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Isolation and production studies of Uricase producing bacterial strains from poultry waste

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***Abstract*— Urate oxidase or uricase is an enzyme that catalyses the oxidation of uric acid to allantoin and plays important role in purine metabolism.. The precipitation of uric acid can leading to gout symptom. Four uricase-producing bacterial isolates were obtained from poultry waste with a medium containing uric acid as the only inducer for the Uricase enzyme production. Based on its morphological and physiological characteristics, as well as 16S rRNA sequence and phylogenetic tree analysis, the most active bacterium was found *Comamonas testosteroni ATCC 11996(T)* .The main purpose of this work is to study the preliminary culture conditions for maximum uricase production by *Comamonas testosteroni ATCC 11996(T)*. The parameters studied were inducer concentration (0.5%-3%),the agitation rate(150 to 250 rpm),different carbon and nitrogen sources. The *Comamonas testosteroni* were inoculated in peptone media with sucrose and incubated for 48 hours at 150 rpm and 30°C. The maximum enzyme activity obtained from the experiment is 0.15 U/ml at pH 7, 250 rpm and cellulose as carbon and peptone as nitrogen sources.**

IndexTerms*——*Comamonas testosteroni ATCC 11996(T), gout, uric acid,uriacase

1.Introduction

Uricase (Urate oxidase E.C.1.7.3.3) is a therapeutic enzyme belonging to the class of oxido-reductases and catalyses the oxidation of uric acid to allantoin, carbon dioxide and hydrogen peroxide[1]It plays a vital role in the purine metabolic pathway. Uric acid is recognised as as antioxidant and it has been hypothesized to be an important antioxidant in the plasma. Allantoin the breakdown product of uric acid is five to ten times more soluble than uric acid and gets filtered more easily from the body through the kidneys[2]. Excessive concentration of uric acid in the blood (hyperuricemia) and in the urine (hyperuricosuria)can lead to painful arthritis (Gout) and renal failure . The normal concentration of uric acid in the blood serum of an adult male should be less than 7.0 mg/dL and that for female it would be less than 6.8mg/dL[3] . uric acid may have a pathogenic role in the development of hypertension, vascular disease, and renal disease.[4]. Uricase has many other applications including usage as a clinical reagent for the determination of blood and serum uric acid concentrations, as a uric acid biosensor in its immobilized form. Many organisms including animals, plants and microbes have the ability to produce uricase, but uricase producing microbial sources provide the advantages of higher growth rates, easy medium optimization and cost effective bio-processing. Since birds dropping are rich in uric acid, the ideal source for microbial isolation is the poultry droppings. More than half of the poultry dropping contains 63 to 87% of uric acid, which is the sole source of nitrogen for the aerobic bacterial action[5]. In this present study uricase producing bacterial strains was isolated from poultry waste, and have done the studies on operating conditions for the production of extra cellular uricase enzyme

2. Materials and Methods

2.1. Sample collection

The samples of deep litter and dropping from poultry farm at Manglore,India were collected and brought to the laboratory in sterile plastic bag and stored in refrigerator. Sample transferred to nutrient broth for the growth of micro organisms present in the sample. Nutrient broth was prepared according to the manufacturer's instructions by dissolving 2.8gm nutrient broth in 100mL of distilled water in 250mL conical flask.. The medium was sterilised by autoclaving at 121°C,15Psi for 15 minutes. After cooling 1gm of samples were added to the broth and the flask was

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incubated on a rotary incubator shaker having rpm of 150 for 48 h at 30°C.

