J Ind Microbiol Biotechnol (2008) 35:35–40
DOI 10.1007/s10295-007-0263-z

ORIGINAL PAPER

Acrylamide synthesis using agar entrapped cells of *Rhodococcus rhodochrous* PA-34 in a partitioned fed batch reactor

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Received: 19 April 2007 / Accepted: 19 September 2007 / Published online: 10 November 2007

Abstract The nitrile hydratase (Nhase) induced cells of *Rhodococcus rhodochrous* PA-34 catalyzed the conversion of acrylonitrile to acrylamide. The cells of *R. rhodochrous* PA-34 immobilized in 2% (w/v) agar (1.76 mg dcw/ml agar matrix) exhibited maximum Nhase activity (8.25 U/mg dcw) for conversion of acrylonitrile to acrylamide at 10°C in the reaction mixture containing 0.1 M potassium phosphate buffer (pH 7.5), 8% (w/v) acrylonitrile and immobilized cells equivalent to 1.12 mg dcw (dry cell weight) per ml. In a partitioned fed batch reaction at 10°C, using 1.12 g dcw immobilized cells in a final volume of 1 l, a total of 372 g of acrylonitrile was completely hydrated to acrylamide (498 g) in 24 h. From the above reaction mixture 87% acrylamide (432 g) was recovered through crystallization at 4°C. By recycling the immobilized biocatalyst (six times), a total of 2,115 g acrylamide was produced.

Keywords *Rhodococcus rhodochrous* PA-34 · Immobilized biocatalyst · Agar · Acrylonitrile · Acrylamide · Partitioned reactor

Introduction

Acrylamide is an important commodity chemical used as a coagulator, soil conditioner, stock additive for paper treatment and in leather and textile industry [10]. It can be synthesized both chemically and enzymatically [18]. Enzymatic synthesis involves nitrile hydratase (Nhase) mediated hydration of acrylonitrile and more than 95,000 tonnes per annum is being produced through this process [15]. In 2000, the worldwide demand for acrylamide was estimated to be 400,000 tonnes per annum and was expected to grow at 3–5% per year from 2000 through 2005 [13].

In most of the reports pertaining to acrylamide synthesis, free cells of microorganisms containing Nhase activity have been used [1, 5, 16, 18]. In order to harness the maximum catalytic potential of the cells to convert acrylonitrile to acrylamide, immobilization becomes highly desirable. Very few studies have been carried out using microbial cells immobilized in alginate, polyacrylamide and diethylaminoethyl (DEAE) cellulose for conversion of acrylonitrile to acrylamide [4, 7, 8, 12, 14]. Agar has also been used for the immobilization of cells and enzymes [2]. However, agar immobilization of whole cells to be used for biotransformation of nitriles to amides or acids has not been studied.

R. rhodochrous PA-34 had exhibited very high conversion of acrylonitrile to acrylamide (55 U/mg dcw) and led to an accumulation of 600 g acrylamide in a fed batch reaction at 1 l scale [17]. These cells retained high Nhase activity after the reaction and it would be worthwhile to recycle these cells. Recycling can be easily accomplished if the cells are immobilized. In the present communication, the use of agar immobilized cells of *R. rhodochrous* PA-34 for conversion of acrylonitrile to acrylamide is being reported for the first time.

Materials and methods

The nitriles and amides used in the present study were purchased from Lancaster Synthesis, England. The culture media ingredients were from HiMedia, Mumbai, India. All other chemicals were of analytical grade and procured from various commercial sources.

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Microorganism and culture conditions

Rhodococcus rhodochrous PA-34 was from Nippon Mining Co., Ltd, Japan (now Japan Energy Corporation). Cells were cultured as described previously for induction of Nhase [17]. The culture broth was centrifuged at 5,000*g* for 20 min, the cells were resuspended in 0.1 M potassium phosphate buffer (pH 7.0) such that it contained 22 mg dcw/ml. The resuspended cells were stored at 4°C till further use.

