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RESEARCH ARTICLE

PRODUCTION OF URICASE A THERAPEUTIC ENZYME FROM *Pseudomonas putida* ISOLATED FROM POULTRY WASTE

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Abstract

Urate oxidase or uricase is the enzyme that catalyzes specifically the oxidation of uric acid to allantoin and plays an important part in nitrogen metabolism. The soil samples were collected from poultry farms and the uricase producing microorganism were isolated. From the uric acid amended plates 5 strains were selected based on different morphology. They were purified and checked for their potential of uricase production by using well assay method. The potential strain which showed a zone of clearance of 14mm on uric acid agar plate was selected and it was to subjected to biochemical tests. The isolated strain was optimized to parameters like temperature, pH, incubation time, carbon sources and nitrogen sources by adopting one parameter at a time method. The study was conducted for a period of hours where analysis was done for every 6hrs interval. The isolated protein was estimated and its was separated by SDS –PAGE. uricase producing gene was isolated and amplified by PCR.The Purified protein Sample was subjected to FTIR spectroscopy. The isolated strain was identified as *Pseudomonas putida*. The optimized parameters, tem 37^0 C, pH-7.0, 36 hrs of incubation time are found suitable for higher production, 1% starch and 0.2% ammonium nitrate were found to be ideal for growth. The highest level of uricase activity attained for the crude enzyme was 38U/ml/min, whereas after purification 67.0U/ml/min. SDS-PAGE analysis revealed the protein profile of partially purification a 66kDa protein was obtained. The amplified uricase gene of *Pseudomonas putida* showed 240bp length. FTIR results also showed characteristic positive vibrations for uricase. Thus the present study contributes much new information on microbial uricase and proved its potential as a industrial scale producer.

Introduction

Urate oxidase or uricase (urate:oxygen oxidoreductase ,Ec 1.7.3.3), is the enzyme that catalayses specifically the oxidation of uric acid to allantoin and plays an important part in nitrogen metabolism (Wakamiya *et al* ., 1994) .Uric acid is a powerful antioxidant and a scavenger of free radicals .Therefore ,a high serum uric acid level caused by the loss of urate oxidase activity may have contribute to a decreased cancer rate and a lengthened hominoid life span (Friedman *et al* ., 1985). Thus, uricase is a promising enzyme with high specificity towards uric acid and usually needed in large quantities for medical uses including analysis of human serum or urine for uric acid and using as a protein drug to reduce toxic urate accumulation.

Hyperuricemia is not only a direct cause of gout and related disease but also an independent risk factor for certain kidney and cardiovascular disease (Hosoya *et al* ., 2011). Therefore, it is essential to reduce the level of uric acid in blood and tissues to prevent and treat many uric acid-related diseases.
Uricase is mainly localized in liver of animals and in Microorganisms especially bacteria such as. *Pseudomonas aeruginosa, Arthrobacter globiformis, Bacillus subtilis, Bacillus fastidious, Nocardia farcinica* and *Microbacterium sp*.

MATERIALS AND METHODS

Isolation and identification

Soil samples were collected from poultry farm and inoculated on the sterile nutrient agar containing 0.3% uric acid plates .The plates were incubated for 2-3 days at room temperature .Identification was done based on morphological, cultural, biochemical and physiological characteristics based on Bergey's manual of determinative Bacteriology (Buchanam *et al* ., 1974).

Optimization of growth

The identified potential strain was optimized for parameters like pH (5,6,7,8,9and 10),temperature (25,30,35,37and 40 ^{0}C) , incubation time (12-96 hrs) carbon sources (starch ,sucrose ,maltose and glucose) and nitrogen sources (beef extract ,peptone ,yeast extract and ammonium nitrate).Strain with maximum uricase production was identifies as *Pseudomonas putida* . Finally the optimized parameters were used to produce uricase enzyme in Shake flask.
Uricase activity was measured according to the procedure described by Adamek *et al.,* 1989. To 2ml of a solution containing uric acid (10µg per ml of borate buffer 0.2M, pH 8.5), 0.8ml of water and 0.1ml of crude enzyme were added at 25°C. After 10 min. 0.2ml of 0.1M potassium cyanide solution was added to the mixture to stop the enzyme reaction. In the reference sample, the solution of potassium cyanide was added to the mixture before the addition of the crude enzyme. The absorbance of samples was measured at 293nm. One unit of uricase enzyme was equal to the amount of enzyme which converts 1µ molar of uric acid to allantoin per min. at 30°C.
The protein concentration of the sample was determined by Lowry's method (Lowry *et al.,*1951) using bovine serum albumin as the standard.

