{max} of 41.86 μmol min^{-1} mg^{-1} DWC, whereas the acrylonitrile and acrylamide induced NHase had a V_{max} of 51.04 and 56.19 μmol min^{-1} mg^{-1} DWC, respectively. The measured activation energy, 28.6 KJ mol^{-1}, indicated a partial control by mass transport through the cell wall.

Keywords: *Brevibacterium imperialis* growth; Nitrile hydratase; Acrylonitrile biotransformation; Enzyme kinetics

1. Introduction

Nitrile hydratase (NHase) (EC 4.2.1.84), which is produced by a number of plants, bacteria and fungi (including *Arthrobacter* sp., *Pseudomonas chlororaphis*, *Rhodococcus* sp., *Brevibacterium* sp. and *Bacillus* sp.), possesses a great potential as a catalyst for the hydration under mild conditions of a large number of diverse nitriles to the corresponding higher-value amides. The use of this biocatalyst represents an alternative to the chemical hydrolysis of nitriles, which usually requires harsh conditions. Cells with NHase activity have been used efficiently for a number of years in the industrial production (≈30,000 t per year world-wide) of acrylamide from acrylonitrile [1–3], the small-scale production of nicotinamide [4,5], and applications in fine chemical and pharmaceutical synthesis, where stereoselectivity provides an additional advantage [5–9]. Nitrile converting enzymes (nitrile hydratase and nitrilase) have been studied recently for

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specific environmental applications [10–12]. These relate to the wide use of nitriles as chemical solvents, recrystallising agents and pesticides which result in soil and water pollution. The demand for efficient biocatalysts and bioprocesses for nitrile degradation is considerable. Much academic and industrial research is focused on this issue, and reports on the optimisation of culture conditions, both for the natural and various mutants species of *Brevibacterium*, are widely reported in the literature [13–17].

In this study, the strain *Brevibacterium imperialis* CBS 498-74 (new classification, *Microbacterium imperiale*) was used for acrylamide production by means of acrylonitrile hydrolysis. This strain produces an intracellular constitutive nitrile hydratase with a specific activity for acrylonitrile bioconversion some 3000 times larger than that of amidase for acrylamide biotransformation. As very little of the produced acrylamide is transformed into the undesired acrylic acid in the course of the reaction, a theoretical conversion approaching 100% becomes possible, thereby minimising the downstream processing operations [18,19].

This study follows previous ones [20–22] on the use of *B. imperialis* resting cells confined in membrane reactors. The fermentation strategy was modified so as to obtain a peak production of NHase during the exponential phase. A subculture was prepared for the inoculation of the fermentation flasks. The two parameters optimised in the experiments were the fermentation time and the glucose concentration. The addition to the culture medium of other C- and N-sources, such as acrylonitrile, acrylamide and methacrylamide, was also investigated: NHases are reported to be inducible by various nitriles, amides and acids [23]. The kinetic behaviour of NHase in acrylonitrile biotransformation was better characterised, and the coefficients of yield and maintenance were determined, from the time course of *B. imperialis* growth and glucose consumption.

2. Materials and methods

2.1. Micro-organism culture conditions

The strain *B. imperialis* CBS 498-74 (kindly supplied by Istituto G. Donegani, Italy) was utilised throughout this study. A 100 ml nutrient broth culture was used to inoculate 500 ml flasks in a rotary shaker (G25-KC, obtained from New Brunswick Scientific, USA). All fermentations were carried out at 28 °C and 220 rpm 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), $0.01\,g\,l^{-1}$ of $FeSO_4{\cdot}7H_2O$ and $0.885\,g\,l^{-1}$ of $MgSO_4{\cdot}7H_2O$. 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. Following sterilisation, the medium pH was adjusted, where required, to 7.0. pH variations during the fermentation periods gave rise to negligible differences in cell growth and NHase units, and NHase activity and deactivation kinetics were also unaffected [22]. Sterile glucose, at initial concentrations ranging from 1 to $40\,g\,l^{-1}$, was added separately to the cultures.

