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Chemoenzymatic production of 1,5-dimethyl-2-piperidone

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Abstract

A chemoenzymatic process for the preparation of 1,5-dimethyl-2-piperidone (1,5-DMPD) from 2-methylglutaronitrile (MGN) has been demonstrated. MGN was first hydrolyzed to 4-cyanopentanoic acid (4-CPA) ammonium salt using the nitrilase activity of immobilized *Acidovorax facilis* 72W cells. The hydrolysis reaction produced 4-CPA ammonium salt with greater than 98% regioselectivity at 100% conversion, and at concentrations of 170–210 g 4-CPA/l. Catalyst productivities of at least 1000 g 4-CPA/g dry cell weight (dcw) of immobilized cells were achieved by recycling the immobilized-cell catalyst in consecutive stirred-batch reactions. After recovery of the immobilized cell catalyst for reuse, the 4-CPA ammonium salt in the aqueous product mixture was directly converted to 1,5-DMPD by low-pressure catalytic hydrogenation in the presence of added methylamine.

Keywords: Nitrilase; Nitrile hydratase; *Acidovorax facilis*; Immobilization; 1,5-Dimethyl-2-piperidone

1. Introduction

Enzyme-catalyzed hydrolysis of nitriles to the corresponding carboxylic acids [1–3] can be preferable to some chemical methods, since the enzymatic reactions are often performed at ambient temperature, do not require the use of strongly acidic or basic reaction conditions, and do not produce large amounts of undesirable byproducts. Aliphatic nitrilases (EC 3.5.5.7), which directly convert nitriles to the corresponding carboxylic acids without formation of amide intermediates, are produced by a variety of microbes, including *Rhodococcus rhodochrous* K22 [4], *Comamonas testosteroni* NI1 [5], *R. rhodochrous* NCIMB 11216 [6,7], *R. rhodochrous* PA-34 [8], *Fusarium oxysporum* f. sp. *melonis* [9], *Acinetobacter* sp. AK 226 [10], *Alcaligenes faecalis* ATCC 8750 [11], and *Acidovorax facilis* 72W [12].

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An additional advantage of using a nitrilase for the hydrolysis of aliphatic dinitriles is regioselectivity, when hydrolysis of only one of two nitrile groups on a dinitrile is desired [5–7,12].

The nitrilase activity of unimmobilized *A. facilis* 72W cells has been used to convert aliphatic α,ω-dinitriles to the corresponding ω-cyanocarboxylic acid ammonium salts, which were subsequently hydrogenated in aqueous solution to produce five- and six-membered ring lactams [12,13]. 2-Methylglutaronitrile (MGN) was hydrolyzed to produce 4-cyanopentanoic acid (4-CPA) ammonium salt with greater than 98% regioselectively at 100% conversion. When unimmobilized cell suspensions were employed to produce aqueous solutions of 4-CPA ammonium salt, either centrifugation or ultrafiltration was required to recover the catalyst for reuse. At high product concentrations (up to 29 wt.% 4-CPA ammonium salt), the unimmobilized cells lost significant activity with each reuse, and cell lysis was observed.

A variety of methods for the immobilization of microbial cells are known which simplify catalyst recovery and reuse, improve the resistance of cells to lysis, and increase the stability of the enzyme activity of the immobilized cells [14–18]. A chemoenzymatic process for the preparation of 1,5-dimethyl-2-piperidone (1,5-DMPD) has now been demonstrated (Scheme 1) which uses immobilized *A. facilis* 72W cells for the production of 4-CPA ammonium salt. A significant improvement in the stability of the nitrilase activity of the immobilized cells when recycled in consecutive batch reactions was achieved when compared to using unimmobilized cells. Hydrogenation of the 4-CPA ammonium salt produced 1,5-DMPD, which has properties similar to those of the industrial solvent *N*-methyl-2-piperidone, and which may find use in electronics, coatings and solvent applications.

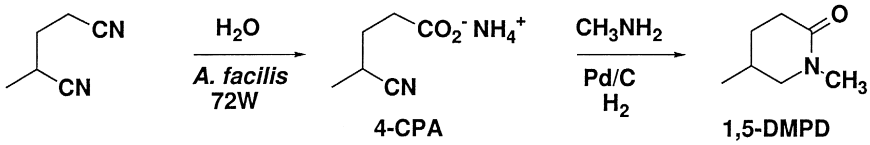
2. Experimental

2.1. Materials and methods

All chemicals and hydrogenation catalysts were obtained from commercial sources and used as received unless otherwise noted. ISAGEL RG 300 carrageenan was obtained from Pronova. MGN was purified by filtration through a column of basic, activity 1 alumina (5 parts MGN:1 part alumina), or by stirring with 5 wt.% basic, activity 1 alumina for 18 h at 25°C. Water was distilled and deionized.

Hydrogenations were performed in a 300-ml stainless steel Autoclave Engineers magnedrive packless autoclave. The percent recovery of MGN and the percent yields of the hydrolysis products formed in nitrilase-catalyzed reactions were based on the initial amount of MGN present in the reaction mixture, and were determined by HPLC using a Supelcosil LC-18 DB column (15 cm × 4.6 mm diameter), 10 mM acetic acid/10 mM sodium acetate in 7.5% methanol/water as mobile phase, and a refractive index detector. The yields of lactams and *N*-methyl-lactams produced by the hydrogenation of aqueous solutions of 4-CPA ammonium salts were based on the initial concentration of 4-CPA ammonium salt present in the reaction mixture, and determined by gas chromatography using a DB-1701 capillary column (30 m × 0.53 mm i.d., 1 μm film thickness).