2.2Isolation, Screening and Identification of Uricase producing bacteria

After 48 h of growth, one ml of turbid broth was inoculated in to a selective medium. The composition of the selective medium is as follows (g/L): Sucrose(20.0); Uric acid(2.0); di-Potassium hydrogenPhosphate(1.0); magnesium sulphate (0.5); ferrous chloride(0.01); sodium chloride(0.5).All the components was sterilized separately and mixed in the laminar airflow chamber. One hundred ppm of amphotericin (antifungal agent) was used to the selective medium to suppress the possible fungal growth. The pH of the medium was adjusted to 7, using 0.1 M NaOH solution. The cultures showing growth in 24 hours were transferred to fresh selective medium. After three consecutive transfers, 24 hour-old turbid broth was serially diluted and spread plated onnutrient agar plate having 1.5 g/L of uric acid and incubated at 35°C for 24 hours. The uric acid powder was separately sterilized in a testube and added to the moulted nutrient agar.After 24 hours of incubation individual colonies were obtained on the 10^{-7}dilution plate and were picked up based on the difference in colony morphology, colony margin, colour, shape and size.Discrete colonies showing clear zones in the vicinity of the colonies, due to production of extracellular uricase were picked up and preserved at 4°C on nutrient agar slants. Individual colonies were streaked for isolation on the uric acid agar plates and were subjected to gram staining procedure. Isolated strains were named as Small Rod, Large rod, Large diplo cocci, Small diplococci(SR,LR,LD,SD).First three bacterial colonies showed large clear zones ,which formed due to extra cellular uricase production in 24 hours, were preserved in 20% glycerol stock.

The isolated bacteria were identified by morphological and physical characteristics and finally using 16SrRNA gene sequencing approach. The isolated ,and screened bacterial cultures were sent for identification to Agharkar Research Institute ,Pune.

2.3 Inoculum Development and Production Medium

In order to induce the production of Uricase,the bacterial cells are allow to grow in a inoculums development medium. The composition of inoculums development media is as follows (g/L):Sucrose 20.0;Uric acid.1.0;di-hydrogen potassium phosphate,1.0;magnesiumsulphate,0.5;sodiumchloride,0.5;ferrous sulphate hepta hydrate,0.01.The standard solutions of 100ml were autoclaved separately and allow to cool down. Uric acid sterilised separately and added to the medium, was used as inducer and sole nitrogen source for uricase production. The pH of the medium was adjusted to 7 using NaOH. A loopful of bacterial strain small rod (SR),was transferred to 50mL of inoculum medium, and incubated on a rotary shaker at 30°C for 24 hours.24-hour old inoculum with an inoculums size of 2% added to 100mL production medium in 250mL conical flask. The production medium has following composition(g/L): peptone,10.0; Sucrose,20.0; di-hydrogen potassium phosphate,1.0; magnesiumsulphate,0.5; sodium chloride,0.5; ferrous sulphate hepta hydrate,0.01; and uric acid,1.5.Each components sterilised separately and mixed in laminar airflow chamber to obtain 100mL.The production medium was incubated on an orbital shaker of 150 rpm for 24 hours at 30°C.

2.4Time Course Studies of Uricase Production

5mL of samples was drawn at every four hours interval from the production media for the analysis of uricase activity and bacterial growth. Sampling was carried out till death phase reached. The samples collected were then centrifuged at 10,000 rpm for 10minutes.The supernatant was taken for crude enzyme analysis. The biomass of the bacterial cells was determined by measuring the absorbance of the broth at 660nm.

2.5Enzyme assay

The enzymatic assay was carried out by the method described by Mahler *et al*.[6] .3mL of 20 mM Boric Acid Buffer(pH 9.0), 0.1mL of 3.57 mM Uric Acid Solution and 0.1mL of crude enzyme was taken. Total protein estimation was carried out using Folin Lowry method.

2.6Effects of operating Parameters

The uricase producing microbes were allowed to grow in 100 ml production medium of pH 7 at 30ºC inside a conical flask of 250 ml capacity. Required temperature and suitable atmospheric conditions for microbial growth were generated by using incubator. As the microbes grow the pH of the culture medium was recorded by the pH meter . To determine the effect of agitation on the activity of the crude enzyme different rpm of 150,200,250 were used..Different carbon sources (glucose,maltose,lactose and carboxy methyl cellulose sodium salt) and different nitrogen sources (Yeast extract,casein hydrolysate,asparagines,beef extract and ammonium nitrate) were tested in order to determine their influences on the uricase activity. To determine optimal inducer concentration, the bacteria were cultured in production medium,with different concentrations of uric acid like 0,0.5,1,1.5,2 g/L.

