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Uricase production from *Bacillus subtilis* isolated from Poultry waste

[*1]K. Jagathy, A. Pushparaj[2] and J. Ronald[3]

[*1]Assistant Professor, Department of Microbiology, Sri Akilandeswari Women's college, Wandiwash, Tamilnadu, India.
[2]Asst. Professor, Department of Zoology, T.D.M.N.S College, T. Kallikulam - 627 113.
[3]Asst. Professor, Research Department of Zoology, St. Xavier's College, Palayamkottai.
*Corresponding Author

Abstract

The present study dealt with the production of uricase enzyme by a bacterial strain isolated from poultry waste. The study proved that uricase producers are abundant in poultry waste examined and the density of uricase producers was 1.6 X 10^8 CFU/g. The most potential strain was identified as *Bacillus subtilis* based on biochemical tests. The optimum cultural conditions such as pH-7, temperature - 30°C, sucrose -1% and peptone - 0.2% and incubation time 24hrs were found to be ideal for growth of the potential strain. Under optimum growth conditions in shake flask, the highest level of uricase activity attained was 32U/ml/min and after partial purification, the observed activity was 60.0U/ml/min. SDS-PAGE analysis revealed that the molecular size of the partially purified uricase enzyme was 48kDa.

Keywords: Uricase, *Bacillus subtilis*, poultry waste, gout, urate oxidase.

Introduction

Urate oxidase or uricase (urate: oxygen oxidoreductase, EC 1.7.3.3) is an enzyme that catalyse the oxidation of uric acid to allantoin and plays an important role in purine metabolism (Wu *et al.*, 1994). It catalyzes the oxidative opening of the purine ring of urate to yield allantoin, carbon dioxide, and hydrogen peroxide. This enzyme is widely present in most vertebrates but is absent in humans (Schiavon *et al.*, 2000). It was first found in bovine kidney.

Recently, interest has been concentrated on production of uricase from various microbial sources such as bacteria (Mansour *et al.*, 1996), fungi (Farley and Santosa, 2002) and eukaryotic cells (Montalbini *et al.*, 1997). Although several microbial sources of uricase have been proposed for gout disease, only one has actually been used commercially under the trade mark of uricozyme and is isolated and purified from *Aspergillus flavus.*

Gout is a painful disorder, characterized by uricemia, recurrent attacks of acute arthritis, deposition of sodium urate in and around joints, and in many cases, formation of uric acid calculi (Lee *et al.*, 1988). Gout was historically known as "the disease of kings" or "rich man's disease". It is caused by elevated levels of uric acid in the blood which crystallizes and the crystals are deposited in joints, tendons, and surrounding tissues.

Worldwide about 1% of the population is suffering from gout disease. The rate of gout has increased in recent decades, not only in America but also in other developed countries. The increase is possibly due to dietary and lifestyle changes, greater use of medications that cause hyperuricemia, and aging populations. Gout is very common in developing countries now.

Diagnosis is confirmed clinically by the visualization of the characteristic crystals in joint fluid. Determining the urate concentration in blood and urine is effective for diagnoses of gout since urate accumulation is a causative factor of gout in humans (Nishiya *et al.*, 2002). Uricase is useful for enzymatic determination of urate in clinical analysis by coupling it with a 4-amino-antipyrine-peroxidase system (Gochman and Schmitz, 1971).

Gout treatment generally includes allopurinol, which is a potent competitive inhibitor of xanthine dehydrogenase, an enzyme which catalyses the conversion of hypoxanthine to xanthine and xanthine to uric acid. Treatment with nonsteroidal anti-inflammatory drugs (NSAIDs), steroids, or colchicine improves symptoms. Once the acute attack has subsided, levels of uric acid are usually lowered via lifestyle changes, and in those with frequent attacks allopurinol or probenecid provide long-term prevention.

Uric acid is the end product of purine metabolism and its concentration is mainly controlled by endogenous metabolism. Human beings lack uricase, the enzyme responsible for converting uric acid into its soluble and excretable form. Renal excretion of urate accounts for most hyperuricemia related diseases and gout. As only few drugs are available to treat this diseases and hypersensitivity developed to few those drugs in use, the present situation warrants new sources of uricases.