A. facilis 72W cells (ATCC 55746) were prepared as previously described [12,13], and stored frozen at −80°C. Wet cell weights of 72W cells employed in reactions, immobilizations, or assays were obtained from cell pellets prepared by centrifugation of either fermentation broth or cell suspensions in buffer. Dry cell weight (dcw) was determined by lyophilization of wet cells, and the ratio of dcw to wet cell weight (wcw) was typically 0.25.



Scheme 1.

2.2. Nitrilase assay

A 50-mg (dcw)/ml cell suspension was prepared in 0.10 M potassium phosphate buffer, and the resulting suspension heated at 50°C for 0.5 h. The suspension was then cooled to 25°C in an ice/water bath, then maintained at 25°C until assayed. Into a 20-ml glass scintillation vial equipped with a magnetic stir bar was added 3.0 ml of an aqueous solution of 0.40 M MGN at 25°C. With stirring, 1.0 ml of the cell suspension at 25°C was added. At 1, 5, 10 and 15 min after the addition of the cell suspension, a 180-μl aliquot was removed from the assay mixture, mixed with 5 μl of 6.0 N HCl and 20 μl of 0.75 M *N*-methylpropionamide in water (HPLC external standard), the resulting mixture centrifuged and the supernatant analyzed by HPLC for the rate of production of 4-CPA ammonium salt. A unit of nitrilase activity (IU) was equivalent to production of 1 μmol 4-CPA ammonium salt/min.

2.3. Cell immobilization in polyacrylamide gel (PAG)

Into a 400-ml beaker containing 30.0 g (wcw) *A. facilis* 72W (no prior heat treatment at 50°C) and 82.1 ml of 0.10 M potassium phosphate buffer (pH 7.0) at 10°C was added with stirring a solution of 13.8 g acrylamide and 1.20 g methylenebisacrylamide dissolved in 15.0 ml of water at 25°C. After ca. 30 s, 0.45 ml of tetramethylethylenediamine was added, followed by 7.5 ml of 5% (w/v) potassium persulfate in water at 25°C. The resulting mixture gelled in 1–2 min, and the gel temperature increased to ca. 30°C. The gel was cooled for 1 h at 10°C in an ice/water bath, then cut into $2 \times 2 \times 25$ mm strips using a food processor equipped with a fine shredding blade. The 72W/PAG catalyst was mixed in 300 ml of 50 mM phosphate buffer (pH 7.0) at 50°C for 1 h (to deactivate nitrile hydratase), then cooled to 5°C. The catalyst was next washed twice by mixing at 10 wt.% catalyst suspension in 50 mM potassium phosphate buffer (pH 7.0) at 5°C for 15 min, then stored in this same buffer at 5°C.

2.4. Cell immobilization in carrageenan gel

To a 600-ml stainless steel beaker was added 128.8 ml of water, which was then heated to 50°C. A four-bladed impeller was used to rapidly stir the resulting mixture as 6.75 g of carrageenan was slowly added. The mixture was heated with stirring at 75–80°C until the carrageenan was completely dissolved, then the solution was slowly cooled to 55–56°C (gelling temperature ca. 52°C). With stirring, a suspension of 46.9 g (wcw, 11.3 g dcw) of *A. facilis* 72W cells in 43.1 g of 0.30 M sodium bicarbonate buffer (pH 7.3) at 50°C, which had previously been heated to 50°C for 1 h to deactivate nitrile hydratase, was added. After the cells were fully dispersed in the carrageenan solution, the mixture was cooled in an ice/water bath and maintained at 5°C for 1 h. The resulting gel was shredded into $2 \times 2 \times 25$ mm strips using a food processor equipped with a fine shredding blade. The catalyst was suspended with gentle mixing in 1 l of 1.0 M ammonium bicarbonate (pH 7.2) at 5°C for 18 h, then the buffer was decanted and replaced with fresh buffer and the catalyst stored at 5°C.

2.5. Preparation of *A. facilis* 72W / carrageenan beads

Into a 250-ml media bottle equipped with magnetic stir bar and containing 64 g of water at 50°C was slowly added 3.38 g of carrageenan with rapid stirring. The mixture was heated to 75–80°C with rapid stirring until the carrageenan was completely dissolved, and the resulting solution cooled to 55–56°C (gelling temperature ca. 52°C) in a thermostated water bath. A suspension of 23.4 g of *A. facilis* 72W cells (wcw, 5.62 g dcw) in 21.56 g of 0.30 M sodium bicarbonate buffer (pH 7.3) was heated to 50°C for 1 h to deactivate nitrile hydratase. The cell suspension at 50°C was then added to the carrageenan solution at 55–56°C with stirring, then the cell/carrageenan mixture was immediately added slowly to 450 ml of soybean oil at 50°C with stirring using an overhead stirrer. After cell/carrageenan droplets of the desired size were produced in the oil by controlling the stirring rate, the temperature of the oil was reduced to 35°C to gel the droplets, and the oil decanted from the resulting beads. The beads were washed with 500 ml of 1.0 wt.% Tween 80 in 1.0 M ammonium bicarbonate buffer (pH 7.3), then stored in 1.0 M ammonium bicarbonate (pH 7.3) for at least 18 h at 5°C to further harden the beads.

