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Immobilization of *Rhodococcus* AJ270 and Use of Entrapped Biocatalyst for the Production of Acrylic Acid

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Summary. *Rhodococcus* AJ270 is adsorbed by Dowex 1 at 15.4 mg dry weight per g resin with maximum amidase specific activity observed at lower loadings. Bacteria form a monolayer on the resin surface, and adsorption is complete within 2 min. AJ270 can be entrapped in agar and agarose gels (optimum loading: 20 mg dry weight bacteria per cm^3 gel). Adsorption and entrapment improve amidase thermal stability 3–4 fold, and entrapment shifts the *pH* optimum from 8 to 7. Adsorbed and free bacteria show similar values for K_m and V_{max}, but entrapped bacteria have higher K_m values. Compared with bacteria adsorbed to Dowex, the activity per cm^3 of matrix of agar-entrapped AJ270 is eight-fold higher. In stirred-tank reactors, exposure to acrylic acid reduces the amidase activity of the biocatalyst in the hydrolysis of acrylamide. In column reactors, entrapped AJ270 suffers little reduction in amidase activity against 0.25 *M* acrylamide over 22 h continuous operation.

Keywords. Acrylamide; Amidase; Acrylate; Biocatalysis; *Rhodococcus*.

Introduction

When grown on acetamide, *Rhodococcus* AJ270 expresses high specific activities of nitrile hydratase and amidase [1], enzymes with broad specificity against a wide range of nitriles and amides [2,3]. Washed suspensions of AJ270 have been used for the enantioselective hydrolysis of (*R,S*)-2-phenylbutyronitrile [2] and of racemic ibuprofen amide, in the latter case yielding the pharmacologically active non-steroidal anti-inflammatory agent *S*-(+)-ibuprofen in 94% *e.e.* [1]. This versatile biocatalyst also catalyses the regioselective hydrolysis of certain dinitriles [2,4]. These applications used the rhodococcal biocatalyst in a 'one-shot' batch mode, whereas immobilization of the bacteria would allow multiple re-use of the catalyst, possibly in continuous reactors, and would certainly facilitate product recovery. This paper describes methods for the adsorption and entrapment of AJ270, reports changes in the properties of the amidase activity resulting from immobilization, and investigates the use of entrapped bacteria for the hydrolysis of acrylamide. This latter biotransformation is of commercial significance for the

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Table 1. Adsorption of AJ270 to anionic exchange resins. Bacteria (4.2 mg dry weight) were immobilized to the anionic exchangers (3 g wet weight), and the % of bacteria adsorbed determined as described in the Experimental section. For the data in the second column, the bacteria and resins were washed with buffer containing 0.01 *M EDTA* prior to immobilization

	% of bacteria adsorbed	
Resin	*−EDTA*	*+EDTA*
Amberlite IRA 904	6	22
Amberlite IRA 400	21	24
Amberlite IRA 402	2	7
Amberlite IRA 440c	6	7
Amberlite IRA 900	4	14
Dowex 1X8-400	96	94
Dowex 2X8-400	60	50

production of acrylates, commodity chemicals produced globally on a large scale for the manufacture of a wide variety of polymers [5,6].

Results and Discussion

Adsorption of AJ270 to anionic exchange resins

Since bacteria generally carry an overall negative charge at physiological *pH*, a variety of anionic exchange resins were tested as adsorption matrices. Most of them except Dowex 1 and 2 showed poor adsorption of AJ270, with Dowex 1 having the highest capacity (Table 1). Treatment of the bacteria with *EDTA* to remove cations from the bacterial surface improved attachment to some of the Amberlite resins significantly, but Dowex 1 remained the best adsorption matrix and was used for all further experiments. Three different Dowex 1 resins were tested, all of mesh size 200–400 but differing in the degree of cross-linkage (2, 4, 8%). For each, the dry weight of AJ270 adsorbed increased with the dry weight of bacteria added over the range of 2–8 mg dry weight per cm^3 settled resin, but little further binding was observed on increasing the amount of bacteria added further. Dowex $1 \times 4-400$ (4% cross-linked) bound slightly more bacteria (7.6 ± 0.3 mg dry weight AJ270 per cm^3 settled resin; 15.4 mg dry weight per g dry resin) than the other two resins (7.0 ± 0.2 mg/cm^3 for 2% cross-linked and 6.4 ± 0.2 mg/ml for 8% cross-linked), and the data below were obtained with this matrix. The specific amidase activity of the bound AJ270 decreased as the bacterial loading increased so that resin loaded with 7.6 mg dry weight bacteria per cm^3 had only half the specific activity of resin carrying 2 mg dry weight per cm^3 (0.9 and 1.6 units per mg dry weight, respectively).

