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Hydration of cyanopyridine to nicotinamide by whole cell nitrile hydratase

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SUMMARY

3-cyanopyridine was hydrated to nicotinamide by whole cells of *Brevibacterium* R-312 containing nitrile hydratase. Cells used for kinetic studies had an initial activity of 0.30 mg nicotinamide/mg cells(dry)-min and storage half-lives (pH 8) of approximately 100 days, 10 days, 5 days and less than 1 day at 4°C, 10°C, 25°C, and 30°C respectively. Temperature and pH maxima were 35°C and 8.0, respectively. Fermentations gave a maximum total hydratase activity of 1.25 mg nicotinamide/min, but at this maximum the amidase activity was unacceptably high (25% of the hydratase activity): nicotinamide was converted too rapidly to nicotinic acid. But systematic fermentation studies (7 l) showed that harvesting at mid-log phase (18–20 h) prior to the attainment of maximum total activity gave reasonably high levels of hydratase (0.3 mg nicotinamide/mg cells-min) and acceptable levels of amidase (0.03 mg nicotinic acid/mg cells-min).

INTRODUCTION

Commercially important amides (e.g. acrylamide, nicotinamide) are produced currently by hydration of the corresponding nitriles via a variety of harsh synthetic reactions (e.g. acid/base hydrolysis, ammoxidation). These share some common disadvantages: formation of large quantities of salts, high energy demands, deleterious side reactions, critical purification problems, and inability to obtain optically-active products.

Enzymatic hydration of nitriles to amines may provide an attractive alternative to chemical syntheses for some products. Nitrile hydratase has been shown to effect under mild conditions single step hydrations to amides of many nitriles, and published results [1–5,7,9–11,14–25] indicate potential applications ranging from commodity chem-

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icals to specialty chemicals. But there are some formidable problems: rapid enzyme decay at temperatures higher than 10–20°C; the presence of amidase enzymes which convert amides to the corresponding carboxylic acids, and which are produced by most organisms producing nitrile hydratase; and substrate and product inhibitions of the hydratase.

To date, only the enzymatic hydration of acrylonitrile to acrylamide has received any in-depth study or attention. Several patents [15–20] have been issued to Nitto Chemical Industry describing processes in which aqueous solutions of acrylonitrile are hydrated by poly-acrylamide entrapped cells.

The patents claim essentially quantitative conversion in batch, fed-batch and multiple packed-bed reactors at final concentrations up to 15%. Temperature, pH and catalyst stability are noted as critical variables. Optimum temperature and pH are given as 10°C and 7.5, respectively. The patents describe genera of microorganisms including *Nocardia, Microbacterium* and *Corynebacterium*. A second-generation *Pseudomonas* with better catalytic activity has been developed at Kyoto University [21]. Nitto has constructed a 400 metric tonne per year pilot plant to perform the hydration [21] but no significant information has been published.

In general, the literature provides very little of the information needed for rational evaluations of technical and economic feasibilities of proposed processes. Also, details of routine, reliable fermentation methods for enzyme production have not been published for anything other than shake-flask cultures. In addition, much remains to be done (1) to determine the spectrum of commercially important substrates for, and products of, hydratase hydrations, (2) to find new and better sources of the enzyme and (3) to develop means (e.g. immobilization) to apply them economically.

The objectives of the work presented here were to develop reliable fermentation protocols which would:

1. produce enough cells for extensive evaluation of the enzymatic properties of whole cells and for the production and evaluation of crude extracts and immobilized cells and 2. produce cells containing as little amidase as possible but containing reasonably high concentrations of hydratase.

The details of the fermentation and the enzymatic characteristics (hydratase and amidase) of the whole cells (non-growing) are reported in this paper.

MATERIALS AND METHODS

Strain selection

Brevibacterium R-312, the A_4 mutant of R-312, *Corynebacterium* N771 (Ferm-PNO 4445), *Corynebacterium* N774 (Ferm-PNO 4446), and *Nocardia* N775 (Ferm-PNO 4447) were evaluated on the basis of cell suspension hydratase and amidase activities under fixed conditions [8]. All of these strains were known to have hydratase activity for aliphatics [3,6,15].

