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Isolation And Molecular Characterisation Of Uricase Producing Bacteria From Poultry Droppings Soil

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ABSTRACT

Uricase (urate oxidase, EC 1.7.3.3) is a crucial enzyme that plays an important role in purine metabolism by oxidising uric acid into more soluble and less toxic compounds allantoin, carbon dioxide, and hydrogen peroxide. It is found in many animals other than primates and is also produced by various microorganisms like bacteria, fungi, etc. Due to a genetic mutation that occurred during evolution, humans lack a functional uricase, which leads to diseases like gout and hyperuricemia in certain abnormal conditions. Because of this therapeutic challenge, the current study focused on the isolation and molecular characterisation of uricase-producing bacteria from poultry droppings soils. To isolate the extracellular uricase producers, the samples spread plated on 0.3% uric acid containing nutrient agar (UNA) media. Primary screening done based on relative enzyme activity (REA) on the UAA medium. Further screening done by uric acid assay (Caraway method) to select the potent isolate. The selected isolate was subjected to molecular characterisation by 16S rDNA gene sequencing and identified as *Pseudomonas aeruginosa* SSB with an enzyme activity of 0.08 micromole/minute.

Keywords: *Uricase, Gout, Uric acid nutrient agar*

1. INTRODUCTION

Enzymes are biocatalysts found in living beings catalyses, outperform their chemical counterparts since they are carried out with far higher fidelity, under mild reaction conditions, and with fewer steps (Meghwanshi *et al.,* 2020).Uric acid, or more accurately (at physiological pH levels), its monoanion urate, is a biologically inert end-product of purine metabolism in man and has shown to be a selective antioxidant, especially when reacting with hydroxyl radicals and hypochlorous acid, and it may also be transformed into harmless compounds such allantoin, allantoate, glyoxylate, urea, and oxalate. (Becker *et al.,* 1993).

Uricase, also known as urate oxidase (EC 1.7.3.3, UC), catalyzes the conversion of uric acid, a byproduct of purine catabolism, to allantoin, a molecule that is more soluble and excretable than the original substance (Schiavon *et al.,* 2000). This enzyme plays a crucial role in nitrogen metabolism across various organisms, including bacteria, fungi, plants, and animals. Humans lack uricase enzyme, despite it being extensively found in the majority of animals (Domagk *et al.,* 1968). Studies showed that the uricase gene was pseudogenized (silencing) in humans as a result of several mutations during the early stages of primate evolution (Hafez *et al.,* 2017, Pustake *et al.,* 2019, Ghasemian *et al.,* 2015).

Microbial uricase production has gained significant interest due to its potential for large-scale fermentation and biotechnological applications. Compared to plant and animal-derived uricase, microbial sources have advantages like rapid growth rates, ease of genetic manipulation, optimization of media components, and feasibility of large-scale enzyme production. (Thillainayagi *et al.,* 2021, Caskey, *et al.,* 1965). Uricase producing microorganisms inhabit diverse ecological niches, including soil, water, plant surfaces, animal gastrointestinal tracts, and decomposing organic matter. Poultry farm soil is an enriched source of uricase producing bacteria, as poultry waste contains high concentrations of nitrogenous compounds, including uric acid (Abdel-Fattah *et al.,* 2002). The diversity of uricase producing bacteria in poultry environments varies based on management practices, poultry species, and geographical factors. Some bacteria convert uric acid to ammonia, while others degrade it into urea (Schefferle *et al.,* 1965). Studies have shown that 63–87% of uric acid present in poultry faeces is metabolized by microbial communities in the litter under aerobic conditions underscoring the potential of this habitat as a reservoir for uricase producing microorganisms. (Caskey *et al.,* 1965, Tinsley *et al.,* 1959).

Bacteria isolated from environments rich in uric acid, such as poultry litter, have been identified as potent uricase producers. For instance, *Bacillus cereus* strain DL3, isolated from poultry sources, demonstrated notable extracellular uricase activity. (Nanda, P., & Jagadeesh Babu, P. E. 2013). Microorganisms such as *Pseudomonas* spp., *Bacillus* spp., *Micrococcus* spp., *Streptomyces* spp., *Candida* spp., and *Gliomastix gueg* have been reported to produce uricase (Khade *et al.,* 2016).

