

## Bioconversion of Acrylonitrile to Acrylic Acid by *Rhodococcus ruber* Strain AKSH-84

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**A new versatile acrylonitrile-bioconverting strain isolated from a petroleum-contaminated sludge sample and identified as *Rhodococcus ruber* AKSH-84 was used for optimization of medium and biotransformation conditions for nitrilase activity to produce acrylic acid. A simple and rapid HPLC protocol was optimized for quantification of acrylic acid, acrylamide, and acrylonitrile. The optimal medium conditions for nitrilase activity were pH of 7.0, temperature of 30°C, agitation of 150 rpm, and inoculum level of 2%. Glycerol as a carbon source and sodium nitrate as the nitrogen source provided good nutritional sources for achieving good biotransformation. Nitrilase activity was constitutive in nature and was in the exponential growth phase after 24 h of incubation under optimal conditions without addition of any inducer. The substrate preference was acrylonitrile and acetonitrile. The present work demonstrates the biotransformation of acrylonitrile to acrylic acid with the new strain, *R. ruber* AKSH-84, which can be used in green biosynthesis of acrylic acid for biotechnological processes. The nitrilase produced by the isolate was purified and characterized.**

**Keywords:** *Rhodococcus ruber*, acrylic acid, acrylonitrile, nitrilase, biotransformation

Acrylic acid that is used traditionally for production of diverse acrylic esters, namely methyl acrylate, ethyl acrylate, butyl acrylate, and 2-ethylhexyl acrylate [20], which find major application in diverse industries related to industrial coatings, adhesives, decoratives, masonry, paper, textile products, resins, flocculants, targeted drug delivery systems, etc. [8, 16, 22]; poly(acrylic acid) (PAA) gels used in the fabrication of BioMEMS devices as sensors and actuators [11]; and superabsorbent polymers [23] and detergent polymers [21]. There is a demand for crude acrylic acid, and the market reached around 3.2

million tons in 2005, with an expectation to reach around 3.7% by the year 2011 [4, 5]. The current industrial demand for acrylic acid is catered by the chemical production process, which is based on gas-phase catalytic oxidation of propylene *via* acrolein in a single or two-step process. The drawbacks of these methods include side reactions, very high temperatures, and risk of the radical-initiated exothermic polymerization with possibility of dimerization of acrylic acid [14]. The environmental safety and cost efficacy are other serious concerns. Biotechnological and biocatalytic processes have been found to be more economical, green, and safe [1]. We isolated novel microorganisms from petroleum-contaminated sludge samples capable of performing biotransformation of acrylonitrile to acrylic acid. Furthermore, a cost-effective and reliable reverse-phase HPLC protocol for the detection and quantification of acrylic acid, acrylamide, and acrylonitrile, using a C<sub>18</sub> column with water–acetonitrile as a mobile phase, was optimized. The results were confirmed using LC–MS. The environmental and nutritional conditions for nitrilase activity were optimized for a newly isolated strain of *Rhodococcus ruber* AKSH-84 using whole cells. This is the first report on the isolation and identification and the medium optimization for nitrilase activity of a *Rhodococcus ruber* strain that is able to convert acrylonitrile to acrylic acid. The biotransformation conditions from acrylonitrile to acrylic acid by whole resting cells and purified nitrilase from the strain AKSH-84 were also optimized.

### MATERIALS AND METHODS

#### Samples and Chemicals

Petroleum-contaminated sludge samples obtained from the Petroleum Refinery Unit, Essar Oil Limited, Vadinar, Jamnagar, Gujarat, India were screened for isolating nitrile-degrading microorganisms. Acrylic acid, acrylamide, acrylonitrile, and all other chemicals and solvents (analytical grade) were purchased from Sigma, St. Louis, MO, USA. Acrylic acid was used after distillation and stored at 13°C. Solvents for HPLC analysis such as acetonitrile, methanol, and water of HPLC

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grade were obtained from Rankem Fine Chemicals, New Delhi, India. The HiPrep 16/10 DEAE FF and Sephadex G-200 superfine columns, marker protein for molecular mass determination, and Coomassie Brilliant Blue R-250 were obtained from GE Biosciences, USA. All the solutions prepared for the purification were filter-sterilized by passing through 0.2- $\mu$ m filters and stored at 4°C.