The inoculum preparation of *B. imperialis* was performed in YMP-medium without added glucose (in order to avoid its carryover from inocula to production fermentations), and was started aseptically by picking one colony off an agar nutrient plate, where *B. imperialis* was routinely maintained. After 24 h incubation at 28 °C, 10 ml of inoculum were transferred to the fermentation flask. The average dry cell yield in the starter culture was 0.97 ± 0.18 mg DWC ml^{-1}, and the specific activity was 25.41 ± 3.0 U NHase mg^{-1} DWC. All runs were replicated at least twice and average values are reported in this work.

2.2. Induction of nitrile hydratase activity

Induction of NHase activity was performed by growing *B. imperialis* with acrylonitrile, acrylamide or methacrylamide in the concentration range: 0–50 mM as the C- and N-sources. The chemicals were added aseptically to the YMP-medium before inoculating the shake flasks. Runs were also carried out in the presence of glucose at $5\,g\,l^{-1}$. Cells were analysed for growth and enzyme activity 24 h after inoculation.

2.3. Growth determination

This measure was carried out either by dry-weight estimation or spectrophotometrically. The broth was sampled at regular time intervals and centrifuged at 11,400 rpm for 15 min at 4 °C. The supernatant was

discarded. The biomass was then washed twice with 100 ml distilled water to eliminate the spent growth medium. Finally, the cells were dried to constant weight (at 80 °C for 24 h) and weighed.

The growth of *B. imperialis* culture over the fermentation period was also assessed by means of optical density (OD) measurements at 610 nm (spectrophotometer model Lambda 2, Perkin-Elmer, USA), the cell paste having previously been diluted to give an absorbance below 1.0. Zero absorbance was set using water, and a calibration chart was prepared to correlate absorbance with weight by drying a solution of known optical density: this showed a unit of optical density (at 610 nm) to correspond to 0.26 mg DWC ml^{-1} of *B. imperialis* suspension.

2.4. Enzyme activity assay

NHase activity was measured for 15 min at 20 °C by direct assay of whole cells using 50 mM of acrylonitrile (Aldrich, Germany) as substrate, and a quantity of cells in the range 0.1–0.2 mg DCW, depending on the specific activity. The reaction medium (2 ml volume) was buffered with 50 mM Na_2HPO_4/NaH_2PO_4 buffer, pH 7.0, and stirred continuously at 250 rpm. The reaction was halted 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 (U) of NHase activity was defined as the amount of resting cells that catalyses the formation of 1 μmol of acrylamide min^{-1} under the adopted conditions. Specific activity was expressed as U mg^{-1} DCW. All tests were performed in duplicate and the mean activity value reported.

2.5. Analytical methods

Glucose was determined in the supernatant of broth samples with the GOD-Perid kit (Boehringer-Mannheim, Germany), a calibration curve being first produced using known glucose concentrations in a broth medium.

Quantitative analysis of acrylonitrile and acrylamide in the reaction system was performed by HPLC (Perkin-Elmer series 2, USA) and a reversed-phase, 25 cm × 4 mm column (Merck C18, USA) operating at 30 °C at a flow rate of 0.5 ml min^{-1}. The mobile phase was prepared with pure acetonitrile and KH_2PO_4/H_3PO_4 buffer (10 mM, pH 2.8) at 1–10 (v/v). As the retention times of acrylonitrile and acrylamide are quite different, the absorbance at 220 nm allowed for the determination of both concentrations. For rapid analysis, acrylamide concentration was also measured spectrophotometrically at 235 nm, a wavelength at which neither the acrylonitrile nor the buffer interfere with the readings.

3. Results and discussion

Optimal growth conditions in the presence of 2% (w/v) glucose, and the relationship between *B. imperialis* cell growth and NHase production have been assessed in previous experiments [20,21]. A significant production of NHase occurred in the late stationary phase during 120 and 144 h of fermentation. Cells for laboratory use were harvested at the maximum activity peak (144 h) and tested in diverse configurations of small-scale bioreactors [20–22]. For large scale applications, however, the achievement of a high activity after a long period of incubation must be offset against the cost of a long fermentation. New growth conditions were, therefore, explored to identify a more practical activity peak during the exponential growth phase—potentially useful for enzyme production in a continuous process. Protease activity was also taken into account: this is known to be maximal at the end of the exponential phase, and should be limited.

The appearance of a NHase peak in the late stationary phase, when the glucose in the medium should be completely exhausted, suggested the exploration of the dependence on glucose concentration in the broth of both biomass growth and NHase production. The effect of acrylonitrile, acrylamide and methacrylamide as alternative C-sources on the volumetric production of NHase was also investigated.