Cell/carrageenan beads were optionally chemically crosslinked by reaction with glutaraldehyde (GA) and polyethylenimine (PEI). After decantation of the soybean oil, the beads were washed with 0.3 M potassium chloride in 50 mM potassium phosphate (pH 7.0). The beads were then resuspended in 250 ml of this same buffer, and 2.0 g of 25 wt.% GA in water was added and the beads mixed for 0.5 h at 25°C. To the mixture was then added 9.0 g of 11 wt.% PEI (average Mw ca. 750,000) in water, and the beads mixed for an additional hour at 25°C. The beads were then washed with 1.0 M ammonium bicarbonate (pH 7.3) and stored in this same buffer for at least 18 h at 5°C to further harden the beads.

2.6. 4-CPA production with immobilized A. facilis 72W cells

In a typical reaction, 16.5 g of immobilized cell catalyst was placed into a 125-ml Celstir™ (Wheaton) jacketed reaction vessel that was temperature-controlled at 25°C with a recirculating temperature bath. To the reaction vessel was added 72.1 ml of water and 11.40 ml (10.83 g, 1.0 M) of MGN, and the mixture stirred at 25°C. Samples (0.100 ml) were mixed with 0.400 ml of water, then 0.360 ml of a diluted sample was mixed with 0.040 ml of 0.75 M *N*-methylpropionamide in water and 0.020 ml of 6.0 N HCl. The resulting mixture was filtered (0.22 μm) and the filtrate analyzed by HPLC. After complete conversion of MGN, the product mixture and catalyst were decanted to a tared 250 ml beaker and the aqueous product mixture decanted from the catalyst. The remaining catalyst was weighed and the weight recorded, then water was added to the catalyst to a final total weight (water and catalyst) of 88.6 g. The catalyst suspension was transferred back to the reaction vessel, 11.4 ml of MGN added, and the reaction repeated.

2.7. Scale-up of 4-CPA production

To 32 l of 0.15 M sodium bicarbonate (pH 7.3) at 50°C was added 1.69 kg of carageenan with stirring, the resulting mixture heated to 80°C for 0.5 h, and the resulting solution cooled to 55–56°C. A suspension of 12.3 kg of *A. facilis* 72W cells (wcw, 2.89 kg dcw) in 10.8 l of 0.15 M sodium bicarbonate buffer (pH 7.3) was heated at 50°C for 1 h, and immediately added to the carrageenan solution at 55–56°C with stirring. The resulting mixture was stirred for ca. 5 min, then cast into stainless steel trays to a depth of ca. 5 cm. The trays were placed in ice/water baths to cool to 15°C for 1–2 h. The resulting gels were cut into 5-cm strips and then shredded using a food processor equipped with a fine-shredding blade. The shredded gel was transferred to a 150 gal vessel containing 225 l of 1 M ammonium acetate (adjusted to pH 7.0 with acetic acid or ammonium hydroxide) at 5°C. The catalyst was hardened in this buffer with occasional agitation for 18 h at 5°C.

To start the conversion of MGN to 4-CPA, the hardening solution was first decanted and 225 l of water added to the reactor at 25°C. The catalyst was mixed for 15 min, then the resulting solution was decanted and 22.5 l of 1.2 M 4-CPA ammonium salt (pH 7.0), 202.5 l of water, and 32.4 kg of MGN added to the reactor. The resulting mixture was stirred at 25°C until the reaction was complete, then the product mixture was decanted from the catalyst and an additional charge of 225 l of water and 32.4 kg of MGN added to the reactor. After the second reaction, the charge of water and MGN added after decantation of the product mixture was increased to 330 l and 53.2 kg, respectively. Product mixtures were filtered to remove particulates, and concentrated by evaporation under reduced pressure to produce 50 to 70 wt.% aqueous 4-CPA, which was stored at 5°C prior to hydrogenation.

2.8. Hydrogenation of 4-CPA to 1,5-DMPD

In a typical batch reaction, 60 g (0.33 mol, 69% acid basis) of aqueous 78 wt.% 4-CPA ammonium salt and 24 g (0.31 mol) methylamine were charged to a 300-ml autoclave equipped with a thermocouple, cooling coils, sample dip tube containing a stainless steel 5 μm Mott filter, and Dispersimix turbine type draft tube agitator equipped with a rotating impeller. A 5% Pd/C hydrogenation catalyst (1.0 g, dry basis, Engelhard) was next charged to the reactor. After closing, the reactor was purged 3 times with hydro-

gen, then the temperature was raised to 150°C under 50 psig hydrogen with very slow stirring. At reaction temperature, the pressure was raised to 600 psig with hydrogen and maximum (~1200 rpm) stirring commenced. Under these conditions, reduction of 4-CPA required 2 h to reach 98.5% conversion. Samples were taken with time through the Mott filter and analyzed by gas chromatography. Analysis of the product after 2 h showed 89.5% selectivity to a 99.5:0.5 mixture of 1,5- and 1,3-DMPD, and 7% selectivity to 5-methyl-2-piperidone. Other by-products observed were bis(*N*-methylamino)-2-glutaramide and *N*-methylamino-2-methylglutaramide in selectivities of 1.1% and 1.2%, respectively.

3. Results and discussion

3.1. Nitrilase stability during heat treatment of A. facilis 72W suspensions

Heating *A. facilis* 72W cell suspensions at 50°C was required to inactivate an undesirable nitrile hydratase activity [12,13]. Heating just prior to immobilization produced an immobilized cell catalyst with significantly higher specific activity than when using cells that were first heated to 50°C at the end of the fermentation, and then frozen and stored until immobilized. For example, using cells heated at 50°C for 1 h in the fermentation broth prior to collection by centrifugation, the resulting immobilized cell/carrageenan gel had a specific activity of 5.1 IU/g, while a carrageenan gel prepared using cells which were not heated at the end of fermentation had 12.1 IU/g gel (with heat treatment just prior to immobilization). Similar results were obtained in PAG immobilizations. Because the heat treatment step at the end of a fermentation was problematic, this step was routinely performed immediately prior to or after immobilization.