### Isolation and Screening for Acrylonitrile-Converting Microorganisms

Enrichment of the sludge samples was carried out by suspending 1.0 g of each sludge sample in 100 ml of enrichment medium with the following composition: glucose 10 g, yeast extract 0.2 g,  $\text{KH}_2\text{PO}_4$  0.5 g,  $\text{K}_2\text{HPO}_4$  0.5 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.5 g,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.01 g (per liter) spiked with 2% (v/v) acetonitrile as an inducer at pH 7.0. The flasks were incubated at 37°C with agitation at 150 rpm for about 30 days. The second and third enrichments were followed by transferring culture suspension (1.0 ml) to the same fresh medium and incubating for 15 days each. Serial 10-fold dilutions were performed on enrichment agar plates containing acrylonitrile (200 mM concentration) as substrate and bromothymol blue (0.01%); these indicator plates were incubated at 37°C for 48 h. Acrylonitrile-converting microorganisms produced yellow-colored halos around the colonies against a dark blue background, and these isolates were purified for 2–3 times on nutrient agar plates and stored as nutrient agar slants (4°C) and glycerol stocks (–70°C).

### Identification of the Nitrilase-Producing Strain

Morphological characteristics of the strain AKSH-84 were observed via a light microscope (Model BX51, Olympus Corporation, Tokyo, Japan). The culture was cultivated in nutrient broth and the cells were separated by centrifugation at 10,000 rpm for 10 min at 4°C and washed with distilled water. The cells were fixed for 1 h in 4% glutaraldehyde in 0.2 M phosphate buffer, pH 6.9, for scanning electron microscopy (SEM). The gold-coated stubs were scanned and micrographs taken on a SEM Model S-3000N (Hitachi, Japan) at the accelerating voltage of 10 kV. Routine physiological and biochemical characterizations were carried out following the methods listed in *Bergey's Manual of Determinative Bacteriology* [10]. The 16S rDNA gene sequencing was carried out using universal primers: forward primer 27f (5'-AGA GTT TGA TCM TGG CTC AG-3') and reverse primer 1492r (5'-ACG GTT ACC TTG TTA CGA CTT-3'). The phylogenetic tree was constructed using MEGA4 (version 4.0) software [19].

### Optimization of Medium and Reaction Conditions for Acrylic Acid Production by *Rhodococcus ruber* Strain AKSH-84

The APY medium [adjusted to pH 7.0 with following composition (per liter): ammonium acetate 10 g, peptone 5 g, yeast extract 5 g,  $\text{K}_2\text{HPO}_4$  5 g, NaCl 1 g,  $\text{MgSO}_4$  0.2 g,  $\text{FeSO}_4$  0.03 g] was evaluated for optimization of acrylic acid production based on different environmental parameters such as pH (4.0 to 10.0 units), temperature (20–40°C), agitation (100, 150, 200, and 250 rpm), inoculum level (1–5%), and nutritional parameters such as carbon and nitrogen sources. The reaction conditions for acrylic acid production were also optimized with respect to reaction buffer (pH 6.0–7.5), incubation temperature (10–50°C), and substrate concentration (0.1–0.5 M). The substrate affinity was also studied at optimized reaction conditions against 100 mM of different nitriles to produce the corresponding acids and ammonia via the nesslerization method [2].