3.1. Effect of glucose

In a first series of experiments, fermentation time courses were followed in duplicate flask cultures containing YMP-medium and sterile glucose. Initial glucose concentrations were 2.5, 5.0, 7.5 and 10.0 g l^{-1}. Ten millilitres of inoculum (25.77 U mg^{-1} DCW; 1.1 mg DCW ml^{-1}) from a 24 h old *B. imperialis* culture was produced in a subculture as reported

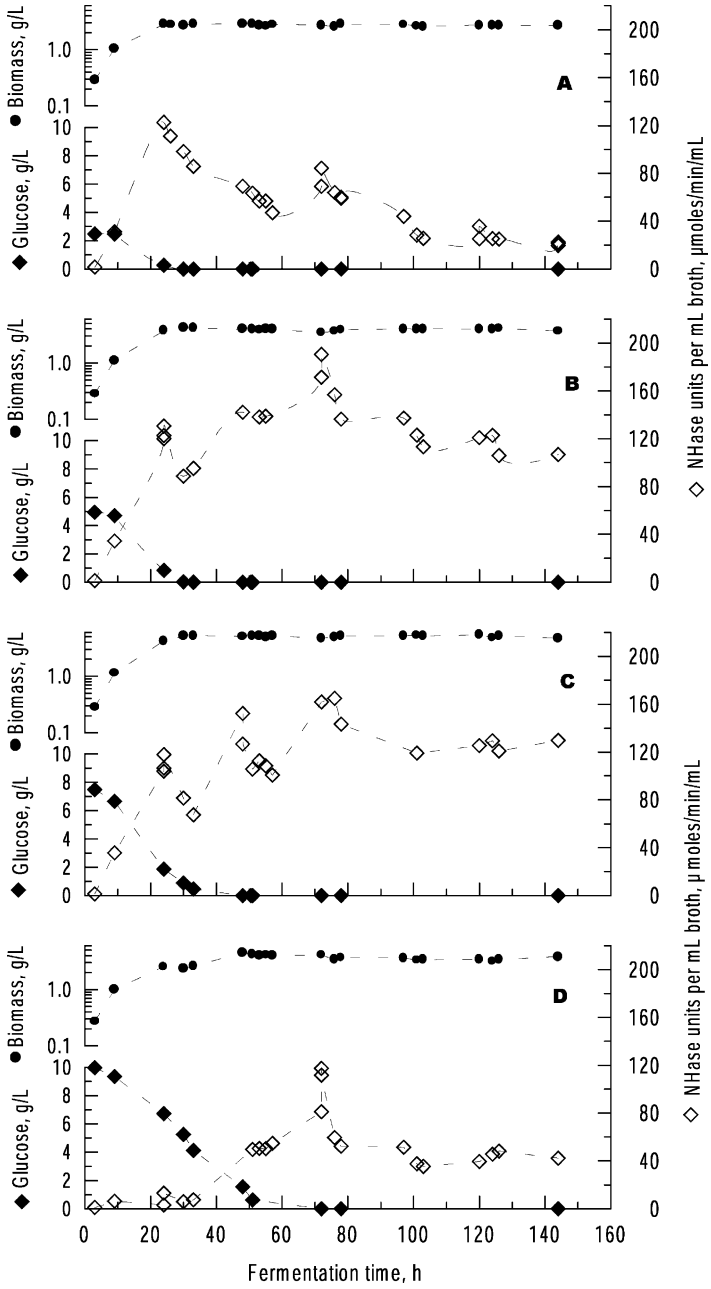

Fig. 1. Time course of NHase volumetric production (◇) on right-hand axis; glucose consumption (◆) on lower left-hand axis; and biomass production (●) on upper left-hand axis. Initial glucose concentration are: (A) 2.5 g l^{-1}; (B) 5.0 g l^{-1}; (C) 7.5 g l^{-1}; (D) 10.0 g l^{-1}. Dashed lines are added to easily follow the data points.

in Materials and Methods. Each flask was sampled under aseptic conditions to determine NHase activity and the biomass and glucose contents. The NHase activity of the cells was monitored following the conversion of acrylonitrile into acrylamide. The culture filtrate did not exhibit NHase activity, indicating that the enzyme was intracellular. Fig. 1 shows the time course, over a period of 144 h, of fermentations carried out with glucose at the four initial concentrations. Residual glucose concentration ($g\,l^{-1}$) is reported on the lower left-hand axis, the biomass produced ($g\,l^{-1}$) on the upper left-hand axis, and the NHase units ml^{-1} of broth ($\mu mol\,min^{-1}\,ml^{-1}$) on the right-hand axis.