Immobilization of *R. rhodochrous* PA-34 cells in agar

The Nhase induced cells of *R. rhodochrous* PA-34 were immobilized following the modified method of Kierstan and Coughlan [11]. Agar slurry of 2*X* concentration was prepared in 0.1 M potassium phosphate buffer (pH 7.0) by melting at 100°C and then cooled to 50°C in a water bath (*X* = agar concentration, w/v, in the immobilized biocatalyst). The cell suspension (22 mg dcw/ml) was diluted to 2*Y* concentration (*Y* = cell suspension concentration, v/v, in the immobilized biocatalyst) and mixed thoroughly in equal volume of the molten agar. This slurry was poured into a Petri dish and kept for 30 min at 4°C for further solidification.

The solidified agar gel was cut into small discs (1 cm diameter and 0.5 cm thickness), washed with 0.1 M potassium phosphate buffer (pH 7.0) and stored at 4°C till further use.

Assay for nitrile hydratase of agar immobilized cells of *R. rhodochrous* PA-34

The Nhase assay mixture contained (per ml) 100 μmol potassium phosphate buffer (pH 7.5), immobilized biocatalyst equivalent to 0.5 mg dcw and 0.02 g acrylonitrile. The reaction was carried out at 10°C for 1 h and stopped by the separation of immobilized cells from the reaction mixture. The amount of acrylamide produced in the reaction mixture was determined from the absorbance at 235 nm (ε_{235} = 0.935 μmol/ml/cm) using UV–VIS spectrophotometer (Shimadzu 160A, Japan) [9]. One Unit of Nhase activity was defined as hydration of 1 μmol of acrylonitrile to acrylamide per min by the immobilized biocatalyst under the assay conditions.

Analytical methods

The acrylonitrile, acrylamide and acrylic acid in the reaction mixture were estimated by gas chromatography (GC; NETEL make, India) using Porapek QS Column (80–100 mesh, 0.32 cm × 2.0 m) and flame ionization detector (FID). The temperatures of column, injector and FID detector were 220, 270 and 300°C, respectively, and nitrogen was used as carrier with a flow rate of 25 ml/min. The retention times for acrylonitrile, acrylic acid and acrylamide in this assay were 1.23, 2.09, and 5.47 min, respectively.

Optimization of agar and resting cells concentrations for immobilization

To optimize the agar and cell concentration for immobilization (entrapment) of *R. rhodochrous* PA-34 cells in agar, the concentration of agar (1–5% w/v) and cells (4–20% v/v of 22 mg dcw/ml cell suspension) were varied and the Nhase activity of the immobilized cells was assayed.

Optimization of reaction conditions for conversion of acrylonitrile to acrylamide using agar immobilized cells

The conversion of acrylonitrile to acrylamide was carried out in 0.1 M potassium phosphate buffer at different pHs (pH 5.5–8.5) and at different temperatures ranging from 10 to 50°C. To work out the optimum enzyme (agar immobilized cells) and substrate concentrations for the conversion of acrylonitrile to acrylamide, the amounts of acrylonitrile (2–20% w/v) and immobilized biocatalyst (equivalent to 0.28–1.40 mg dcw/ml reaction mixture) were varied in the reaction mixture. The conversion of 8% (w/v) acrylonitrile to acrylamide was studied up to 4 h to find out the time required for the complete conversion of added substrate in the reaction mixture.

Acrylamide synthesis using agar entrapped cells of *R. rhodochrous* PA-34 in a partioned fed batch reactor

The optimized acrylamide production at 1 l scale (final volume) was carried out at 10°C in a fed batch mode using BioFlo C-32 Fermenter (New Brunswick Scientific, USA) as reaction vessel with some modifications (Fig. 1). The reaction mixture initially comprised 428 ml of 0.1 M potassium phosphate buffer (pH 7.5), and immobilized biocatalyst corresponding to 1.12 g dcw between the nylon net partition and the vessel wall. In each feed, acrylonitrile was added to a concentration of 8% (w/v) in the reaction mixture and a total of 372 g of the substrate was added in seven feeds after every 3 h (0 h: 38 g; 3 h: 42 g; 6 h: 47 g; 9 h: 52 g; 12 h: 58; 15 h: 64 g; 18 h: 71 g). The water consumed for the hydration of acrylonitrile during the reaction was also compensated accordingly during each feed. The impeller speed was set to 200 rpm and the reaction was allowed to proceed for 24 h. Samples (0.1 ml) were withdrawn at intervals of 3 h and analyzed by gas chromatography for the amount of acrylonitrile, acrylamide and acrylic acid (if any) in the reaction mixture.