Cell suspensions were prepared as follows: 100 ml of fermentation medium (see below) in a 500-ml flask was inoculated aseptically from an agar slant. The flasks then were incubated in a rotary shaker bath (New Brunswick Scientific Co., Model G-28) operated at 200 RPM and 30°C for 16, 24 or 36 h. After incubation, the broth was centrifuged at 18 000 RPM and 4°C. Cells then were washed in pH 8 phosphate buffer and were recentrifuged. This step was repeated. Finally, cells were assayed by the standard method described below.

The *Brevibacterium* R-312 strain was selected [8] (see Results), and was used throughout this study. It was maintained at 4°C on agar slants containing: Difco peptone, 3%; Difco yeast extract; Difco agar, 1%.

Fermentation medium

The fermentation medium contained (g/l in dionized water): glucose, 10; casamino acids, 10; yeast extract, 1.0; $MgSO_4 \cdot 7H_2O$, 1.0; $(NH_4)_2SO_4$, 5.0; KH_2PO_3, 1.0; NaCl, 1.0; $CaCl_2 \cdot 2H_2O$, 0.1; $CuSO_4$, 0.04; KI, 0.1; $FeCl_3 \cdot 6H_2O$, 0.1; $MnSO_4 \cdot 4H_2O$, 0.2; Na_2MoO, 0.2; $ZnSO_4 \cdot 6H_2O$, 0.4 [8].

Fermentation procedure

All fermentations were conducted according to the following protocol [8]. Cultures first were trans-

ferred to new agar slants, and were incubated for 48 h at 30°C. 100 ml of fermentation medium in a 500-ml flask (pre-sterilized at 121°C for 20 min) then was inoculated aseptically from one agar slant. The flasks were incubated in the shaker bath at 30°C for 24 h. Next, 5 liters of fermentation medium was placed in a 7 liter, glass fermentor (New Brunswick Scientific Co. Microferm). The vessel and its contents were autoclaved at 121°C for 1 h, and then were cooled to room temperature. Thiamine hydrochloride was added via a 0.45 sterile filter (Nucleopore) to the cooled fermentor. The fermentor then was inoculated aseptically with the contents of two shake flasks.

The operating conditions were: temperature was held constant at 30°C throughout the fermentation; the initial agitation rate was 300 RPM; aeration was constant at 5 SLPM; pH was maintained at 7.0 by automatic addition of 0.4 N KOH (controlled by a Horizon Model 5997 pH controller coupled to an Ingold pH probe); dissolved oxygen was held above 10% (relative to saturation at atmospheric conditions) by increasing manually the agitation rate (450 maximum) in response to the reading from a Fermentation Design D.O. monitor coupled to an ABEC D.O. probe.

Samples were taken aseptically by first flushing the sample line with about 50 ml of broth (discarded), and then taking the actual sample which was refrigerated until it could be analyzed. All samples were analyzed for cell mass and glucose concentrations and for hydratase and amidase activities.

Cell harvest

Cell harvest was accomplished in two steps: foam fractionation followed by centrifugation. Initial studies showed that enough foam could be generated in the fermentor to carry out almost all of the cell mass and concentrate it. This was used to advantage to reduce dramatically the volume prior to centrifugation. This was accomplished by promoting a 'foam-over' into a sterile, large trap placed between the fermentor and the air exhaust filter. During this stage of the harvest, we increased the air flow and agitation rate to their maxima (10 SLPM and 450 RPM, respectively) to generate as much foam as possible.

The foamed-over broth was centrifuged at 18 000 RPM at 4°C, and then was washed with 0.05 M phosphate buffer. Centrifugation and washing were repeated.

Cell mass determination (dry weight)

A pan and filter were dried and tared. Whole culture broth was filtered (0.45 Nucleopore filter), and then was washed with distilled water. The filter was then transferred to the weighing pan, and the pan, the filter and the washed cells were then dried to constant weight. All other dry weights (e.g. for assays) were determined similarly.

Glucose determination

A YI glucose analyzer (Model 23, Yellow Springs Instruments, Yellow Springs, OH) was used to determine glucose. Fermentation samples were diluted appropriately with deionized water. A 1 μl diluted sample was then injected directly into the instrument which gave the glucose measurement directly in mg/dl. The instrument was calibrated frequently with standard glucose solutions.