Under the binding conditions used, the resin reached 80% of capacity within 1 min. and full capacity after 2 min. Scanning electron microscopy of resin loaded with 7.6 mg dry weight AJ270 per cm^3 showed the adsorbed biocatalyst to consist of a monolayer of single, rod-shaped bacteria attached along their longest axis and

Table 2. Thermal stability of amidase at 60°C in AJ270 adsorbed to Dowex 1, entrapped in agar or in free suspension. Samples (1 cm^3) of AJ270 in free suspension, adsorbed to Dowex 1X4-400 at 2 mg dry weight bacteria per cm^3 resin or entrapped in agar at 20 mg dry weight per cm^3 gel were quickly heated to 60°C and held at that temperature for timed intervals. They were then rapidly cooled in an ice-water bath and assayed for amidase activity against acetamide. 100% = 3.1 units per mg dry weight for free bacteria, 1.7 units per mg dry weight for adsorbed bacteria, or 1.6 units per mg dry weight for entrapped bacteria

	% of initial amidase activity		
Time (min)	Free bacteria	Adsorbed bacteria	Entrapped bacteria
0	100	100	100
5	74	96	98
10	35	61	70
20	31	53	62
40	26	44	47
60	19	34	38

evenly covering the surface of the resin beads. This suggests that the groups involved in attachment are evenly distributed over the bacterial surface whilst cell-to-cell interactions are not important in attachment. Transmission electron microscopy of bead cross-sections confirmed that the bacteria did not penetrate to the interior of the resin beads.

Effect of salt, temperature, and pH value on the amidase activity of adsorbed AJ270

Adsorbed amidase activity was not eluted by prolonged washing of the immobilized biocatalyst in a buffer containing 2.5 *M* NaCl indicating that factors other than charge are important in attachment. The temperature profile for the amidase activity of immobilized bacteria (tested at 0.2 cm^3 resin or 0.4 mg dry weight AJ270 per cm^3 buffer) was very similar to that of bacteria in free suspension (0.4 mg dry weight per cm^3 buffer), activity increasing steadily to a maximum at about 55°C with only 60% remaining at 70°C. The amidase activity of the adsorbed bacteria was significantly more stable to high temperature than that of bacteria in free suspension (Table 2) with a significant three-fold increase in $T_{0.5}$ from 7 min to 22 min. The *pH* profiles for adsorbed and free bacteria were very similar at high *pH* values with clear optima at $pH = 8.0$, but the adsorbed bacteria were significantly less active at acid *pH* values (Fig. 1). This effect was not due to elution of adsorbed bacteria with decreasing *pH* since washing the immobilized biocatalyst with buffers of *pH* value from 5.5 to 9.0 and then measuring their amidase activity at *pH* 8.0 resulted in losses of amidase activity not exceeding 4%.