Extraction and purification of enzyme

Harvested culture broth was centrifuged at 10000 rpm for 30 mins and the cell free supernatant was used for enzyme purification .Concentration of protein was done by ammonium sulphate precipitation (40, 60, 70 and 80 %).The resultant precipitate was dissolved in 5 ml of 0.02 M tris Hcl buffer pH 8.5 and dialysed over night against 2 L of the same buffer in a cellophone bag (Saeed *et al* ., 2004).
SDS-PAGE analysis is carried out to reveal the protein profile. Uricase gene of *Pseudomonas putida* was Isolated and Amplified by PCR technique. The PCR product was detected by agarose gel electrophoresis.
Infra-red spectrum of purified sample was analyzed by using Fourier transferred infrared spectrophotometer. Infrared spectral analysis of biological material was utilized to investigate their chemical constituents. (Sachdev *et al*., 2009).

RESULT AND DISCUSSION

From the uric acid amended plates 5 strains were selected based on different colony morphology. The potential strain SBSUC2 was selected as the most potential as it resulted in a zone of clearance was 14mm. The most potential strain was identified as *Pseudomonas putida* based biochemical tests (Table 1). Bacteria capable of producing uricase that have been documented are only few like *P. aeruginosa* (Frank and Hahn, 1955), *P. acidovorans* (Sin, 1975),

Optimal Culture conditions like pH-7 ,Lofty, 2008 observed highest enzyme production at pH 7.0 in a *Bacillus thermocatenulatus* strain isolated from Egyptian soil (Graph1) and temperature 37^{o}C,(Graph2) Li *et al.,* 2006 found that temperature around 30-37ºC was suitable for the uricase they have produced in *E.coli* host. Nutrients like carbon (starch -1%) (Graph3)and nitrogen sources (0.2% ammonium nitrate) (Graph4) were found influence for growth. The inhibitory protein was found to be maximum at 80% ammonium sulphate precipitation.

The desalted protein sample was further purified on DEAE-Sephadex A-50 column. The enzyme activity for crude and purified enzyme was 38U/ml, 67U/ml. whereas, the specific activity was 36U/ml and 70U/ml for crude and purified enzyme respectively (Fig 1).

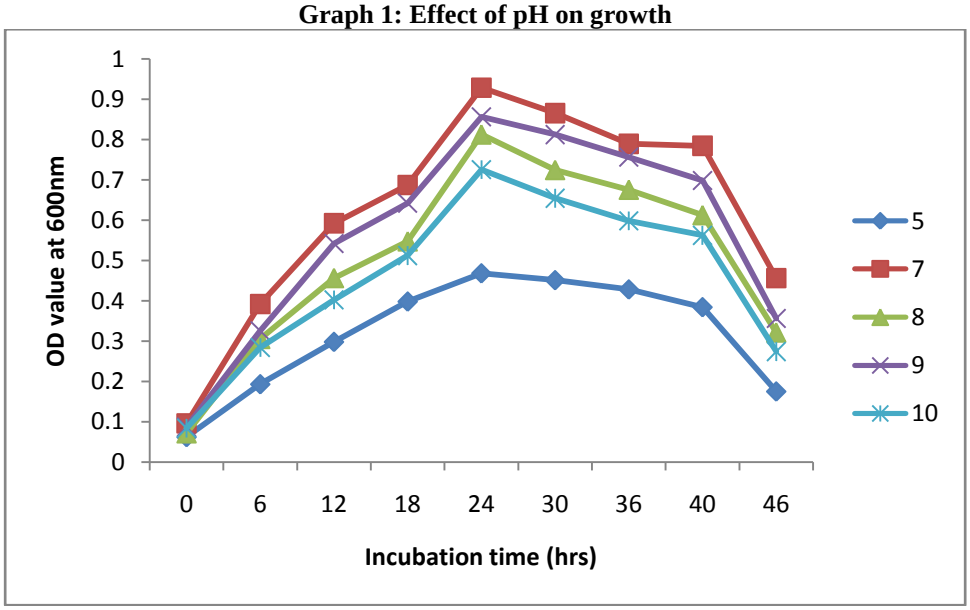
SDS-PAGE analysis of purified fractions revealed five peptides with a molecular weight of 18, 36, 42 and 66 and 116 kDa. Among five peptides 66 kDa showed zone of clearance on uric acid agar plate. However in *Bacillus subtilis*, Pollyana *et al.*, 2010 reported 60 kDa protein as uricase. The column fractions were checked for uricase activity using Well diffusion assay (Fig:2). The detection of uricase gene using PCR, reveled an amplified uricase gene size of 240 bp (Fig:3). Cheng *et al.*, 2008 amplified the known *Candida utilis* urate oxidase gene sequence and the PCR-amplified urate oxidase gene's size of 0.9 kb.

