PRODUCTION, OPTIMIZATION AND THERAPEUTIC APPLICATIONS OF URICASE DERIVED FROM *BACILLUS SUBTILIS*

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DOCTOR OF PHILOSOPHY IN MICROBIOLOGY

Submitted by

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CERTIFICATE

This is to certify that the thesis entitled "**PRODUCTION, OPTIMIZATION AND THERAPEUTIC APPLICATIONS OF URICASE DERIVED FROM *BACILLUS SUBTILIS***" submitted by **Mrs. V. Manimekalai** for the degree of **Doctor of Philosophy in Microbiology** to Bharathidasan University, Tiruchirappalli is based on the results of studies carried out by her under my guidance and supervision. This thesis, or any part thereof, has not been submitted elsewhere for any other degree.

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DECLARATION

I hereby declare that the thesis entitled "**PRODUCTION, OPTIMIZATION AND THERAPEUTIC APPLICATIONS OF URICASE DERIVED FROM *BACILLUS SUBTILIS***" has been originally carried out by me under the guidance and supervision of **Dr. B. ANANDHARAJ, M.Sc., M.Phil., Ph.D.,** Research Department of Microbiology, M. R. Government Arts College, Mannargudi - 614 001, Thiruvarur Dt., Tamilnadu, India and this work has not been submitted elsewhere for any other degree, diploma or other similar titles.

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V. MANIMEKALAI

Dedicated to my Adorable Grandfather

Late. Mr. M. Ramalingam

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List of Abbreviation

BLAST	-	Basic Local Alignment Search Technique
BSA	-	Bovine Serum Albumin
CFU	-	Colony Forming Unit
DEAE	-	Diethylaminoethyl
DMSO	-	Dimethylsulfoxide
DNA	-	Deoxyribonucleic acid
dNTP	-	Deoxynucleotide Tri Phosphate
FASTA	-	fast-all/FastA
IL-10	-	Interleukin 10
MTT	-	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl 2H-tetrazolium bromide
NCBI	-	National Center for Biotechnology Information.
OD	-	Optical Density
PAGE	-	Poly Acrylamide Gel Electrophoresis
PBS	-	Phosphate Buffer Solution
PCR	-	Polymerised Chain Reaction
PEG	-	Poly Ethylene Glycol
RAW	-	Murine macrophage cell line
rpm	-	Revolutions Per Minute
rRNA	-	Ribosomal Ribonucleic acid
SDS	-	Sodium Dodecyl Sulphate
Sp.	-	Species
SUA	-	Serum Uric Acid
TCA	-	Tri Carboxylic Acid
TNF	-	Tumour Necrosis Factor
XO	-	Xanthine Oxidase

List of Symbols

°C	-	Centigrade
Df	-	Dilution factor
µl	-	Microlite
µg	-	Microgram
mM	-	Millimolar
ml/min	-	ml/min
%	-	Percentage.
pH	-	Potential of hydrogen,

Chapter – 1

INTRODUCTION

CHAPTER – 1

INTRODUCTION

Uricase as a therapeutic and diagnostic enzyme

Urate oxidase, also known as uricase (urate: oxygen oxidoreductase, EC 1.7.3.3), is a nitrogen-metabolizing enzyme which catalyses the oxidation process of uric acid to allantoin **(Wakamiya *et al.*, 1994).** Greater primates (apes and humans) expel uric acid as a derivative of purine degradation because they lack functional uricase **(Friedman *et al.*, 1985)**. Gout symptoms can occur in some people when uric acid crystallises.

This enzyme was first discovered in the kidney of a cow and is now found in nearly all vertebrates **(Schiavon *et al.*, 2000)**. Natural sources of uricase were discovered in bacteria **(Mansour *et al.*, 1996),** fungi **(Farley and Santosa, 2002)**, as well as eukaryotic cells **(Montalbini *et al.*, 1997).**

Uricase was first extracted from mammals, but more recently, microbial resources like fungai, bacteria and yeast were taken for a variety of reasons, including: (i). a quicker generating time. (ii). The ability to work with a variety of different substrates, including those that are less expensive. (iii). Technically, genetic manipulation is easier. (iv). They are easier to culture in fermentors under controlled circumstances since they are smaller. (v). Variations in the seasons can be managed (vi). There are no ethical issues (vii). Technical expertise and procedures developed in-house.

Uricase is a purine degradation enzyme that catalyses the oxidative disintegration of uric acid caused to allantoin. Uric acid, exsit in bodily fluids like blood and urine, is due to the end product of purine metabolisam. Also a sequence of medical illnesses had raised the amount uric acid in the biological fluids. These circumstances can lead to chronic renal disease, specific organic acidemias, and Lesch–Nyhan syndrome.

Several efforts have been made to develop uric acid sensors employing uricase as a biocatalyst **(Yutaka *et al.*, 1992).** The uricase molecule catalyses the in vivo oxidation of uric acid in the presence of oxygen, converting uric acid to allantoin and CO2, leaving hydrogen peroxide as the reduction product of O2. The vast majority of these enzymes are either thermally stable or active over a wide pH range. An isolated uricase from *Bacillus* sp. TB-90 was found to have high activity and thermal stability over a wide pH range (pH 6–9). As a result, more innovative sources with desirable characteristics are required.

The biological origin of impaired urate oxidase activity in humans as well as certain animals is unclear. Contrary to one perspective, this deficit has resulted in a net gain, because uric acid is potent antioxidant and free radical scavenger. A high serum uric acid level due to the lack of urate oxidase activity might have resulted to a lower cancer rate and a longer hominoid lifespan **(Friedman *et al.*, 1985).** Uricase, on the other hand, is necessary when the human body develops a hyperuricemic illness.

Uricase and hyperuricemia

Hyperuricemia seems to be a reason for various renal and cardiovascular disorders as well as an actual cause of gout and other associated diseases

(Hosoya *et al.*, 2011). As a result, reducing uric acid levels in the blood and tissues is necessary for preventing and treating a variety of uric acid-related disorders. Since the human body lacks the ability to produce urate oxidase on its own, controlling uric acid levels necessitates protracted or perhaps perpetual medication. However, numerous uric acid management medicines now inflict varying degrees of harm towards the human body, making them unsuitable for long-term usage.

In many people, gout is a painful condition characterised by hyperuricemia, recurrent acute arthritic episodes, sodium urate buildup throughout the joints, and the formation of uric acid gallstones **(Laemmli *et al.*, 1970)**. Allopurinol is a strong inhibitor of xanthine dehydrogenase, an enzyme that converts hypoxanthine to xanthine and xanthine to uric acid. It is frequently used to treat gout **(Massey *et al.*, 1970).** In the case of gout associated with renal complications, direct injection of urate oxidase allows for much faster absorption of urate nephrolithiases, and this is intended to mitigate or alleviate hyperuricemia abnormalities that may emerge while undergoing chemotherapy. Pegloticase is a recombinant uricase that could be used to lower uric acid levels in humans by catalysing uric acid oxidation.

Gout affects around two million people, with 75-90% of those affected being middle-aged males. Women usually acquire gout after menopause, presumably as a result of oestrogen deficiency. Gout is caused by a multitude of factors, including alcohol intake, a purine-rich nutrition, physical intimacy (men are more affected), genetic inheritance, some health complications such as hypertension, thyroid problems, skin problems, hemolytic anaemia, or even cancer, Kelley-Seegmiller Syndrome or Lesch-Nyhan Syndrome, lead exposure, obese and renal disease, as well as medications

such as cyclosporine, furosemide, hydrochlorothiazide, metolazone, levodopa, and aspirin. Symptomless hyperuricemia, acute gout, interim or intercritical gout, and chronically tophaceous gout are the four phases that gout can proceed through. The medications of choice include Allopurinol, Febuxostat, Pegloticase, and Probenecid. Apart from these four medications, there is no alternative treatment for hyperuricemia-related disorders. This circumstance necessitates more investigation into this crucial enzyme. When compared to allopurinol, uricase offers benefits in the treatment of gout. Uricase could also be used in diagnostic tool to predict urate enzymatically using the 4-aminoantipyrine peroxidase method **(Klose *et al.,* 1978).** Hyperuricemia and tumour lysis syndrome are significant side effects which can occur following hematologic cancer treatment.

Uricase enzyme therapy

Rasburicase (Fasturtec/Elitek) is a brand name for a clinically authorised urate oxidase from Aspergillus flavus produced in Saccharomyces cerevisiae. Rasburicase is a mono polypeptide chain of 301 amino acids that would be an effective and safe substitute to allopurinol for decreasing the levels of uric acid.

Allopurinol inhibits xanthine dehydrogenase, an enzyme that catalyses the change of hypoxanthine to xanthine and xanthine to uric acid. It is frequently used to treat gout **(Massey *et al.,* 1970)**. Uricase has been used as a diagnostic tool in therapeutics and clinical biochemistry to assess uric acid quantities in blood and other biological fluids **(Adamek *et al.,* 1989).**

1.1 Aim and objectives of the present study

Aim

- ✓ To identify uricase-producing bacterium from poultry waste soil and to analyse therapeutic application of uricase.

Objectives

- To isolate uricase producing bacteria from poultry fecal contaminated soil.
- To screen uricase producing bacteria by primary and secondary screening methods and determine its activity.
- To identify the bacteria by morphological, biochemical and molecular methods after selection.
- To optimize and purify uricase producing bacterial culture by physicochemical parameter and chromatography.
- To determine the molecular weight and characterize the physical and chemical characters of the enzyme.
- To evaluate the uricase enzyme potential in RAW 264.7 cell line.

Chapter – 2

REVIEW OF LITERATURE

CHAPTER – 2

REVIEW OF LITERATURE

2.1 Isolation, optimization, and characterization of uricase-producing microorganisms

Thillainayagi *et al.*, (2021) obtained uricase-producing *Alcaligenes faecalis* from the vicinity of poultry litter farms. They noticed that the enzyme was stable over a variety of pH and temperature ranges. They also employed poultry waste as a less expensive alternative substrate, which might greatly reduce the economic burden by lowering manufacturing costs and is a readily available substrate supply.

Pustake *et al.*, (2019) after extensive screening, used 16s rDNA sequencing to identify *Bacillus subtilis* strain SP6 as a powerful uricase producer. To increase the production of uricase from the recently discovered *Bacillus subtilis* strain SP6, response surface methodological optimization was used. In media optimization studies, Plackett Burman (PB) design was used to determine important media components, which were then further optimised using central composite design (CCD). Lactose, soya peptone, uric acid, and $Fe_{SO4}.7H_{2O}$ have all been identified as important factors in enzyme synthesis.

Shweta V. Pawar *et al.*, (2017) investigated a unique idea of *Bacillus licheniformis* coproducing uricase and alkaline protease in a single step utilising a single substrate. Uricase synthesis was tested in seven local bacterial strains, with *B. licheniformis* producing the most uricase and alkaline protease. The effects of several parameters on *B. licheniformis'* maximal enzyme coproduction are optimised.

Abbas (2016), researched the synthesis, extraction, and optimization of extracellular fungal uricase. Soil was used to isolate the fungal flora. The following optimization settings resulted in the highest specific activity value for uricase synthesis: (i.e.). Uric acid and 2% yeast extract media yielded 14.83 U/mg, lactose as a carbon source yielded 8.23 U/mg, uric acid as a nitrogen source produced 14.05 U/mg, NaH2PO4 as a phosphorus source produced 7.1 U/mg, and mangnous sulphate as an elemental source yielded 12.64 U/mg, ascorbic acid as a vitamin source yield.

Ravichandran *et al.*, (2015) identified *Sphingobacterium thalpophilum* (VITPCB5), extracellular uricase-producing bacteria from a duck farm's soil in Chidambaram, Tamil Nadu, India. Uric acid was utilised as a powerful inducer. Km and Vmax of the enzyme were found to be 0.28 mM and 0.92 M/min ml, respectively. The utmost uricase was formed by using lactose a carbon source. Urea produced the greatest uricase of all the nitrogen sources studied. The enzyme's ideal temperature and pH were found to be 25 - 45°C and pH 8. Cu^{2+} ions increased enzyme activity whereas Ca^{2+}, Fe^{2+}, Zn^{2+}, and Ni^{2+} ions hindered it slightly.

Akhtar *et al.*, (2014) investigated the synthesis and activity of catalase, pectinase, lipase, and urease enzymes by various *A. niger* isolates that were comparable morphologically but genetically distinct.

Hatijah *et al.*, (2013) optimised the growth conditions for *Aspergillus flavus* in order to maximise uricase production. At pH 6, 200 rpm, and 30 g/l sucrose content, the maximal enzyme activity measured was 0.03974 U/ml.

Nanda *et al.*, (2012) evaluated five distinct fungal strains from the *Gliocladium* and *Gliomastix* species for their ability to produce uricase, with *Gliocladium viride* MTCC 3835 producing highly active uricase. Statistical designs were used to improve

uricase production. Using the Plackett–Burman design, it was discovered that peptone, yeast extract, and $CuSO_4$ all have a significant effect on enzyme activity. Using the Box-Behnken design, the optimal concentrations of the following important ingredients were determined: peptone (12.71g/L), yeast extract (10.57g/L), and $CuSO_4$-0.0762g/L. The maximum activity measured in the laboratory was 84.92U/ml, which was 1.344 times greater than the activity measured in the basal medium.

Cheng *et al.*, (2012) engineered the urate oxidase gene into *Lactobacillus bulgaricus* and demonstrated that it synthesized urate oxidase to degrade uric acid. The molecular weight of the urate oxidase subunit produced by the genetically changed bacteria was around 34 kDa, and the in vitro enzymatic activity of the bacterial preparation was up to 0.33 U/mL, according to SDS-PAGE.

Anderson and Vijayakumar (2011) isolated and purified uricase from *Pseudomonas aeruginosa* using ammonium sulphate precipitation. The molecular weight of pure uricase, which was computed as KDa, was determined using SDS-PAGE. The influence of various metal ions with uricase activity, as well as the ideal temperature and pH, were examined. The results showed that uricase activity was 40.0 U/ml higher at 70% ammonium sulphate concentration than at other concentrations and in cell free supernatant. On the uricase enzyme, the effects of physical, chemical, and metal ions were studied.

Geweely and Nawar (2011) studied the synthesis, extraction, and purification of intracellular and extracellular uricase from fungal organisms. *Aspergillus niger*, *Rhizopus stolonifer*, *Penicillium chrysogenum*, and *Fusarium moniliforme* were isolated and uricase was synthesized under optimal circumstances. After the purification process three extracellular uricases namely UI, UII and UIII along with one

intracellular uricase UIV were recovered with specific activity of 105.9, 81.25, 101.96, and 9.66, respectively.

