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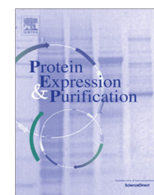

Purification and characterization of an extracellular uricase from a new isolate of *Sphingobacterium thalpophilum* (VITPCB5)

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ABSTRACT

An extracellular uricase producing bacterium (VITPCB5) was isolated from soil of the duck farm near Chidambaram, Tamilnadu, India and it was identified based on its 16S rRNA as *Sphingobacterium thalpophilum*. Uric acid was used as an effective inducer. The enzyme kinetics was studied using uric acid as a substrate. The K_m and V_{max} for the enzyme was found to be 0.28 mM and 0.92 μM/min ml, respectively. Maximum uricase production was observed when lactose was used as a carbon source. Among the nitrogen sources tested, urea gave the maximum uricase production. The enzyme was successfully purified using a weak cation exchange convective interaction media carboxy methyl (CIM-CM) monolith column with a recovery of 79.7% ± 0.1 and 14.2 ± 1.8-fold purification. The optimal reaction temperature of the enzyme was observed between 25 and 45 °C. The pH optimum of the enzyme was 8.0. The enzyme activity was enhanced by copper and partially inhibited by calcium, iron, zinc and nickel ions. Treatment with ethylene diamine tetraacetic acid completely inhibited the enzyme activity. The in-gel trypsin digested peptides of 48-kDa uricase when analyzed using mass spectrometry, gave 32% sequence coverage with the uricase (30-kDa) from *Cyberlindnera jadinii*.

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1. Introduction

Urate oxidase or uricase catalyzes the oxidation of uric acid to allantoin and plays an important role in purine metabolism [1]. Urate accumulation in human blood is a causative factor of gout [2]. Uricase is useful for the enzymatic determination of urate in clinical analysis by coupling with 4-aminoantipyrine-peroxidase system [3]. Direct injection of uricase allowed a much more rapid resorption of urate nephrolithiases in the case of gout associated with renal complications [4]. Infusion of *Aspergillus flavus* uricase (Rasburicase; Sanofi Synthelabo) is used to prevent acute uric acid nephropathy caused by tumor lysis in patients with leukemia and lymphoma [5,6]. Rasburicase has been utilized for the management of anticancer-therapy-induced hyperuricemia in pediatric patients in the EU and USA and in adult patients in the EU [7].

The uricase preparations from various microbial sources like fungi, yeast and bacteria have been reported [8–11]. But its increasing importance in treatment and diagnosis, necessitate to screen new sources for production, which are more economical, and may have unique properties to expand its usefulness. To date, pure cultures of bacteria capable of producing uricase that have been reported are *Pseudomonas aeruginosa* [12], *Micrococcus*, *Brevibacterium* and *Escherichia coli* [13], *Proteus vulgaris* [14], *Streptomyces albosriseolus* [15,16], *E. coli*, *Klebsiella* and *Serratia* [17]. However, to the best of our knowledge, there are no reports on the uricase from *Sphingobacterium thalpophilum*. In this study, we describe the production, purification and characterization of an extracellular uricase from the bacterium *S. thalpophilum* (VITPCB5), isolated from poultry soil sample.

2. Materials and methods

2.1. Source of bacterium

The bacterium (VITPCB5) used in this study was isolated from soil samples of a duck farm in Chidambaram, Tamilnadu, India. The basal agar medium used for the bacterial isolation consisted of 1% uric acid, 0.1% yeast extract, 0.1% glucose and 2.0% agar. This bacterium showed formation of clear zones around the colony after incubation for 1–3 days in plate assay which indicated the production of extra cellular uricase.

2.2. Strain identification

The micro organism was examined for morphological and biochemical characteristics [18]. The strain was found to be positive

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for catalase, oxidase and negative for gram staining, sporulation, H_2S and indole production and further based on 16S rRNA gene sequencing, the strain VITPCB5 was classified as *S. thalpophilum*. The DNA from the bacteria was isolated and purified according to [19]. The forward primer 27F (5′ GAGTTTGATCCTGGCTCAG-3′ and reverse primer 1492R (5′-GGTTACCTTGTTACGACTT-3′ were used to amplify 16S rRNA genes from the genomic DNA. The amplified products were sequenced by dideoxy chain termination method [20]. The sequence results gave a total of 1335 nucleotides and base pair similarity was compared with a BLAST search database and the 16S rRNA gene coding nucleotide sequences were deposited in GenBank (accession number JX083378).

2.3. Medium

The composition of pre-culture medium was 1% peptone, 2% glucose, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.5% NaCl. The pH of the medium was adjusted to 8.0. The production medium was prepared by the addition of 0.05% uric acid to the pre-culture medium.

2.4. Cell growth and uricase production

1% (v/v) of pre-cultured organism was inoculated into a 3000 ml Erlenmeyer flask containing 500 ml of sterilized production medium. The flask was incubated in an orbital shaker incubator for 48 h at 175 rpm maintained at 37 °C. Samples were taken periodically to determine the cell biomass and uricase production. The growth of the organism was monitored at 600 nm for every 4 h using a spectrophotometer (Beckman Coulter, USA). The un-inoculated medium was used as blank.