### Purification of Nitrilase

The *Rhodococcus ruber* strain AKSH-84 was cultured in 1 l of production medium (pH 7) with 3% inoculum in a 5-l Erlenmeyer flask and incubated at 30°C with shaking at 150 rpm on an orbital shaker (Innova 4230, New Brunswick Scientific, USA). The cells at log phase were harvested by centrifugation (10,000 rpm/4°C) for 10 min. The cell pellet was washed with 0.1 M phosphate buffer containing 10 mM dithiothreitol and 10 mM  $\beta$ -mercaptoethanol to remove traces of medium constituents, and the cell pellet was later mixed with the same buffer above. The washed cells were further suspended in 0.1 M potassium phosphate buffer and then disrupted with a Vibram Cell ultrasonic oscillator (Model VC505, Sonics and Materials, Inc., USA) at 19 kHz frequency for 10 min with a burst interval of 30 s each. The mixture was centrifuged at 10,000 rpm for 10 min and the resultant supernatant was referred to as the cell-free extract. The cell-free crude extract was precipitated with solid ammonium sulfate (45–70% saturation), and the precipitate was collected after centrifugation at 12,000 rpm/4°C for 15 min, resuspended in phosphate buffer (0.1 M), and dialyzed against the same buffer. The dialysate was desalted using PD10 columns (GE Biosciences, USA) and loaded on a HiPrep 16/10 DEAE FF ion-exchange column (GE Biosciences, USA) equilibrated with the loading buffer containing 50 mM HEPES (pH 7.5) and 1 mM EDTA and was interfaced to a Biologic Duoflow Fast Precision Liquid Chromatography (FPLC) System (Bio-Rad, USA). The enzyme was loaded after equilibration of the column with loading buffer and washed with 100 ml of the loading buffer to remove the unbound protein. The enzyme was eluted with a step-wise gradient of 0–400 mM KCl in 50 mM HEPES buffer (pH 7.5) containing 1 mM EDTA. The eluted enzyme fractions were assayed for nitrilase activity and protein content using the Bradford method [7]. The active fractions were pooled and concentrated. The concentrated enzyme solution obtained was passed through a Sephadex G-200 superfine column (1.5 $\times$ 23 cm) equilibrated with 0.1 M potassium phosphate buffer containing 0.1 M KCl and 1 mM EDTA, and the eluted active fractions were pooled and concentrated using Centriplus concentrators (Amicon Inc.) and stored at –10°C until further use. All the purification steps were performed at 4°C.

### Biotransformation of Acrylonitrile to Acrylic Acid by Whole Resting Cells Exhibiting Nitrilase Activity

The nitrilase activity was assayed using acrylonitrile as substrate. The resting cell suspensions were prepared by suspending the cell pellet in 10 mM phosphate buffer (pH 7.2), so as to get a concentration of 200 mg/ml. The reaction mixture was prepared by using 875  $\mu$ l of 10 mM phosphate buffer, 25  $\mu$ l of 500 mM acrylonitrile substrate, and 100  $\mu$ l of cell suspension so as to get a final concentration of 20 mg/ml cell pellet and 100 mM of substrate. The reaction was performed at 37°C for 2 h with agitation at 150 rpm; the reaction was terminated by removal of the cells by centrifugation, and the supernatant was then analyzed by HPLC. The biotransformation of acrylonitrile to acrylic acid was also performed using the purified nitrilase. The acrylic acid was recovered from the reaction mixture supernatant by ethyl acetate extraction and then concentrated under reduced pressure on a rotary vacuum evaporator (Rotavapor R-205, Büchi, Bern, Switzerland). A representative calibration graph of peak area versus acrylic acid concentration in the range of 0.1 to 1 mg/ml resulted in a regression equation,  $y=872927x+9805.8$  ( $R^2=0.9997$ ), which was used for quantification of acrylic acid.

### Analytical Methods

Analytical-grade acrylic acid, acrylamide, and acrylonitrile were dissolved in HPLC-grade water at a concentration of 1 mg/ml and scanned from 190–800 nm employing a UV/Visible spectrophotometer (Lambda 25; Perkin Elmer, Shelton, CT, USA). HPLC (Waters, Milford, MA, USA) equipped with a Spherisorb ODS2 C<sub>18</sub> column (4.6×250 mm, 5 μm particle size; Waters, USA) was used for the quantification of nitrile, amide, and acid in the cell-free supernatant. The detection of these compounds was carried out with a Model 2998 photodiode array (PDA) detector set at a wavelength of 220 nm and using a mobile phase of 70% HPLC-grade water and 30% acetonitrile in combination with 0.05% formic acid at a flow rate of 1.0 ml/min in isocratic mode. The HPLC system was interfaced with Empower 2 software (Waters, Milford, MA, USA) for system and data management. All solvents used for mobile-phase optimization were degassed before use. The acrylic acid formation was confirmed by LC–MS (LCQ–ESI ion-trap mass spectrometer; ThermoFinnigan, San Jose, CA, USA) on a Spherisorb ODS2 C<sub>18</sub> column under negative-mode conditions. One unit of nitrilase activity (1 U) was defined as the amount of enzyme that catalyzed the formation of 1 μmol of acrylic acid per minute under standard conditions. The values were an average of three independent assays. SDS–PAGE was performed on 10% polyacrylamide slab gels using the Tris-glycine buffer system [17]. Proteins were stained with Coomassie Brilliant Blue R-250 and destained in a methanol:acetic acid:water mixture [9:2:9 (v/v)]. The molecular mass of the enzyme was compared with the relative mobilities of standard proteins.