Neveen *et al.*, (2011) investigated the synthesis, extraction, and purification of fungal intracellular and extracellular uricase. Fungal organisms such as *Aspergillus niger*, *Rhizopus stolonifer*, *Penicillium chrysogenum*, and *Fusarium moniliforme* were isolated from soil. Optimization analysis revealed that 0.1 % uric acid and 0.2% sodium phosphate seem to be the most effective inducers of *A. niger* uricases.

Khucharoenphaisan and Sinma (2011) isolated an actinomycete strain known as PNR11 from termite guts and tested it for its ability to degrade uric acid. The strain was able to synthesise an intracellular uricase when grown in fermentation media containing uric acid as a nitrogen source. This strain was identified as Saccharopolyspora sp. based on morphological characteristics and 16S rDNA genome analysis.

Atalla *et al.*, (2009) investigated the production of uricase in 19 microbial strains. Gliomastix gueg was found to produce a large amount of the enzyme. The uric acid medium was found to be the most suitable, with additional advantages such as 30°C, pH-8, 150 rpm, and an incubation time of 8 days. Sucrose was discovered to be the best carbon source, while uric acid was discovered to be the best nitrogen source. When given dipotassium hydrogen phosphate and ferrous chloride, as well as certain vitamins, *Gliomastix gueg* produced the most uricase.

Yazdi *et al.*, (2006) isolated 165 strains of microorganisms from soil samples that could grow in a uric acid-containing medium.

Azab *et al.*, (2005) looked at uricase synthesis in *Proteus vulgaris* 1753 then B-317-C in addition to found that 37^{o}C was the optimal temperature for uricase induction.

*Streptomyces graminofacien*s and *Streptomyces albidoflavus* were 28°C, while *Saccharopolyspora sp*. PNR11 was 30°C. They discovered that *Proteus* and *Streptomyces* uricase could be precipitated from culture broth using 80% ammonium sulphate.

Abdel-Fattah *et al.,* (2005) used a sequential optimization technique based on statistical experimental design to boost the production of uricase from *Pseudomonas aeruginosa*. The addition of glucose to the basal media suppressed the synthesis of uricase. pH, $CuSO_4$, and $FeSO_4$ were shown to have a very significant influence among the 15 factors evaluated. To obtain the optimal process conditions, response surface approach was used. The optimal uricase activity was projected to be 7.1U/ml/min, which was 16.5 times greater than that of the baseline medium.

Zhou *et al.,* (2005) recovered uricase producing *Microbacterium* from soil samples collected in Hangzhou, Zhejiang Province, China. It was shown to be stable, and after half an hour of heat treatment at 70°C, the enzyme activity was restored to 100% of its original level. It remained stable for 40 days at a pH of 8.5 with a temperature of 37°C. Uric acid was revealed to be an uricase inducer. The strain produced a total volume of 1U/ml under ideal circumstances.

Ghasemi *et al.,* (2004) investigated the synthesis of urease in 13 *Aspergillus niger* strains, seven of which were obtained from soils in the Semnan area of Iran and six from the Persian Type Culture Collection (PTCC). In two immersed mediums, the production of enzyme was quantified. In submerged culture, the maximal enzyme production of the PTCC 5011 and S31 strains was 106 and 106 U.g-1dry mass, respectively. The latter was employed for mass

screening of urease production by filamentous fungi due to satisfactory correlations in between two procedures.

Saeed *et al.*, (2004) found that one subunit of the isolated uricase using a molecular weight of 68.0 kDa through exploiting SDS-PAGE analysis. The uricolytic activity of the purified enzyme peaked at pH 9.0. They also observed that the ideal temperature for pure uricase action was 35°C.

Fattah *et al.*, (2002) observed that *Aspergillus flavus* synthesized the maximum uricase in a sucrose-containing medium. After four days of incubation, they were able to produce uricase from *Aspergillus flavus*, *Aspergillus terreus*, and *Trichoderma* sp.

Zhu *et al.*, (2001) found that purified *Candida utilis* uricase enzyme was heat sensitive.

Ertan and Aksz (2000) looked at the production of uricase enzyme from *Aspergillus niger*, as well as the impact of different circumstances on its activity.

El-Dein and El-Fallal (1996) examined with the uricase activity of twenty-nine fungal species. Only twenty-two of them are capable of producing uricases. Basidiomycotina contains the majority of the fungi that are unable to synthesize uricases. *Aspergillus carbonarius*, *Botrytis fabae*, and *Aspergillus sydowii* produced the most uricase (0.16, 0.13, and 0.093 units/ml/min). Uricase activity was found in all of the farmed mushrooms examined. Among all the cultivated mushrooms examined, *Pleurotus sajor-caju* showed the greatest uricase activity. The mycelial dry weight and uricase production were shown to be unrelated in the examined fungi.

Ammar *et al.*, (1988) identified an Egyptian soil actinomycete named *Streptomyces albosriseolus* effectively synthesize uricase.

El-Desouky (1981) secluded and discovered uric acid degrading microorganisms using soil samples and examined their distribution as well as its uricolytic abilities. He believed that uricase might potentially be employed as a protein therapy to minimise toxic urate buildup.

Asano *et al.*, (1971) obtained uricase from thermophilic *Bacillus sp*. and it was the first experiment on heat stable uricase.

From pH 6–9, the enzyme was found to have great heat stability and activity. Uricase was synthesized by using bacteria like *Micrococcus* and *Brevebacterium.* **(Kida and Kunihisa, 1966)**, *Bacillus pasteurii* **(Cheristians and Kaltwasser, 1986),** *Proteus mirabilis* and *E. Coli* **(Nakagawa *et al.*, 1996)**, as well as actinomycetes such as *Streptomyces* (**Watanabe and Fukumoto, 1970 and Watanabe *et al.*, 1969**).

Uricase has been discovered in human, plants and microbial cells by **(Wu *et al.*, 1989)**, **(Suzuki and Verma, 1991**) and **(Yazdi *et al.*, 2006 and Lotfy, 2008).** Uricase is present in bacteria, yeast, and filamentous fungi, among other microbes.

Kaltwasser (1968 and 1969) observed an increase in uricase activity after transplanting *Alcaligenes eutrophus (Hydrogenomonas H16), Pseudomonas aeruginosa*, and *Micrococcus denitrificans* into uric acid-containing medium. *Bacillus fastidious* could only grow in media that contained uric acid, allantoin, or allantoate as the primary or sole carbon, nitrogen, and energy source.

2.2 Production and stability of uricase

Neda Motamedi *et al.*, (2021) discovered that immobilising *Aspergillus flavus* urate oxidase (Uox) on the surface of a Ni-based magnetic metal–organic framework (NimMOF) nanomaterial improved its enzyme activity. The physicochemical properties of NimMOF and its application as an enzyme stabilising support were investigated, and it was discovered that immobilisation on NimMOF Uox@NimMOF resulted in a significant improvement in its stability. While the free Uox enzyme lost almost all of its activity at 40–45°C, the immobilised Uox@NimMOF retained roughly 60% of its initial activity even at 70°C.

Neveen *et al.*, (2011) investigated the synthesis, extraction, and purification of intracellular and extracellular uricase in fungal organisms. *Aspergillus niger*, *Rhizopus stolonifer*, *Penicillium chrysogenum* and *Fusarium moniliforme* were isolated from soil and they were provided with various nutritional and physical parameters in the basal medium to maximize the synthesis of extracellular and intracellular uricases. *A. niger* uricase was found to be triggered by 0.1 % uric acid and 0.2 % sodium phosphate.

Tan *et al.*, (2010) used reverse phase evaporation to encapsulate uricase in lipid vesicles. At 4°C in a borate buffer with a pH of 8.5, the storage stability of uricase lipid vesicles was significantly higher than that of free uricase. At 55°C, thermostability has also been improved. An in vitro study discovered that uricase entrapped in lipid vesicles had greater uricolytic activity than native uricase.

Du *et al.*, (2010) reported that uricase is a putative thioredoxin target in the control of nodule growth. According to them, early communication between host and rhizobia during symbiotic nodule growth in legume roots was a significant factor for

the appropriate initiation of nodule morphogenesis, nitrogen fixation, and assimilation. Affinity chromatography and mass spectrophotometry were used to identify the thioredoxin target protein. The findings suggested that thioredoxin performs a new function in the control of enzyme activity in nodule growth and nitrogen fixation. Thioredoxine was discovered to be a target of noduline-35, a uricase subunit.

Leplatois *et al.*, (1992) found that uricase from *A. flavus* was expressed in both soluble and active forms in the yeast *Saccharomyces cerevisiae*. However, they claimed that in medical applications, recombinant uricase *after E. Coli* or *S. cerevisiae* however faced the difficult of poor efficiency and commercial separation **(Li *et al.*, 2006).**

2.3 Uric acid accumulation disorders and therapeutic approaches

Liming Cai *et al.*,(2020) reported a novel method for treating hyperuricemia that involves uricase released by intestinal bacteria expressing a recombinant uricase gene digesting uric acid. The uricase synthesized by uricase-expressing engineered bacteria, which were created using gene recombination technology, favoured the conversion of intestinal uric acid into allantoin, which not only prevented intestinal uric acid from being absorbed into the bloodstream but also accelerated the diffusion of SUA into the intestine, lowering SUA levels.

Sherman *et al.*, (2008) investigated the use of PEG-functional uricases in the treatment of treatment-resistant gout and hyperuricemia. The PEG conjugation of recombinant porcine-like uricase, which was shown to reduce plasma urate concentrations persistently in a phase II experiment, was discussed in this review. In addition to the primary end point of lower plasma urate concentration, this evaluation includes two additional phase III studies that included systematic assessments of gout symptoms, tophus resolution, and quality of life.

Johnson *et al.*, (2002) reported that if uric acid actually causes hypertension, there should be proof that allopurinol therapy may reduce blood pressure. They claim that they were unable to find any controlled research that looked at this potential. They believed that future research to determine if allopurinol can reduce blood pressure should be carried out with caution.

Mazzali *et al.*, (2001) hypothesised that allopurinol (a xanthine oxidase inhibitor) or benziodarone could reduce the rise in blood pressure caused by uric acid levels (a uricosuric agent). The increase in blood pressure in hyperuricemic rats was found to be partially mediated by activation of the rennin angiotensin system.

Distasio *et al.*, (1976) inferred the variations in hepatic urate oxidase activity were linked to uric acid levels in monkeys. Despite the fact that no studies have been conducted to determine the location of the mutations responsible for uricase loss in New World monkeys, they discovered that the mutations were likely self-reliant from those seen during hominoid evolution, which should be taken as proof that the Platyrrhini infra order (New World monkeys) diverged from the Catarrhini (Old World monkeys and hominoids) approximately 40 million years ago.

Alderman *et al.*, 1999 investigated while higher serum uric acid levels may have been beneficial for regulating blood pressure during low -salt dietary conditions, chronic salt sensitivity is expected to result in hypertension in modern civilization with its high-salt diet. It's worth noting that hyperuricemia is a strong predictor of ocular hypertension and has a strong link to cardiovascular disease.

Hochberg *et al.*, 1995 and Johnson *et al.*, 1999, reported men and postmenopausal women (since oestrogen is uricosuric), obese insulin-resistant individual people (because insulin may accelerate uric acid reabsorption), blacks, and

sick people with renal disease all had higher serum uric acid levels, (secondary to decreased excretion).

Wu *et al.*, (1992), inferred most mammals produce uric acid, which is then metabolised into allantoin by the hepatic enzyme uricase (urate oxidase), consequential in blood uric acid intensities ranging from 0.5 to 1.5 mg/dL. Ominoids (apes and humans), as well as some New World monkeys, have higher blood uric acid levels. Nonfunctional mutations in the uricase gene were found to be the cause of the uric acid increase. In humans, Chimpanzees (Pan), and Gorillas, a nonsense mutation in codon 33, a nonsense mutation in codon 187, and a splice mutation in exon 3 have all been discovered (Gorilla).

Harper (1977) discovered that uric acid is the most common purine catabolite in humans, making it a useful marker for purine metabolism disorders like gout, hyperuricemia, and the Lesch–Nyhan syndrome.

Wacker (1970) hypothesised that the urate oxidase found in Old World monkeys (Cercopithecoidea) was less stable than that seen in other mammalian (nonprimate) species. To some extent, the evolutionary processes that inhibited urate oxidase function may have also been active in this superfamily.

Because uric acid could act as an antioxidant, the majority of researchers believed that uricase genetic changes supplied an evolutionary advantage. Although the antioxidant function of uric acid can discuss some of the visible effects of the uricase mutation, a further possibility was that uric acid could regulate blood pressure. This assumption stemmed from past research, that also discovered that serum uric acid was connected to blood pressure and predicted the development of hypertension in population-based studies.

2.4 Gene responsible for uricase

Shaaban *et al.*, (2015) investigated the production of uricase in 127 clinical isolates of *Pseudomonas aeruginosa*. The greatest amount of natural uricase enzyme expression was seen in *Pseudomonas Ps43*. *Ps43* was used to amplify the uricase enzyme gene, which was then cloned into the expression vector pRSET-B and expressed in *E. Coli* BL21 (DE3). The nucleotide sequence of *Pseudomonas aeruginosa* PAO1's uricase gene puuD was determined to be comparable to the coding sequence of *Pseudomonas aeruginosa* PAO1's uricase gene puuD. In *E. Coli*, researchers discovered an induced protein with a molecular mass of 58 kDa.

Sankari *et al.*, (2015) used disc diffusion to establish the capacity of an *A. flavus* culture to thrive on uric acid, and molecular biology methods validated the presence of a urate oxidase gene.

Fazell *et al.*, (2014) expressed the *Aspergillus flavus* urate oxidase gene in *P. pastoris* after cloning it in the pPICZA expression vector. The secretory form of recombinant urate oxidase was validated by RT-PCR, SDS-PAGE analysis, and western blotting. A colorimetric test was used to evaluate enzyme activity. The production yield of culture supernatant was 0.43 U/ml.

Hesham *et al.*, (2004) for the first time isolated and characterized a *Pseudomonas aeruginosa* urate oxidase enzyme gene. They recovered and cloned a 1.350 kilobase pair DNA fragment expressing a potential urate oxidase gene from the genomic library of *Pseudomonas aeruginosa* Ps-x. The nucleotide sequence of the cloned DNA insert revealed an open reading frame encoding a 54.0 kDA protein. The cloned DNA fragment demonstrated uricolytic activity when transcribed in *E. Coli* DH5.

Koyama *et al.*, (1996) transcribed the uricase gene (UOXu) of *C. utilis* in *E. Coli*. The genetic factor had 909 origin pairs and no introns, and it encoded a protein with 303 amino acid deposits and a mass of 34,1463 Da.