Entrapment of AJ270 in agar and agarose beads

There are several gels that may be used for the entrapment of bacteria differing in their properties and method of preparation. In this study, agar was chosen for its

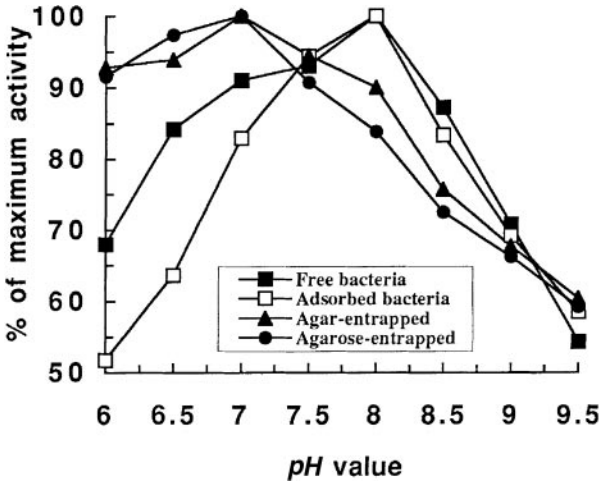

Fig. 1. Effect of *pH* on the amidase activity of *Rhodococcus* AJ270. AJ270 adsorbed to Dowex 1X4-400 beads at 2 mg dry weight per cm^3 of resin entrapped in agar or agarose beads at 20 mg dry weight per cm^3 gel or free in suspension were assayed for amidase activity against acetamide in buffers of different *pH* value. 100% = 3.3 units per mg dry weight for free bacteria, 1.7 units per mg dry weight for adsorbed bacteria, or 1.6 units per mg dry weight for entrapped bacteria

relatively low cost and ease of handling, and compared with agarose, its more expensive counterpart. When bacteria are entrapped within a carrier gel, the rate of reaction is dependent not only on the specific activity of the bacteria but also upon mass transfer limitations imposed by the topography of the carrier matrix. If the mass of bacteria within the gel is increased, there will be a point at which the rate of reaction becomes diffusion limited, and further increases in bacterial loading will have little effect. This point occurred above 20 mg dry weight bacteria (per cm^3 gel), equivalent to 1.33 g dry weight (per g dry matrix) with agar and agarose gels, under the conditions used in this study (1.5% w/v gels; bead size < 1 mm diameter; 50 m*M* acetamide; assay conditions as described in the Experimental section) (Fig. 2). Bacterial loadings in excess of 80 mg dry weight (per cm^3 gel) were readily achieved with agar and agarose gels but with no further increase in amidase activity per g gel.

Effect of temperature and pH on the amidase activity of entrapped AJ270

The temperature-activity profiles for the amidase activity of bacteria entrapped in agar or agarose (tested at 0.2 cm^3 gel or 4 mg dry weight AJ270 per cm^3 buffer) were almost identical to those reported above for bacteria in free suspension or for bacteria adsorbed to Dowex 1. Amidase activity was most stable in agar-entrapped bacteria with $T_{0.5}$ at 60°C = 34 min, a more than four-fold increase over the $T_{0.5}$ of free bacteria (Table 2). The *pH* optimum of amidase activity was shifted to lower *pH* values in both agar- and agarose-entrapped bacteria (Fig. 1) with highest activities observed at *pH* values between 6 and 7, whereas the activity of bacteria in free suspension was highest at *pH* 8.0 and was 32% lower at *pH* 6.

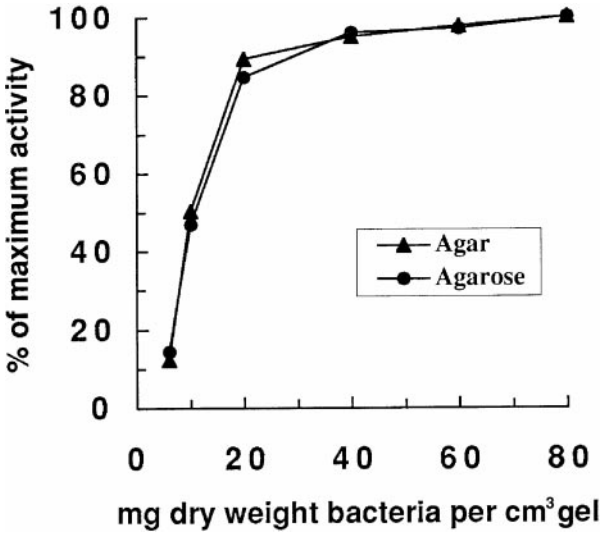

Fig. 2. Effect of bacterial loading on the specific amidase activity of *Rhodococcus* AJ270 entrapped in agar beads; 100% = 32 units per cm^3 gel)