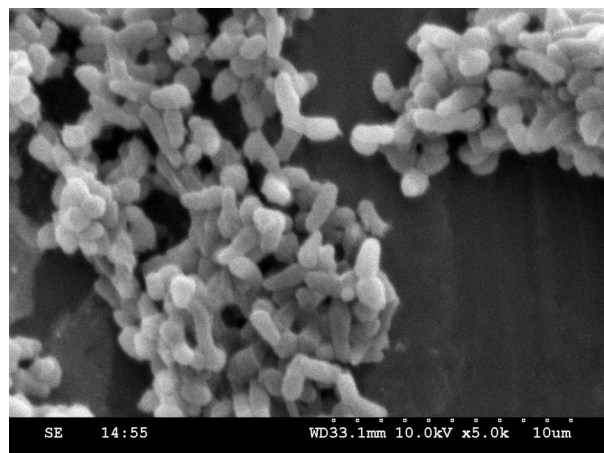
## RESULTS AND DISCUSSION

### Screening of Acrylonitrile-Converting Microorganisms

Different microbial strains were isolated from the sludge samples through enrichment technique. Using a bromothymol blue indicator plating method, 11 isolates exhibiting positive nitrilase activity were selected out of 108 isolates (data not shown). Among these, the isolate AKSH-84 exhibiting maximum acrylonitrile bioconversion was found to be promising (Fig. 1) and used for further studies. The 16S rDNA sequence of strain AKSH-84 was deposited in the EMBL database under the accession number FM995614. Based on the phenotypic characterization (Supplementary Table S1) and 16S rDNA sequencing (Supplementary Fig. S1), the promising strain was identified as *Rhodococcus ruber*.

### Optimization of Mobile Phase for Detection of Acrylonitrile, Acrylamide, and Acrylic Acid Using HPLC

One of the serious issues in the handling of acrylic acid is its high instability due to its sensitivity to various conditions like light, weak acid and basic conditions, and free radicals, which would readily initiate its polymerization. A mobile phase of acetonitrile and water (20:80) along with 0.05% formic acid was optimized for the separation and detection of acrylonitrile, acrylamide, and acrylic acid at 220 nm. It is reported that formic acid is an ion-pairing

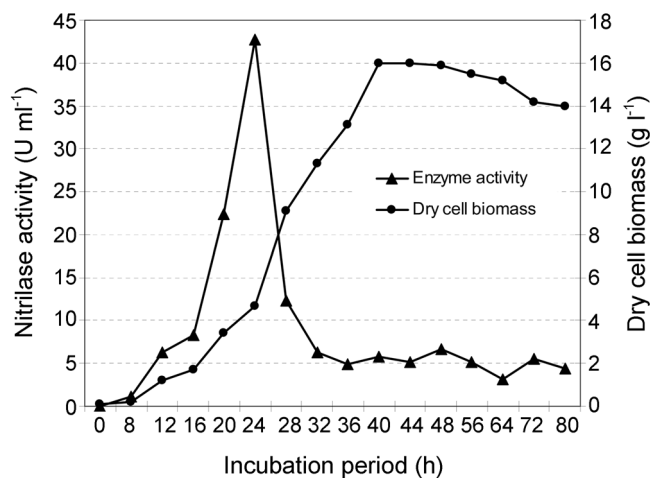


**Fig. 1.** Scanning electron micrograph of *Rhodococcus ruber* strain AKSH-84.

agent and, when used at high concentrations in the mobile phase for reverse-phase LC–MS, resulted in the suppression of the analyte signal [9]. This optimized mobile phase was further confirmed on a reverse-phase LC–ESI–MS in negative mode for ion generation using the same protocol and column. The ions at  $m/z=71$  [M-H]<sup>-</sup> (see Supplementary Fig. S2a and S2b) suggested the presence of acrylic acid at a retention time of 3.68, which correlated with the retention time of the LC chromatogram.