As far as biomass growth is concerned, the time needed to attain the stationary phase increased progressively from 20 to 40 h with increasing initial glucose concentrations. Cell growth exhibits little, if any, initial lag period. Growth appears to proceed exponentially only in the first 15 h with a specific rate of approximately $0.38\,h^{-1}$, and reaches the stationary phase when glucose is exhausted. Cell concentrations were 2.7, 3.9, 5.3 and 3.7 $g\,l^{-1}$ at 24 h for 2.5, 5.0, 7.5, 10.0 $g\,l^{-1}$ initial glucose concentration, respectively. Fig. 1 shows firstly that the time at which the main peaks in NHase production occurred depended on the initial glucose concentration in the culture: the higher the initial glucose concentration, the longer the lag period before the appearance of the main NHase peak production. Secondly, the presence of NHase activity in the cells generally appeared after the glucose concentration had dropped to 2 $g\,l^{-1}$ or less. The highest volumetric production of enzyme (122–131 U ml^{-1}) was achieved after 24 h fermentation at low glucose concentration, 0.25 and 0.50% (w/v). These results clarified the importance of glucose for cell growth but also indicated the possibility that NHase production might be repressed by glucose. The observed periodicity of enzyme production during culture might be due to the poor NHase stability of *B. imperialis*. This latter was previously determined at fermentation temperature, 28 °C, and the first-order deactivation mechanism exhibited an operational half-life equal to 2.11 h. Besides, differences in medium composition and in the time of harvesting did not affect enzyme kinetic parameters, and likely neither metabolites nor proteases, which could alter NHase stability were released from cells during the stationary phase [22]. Therefore, two opposing effects—production and deactivation of the enzyme—determine the effective instantaneous NHase activity in cells collected at different times. Moreover, if the hypothesis of glucose repression is sound, a long fermentation period should elapse before the major enzyme production rates are reached, and during this time a large part of the activity of the already produced enzyme will have been curtailed by deactivation. A maximum in the curve of NHase production as a function of initial glucose concentration is, therefore, to be expected.

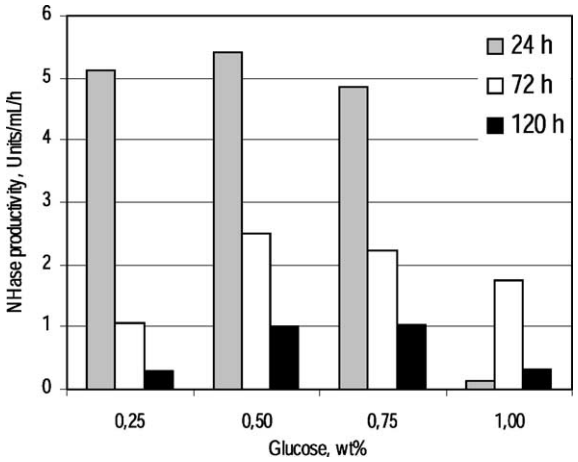

Fig. 2. Effect of various initial concentrations of glucose on the NHase volumetric productivity. Fermentation time: shadow bar, 24 h; open bar, 72 h; dark bar, 120 h.

This reasoning is in accord with the data shown in Fig. 2, where nitrile hydratase productivity is reported as function of the initial glucose concentration after three diverse fermentation periods. The highest values were attained at 24 h of growth, the only exception being for experiments conducted at the highest initial glucose concentration of 10 $g\,l^{-1}$ glucose—where the glucose concentration in the broth after 24 h was still too high ($\approx$6.6 $g\,l^{-1}$) to overcome the repression of NHase formation. Fig. 2 also shows maxima in the NHase productivity after 72 and 120 h of fermentation, though much smaller ones than at 24 h. It would appear that enzyme deactivation weighs heavily during the time elapsed before the appearance of these NHase production peaks.