Kinetics of amidase activity in free and immobilized AJ270

Apparent K_m and V_{max} values for acetamide and acrylamide hydrolysis by AJ270 washed suspensions and by Dowex-adsorbed, agar- and agarose-entrapped AJ270 are given in Table 3. The K_m values for free bacteria and for adsorbed bacteria were

Table 3. Kinetic data for amidase activity in free and immobilized AJ270. K_m and V_{max} values were determined by *Lineweaver-Burke* double reciprocal plots of specific amidase activity as a function of substrate concentration; n.a.: not applicable, n.d.: not done

	Immobilisation Matrix			
Parameter	None	Dowex 1	Agar	Agarose
Loading (mg dry weight per cm^3)	n.a.	20	20	20
Apparent K_m for acetamide (m*M*)	6.7±0.4	7.3±0.5	20.4±0.8	20.1±0.9
Apparent V_{max} for acetamide (*U* per mg dry wt. bacteria)	3.9±0.3	2.4±0.3	2.2±0.2	2.1±0.3
Apparent V_{max} for acetamide (*U* per cm^3 matrix)	n.a.	5	48	42
Apparent K_m for acrylamide (m*M*)	9.2±0.3	11.1±0.5	24.7±0.9	n.d.
Apparent V_{max} for acrylamide (*U* per mg dry wt. bacteria)	1.1±0.2	0.8±0.2	0.7±0.2	n.d.
Apparent V_{max} for acrylamide (*U* per cm^3 matrix)	n.a.	1.6	14	n.d.

very similar, presumably because the monolayer of bacteria adsorbed to the beads of anionic exchanger does not significantly affect the free diffusion of substrate to the bacteria. In contrast, bacteria entrapped in gel beads are impeded in their access to substrate, and this is reflected in the three-fold higher apparent K_m value. Nevertheless, because of the higher bacterial loadings achieved by entrapment, this process resulted in an immobilized biocatalyst with at least 8-times the activity (per unit volume) achieved with Dowex-adsorbed bacteria (based on V_{max} values).

We conclude that adsorption has little effect on amidase kinetic parameters, but significantly improves the temperature stability. As might be expected, entrapment in agar or agarose beads has a greater effect on kinetic parameters producing a biocatalyst with higher K_m. However, this disadvantage is outweighed by (*i*) much higher bacterial loadings, (*ii*) greater temperature stability, and (*iii*) a shift in the *pH* optimum from 8 to 7. As pointed out by *Brennan et al*. [5], *pH* 7 is ideal for ammonium acrylate production. In addition, the physical characteristics of the gel-entrapped biocatalysts are better suited to continuous column reactors than Dowex 1, allowing for higher flow-rates at lower pressure differentials. No advantage was found in using the more expensive agarose, rather than agar, as the immobilization matrix.

Biotransformation of acrylamide to acrylic acid in an STR

The kinetic data for acrylamide hydrolysis by agar-entrapped AJ270 (Table 3), in comparison with the data for acetamide hydrolysis, indicated a somewhat poorer affinity for this substrate and a significantly lower reaction rate. Nevertheless, the data suggest that entrapped AJ270 could be a very useful biocatalyst for acrylic acid production. In order to test this, the hydrolysis of 250 m*M* acrylamide to acrylic acid was attempted using 10 cm^3 of agar-entrapped bacteria (20 mg dry weight bacteria per cm^3) in a 50 cm^3 batch STR at 30°C (Fig. 3). Under the conditions used (see Experimental), acrylamidase activity was independent of stirrer speeds between 100 and 500 rpm. A STR operated in batch mode may be subject to diffusional limitations represented by the stationary boundary layer between the bulk fluid and the support surface. Clearly even the lowest stirrer speed used in this study was sufficient to overcome this boundary layer effect. About one fifth (2.44 mmol) of the starting acrylamide (12.5 mmol) was converted to acrylic acid after 30 min (specific activity for acrylamide hydrolysis = 0.4 units per mg dry weight bacteria), but the reaction then slowed down very significantly so that after 210 min only 59% of the acrylamide had been hydrolyzed. The values quoted are the mean values of three separate experiments, and in each case at least 95% of the acrylamide hydrolyzed was accounted for as acrylic acid.