Enzyme activity determinations

A 0.2 ml cell suspension having a known dry weight was added to 2.0 ml of 8.0 g/l (76.84 mM) 3-cyanopyridine (Aldrich Chemical Co.) buffered at pH 8 with 0.05 M phosphate buffer. The mixture was agitated in a reciprocating shaker bath operated at 125 strokes per minute, at 25°C. 0.1 ml samples were diluted with 1 ml or 2 ml of distilled water at pH 2.3–3.0 (HCl) to stop the reaction. The diluted samples were kept in an ice bath until analyzed.

Analyses were done by means of an IBM Liquid Chromatograph (Model 9533 Ternart Gradient LC) with an IBM Instruments computer system. A Chromatography Applications Program (IBM CAP Version 3) was used with the computer system. A reverse phase column (Waters Associates Nova Pack C18, 5 spherical silica packing) was used to separate nicotinamide, nicotinic acid and 3-cyanopyridine (see [8] for further details).

Specific hydratase activity, v, of cell suspensions was defined as follows:

$$v = \frac{\text{mg nicotinamide formed}}{\text{mg cell dry weight-min}}$$

Specific amidase activity was defined similarly.

Storage stability of whole cell suspensions

Washed cells were stored in pH 8 phosphate buffer at 4°C, 10°C, 25°C, and 30°C, and were assayed periodically (as per the assay method, above). The results (Fig. 1) show that the half-lives of cells so stored are approximately 100 days, 10 days, 5 days, and less than 1 day at 4°C, 10°C, 25°C, and 30°C, respectively. On the basis of these results, cells were stored routinely at 4°C and were discarded after a maximum of 60 days.

Routine assay studies were not done at temperatures higher than 25°C because hydratase half-lives at higher temperatures were too short (e.g., less then 1 day at 30°C): it did not appear to be practical to use cells having such limited stability.

RESULTS AND DISCUSSION

Strain selection

Brevibacterium R-312 had the highest 3-cyanopyridine hydratase among the five strains tested (see Table 1). Also, all strains which had hydratase activity for 3-cyanopyridine had amidase activity for nicotinamide. This is important particularly with regard to the A_4 mutant of R-312 which has been reported not to exhibit amidase activity—at least for a wide range of (primarily) aliphatic amines [3]; it implies that the A mutation alters the structure of the enzyme, but does not eliminate it.

Table 1

Screening test results

	Hydratase activity*			Amidase activity*		
	16 H	24 H	36 H	16 H	24 H	36 H
Nocardia N775	nd**	nd	nd	nd	nd	nd
Corynebacterium N774	nd	nd	nd	nd	nd	nd
Corynebacterium N771	0.13	0.08	0.05	0.01	0.05	0.05
Brevibacterium R-312	0.20	0.12	0.09	0.02	0.06	0.09
Brevibacterium A_4	0.15	0.09	0.07	0.01	0.04	0.06

* Specific activity as defined in Materials and Methods.
** No activity detectable.

Fermentation

Typical fermentations (Figs. 2 and 3) gave a maximum cell mass concentration of only 5.6 gm (DW)/l (Fig. 2) and an apparent cell yield of 0.79 gm cells/gm glucose used. The surprisingly high yield seems to indicate that the cells grew on both glucose and on cassamino acids (CA) (not monitored). This is supported by Arnaud's [16] results which show that *Brevibacterium* R-312 can use CA as a sole carbon source for growth. In the present case, however, the organism appeared to use glu-

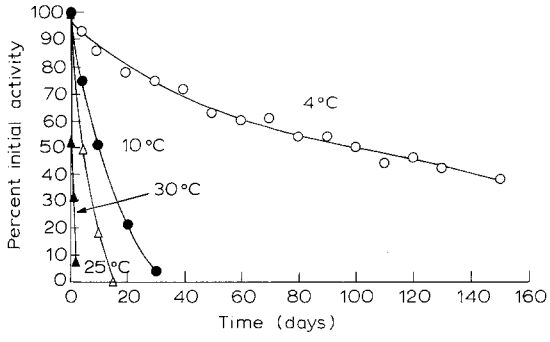

Fig. 1. Whole cell (resting) storage stability in pH 8 buffer.

