



# Bioconversion of acrylonitrile using nitrile hydratase activity of *Bacillus* sp. APB-6

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## Abstract

*Bacillus* sp. APB-6 harboring nitrile hydratase was used in the production of acrylamide from acrylonitrile. *Bacillus* sp. APB-6, for maximum production of Co<sup>++</sup> containing nitrile hydratase, was cultured in the medium containing lactose (18.0 g l<sup>-1</sup>), peptone (1.0 g l<sup>-1</sup>), yeast extract (2.0 g l<sup>-1</sup>), MgSO<sub>4</sub> (0.5 g l<sup>-1</sup>), K<sub>2</sub>HPO<sub>4</sub> (0.6 g l<sup>-1</sup>), urea (9.0 g l<sup>-1</sup>), and CoCl<sub>2</sub> (0.01 g l<sup>-1</sup>), pH 7.0, and incubated at 35 °C for 24 h in an incubator shaker (160 rpm). Nitrile hydratase exhibited relatively high specificity for aliphatic nitriles. Free cells were immobilized using 2% (w/v) agar solution to enhance enzyme stability and reusability in repetitive cycles of acrylamide production. Under optimized conditions, nearly complete bioconversion of acrylonitrile was achieved with a fair recovery of 85% using free and immobilized cells equivalent to 500 mg dcw l<sup>-1</sup>. An efficient nitrile hydratase-mediated bioconversion of acrylonitrile to acrylamide at 1-l scale was achieved with time and space productivity of 426 g h<sup>-1</sup> g<sup>-1</sup> dcw using free cells.

**Keywords** Nitrile hydratase · Bioconversion · Immobilization · Acrylonitrile · Acrylamide

## Introduction

Application of microorganisms in the production of chemical commodities has been carried out for thousands of years. In the last few decades, the development of biocatalytic methods has emerged as valuable alternative to the conventional chemical methods. In the same pipeline of biocatalytic methods, bioconversions of nitriles provide an important option to customary chemical methods which involve high energy input, low yield of primary products, and high production rate of by-products. Nitrile degrading enzymes are of considerable importance in industrial bioconversion, being used as an alternative to chemical methods to afford a broad spectrum of useful carboxylic acids and amides along with their prominent application in the treatment of nitriles containing industrial effluents. Nitrile hydratase (Nhase; EC

4.2.1.84), a prominent enzyme of the lyase group, has drawn an increasing attention in the last few decades owing to catalytic role in potential conversions and production of amides (Egelkamp et al. 2017; Gilligan et al. 1993; Kobayashi and Shimizu 1998; Martinez et al. 2017; Prasad and Bhalla 2010; Pratush et al. 2017; Singh et al. 2016; Wang et al. 2017).

Acrylamide is used for a range of applications including synthesis of polyacrylamide. Polyacrylamide has found several applications as flocculants for wastewater treatment, soil conditioner to cut soil erosion, stock additives in paper and pulp industries, and in petroleum industry for enhanced oil recovery. Other uses of acrylamide include production of plastic, dye, adhesive, in food processing, cosmetic additive, and in research laboratories for protein separation through electrophoresis (Friedman 2003; Raj et al. 2006). The water management, petroleum, and paper industries account for about 77–84% of worldwide acrylamide consumption (Acrylamide-Chemical Economics Handbook). The global polyacrylamide market was estimated to grow at a compound annual growth rate of 6.3% during 2017–2025 and reach nearly \$7.65 billion by 2025 (Polyacrylamides market).

Nitrile hydratase-mediated production of acrylamide is the first bioconversion process used successfully for the industrial production of commodity chemical (Yamada and Kobayashi

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1996). Nhase from *Rhodococcus* sp. N774 and *Pseudomonas chlororaphis* B23 have been used as the first- and second-generation catalyst for commercial production of acrylamide. Nhase-mediated production of acrylamide is now performed by the third-generation catalyst from *Rhodococcus rhodochrous* J1 (Kobayashi and Shimizu 1998; Lan et al. 2017).