Materials and Methods

Collection of Samples

Soil samples contaminated with chick faecal materials were collected from chick farm located in Chidambaram.

Isolation of Bacteria

Serially diluted samples were plated on the surface of nutrient agar containing 0.3% uric acid (Hesham *et al.*, 2004). After 2-3 days of incubation at room temperature, the colonies with uricotytic activity was selected for further screening.

Screening for potential strain

The potential uricase producing strain was selected based on the screening on uric acid plate by the measurement of zone of clearance using well diffusion assay in uric acid incorporated plates in which 50µl of cell free extract of each bacterial strain was filled in 8mm diameter wells cut using a well cutter and the inoculated plates were incubated for 24-48hrs at 37°C, and the diameter of the zone of clearance was measured. Strain showing highest zone of clearance was selected for further study.

Identification and confirmation of potential strain

Identification was done based on morphological, cultural, biochemical and physiological characteristics based on Bergey's Manual of Determinative Bacteriology (Buchanan *et al.*, 1974).

Optimization of growth for potential strains

The identified potential strain was optimized for growth parameters like pH, temperature, salinity, carbon and nitrogen sources. Different pH ranged from 5-10 (5, 6, 7, 8, 9 and 10), temperatures (25, 30, 35 and 40°C) were tested. Carbon sources like starch, cellulose, sucrose, maltose and glucose were incorporated separately at the rate of 1% in nutrient broth and their influence on growth was studied in 250ml Erlenmeyer flasks. Likewise nitrogen sources like beef extract, peptone, yeast extract and ammonium nitrate were added at the rate of 0.2% in nutrient broth. In all flasks 0.3% of uric acid was incorporated. Growth was estimated at 600nm (Olesen *et al.*, 2000) in a UV spectrophotometer (Spectronics, India). The influencing parameters and nutrients were studied by selecting one parameter at a time at which other factors were kept as constant. Finally the optimized parameters were used to produce uricase enzyme in shake flasks.

Uricase enzyme activity assay

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 at 25°C were added. 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. The difference between absorbance of the sample and reference is equivalent to the decrease in uric acid during the enzyme reaction. One unit of uricase enzyme was equal to the amount of enzyme which converts 1µmol of uric acid to allantoin per min. at 30°C.

Extraction and partial purification of uricase

The cell free culture broth from the mass scale shake flask was harvested by centrifuging at 10000rpm for 30min. The uricase enzyme in the cell free supernatant was further concentrated by ammonium sulphate precipitation followed by dialysis. Different concentrations of ammonium sulphate (40, 60, 70, and 80% w/v) were used for fractional precipitation of uricase. Solid ammomium sulphate was added slowly to the culture filtrate with gentle stirring on ice bath until the required saturation of ammonium sulphate was reached after which the mixture was allowed to stand at 4ºC overnight. The mixture was then centrifuged at 8000 rpm for 30 min. at 4ºC. Further ammonium sulphate was added to the culture filtrate and the process repeated until the final saturation reached 80%. Enzyme activity was determined for each separate fraction. This purification step was carried out to remove the traces of ammonium sulphate. The resultant precipitate was dissolved in 5 ml 0.02 M tris-HCl buffer pH 8.5 and dialyzed overnight against 2 liters of the same buffer in a cellophane bag (Saeed *et al.*, 2004).

Protein Separation- SDS-PAGE-(Laemmli, 1970)

The molecular size of the partially purified uricase was determined according to the method Laemmli, 1970.

Results and Discussion

In the present study samples were collected from poultry farm and the bacterial density was found to be 1.6 X 10^8 CFU/g. From the uric acid amended plates 6 strains were selected based on different morphology (Fig.1). They were purified and checked for their potential of uricase production using well assay. About 6 strains were selected based on the measurement of zone of clearance (Table-1). The most potential strain (UCSBS5) was selected based on the maximum zone of clearance of 15mm (Fig.2) and it was used for further study.