Izuhara *et al.*, (1995) studied the uricase encoding gene in rat liver. The uricase-producing gene (UOX) was shown to have a higher mRNA level than genes encoding other peroxisomal enzymes including catalase, acyl-CoA oxidase, and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase. The enhancer activity of the isolated 5'- untranslated region of the gene was measured to see if cAMP was involved in the glucagon-induced transcription of rat UOX.

Christen *et al.*, (1971), the uricase gene was altered similarly in New World (*Ceboidea*) and Old World (*Cercopithecoidea*) monkeys. Some New World monkey species have higher serum uric acid levels, such as the tamarin (*Saguinus*), capuchin (*Cebus*), and woolly monkey (*Lagothrix*), while squirrel monkeys (*Saimiri*) and owl monkeys have lower levels (Aotus) uricase induction.

2.5 Induction of uricase

Lotfy *et al.*, (2008) explored that *Bacillus thermocatenulatus,* 0.3 % uric acid boosted uricase expression by 0.45U/ml.

Farley and Santosa (2002) inferred uricase mobility boosted 10 to 40 times beneath derepression and remained enhanced by exogenous uric acid (60 to 78 fold).

Tanaka *et al.*, (1977) found that, in additive to uric acid, xanthine, guanine, adenine, and hypoxanthine remained efficient in generating uricase in *Candida tropicalis*.

Watanabe and Fukumoto (1970) demonstrated that uricase could be induced in *Streptomyces* sp.

Fitzpatrick *et al.*, (1975) stated that uricase is an essential medicinal enzyme. They conducted cross-reaction investigations using enzyme preparations from three microbes such as *Candida utilis*, *A. flavus* and *Bacillus fastidious* and discovered that they were antigenically independent.

Rouf and Lomprey (1968) noted that the capacity of microorganisms to digest uric acid and utilise it for growth was thought to be an inducible trait in diverse bacteria. Decomposition of uric acid was discovered to be an induced feature in organisms such as *Klebseilla pneumonia*, *Pseudomonas aeruginosa*, and *Serratia marcescens* (MC75). Uricases of *Bacillus fastidious* **(Bongaerts and Vogels, 1976)** and *Aspergillus nidulans* **(Scazzocchio and Darlington 1968)** have been found to be triggered by uric acid.

2.6.Biosensor

Pan *et al.*, (2006) produced a uricase biosensor based on an o-aminophenol aniline co-polymer. The biosensor was analyzed through FTIR, UV-VIS, and SEM.

Zhang *et al.* (2004) designed a reagent less uric acid biosensor by arresting uricase on ZnO nanorods. Thermodynamic durability and direct electrochemistry were investigated. The sensor displayed thermal stability up to 85°C and electrocatalytic activity for uric acid oxidation in the absence of an electron mediator.

Zhang *et al.*, (1998) established a uric acid biosensor by immobilising uricase in a silk fibroin membrane. It was useful in identifying 60-70 human samples per hour and storing them for more than 2 years after being checked for biosamples over 1000 times.

Jinqing *et al.*, (1992) devised a polyaniline uricase electrode to evaluate uric acid levels in various samples.

2.7 Clinical analysis

Dwivedi *et al.*, (2012) established a new approach for screening microorganisms for the synthesis of uricase and estimating the amount of uricase produced. This was accomplished through using the datum that uric acid degrades when it is treated with uricase. The suggested approach was deemed to be a unique, low-cost, easy and sensitive strategy for uricase screening and estimation.

Dmytruk *et al.*, (2011) measured uric acid levels in human physiological fluids, which is important in the diagnosis also treatment of patients misery after a variety of illnesses associated through abnormal purine metabolism, maximum notably gout plus hyperuricasemia. The development of low-cost, dependable urate-selective amperometric biosensors stands a demanding task.

Chohan and Becker (2009) discovered by taking advantage of the fact that uric acid degrades when it is treated with uricase.

Bordelon *et al.*, 2006 published the 2.3°A resolution framework of the *Deinococcus radiodurans* R1 uricase regulator (HucR) in 2006. Because uric acid is a powerful scavenger of reactive oxygen species and *D. radiodurans* is known for its exceptional tolerance to DNA-damaging chemicals, they discovered a novel oxidative stress response mechanism.

Wilkinson and Grove (2004) examined the special effects of uric acid going on the transcriptional regulator HucR in *Deinococcus radiodurans*, concluding that uric acid regulates HucR to ensure normal cellular levels of this free radical scavenger in response to oxidative stress including DNA damage.

Huang *et al.*, (2004) proposed a modified colorimetric test for uricase activity using the uric acid/horseradish peroxidase/4- aminoantipyrine/3,5- dichloro-2-hydroxybenzene sulfonate colorimetric responses of uricase on adjustable 96-well microtiter plates.

Marinello *et al.*, (2002) investigated uric acid and allontoin were labelled with 14C-formate to study purine nucleotide breakdown in rat liver. The radioactivity incorporation into uric acid and allontoin did not go as planned.

Zhu et al. (2001) employed urate oxidase obtained via cloning for serum uric acid analysis, **Schiavon *et al.*, (2000)** used linear PEG, branched PEG, and poly N- acryloylmorpholine to covalently link the uricase isolated from *Bacillus fastidiosus*. Minor modifications in the inhibitory constant, ideal pH, temperature resistance, and affinity for substrate were identified in Uc conjugates, as well as greater resistance to proteolytic digestion. The results suggested that PAcM, a less well-known amphiphilic polymer, may be used instead of PEGs to modify enzymes for treatment purposes.

Itiaba *et al.*, (1975) stated that uricase is critical for determining uric acid in biofluids. They discovered that the reaction is not only distinct, but it can also be detected at 292 nm, 340 nm, or colorimetrically via associated chromogenic response. They also discovered that turbidity and the presence of aspirin, ascorbic acid, glutathione, paracetamol, and several antibiotics interfered with non-enzymatic techniques.

Gochman and Schmitz (1971) uttered that uricase is beneficial for enzymatic assessment about urate in diagnostic purposes when combined with a 4- aminoantipyrineperoxidase system. As a copper protein, this enzyme remained

assumed near a tetramer consisting of two distinct subunits with a total molecular mass in 145–150 kDa range. According to the cDNA sequence, the subunit size was 35 kDa. Uricase can also be utilised as a protein medicine to minimise excessive urate buildup (**Nishiya *et al.*, 2002**).

To determine uric acid in biofluids, uricase from diverse sources was immobilised on dextran and polyethylene glycol **(Yasuda *et al.*, 1990),** nylon tubing polyamide tubing **(Werner *et al.*, 1979**), elastin (**McCarthy and Johnson, 1977)**, cellulose acetate membrane.

2.8 Application of Uricase

Abdullah *et al.*, (2015) studied the in vivo impact of isolated uricase from *Pseudomonas aeruginosa* on hyperuricemia. The purified uricase did not produce hemolysis or erythrocyte agglutination when tested at 37 °C, according to the findings of this investigation. Also, after 1 to 2 hours of incubation in the presence of plasma, the enzyme sustained around 90-95% of its initial activity, respectively. However after 6 hours of incubation in the presence of plasma, only about 40% of the activity was sustained. In an in vivo research, uricase was found to have the ability to reduce plasma uric acid levels from a high concentration (298.70 34.91mol/L) to a low concentration (132.60 14.39mol/L) in less than an hour.

Lin *et al.*, (2012) investigated the prevalence of hyperuricemia and its association with antihypertensive medication. In Taiwan, they discovered that diuretic users had significantly higher blood uric acid and serum ureatine levels than non-users. Hyperuricemia was found in 44% of the those given thiazides, 56% of those given loop diuretics, and 57% of those given aldosterone receptor blockers. They were unable to

determine whether diuretics cause kidney problems by increasing the blood flow uric acid levels..

Hink *et al.,* (2002) discovered that uric acid has a function in modifying in vivo activity, leading in the antioxidant property of extracellular superoxide dismutase.

Kean *et al.,* (2000) investigated the anti-inflammatory impact of uric acid in experimental allergic encephalanyelitis.

Pui *et al.,* (1997) investigated the use of uricase produced from *Aspergillus flavus* in the treatment of malignant hyperuricemia during chemotherapy. However, they believe that the utility of the fungal enzyme for chronic gout treatment may be restricted due to its quick elimination, and that it may require daily infusion. The creation of immunogenicity has also been identified as a restriction.

O'Neill and Lowry (1995) used in vivo voltammetry and microdialysis to quantify the amount of uric acid in extracellular fluid, and data was compared for probes of various sizes from the day of implantation over several days following surgery.

Rao et al. (1991) also demonstrated that Uric acid and allontoin were labelled with 14C-formate to study purine nucleotide breakdown in rat liver. The radioactivity incorporation into uric acid and allontoin did not go as planned. They also discovered that uric acid-induced cyclooxygenase-2 (COX-2) expression was linked to smooth muscle cell proliferation via the production of thromboxane.

Chapter – 3

MATERIALS AND METHODS

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3.1 Sample collection

Four different soil models were procured with diverse habitats, including bird faecal contaminated soil (pigeon, turkey, parrot, and chicken farms) in Hosur, Krishnagiri District, Tamilnadu. A 5cm depth soil samples were scooped aseptically into sterile zip lock plastic bags using a sterile disposable spatula.

3.2 Isolation of uricase producing bacteria

In 9 ml of sterile double distilled water, one gram of soil sample was suspended. It was diluted in 9 ml of sterile distilled water and labelled as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and so on. The dilutions of 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} were chosen for spread plating, so as to isolate uricase producing bacterial isolates.

3.3 Qualitative assay of uricase by plate assay method

3.3.1 Primary screening

The primary screening for uricase activity was done by using plate assay method, by inoculating 0.1 ml of the dilution from various dilutions onto agar plates containing uric acid - 0.8%, dipotassium hydrogen orthophosphate - 0.2 %, potassium dihydrogen orthophosphate - 0.05 %, magnesium sulphate - 0.01 %, sodium chloride - 0.01 %, calcium chloride - 0.01 %, agar - 2.0 %, distilled water - 100 ml, pH - 7.0 **Xue-lai Zhou *et al.*, (2005)**. The media was autoclaved at 121°C for 15 minutes to sterilise it. The inoculated plates were incubated for 72 hours at 37°C. The presence of clear zones encircling the colonies indicates that uricase enzyme is being produced.

3.3.2 Secondary screening

The isolate that showed a clear zone around the colonies were selected and re- inoculated on to fresh uric acid medium to ensure enrichment of uricase production by the isolate. The isolates that displayed clear zones were determined to be uricase positive.

3.4 Quantitative assay of uricase by submerged fermentation process

Individual isolates with the highest zone of clearance were grown in 20 ml Uric acid medium [composition: uric acid - 0.8%, Dipotassium hydrogen orthophosphate - 0.2%, potassium dihydrogen orthophosphate - 0.05%, magnesium sulphate - 0.01%, sodium chloride - 0.01%, calcium chloride - 0.01%, distilled water - 100ml and pH adjusted to 7.0] and incubated at 37^{o}C for 24 hours for the preparation of standard inoculum. Inoculums for the fermentation medium were made from this. The fermentation media was identical to the inoculum medium in terms of composition. Fermentation was done in 250 ml Erlenmeyer flasks with 100 ml sterile uric acid medium and 5% standard inoculums. The flasks were shaken for 48 hours at 150 RPM at 37^{o}C in a rotary shaker **Mona *et al.*, (2015)**.

3.5 Preparation of crude enzyme

The cultures were centrifuged for 20 minutes at 1600 RPM at 4°C after incubation, and the supernatant was used as a source of crude enzyme. The crude enzyme solution was used to measure the enzyme activity **Atty, F. K., *et al.*, (2016).**

3.6 Uricase enzyme assay

To begin, a 3ml quartz cuvette was filled with 2.5ml of 0.001% uric acid solution and 0.1mol/L of boric acid buffer solution (pH 8.5). Then, about 0.5 ml

solution diluted enzyme was added and stirred. Then, at 293 nm and 25°C, the absorbance variations were continually monitored, and the activity was calculated using the method below. Activity (μ/ml) = (ΔOD293 nm × df × 0.5)

The enzyme dilution factor is denoted by df, the absorbance change is denoted by OD293 nm, and the time in minutes is indicated by t. The quantity of enzyme required to catalyze 1 mol about uric acid oxidation each minute at 25°C and pH 8.5 was defined as an enzyme activity unit **Cheng *et al.*, (2012**).

3.7 Estimation of protein

The protein concentration of the uricase enzyme was ascertained utilising UV absorption coefficient at 660 nm and BSA as a standard, and the protein content was determined by calculating gravimetrically using the standard curve **Lowry *et al.*, (1951).**

3.8 Identification of bacteria

3.8.1 Morphological and physiological characterization

Based on conventional, morphological, biochemical and physiological characterization the isolates of bacteria were identified. Under the morphological characteristics, shape, size, margin, elevation, opacity, texture and pigmentation, gram's reaction was analyzed while in physiological characterization, culture growth under different temperatures and pH was studied as recommended by Bergey's Manual of determinative bacteriology and by **Bergey et al., 2000.**

3.8.2 Molecular identification of *Bacillus* sp.

The DNA from *Bacillus* sp. isolate was isolated as recommended by **Wright *et al.*, (2017)** was subjected to PCR so as to amplify the fragment region of the DNA.

3.8.3 PCR amplification

During PCR, in a 0.5 ml PCR tube, 10 µl of each downstream primer (5‟- AGAGTTTGATCCTGGCTCAG3‟), upstream primer (5‟-GGGCTACCTTGTT ACGACTT 3"), 10 µl 10X PCR buffer, 25mM $MgCl_2$ 8 µl, dNTP mix 6 µl, template DNA (50 ng), 5 µl and Taq DNA polymerase (3U/µl), sterile distilled deionized water 49 µl was taken. The total 100 µl mixture in a tube was gently spun for 10 seconds and allowed to settle the contents. The total mixture was kept in PCR thermal cycler. The amplification was done in 35 cycles, (denaturation for 60 seconds at 92°C, primer annealing for 60 seconds at 54°C and polymerization for 90 seconds at 72°C), after polymerization 10 µl of PCR products with 2 µl of loading dye was mixed and loaded on a 1.5% agarose gel and analysed electrophoretically at 60 volts for 45 minutes. The gel was visualized on UV - transilluminator and analysed with 1kb DNA ladder. In a 1% agarose gel the PCR products along with 5 µl of DNA molecular weight marker was electrophoresed at 40 mA and were eluted.

3.8.4 NCBI-BLAST for sequences

The PCR product was then sequenced using an automated sanger sequence technique with the study's PCR primers. The same primers as previously reported were used for sequencing. The isolated *Bacillus* strain's sequence similarity to the study's reference strain was also compared using NCBI-BLAST **www.ncbi.nlm.nih.gov/blast.** The test *Bacillus* 16S rRNA sequence was assigned a sequence accession number by NCBI. In this study, the NCBI BLAST was used to generate the phylogenetic tree from the bootstrapped data set.