The N-H stretching vibrations at 3427 cm^{-1} in the FT-IR spectrum showed N- H stretch of the protein compound(Graph :5). The CH_2 vibration was obtained at 2912 cm^{-1}. The uricase enzyme showed a characteristic peaks 1612 and 1595 cm^{-1} attributed to the primary and secondary amide linkages of enzyme molecules. C-H vibration was obtained at Wave number 1421cm^{-1}.

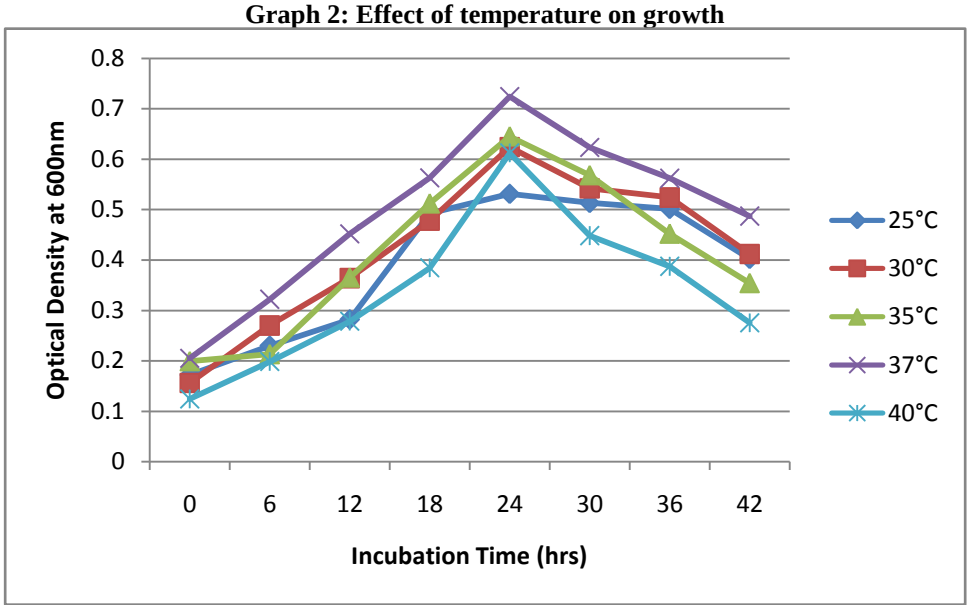
Table1: Biochemical characteristics of *Pseudomonas putida*

BIOCHEMICAL TEST	RESULT
Indole	Negative
Methyl red	Negative
Voges Proskauer	Negative
Citrate	Positive
Nitrate reduction test	Positive
Oxidase test	Positive
Catalase test	Positive