Inhibition of amidase activity by acrylic acid

The results in Fig. 3 suggest that contact with high concentrations of acrylamide or acrylic acid inhibits the amidase activity of the immobilized biocatalyst. Exposure to high acrylamide concentrations was reduced by stepwise addition of the substrate (Fig. 3). This increased the final concentration of acrylic acid achieved, but only slightly improved the proportion of acrylamide hydrolysed (from 59 to

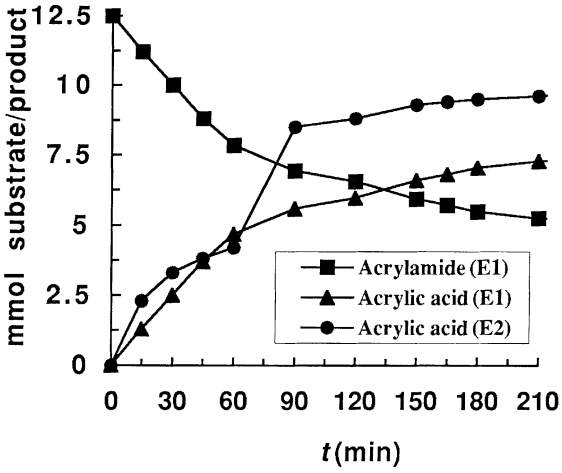

Fig. 3. Hydrolysis of acrylamide by agar-entrapped *Rhodococcus* AJ270 in a STR. In the first experiment (E1), agar-entrapped AJ270 (10 cm^3; 20 mg dry weight per cm^3 gel) was incubated with 12.5 mmol acrylamide in a 50 cm^3 STR. In the second experiment (E2), the STR contained 5 mmol acrylamide, and further 5 mmol were added at 60, 120 and 180 min. Acrylamide and acrylic acid concentrations were determined by GC

66%), suggesting that acrylic acid is the major inhibitor. This was confirmed (Table 4) by incubating entrapped bacteria for 20 min with 0–250 m*M* acrylic acid, then washing the biocatalyst with buffer, and measuring its amidase activity against acetamide. Inhibition by acrylic acid in the experiments described above was not due to a significant fall in *pH*.

Continuous acrylamide hydrolysis/acrylic acid production in a column reactor

In a continuous column reactor fed with an acrylamide solution, only the biocatalyst at the outlet end of the column is exposed to high concentrations of product and therefore subject to inhibition by acrylic acid. The amidase activity of such a column is unlikely to suffer the dramatic losses observed in the STR.

Continuous column reactors containing 40 cm^3 of agar-entrapped AJ270 were challenged with acrylamide solutions of different concentrations at various flow

Table 4. Inhibition of amidase activity in entrapped AJ270 by acrylic acid. Samples of AJ270 entrapped in agar beads at 20 mg dry weight per cm^3 gel were incubated at 4°C for 20 min in buffer containing various concentrations of acrylic acid. The beads were then spun, washed in buffer, and assayed for amidase activity against acetamide. 100% = 31 units per cm^3 gel.