### Effect of Medium Conditions for Acrylic Acid Production by *Rhodococcus ruber* strain AKSH-84

When *R. ruber* strain AKSH-84 was cultivated in APY medium with pH adjusted from 4.0 to 10.0, the nitrilase activity was detected only at a pH range of 7.0–9.0. Maximum nitrilase activity (9.39 U/ml) was observed at pH 7.0. This observation corroborates the result of Khandelwal *et al.* [12], who also observed an optimal pH of 7.0 for the nitrilase activity produced by *Streptomyces* sp. MTCC 7546. In the present study, the enzyme activity lowered to 0.91 U/ml with an increase in the pH to 9.0. At pH 8.0, the enzyme activity was 35% as compared with pH 7.0. Interestingly, it was observed that pH values lower than 7.0 did not support the growth of the organism. The optimum temperature recorded for nitrilase activity was 30°C, suggesting that *R. ruber* strain AKSH-84 was mesophilic in nature. In general, most nitrile-degrading microorganisms exhibited nitrilase activity at around 30°C [6]. The optimal condition for agitation was 150 rpm that favored nitrilase activity. Under shake-flask conditions, a maximum yield of 12.2 U/ml was recorded for *R. ruber* strain AKSH-84 with an inoculum level of 3% after 24 h incubation at 30°C. Glycerol (10 g/l) exhibited good nitrilase activity for the bioconversion of acrylonitrile to acrylic acid as compared with other carbon sources (Supplementary Fig. S3). Sodium nitrate and casein showed more or less equal



**Fig. 2.** Time course of nitrilase production from *Rhodococcus ruber* strain AKSH-84.

The bacterium was grown in production medium for 80 h. Samples were withdrawn periodically for the estimation of cell growth and nitrilase activity using whole resting cells.

nitrilase activity of 14.14 and 13.74 U/ml as compared with the other nitrogen sources tested (Supplementary Fig. S4).

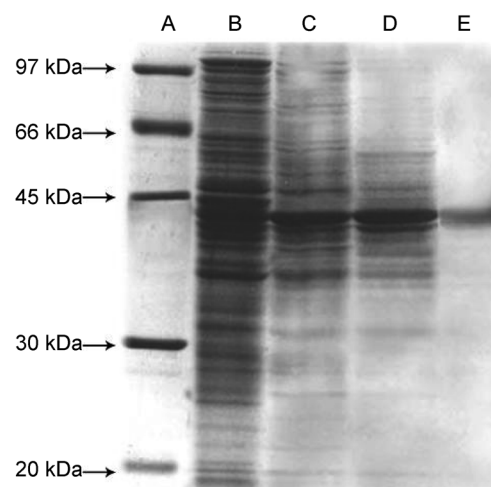
The acrylonitrile to acrylic acid bioconversion ability of *R. ruber* strain AKSH-84 in the APY medium under optimal conditions without inducer was very low during the initial lag phase of growth (0–8 h) and gradually increased in the early exponential growth phase (8–16 h), which increased steeply owing to the increase in the enzyme activity and the maximum nitrilase activity recorded was 42.8 U/ml when the cells were still in the early exponential phase at 24 h (Fig. 2), suggesting a constitutive enzyme secretion. Subsequently, a rapid decrease in the nitrilase activity was observed when the cells were in the mid-exponential phase at 32 h and the decrease was more or less constant beyond 40 h of incubation. The reduction in nitrilase activity on prolonged incubation may be due to the degradation of the nitrilase by intracellular proteases. However, *Arthrobacter* sp. strain C-38 exhibited maximum activity at 60 h; the nitrile hydratase activity increased until 30 h and a loss in enzyme activity was observed on further incubation [3].

### Purification of Nitrilase

The results on the purification of the nitrilase from *Rhodococcus ruber* AKSH-84 are summarized in Table 1.

