Encapsulation of resting cells of *Nocardia globerula* in agar gel beads and optimization of process parameters for amidohydrolase activity

Deepak Pandey[a], Duni Chand[a*], Rajendra Singh[a] and Tek Chand Bhalla[a]

[a]Department of Biotechnology, H. P. University, Summer Hill, Shimla- 5, Himachal Pradesh, India
*****Corresponding author. Email: dunichand_2000@yahoo.com

Introduction

Amidase [EC 3.5.1.4, amidohydrolase], an amide-hydrolyzing enzyme has a great potential for the hydrolysis of amides to corresponding acids. These compounds are widespread in nature (both in biotic and abiotic components of ecosystem) and are extensively manufactured and used by a large number of industries as organic solvents, herbicides, organic feed stocks, extractants, recrystallizing agents, precursors in the synthesis of plastics, synthetic fibers, resin, dye stuffs, emulsifiers and pharmaceuticals (Ramakrishna *et al.* 1999).
Among the nitrile degrading enzymes, amidases have gained importance because of their potential applications in neurobiochemistry, plant physiology, applied microbiology and bioremediation (Kobayashi *et al.* 1998). These enzymes are generally found in bacteria viz. N*ocardia, Rhodococci and Arthrobacteria* (Koltova *et al.* 1998).

$$\underset{\text{(amide)}}{R-CONH_2} + H_2O \xrightarrow{\text{Amidohydrolase}} \underset{\text{(carboxylic acid)}}{R-COOH} + \underset{\text{(ammonia)}}{NH_3}$$

Amidase also exhibit an acyl transfer activity (acyltransferase) in the presence of hydroxylamine (Fournand *et al.* 1998).

$$\underset{\text{(amide)}}{R-CONH_2} + \underset{\text{(hydroxylamine)}}{NH_2OH} \xrightarrow{\text{Acyltransferase}} \underset{\text{(hydroxamicacid)}}{R-CONHOH} + \underset{\text{(ammonia)}}{NH3}$$

Here, we describe the optimization of reaction parameters for amidohydrolase activity of resting cells of *Nocardia globerula* entrapped in agar gel beads.

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 *Nocardia globerula* 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.

General assay conditions and determination of enzyme activity

Enzyme activity was assayed with different substrates by the method described by Fawcett and Scott (1960). If not stated otherwise, around 120 µg dry weight of cells per ml of reaction mixture were applied for the degradation of acetamide that was taken as standard substrate at a concentration of 312.5 mM. 100 mM potassium phosphate buffer pH 8.5 was applied to carry out the reactions and the assays were performed at 55 ^{0}C. Reactions were stopped after incubation of 5 min. by the addition of hydrochloric acid.

Immobilization of Nocardia globerula in agar gel discs

Whole resting cells were immobilized into agar gel discs by the method described by Kierstan and Coughlan (1985). However, concentrations of constituents were optimized with respect to optimal amidase activity. In contrast to the standard protocol (Kierstan and Coughlan, 1985), 2.5% agar was applied.

To 9 ml of normal saline, 250 mg of agar was added and heated at 100^{O}C to 110^{O}C and then cooled to 50^{O}C by keeping in water bath. Added 1 ml of resting cell suspension (O.D 35) to the agar solution at 50^{O}C and thoroughly mixed. The mixture was immediately poured into a petridish and kept at 5^{O}C for gelling. The gel was cut into discs of 1 cm diameter and stored in phosphate buffer for further use.

Optimization of reaction parameters for gel entrapped resting cells

The hydrolysis of acetamide was carried out using agar gel entrapped resting cells of *Nocardia globerula* in selected 100 mM potassium phosphate buffer at different pH value from 5.0 to 10, at temperature between 30 to 65 ^{0}C and varied concentrations of acetamide from 125 µmoles to 1,000 µmoles. The gel beads containing an equivalent enzyme present in 25 µl cell suspension were added in each reaction mixture and the reaction was stopped by removing the beads from reaction mixture instead of adding 0.1N HCl. Substrate affinity of acyltransferase activity of immobilized cells was tested using a number of substrates. The thermostability and shelf life of the immobilized cells were also studied. The reusability of the agar gel beads was also tested.

Results and Discussion

Optimization of reaction parameters for agar entrapped resting cells

The maximum amidohydrolase activity was obtained in 100 mM potassium phosphate buffer at pH 8.5 and a temperature of 55 ^{0}C. Gel discs showed a maximal amidohydrolase activity when

concentration of acetamide was 625 μmoles. Agar gel immobilized cells were recycled for 10 times (Fig. 2) and no loss of activity was observed. Instead, progressive increase in enzyme activity on recycling of gel beads up to fifth recycle was recorded and afterwards activity remained almost constant. Initially entrapped resting cells exhibited about 70% activity with respect to free cells, however, it was increased to about 98% after fifth recycle. It might be possible that after repeated use of immobilized biocatalyst, a certain level of substrate and product is maintained within the immobilized cell system which favours the reaction.