The culture conditions like production pH, temperature, and time have profound effect on the production of enzyme by microorganism. Majority of the Nhase producing microorganisms have been reported to grow well in the pH range 7.0–8.0 and most of them are mesophiles (Gilligan et al. 1993; Kobayashi et al. 1996; Lee et al. 1993; Nagasawa et al. 1986, 1988; Raj et al. 2006; Watanabe et al. 1987). Besides, thermophilic microbes with Nhase activity have also been reported (Prasad and Bhalla 2010; Yamaki et al. 1997). The time course of growth and Nhase production suggests that Nhase is produced from early exponential phase to stationary phase. Maximum production of Nhase has been observed at 48, 45, 76–80, and 24 h of incubation/growth, respectively, for *Agrobacterium tumefaciens* (Kobayashi et al. 1996), *Brevibacterium* sp. R 312 (Nagasawa et al. 1986), *R. rhodochrous* J1 (Nagasawa et al. 1988), and *R. rhodochrous* NHB-2 (Sankhian et al. 2003).

In most of the biocatalytic processes for nitrile hydratase-mediated synthesis of amides, free/unimmobilized cells have been used widely. To improve operational stability and reusability of free cells, the immobilization of cells turn out to be very advantageous. In addition, immobilization has also been reported to catalyze a broader range of nitrile substrate than free cells. Different immobilization methods, such as, adsorption, entrapment, cross-linking, and membrane immobilization have been used for nitrile degrading processes utilizing free cells and enzymes (Velankar et al. 2010). Agar, alginate, di-ethylaminoethyl cellulose, polyacrylamide, and sol–gel matrix (Martinez et al. 2014; Mersinger et al. 2005; Raj et al. 2008, 2010) have been used previously for immobilization of biocatalyst containing cells to develop an enzymatic process for bioconversion of acrylonitrile to acrylamide.

*Bacillus* sp. APB-6, isolated in our laboratory (Pandey et al. 2011), was investigated and proved as a potential source of Nhase activity. In the present communication, optimization of culture conditions for production of Nhase by *Bacillus* sp. APB-6 and bench scale production of acrylamide using free and immobilized cells are being reported.

## Materials and methods

### Chemicals

The nitriles and amides were purchased from Alfa Aesar, A Johnson Matthey Company (earlier Lancaster Synthesis).

Media components were procured from HiMedia. For high-performance liquid chromatography, HPLC grade solvents were procured from Merck. All other chemicals and inorganic salts were of analytical grade and procured from different sources.

### Microorganism

*Bacillus* sp. APB-6 was used as a source for Nhase production. The organism was grown aerobically in 250 ml Erlenmeyer flasks containing 50 ml medium containing beef extract (3.0 g l<sup>-1</sup>), peptone (5.0 g l<sup>-1</sup>), glucose (10.0 g l<sup>-1</sup>), and yeast extract (1.0 g l<sup>-1</sup>) (pH 7.5), and incubated at 30 °C, 160 rpm in an incubator shaker for 24 h to prepare preculture for the production of Nhase. Preculture was inoculated into growth medium and incubated at 30 °C and 160 rpm for 24 h. The biomass was harvested by centrifugation at 8000g for 5 min at 4 °C. The cells were washed twice and suspended in potassium phosphate buffer (100 mM, pH 7.0) and stored at 4 °C until further use.

To determine the dry cell weight, the culture broth was appropriately diluted in pre-weighed eppendorf tubes and the absorbances were measured at 600 nm. The culture in each tube was centrifuged at 10,000g for 15 min, supernatant decanted, and the pellet was dried at 80 °C until constant weight was reached. A graph was plotted between dry weight of cells and their absorbance at 600 nm.

### Immobilization

The free cells of *Bacillus* sp. APB-6 were immobilized by the method of Kierstan and Coughlan (1985). 2% (w/v) agar solution was prepared in potassium phosphate buffer (100 mM, pH 7.0) by vigorous shaking at 100 °C and then cooled down to 50 °C in water bath. The cell suspension containing appropriate amount of dry cells was added to melted agar and mixed thoroughly. This mixture was immediately poured into clean petri dish and kept for 30 min at 4 °C for solidification. The enzyme containing solidified agar gel was cut into small beads of equal size using a metallic cork borer and washed with potassium phosphate buffer (100 mM, pH 7.0) to remove untrapped or loosely trapped enzyme. The immobilized cells were stored at 4 °C till further use.