**Table 1.** Purification scheme of *Rhodococcus ruber* AKSH-84 nitrilase.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Crude cell-free extract	10,050	1,560	6	100	1
Ammonium sulfate	4,690	106	44	47	7
DEAE-FF fraction	4,300	30	143	43	23
Sephadex G200	4,200	24	175	42	27



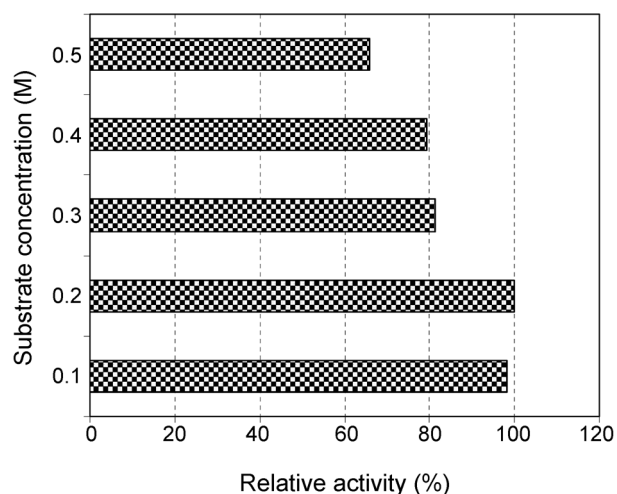
**Fig. 3.** SDS-PAGE of the nitrilase.

Lane A: Molecular mass standard proteins: Soyabean trypsin inhibitor (20 kDa), carbonic anhydrase (30 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), phosphorylase B (97 kDa). Lane B: Crude cell-free extract; Lane C: Ammonium-sulfate-precipitated enzyme; Lane D: DEAE FF column eluate; Lane E: Purified enzyme obtained from Sephadex G-200 gel filtration.

The ammonium-sulfate (45–70% saturation)-precipitated enzyme, when chromatographed on a DEAE-FF column, showed a 23-fold increase in nitrilase-specific activity. After the gel filtration step, the enzyme was purified to about 27-fold with a specific activity of 175 U/mg and a final yield of 42% (Table 1). The various fractions collected at each step of the purification process were subjected to SDS-PAGE, which showed a major band that closely corresponded to the molecular mass value of ovalbumin (45 kDa) (Fig. 3). The deduced molecular mass value of the enzyme was approximately 41 kDa, which is similar to the molecular mass reported for nitrilase from *Rhodococcus rhodochrous* K22 [13]. The purified enzyme catalyzed the hydrolysis of acrylonitrile to acrylic acid at 150  $\mu\text{mol}/\text{min}/\text{mg}$  protein under standard reaction conditions.

### Partial Characterization of the Purified Nitrilase

The *Rhodococcus ruber* AKSH-84 nitrilase showed a broad peak of activity between a pH range of 5.5 to 8.5 with an optimum at pH 7.0. The nitrilase exhibited an optimal activity at a temperature of 30°C and the enzyme also maintained its activity at 4°C. However, at a temperature of above 35°C, the enzyme activity was rapidly lost. Under



**Fig. 4.** Effect of substrate concentration on the nitrilase activity of *Rhodococcus ruber* strain AKSH-84.

The bacterial cells were cultivated in the production medium for 24 h and nitrilase activity was measured using whole resting cells. The absolute nitrilase activity at 100% relative activity is 6 U.

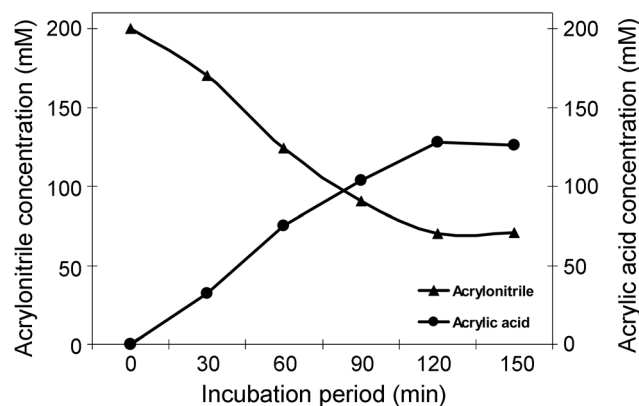
the specified incubation conditions, the nitrilase exhibited the following half-lives of irreversible inactivation: 18 h at 30°C, 12 h at 40°C, 2 h at 50°C, and 2 min at 60°C (data not shown). The  $K_m$  of the enzyme was calculated as  $20.5 \pm 2.8$  and the  $V_{max}$  was observed to be  $250 \pm 5.5$ . The double-reciprocal plot is shown in Supplementary Fig. S5.

