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# Purification and characterization of amidase from *Paracoccus* sp. SKG: Utilization of amidase-inhibited whole cells for bioconversion of acrylonitrile to acrylamide

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ABSTRACT

A bacterial strain, *Paracoccus* sp. SKG, capable of utilizing aliphatic nitriles as a sole source of carbon and nitrogen was isolated. Degradation of nitriles follows a bi-enzymatic pathway with the successive action of nitrile hydratase (NHase) and amidase. The inducible enzyme, amidase from cell-free extract of *Paracoccus* sp. SKG, was purified and characterized. The amidase was purified to 43.7 fold with a recovery of 46.5% yield and an estimated molecular mass of 90 kDa. The purified amidase exhibits the highest activity at pH 7.5 and temperature of 50 °C. This enzyme is highly specific to aliphatic amides with the highest activity to acetamide, propionamide and acrylamide, but not to aromatic amides. Among the metal ions studied, $Mn^{2+}$, $Mg^{2+}$ and $Ni^{2+}$ ions increase the enzyme activity, whereas $Cu^{2+}$ completely inhibits it. *In vitro* inhibition of amidase was confirmed by preincubation of the whole cells with $Cu^{2+}$. The use of whole cells of *Paracoccus* sp. SKG was demonstrated as a biocatalyst for the bioconversion of acrylonitrile to acrylamide in a batch reaction. The bioconversion of acrylonitrile to acrylamide resulted in 27 g/L of acrylamide with 65% of conversion under optimal conditions of pH (7.5) and temperature (30 °C).

## 1. Introduction

A wide range of bacteria are able to utilize nitriles and amides as sole sources of carbon and nitrogen for growth. These bacteria have the ability to hydrolyze nitriles and amides to the corresponding acids with the release of ammonia. Amidases or amidohydrolases (EC 3.5.1.4) are ubiquitous enzymes in the living world and can be divided into two types. The first type includes aliphatic amidases and the second the aromatic and mid-chain amidases such as acrylamide, aminoamide and hydroxyamide hydrolyzing enzymes. All these amidases exhibit an acyl transferase activity leading to the formation of hydroxamic acids (Fournand and Arnaud, 2001). Amidases belong either to the amidase signature or nitrilase superfamily (Chebrou et al., 1996). Those amidases that contain a GGSS motif in the primary structure share a conserved stretch of about 130 amino acids located in the center of the protein and belong to the amidase signature family (Mayaux et al., 1990). Aliphatic amidases belong to the nitrilase superfamily and typically hydrolyze short-chain aliphatic amides (Pace and Brenner, 2001). Members of the nitrilase superfamily have subunits of 30–44 kDa and exist as active dimers, tetramers, hexamers, octamers and longer oligomeric spirals (Dakshina et al., 2003). Amidases act as versatile biocatalysts in white and green chemistry. These enzymes find a place as industrial catalysts in organic synthesis (Rahim et al., 2003) making them a good candidate not only for environmental remediation but also for the biosynthesis of novel compounds. Purification and characterization of amidases are a significant step in biotechnological applications in the production of enantiomerically pure intermediates used in the synthesis of herbicides and pharmaceuticals as well as commodity chemicals such as acrylic acids, hydroxamic acids, amino acids and penicillin derivatives (Fournand and Arnaud, 2001).

Microorganisms having NHase and amidase activity are used for the bioconversion of acrylonitrile to acrylamide and acrylic acid, whereas organisms having nitrilase activity are used for the conversion of acrylonitrile to acrylic acid (Jonathan et al., 1998). Acrylamide is an important chemical used as coagulator, soil conditioner, and stock additive for treatment in leather and textile industry. Acrylamide can be synthesized both chemically and enzymatically. Its chemical synthesis has some disadvantages, such as the rate of formation of by-product,

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acrylic acid, is higher than acrylamide and requires high-energy input (Yamada and Kobayashi, 1996). Purified NHase from microbes is also used for the bioconversion of nitrile to amide. However, the purified NHase is not preferred as the deactivation rate constant of free enzyme is higher (265 times) than that of free cells (Sun et al., 2009). Recombinant expression of NHase is a possible strategy but the stability of NHase recombinant *Escherichia coli* cells is not as good as that of *Rhodococcus* cells (Shi et al., 2004). In recent years, microbial bioconversion of acrylonitrile using whole cells having NHase has received much attention because of its environment-friendly features (Okamoto and Eltis, 2007). Some strains of *Pseudomonas* (Wu et al., 1997), *Bacillus* sp. (Kim and Oriel, 2000) and *Nocardia* (Shi et al., 2004) with considerable activity of NHase have been successfully applied for the production of acrylamide. Further, during biosynthesis, using whole cells as biocatalyst, acrylamide further transforms into acrylic acid through amidase catalysis (Banerjee et al., 2002), which is an undesirable feature. Thus, there is a need for reduction in the formation of acrylic acid for further intensification of increased production of acrylamide using microbial systems (Brady et al., 2004).

Our previous study has demonstrated that the bacterial strain *Paracoccus* sp. SKG can degrade aliphatic nitriles and amides (Santoshkumar et al., 2011). NHase and amidase are key enzymes in the degradation of nitriles. Therefore, in this study we have presented the purification and characterization of amidase. Further, we have explored the use of amidase-inhibited whole cells of *Paracoccus* sp. SKG as biocatalyst for the production of acrylamide in a batch reaction and also evaluated the optimal conditions required for bioconversion.

## 2. Materials and methods

### 2.1. Chemicals, microorganism and culture conditions

Acetonitrile and acrylonitrile were procured from s d fine chemicals, India. Acetamide, acrylamide, propionamide, valeramide, benzamide and hydroxylamine hydrochloride were obtained from Sigma Aldrich, Steinheim, Germany. Media ingredients were purchased from Himedia, Mumbai. The other chemicals used in the study were of analytical grade and of higher purity.