### Enzyme assay

Nhase activity of *Bacillus* sp. APB-6 was assayed using the method developed by Hjort et al. (1990). The assay reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), appropriate amount of resting cells' suspension, and acrylonitrile. The reaction was carried out at 55 °C for 20 min and quenched by adding an equal volume of 0.1 N

HCl. The amount of acrylamide produced in the reaction mixture was determined by measuring the absorbance at 235 nm. One unit of nitrile hydratase activity was defined as the amount of enzyme that catalyzed the formation of 1  $\mu\text{mol}$  of acrylamide per min under standard assay conditions. The specific activity of the enzyme was expressed as units per milligram of dry cell weight ( $\text{U mg}^{-1} \text{dcw}$ ). All the experiments were performed independently in triplicate. The values were presented as mean  $\pm$  SD.

### Analytical method

High-performance liquid chromatography (HPLC) was performed using *Series 200 Ic pump* (Perkin Elmer) equipped with Oyster ODS3 column ( $150 \times 4.6 \text{ mm}$ ,  $5 \mu\text{m}$  particle size; Merck) and *785A Programmable Absorbance Detector* (Applied Biosystem). The absorbance was monitored using NetWin Software (Netel Chromatographs, India). The reaction mixture was centrifuged at  $10,000g$  for 5 min and the analysis of acrylamide and acrylic acid was done at a flow rate of 1.0 ml per min at ambient temperature using acetonitrile with 5 mM orthophosphoric acid (5:95 v/v) as mobile phase at 235 nm.

### Optimization of culture conditions for Nhase production

*Bacillus* sp. APB-6 was grown in 13 media (pH 7.5) at  $30^\circ\text{C}$ , and the medium with optimal Nhase production was selected for subsequent experiments. The composition of different media is shown in Table 1. To determine optimal medium components and culture conditions for the

production of Nhase of *Bacillus* sp. APB-6, medium constituents and culture conditions were studied by changing one factor at a time. The parameters explored were selection of carbon source (dextrose, fructose, galactose, sorbitol, rhamnose, xylose, arabinose, sucrose, maltose, lactose, and melibiose; each at a constant concentration of  $55 \text{ mM}$ ); optimum concentration of carbon source ( $2.0\text{--}30.0 \text{ g l}^{-1}$ ), peptone ( $1.0\text{--}10.0 \text{ g l}^{-1}$ ), yeast extract ( $0.5\text{--}5.0 \text{ g l}^{-1}$ ), Urea ( $1.5\text{--}15.0 \text{ g l}^{-1}$ ), mineral elements [ $\text{K}_2\text{HPO}_4$  ( $0.1\text{--}1.0 \text{ g l}^{-1}$ ),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  ( $0.1\text{--}1.0 \text{ g l}^{-1}$ )] and physical conditions [production pH (pH 6.0–10), production temperature ( $25\text{--}60^\circ\text{C}$ ), inoculum size (2–16% v/v), and inoculum age (8–36 h)]. All factors were optimized sequentially using one factor at a time approach and the optimized value for each factor was used as control for subsequent experiment.

### Reactions parameters for bioconversion

The reaction parameters, such as time course of enzyme reaction, substrate concentration, and enzyme–substrate concentration, for higher bench scale production of acrylamide were optimized. Reusability potential of enzyme entrapped in agar discs was also assessed by recycling the discs.

### Bioconversion of acrylonitrile

The Nhase-mediated bench scale conversion of acrylonitrile was performed at 1 l scale in BIOFLO C-32 Fermenter (New Brunswick Scientific, USA). The reaction mixture consisted of acrylonitrile, Tris buffer, and appropriate amount of free/immobilized cells.