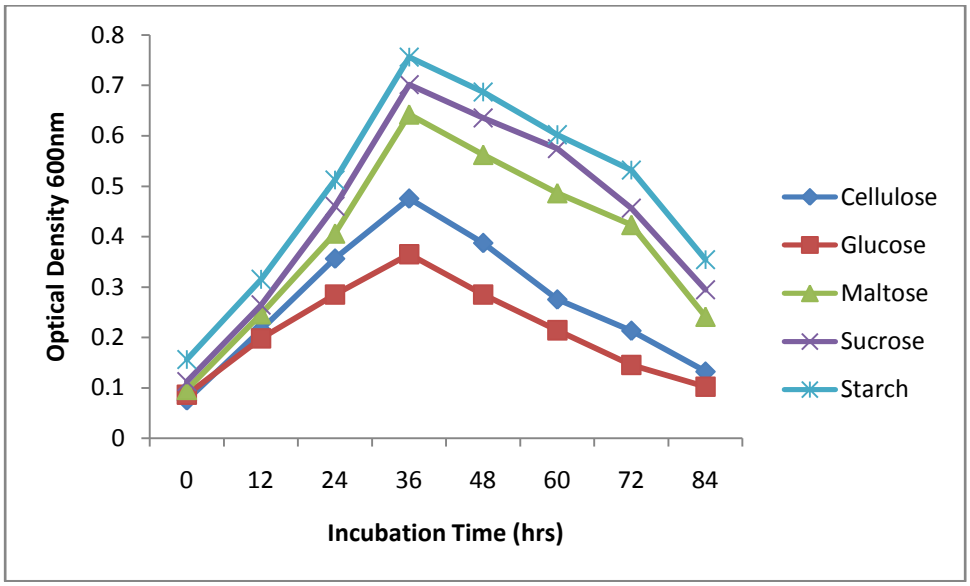
Graph 1: Effect of pH on growth



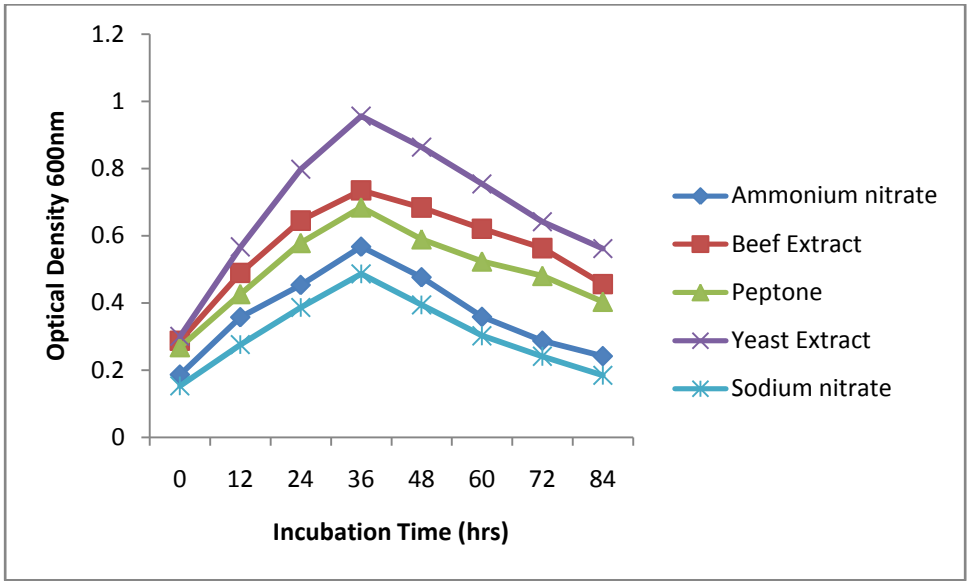
Graph 2: Effect of temperature on growth