The minimal mineral salts medium (MM1) used in the production of amidase comprised (g/L): $K_2HPO_4$, 6.8g; $KH_2PO_4$, 1.2g; $MgSO_4{\cdot}7H_2O$, 0.1g; $MnSO_4{\cdot}4H_2O$, 0.1g; $CaCl_2{\cdot}2H_2O$, 0.1g; $FeSO_4{\cdot}7H_2O$, 0.1g; $Na_2MoO_7{\cdot}2H_2O$, 0.006g. Filter-sterilized acetonitrile (1.5% v/v) was added to the above medium as the sole source of carbon and nitrogen. *Paracoccus* sp. SKG capable of degrading acetonitrile was isolated from chemical waste samples by acetonitrile-enrichment culture technique using MM1 medium (Santoshkumar et al., 2010). *Paracoccus* sp. SKG was incubated in 250 ml flasks containing MM1 medium and acetonitrile at 30 ± 2 °C on an orbital shaking incubator at 180 rpm. The strain is deposited with the National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India, with the accession number **NCIM 5314**.

### 2.2. Preparation of cell-free extract

Cell-free extract was prepared from the bacterium grown in MM1 medium containing acetonitrile (1.5% v/v). Fresh cells from the mid-logarithmic growth phase were harvested by centrifugation at 5500 × *g* for 10 min at 4 °C and the resulting cell pellet was washed twice with 50 mM phosphate buffer (pH 7.0) and resuspended in the same buffer. The cell suspension was disrupted by sonication (Vibracell Ultrasonicator model 375, USA) at a nominal power of 70 W for 6 min periods; each period of disruption was followed by 1 min off cycle during which the disrupted cells and oscillator probe were cooled in ice. Unbroken cells and cell debris were removed by centrifugation at 19,500 × *g* for 30 min at 4 °C. The cell-free supernatant thus obtained served as an enzyme source for purification.

### 2.3. Purification of amidase

Amidase was purified employing ammonium sulphate fractionation, ion exchange and gel permeation chromatography techniques. All purification procedures were performed at 4 °C. The cell-free extract obtained as described above was used for ammonium salt precipitation, where 60% ammonium sulphate was added to the cell-free extract. The precipitate was separated by centrifugation at 19,500 × *g* for 30 min. Additional ammonium sulphate was then added to the supernatant to reach 70% saturation. The resulting precipitate was collected by centrifugation and dissolved in 50 mM potassium phosphate buffer (pH 7.2). The solution was then dialysed overnight against 100 volumes of 50 mM potassium phosphate buffer (pH 7.2) at 4 °C. The dialysed protein sample was further applied to a DEAE Sepharose column (XK 16/40) operated on an AKTA basic FPLC system (GE Healthcare) equilibrated with 20 mM potassium phosphate buffer (pH 8.0). Later, the column was washed thoroughly with 20 mM potassium phosphate buffer, followed by an elution step with 300 ml of an increasing linear gradient of 0–0.5 M NaCl in potassium phosphate buffer (pH 8.0) at a flow rate of 1 ml per min and collected as 5 ml fractions. The fractions having amidase activity were pooled and the protein solution was concentrated 10 fold by ultrafiltration through a 10 kDa cut-off membrane (Amicon). The concentrated sample was loaded to Sephacryl S-200 h columns (XK 16/100). The column was equilibrated with 20 mM potassium phosphate buffer (pH 7.2) with 1% glycerol and 50 mM NaCl. The amidase enzyme was eluted with the same buffer at a flow rate of 0.5 ml per min and collected as 2 ml fractions. The fractions containing amidase activity were pooled and used for checking homogeneity, zymogram assay and characterization. The protein concentration was determined by using Bradford method (Bradford, 1976) with bovine serum albumin (BSA) as standard.

### 2.4. Molecular mass determination of purified amidase

Homogeneity and molecular mass of amidase subunits were determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by coomassie brilliant blue staining. The molecular mass of amidase was determined by gel-filtration on a Sephacryl S-200 h column using 20 mM potassium phosphate buffer (pH 7.2) containing 1% glycerol and 50 mM NaCl. The flow rate was 0.5 ml per min and the column was calibrated with the following standards: ribonuclease (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), albumin (67 kDa), alcohol dehydrogenase (150 kDa) and blue dextran (200 kDa) (GE Healthcare).

### 2.5. Zymogram staining for purified amidase

Amidase activity in native polyacrylamide gels (7.5%) was examined by modified method of Egorova et al. (2004). Zymogram staining technique was carried out by using purified amidase. After electrophoresis, the gels were incubated in 20 mM potassium phosphate buffer (pH 7.2) containing 10 mM acetamide and 0.7 M hydroxylamine hydrochloride at 37 °C for 10 min. The gels were washed in water and covered with an acid solution of iron chloride (0.1% $FeCl_3$ in 0.5 M HCl). Iron reacts with hydroxamic acid and forms a red-brown band, which corresponds to amidase activity.

### 2.6. Assays of hydrolytic and acyl transferase activity of amidase

The amidase hydrolytic activity was assayed in a reaction mixture containing 0.3 ml of potassium phosphate buffer (50 mM, pH 7.2), 0.1 ml of 10 mM acetamide and 0.1 ml of enzyme solution and incubated for 30 min at 37 °C in a water bath. The reaction was stopped by adding 0.25 ml of 1 M HCl. After 3 min, the solution was neutralized by the addition of 0.25 ml of 1 M NaOH and centrifuged. For the control, the enzyme was added immediately after the addition of HCl.