Acrylic acid (m*M*)	% Activity remaining
0	100
25	86
50	81
100	74
175	61
250	50

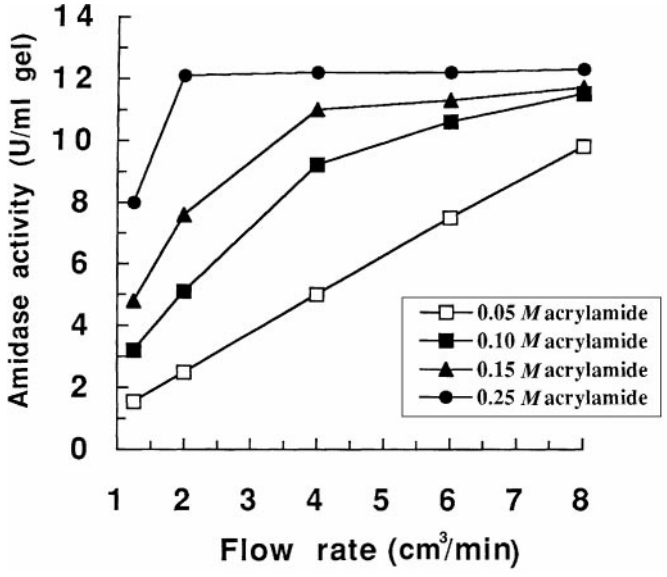

Fig. 4. Effect of flow rate and acrylamide concentration on the amidase activity of agar-entrapped *Rhodococcus* AJ270 in a column reactor. Agar-entrapped AJ270 (40 cm^3 gel; 20 mg dry weight per cm^3) in a column reactor (internal diameter 2.4 cm) was fed with buffers containing 0.05–0.25 *M* acrylamide at various flow rates. Acrylamidase activities were computed from the acrylamide concentration in the input and effluent streams

rates. Specific activity increased with both flow rate and acrylamide concentration until a plateau was reached at about 12.3 units (per cm^3 gel) (Fig. 4). As expected, % conversion rates followed the reverse trend (Fig. 5). The results suggested that a flow rate of 1.3–2.0 $cm^3 \cdot min^{-1}$ would achieve >95% conversion of 0.25 *M* acrylamide.

In a final set of experiments, similar column reactors packed with agar-entrapped AJ270 (40 cm^3 gel; 20 mg dry weight bacteria per cm^3 of gel) and maintained at 30°C were fed continuously with 0.25 *M* acrylamide in 0.1 *M tris*-HCl buffer (*pH* 7) at 1.5 $cm^3 \cdot min^{-1}$ for 30 h. These reactors were operated at >97% conversion of the substrate to acrylic acid for 22 h (specific acrylamidase activity = 0.4 units per mg dry weight bacteria), after which the % conversion fell steadily to 60% at 30 h.

Rhodococci expressing nitrile hydratase and immobilized in polyacrylamide gel are used for the commercial production of acrylamide from acrylonitrile in a plant with a capacity of 30000 t/a [7]. In contrast, a commercial process for the production of acrylate has not been developped, even though this commodity chemical is widely used in the production of polymeric flocculents, thickeners, adhesives, and adsorbents. Several workers have described the isolation of bacteria able to grow on acrylamide [8–11], but most work on acrylamide hydrolysis, as in the present study, has involved bacteria grown on simpler, saturated aliphatic nitriles and amides.

Rhodococcus AJ270 grown on acetamide expresses high amidase activity against acrylamide. However, with 0.25 *M* acrylamide as substrate, exposure to the acrylic acid product slowly inactivates the amidase activity, eventually limiting the

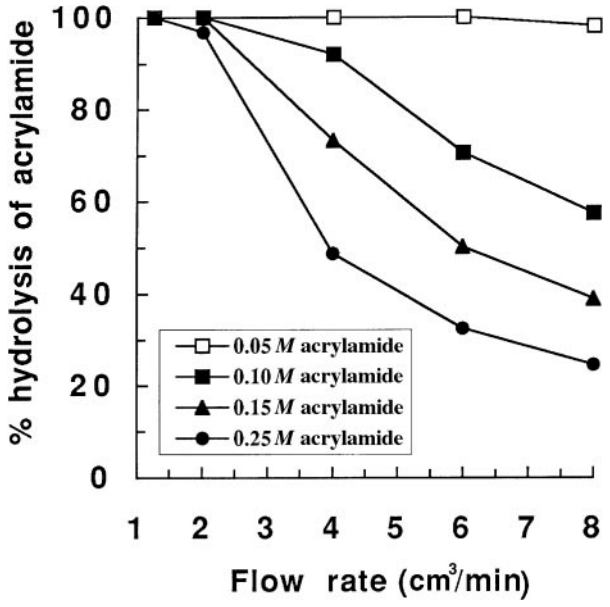

Fig. 5. Effect of flow rate and acrylamide concentration on percent conversion by agar-entrapped *Rhodococcus* AJ270 in a column reactor. The conditions were as for Fig. 4; percent conversion figures were computed from the acrylamide concentrations in the input and effluent streams