The first step in the preparation of immobilized *A. facilis* 72W cells required heating a 12.5% dcw cell suspension at 50°C for at least 1 h to denature the undesirable nitrile hydratase present in 72W cells. This procedure was initially performed by preparing a suspension of cells in 0.88% sodium chloride prior to heating, but a study of the stability of nitrilase activity at 50°C indicated a significant loss of activity over several hours (a length of time which could be required for a commercial-scale immobilization). It was also desirable to maintain < 1 ppm of chloride ion in the 4-CPA product mixture to prevent poisoning of the hydrogenation catalyst during the subsequent conversion of 4-CPA to 1,5-DMPD. Rather than removing chloride ion from immobilized cells by washing, alternatives to sodium chloride were examined as a way to stabilize nitrilase activity, control osmotic pressure and limit cell lysis during the heat-treatment step.

A 12.5% dcw cell suspension rapidly lost nitrilase activity at 50°C in 0.88% sodium chloride, 0.15 or 0.50 M sodium acetate, 0.10 M mannitol, and 0.20 M sorbitol at pH 7.0 over 4–5 h (Fig. 1). The nitrilase activity was significantly more stable at 50°C in 0.15 or 0.30 M sodium bicarbonate (pH 7.3), or in 0.20 to 0.50 M sodium phosphate (pH 7.0). At a higher concentration of bicarbonate (0.50 M), the nitrilase activity was stable but the cell suspension became heterogeneous and unusable. The protocol

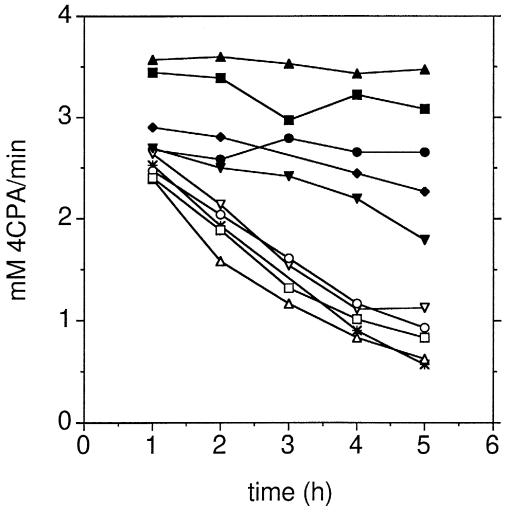


Fig. 1. Stability of nitrilase activity of 50% wcw suspensions of *A. facilis* 72 W in various buffers (pH 7.0–7.3) at 50°C: 0.35 M sodium phosphate (▲), 0.30 M sodium bicarbonate (■), 0.50 M sodium phosphate (●), 0.15 M sodium bicarbonate (◆), 0.20 M sodium phosphate (▼), 0.50 M sodium acetate (▽), 0.20 M sorbitol (○), 0.10 M mannitol (□), 0.15 M sodium acetate (∗), 0.88% sodium chloride (△). Aliquots of cell suspensions at 50°C were assayed at 25°C for mM 4-CPA/min using 12.5% dcw cell suspensions in 0.30 M MGN.

for immobilization in carrageenan was therefore changed to heating cell suspensions in 0.35 M sodium bicarbonate (pH 7.3) for 1 h at 50°C, then adding the cell suspension to a 5% carrageenan solution at 55–56°C prior to gellation.

3.2. Immobilization in PAG or carrageenan

Immobilization in alginate [18] produced gels which were not stable in product mixtures containing high concentrations of 4-CPA ammonium salt, whereas PAG [13] or carrageenan [15] gels were stable when recycled in consecutive batch reactions. Immobilization of 72W cells in carrageenan required the exposure of cells to temperatures greater than 50°C for short periods of time prior to gellation by a reduction in temperature. For immobilization in carrageenan, the gelling temperature of carrageenan solutions was dependent on carrageenan concentration, and increased from 41°C at 2.5% (w/w) to 52°C at 5.0% carrageenan. Cell suspensions at 50°C were therefore added with mixing to 5 wt.% carrageenan solutions at 55–56°C. The stability of a 12.5% dcw cell suspension in 0.50 M sodium phosphate at 50°C, 55°C and 60°C was compared; where there was no loss of nitrilase activity at 50°C after 5 h, a 40% loss in 5 h was observed at 55°C, and an 83% loss in 45 min was observed at 60°C (Fig. 2), so limiting the time at temperatures greater than 50°C was important for good yields of immobilized nitrilase activity.

Initially, gels of PAG or carrageenan containing *A. facilis* 72W cells were cut into small particles using a food-processor equipped with a fine shredding blade. The irregularly shaped particles produced by this method showed more attrition when recycled in consecutive batch reactions than when using gels beads. Gel beads were prepared by first dispersing an aqueous suspension of cells and either PAG prepolymer or carrageenan in soybean oil, and the resulting droplets were gelled either by the addition of a polymerization initiator in the case of PAG [19–22], or by lowering the temperature of the oil below the gelling temperature of the carrageenan [19,23,24]. Microbeads of PAG without cells (less than 0.5 mm diameter) could be prepared in this manner, but attempts to reproducibly prepare beads when cells were included in the mixture were unsuccessful.