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3. Results

The bacterial strains giving biggest zones of clearance within the first 24 hours of incubation were selected for further studies. The morphological and physiological characteristics of these strains are shown in Table1.The bacterial species small rod(SR) was observed to be Gram positive and grew with small,white,shiny colonies.It produced uricase with maximum activity and bigger zones of clearances on the plate and was identified by 16SrRNA gene sequencing procedure as shown in Fig1.The gene sequence of the identified bacterial species as reported by Agharkar Research Institute,Pune was*Comamonas testosteroni ATCC 11996(T)* (SR),*Bacillus flexus IFO 15715(T)(LR), Staphylococcus capitis (SD) Bacillus aryabhattai B8W22(T)*(LD).. The time course of uricase production was tested and found that the maximum activity of *Comamonas testosteroni ATCC 11996(T)*(SR)was 0.05 U/mL at 12 hour for the crude enzyme as shown in fig2. The pH of the medium increases as in fig7.Different agitation rate applied to find out maximum production of uricase enzyme,and found the production was high in 250 rpm as shown in fig3. The results showed that, the large amount of uricase (0.15U/ml) was produced in the medium containing cellulose.The amount of uricase produced by the usage of cellulose was higher than that used by the sucrose as shown in fig4. Different nitrogen sources used to determine the highest production of the enzyme.Sucrose was used as the carbon sources in the production media. It was observed that yeast extract contributed towards activity and biomass production. Yeast gave highest activityof0.058U/Ml as shown in fig5.Uric acid act as the inducer for the production for uricase enzyme. It was found that uric acid concentration of 2g/L gives higher activity compare to other concentration as in fig6. A control medium of 0g/L uric acid was also analysed, which gave less activity. From this its proved that uric acid act as inducer for the production of enzyme.

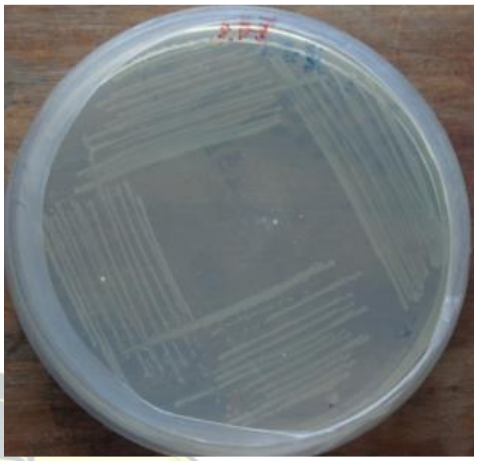

FIGURE 1

(1)