% conversion of the acrylamide substrate. Inactivation of immobilized acetamide-grown *Corynebacterium nitrilophilus* by high concentrations of acrylamide and acrylic acid during the biocatalytic hydrolysis of acrylamide has been noted previously [5] and is thought to be due to the effect of these β-unsaturated compounds on sulfydryl groups essential for amidase activity [12]. In the present study, inactivation was minimized by using a fixed-bed column reactor allowing continuous production of acrylic acid for 22 h at >97% conversion of acrylamide. Improvements on this basic protocol, such as the periodic replacement of the biocatalyst at the outlet end of the reactor, might enable extended production of acrylate using this biocatalyst.

Experimental

Chemicals and immobilization matrices

Acrylic acid (99%) and acetamide (99+%) were obtained from Aldrich (Dorset, UK). Dowex 1X4-400, purified agar, agarose (type 1-A), and all other chemicals and matrices were purchased from Sigma (Dorset, UK). Vegetable oil (pure sunflower oil) was obtained from a large UK retail outlet (ASDA Stores, Leeds). The ion exchange resins used in the study (see Table 5) were all polystyrene based. Amberlite IRA900 and IRA904 are macroreticular, the others are gels.

Analytical methods

Acrylamide and acrylic acid were determined by gas chromatography as described previously [2]. The concentrations of ammonia in assay samples were determined using *Nessler*'s reagent. The

Table 5. Properties of the anionic exchange resins used

Resin	Active group	Mesh size	% Cross linked	Capacity ($meq \cdot cm^{-3}$)	Ionic form
Amberlite IRA904	quaternary ammonium	20–50	–	1.0	Cl^-
Amberlite IRA400	quaternary ammonium	16–50	8	1.4	Cl^-
Amberlite IRA402	quaternary ammonium	16–50	6	1.3	Cl^-
Amberlite IRA440c	quaternary ammonium	16–40	–	1.0	OH^-
Amberlite IRA900	quaternary ammonium	16–50	–	1.0	Cl^-
Dowex 1X8-400	trimethylbenzylammonium	200–400	8	1.2	Cl^-
Dowex 2X8-400	dimethylethanol-benzylammonium	200–400	8	1.2	Cl^-

sample (0.025 cm^3) was diluted with 0.85 cm^3 of water, and then 0.125 cm^3 of reagent (ammonia colour reagent from Sigma, Dorset, UK) were added. The colour was allowed to develop for 10 min before measuring the absorbance at 420 nm. Standard curves were prepared using NH_4Cl (> 99.5%). Bacterial concentrations of washed suspensions were determined turbidometrically by measuring their absorbance at 540 nm and converted to dry weights using a standard curve. The data are the mean values from three separate experiments; in no case the results of single experiments differed by more than 12%.

Bulk growth of Rhodococcus AJ270

AJ270 was grown on 50 m*M* acetamide, and washed suspensions were prepared as described previously [1].

Adsorption on Dowex 1 and other anionic exchangers

Bacteria and resin were both washed three times in 0.1 M *tris*-HCl buffer, *pH* 8.0, prior to use. Bacteria were re-suspended in the same buffer to 22 mg dry weight (per cm^3) and 5 cm^3 of the suspension were added to a Universal bottle containing 3 g of the washed resin. The mixture was agitated for 45 min on a Denley Spiramix, the resin allowed to settle for 5 min, and then the supernatant was removed. The settled resin was washed five times with 0.01 *M tris*-HCl buffer, *pH* 8.0, to remove any unadsorbed bacteria. The amount of bacteria adsorbed was determined by comparing the absorbance (A_{540nm}) of the bacterial suspension before and after adsorption. The basic method was modified by changing the ratio of bacteria to resin, including *EDTA* in the washing buffers, or by using different anionic exchangers.