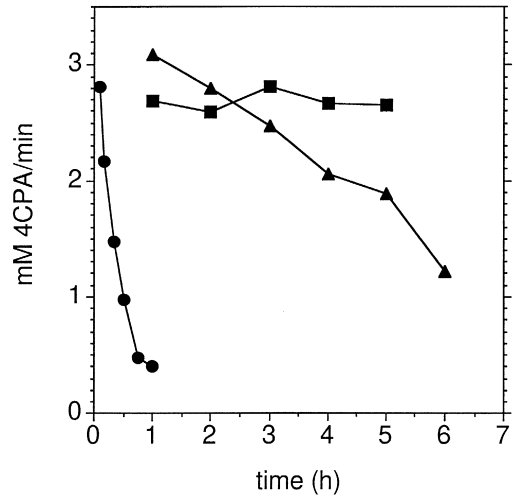


Fig. 2. Stability of nitrilase activity of 50% wcw suspensions of *A. facilis* 72W in 0.50 M phosphate (pH 7.0) at 50°C (■), 55°C (▲), or 60°C (●). Aliquots of cell suspensions were assayed at 25°C for mM 4-CPA/min using 12.5% dcw cell suspensions in 0.30 M MGN.

Either polymerization occurred prematurely (control of polymerization rate was problematic), or polymerization resulted in the production of a heterogeneous mass of fine particles rather than beads.

The preparation of 72W/carrageenan beads was much less problematic; preliminary trials have produced beads having an average diameter of from 0.5 to 3 mm. The beads were separated from the oil phase after cooling below 45°C, then washed and hardened in 1.0 M ammonium acetate or bicarbonate at 5°C for 18 h. A detergent (0.1% Tween 80) was used to remove the soybean oil from the beads during washing, and the detergent was shown to cause no loss in catalyst bead activity.

3.3. Storage stability of unimmobilized or immobilized cells

In the course of preparing and storing unimmobilized or immobilized *A. facilis* 72W cells as suspensions in phosphate buffer or physiological saline, it was found that many of these storage solutions did not prevent microbial contamination of the stored catalyst at temperatures ranging from 5°C to 30°C, or

in those cases where microbial contamination was not observed, a significant loss of nitrilase activity still occurred. Freezing of immobilized cell catalysts typically resulted in the destruction of the immobilization matrix, and while unimmobilized cells could be stored frozen without significant loss of activity for months, a significant cost was associated with this method. A method of storing aqueous suspensions of unimmobilized or immobilized microbial cells for weeks or months prior to use, without loss of activity or microbial contamination, was required.

The effect of various inorganic salts on the storage stability of the nitrilase activity of unimmobilized or immobilized *A. facilis* 72W was examined. Storage solutions containing potassium phosphate or ammonium chloride have been reported to stabilize microbial nitrilase or nitrile hydratase activity of unimmobilized or immobilized cells [25], but various concentrations of these two salts did not result in a prevention of the loss of nitrilase activity of unimmobilized or immobilized *A. facilis* nitrilase activity when stored at 5°C. Salts of organic acids such as acetate or propionate, previously reported to stabilize the nitrile hydratase activity of microbial cells [26], were also examined. Ammonium acetate or ammonium propionate were not effective in preserving the nitrilase activity of unimmobilized or immobilized *A. facilis* 72W cells.

Bicarbonate salts, such as sodium, potassium or ammonium bicarbonate, were effective for both the prevention of significant microbial contamination or putrefaction of suspensions of unimmobilized or immobilized *A. facilis* 72W cells, and the stabilization of the nitrilase activity of these suspensions. Cells were isolated from cultured broth by centrifugation, and the unimmobilized cells then suspended at a concentration of 1–5% dcw in aqueous potassium phosphate (0.10 or 1.00 M), sodium acetate (0.10 or 1.00 M), or sodium bicarbonate (0.10 or 0.30 M) at pH 7.3, and the resulting suspensions stored at 5°C. Samples were removed from the cell suspensions over time and the samples assayed for nitrilase activity. Table 1 lists the relative nitrilase activity of the stored cell suspensions, with the nitrilase activity of the suspensions at day 0 defined as 100%. Cells stored for 92 days in 0.10 or 0.30 M sodium bicarbonate retained a significantly higher percentage of the initial nitrilase activity when compared to storage in either 0.10 or 1.0 M sodium acetate or potassium phosphate.

A. facilis 72W cells (5% dcw) immobilized in carrageenan (3.0 wt.%) were suspended in aqueous 1.0 M potassium phosphate, ammonium acetate, ammonium propionate, ammonium bicarbonate, or in 50 mM potassium phosphate containing 1.0 M ammonium chloride at pH 7.0, and stored at 5°C. Samples were removed over time and the immobilized cells assayed for nitrilase activity. Fig. 3 shows the relative nitrilase activity of the stored immobilized cell suspensions, with the initial activity defined as 100%. Immobilized cells stored for 96 days in 1.0 M ammonium bicarbonate at 5°C showed no significant loss of nitrilase activity when compared to the other buffers examined.

Immobilized cells were also examined for microbial contamination by measurement of the change in optical density (O.D.) at 600 nm. Microbial contamination occurred with cells immobilized in either

Table 1
Stability of nitrilase activity in 5% dcw suspensions of unimmobilized cells stored in various buffers (pH 7.0–7.3) at 5°C

Day	Nitrilase activity (%)					
	KH_2PO_4 0.10 M	KH_2PO_4 1.00 M	NaOAc 0.10 M	NaOAc 1.00 M	$NaHCO_3$ 0.10 M	$NaHCO_3$ 0.30 M
0	100	100	100	100	100	100
39	93	86	87	82	97	
41						106
64	96	86	72	82	104	
69						92
92	81	78	63	76	95	95

The nitrilase activity (IU/g dcw) for individual samples are reported as a percentage relative to the initial nitrilase activity of the cells in each buffer.