In this study, the most potential uricase producing isolate from poultry waste was identified as *Bacillus subtilis* based biochemical tests (Table-2). Bacteria capable of producing uricase that have been documented are only few like *P. aeruginosa* (Frank and Hahn, 1955), *P. acidovorans* (Sin, 1975), *Arthrobacter globiformis* (Nobutoshi *et al.*, 2000), *Bacillus subtilis* (Hunag and Wu, 2004), *Nocardia farcinica* (Ishikawa *et al.*, 2004).

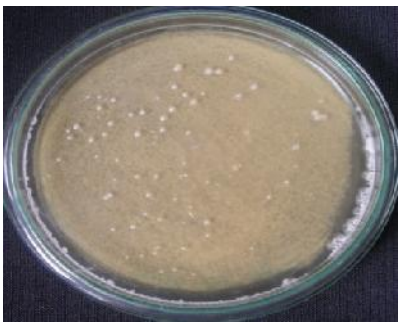

Fig.1: Isolation of uricase producing strains

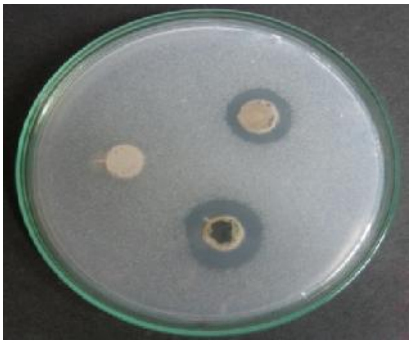

Fig. 2: Screening for uricolytic activity

Table – 1: Uricase activity of strains collected from Chick farm

Strain No.	Zone of Clearance (mm)
UC SBS1	-
UCSBS2	11
UCSBS3	13
UCSBS4	-
UCSBS5	15
UCSBS6	12

Table-2: Physiological and biochemical characteristics of *Bacillus subtilis*

Characteristic	Results
Shape	Rod
Spore	+
Gram reaction	+
Motility	-
Carbohydrate utilization	
Arabinose	-
Xylose	-
Mannitol	-
Starch	+
Gelatin	-
Casein	-
Cellulose	+
Glucose	+
Growth at 5% NaCl	-
Growth at 7% NaCl	-

Culture conditions like pH, temperature, salinity, nutrients like carbon and nitrogen sources were found to influence the growth. The identified optimized parameters were pH-7, temperature-30°C, sucrose-1% and peptone-0.2% and incubation time 24hrs for the strain *Bacillus subtilis* used in the present study (Fig. 3-6).