At this stage of investigations, the periodic production of enzyme during culture is difficult to explain on other evidences. Experiments performed in

YMP-medium without glucose (data not reported) did not show significant enzyme peak production thus suggesting that the other compounds from the complex media are irrelevant. A major peak was obtained at 20 h with a productivity of 35 U ml^{-1}. On the other hand, cultures in a simple mineral salt medium with glucose 5 g l^{-1} gave rise again to several peaks with a major one of 12 U ml^{-1} at 24 h.

These initial findings suggested a more detailed study of the phenomenon of glucose repression of NHase production. Further fermentations (run in triplicate) were, therefore, carried out, enlarging the initial glucose concentration range from 0.1 to 4% (w/v). Twenty-four hours after the addition of the inoculum to the broth, the culture were halted. The enzyme production during this period was not greatly affected by deactivation. Fig. 3 illustrates the data of NHase specific activity, NHase productivity and biomass growth as functions of initial glucose concentration. The NHase productivity improved continuously with initial glucose concentrations from 1.0 to 5.0 g l^{-1} and fell above 7.5 g l^{-1}. In the interval from 1 to 7.5 g l^{-1} biomass production increased continuously as a function of glucose concentration. For glucose concentrations higher than 10.0 g l^{-1}, NHase production was practically nil and the growth of *B. imperialis* was considerably lowered. However, the different dependencies of NHase production and biomass growth on glucose concentration led to the highest specific activity in the cells, U mg^{-1} DWC, when the culture was started with 2.0 g l^{-1} nutrient.

3.2. Effect of other carbon source

The effect of different C-sources on enzyme production was also explored. To this end, another series of fermentations was carried out using acrylonitrile, acrylamide, and methacrylamide, in the concentration range 0–50 mM, as the sole C-source in the reference YMP-medium without glucose. These chemicals have been successfully used as inducers in other studies [23]. As both biomass growth and NHase production were found to be practically nil in these cultures, the conclusion was reached none of them is suitable as a sole C-source (data not shown). However, fermentations were run supplementing the YMP-broth prepared with the optimum glucose concentration (5.0 g l^{-1}) with each of these three chemicals in the concentration range 0–50 mM. The flask cultures (at least in triplicate) were started with 10 ml of inoculum.

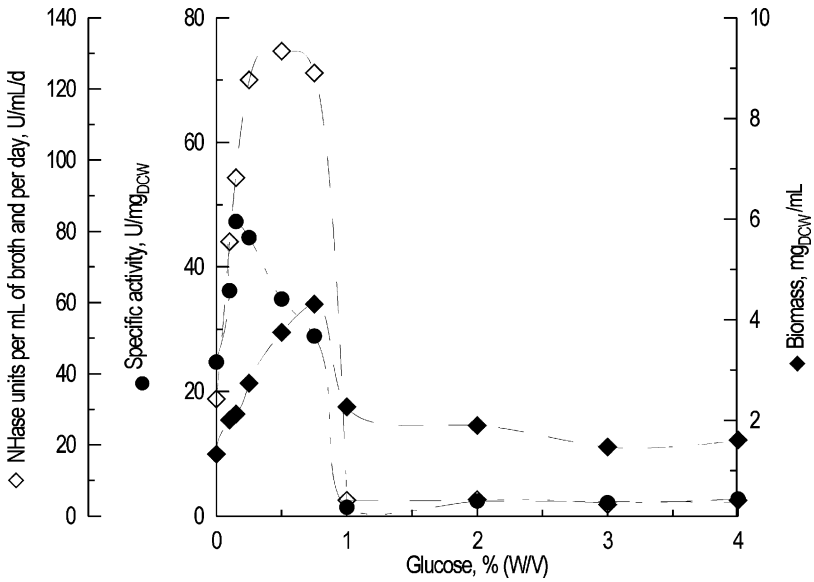

Fig. 3. Effect of various initial concentrations of glucose on the daily volumetric productivity of NHase (left-hand axis, ◇) on the specific activity (left-hand axis, ●) and on the biomass growth (right-hand axis, ◆). Dashed lines are added to easily follow the data points.