#### Whole Cell Biotransformation of Acrylonitrile to Acrylic Acid by *Rhodococcus ruber* Strain AKSH-84

The optimum pH for nitrilase activity was observed in phosphate buffer at a pH of 7.2. It was earlier reported that the resting cells of *Rhodococcus rhodochrous* J1 employing acrylonitrile as substrate reacted at a pH of approximately 7.8 [15]. Reaction temperature is another critical factor for nitrilase activity, and the optimum temperature for nitrilase activity was at 30°C. However, the optimum temperature for nitrilase activity was 40°C using 0.2 and 0.4 g of wet cells of *Arthrobacter nitroguajacolicus* ZJUTB06-99 [18].

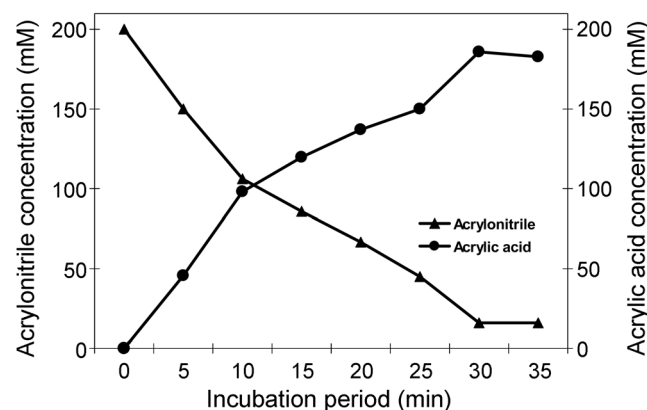
**Table 2.** Substrate affinity on different nitriles by resting cells of *Rhodococcus ruber* AKSH-84.

Nitriles	Relative activity (%)
Acetonitrile (aliphatic mononitrile)	100
Acrylonitrile (aliphatic mononitrile)	86.59
Succinonitrile (aliphatic dinitrile)	64.94
Fumaronitrile (aliphatic dinitrile)	61.85
Adiponitrile (aliphatic dinitrile)	36.59
2-Cyanopyridine (heterocyclic mononitrile)	14.43
3-Cyanopyridine (heterocyclic mononitrile)	45.36
Indole-3-acetonitrile (aromatic mononitrile)	53.09
Mandelonitrile (aryl acetonitrile-mono)	22.40



**Fig. 5.** Biotransformation profile of acrylonitrile to acrylic acid using whole resting cells of *Rhodococcus ruber* strain AKSH-84.

It is suggested that the reaction rates increased with the increase in reaction temperature, since the risen temperature increases the molecular free energy, which makes more efficient collisions between the molecules. However, at very high temperatures, the enzyme structure gets destabilized, which results in the loss of enzyme activity [18]. The substrate concentration required for optimal nitrilase activity in *Rhodococcus ruber* strain AKSH-84 was 200 mM (Fig. 4). The activity of nitrilase produced by the isolate was very high at low concentrations of acrylonitrile. The decreased activity at higher concentrations of acrylonitrile might be due to limitations on the solubility of the acrylonitrile in the buffer. The nitrilase from *Rhodococcus ruber* strain AKSH-84 exhibited wide substrate specificity and was able to hydrolyze different nitriles (aliphatic mono- and dinitriles, aromatic, heterocyclic) at 100 mM concentration (Table 2); higher substrate affinity was observed towards aliphatic mononitriles (acetonitrile and acrylonitrile) followed by succinonitrile and fumaronitrile, whereas lower affinity was observed towards mandelonitrile and 2-cyanopyridine.



**Fig. 6.** Biotransformation profile of acrylonitrile to acrylic acid using purified nitrilase from *Rhodococcus ruber* strain AKSH-84.

Employing the optimum conditions for the biotransformation of acrylonitrile to acrylic acid by *Rhodococcus ruber* strain AKSH-84, the yield of bioconversion using whole resting cells was recorded to be 63% (acrylic acid concentration was 126 mM) after 120 min (see Fig. 5), which is plausibly due to the slower mass transfer of the substrates and products into and out of the cells. The yield of bioconversion using purified nitrilase (50 U/mg) was observed to be 92% (acrylic acid concentration was 183 mM) after 30 min (see Fig. 6). Further studies are in progress to scale up the production and biotransformation processes. This is the first report on the bioconversion of acrylonitrile to acrylic acid using *Rhodococcus ruber*.

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