**Table 1** Composition of media used for production of Nhase of *Bacillus* sp. APB-6

Medium	Composition ( $\text{g l}^{-1}$ )	References
1	Glycerol (10.0), $\text{KH}_2\text{PO}_4$ (0.5), $\text{K}_2\text{HPO}_4$ (0.5), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1), yeast extract (1.0), peptone (5.0)	Kobayashi et al. (1993)
2	Glucose (10.0), yeast extract (1.0), peptone (3.0), $\text{K}_2\text{HPO}_4$ (0.5), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5), urea (7.5), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.01)	Nagasawa et al. (1991)
3	$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (2.5), $\text{KH}_2\text{PO}_4$ (2.0), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.03), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.06), yeast extract (0.1)	Bhalla et al. (1992)
4	Glucose (10.0), peptone (3.0), yeast extract (1.0), $\text{K}_2\text{HPO}_4$ (0.5), $\text{KH}_2\text{PO}_4$ (0.5), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.005), yeast extract (1.0), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.005), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.005)	Kobayashi et al. (1996)
5	$(\text{NH}_4)_2\text{HPO}_4$ (5.0), peptone (5.0), yeast extract (3.0), $\text{K}_2\text{HPO}_4$ (5.0), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.02)	Bhalla et al. (1992)
6	Tryptone (30.0), yeast extract (15.0), NaCl (5.0)	Piotraschke et al. (1994)
7	Peptone (20.0), NaCl (5.0), glucose (2.0), beef extract (3.0), yeast extract (3.0)	Robas et al. (1993)
8	Glycerol ( $10 \text{ ml l}^{-1}$ ), peptone (5.0), malt extract (3.0), yeast extract (3.0), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.01), acetonitrile (0.2% v/v)	Modified MY medium
9	Glucose (10.0), peptone (5.0), malt extract (3.0), yeast extract (3.0), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.01)	Modified MY medium
10	Glycerol ( $10.0 \text{ ml l}^{-1}$ ), peptone (5.0), malt extract (3.0), yeast extract (3.0), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.01)	Modified MY medium
11	Glucose (10.0), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5), urea (7.5), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.01)	Self-formulated
12	Glucose (10.0), urea (7.5), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.01)	Self-formulated
13	Glucose (10.0), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5), urea (7.5), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.01)	Self-formulated

## Results and discussion

The optimal production of enzyme depends on the culture conditions, whereas the development of a bioprocess to produce commodity chemical is essentially a shared result of optimal reaction parameters. Different parameters of culture conditions and medium composition were optimized using one factor at a time approach. The following are the results of the experiments performed for nitrile hydratase production and bench scale bioconversion of acrylonitrile to acrylamide using free and immobilized cells of *Bacillus* sp. APB-6.

### Optimization of culture conditions

The *Bacillus* sp. APB-6 was grown in 13 media (pH 7.5) at 30 °C and the results are illustrated in Fig. 1. Among the media used, Medium No. 2 containing glucose, yeast extract, peptone,  $K_2HPO_4$ ,  $MgSO_4$ , urea, and  $CoCl_2$  (Nagasawa et al. 1991), proved to be the best medium for the production of nitrile hydratase ( $37.04 \pm 2.85$  U  $mg^{-1}$  dcw and  $60 \pm 2.5$  U  $ml^{-1}$ ) with maximum microbial growth.

It is evident from the inference that the Nhase of the *Bacillus* sp. APB-6 is  $Co^{++}$  containing enzyme as it was only expressed in the presence of  $CoCl_2$ . This nature of enzyme has been reported by many researchers in earlier investigations (Bhalla et al. 1992; Mauger et al. 1988; Prasad and Bhalla 2010; Watanabe et al. 1987). Carbon is a main constituent of cellular material of prokaryotes and eukaryotic cells; therefore, selection of carbon source has a profound effect on the growth of microorganisms. Although the bacterium was able to utilize all the carbon sources for its growth, addition of lactose exhibited maximum enzyme production (Fig. 2a). To the best of our knowledge, first time lactose has been observed as optimal carbon source for nitrile hydratase production. Most of the earlier studies have reported glucose as an optimal carbon source for

Nhase production from *Bacillus subtilis* ZJB-063 (Zheng et al. 2008), *Brevibacterium* sp. R312 (Nagasawa et al. 1986), *Rhodococcus* sp. N-774 (Watanabe et al. 1987), and *Serratia marcescens* ZJB-09104 (Jin et al. 2010). Besides, glycerol has also been used as a carbon source previously for production of Nhase (Raj et al. 2006).