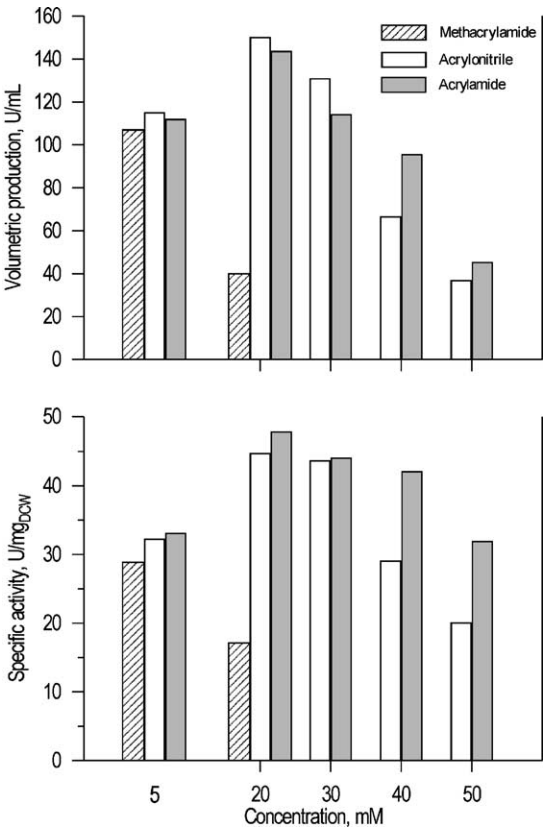

Fig. 4. Effect of methacrylamide, acrylonitrile and acrylamide on the volumetric production of NHase (upper plot) and on the specific activity of NHase (lower plot) in cells grown in the contemporary presence of glucose 0.5%.

Biomass concentration and NHase specific activity were evaluated in cultures terminated after 24 h of fermentation. Volumetric production (U ml^{-1}) and specific activity (U mg^{-1} DWC) are reported in the upper and lower plots of Fig. 4 as function of the chemical concentration. The addition of methacrylamide was always detrimental and inhibited completely NHase production at concentrations above 30 mM. In contrast, the addition of acrylonitrile and acrylamide (in 20–30 mM concentration range) induced a higher NHase activity in the cells. Specific NHase activity reached the value of 43 U mg^{-1} DWC with acrylonitrile, and 47 U mg^{-1} DWC with acrylamide, which compares with approximately 35 U mg^{-1} DWC in cultures with solely 5.0 g l^{-1} of glucose. As biomass growth also depended on the added chemical and its concentration, the U ml^{-1} of broth varied in the diverse tested conditions. Biomass content, after 24 h of culture, was 3.75 mg DWC ml^{-1} with solely 5.0 g l^{-1} of glucose, and 3.4 and 3.0 mg DWC ml^{-1} upon the addition of the optimum concentration (20 mM) of acrylonitrile and acrylamide, respectively. These led to only slightly different volumetric productions of NHase (U ml^{-1}) as shown in the upper plot: 146 U ml^{-1} with acrylonitrile, and 141 U ml^{-1} with acrylamide, as compared to 131 U ml^{-1} in the reference culture. These experimental results clearly indicate that acrylonitrile and acrylamide have similar NHase-inducing properties. However, this effect could be attributable to acrylamide only. In fact, the specific activity of the inoculum should result in the complete transformation of the initially present acrylonitrile in roughly 5.4 min. Therefore, biomass growth and NHase production occurred always in the presence of acrylamide. It is impossible in practice to keep the acrylonitrile concentration constant by means of its continuous addition without also varying simultaneously the concentration of the formed acrylamide.

3.3. Kinetic and culture parameters

The kinetic parameters and the activation energy of acrylonitrile conversion to acrylamide were determined by varying the substrate concentration from 2 to 50 mM and the temperature from 5 to 20 °C. The Michaelis–Menten equation described well the rate of the reaction catalysed by NHase within the cells for three different culture conditions. The kinetic parameters, quoted in Table 1, are very similar, indicating that the addition of acrylonitrile and acrylamide to the *B. imperialis* culture did not alter the substrate transport through the cell wall and the enzyme specificity toward the substrate. Reaction rates were found

Table 1
Effect of carbon source on the kinetic parameters of acrylonitrile bioconversion

Carbon source	K_M (mM)	V_{max} (μmol min^{-1} mg^{-1} DWC)
0.5% (w/v) Glucose	9.80	41.86
0.5% (w/v) Glucose plus 20 mM acrylonitrile	9.35	51.04
0.5% (w/v) Glucose plus 20 mM acrylamide	9.78	56.19

to follow the Arrhenius law, with an activation energy of 28.6 KJ mol^{-1} for the runs with 50 mM acrylonitrile and 0.078 mg DWC ml^{-1} of *B. imperialis* cells. This rather low activation energy indicates the overall biotransformation rate to be partially controlled by mass transport through the cell wall. The body of these results leads to the conclusion that differences in the catalytic behaviour can be solely attributed to the cell enzyme content and the kinetic parameters of the Michaelis–Menten equation are apparent.