3.9 Preliminary growth parameters optimization

To evaluate the basic optimal growth parameters required for the better growth and synthesis of uricase were studied as follows **Tork, S.E., *et al.*, (2018).** The one-factor- at-a-time method was applied for each factor such as pH 5-9, temperature 30-70˚C, 2% of carbon source: glucose, sucrose, maltose, fructose, and lactose, 1% about nitrogen source: yeast extract, peptone, ammonium nitrate, ammonium chloride, and casein, the various concentration of uric acid (0.12, 0.22, 0.32, 0.42, and 0.52%), and 5% of natural uric acid enriched organic sources (dried and sterilized powdered form of wheat bran, beans, cauliflower, and pigeon fecal) with standard growth conditions in 100 ml of uric acid broth medium containing 250 ml conical flask individually. About 5 ml (%) of 24 hours old *Bacillus subtilis* MM13 (OD_{600}: 1.5) was inoculated on each flask and incubated along a shaker incubator with 150 rpm with a duration about 48 hours. The standard uricase assay activity was performed after incubation of each growth parameter **Aly *et al.*, (2013).**

3.10 Secondary growth parameters optimization

The preliminary growth parameters optimization studies results revealed that the *Bacillus subtilis* MM13 showed excellent uricase enzyme activity at pH 7.0, 40˚C, sucrose, yeast extract, and 0.32% of uric acid. Hence, in this secondary optimization study, the concentrations of sucrose (0.25, 0.5, 0.75, 1.0, and 1.5%) and yeast extract (0.2, 0.3, 0.4, 0.5, and 0.6%) required for attaining maximum yield from *Bacillus subtilis* MM13 were optimized through submerged fermentation process one-factor-at-a-time approach in 100 ml of uric acid broth in 250 ml of the conical flask. The culture inoculated flask was incubated through a shaker incubator by 150 rpm for

48 hours, and a uricase assay was performed to calculate the uricase activity **(Kotb E., 2016).**

3.11 Extraction and purification of uricase enzyme

Bacillus subtilis was cultivated in 1 litter of uric acid medium at 37°C for 18 hours with a rotator at 200 rpm until late exponential phase. After that, the culture was collected by spinning it at 8000 rpm for 20 minutes at 4°C and thoroughly rinsing it with 50 mM phosphate buffer (pH 7.5). At 4°C, the bacteria were well mixed in 0.1 M (pH 7.0) phosphate buffer and disturbed only with an ultrasonic wave set to 4°C (5 % amplitude). **Ghosh, T., *et al., (*2014)**. The uricase enzyme was then isolated and purified using ammonium sulphate precipitation followed by dialysis. Solid (80%) ammonium sulphate was used to completely saturate the extract. The reaction mixer was gently stirred on an ice bath after adding solid ammonium sulphate, and the blend remained at 4°C around 24 hours before being centrifuged at 12000 rpm for 30 minutes by 4°C. The pellets were dispersed available 5ml as to 0.02M Tris-HCl pH 8.5.The precipitate obtained from the saturation of 80% fraction was spun as well as the sediment was collected for supplementary evaluation. The collected enzyme mix was dispersed in 2:1 ratio of a buffer and enzyme, transmitted to a column dialysis, and dialyzed against sodium phosphate buffer (1X PBS), as well as vortexed with a magnetic stirrer overnight at 4^{o}C. Moreover, to achieve proper purification, the phosphate buffer was exchanged 3 times at the dialysis. The purified enzyme was then saved for further examination.

3.11.1 Purification of uricase using ion exchange chromatography on DEAE-cellulose

A 2.5x7cm column of DEAE-cellulose was packed **Ghosh, T., *et al.*, (2014).** The dialyzed cell supernatant was added to a column that had been pre-stabilized with

20mM Tris-HCl before use (pH 8.5). The column was cleansed three times with three beds of 20mM Tris-HCl pH 8.5 at a flow rate of 60 ml/h, and the bound enzymes (proteins) were eluted in the same buffer via a linear NaCl gradient (0.75M). The accumulated fraction contained 5 ml, and their optical density was measured at 280 nm, as well as uricase activity for an active fraction. To concentrate the fraction containing uricase enzyme activity, polyethylene glycol was utilised.

3.11.2 Purification of uricase by sephadex G-50 column

DEAE-concentrated cellulose's and active fraction was loaded onto a 1.5x45cm sephadex A-50 column that had been stabilised with 0.02M Tris-HCl pH 8.5. **An, *et al.*, (2017).** Slow flow rates (0.6ml/min) were used to permit the ideal separation of enzyme from other proteins. Polyethylene glycol was used to concentrate uricase activity-containing fraction.

3.11.3 Molecular mass determination

SDS-PAGE with 10% polyacrylamide gels had been used to determine the uricase's relative molecular weight (MW) **Kai, L., *et al.*, (2008).** Protein size was calculated by comparing them to known MW marker proteins. Samples of pure enzyme fractions of 0.8 mg/ml and 0.6 mg/ml of DEAE sephadex A-50 column purified uricase enzyme solution were placed in the well and run for 45 minutes at a fixed voltage of 150 V. After 1 hour of staining with Pierce Imperial^{TM} Protein Stain, the protein was de-stained in distilled water overnight.

3.11.4 Measurement of enzyme activity

Purified 0.1ml of uricase was mixed with 0.6ml of 0.1M sodium borate buffer (pH 8.5) containing 2mM uric acid, 0.15 ml of 30 mM 4-aminoantipyrine,

0.1ml of 1.5% phenol, and 0.05 ml of peroxidase (15U/ml) for 20 minutes at 37^{o}C. **Kai, L., *et al.*, (2008)**. By adding 1.0 ml of ethanol to the reaction, it was slowed down, the absorbance at 540 nm was then measured against a blank using a spectrophotometer. One unit of enzyme occurs expressed as the quantity of enzyme capable of generating 1.0 mmol of H_2O_2 per minute under the conditions evaluated.

3.12 Enzyme characterization

3.12.1 Temperature effects on uricase activity and stability

The purified enzymatic reaction blend was incubated for 30 minutes at different temperatures (25, 35, 45, 55, and 65°C) to investigate the effect of temperature (°C) on uricase activity **D. da Silva Freitas., *et al.*, (2010).**

3.12.2 pH effects on uricase activity and stability

The enzyme was tested at various pH levels to see how pH affects uricase enzymatic activity: 5.0, 6.0, 7.0, 8.0, 9.0, and 10. For pH values 6.0–8.0, phosphate buffer was utilised, and for pH values 8.0–10.0, borate buffer was employed. Furthermore, to investigate the impact of pH on uricase consistency (stability), 0.1ml of uricase was incubated for 30 minutes with the same amount of various buffer solutions at different pH values: 6.0, 7.0, 8.0, 9.0, and 10.0, after which enzyme activity was measured at the ideal temperature and optimum pH value as previously described.

3.12.3 Metal ion effects on uricase activity and stability

Following established techniques, the effects of metal ions (Co^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, and Zn^{2+}) on uricase activity were investigated. Metal ions remained mixed to the solution at a concentration of 1 mM. The uricase was properly mixed with metal ion solutions before incubation at 37°C for 20 minutes. An enzyme mix devoid of

metal ions was employed as a control. After the incubation time, the activity of uricase was compared to a control (without metal ions).

3.13 Cell culture

ATCC's cell repository was used to obtain RAW 264.7 cells. These cell lines are developed in 1X Dulbecco's Modified Eagle's Medium (DMEM) augmented with 10% FBS plus 1% penicillin/streptomycin (PenStrep) and kept by 37°C now a humidified environment per 5% CO_2. Cells were passaged and used for future experiments in an exponential development stage until they were confluent (80% confluency).

3.13.1 Cytotoxicity evaluation

The effects of uricase were assessed using a modified MTT test **Sylvester, P.W., (2011),** which involves the reduction of 3-(4,5-dimethylthizaol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan in the presence of mitochondria. RAW 264.7 After being stimulated with LPS (500 ng/ml), cells (5104 cells/well) were grown in 96-well plates and treated with uricase at 10 different concentrations (10 to 100 µg/ml). After 24 hours, the cells were treated with 15 µl of MTT solution (0.5 mg/ml) and incubated at 37^oC for 4 hours. The cells were given 100 µl of DMSO to dissolve the for mazan after discarding the supernatant. After stirring up the plates for 10 minutes at room temperature, the absorbances was measured but use a microplate reader at wavelengths of 490 nm and 630 nm. The control group, which involved of unprocessed cells, was assumed to be 100% viable. The inhibition rate was calculated using the formula below.:

$$\% \text{ Inhibition Rate} = \{1- [A_{490} - A_{630} (\text{Treated}) / A_{490} - A_{630} (\text{control})] \times 100\}$$

3.13.2. Trypan blue exclusion assay

The dye exclusion test is exploited to establish the amount of viable cells in a cell suspension. It is formed on the assumption that living cells must intact cell membranes. The trypan blue exclusion test was used to observe into the outcome of uricase on LPS-induced RAW264.7 cells **(Crowley *et al.*, 2016).** Cells were seeded in 6-well plates, stimulated with LPS, then treated for 24 hours (5% CO_2, 37°C) with 10 different doses of uricase (10 to 100 µg/ml). The cells were trypsinized and resuspended in a mixture of culture media and trypan blue dye (5 mg/ml). The numbers of viable (unstained) and nonviable (blue-stained) cells were counted using a hemocytometer, and the results are shown below.

$$\text{The average number of cells per field} = \frac{\text{Sum of cells per field}}{\text{Number of fields}}$$

Total number of cells per ml ($x* 10^4$/ml) = Average number of cells per field × df

(The cells were previously diluted with equal quantities of trypan blue, therefore the dilution factor is 2).

%Viability = Number of colourless cells/Total number of cells × 100

3.13.3. Morphological analysis - phase contrast microscopy

RAW 264.7 was induced by LPS. In tissue culture dishes, 7 cells (5 × 105 cells /well) were cultured for 24 hours with four different doses of uricase (0, 30, 50, and 100 µg/ml) (60 mm diameter). The removed media after incubation, and then the cells were washed once using Phosphate Buffer Saline (1X PBS). At a magnification of 200X, the morphological changes were observed **Moongkarndi *et al.*, (2004).**

3.13.4. Xanthine oxidase (XO) activity assay

The Xanthine oxidase activity was carried out by **Zahide Esra Durak, *et al.*, (2014)**. Xanthine oxidase levels were measured, an increase in the absorbance indicates the formation of uric acid from xanthine. Briefly, RAW 264.7 cells were plated at 5×10^5 cells/well. After induction, the cells were preserved with 30, 50, and 60 g/ml uricase for 24 hours. The samples were centrifuged were isolated and pre-incubated at 37 °C for 40 minutes after the treated cell pellets were combined with assay buffer. Working solutions comprising xanthine, buffer, and an appropriate amount of enzyme (supernatant) were added, and the initial absorbance (A1) was measured at 293 nm after 30 minutes of incubation at 37°C. The identical reaction mixture was used in the blanks, but without the xanthine. A volume of 0.1 ml of 100% (w/v) trichloroacetic acid (TCA) was added to the working solution, centrifuged at 5000 x g for 10 minutes, and the absorbance (A2) at 293 nm was measured once more. The absorbance change (A) is calculated using the following formula:

A = [(A2) assay - (A2) blank] - [(A1) assay - (A1) blank]

Note: A/min =A/30

3.13.5 Uric acid Estimation

According to the manufacturer's instructions, uric acid was measured with a commercial Uric Acid Kit (Uricase - PAP technique, Tulip Diagnostics). RAW264.7 cells were plated at a density of 5 x 10^5 cells per well for this experiment. The cells were given 30, 50, and 60 µg/ml of uricase for 24 hours after being inducted for 4 hours. Mix the materials of the kit (4 parts buffer reagent, 1 part enzyme reagent) to make a working reagent. Blank (B), Standard (S), and Test (T) were the labels on dry test tubes, which were preceded as follows:

Addition Sequence	Blank (B) (ml)	Standard (S) (ml)	Test (T) (ml)
Working reagent	1.0	1.0	1.0
Distilled water	0.2	-	-
Uric acid Standard	-	0.2	-
Sample	-	-	0.2

The components were carefully mixed previously being incubated intended for 5 minutes at 37°C, after which the absorbance was measured at 520 nm and computed as follows:

$$\text{Uric acid in mg/dL} = \frac{Absorbance\ at\ T}{\text{Absorbance at S}}\ X\ 8$$

3.13.6 Gene expression analysis

RAW 264. 7 cells were planted in culture flasks and treated with 30, 50, and 60 µg/ml of uricase for 24 hours after being stimulated with 500 ng/ml LPS for 4 hours. DNA was quarantined exploiting a Nucleospin DNA isolation kit as directed by the manufacturer, and PCR was performed using the primers listed below:

β-Actin

5’ TCAAGGTGGGTGTCTTTCCTG 3’ (F)

5’ ATTTGCGGTGGACGATGGAG 3’ (R)

TNF- alpha

5’ACACAGAAGACACTCAGGGA 3’ (F)

5’ CCGGTACTTAACCCTACCCCC 3’(R)

IL10

5’AATCACGGCTCAGTTCTCCC 3’(F)

5’ GGTCACACCATTTGCTGGGT 3’(R)

Initial denaturation - 1 cycle at 95°C for 2 minutes, DNA denaturation - 95 °C for 1 minutes, Annealing Temperatures - 54°C (-Actin), 54.6°C (IL-10) and 55.4°C (TNF-alpha) for 30 s and Extension - 72°C for 1 minutes, Final extension - 72 °C for 5 minutes were used in the amplification sequence protocol.

Chapter – 4

RESULTS

CHAPTER – 4

RESULTS

4.1 Collection of samples and the total heterotrophic bacterial density

In the current study, there were a total of four different birds fecal contaminated soil samples were collected from pigeons, turkey, parrot, and poultry farms in Rayakota, Berigai, Perandapalli, and Kumudepalli of Hosur, Krishnagiri District. The soil samples which were serially diluted were spread on uric acid agar medium for assessing their microbial density and diversity (Plate 1). The microbial density and diversity of the fecal contaminated soil samples were listed in (Table 1).

4.2 Screening and isolation of uricase producing bacteria

Four separate soil samples yielded a total of 13 isolates. Ten of the thirteen isolates that showed a broad zone encircling the colonies were identified on uric acid agar medium and named as MM01, PM16, SC21, EC04, MM20, KS17, EC07, MM08, MM13, and SC14 respectively. The zone measurements of the selected isolates were provided in (Table 2). The MM13 isolate was selected among the 10 isolates because of its wide zone formation of about 37 mm (Plate 2).