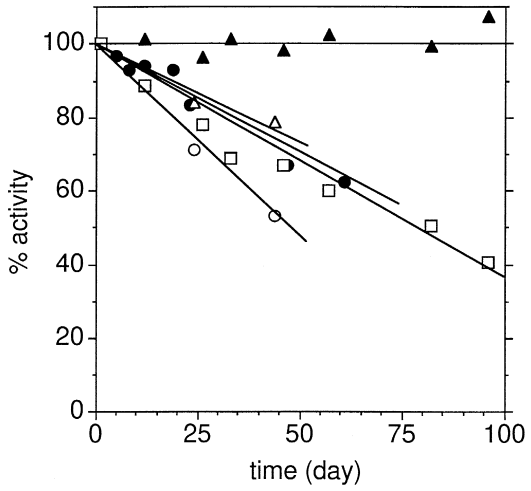


Fig. 3. Nitrilase stability of *A. facilis* 72W cells (5% dcw) immobilized in carrageenan (3.0 wt.%) and suspended in aqueous 1.0 M potassium phosphate (○), ammonium acetate (□), ammonium propionate (△), ammonium bicarbonate (▲), or in 50 mM potassium phosphate containing 1.0 M ammonium chloride (●) at pH 7.0 and 5°C. The nitrilase activity (IU/g dcw) for individual samples are reported as a percentage relative to the initial nitrilase activity of the immobilized cells in each buffer.

PAG or carrageenan when stored in 50 mM potassium phosphate, while no significant microbial contamination or putrefaction was observed for cells stored in 1.0 M ammonium acetate, 1.0 M ammonium bicarbonate, or 1.0 M ammonium chloride/50 mM potassium phosphate. Of the three storage suspensions which showed no microbial contamination, only the immobilized cells stored in 1.0 M ammonium bicarbonate showed no significant loss of nitrilase activity.

No loss of enzyme activity was observed when storing the purified nitrilase (isolated from 72W cell extracts) in 20 mM phosphate buffer (pH 7.0) at 5°C for up to 6 months, so it appeared that bicarbonate was not directly protecting the nitrilase from inactivation, but interfering with another mechanism of deactivation that occurs in the non-growing but metabolically active unimmobilized cells, or immobilized cells. The stability of immobilized cells stored in 1.0 M ammonium bicarbonate at 25°C was also determined; after 38 days at room temperature, cells immobilized in carrageenan retained 92% of their initial activity.

3.4. Effect of MGN purity on immobilized cell nitrilase activity in recycle reactions

When *A. facilis* 72W cells immobilized in PAG were first recycled in consecutive batch reactions for the conversion of MGN to 1.0 M 4-CPA ammonium salt, significant loss of nitrilase activity was observed with each consecutive reaction. A set of recycle reactions was performed to compare using unpurified MGN, unpurified MGN with 1 mM added EDTA, and purified MGN which had been distilled from powdered NaOH and then redistilled. After four reactions with catalyst recycle, the catalyst in reactions using unpurified MGN had each lost ca. 65% of initial activity, while the catalyst in reactions using purified MGN lost only 16% of initial activity, indicating an impurity present in the MGN was responsible for a major portion of activity loss in the immobilized cell catalyst.

As an alternative method of purification, MGN was filtered through a column of basic alumina (activity 1). This alumina-treated MGN (5 parts MGN:1 part alumina (wt/wt)) was compared to NaOH-distilled/redistilled MGN, distilled MGN, and unpurified MGN used in combination with added 5 mM sodium metabisulfite, 1 mM sodium dithionite, or 10 mM dithiothreitol (DTT) in recycle reactions under nitrogen (Fig. 4). After four reactions, catalyst in reactions which used the alumina-treated MGN had retained almost as much nitrilase activity as reactions employing NaOH-distilled/redistilled MGN or reactions run with added DTT. Stirring the MGN with 5 wt.% activity 1 basic alumina for 18 h produced a similar improvement in catalyst stability, and either alumina-treatment method was used to routinely purify MGN used in subsequent reactions. No difference in nitrilase stability was seen when comparing reactions run under nitrogen or air. DTT was an effective catalyst poison in subsequent hydrogenation reactions, and was not used.

3.5. Optimization of reaction conditions

A time course for the production of 1.0 M 4-CPA using 72W/PAG catalyst in 20 mM phosphate buffer (pH 7.0) at 25°C is illustrated in Fig. 5. As the solubility of MGN in water at 25°C is only ca. 0.50

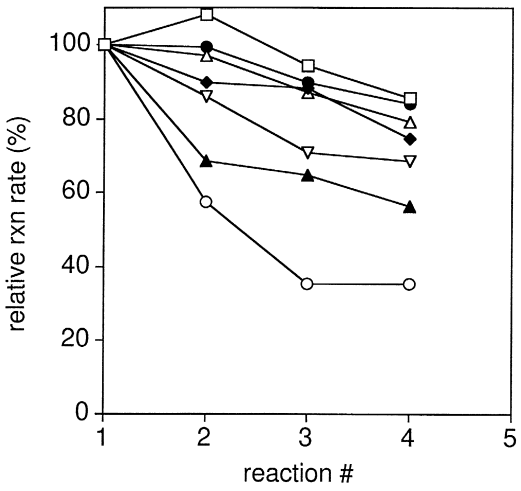


Fig. 4. Effect of various additives or MGN purifications on recovered nitrilase activity in consecutive batch reactions using 72W/PAG catalyst (16.5 wt.% catalyst loading) to produce 1.0 M 4-CPA (20 mM potassium phosphate at pH 7.0, 25°C, under nitrogen): 10 mM dithiothreitol (□), NaOH-distilled/redistilled MGN (●), alumina-filtered MGN (△), 1 mM sodium dithionite (◆), distilled MGN (▽), 5 mM sodium metabisulfite (▲), unpurified MGN (○).