Maximum Nhase production was observed with  $18.0$  g  $l^{-1}$  lactose (Fig. 2b), while further increase in concentration of lactose leads to decrease in the enzyme production, possibly due to either catabolic repression as increase in concentration of sugar in medium exhibits repression effect on bacterial operon due to decrease in the concentration of c-AMP or due to increase in osmotic concentration of the medium as compared to cell. Maximum Nhase production was observed in the presence of lactose ( $18.0$  g  $l^{-1}$ ), peptone ( $1.0$  g  $l^{-1}$ ), yeast extract ( $2.0$  g  $l^{-1}$ ), urea ( $9.0$  g  $l^{-1}$ ),  $K_2HPO_4$  ( $0.6$  g  $l^{-1}$ ), and  $MgSO_4 \cdot 7H_2O$  ( $0.5$  g  $l^{-1}$ ) (Fig. 2b, c).  $CoCl_2$  is the vital component in production medium and  $0.01$  g  $l^{-1}$   $CoCl_2$  was observed optimum for Nhase production in *Bacillus* sp. APB-6 (data not shown). Some previous studies have also used similar concentration of  $CoCl_2$  in the growth medium for Nhase production (Gilligan et al. 1993; Mauger et al. 1988; Nagasawa et al. 1991; Yamaki et al. 1997). Nhase of *R. rhodochrous* J1 (Nagasawa et al. 1988), *Rhodococcus rhodochrous* M8 (Pogorelova et al. 1996), and *R. rhodochrous* NHB 2 (Sankhian et al. 2003) also contained Co as prosthetic group. Optimum Nhase production was achieved when *Bacillus* sp. APB-6 was grown in selected production medium at pH 7.0 and 35 °C for 24 h using 8% (v/v) 20 h grown preculture (Fig. 2d). Similarly, several studies have been reported for optimal Nhase production in the pH range of 7.0–8.0 and majority of the organism were mesophiles (Gilligan et al. 1993; Kobayashi et al. 1996; Lee et al. 1993; Nagasawa et al. 1986, 1988; Raj et al. 2006; Watanabe et al. 1987).

### Substrate specificity of the nitrile hydratase of *Bacillus* sp. APB-6

Nhase activity of *Bacillus* sp. APB-6 was used for the hydrolysis of various nitriles (acrylonitrile, adiponitrile, benzonitrile, butyronitrile, propionitrile, and valeronitrile) to evaluate its substrate specificity. All selected nitriles were used at a similar concentration (150 mM) in different reactions keeping other ingredients of the reaction mixture constant. The reactions were performed as described in preceding section and each product was analyzed through HPLC using their respective standards. Nhase of *Bacillus* sp. APB-6 was observed to be more active for aliphatic substrates in comparison to aromatic nitriles (Fig. 3). The previous studies have reported Nhase with diverse substrate specificities. Nhase with broad substrate specificity have been reported from *R. rhodochrous* NHB-2 (Sankhian et al.