4.3 Identification of potential uricase producer

4.3.1 Cultural, morphological and biochemical characteristics of MM13

The cultural characteristics of the isolate MM13 on nutrient agar plates, staining and biochemical reactions showed that the bacterium belongs to the genus *Bacillus*. The results were tabulated in (Table 3 and Plate 3 - 7).

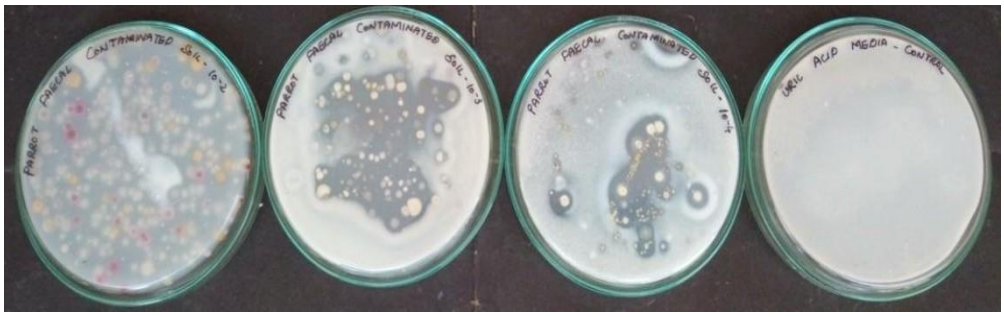
Table 1: Microbial density of uricase producing bacteria in faecal contaminated soil samples

S. No.	Type of soil sample collected	Place of soil sample collection	Microbial density of the collected soil samples (CFU/g)	Diversity of the organisms obtained from soil samples	No. of positive isolates	% of positive isolates
1.	Pigeon faecal contaminated soil	Rayakottai	$2.1x10^3$ - $2.4x10^4$	4	3	75
2.	Turkey faecal contaminated soil	Berigai	$2.4x10^2$ - $2.6x10^6$	3	2	66
3.	Parrot faecal contaminated soil	Perandapalli	$2.6x10^4$ - $2.8x10^6$	3	3	100
4.	Poultry faecal contaminated soil	Kumudapalli	$2.2x10^2$ - $2.5x10^5$	3	2	66
Total no. of bacterial isolates				**13**	**10**	**-**

Table 2: Uricase activity among the test bacterial isolates and diameter of zone of clearance

S. No.	Type of soil sample	Isolate	Diameter and zone of clearance in uric acid media (mm)
1.	Pigeon faecal contaminated soil	Isolate MM13	37
		Isolate PM16	20
		Isolate SC21	16
2.	Turkey faecal contaminated soil	Isolate EC04	14
		Isolate MM20	18
3.	Parrot faecal contaminated soil	Isolate KS17	19
		Isolate EC07	10
		Isolate MM08	14
4.	Poultry faecal contaminated soil	Isolate MM01	12
		Isolate SC14	16

Plate 1. Microbial density of uricase producing bacteria in faecal contaminated soil samples



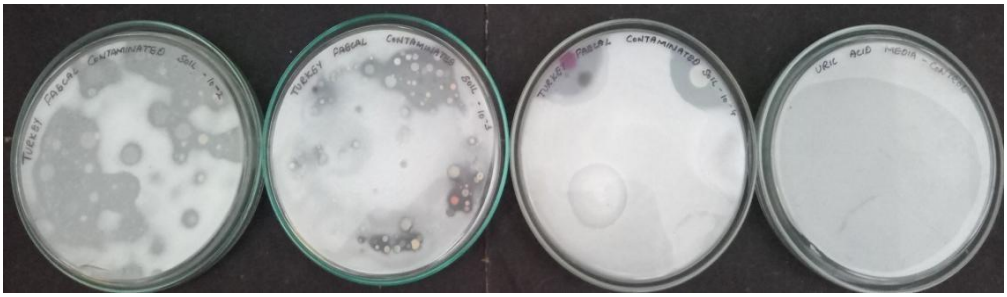


Plate 2: Isolate MM13 showing 37 mm clear zone

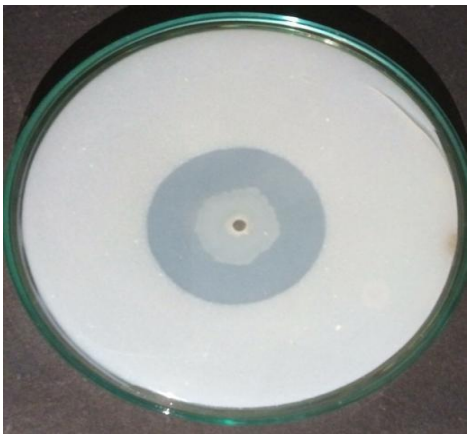
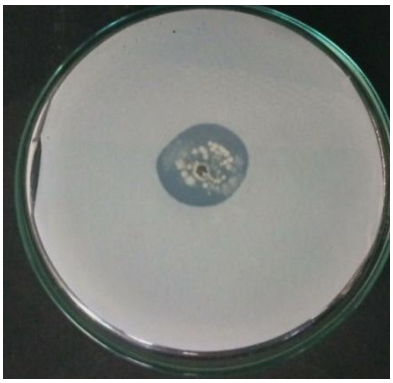
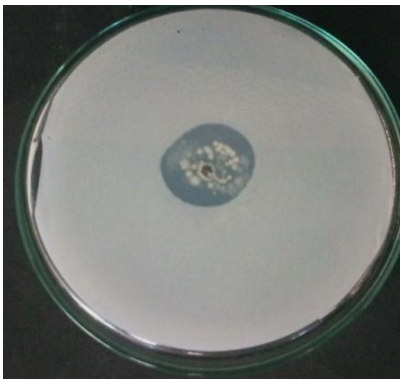
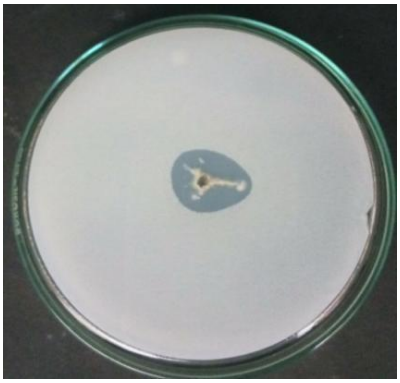


Plate 3: Zone of clearance formed by various isolates







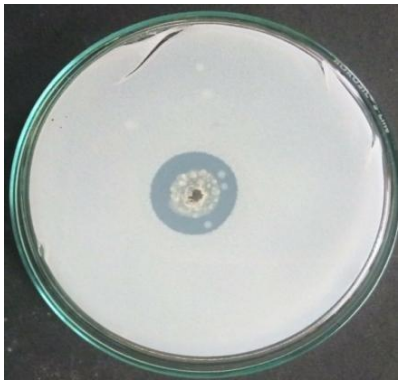


Plate 4: Cultural characteristics of MM13 on Nutrient Agar plate

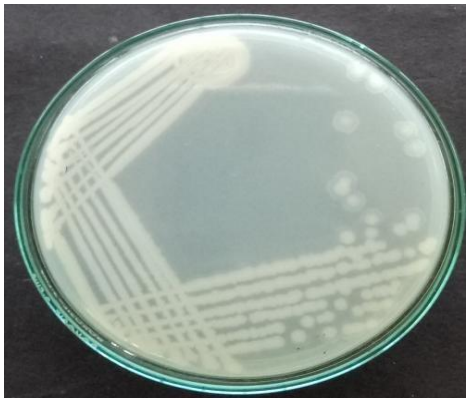


Plate 5: Gram's staining of MM13

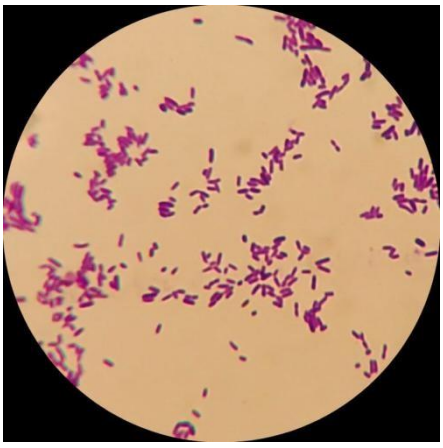


Plate 6: Spore staining of MM13

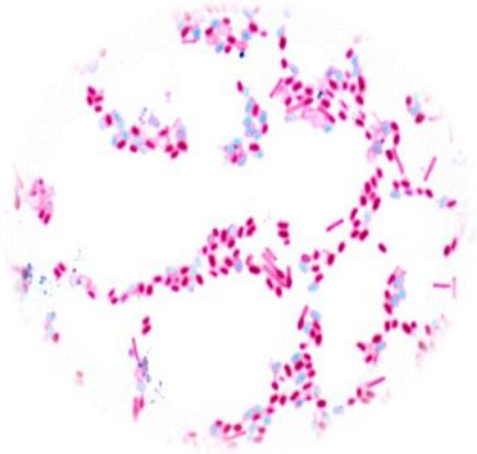
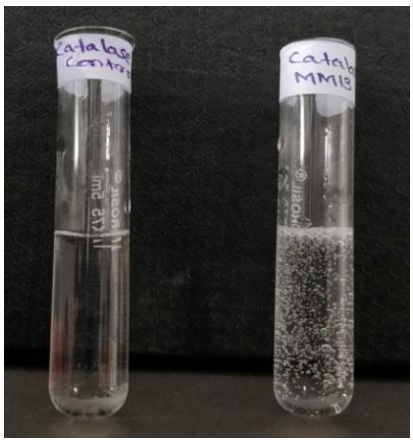
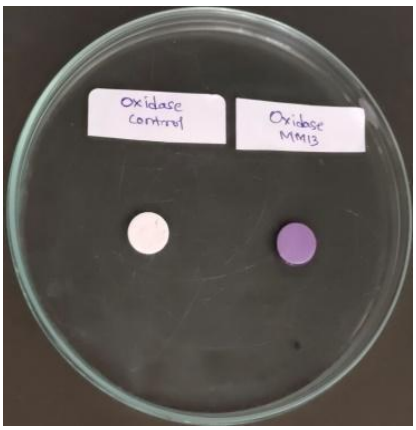


Plate 7: Biochemical characteristics of MM13

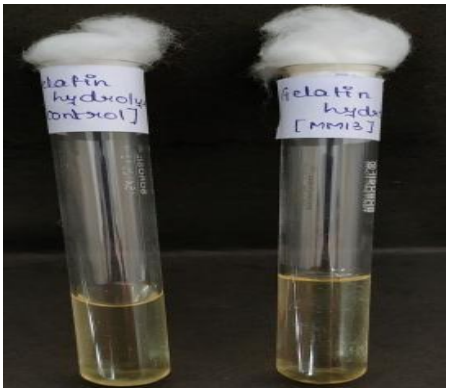


Catalase Test



Oxidase Test

Starch hydrolysis Test



Gelatin hydrolysis Test

Table 3: Cultural, morphological and biochemical characteristics of MM13

S. No.	Name of the test	Result of MM13
1	Colony appearance	Rough, opaque and fuzzy white colonies
2	Motility	Positive
3	Grams staining	Positive rods
4	Spore staining	Positive
5	Indole test	Negative
6	Methyl Red test	Negative
7	Voges-Prouskaur test	Positive
8	Citrate utilization test	Positive
9	TSI	Positive
10	Urease	Negative
11	Catalase	Positive
12	Oxidase	Positive
13	Nitrate Reduction	Positive
14	Casein hydrolysis	Positive
15	Lipid hydrolysis	Positive
16	Starch hydrolysis	Positive
17	Gelatin hydrolysis	Positive
Carbohydrate fermentation test		
18	Glucose	Positive
19	Lactose	Positive
20	Maltose	Positive
21	Mannitol	Positive
22	Sucrose	Positive

4.3.2 Molecular characterization of MM13

Using 16S rRNA studies, the MM13 isolate was genotypically defined. The sequence-based MM13 16S rRNA genes have been deposited to Genbank http://www.ncbi.nlm.nih.gov/genbank on under NCBI accession number MM13 Bacillus subtilis (MK503710) (Figure 1 and 2). The *Bacillus subtilis* MK503710 isolate's sequence was compared to test sequences of *Bacillus* sp. from the BLAST database to evaluate phylogenetic relatedness using the neighbour joining tree technique (Figure 3).

The *Bacillus subtilis* (MK503710) isolate contained a 680bp sequence that was identical in every way. *Bacillus subtilis* (MK503710) was grouped together as one clade segment in the phylogenetic tree, which was represented by bars corresponding to an evolutionary distance of 0.0006 (Figure 4). The numbers above the branches indicate that the bootstrap value is more than 99% (Figure 5).

4.4 Qualitative estimation of uricase

Furthermore, the strain MM13 was exposed to quantitative estimation of uricase using a liquid medium, then the uricase activity was evaluated by plotting on a uric acid standard graph and was determined to be 1.75 U/ml.

4.5 Protein estimation

The protein estimation of MM13 was performed by Lowry method and estimated as 5.18 mg/ml. Similarly, the specific activity was estimated as 0.33 U/mg.

4.6 Optimization of growth parameters

The optimal essential growth parameters are required to obtain the maximum uricase enzyme activity of *Bacillus subtilis* MM13. The metabolic activity and growth rate of bacteria can be influenced by essential growth factors such as pH, temperature, carbon and nitrogen sources.