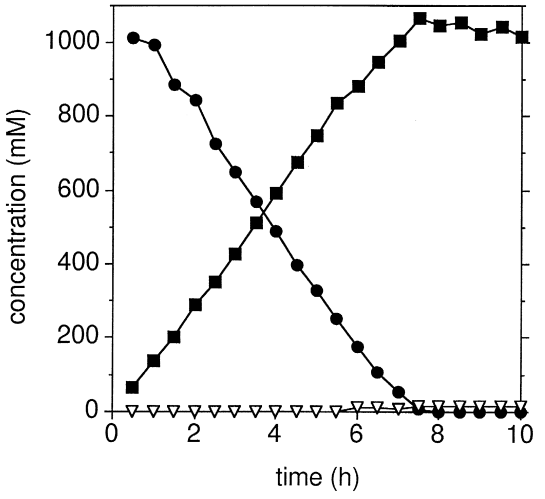


Fig. 5. Time course for production of 1.0 M 4-CPA using 16.5 wt.% 72W/PAG immobilized catalyst in 20 mM potassium phosphate (pH 7.0), 25°C: MGN (●), 4-CPA (■), MGA (▼).

M, reactions run to produce 4-CPA at concentrations greater than 0.50 M were initially two-phase mixtures of undissolved MGN dispersed in the aqueous phase. Recycle reactions were routinely performed with no added buffer, adding only water and MGN to the reaction heel remaining after decantation of the product mixture from the catalyst (ca. 20% heel, catalyst plus product mixture) from the previous reaction. No pH change over the course of recycle reactions was observed when generating from 1.2–1.5 M 4-CPA ammonium salt and using the reaction product, 4-CPA ammonium salt, as buffer (pH of reaction mixtures ca. 7.3). During and after complete conversion of MGN to 4-CPA, there is a slow rate of conversion of 4-CPA to 2-methylglutaric acid (MGA) by the nitrilase; this rate is ca. 1.5% the rate of hydrolysis of MGN to 4-CPA. No effect of catalyst particle size on reaction rate or catalyst stability was observed when comparing catalyst particle diameters of from 200–500 μm to 2–3 mm.

The dependence of reaction rate for 4-CPA production on the concentration of 4-CPA in the reaction mixture was measured using *A. facilis* 72W/carrageenan beads as catalyst (Fig. 6). Reaction rates were measured for the production of 1.0 M 4-CPA in reaction mixtures where the initial 4-CPA concentration ranged from 72 to 1442 mM. At an initial 4-CPA concentration of 1442 mM, the reaction rate was 92% of the rate at 72 mM. Unimmobilized cells and cell extracts had lower relative rates of reaction with increasing 4-CPA concentration.

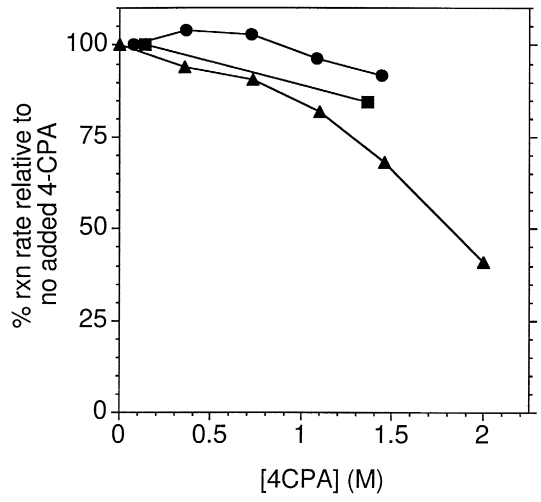


Fig. 6. Relative reaction rate vs. initial added 4-CPA concentration for production of 1.0 M 4-CPA (50 mM potassium phosphate or 4-CPA buffer, pH 7.5, 25°C) when using 72W/carrageenan beads (●), unimmobilized cells (■), and cell extract (▲) as catalyst.

Reaction rates during the production of from 1.0 to 1.5 M 4-CPA are typically zero order in MGN over at least 95% conversion, so product inhibition did not appear to be a significant problem.

Increasing the reaction temperature from 25°C to 30°C in comparative sets of recycle reactions using 72W/PAG catalyst produced the expected increase in reaction rate (ca. 40% increase in rate for 1.2 M 4-CPA production). The percent loss of catalyst activity with catalyst recycle was almost identical for two sets of recycle reactions run at 25°C and 30°C.

3.6. Scale-up of 4-CPA production

A total of 383 kg of 4-CPA (434 kg of 4-CPA ammonium salt) was produced in seven consecutive batch reactions with catalyst recycle run in a 150-gal reactor (as described in Section 2.7). The reaction rates for reactions 4–7 were all identical, demonstrating excellent catalyst stability, and reaction times averaged 18 h. The average yield of 4-CPA for the seven reactions was 98.7% at 100% conversion. Decanted product mixtures were filtered and concentrated by distillation to produce 52–63 wt.% 4-CPA for hydrogenation to 1,5-DMPD.