In humans, the absence of functional uricase sometimes leads to the accumulation of uric acid, which can result in the clinical condition hyperuricemia, associated with purine-rich diets, metabolic disorders, and renal dysfunction. Hyperuricemia contributes to the formation of urate crystals, leading to gout - an inflammatory arthritis predominantly affecting the joints (Anderson *et al.,* 2019). In this study, an attempt was made to isolate extracellular uricase producing bacteria from the premises of poultry dropping soils.

2. MATERIALS AND METHODS

2.1 Isolation of uricase producing bacteria

2.1.2 Sample collection

Soil samples were collected from the premises of different poultry farms in different localities of Malappuram district, Kerala. Samples were collected in sterile polythene bags, stored in cold conditions, and processed within 24 hrs of collection.

2.1.3 Primary isolation

10 g of the soil sample was suspended in 90 ml of sterile distilled water and shaken vigorously for 5 minutes in the shaker and rested for 10 min. The soil suspension was serially diluted and spread plated on nutrient agar plates containing 0.3 % uric acid (UNA) media that support the growth of uricase producing bacteria. Triplicates of plates were prepared and incubated at room temperature for 48-72 hrs. After incubation, the plates showing clear zones around the colonies were considered as potential uricase producers due to their ability to degrade uric acid.

Colonies showing prominent clear zones were sub cultured on fresh UNA plates to obtain pure cultures. Around thirteen bacterial isolates SA, SB, SC, SD, SE, SF, SG, SH, SI, SJ, SK, SL, and SN were obtained. The pure isolates were maintained on nutrient agar slants and stored at 4°C for further studies.

2.1.3.1 Spot inoculation

To confirm uricase production the primary isolates were further screened by spot inoculating into UAA medium (Uric acid 0.3%, K_2HPO_4 0.25%, KH_2PO_4 0.5%, agar 2%, salt solution 0.1ml in 100 ml) (salt solution: $MgSO_4.7H_2O$ 0.025g, $MnSO_4$ 0.17g, $FeSO_4$ 0.028g, NaCl 0.0006g, $CaCl_2.2H_2O$ 0.001g, $ZnSO_4$ 0.0006g in 1000 ml distilled water.) containing uric acid as the sole source of carbon and nitrogen. The plates were incubated at room temperature for 48-72 hrs, with clear zones around the colonies indicating the ability of the isolates to utilize uric acid. The zone diameter around colonies was measured and relative enzyme activities were calculated.

2.1.3.2 Conformation of extracellular nature of uricase

To confirm their extracellular nature, the selected primary isolates were inoculated into a 100 ml Uric acid broth (UAB) medium were uric acid as the sole source of carbon and Nitrogen. The inoculated broths were Incubated in a rotary shaker (Labline Orbital shaker) at 150 rpm for 48-72 hrs. After incubation, the UAB was centrifuged at 7000 rpm (Remi C24 plus) for 10 min.

The cell free supernatants were tested for extracellular uricase activity using the agar well diffusion method. Wells of 4 mm diameter were made on UAA plates using a sterile well borer.100 microlitre of supernatant was added aseptically into respective wells and incubated at room temperature for 24 hrs. A clear zone around the well indicates the extracellular nature of the enzyme.

2.1.4 Secondary screening

2.1.4.1 Quantitative uric acid assay

Secondary screening involved quantitative uric acid assay to measure uricase activity by the Caraway method (Henry *et al.,* 1974). Phosphotungstic acid in an alkaline medium oxidizes uric acid to allantoin. During this reaction, Phospho tungstic acid gets reduced to tungsten blue which is estimated colorimetrically at 710 nm.

The selected isolates were cultured in UAB, and the supernatants were mixed with the standard uric acid solution. After incubating for 1 hour, 0.6 ml sodium carbonate solution and phosphotungstic acid were added and incubated at 25^0C for 30 minutes. The OD was measured, and uricase activity was calculated. Among the isolates, strain SB exhibited the highest uricase activity and was selected for further studies.

2.2. Identification of the selected isolate

2.2.1. Morphological and Biochemical Characterisation

The selected isolate was subjected to gram staining, capsule staining, and spore staining. Colony morphology was studied based on size, shape, pigmentation, and other parameters. Biochemical tests including IMViC, Carbohydrate fermentation, oxidase, catalase, H_2S and urease tests were performed.