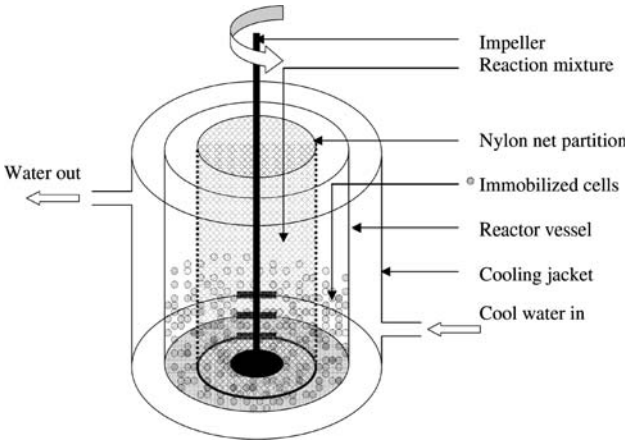

Fig. 1 Schematic diagram of the reaction vessel used for the bioconversion of acrylonitrile to acrylamide by the agar immobilized cells of *R. rhodochrous* PA-34. The physical contact of the immobilized cells (agar discs) with the impeller during agitation was prevented by placing them between the reactor vessel and nylon net partitioning. The reaction mixture had free contact with the immobilized cells and the temperature of the reactor was maintained at 10°C by circulation of chilled water through the cooling jacket

Recycling of immobilized cells of *R. rhodochrous* PA-34

The agar discs containing cells of *R. rhodochrous* PA-34 (immobilized biocatalyst) separated from the above reaction mixture were washed thoroughly in potassium phosphate buffer (pH 7.5) and recycled six times for performing biotransformation of acrylonitrile to acrylamide in similar reaction conditions as mentioned above. After each cycle, the residual nitrile hydratase activity of the immobilized cells was also determined.

Recovery of acrylamide

After separating out the immobilized biocatalyst, the reaction mixture was kept at 4°C until the acrylamide in the mixture formed crystals. The acrylamide crystals were harvested by filtration at 4°C and dried at room temperature and weighed. This simple procedure, which took 16 h, allowed for an 87% recovery of the acrylamide.

Results and discussion

Immobilization of *R. rhodochrous* PA-34 cells in agar

The entrapment of cells in agar is a very simple technique used for the immobilization or entrapment of cells in the form of beads, blocks, discs and membranes. Therefore, agar was selected as matrix for entrapment of *R. rhodochrous* PA-34 cells. The concentration of agar was varied from 1–5% w/v. The cells entrapped in 2% w/v agar exhibited maximum Nhase activity (results not shown) and thus the *R. rhodochrous* PA-34 cells entrapped in 2% (w/v) agar gel were used in the rest of the experiments. Higher concentrations of agar in the gel decreased the Nhase activity of immobilized cells probably due to smaller pore size in the discs, which might have interfered with the diffusion of substrate and product in the gel [6]. The amount of cell suspension (22 mg dcw/ml) was varied from 4–20% v/v and the Nhase activity was recorded to be maximum at 8% cell concentration in 2% w/v agar, corresponding to 1.76 mg dcw/ml agar matrix (Fig. 2). There was an increase in the Nhase activity from 4 to 8%, but the Nhase activity declined with further increase in cell concentration probably due to limitation of substrate availability. At higher concentration of cells (i.e., Nhase in the matrix or Vmax), the diffusion of substrate became a rate limiting factor, which resulted in decrease of overall activity of Nhase of agar immobilized cells. The agar discs used in further reactions were thus prepared from 8% (v/v) cell suspension (22 mg dcw/ml) of *R. rhodochrous* PA-34 in 2% (w/v) agar and each of them had a volume of 0.4 ml and contained 0.7 mg dcw approximately.