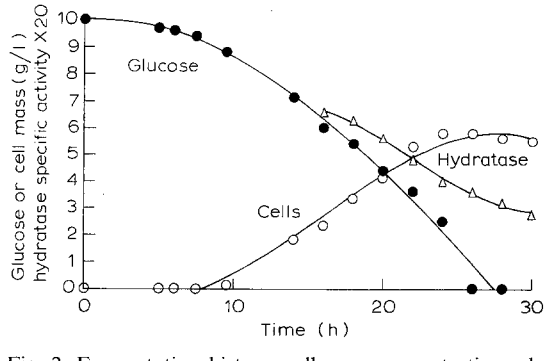

Fig. 2. Fermentation history: cell mass concentration, glucose and hydratase specific activity.

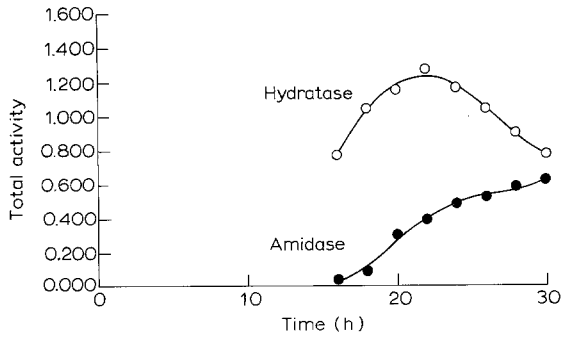

Fig. 3. Fermentation history: total activities of hydratase and amidase.

cose and CA simultaneously for growth.

The maximum total hydratase activity of 1.25 mg nicotinamide/min (25°C), corresponding to a specific activity of 0.25 mg nicotinamide/mg cells-min, was reached usually at 20–22 h (see Fig. 3); however, amidase activity at that time was unacceptably high (25% of the hydratase activity). Fermentations were, therefore, harvested at 18–20 h when the specific and total hydratase activities were 0.3 mg nicotinamide/mg cells-min and about 1.5 mg nicotinamide/min, respectively, and the amidase activity was only 10% of the hydratase activity which was low enough for kinetic experiments. This procedure is satisfactory for basic studies, but it is unlikely to be acceptable for practical applications; it probably will be necessary to find other ways to limit amidase activity (see below).

The time dependencies of the total enzyme activities can be related directly to the histories of the specific enzyme activities, and to their dependencies on cell specific growth rate (Fig. 4 and 5). The cell specific growth rate and the hydratase specific activity decreased with time, while the amidase specific activity increased (Fig. 4). Also, the hydratase specific activity decreased and the amidase specific activity increased with decreasing cell specific growth rate (Fig. 5). The reasons are not clear partly because of the batch nature of the fermentations.

It is possible that the decline in hydratase specific activity hydratase arises from thermal decay of the enzyme as would be inferred from the storage stability characteristics of cell suspensions (Fig. 1). But it must be recalled that the storage data is for non-growing cells in buffer. Also, it is possible that the hydratase is degraded by a protease as reported by Arnaud [3]. But the effect of temperature on the protease activity is not known; therefore, it is not yet possible to determine the relative contributions of intrinsic thermal decay and protease activity to the *observed* thermal decay characteristics.

Continuous fermentation studies might clarify these time and growth rate dependencies, and might help point the way toward conditions required for producing higher cell mass and higher hydratase titer with minimal production of protease and amidase. The studies should include medium development and determinations of the effects of environmental conditions on cell specific growth rate and on specific activities of the enzymes as functions of specific growth rate and of time; little has been reported about either. Such work might be particularly fruitful if done also with *Rhodococcus rhodochorous* J1, which has been reported recently

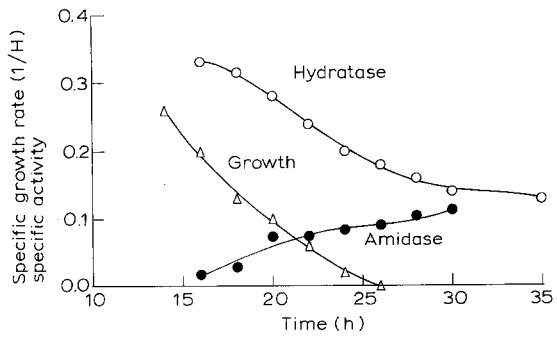

Fig. 4. Fermentation history: specific growth rate and specific activities of hydratase and amidase.