Figure 1: 16s rRNA partial sequence submission on NCBI *Bacillus subtilis*

(MK503710)

GenBank

Bacillus subtilis strain MM13 16S ribosomal RNA gene, partial sequence

GenBank: MK503710.1

FASTA Graphics

Go to:

```
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DEFINITION  Bacillus subtilis strain MM13 16S ribosomal RNA gene, partial
            sequence.
ACCESSION   MK503710
VERSION     MK503710.1
KEYWORDS    .
SOURCE      Bacillus subtilis
  ORGANISM  Bacillus subtilis
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REFERENCE   1  (bases 1 to 630)
  AUTHORS   Anandharaj,B. and Manimekalai,V.
  TITLE     Direct Submission
  JOURNAL   Submitted (11-FEB-2019) Microbiology, M.R.Government Arts College,
            Mannargudi- 614001, Government Arts College Road, Mannargudi, TAMIL
            NADU 614001, India
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            Sequencing Technology :: Sanger dideoxy sequencing
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//
```

Figure 2: FASTA sequence on NCBI *Bacillus subtilis* (MK503710)

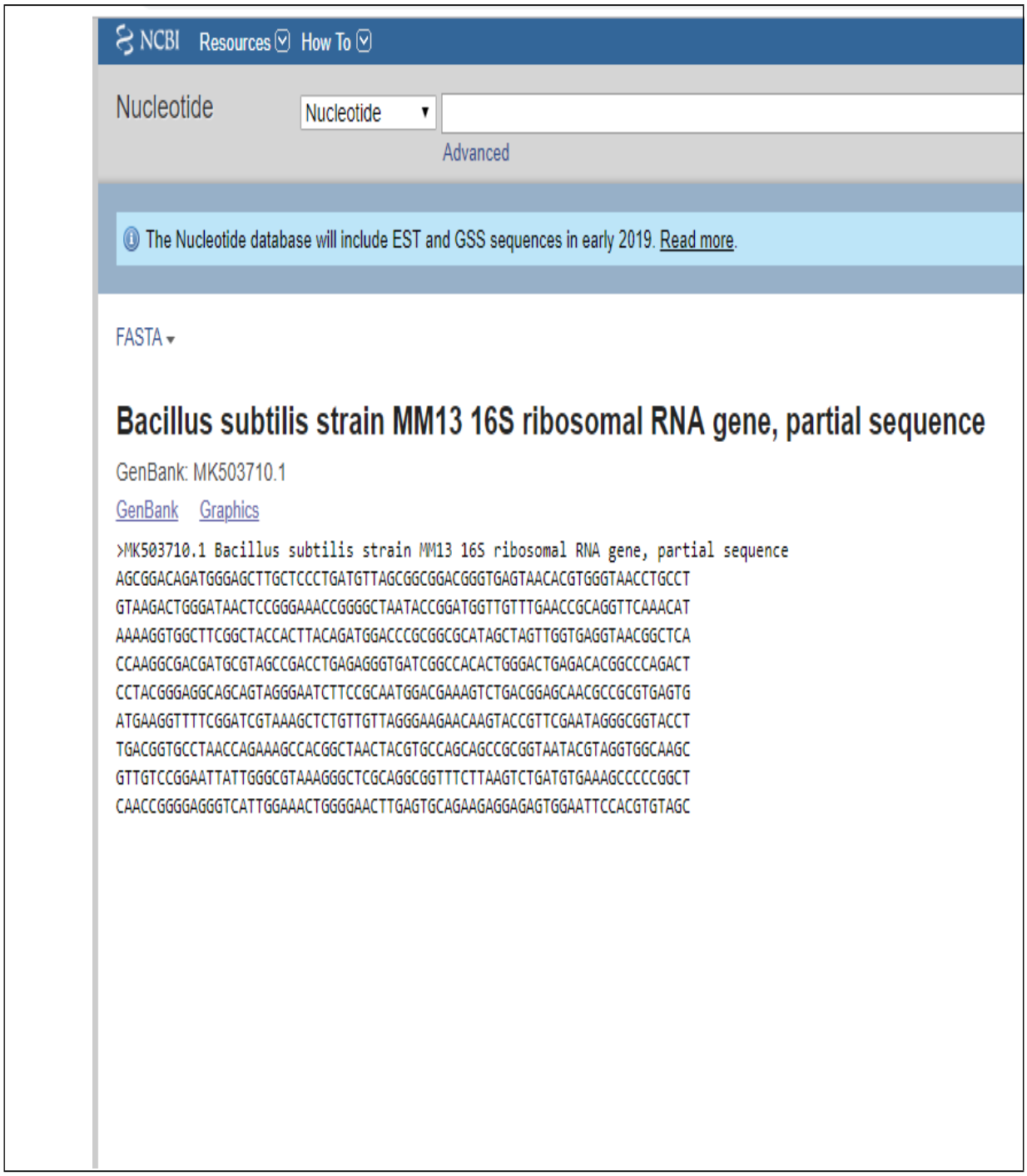


Figure 3: BLAST database of the *Bacillus subtilis* (MK503710)

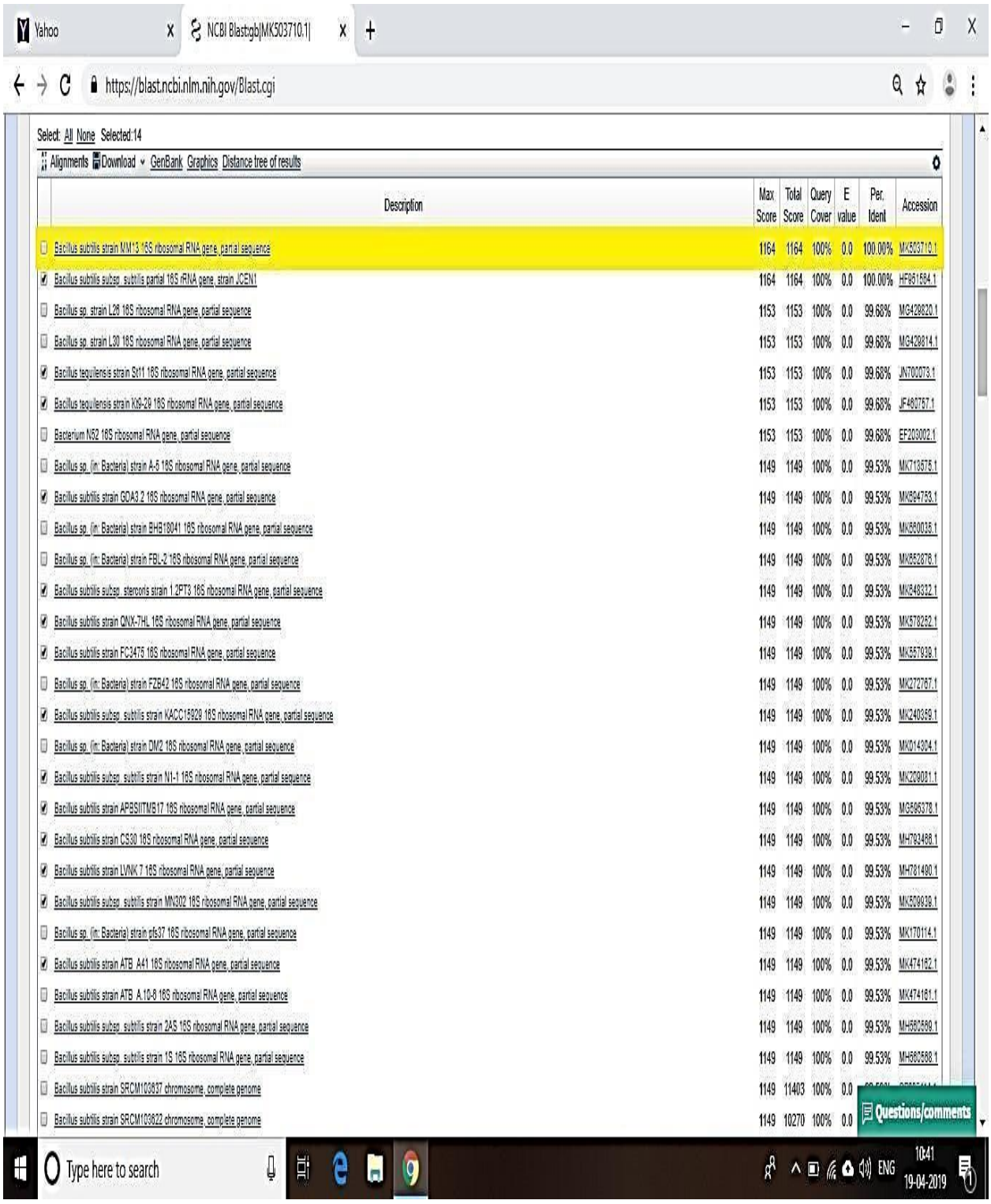


Figure 4: Phylogenetic tree showing phylogenetic position of the *Bacillus subtilis*(MK503710) neighbour joining method

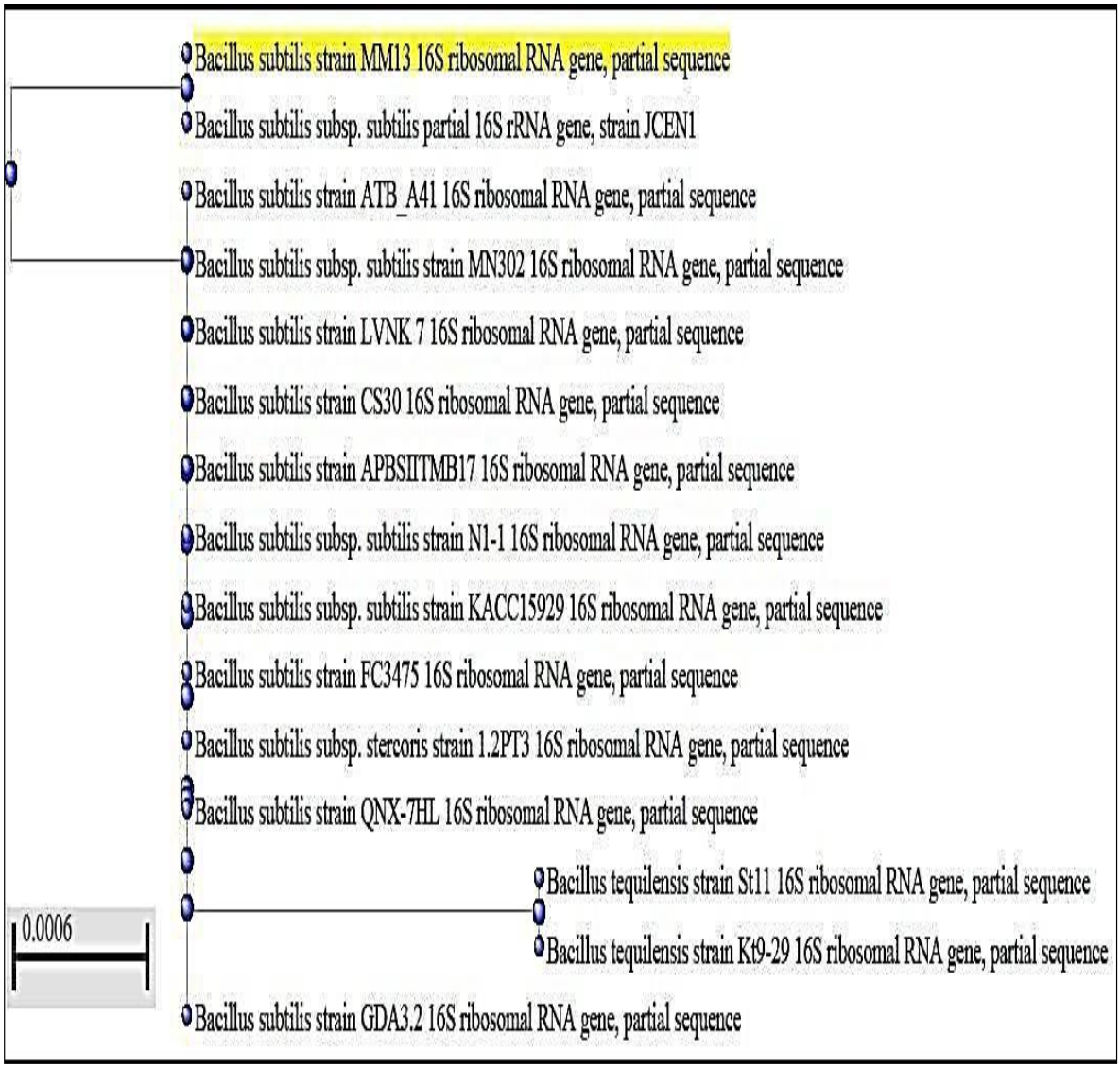
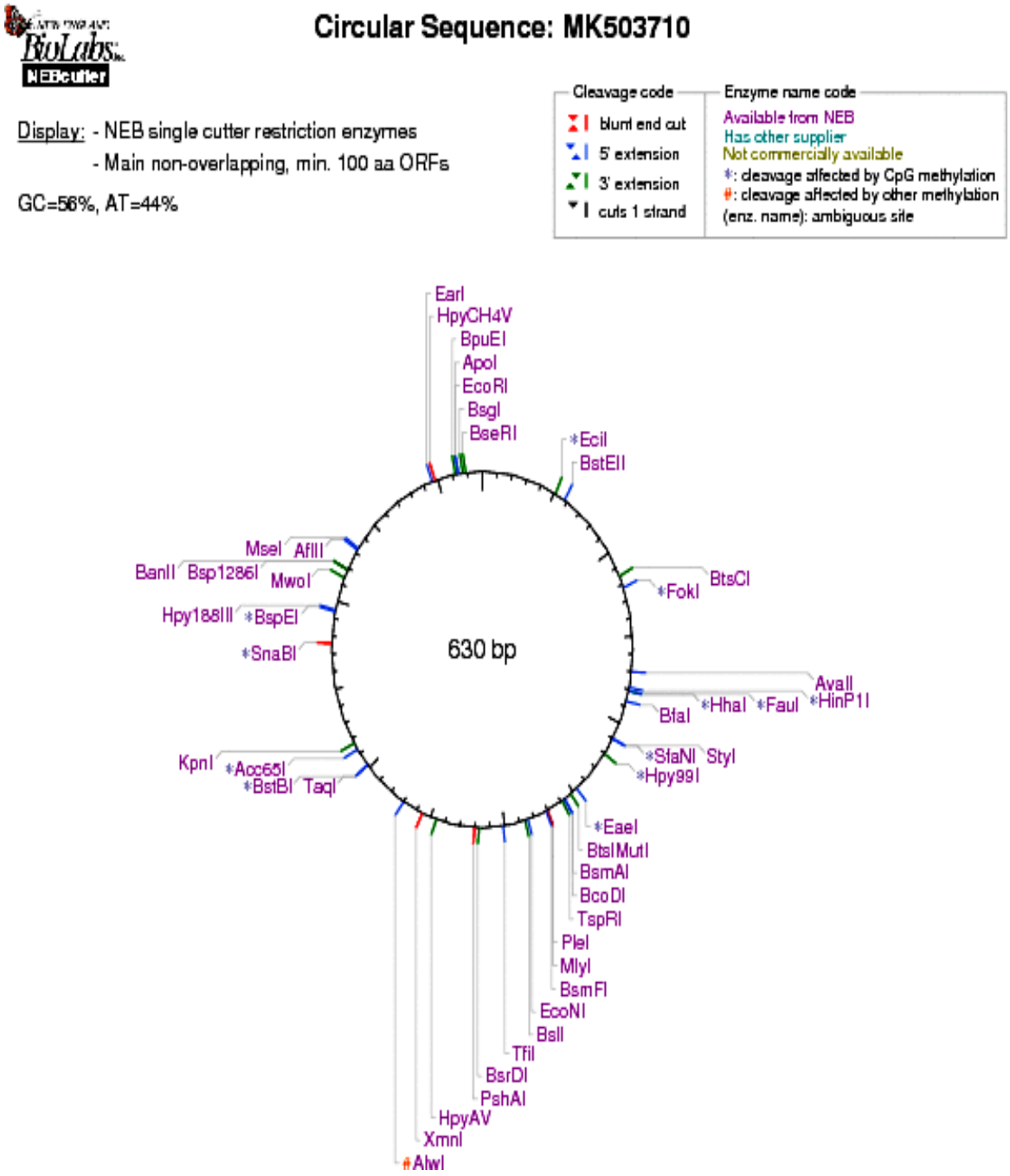


Figure 5: Circular sequence of *Bacillus subtilis* MM13 represents the GC content and restriction sites



4.6.1 pH and temperature influence on uricase activity

The optimal physical factors such as pH and temperature are required for producing maximum uricase enzyme venture. The results obtained indicates uricase enzyme activity happened at 1.85 and 1.25 U/ml at pH 7.01.25 U/ml and 40°C respectively at 48 hours of the submerged fermentation process, and these were statistically significant at $P<0.05$ (Figure 6 and 7). Furthermore, the uricase enzyme activity was significantly reduced at pH 5, 6, 8, and 9 and at 30, 50, 60, and 70˚C, respectively.