Optimization of reaction conditions for conversion of acrylonitrile to acrylamide using agar immobilized cells

The reaction conditions for conversion of acrylonitrile to acrylamide using agar immobilized cells were optimized. The conversion of acrylonitrile to acrylamide was carried

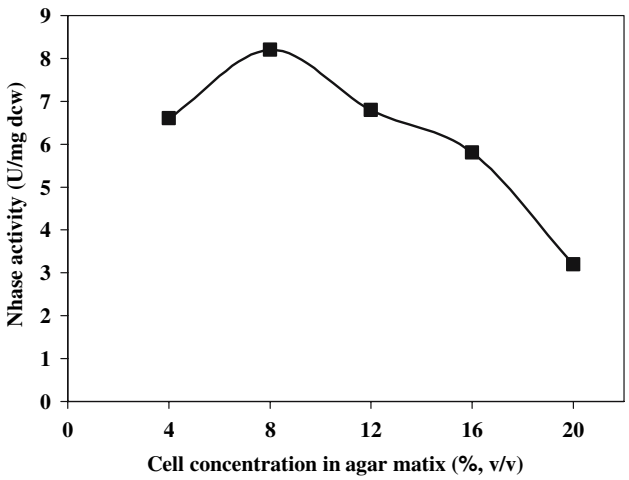

Fig. 2 Nhase activity of various concentrations of immobilized biocatalyst in 2% agar (w/v). The cell suspension (22 mg dcw/ml) was diluted accordingly (e.g., for 4% final concentration, the original cell suspension was diluted to 8% v/v) and added in equal volume to 4% w/v of agar gel to prepare the immobilized biocatalyst having different percentage of cell suspension (v/v) in 2% agar matrix Each *data point* in the figure represents the average of the values for reactions carried out in triplicate

out in potassium phosphate buffer (0.1 M) with pH ranging from 5.5 to 8.5, and pH 7.5 turned out to be optimum for this reaction (8.25 U/mg dcw; Fig. 3). This shift in optimum pH, i.e., 7.0 for free cells [17] to 7.5 for agar entrapped cells might be due to partitioning of hydrogen ions in the agar matrix [6]. It means that hydrogen ion concentration was higher in agar matrix as compared to bulk phase i.e., the actual pH of the agar discs was lower than the bulk phase (reaction medium), and in order to have optimum operational pH for Nhase, the bulk phase pH had to be higher than the actual pH of the agar matrix [11]. Similarly, Nhase of *Rhodococcus* S6 showed optimum pH of 7.7 and 8.5 for the free and immobilized cells in polyacrylamide, respectively [3].

The effect of temperature on the activity of Nhase of *R. rhodochrous* PA-34 was also studied by carrying out the reaction at 10–50°C. At 10°C, maximum transformation of acrylonitrile to acrylamide was recorded (8.25 U/mg dcw, Fig. 4). Above 10°C, formation of acrylic acid along with acrylamide was detected; thus, decrease in Nhase activity was observed at >10°C. This is due to the activation of amidase present in *R. rhodochrous* PA-34 at >10°C, which hydrolyzes the acrylamide formed by the Nhase activity into acrylic acid [16, 17].

The enzyme and substrate ratio for conversion of acrylonitrile to acrylamide by *R. rhodochrous* PA-34 was optimized by varying concentrations of substrate (acrylonitrile, 2–20% w/v) and immobilized biocatalyst (0.28–1.40 mg dcw/ml). The maximum Nhase activity (8.25 U/mg dcw) for conversion of acrylonitrile to acrylamide was recorded when immobilized cells corresponding to 1.12 mg dcw/ml and 8% w/v (~1500 mM) acrylonitrile were used in the reaction (Fig. 5). Under optimized conditions, the conversion of 8% (w/v) acrylonitrile to acrylamide was followed up to 4 h using agar immobilized cells equivalent to 1.12 mg cells DW per ml reaction mixture. Acrylonitrile (8% w/v) was completely converted to acrylamide in 3 h (Fig. 6), while in a previous study the free cells (1.6 mg dcw/ml) completely converted acrylonitrile (8% w/v) in 1 h [17]. Lower Nhase activity of immobilized cells compared