2.5. Uricase assay

The uricase activity was determined based on the method of [21]. The Standard reaction mixture contained 0.6 ml of 2 mM uric acid dissolved in 0.1 M sodium borate buffer (pH 8.5), 0.1 ml of 1.5% phenol, 0.15 ml of 30 mM 4-aminoantipyrine, 0.05 ml of 15 U/ml peroxidase from horseradish, and 0.1 ml of enzyme solution. The mixture was incubated at 25 °C for 20 min. The reaction was terminated by the addition of 1 ml of ethanol, and the absorbance at 540 nm was read against the blank by a spectrophotometer. One unit of uricase was defined as the amount of enzyme that produces 1.0 μmol of H_2O_2 per minute under the standard assay conditions. To determine the uricase activity, samples of the culture were withdrawn, and the cells were centrifuged at 3000*g* for 10 min and filtered through 0.2 μm. The filtered supernatant (crude enzyme solution) was used for the analysis of enzyme activity.

2.6. Micro well urate oxidase activity

To study the time course of cell growth and uricase production, 50 μl of the cell free supernatants (0.2 μm filtered) obtained at different incubation periods (12, 24, 36 and 48 h) were added to the wells of the uric acid agar plate. After 12 h of incubation, the zone of clearance of uric acid was observed around the wells [22].

2.7. Uricase zymography

The zymography was carried out with the polyacrylamide gels and the enzyme activity was visualized by immersing the gel in 0.1 M Tris–glycine buffer (pH 8.0) with 3 mM 4-aminoantipyrine and 0.1 mM uric acid along with 10 U of horse radish peroxidase [23]. The gel was incubated for 30 min in the buffer and stained with amido black dye to visualize the uricase activity as a clear white band.

2.8. Effect of different carbon and nitrogen sources

The effect of carbon sources was studied with the 100 ml of basal medium containing K_2HPO_4 0.1 g; uric acid 0.05 g; NaCl 0.5 g; $MgSO_4 \cdot 7H_2O$ 0.05 g with 1% of different carbon sources (dextrose, maltose, lactose, sucrose, starch). Uricase assay was done after 24 h of cultivation.

The effect of nitrogen sources was studied with the 100 ml of basal medium containing 1% of different nitrogen sources (beef extract, yeast extract, urea and sodium nitrate). The nutrient broth containing 0.05 g uric acid was kept as the control medium in each case. Uricase assay was done after 24 h of incubation.

2.9. Purification of uricase enzyme using convective interaction media carboxy methyl (CIM-CM™) monolith column

A total of 2.7 ± 0.21 gram wet biomass of cells per 500 ml of culture broth (24 h) were harvested and cells were pelleted by centrifugation at 10,000*g* for 10 min. at 4 °C. The cell free supernatant containing the extracellular enzyme was subjected to 70% saturated ammonium sulfate precipitation, followed by centrifugation at 14,000*g* for 30 min. at 4 °C. The pellet was dissolved and dialyzed against sodium borate buffer (pH 8) for a period of 12 h at 4 °C.

Monolith CIM-CM disk (BIA Separations, Ljubljana, Slovenia) of dimensions 12 mm × 3 mm i.d. (0.34 ml) was used for rapid separation of uricase from the crude enzyme. Prior to purification, the crude enzyme was concentrated to ~1 ml using a 3-kDa MW cutoff membrane filter (Amicon, Millipore, USA) and buffer exchanged with the optimized binding buffer system containing 25–50 mM MES–MOPS–acetate (MMA), pH 6. The elution was done with increasing NaCl step gradient 0.25, 0.5 and 1 M NaCl in the binding buffer. A sample load of 10 mg total crude enzyme was injected into the column at a flow rate of 3 ml/min. Chromatographic experiments were done on a fully automated AKTA FPLC system (Amersham Bioscience, Uppsala, Sweden) consisting of a P-920 pump and a UPC-900 monitor for measurement of UV absorption (wavelength set at 280 nm), pH and conductivity. The purified enzyme was used in studying the kinetic constants, effect of pH, temperature and metal ions on enzyme activity.

2.10. Effect of pH and temperature on uricase activity

The effect of pH on uricase activity was determined by incubating the purified enzyme sample in different pH solutions (pH 4, 5, 6, 7, 8, 9 and 10). The uricase solution was made in 50 mM sodium acetate buffer for pH 4 and 5. Sodium phosphate buffer (50 mM) was used for pH 6 and 7, and Tris–HCl (50 mM) was used for pH 8 and 9. Glycine buffer (50 mM) was used for pH 10. The samples were then incubated for 30 min at 25 °C. After incubation in respective pH buffers, the solution was neutralized in sodium borate buffer, pH 8 and enzyme activity retained was determined by the standard assay procedure.

The optimal temperature for uricase activity was determined by incubation of the enzyme at temperatures from 20, 30, 40, 50, 60, 70 and 80 °C. To determine the temperature stability of the enzyme, it was incubated at 60 °C for 30 min. and 1 h and the residual enzyme activity was evaluated.

2.11. Effect of some metal ions on uricase activity

The effect of some metals and inhibitor on enzyme activity was carried out with 1 mM concentration of different metals/inhibitor

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