2.2.2. Molecular Characterization

The isolate was identified through molecular techniques, including PCR amplification and 16S rDNA gene sequencing. Genomic DNA was extracted using the NucleoSpin® Tissue Kit. PCR was performed using 16S rRNA-specific primers, and the amplified product was analyzed using agarose gel electrophoresis. Sequencing was carried out with the BigDye Terminator v3.1 Cycle Sequencing Kit.

2.2.3. Sequence Analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1

2.2.4. Phylogenetic tree construction

The sequence obtained after sequence analysis was used to carry out nucleotide BLAST analysis in the NCBI database. Multiple sequence alignment was performed using ClustalW and evolutionary history inferred by the neighbourhood - joining method. The phylogenetic tree was constructed using the MEGA X11 software tool.

3. RESULT AND DISCUSSION

3.1 Primary Screening

The enzyme uricase, plays a crucial role in catalysing the oxidation of uric acid to allantoin, an essential metabolic process in many organisms, including some bacteria, fungi, and animals. However, humans and other primates lack functional uricase due to evolutionary gene silencing, which has led to its exploration as a therapeutic agent, especially in managing hyperuricemia and gout.

3.1.1 Isolation of extracellular uricase producers

This study focuses on the successful isolation and screening of extracellular uricase producing bacteria from poultry droppings soil, an environment rich in uric acid. Most of the studies conducted so far were on intracellular uricases with a few exceptions like *Alcaligenes faecalis* and *Pseudomonas aeruginosa* which were found to be producing extracellular uricase enzyme. (Ajay Kumar *et al.,* 2016, Heshham *et al.,* 2004.)

3.1.2. Primary screening

The primary screening was done on 0.3% uric acid nutrient agar (UNA) media, which allowed for the growth of uricase-producing colonies. Out of 13 isolates, labelled SA to SN, exhibited significant uricase activity by forming clear zones around colonies by spot inoculation on UAA medium where uric acid was given as the sole source of carbon and nitrogen source. Previous findings from Ajay Kumar *et al., (*2016), isolated *Alcaligenes faecalis* from similar poultry farm environments and demonstrated its high uricase activity when cultured in uric acid-rich media. Here after primary screening, 9 isolates exhibited uricolytic activity on UAA medium (Table1 and Figure1).

Isolates	Diameter of clearance in mm	Colony diameter in mm	REA
A	8	5	1.6
B	18	6	3
C	13	6	2.2
D	1.0	5	2
E	8	5	1.6
F	13	6	2.2

G	13	7	2.6
I	8	5	1.6
J	17	8	2.1

Table 1: shows the relative enzyme activity of the selected isolate

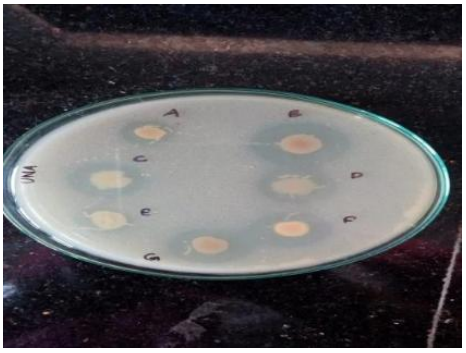

Figure 1: isolates showing uricolytic activity on UAA medium

3.1.3. Conformation of extracellular nature of enzyme

The extracellular nature of the 9 isolates were tested by the agar well diffusion method. Based on the extracellular nature of the enzyme, five isolates were selected for secondary screening. It has been reported that the different sources of microbial uricase such as *Nerosporacrassa, A.flavus, C.albicans, and Streptomyces cyanogenus* (Yokoyam *et al.,* 1998), *M.leutius* NRRL-B-8166, *M.roseus* NRRLB 1219 etc (Oliveri *et al.,* 1983, Snoke *et al.,* 1977) produce the intracellular enzyme. However, some microbial resources *Corynebacterium uratoxidans* and *Pseudomonas aeruginosa* produce extracellular uricase with no need for cell disruption to be reported. (Heshham *et al.,* 2004,). Another study of extracellular uricase producing bacteria, *Sphingobacterium thalpophilum* was isolated in Tamil Nadu, India (Ravichandran *et al.,* 2015).