Agar immobilized cells exhibited poor thermostability (Fig 3) as compared to non-immobilized cells of *Nocardia globerula*. These results can be explained on the basis that during immobilization resting cells are exposed to heat shock, which inactivated resting cells and effect of heat on cells is more in the gel then in liquid phase and this might have inactivated immobilized cells faster then free cells on exposure to higher temperature. There was sharp decline in amidase activity above 55^{O}C preincubation.

Agar gel entrapped cells of *Nocardia globerula* were stored both at 4^{O}C and room temperature to investigate their storage stability. They have exhibited lower storage stability in comparison to free cells. After 30 days residual activity retained by agar gel entrapped resting cells was 48% and 60% at room temperature and 4^{O}C respectively.

Relative hydrolysis of various amides by agar-immobilized cells decreased as compared to free cells. Although substrate affinity pattern remained the same except that in case of agar gel beads no activity could be detected in caprolactam, N-ethylacetamide, N-methylacetamide, trimethylacetamide and methacrylamide (Table 1). This reduced substrate specificity might have occurred due to poor accessibility of entrapped resting cells to larger size of these substrates in comparison to acetamide.

Fig. 1 Agar gel discs containing resting cells of *Nocardia globerula.*

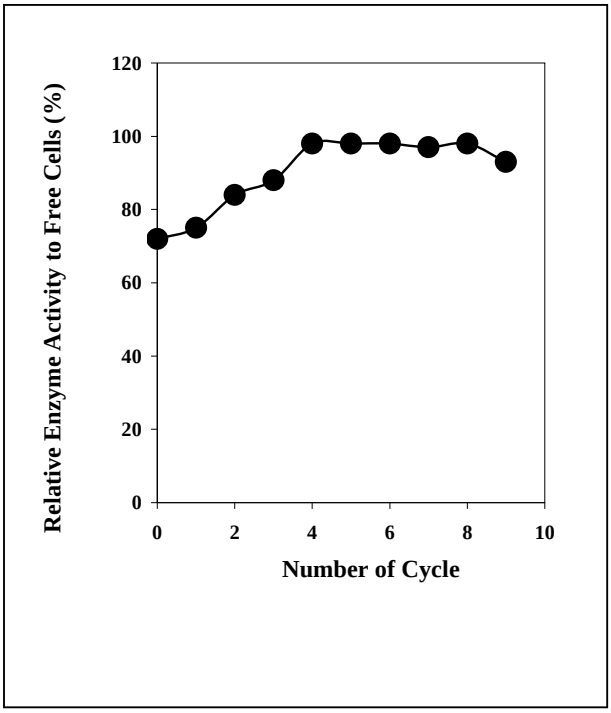

Fig. 2 Reusability of agar gel entrapped cells of *Nocardia globerula.*

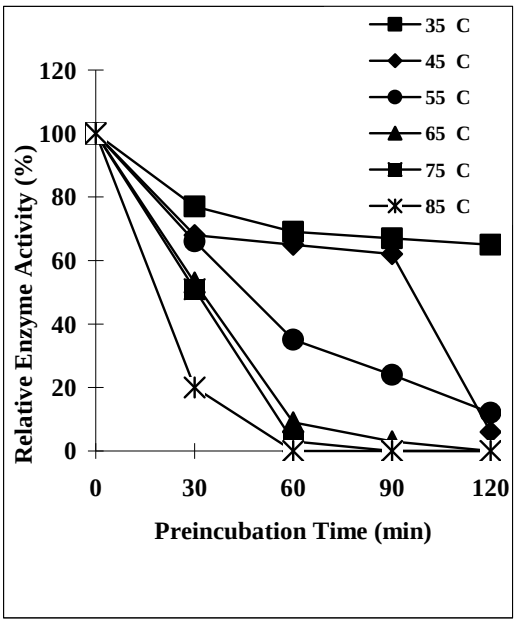

Fig. 3 Thermostability of amidase activity of resting cells of *Nocardia globerula* immobilized in agar gel.

Table 1 Substrate affinity of amidase of agar gel entrapped cells of *Nocardia globerula.*

Substrates	Relative Enzyme Activity (%)	Substrates	Relative Enzyme Activity (%)
Acetamide	100*	Cyanoacetamide	16
Acrylamide	89	Trimethylacetamide	ND
Nicotinamide	23	Thioacetamide	23
Benzamide	10	Lactamide	3
Caprolactam	ND	Propionamide	230
Methacrylamide	ND	Malonamide	16
N-Methylacetamide	ND	Butyramide	98
N-Ethylacetamide	ND		

* Corresponds to 2.080 units mg^{-1} dry cell weight.
One unit of enzyme activity is defined as one μmole of NH_3 released min^{-1} under assay conditions.

Conclusions

The results obtained in this investigation show that *Nocardia globerula* entrapped in agar gel discs can be repeatedly used for the hydrolysis of a variety of amides.

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