Acrylic acid synthesis using amidase activity of polyacrylamide gel entrapped resting cells of *Rhodococcus rhodochrous* in a stirred tank reactor

Duni Chand[a], Frank Vitzthum[b] and Tek Chand Bhalla[a]*

[a]Department of Biotechnology, H. P. University, Summer Hill, Shimla- 5, Himachal Pradesh, India
[b]Dade Behring Marburg GmbH, Emil-von-Behring-Strasse 76, D-35041 Marburg, Germany
*Corresponding author. Email: tcbhalladbthpu@gmail.com

INTRODUCTION

Acrylic acid is a commercially very important commodity compound that has been reported to be useful for a variety of industrial applications including plastics, textiles, thickening agents, dispersing agents, surfactants, chelating agents, adhesives, water-based coatings, water treatment. Acrylic acid is traditionally manufactured by gas phase oxidation of propylene and isobutylene in the presence of oxide catalysts at a very high temperature. But, chemically synthesized acrylic acid contains undesirable impurities like dimeric acrylic acid (Armitage *et al.* 1999).

As an alternative to the chemical synthesis, enzymatic transformations needs less severe pH and temperature conditions and are stereo-selective and produce pure products and also facilitate otherwise difficult reactions under mild conditions (Kobayashi *et al.* 1993). Alternatively, pure acrylic acid can also be produced by biotransforming either acrylonitrile (by NHase-amidase system, Nawaz *et al*. 1989) or acrylamide (by amidase).

$$\underset{\text{(Acrylonitrile)}}{CH_2{=}CH{-}CN} \xrightarrow{\text{NHase}} \underset{\text{(Acrylamide)}}{CH_2{=}CH{-}CONH_2} \xrightarrow{\text{Amidase}} \underset{\text{(Acrylic acid)}}{H_2{=}CH{-}COOH}$$

OR

$$\underset{\text{(Acrylamide)}}{CH_2{=}CH{-}CONH_2} \xrightarrow{\text{Amidase}} \underset{\text{(Acrylic acid)}}{CH_2{=}CH{-}COOH}$$

(Bioconversion of acrylonitrile and acrylamide to acrylic acid)

Here, we describe, a laboratory scale batch and fed-batch mode synthesis of acrylic acid using amidase activity of resting cells of *Rhodococcus rhodochrous* entrapped in polyacrylamide gel discs.

Materials and methods

Chemicals

The nitriles and amides used in the present study were purchased from Lancaster Synthesis, England. The media components were obtained from HiMedia, Mumbai, India. All other reagents and chemicals used were of highest analytical grade available.

Microorganism, culture conditions and preparation of resting cells

The bacterial isolate *Rhodococcus rhodochrous* has been procured from the culture collection of the Department of Biotechnology, Himachal Pradesh University, Summer Hill, Shimla-5, India. It had been isolated earlier from the soils of Himachal Pradesh as a nitrile-metabolizing bacterium (Bhalla *et al.* 2005). The bacterium was routinely subcultured on nutrient agar slants at 30 ^{0}C and

maintained at 4 ^{0}C. Preculture was prepared by inoculating a loop full of culture from the slant to 2 ml of seed medium containing 0.5% peptone, 0.3% beef extract, 0.1% yeast extract and 1% glucose (pH 7.5) at 30 ^{0}C, 160 rpm for 24 h. This 2 ml of seed culture was added to 50 ml of seed medium and grown under the same conditions. These 24 h preculture were added to 50 ml of production medium containing 3% Tryptone, 1.5% yeast extract, 0.5% NaCl, pH 8.5 (Piotraschke *et al.* 1994) and 0.2% acetonitrile (v/v) as an inducer, followed by incubation at 30 ^{0}C for 24 h in an incubator shaker at 180 rpm.

Cells were harvested by centrifuging the cultures at 5000 x g for 20 min at 0-4 ^{0}C. The pellets were suspended and washed twice with potassium phosphate buffer (pH 8.5) and finally re-suspended in the same buffer and were referred to as 'whole resting cells'. The whole resting cells were assayed for amidase activity and used for further investigations.

Amidase assay

The amidase activity was determined spectrophotometrically measured by the method described by Fawcett and Scott (1960). If not stated otherwise, the assay was performed in 2 ml of reaction mixture containing resting cells, acrylamide solution 100mM potassium phosphate buffer (pH 8.5) at 55^O C for 10 min. The absorption was measured at 640 nm and related to the amount of acrylic acid formed in reaction by comparison with a standard.