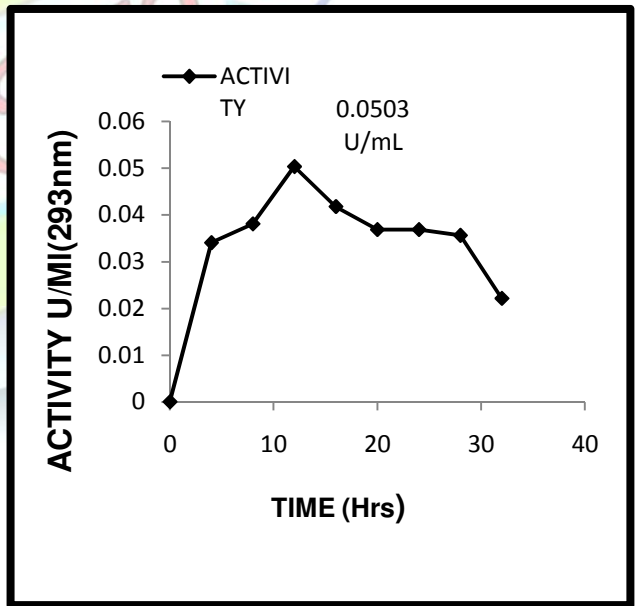

FIGURE2

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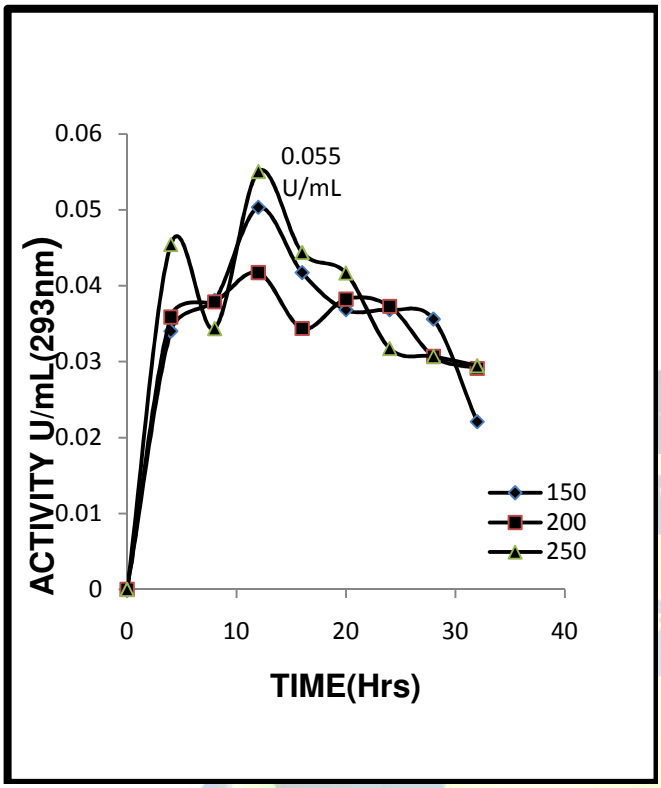

FIGURE 3

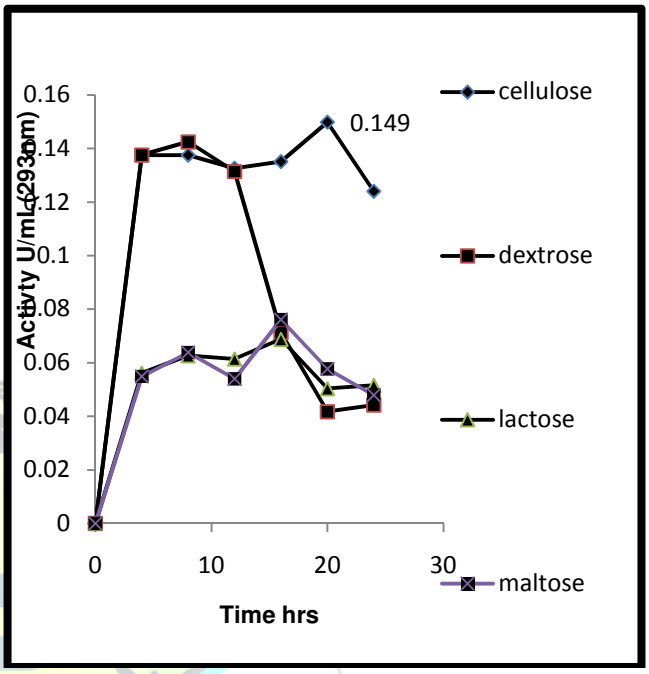

FIGURE4

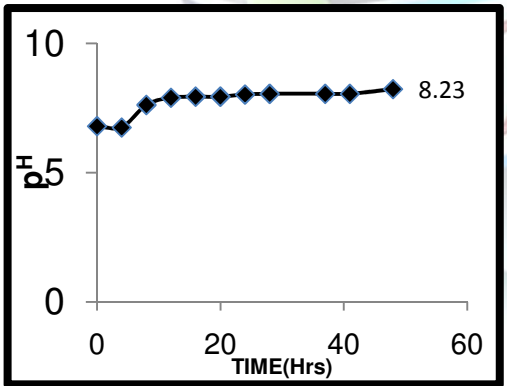

FIGURE7

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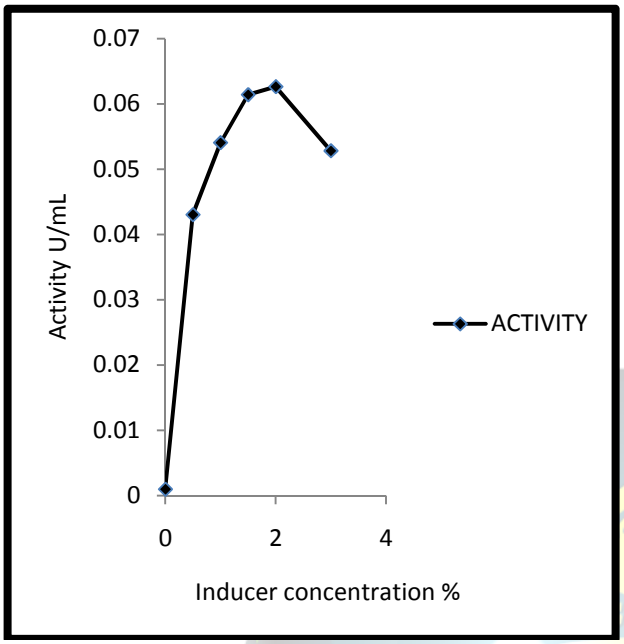