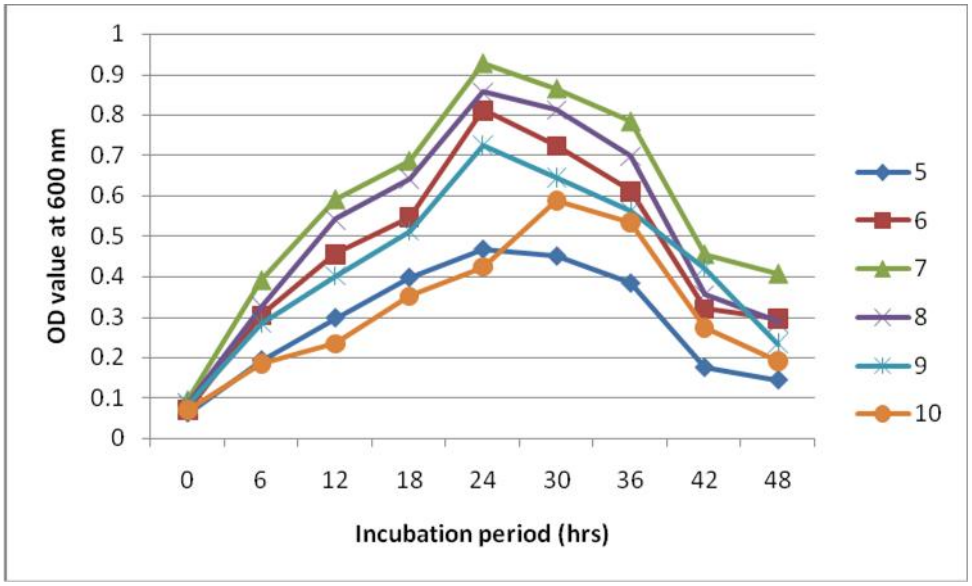

Fig. 3: Effect of pH on growth

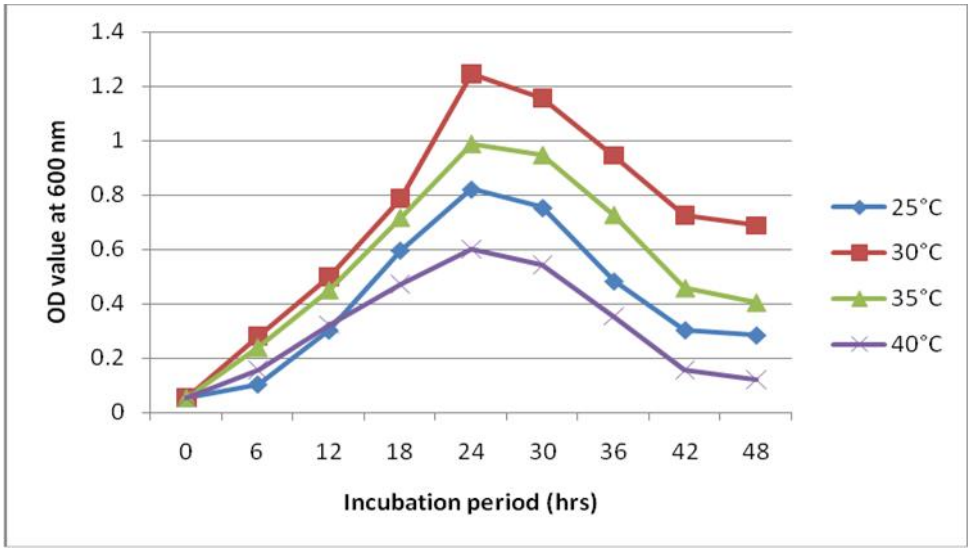

Fig.4: Effect of temperature on growth

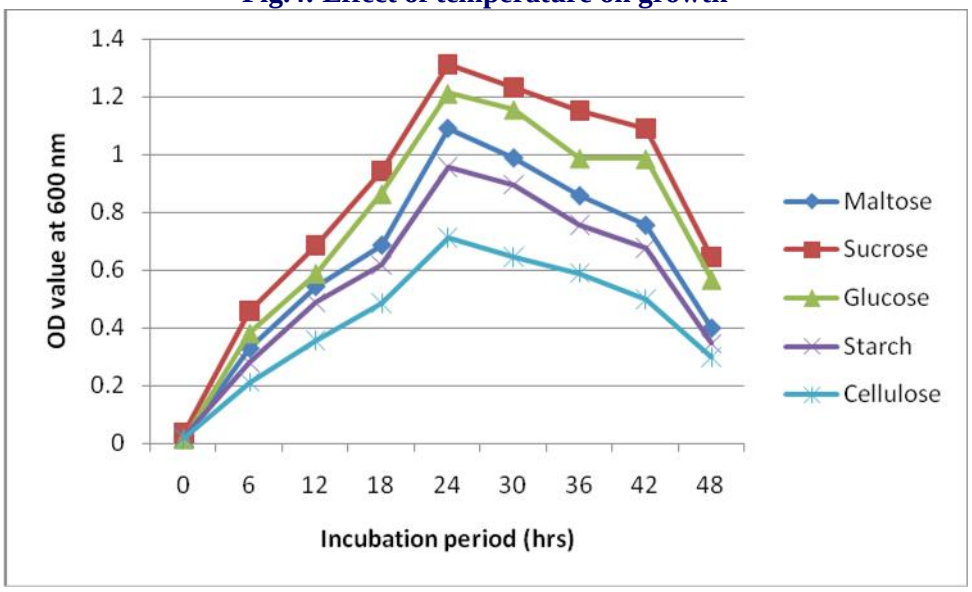

Fig. 5: Effect of carbon sources on growth

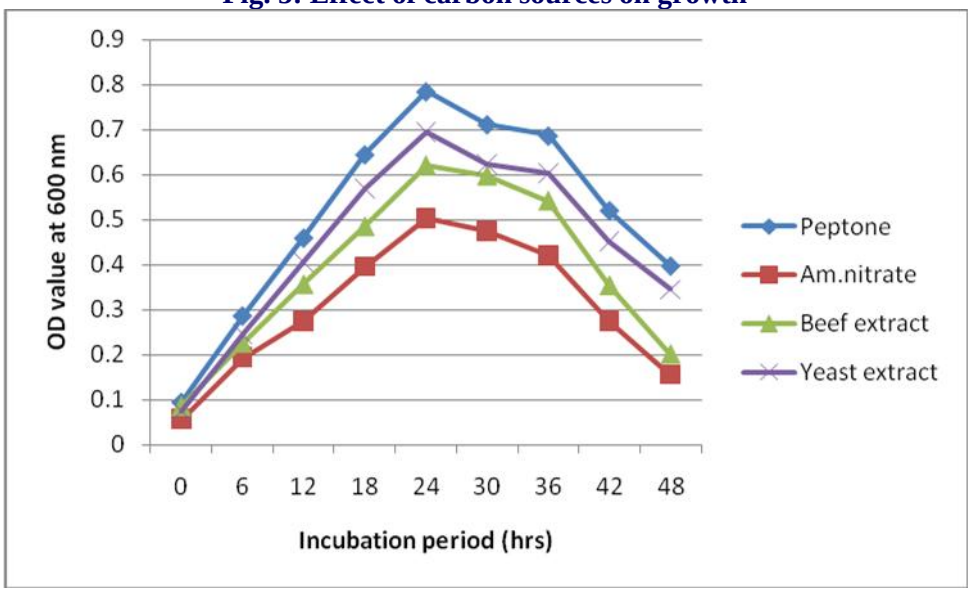

Fig.6: Effect of nitrogen sources on growth

The initial medium pH ranging from 5 to 10 was studied to detect the effect on uricase production by *Bacillus subtilis*. In the present study optimum pH observed for maximum activity was pH-7. Li *et al.*, 2006 and Abdel-Fattah and Hesham (2005) however found a relatively lower pH (i.e.) 5.5 as the most suitable pH for uricase activity.

In the present study 30ºC was found to be optimum for production of enzyme. Li *et al.*, 2006 found that temperature around 30-37ºC was suitable for the uricase they have produced in *E.coli* host. These results were in agreement with those of Ammar *et al.*, 1988), Tohamy and Shindia (2001), Abdel-Fattah and Yazdi *et al.*, 2006 found that the optimum temperature for uricase production as 30°C to 35ºC.

The influence of different carbon sources was studied and the results as follows: Sucrose>Glucose> Cellulose > >Starch> Maltose. In another study on the effect of carbon sources on the uricase production enzyme productivity decreased in the following order: pretreated cane molasses>sucrose> starch>glucose>citric acid and lactose. Cane molasses, a by-product of cane sugar industry, contains trace elements such as, potassium, sodium, calcium, zinc, iron and copper (Pazouki *et al.*, 2000) that may act as macro or micro nutrient for the bacterial strain and therefore encouraged the uricase production.

In various microorganisms, uricase synthesis seemed to be regulated by components of the growth medium and the ability to degrade uric acid and to use it for growth is an inducible property of these microorganisms (Vander Drift and Vogels, 1975). Moreover, it was suggested that uricase formation might be controlled by a repression in which a metabolite derived from both the nitrogen and carbon sources may participate (Bongaerts *et al.*, 1977).