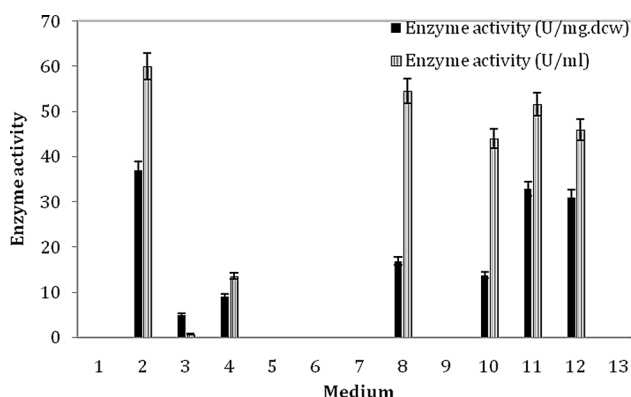
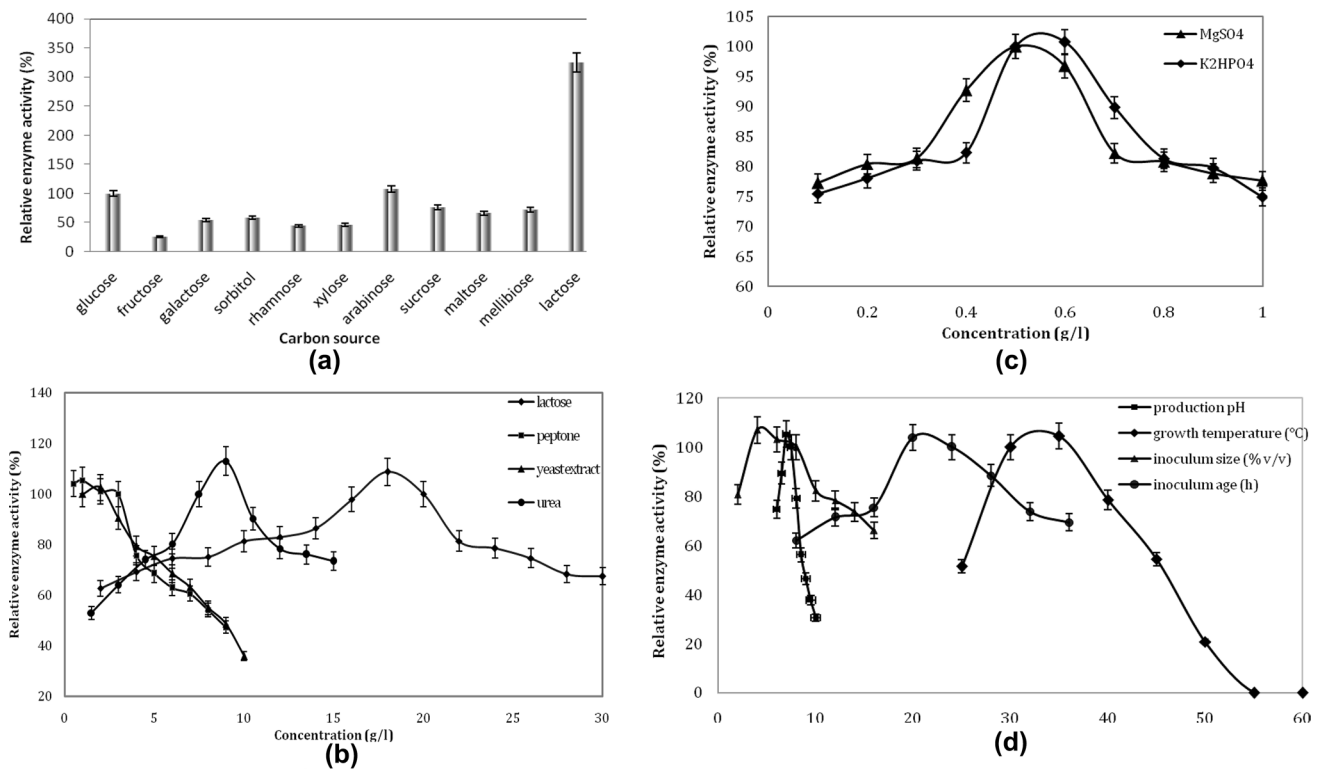
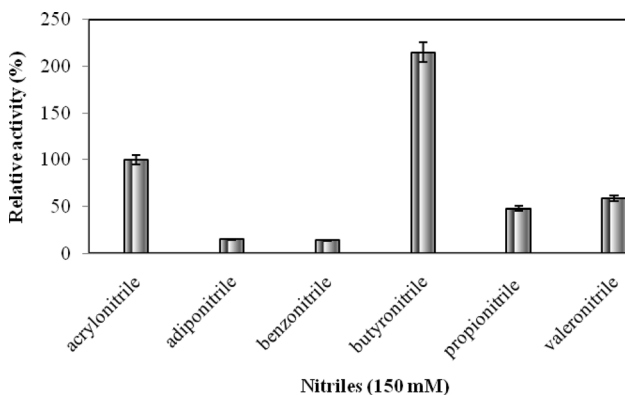


Fig. 1 Selection of growth medium



**Fig. 2** Effect of medium components and culture conditions: **a** carbon source, **b** lactose (2–30 g l<sup>-1</sup>), peptone (0.5–9.0 g l<sup>-1</sup>), yeast extract (1–10 g l<sup>-1</sup>), and urea (1.5–15 g l<sup>-1</sup>); **c** MgSO<sub>4</sub> (0.1–1.0 g l<sup>-1</sup>) and

K<sub>2</sub>HPO<sub>4</sub> (0.1–1.0 g l<sup>-1</sup>); and **d** production pH (pH 6–10), production temperature (25–60 °C), inoculum age (8–36 h), and inoculum size (2–16% v/v)



**Fig. 3** Substrate specificity

2003), *Bacillus subtilis* ZJB-063 (Zheng et al. 2008) whereas *Brevibacterium* sp. R312 (Nagasawa et al. 1986), and *Pseudonocardia thermophila* JCM 3095 (Yamaki et al. 1997) produced Nhase with specificity for aliphatic nitriles.