3.7. GA/PEI treatment of immobilized cells

The use of GA and PEI has been previously reported to significantly stabilize the enzymatic activity of immobilized microbial cells in many applications, including the production of high concentrations of carboxylic acid salts [27–31]. A series of 67 consecutive batch reactions with catalyst recycle were run to produce 1.3 M 4-CPA, where the use of uncrosslinked 72W/carrageenan beads was compared with GA/PEI-crosslinked 72W/carrageenan beads. The GA/PEI-crosslinked beads retained ca. 20% more activity over the course of the 67 runs compared to the uncrosslinked beads (Fig. 7). The measured soluble protein in the first product mixture produced by the crosslinked catalyst was only 3% of the total available soluble protein, while the first control reaction had 7.5% total available soluble protein in the aqueous phase of the product mixture. There was also no measurable loss of catalyst bead

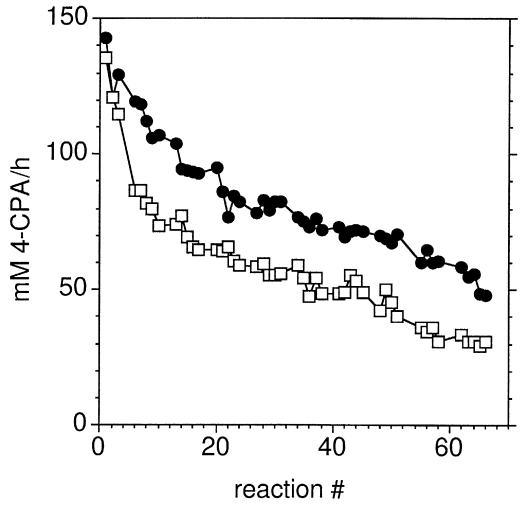


Fig. 7. Reaction rates for consecutive batch reactions with catalyst recycle (16.5 wt.% catalyst loading) for production of 1.3 M 4-CPA (pH 7.3, 25°C), using uncross-linked (□) or GA/PEI-crosslinked (●) 72W/carrageenan beads.

weight over the course of the 67 recycle reactions with the GA/PEI-crosslinked beads, and 1001 g 4-CPA/g dcw 72W cells was produced.

3.8. Hydrogenation of 4-CPA to DMPD

Using a 5% Pd/C slurry as catalyst, hydrogenation of an aqueous solution containing 69 wt.% 4-CPA with methylamine added in a 1:1 mol ratio at 150°C and 600 psig hydrogen produced an 88.1% yield of 1,5-DMPD. A small amount ($< 0.5\%$) of the 1,3-DMPD isomer was also produced. Under the reaction conditions, the nitrile is reductively alkylated with methylamine to produce the intermediate 5-(*N*-methylamino)-2-methylpentanoic acid. This intermediate is most likely present in both the ammonium and methylammonium forms during the hydrogenation, and equilibration to the acid form most likely occurs prior to cyclization at 150°C, where an equivalent of water is lost. The build-up of ammonia in the reaction contributes to competitive hydrogenation of 4-CPA to produce 5-amino-2-methylpentanoic acid, which in turn cyclizes to 5-methyl-2-piperidone in 6.9% yield. Hydrolysis of the nitrile group in the presence of ammonia and methylamine

yields a mixture of methylamino- and bis-methylamino amides of MGA in yields of ~1% each.

4. Conclusions

Optimization of reaction conditions and cell immobilization have produced significant improvements in catalyst stability and productivity in reactions run to convert MGN to 4-CPA at concentrations as high as 210 g/l (as the ammonium salt). The most significant factor affecting the stability of catalyst activity in recycle reactions was the purity of the MGN. Although the trace component(s) in the MGN responsible for nitrilase deactivation were not identified, simple and economical methods of purification were identified; the most preferred method would be to perform a more efficient distillation at the site of MGN manufacture, but alumina-treatment of MGN prior to running the reaction is a feasible alternative.

Immobilized cell catalyst productivities as high as 1000 g 4-CPA/g dcw of immobilized *A. facilis* 72W cells were achieved by recycling the catalyst in consecutive stirred-batch reactions; this level of productivity could not be achieved with unimmobilized cells, which lysed and rapidly lost activity in consecutive batch reactions that produced high concentrations of 4-CPA ammonium salt. Scale-up of the reaction to a 500-l reaction volume using cells immobilized in shredded carrageenan gel produced a total of 434 kg of 4-CPA ammonium salt in seven consecutive batch reactions with catalyst recycle, with no measurable loss of catalyst activity. The problem of catalyst attrition and production of small particles that required removal by ultrafiltration when using this irregularly shaped catalyst was resolved by immobilizing cells in gel beads; the beads were very robust and did not suffer from attrition when stirred continuously in batch reactions for several months. The stability of nitrilase activity under reaction conditions was further improved by crosslinking the gel beads with GA and PEI.

Hydrogenation of concentrated aqueous solutions of 4-CPA ammonium salt which were produced using immobilized cell carrageenan beads produced the desired product, 1,5-DMPD, in high yield. The concentrated 4-CPA ammonium salt solutions required no further purification prior to hydrogenation. 5-Methyl-2-piperidone was produced as the only major byproduct of the hydrogenation, and separation of product from byproducts was easily performed by simple distillation.

Acknowledgements

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