HPLC analysis

Acrylamide and acrylic acid present in the reaction mixture were quantitatively analyzed by high performance liquid chromatography (HPLC), as described by Goldlust and Bohak (1989) using a Perkin Elmer HPLC system equipped with an C-18 reverse phase column (4.6 X 250 mm) at a flow rate of 1 ml min^{-1}, at an ambient temperature (20 to 25 ^{0}C) with mobile phase of 0.5 % acetonitrile and 0.07 % orthophosphoric acid at a flow rate of 1 ml min^{-1}. Spectrophotometric detection was performed at a wavelength of 210 nm. The volume injected was 5µl.
HPLC calibration curves were prepared each for acrylamide (1 to 10mM) and acrylic acid (1 to 10mM). Retention times of acrylamide and acrylic acid were 2.398 and 4.042 respectively.

Immobilization of Rhodococcus rhodochrous in polyacrylamide gel discs

Whole resting cells were immobilized by the method as described by Kierstan and Coughlan (1985). To 5 ml of chilled potassium phosphate buffer (pH8.5), 1.425g of acrylamide, 0.075g of bis-acrylamide and 5mg ammonium persulphate were added and thoroughly mixed. In another test tube, 2 ml of resting cells were mixed with 3 ml of chilled potassium phosphate buffer. Added 50µl of TEMED to the first tube containing polymer, mixed properly the contents of two tubes and immediately poured in a glass cavity and covered and kept for the polymerization to proceed for one hour. Phosphate buffer washed polyacrylamide gel (7.0x5.0x3.0 cm) was cut into beads of 1 cm diameter and stored the beads in the same buffer for further use.

Optimization of reaction parameters for gel entrapped resting cells

The conversion of acrylamide to acrylic acid was carried out using polyacrylamide gel entrapped resting cells of *Rhodococcus rhodochrous* in selected 100 mM glycine-NaOH buffer at different pH value from 5.0 to 11, at temperature between 30 to 70 ^{0}C and varied concentrations of acrylamide. Substrate affinity of amidase activity of immobilized cells was tested using a number of substrates.

Acrylic acid synthesis by batch and fed batch mode supply of substrate

The biotransformation of acrylamide to acrylic was performed in a batch and fed-batch mode in 1.5 litre compact New Brunswick Scientific Fermenter (Fig. 1) using a 500 ml reaction mixture at 45 ^{0}C**.** In batch mode a single feed of 0.5 mole acrylamide in 100mM potassium phosphate buffer pH 8.5 and polyacrylamide gel discs (Fig. 2, containing 50 mg dry cell wt.) were added, whereas in fed-batch mode the substrates were added in 5 feedings, 0.25 mole acrylamide at an interval of 1 hour. The reaction was allowed to proceed for 5 h.

Results and Discussion

Optimization of reaction parameters for gel entrapped resting cells

The maximum turnover of acrylamide to acrylic acid was obtained in 100 mM potassium phosphate buffer at pH 8.5 and a temperature of 55 ^{0}C. Gel discs with 0.8 mg resting cells (dcw) showed a maximal amidotransferase activity when concentrations of acrylamide were 0.625 mmoles. The maximum acrylic acid production was observed at 50 ^{0}C in 2 h of reaction.

Fig. 1 Batch synthesis of acrylic acid using resting cells of *Rhodococcus rhodochrous* entrapped in polyacrylamide gel discs.

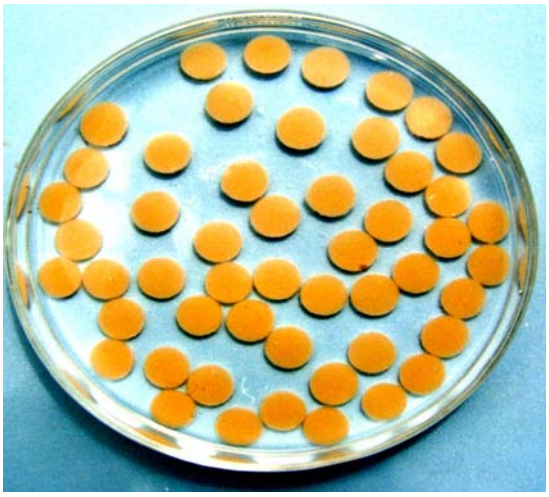

Fig. 2 Polyacrylamide gel discs entrapped resting cells of *Rhodococcus rhodochrous*

Whole resting cells exhibited broad substrate affinity with greater turnover of aliphatic amides as compared to aromatic amides to their carboxylic acids. Similar broad-spectrum amide specificity was reported for amidase of *Rhodococcus* sp. R312 (Fournand *et al*. 1998) and *Rhodococcus rhodochrous* NHB-2 (Chand *et al.* 2004).