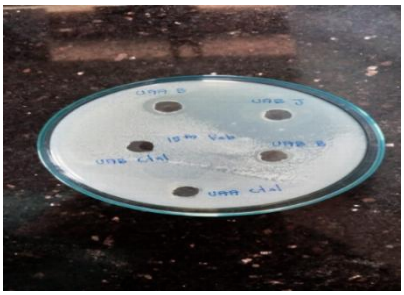

Figure 2: The extracellular uricolytic activity of cell free supernatant on UAA plate

3.2. Secondary screening

The isolates that showed extracellular enzyme activity were further subjected to Secondary screening by the Caraway method (Figure:3). Here, the isolate SB showed the highest activity of 0.08 micromol /min which indicates that isolate SB had the highest enzymatic activity which ensured the selection of potent strain among the other strains isolated and was further subjected to identification procedure. A previous study by Singh *et al.,* (2014) reported similar screening techniques for isolating *Bacillus cereus* strain DL3, achieving substantial uricolytic activity under optimized conditions. The colorimetric approach was used to detect the production of uricase from *Pseudomonas aeruginosa* isolates. (Suhad K *et al* .,2015)

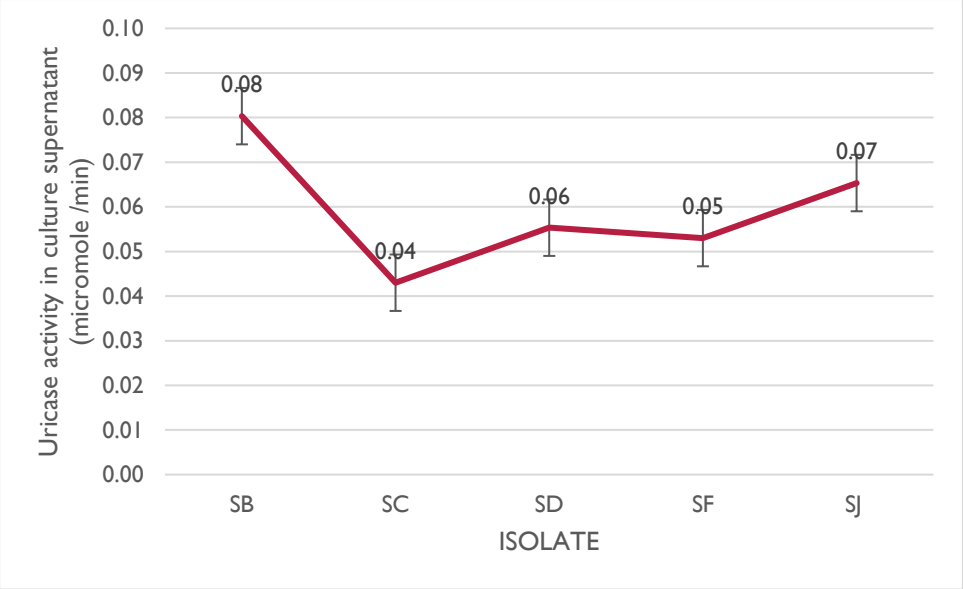

Figure 3: Uricolytic activity of cell free culture supernatant

3.3. Identification of the selected strain

3.3.1. Morphological and biochemical characterisation

Morphological analysis revealed that isolate SB formed small, round opaque colonies with smooth surfaces and slightly brown pigmented colon. Microscopic observations identified SB as Gram-negative rod and motile. Biochemical tests indicated positive results for catalase, oxidase, citrate, nitrate while indole, MR, VP, urease, H_2S and sugars fermentation (glucose, sucrose, mannitol, lactose) tests were negative.