Finally, the glucose consumption was accurately monitored during a culture period of 24 h for the fermentations carried out with 2.5, 5.0, 7.5 and 10 g l^{-1} of initial glucose concentration, and reported in the Fig. 5. Fig. 6 shows biomass data in these fermentations (plain symbols) together two reference fermentations performed in the YMP-medium without added glucose, and in a broth prepared with solely 5.0 g l^{-1} of glucose in the salts medium. The data here reported well evidence the synergistic effect on growth of nutrients in the complete medium. The specific growth rate (μ) and the biomass variable yield ($Y_{X/S}$), which describes the actual amount of biomass produced (gram) per gram of glucose, were determined at diverse fermentation times in YMP-medium containing glucose cultures. The specific growth rate was 0.38 h^{-1} in the first 10 h and ranged from 0.073 to 0.106 h^{-1} after 11 h until the onset of stationary phase. This growth behaviour suggests an environment containing multiple limiting substrates, each serving as both carbon and energy sources, in which the organism grows sequentially on the available substrates and consumes first the substrate that maximises the specific growth rate. The coefficients of yield $Y^{G}_{X/S}$, a constant which represents the fraction of the substrate removed which is channelled into growth function, and of maintenance energy requirement (m), the specific substrate utilisation for energy maintenance, were calculated by means of the well known relation:

$$\frac{1}{Y_{X/S}} = \frac{1}{Y^{G}_{X/S}} + \frac{m}{\mu}$$

where $Y^{G}_{X/S}$ and $Y_{X/S}$ are dimensionless, while μ and m are expressed as h^{-1}. Values of $Y^{G}_{X/S}$ and m were quoted in Table 2. $Y^{G}_{X/S}$ increases progressively with initial glucose concentration until 0.75% then decreases indicating a possible glucose inhibition on growth. This behaviour might be, however, only apparent since at low initial glucose concentration the

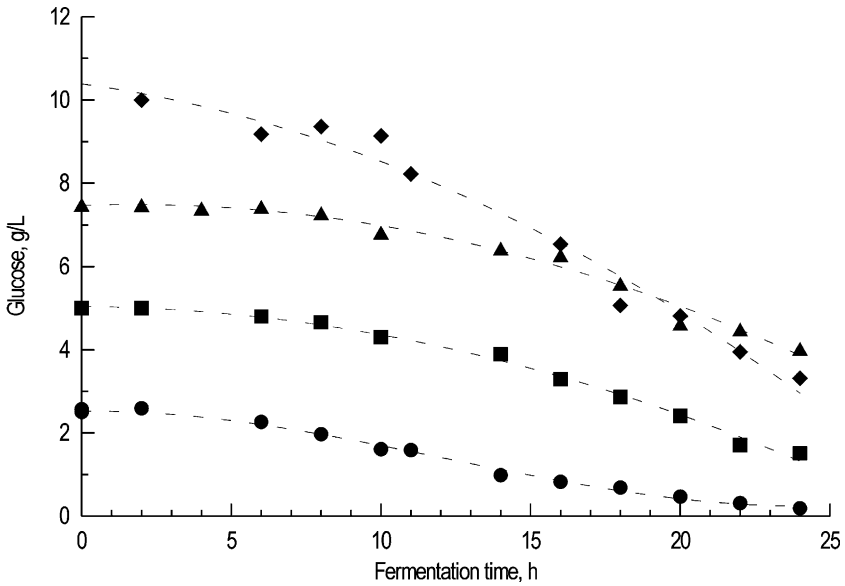

Fig. 5. Time course of glucose consumption as function of initial glucose concentration: (●) 2.5 g l^{-1}; (■) 5.0 g l^{-1}; (▲) 7.5 g l^{-1}; (◆) 10.0 g l^{-1}. Dashed lines are higher-order polynomial curve-fitting.