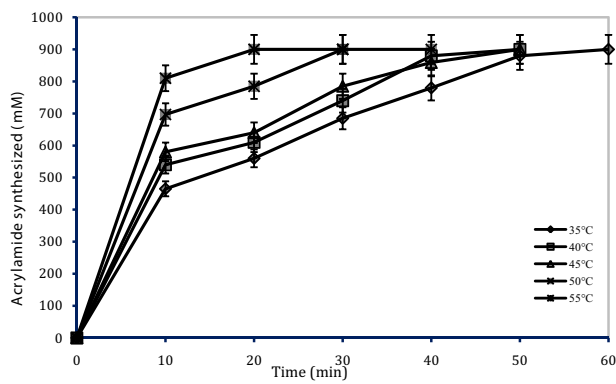
### Reaction conditions

The reaction parameters such as time course of enzyme reaction, substrate concentration, and enzyme–substrate

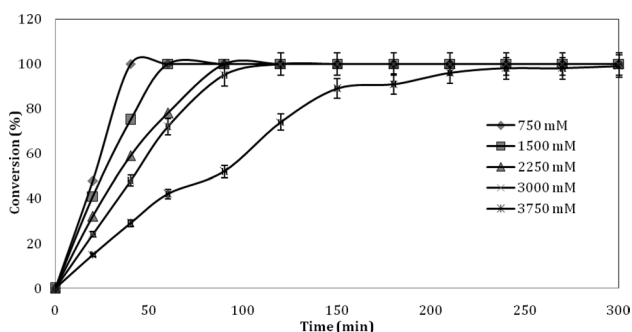
concentration were carried out in 40 mM Tris–HCl buffer (pH 8.0). Selection of buffer system has not been included in this communication as the results of this parameter included in another study (unpublished data).

### Time course of enzyme reaction at different temperatures

To find out the best time and temperature combination for maximum conversion of substrates into product, time course of enzyme reaction at different temperatures (35, 40, 45, 50, and 55 °C) was investigated by terminating the reaction at different intervals of time, viz., 10, 20, 30, 40, 60, 80, 100, and 120 min. Samples were withdrawn on regular interval of time and the amount of product formed (mM) was analyzed by HPLC. It was observed that the complete conversion of acrylonitrile using free cells occurred in 1 h and 20 min at 35 and 55 °C, respectively. Free cells were not stable for long at higher temperature; therefore, temperature 35 °C was selected for batch experiments for their longer operational stability and reusability. The results obtained are summarized in Fig. 4.



**Fig. 4** Time course of enzyme reaction at different temperatures for conversion of acrylonitrile to acrylamide using free cells ( $0.15 \text{ mg dcw ml}^{-1}$ )



**Fig. 5** Effect of substrate concentration on enzyme activity by immobilized cells

### Enzyme–substrate concentration for higher conversion

To achieve higher bench scale productivity of acrylamide production from acrylonitrile, the conversion was carried out in separate experiments using increased substrate and enzyme concentrations. The experiments were carried out in optimized reaction conditions as described in the previous section. The results obtained using immobilized cells are illustrated in Fig. 5. A complete conversion of  $3 \times 10^3 \text{ mM}$  acrylonitrile was achieved in 90 min at  $35^\circ\text{C}$  using immobilized cells equivalent to  $500 \text{ mg dcw l}^{-1}$ . Likewise, complete conversion of the substrate was also achieved in 60 min with same quantity of unimmobilized dry cell weight. However, nearly complete conversion of  $3.75 \times 10^3 \text{ mM}$  substrate was also achieved when incubation time was increased further. The conversion of higher concentration of acrylonitrile hardly increased with reaction time (data not shown).

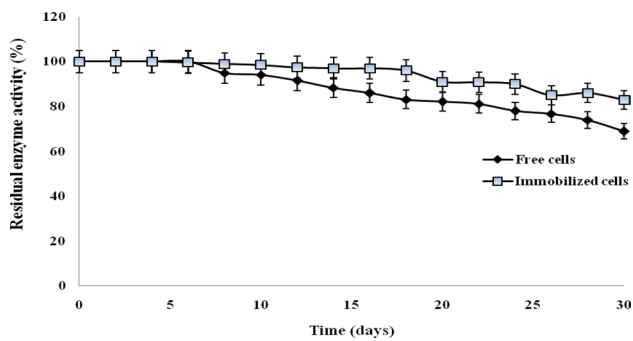
### Bioconversion of acrylonitrile to acrylamide