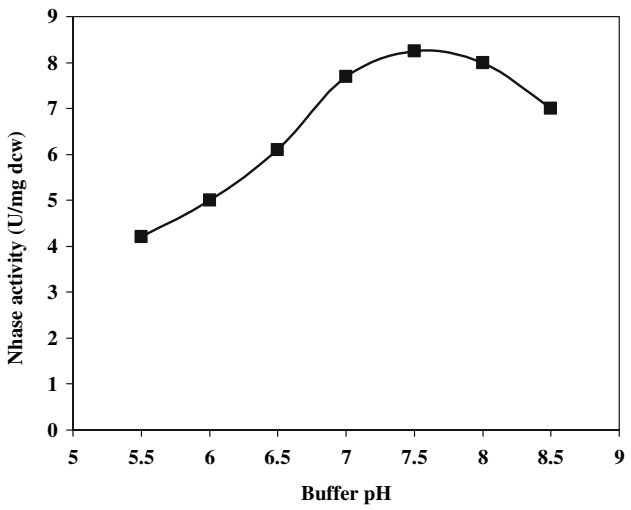

Fig. 3 Effect of reaction buffer pH on Nhase activity of agar immobilized cells of *R. rhodochrous* PA-34. Each *data point* in the figure represents the average of the values for reactions carried out in triplicate

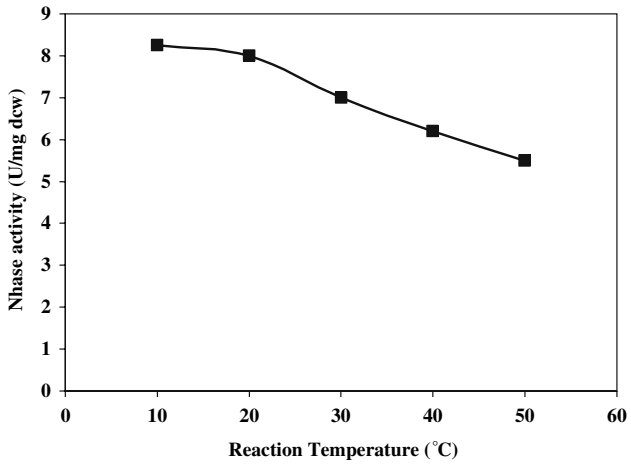

Fig. 4 Effect of incubation temperature on Nhase activity of agar immobilized cells of *R. rhodochrous* PA-34. Each *value* in the figure represents the average of the values for reactions carried out in triplicate

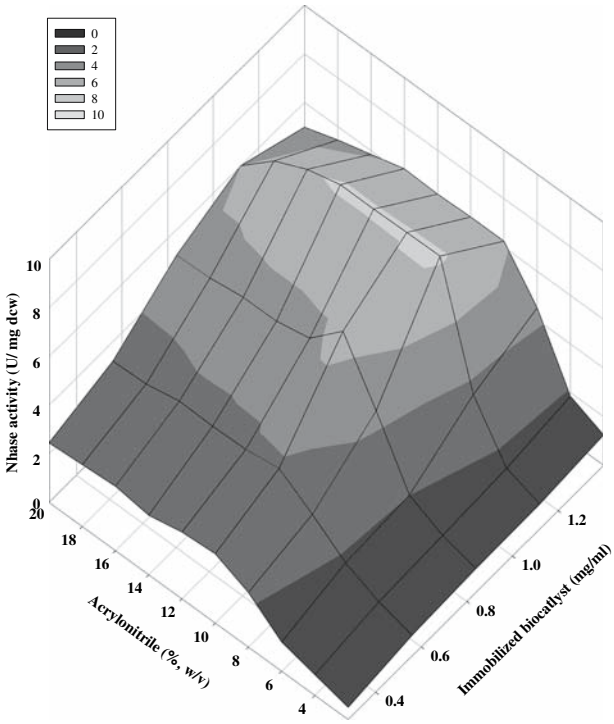

Fig. 5 Nhase activity at different concentrations of both acrylonitrile and immobilized biocatalyst in the reaction mixture Each agar disc contained cells of *R. rhodochrous* PA-34 corresponding to 0.7 mg dcw. Each *data point* in figure represents the average of the values for reactions carried out in triplicate