Acrylic acid synthesis by batch and fed batch mode supply of substrate

In batch mode the rate of formation of acrylic acid gradually increased up to 4 h of incubation time and thereafter no further increase occurred. Maximum synthesis of acrylic acid in batch mode recorded was 232.01 g g^{-1} dry cell weight h^{-1}, whereas when the substrates were added in fed batch manner, the amount of acrylic acid produced was 280.432 g g^{-1} dry cell weight h^{-1}. As evident from HPLC chromatograms (Fig. 3 and 4), fed-batch mode shows higher conversion yield as compared to batch mode.

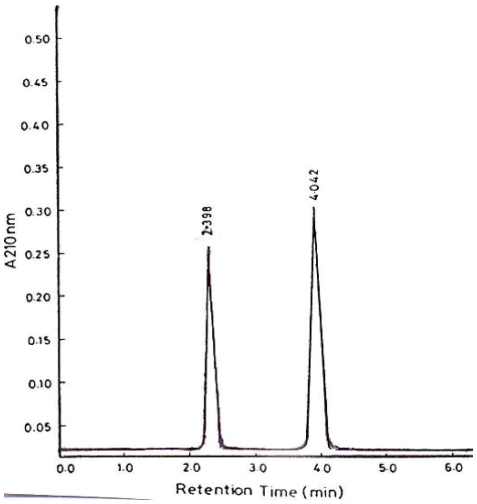

Fig. 3 HPLC chromatogram of a sample from batch reaction

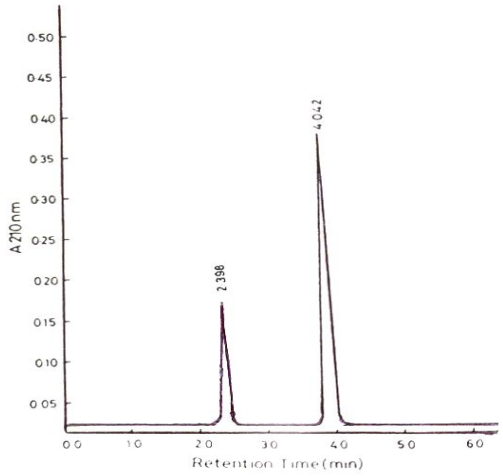

Fig. 4 HPLC chromatogram of a sample from fed-batch reaction

Conclusions

The results obtained in this investigation show that *Rhodococcus rhodochrous* entrapped in polyacrylamide gel discs express thermostable amidase activity that may be used for the synthesis of acrylic acid in batch as well as in fed-batch mode. Biotransformation at commercial scale has a very high potential in contrast to chemical processes for the synthesis of this commodity compound, which is a key compound for a variety of industrial applications including the use in oil treatment chemicals, detergent intermediates, water treatment chemicals, and water absorbent polyacrylic acid polymers (Mannsville 1992).

References

Bhalla T C et al. (2005) *Nocardia globerula* NHB-2: *A versatile nitrile degrading organism*. *Can J Microbiol* 51:705-708.

Chand D et al. (2004) Treatment of simulated waste water containing toxic amides by immobilized *Rhodococcus rhodochrous* NHB-2 using a highly compact 5-stage plug flow reactor. *World J Micro.Biotech.* 20:679-686.

Fawcett J K and Scott J E (1960) A rapid and precise method for the determination of urea. *J Clin Pathol* **13** : 156 – 159.

Fournand D et al. (1998) Studies of the acyltransfer activity of a recombinant amidase overproduced in an *E. coli* strain. Application for short chain hydroxamic acid acid hydrazide synthesis. *J.Mol. Catal.B* 4: 77-90.

Goldlust A and Bohak Z (1989) Induction, purification and characterization of the nitrilase of *Fusarium oxysporum* f sp. *melonis. Biotechnol Appl Biochem* **11** : 581 – 601.

Kierstan M P J and Coughlan M P (1985) Immobilization of cells and enzymes by gel entrapment In : Immobilized cells and enzymes (WOODWARD J Ed) *IRL Press Oxford* : 43-45.

Kobayashi M et al. (1993) Amidase coupled with low-molecular-mass nitrile hydratase from *Rhodococcus rhodochrous* J1. *Eur J Biochem.* 217: 327-336.

Mannsville (1992) Chemical Products Synopsis, Acrylic Acid. Mannsville Chemical Products Corporation.

Piotraschke E et al. (1994) Genetic construction of catalytically active cross- species heterodimer penicillin G amidase. *Biotechnol Lett* 16: 119-124.