*Bacillus* sp. APB-6 Nhase-mediated bench scale conversion of acrylonitrile was performed at 1 l scale in BIOFLO C-32 Fermenter (New Brunswick Scientific, USA). The reaction mixture consisted of acrylonitrile ( $3.0 \times 10^3 \text{ mM}$ ), Tris buffer (40 mM, pH 8.0), and cells equivalent to 500 mg dcw. The amount of water required for the hydration reaction was also added in the reaction vessel. The reaction was carried out at  $35^\circ\text{C}$  and constant agitation of 120 rpm. Samples were withdrawn from the reaction mixture with lapse of time and analyzed by HPLC as mentioned in the preceding section. The cells were separated from the reaction mixture on completion of the reaction; the product formed was isolated and lyophilized (Flexi-Dry MP™, FTS USA) to recover crystallized product. HPLC analysis of reaction mixture on completion of reaction or acrylamide recovered (lyophilized) showed that the product acrylamide was not contaminated with substrate (acrylonitrile) or acrylic acid (a product of amidase activity). A complete conversion of acrylonitrile with approximately 85% yield of white and crystalline acrylamide was achieved in 60 and 90 min using free and immobilized cells, respectively. The productivity achieved using free and immobilized cells (1 batch) was  $426$  and  $284 \text{ g h}^{-1} \text{ g}^{-1} \text{ dcw}$ , respectively. In a previous study, productivity of acrylamide production was  $31.25 \text{ g h}^{-1} \text{ g}^{-1} \text{ dcw}$  using free cells of *R. rhodochrous* PA-34 (Raj et al. 2006). Similarly, acrylamide production was also achieved using Nhase of *Brevibacterium* sp. CH2 with time and space productivity of  $20 \text{ g h}^{-1} \text{ g}^{-1} \text{ cells}$  (Lee et al. 1993). Consequently, the productivity achieved using Nhase of *Bacillus* sp. APB-6 for production of acrylamide was higher; and hence, the *Bacillus* sp. APB-6 Nhase proved to be a potential source for bioconversion of other nitriles to valuable amides.

The immobilized cells of *Bacillus* sp. APB-6 were tested for the reusability so as to make the biocatalysts applicable for repeated cycles of production of acrylamide from acrylonitrile. The decrease in enzyme activity for each cycle was determined assuming activity of beads in the first cycle as 100%. The immobilized cells retained similar activity up to three batches and could be reused up to eight batches making the biocatalyst useful for commercial purposes.

### Shelf life of enzyme

Shelf life of enzyme is an essential factor for long-term operational stability. To study the storage stability, the free and immobilized cells were stored for 30 days at  $4^\circ\text{C}$  and tested for enzyme activity at an interval of 2 day. The results obtained have been shown in Fig. 6. Only 17% loss of activity was observed with immobilized cells on storage at  $4^\circ\text{C}$ , whereas 69% residual activity was retained by unimmobilized nitrile hydratase of *Bacillus* sp.



**Fig. 6** Shelf life of enzyme containing free cells and immobilized cells

APB-6. The results indicated that the immobilized cells have stronger storage stability than free cells.

## Conclusion

*Bacillus* sp. APB-6 Nhas was proved as an important catalyst in the development of an efficient bench scale bioprocess for conversion of acrylonitrile to acrylamide using free and immobilized cells with high yield and productivity. Optimum cultivation of *Bacillus* sp. APB-6 for production of  $\text{Co}^{++}$  containing nitrile hydratase was achieved when organism was cultured in a medium constituting lactose, peptone, yeast extract,  $\text{MgSO}_4$ ,  $\text{K}_2\text{HPO}_4$ , urea, and  $\text{CoCl}_2$ , and incubated at 35 °C for 24 h using 8% (v/v) 20 h grown preculture. The bioconversion of acrylonitrile to acrylamide was performed in BIOFLO C-32 fermenter at 1 l scale in reaction medium containing Tris buffer (40 mM, pH 8.0), dry cell weight concentration 500 mg dcw, and substrate concentration  $3 \times 10^3$  mM. Immobilized cells showed stability and a good level of reusability up to eight batches for bioconversion. The complete bioconversion was achieved successfully using free cells with a recovery of  $362 \text{ g h}^{-1} \text{ g}^{-1} \text{ dcw}$ ; and therefore, the biocatalytic process developed may be suitable for higher scale applications.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interests.

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