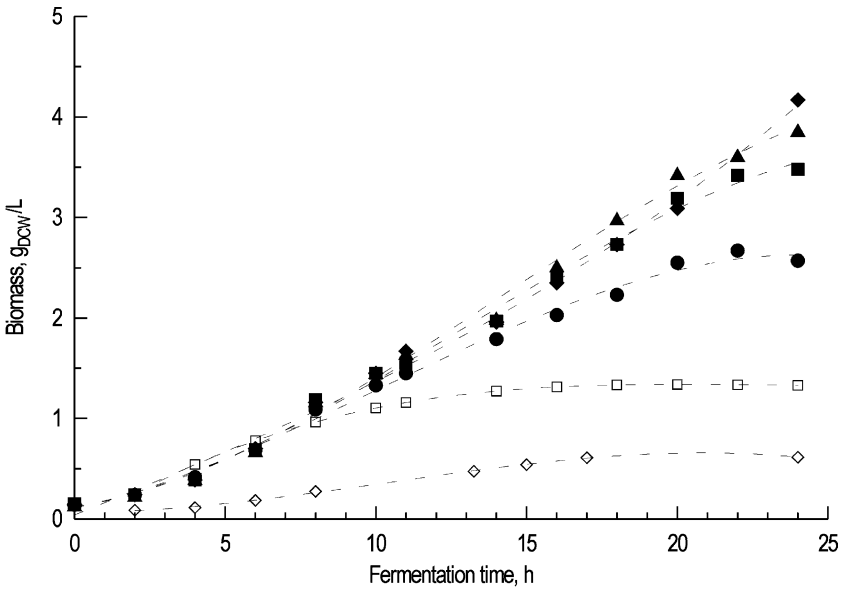

Fig. 6. Time course of biomass growth as function of initial glucose concentration (plain symbols): (●) 2.5 g l^{-1}; (■) 5.0 g l^{-1}; (▲) 7.5 g l^{-1}; (◆) 10.0 g l^{-1}. Reference fermentations: (□) YMP-medium without glucose; (◇) salts medium plus glucose 5.0 g l^{-1}. Dashed lines are higher-order polynomial curve-fitting.

Table 2
Effect of initial glucose concentration on growth yield and maintenance energy requirement

Glucose concentration (%, w/v)	m (h^{-1})	$Y^{G}_{X/S}$
0.25	0.023	1.56
0.50	0.036	1.74
0.75	0.062	3.50
1.00	0.070	0.85

contribute to growth from complex medium might weigh more. The dependence of m was not linear and approached an exponential behaviour. A better quantitative knowledge of this behaviour should be obtained by comparing the biomass and glucose profile generated by different mathematical models reported in the literature.

4. Conclusions

Shake flask cultivations of *B. imperialis* on complete medium containing glucose at different concentrations as the C-source indicated that growth reaches the maximum in cultures starting with 5.0 g l^{-1} of glucose, decreasing at higher initial concentrations. The coefficients of yield and maintenance are increasing functions of initial glucose concentration.

The appearance of a peak of NHase activity in the cells is associated with a residual glucose concentration in the broth below 2.0 g l^{-1}. Because of the important thermal deactivation of the enzyme at the fermentation temperature of 28 °C, the highest production of NHase per litre of broth and per unit weight of dry cell is attained in the exponential phase (24 h). This result is interesting as it may be achieved without incurring the costs of long time fermentation.

Complete substitution of glucose with alternative C-sources (acrylonitrile, acrylamide and methacrylamide) totally depresses cell growth. In contrast, the addition of both acrylonitrile and acrylamide (20 mM) to a broth prepared with 5.0 g l^{-1} of glucose results in a 15% increase of NHase units per litre of culture even though biomass production is slightly impaired with respect to fermentation with only glucose as the C-source. The addition of methacrylamide was found to be detrimental at all tested conditions. The kinetic parameters determined following the bioconversion

of acrylonitrile into acrylamide suggest that the same form of enzyme is produced under the various tested conditions. The culture filtrate does not exhibit NHase activity, indicating that the enzyme is intracellular. The relatively low activation energy indicates that the biotransformation is controlled by mass transport through the cell wall.

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