4.6.2 Suitable carbon and nitrogen sources

Bacterial enzymatic and metabolic activities, as well as growth rate, are based on availability of appropriate carbon and nitrogen sources. In this study, various carbon sources such as glucose, maltose, lactose, sucrose, and fructose were subjected to identify the suitable carbon source for *Bacillus subtilis* MM13 to secrete uricase enzyme activity. The obtained results state that the test isolate preferably used sucrose as a major carbon source than other sugars and showed maximum uricase enzyme activity 2.54 U/ml. This value was statistically significant at $P<0.05$ (Figure 8).

Similarly, the *Bacillus subtilis* MM13 strain effectively utilized the yeast extract as the preferred nitrogen source and yielded the uricase enzyme activity as 2.37 U/ml in a 48 h period of incubation. This was statistically significant at $P<0.05$ (Figure 9). The uricase activity was considerably reduced in other nitrogen resouces such as peptone, ammonium nitrate, ammonium chloride, as well as casein.

Figure 6: Optimization of growth parameters for uricase enzyme activity in *B*

***Bacillus subtilis* MM13 using various pH**

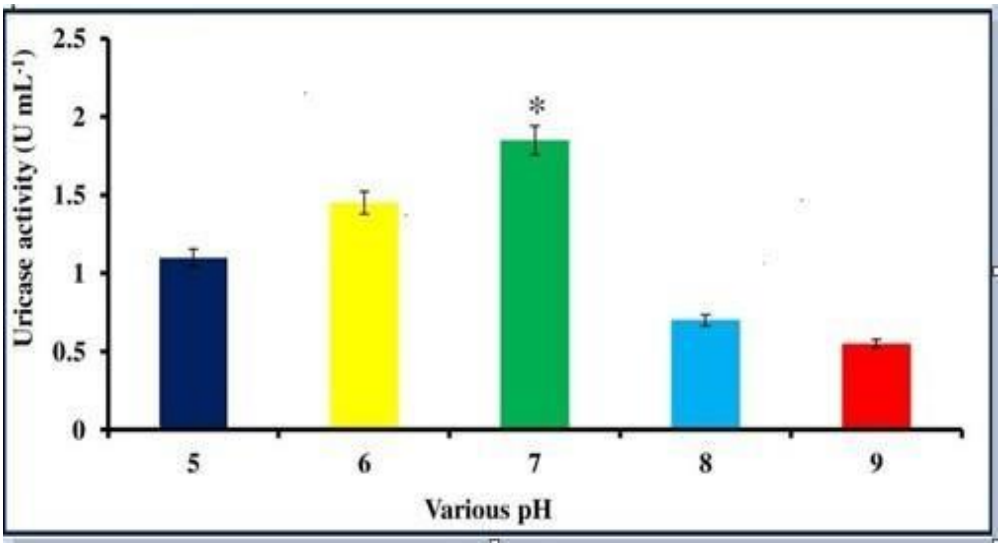


Figure 7: Optimization of growth parameters for uricase enzyme activity in

***Bacillus subtilis* MM13 using various Temperature**

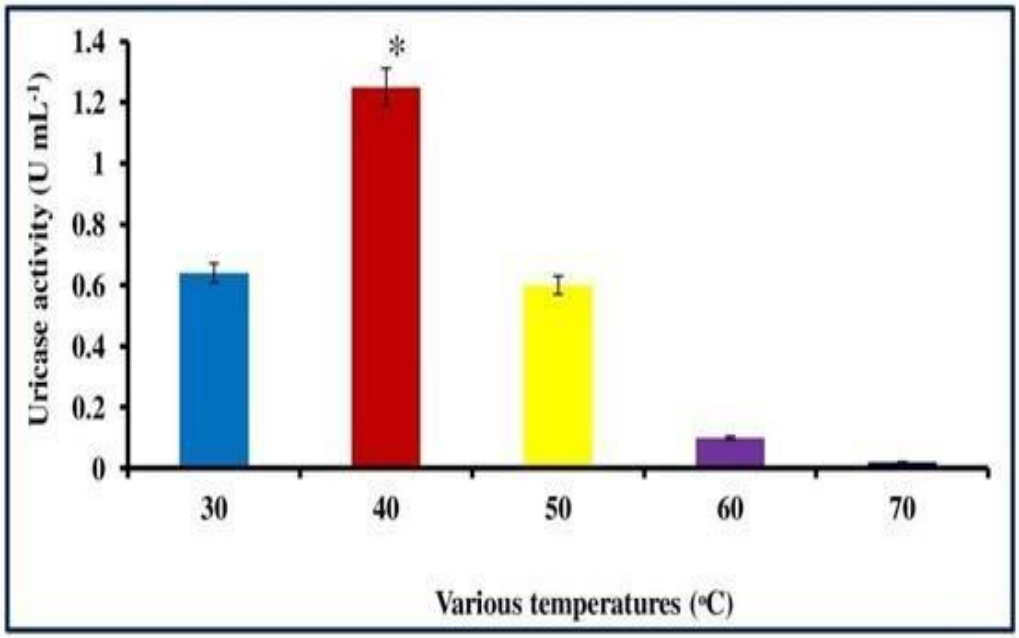


Figure 8: Optimization of growth parameters for uricase enzyme activity in *B.subtilis* MM13 using various carbon sources

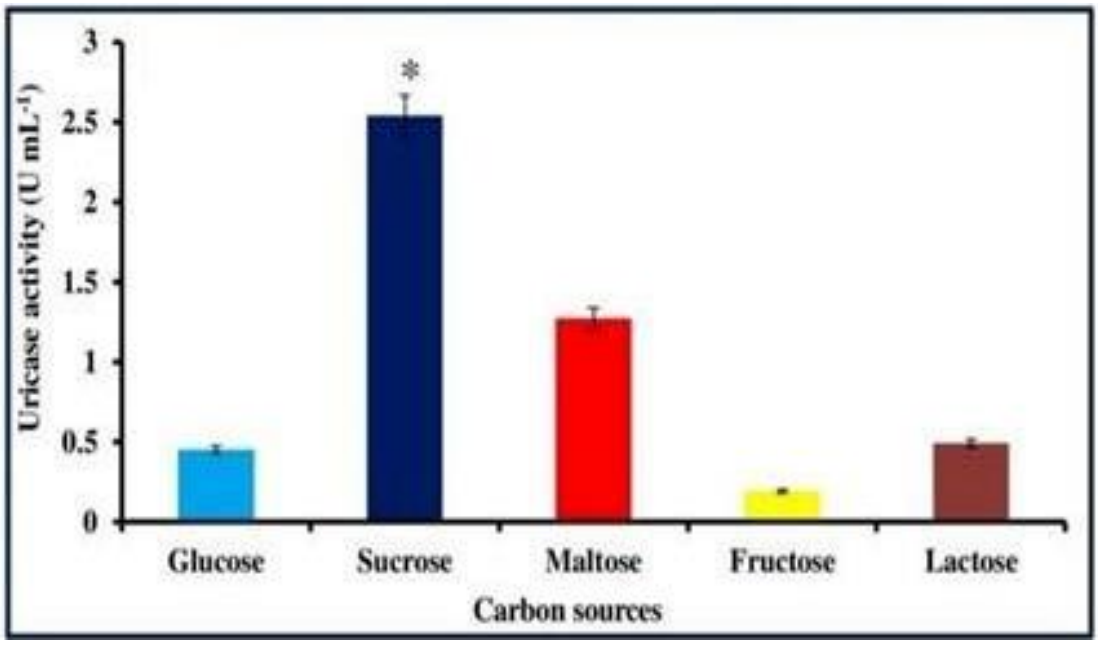
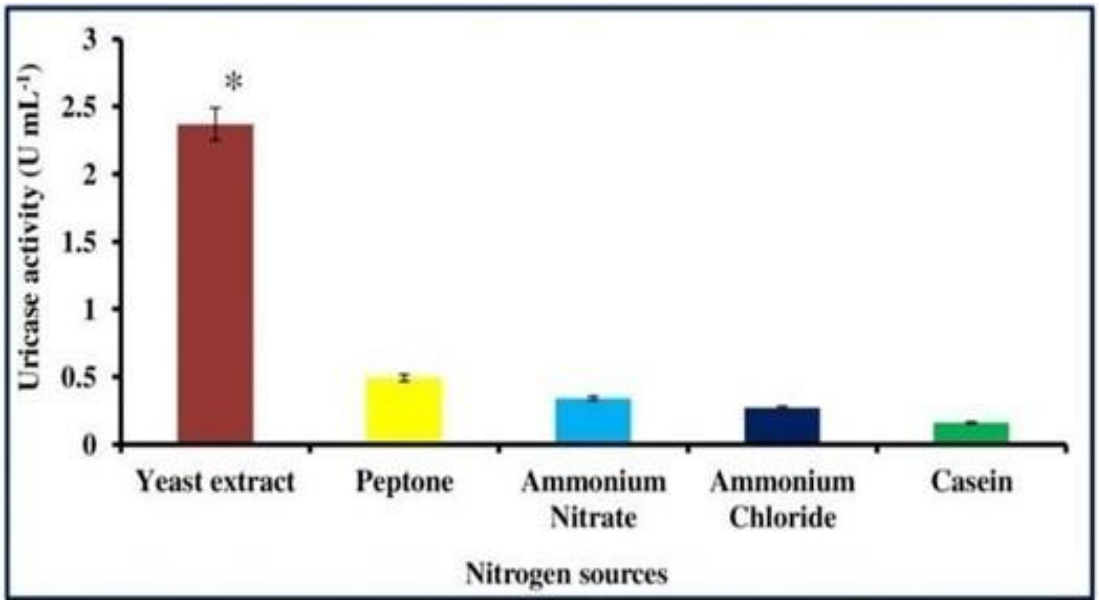


Figure 9: Optimization of growth parameters for uricase enzyme activity in *Bacillus subtilis* MM13 using various nitrogen sources



4.6.3 Optimal uric acid concentration for uricase production

The uric acid served as a primary inducer for uricase activity in bacteria. The ideal uric acid concentration for the uricase enzyme activity of *Bacillus subtilis* MM13 was 0.32%. It yielded about 1.17 U/ml of uricase enzyme in a 48 hours of incubation period and this value was statistical significance at $P<0.05$ (Figure 10).

The dried and sterilized powdered form of uric acid enriched wheat bran, beans, cauliflower, and pigeon fecal were studied and interestingly, the *Bacillus subtilis* MM13 effectively utilized the pigeon fecal matter and showed considerable uricase enzyme activity as 0.7 U/ml, however this was significantly lower than the readymade form uric acid (1.17 U/ml). This result suggests that the tested natural uric acid enriched sources have not significant influence on enzymatic activity in *Bacillus subtilis* MM13. This might be due to the insufficient quantity of uric acid content in these natural sources.

4.6.4 Optimization of suitable concentration of sucrose and yeast extract

The initial optimization study results revealed that the sucrose as well as yeast extract were discovered to be good carbon and nitrogen traces for excellent uricase enzyme activity in *Bacillus subtilis* MM13. Hence, the suitable concentration for these two energy sources for *Bacillus subtilis* MM13 to produce maximum uricase enzyme activity were found as 1% for sucrose and 0.2% for yeast extract and yielded 2.54 and 2.37 U/ml, correspondingly. At $P<0.05$, these results were significant statistically (Figure 11 and 12).

Figure 10: Optimization of growth parameters for uricase enzyme activity in *Bacillus subtilis* MM13 using various uric acid concentrations

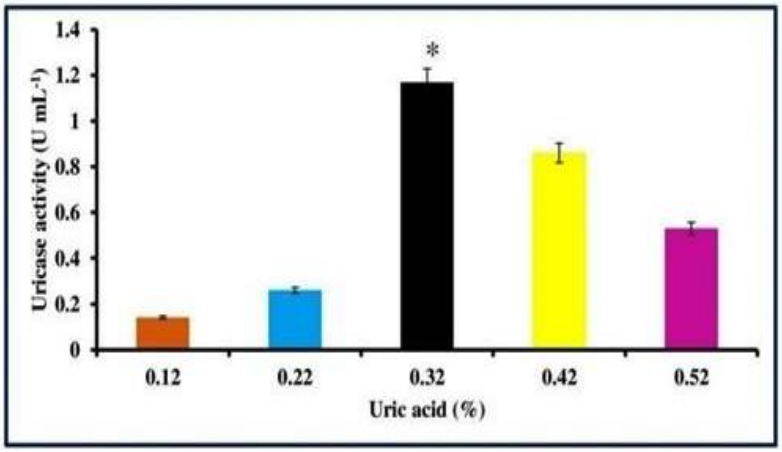


Figure 11: Optimization of growth parameters for uricase enzyme activity in *Bacillus subtilis* MM13 using various sucrose concentrations