Morphology	Rod
Endospore formation	-ve
Colony character	Small, round , smooth , opaque , centrally pointed colony
Pigment production	Slightly brown pigmented colony
Grams reaction	Gram negative
Capsule Staining	Non capsulated
Motility	motile
Catalase	+ve
Oxidase	+ve
Indole	-ve
MR	-ve
VP	-ve
Citrate	+ve
Nitrate	+ve
H_2S	-ve
Glucose	-ve

Lactose	-ve
Sucrose	-ve
Maltose	-ve
Mannitol	+ve
Urease	-ve

Table 2: shows morphological and biochemical characteristics of isolate SB

3.3.2. Molecular Identification and Phylogenetic Analysis

After various morphological and biochemical studies, the selected isolate was tentatively identified as *Pseudomonas* genus. The sequence obtained from 16S rDNA `sequencing showed 99.07 % sequence similarity with the strain of *Pseudomonas aeruginosa* SA3 *by* phylogenic analysis using MEGA X11software. (Figure 4). Based on the above phylogenetic analysis the selected isolate SB was identified as *Pseudomonas aeruginosa* strain. Thus, the isolate was further named *Pseudomonas aeruginosa SSB,* and the nucleotide sequence was deposited in GenBank with nucleotide sequence accession number PV163965. This methodology matches the approach used by Ahmed *et al.,* (2015), where 16S rDNA sequencing was employed to identify *Pseudomonas aeruginosa* as a potent uricase producer and Ajaykumar *et al.,* (2016), also used the method of procedure for the identification of *Alcaligenes faecalis* for isolation of extracellular uricase producers from poultry farms.

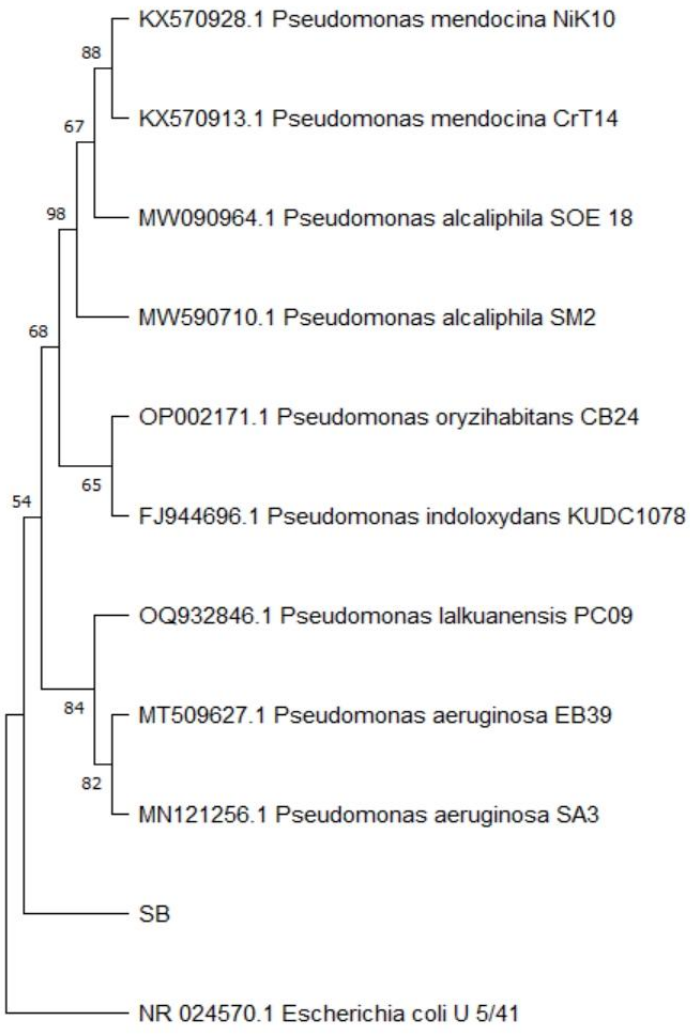

Figure :4 Phylogenetic tree of *Pseudomonas aeruginosa* SSB.

4. CONCLUSION

The present study focuses on extra cellular uricase producing bacteria from the premises of poultry droppings soil. The extra cellular nature of the enzyme was confirmed by agar well diffusion method. The potent strain was selected by secondary screening using uric acid assay method. Molecular studies identified the selected isolate as *Pseudomonas aeruginosa* with an enzyme activity of 0.08 micromole / min. Further studies on optimization and purification of the enzyme will be very useful since uricase is one of the important industrial and clinical enzyme. The enzyme has significant therapeutic applications, particularly in the estimation of serum uric acid, used in tumor lysis syndrome and managing gout disorders, and for Poultry waste management etc. This suggests microbial uricase has potential for both industrial and medicinal uses.

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