FIGURE6

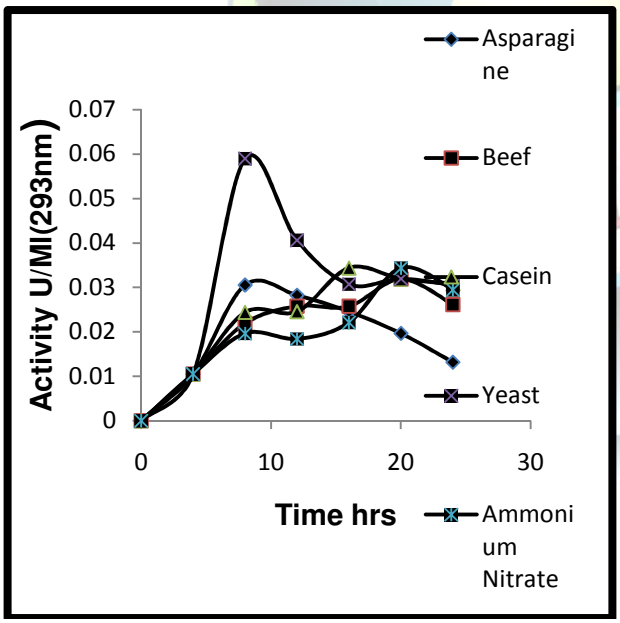

FIGURE5

4. Discussion

In this work four Uricase producing bacteria were isolated from poultry waste.Of which *Comamonas testosteroni ATCC 11996(T)*which identified through 16SrRNA gene sequencingshowed the maximum activity of 0.05 U/mL at 12 hour for the crude enzyme.*Comamonas testosteroni* is an aerobic, motile, non-spore-forming, medium-to-long gram-negative bacillus which occurs singly or in pairs .It is an environmental organism .The amount of uric acid which added initially dissolved completely.Further purification steps will enhance the production of the enzyme. The pH of the medium increases which indicates that uric acid which added as inducer was consumed by bacteria for the extra cellular production of uricase.[7]Magda ALY*et al*found that all uricase-producing bacteria were grown in liquid medium containing uric acid as a nitrogen source.*EAAzab etal[8] found uricacid was the most potent inducer.* M. A. Rouf et al[9]identified nine cultures of aerobic bacteria capable of growing on an elective medium containing uric acid as the only source of carbon, nitrogen, and energy.Maximum production of enzyme were found at 250rpm.Essam A. Azab*et al[8]*proved that uricase induction was markedly affected by agitation rate in both *P. vulgaris* strains and *Streptomyces*. Generally the amounts of induced uricase increased by aeration up to 180 rpm (*P. vulgaris* strains), 200 rpm (*Streptomyces graminofaciens*) and 220 rpm (*S. albidoflavus*), and then decreased.M. Siti Hatijah*et al[10]* found that 200rpm gave higher activity of enzyme followed by250,150,and 100rpm.They have conducted shake flask studeis. H. Ingesson*et al*[11] observed in a shake flask study that excessive mixing can deactivate the enzyme and reduce the conversion yield. It was found that the amount of uricase produced by the usage of cellulose was higher than that used by the sucrose.Dextrose. In the previous reports,the best carbon sources for uricase production of other uricase producing bacteria have been saccharose for *Microbacterium sp* and lactose for *Bacillus thermocatenulatus*. Pooja Nanda *et al*[12] reported that carboxy methyl cellulose gave highest activity of enzyme. Anderson Amirthanathan *et al[13]* reported that sucrose act as best carbon source for the production of highest active enzyme. Anderson Amirthanathan *et al[13]* showed that yeast extract proved to be the best nitrogen source for maximum uricase production ... Further optimization of various production factors and study of variations in pH,effect of various metal ions,effect of temperatures on uricase production are important.

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