Entrapment in agar or agarose

The method was modified from *Wikstrom et al*. [13]. Bacteria were washed three times with 0.1 *M tris*-HCl buffer, *pH* 8.0, resuspended in the same buffer to the desired concentration, and mixed with an equal volume of 3% (w/v) molten agar or agarose at 48°C. The mixture was quickly added to an equal volume of vegetable oil also held at 48°C and agitated with a *Rushton*-style impeller at 250 rpm to form an even emulsion. The temperature of the emulsion was then lowered by immersion of the vessel in an ice-water bath whilst maintaining the agitation. Once the temperature had fallen below 20°C, agitation was stopped, and the two phases were allowed to separate. The aqueous phase consisted of a heterogeneous mix of bead sizes < 1 mm in diameter. The beads were washed with buffer until no oil was visible on the buffer surface.

Bioreactors

The stirred-tank reactor (STR) consisted of a glass vessel of working volume 50 cm^3, fitted with baffles and agitated using a *Rushton*-style impeller at 100–500 rpm. Fixed-bed column reactors consisted of jacketed glass columns (2.4 cm internal diameter) packed with 40 cm^3 of agar-entrapped biocatalyst. Substrate solution was pumped through the columns in an upward direction to prevent compression of the gel. Both types of reactors were kept at constant temperature (30°C) by connecting their water jackets to a circulating water bath.

Determination of amidase activity

For washed suspensions and Dowex 1-immobilized bacteria, samples (0.1 cm^3) and 0.35 cm^3 of 0.1 M *tris*-HCl buffer, *pH* 8.0 were placed in an *Eppendorf* tube and pre-warmed to 30°C in a water bath. Substrate (0.05 cm^3 of 0.5 *M* sodium acetamide unless stated otherwise) was added, and the tube was incubated at 30°C for 5–30 min before stopping the reaction with 0.025 cm^3 of 0.25 M HCl. The mixture was then centrifuged for 2 min in a microcentrifuge, and the clear supernatant was removed for ammonia determination.

Gel-entrapped bacteria were drained of all buffer and 5 g removed to a 50 cm^3 conical flask. The beads were resuspended in 22.5 cm^3 of 0.1 *M tris*-HCl buffer, *pH* 8.0, and warmed to 30°C in a shaking waterbath. Pre-warmed substrate solution (2.5 cm^3 of 0.5 *M* sodium acetamide unless otherwise stated) was added, and samples were removed at timed intervals for ammonia determination.

In experiments where amidase activity was determined at different *pH* values, 0.1 *M* sodium phosphate buffers were used for *pH* 6–7.0 and 0.1 *M tris*-HCl buffers for *pH* 7.5–9.5.

Electron microscopy of adsorbed bacteria

Samples were fixed in 2.5% (w/v) glutaraldehyde in 0.1 M sodium phosphate buffer, *pH* 7.3, at 4°C. The fixed samples were then washed twice with the same buffer before fixing in 1% (w/v) osmium tetroxide for 1 h. After washing twice with water, the samples were fixed in 1% (w/v) uranyl acetate for 30 min and washed in water for 5 min. After fixing, samples were dehydrated by washing with ethanol (50, 70, 90% v/v and then twice with 100% for 15 min each wash).

Samples for TEM were embedded in epoxy resin, sectioned, and the sections mounted on copper grids. Grids were stained with saturated uranyl acetate in 70% (v/v) ethanol for 10 min, washed with water, then with alkaline lead citrate for 10 min, and then with water again before drying. Samples for SEM were mounted on carbon adhesive discs and sputter-coated initially with carbon and then gold/palladium. Specimens were viewed using a Hitachi H7000 microscope and, for SEM, a H7110 scanning attachment.

Acknowledgements

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