Graph 3: Effect of carbon sources on growth



Graph 4: Effect of nitrogen sources on growth



Graph 5: FTIR spectrum of uricase from *Pseudomonas putida*

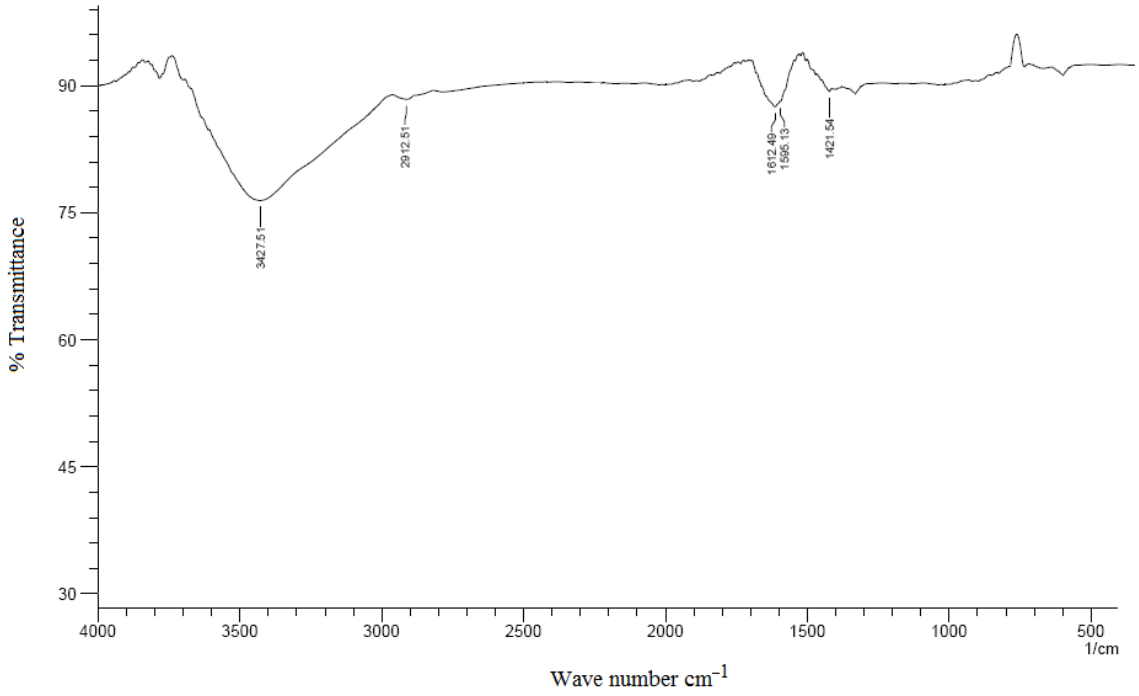


Fig.1: Uricolytic activity of crude and purified uricase

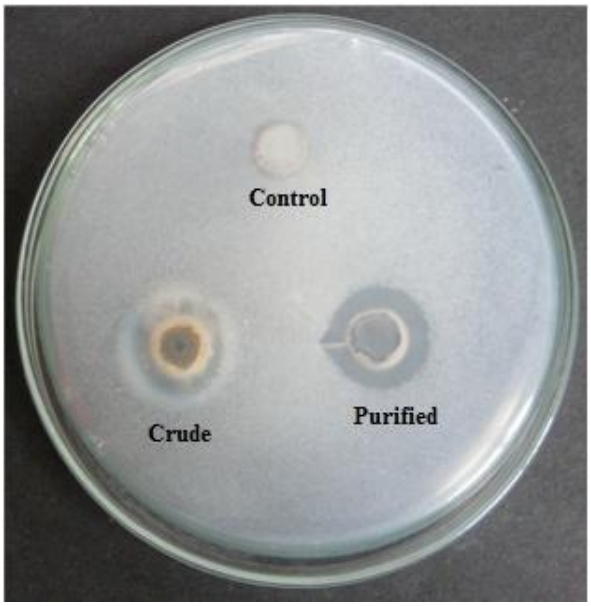
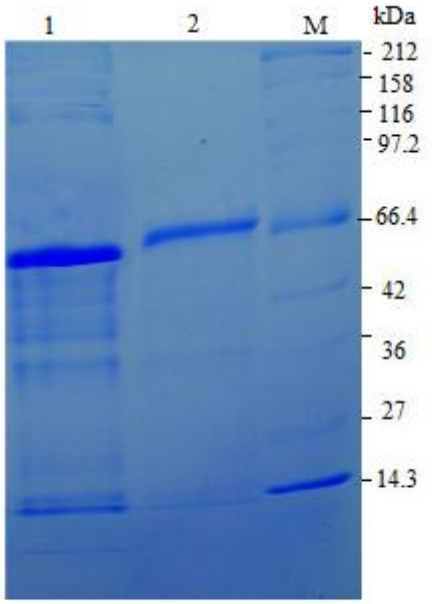
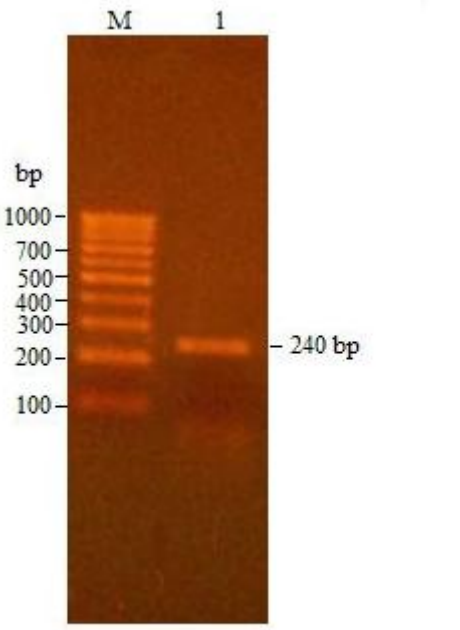


Fig. 2: Protein profile of uricase from *Pseudomonas* putida on SDS-PAGE gel



Lane 1: Crude Uricase enzyme
Lane 2: Purified Uricase
Lane M: Standard Protein molecular weight marker

Fig. 3: Amplification of uricase producing gene of *Pseudomonas putida* using PCR



Lane 1: amplified 240 bp gene of Uricase
Lane M: 100 bp DNA Ladder

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