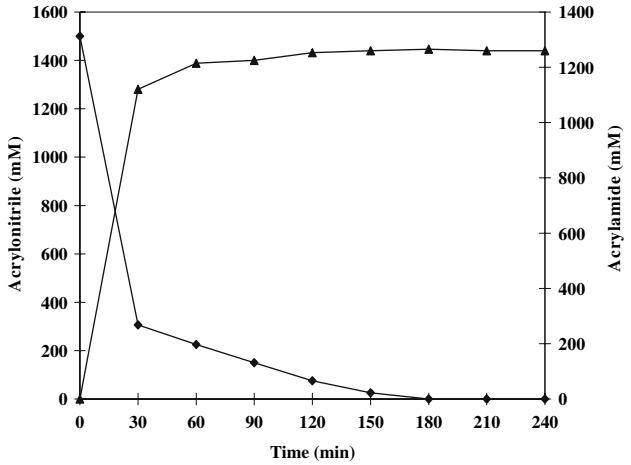

Fig. 6 Conversion of acrylonitrile (8% w/v) to acrylamide in the reaction mixture containing immobilized cells (1.12 mg dcw/ml) of *R. rhodochrous* PA-34. Each *value* in the figure represents the average of the values for reactions carried out in triplicate

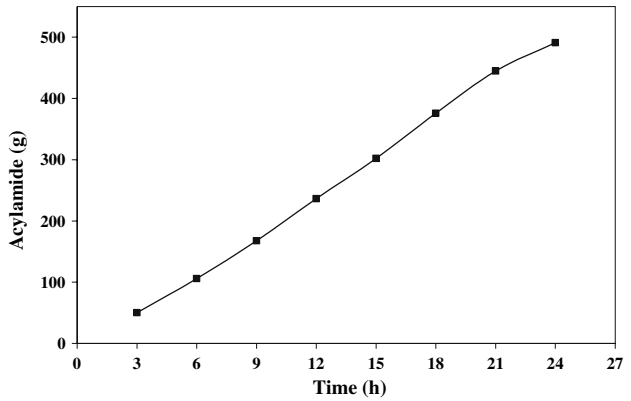

Fig. 7 Amount of acrylamide accumulated during the fed batch reaction using agar immobilized cells (first cycle) of *R. rhodochrous* PA-34

to free cells (55 U/mg dcw) [17] may be due to slow diffusion of acrylonitrile/acrylamide across the agar matrix.

Acrylamide synthesis at 1 l scale using immobilized cells of *R. rhodochrous* PA-34 and recycling of cells

After 24 h of reaction, the crystals of acrylamide started appearing in the reaction vessel and this also hindered the rotation of impeller in the reaction vessel. On removal of immobilized biocatalyst from the reaction vessel, the reaction mixture did not contain any disintegrated agar pieces and showed that immobilized cells could be safely used in a stirred tank reactor for batch or continuous reaction by partitioning them from the bulk reaction mixture to avoid their disruption due to impeller movement.

There was 100% conversion of acrylonitrile to acrylamide (498 g/l) in 24 h and no traces of acrylonitrile/acrylic acid were detected through GC analysis (Fig. 7). The crystallization process employed for the harvesting of acrylamide from the reaction mixture resulted in 87% recovery (432 g) of the total amount of the product formed. While free cells produced 600 g of acrylamide in 12 h, the major objective of immobilization of cells/enzymes is to recycle them until their use is economically viable. The agar immobilized *R. rhodochrous* PA-34 cells were recycled six times (i.e., seven cycles of reactions were carried out) and a total 2,115 g of acrylamide was recovered from the reaction mixture through crystallization (Table 1). The agar discs containing cells remained intact in the nylon net partition even after six recycles.

Table 1 Recycling of agar entrapped cells of *R. rhodochrous* PA-34 for acrylamide production

Biocatalyst	Acrylamide produced (g/l)	Residual activity (%)
Immobilized cells	432	97
First recycle	421	95
Second recycle	330	80
Third recycle	320	70
Fourth recycle	250	60
Fifth recycle	200	50
Sixth recycle	162	15
Total	2115	

Acknowledgments We acknowledge the financial support in the form of Senior Research Fellowship from University Grants Commission (UGC), New Delhi, India to Mr. Jog Raj, from the Council and Scientific and Industrial Research (CSIR) to Mr. Nitya Nand Sharma and from the Department of Biotechnology, Government of India, New Delhi to Mr. Shreenath Prasad. The computational facility availed at Bioinformatics Centre, H. P. University is gratefully acknowledge.

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