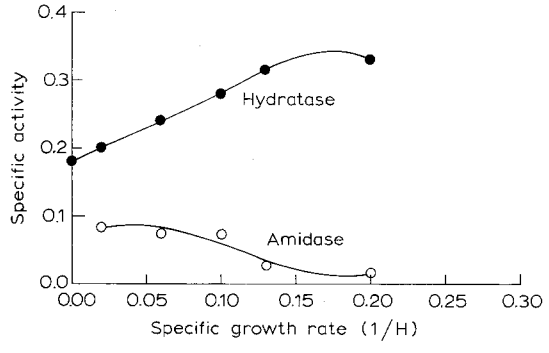

Fig. 5. Specific growth rate dependence of hydratase and amidase during batch fermentation.

[12,13] to be able to hydrate 3-cyanopyridine at high concentrations, and to have very little amidase activity.

In addition, genetic modification should be attempted to improve the intrinsic thermal stability of R-312 hydratase, and to diminish or to eliminate amidase and protease activities. The characteristics of the mutants should be compared with those of *R. rhodochorous* J1.

Temperature and pH profiles

Figs. 6 and 7 give hydratase temperature and pH profiles, respectively. The maximum temperature is 35°C and the maximum pH is 8.0. This agrees reasonably with the results of Arnaud [3]. It is important to recall, however, that at the maximum temperature the enzyme half-life is less than one day: the half-life does not begin to become practical (>10 days) until the temperature is decreased to 10°C. But at such low temperatures the activity is very low (30% of maximum). These observations imply that whole cells of R-312 may not be suitable for practical application, and that the enzyme may have to be used in soluble or (more likely) in immobilized form. Studies concerning such approaches as well as those noted in the previous section will be reported in future publications.

CONCLUSIONS

1. The maximum hydratase activity (1.25 mg nicotinamide/min) was reached as the culture entered stationary phase (20–22 h). But amidase activity at this point was too high (25% of the hydratase activity); therefore, the fermentation had to be harvested before (18–20 h) the maximum total hydratase activity was obtained.

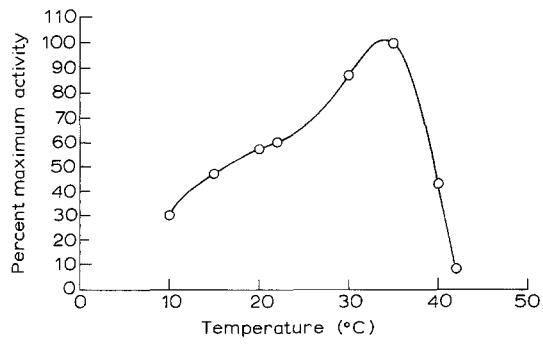

Fig. 6. Resting cell hydratase temperature profile (in pH 8 phosphate buffer).

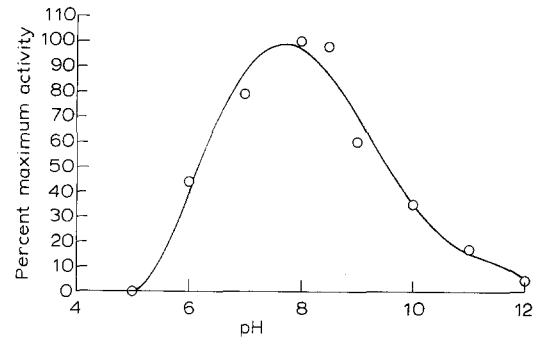

Fig. 7. Resting cell hydratase pH profile (at 15°C).

2. Specific activity of hydratase appeared to decrease with decreasing specific growth rate. This may have resulted from batch fermentation conditions or may have been associated with thermal and/or proteolytic degradation of activity.

3. Cell yield on glucose appeared high (0.78 g dry wt. cells/g glucose). We speculate that simultaneous use of glucose and cassamino acids may have been responsible for this observation.

4. Whole cell R-312 hydratase half-lives are 100 days, 10 days, 5 days and less than 1 day at 4°C, 10°C, 25°C, and 30°C, respectively.

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