In the present study nitrogen sources like beef extract, ammonium nitrate, yeast extract and peptone were examined in which peptone was found to be the most preferable followed by yeast extract, beef extract and ammonium nitrate. Nitrogen sources had a dramatic effect on the production of uricase of strain ZZJ4-1 as per another report. Several inorganic and organic nitrogen sources were evaluated. The results showed that maize milk was the best nitrogen source for uricase production (Zhou *et al.*, 2005).

Mass scale cultivation with optimized parameter was done in shake flask. The culture filtrate was partially purified using ammonium sulphate precipitation and subsequent membrane dialysis. Based on activity assay 60% ammonium sulphate was found to be effective. By optimizing the process parameters the highest level of uricase activity attained was 32U/ml/min in shake flask and after partially purification (membrane dialysis) 60.0U/ml/min was observed. This value was higher than other uricase bacterial strains reported (Abdel-Fattah *et al.*, 2005 and Zhou *et al.*, 2005).

The molecular size of the partially purified uricase from the strain *Bacillus subtilis* was analyzed using SDS-PAGE and it was 48kDa (Fig. 7). However in *Bacillus subtilis*, Pollyana *et al.*, 2010 reported a 60 kDa protein as uricase. Likewise Hunag and Wu (2004) isolated genomic DNA from *Bacillus subtilis*, cloned in *E.coli* and produced uricase of 98 kDa. The uricase from strain ZZJ4-1 as well as *Candida utilis* (Koyama *et al.*, 1996) were of 34 kDa. Ishikawa *et al.*, 2004 observed that the uricase of *Pseudomonas aeruginosa* was of 54kDa.

Uricase catalyzes the oxidative reaction that converts urate to allantoin, a more soluble and easily excreted compound. Due to this property urate oxidase may be used for both therapeutical and diagnostic purposes (Chohan and Becker, 2009). Many sources of urate oxidase are available, but commercial production may be hampered by low productivity and difficulties in protein purification for clinical applications (Chohan and Becker, 2009). In the present study *Bacillus subtilis* strain showed very high enzyme activity and it seems to be suitable for commercial exploitation.

Table -3: Uricase activity at different purification steps

S.No	Purification step	Uricase activity (U/ml/min)
1.	Cell free supernatant (Shake flask)	32.0
2.	Ammonium precipitation	45.0
3.	After membrane dialysis	60.0

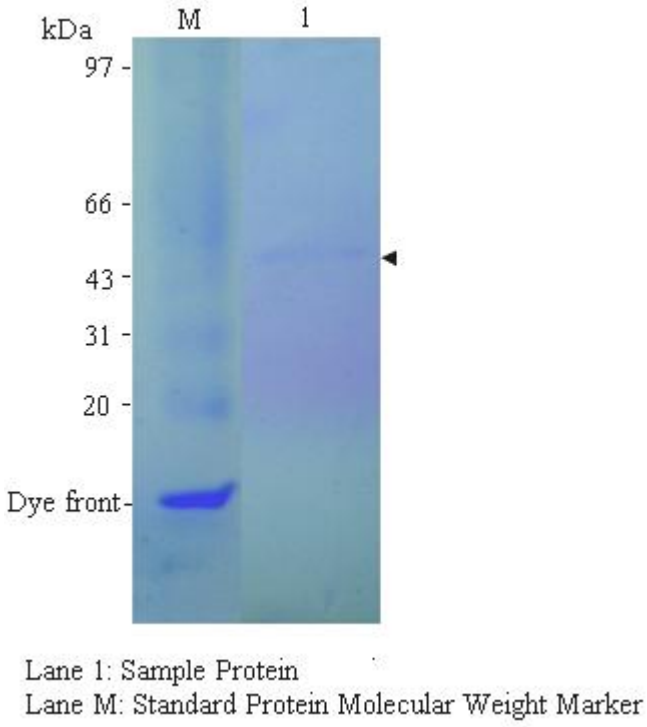

Fig. 7: SDS-PAGE analysis of partially purified uricase (48kDa) from *Bacillus subtilis*

Conclusion

The study proved that uricase producers are abundant in the soil sample collected from poultry farms and they harboured higher density of uricase producers. The isolate *Bacillus subtilis* was found to be an efficient producer of uricase enzyme.

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