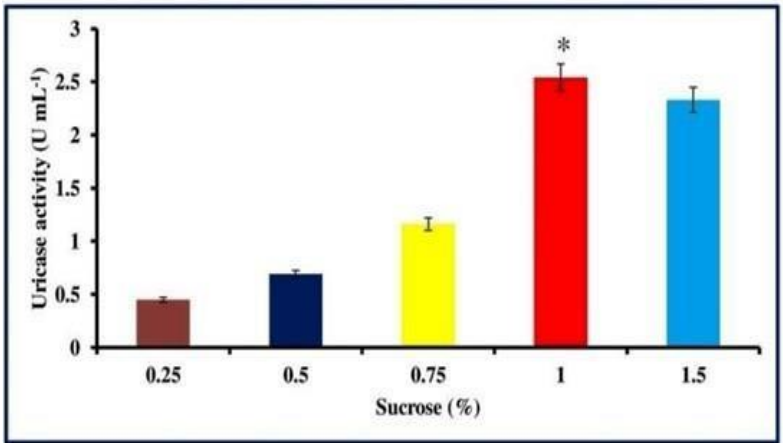
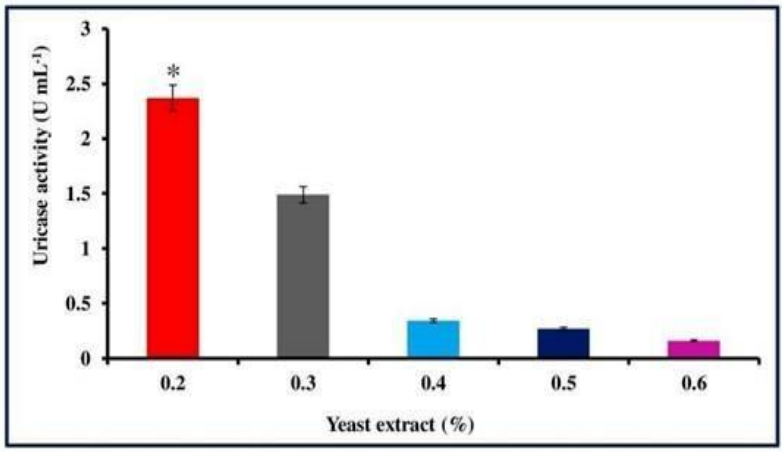


Figure 12: Optimization of growth parameters for uricase enzyme activity in *Bacillus subtilis* MM13 using various concentrations of yeast extract



4.7 Extraction and purification of uricase enzyme derived from *Bacillus subtilis*

The crude enzyme was extracted (Figure 13) from *Bacillus subtilis* at the end of the logarithmic cycle through 80% Ammonium sulphate precipitation. The extracted cell free crude uricase enzyme precipitate was partially and initially purified with a dialysis membrane (Figure 14). Uricase enzyme activity was found as 245.8 U/mg and it was 2.74 fold purified as compared to crude enzyme and yield was calculated as 42.00% and this Ammonium sulphate precipitated (80%) and dialyzed uricase enzyme was considerably higher than specific activity (89.6 U/mg) of crude uricase extract (Table 4).

Table 4: Purification of uricase and relative enzymatic activity

Sample	Volume (ml)	Total Activity (u/ml)	Total protein (mg/mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude	245	2956	28.41	89.6	1	100
Ammonium sulphate precipitation (80%)	31	1241.7	7.3	245.8	2.74	42.00
DEAE cellulose Column	15	1024	2.8	1125	12.55	34.64
DEAE Sephadex A-50 column	7	684.5	1.6	1352	15.08	23.15

Figure 13: Extraction of uricase from *Bacillus subtilis*

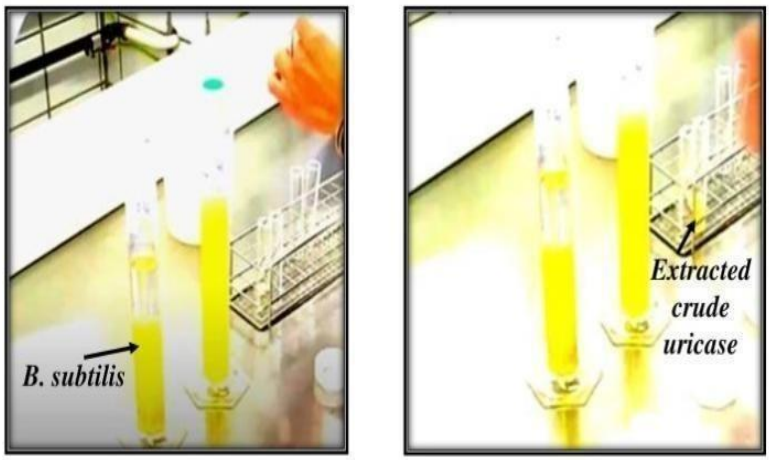
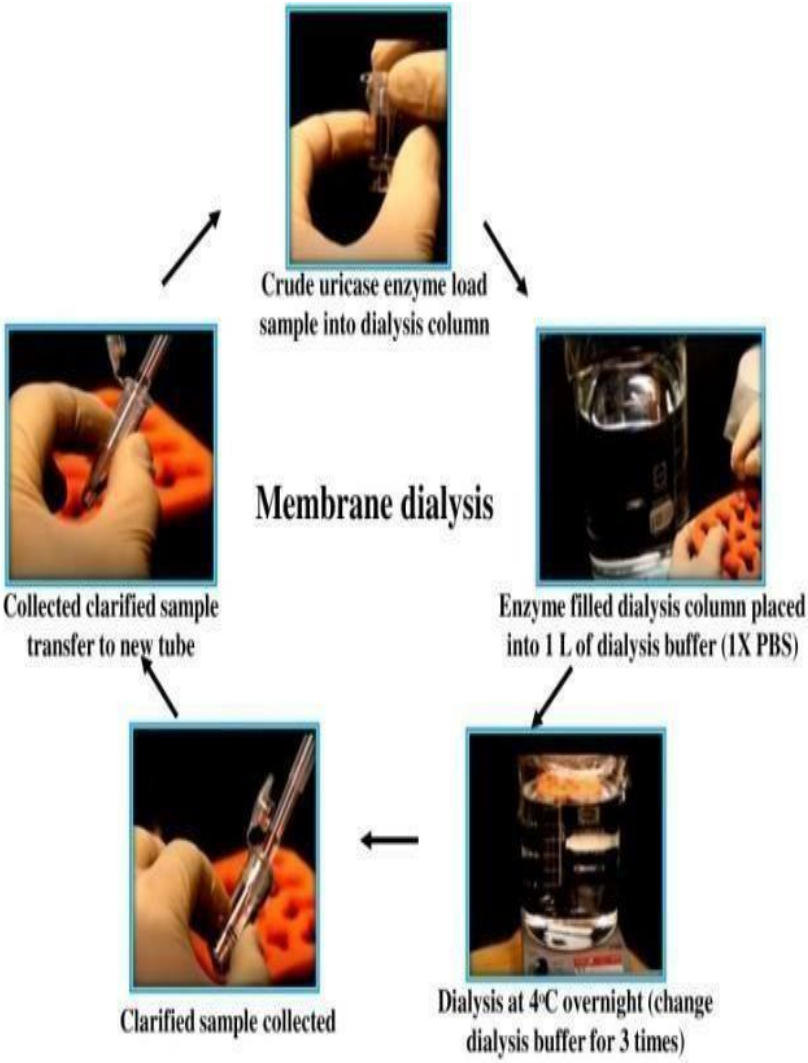


Figure 14: Membrane dialysis of uricase



Furthermore, the DEAE cellulose column base purification (Figure 15) results showed that the uricase enzyme was purified as 12.55 fold and yielded 34.64% with increased specific uricase activity as 1125 U/mg while compared with 80% Ammonium sulphate precipitated and dialyzed uricase enzyme.

The purity of the uricase enzyme extracted from *Bacillus subtilis* was effectively further purified by DEAE sephadex A-50 column (Figure 16). It yielded around 23.15% with increased specific uricase activity as 1352 U/mg and it was 15.08 fold significantly greater than the crude uricase enzyme.

Finally the molecular weight of DEAE sephadex A-50 column purified uricase enzyme was examined through SDS-PAGE by loaded with 0.8 mg/ml (lane 1), and 0.6 mg/ml (lane 2) of enzyme. The obtained result indicated that the MW of uricase (purified) was found as 48 KDa (Figure 17).

Figure 15: DEAE cellulose based purification of uricase

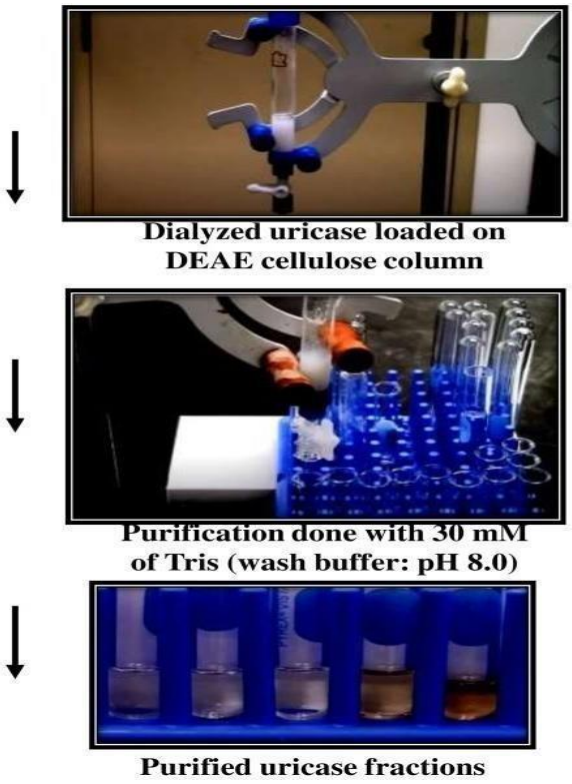


Figure 16: DEAE cellulose Sephadex A- 50 column based purification of uricase

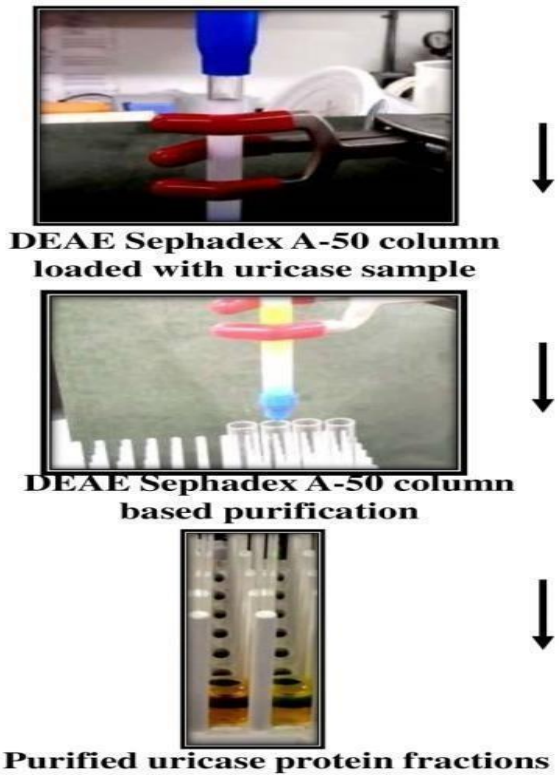
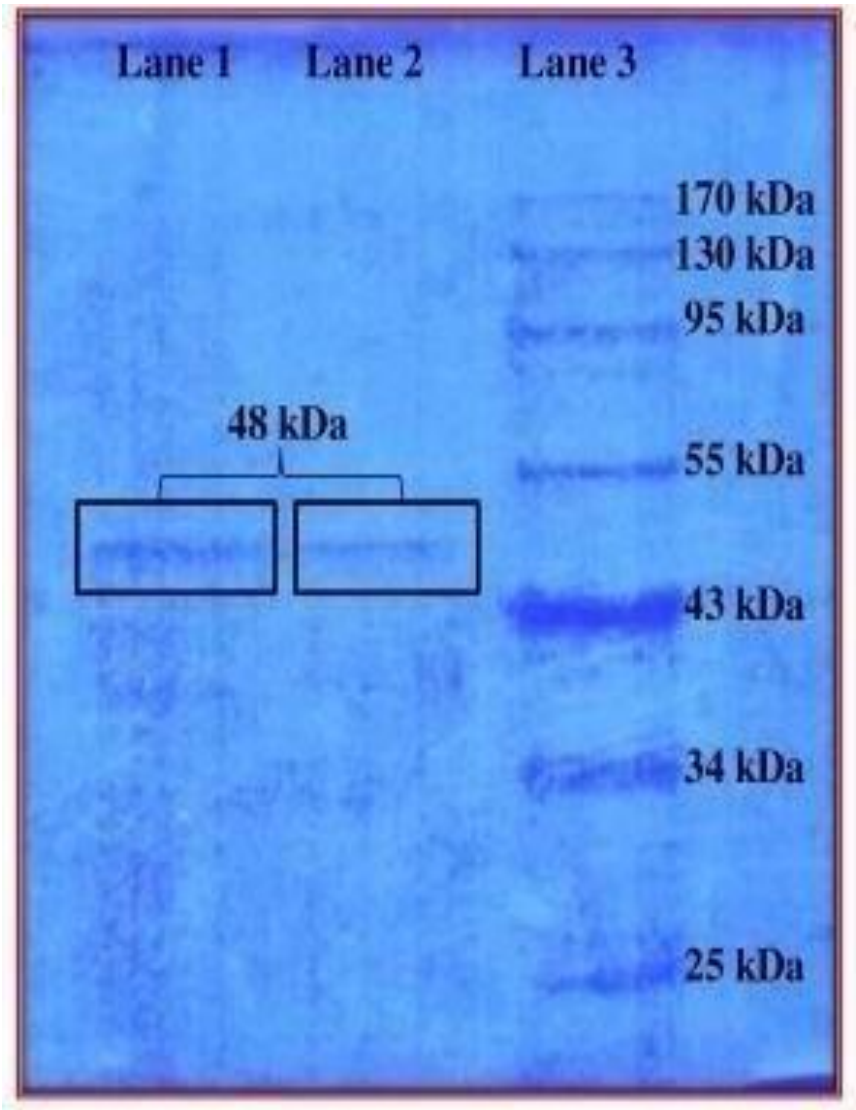


Figure 17: SDS-PAGE analysis of purified uricase enzyme



Legend: Lane 1: 0.8 mg/ml, Lane 2: 0.6 mg/ml, Lane 3: marker protein

4.8 Physical and chemical characterization of purified uricase enzyme

At the suitable physical and chemical factors the hydrogen peroxide is established when uricase interacts with urate and interacts with peroxidase occurs in the presence of 4-aminoantipyrine and phenol. As a result of the enzymatic reaction, quinoneimine dye was produced. The quantity of uric acid deteriorated by uricase has been relative to the amount of dye quinoneimine formed.

4.8.1 Temperature effects in the activity and stability of the uricase enzyme.

The fine purified uricase enzyme activity (100%) and stability (100%) were significantly higher at 35 ºC and it followed by 25ºC (97%) and 45ºC (89%) while, the temperature increasing the enzyme activity was significantly reduced (Figure 18.a). For example, at 55 ºC, the enzyme relative activity was reduced as 42% and further reduced to 17 at 65 ºC. Thus, this *Bacillus subtilis* synthesized uricase showed excellent activity at 35 ºC and stability at 25ºC to 45 ºC.

4.8.2 pH effects in the activity and stability of the uricase enzyme.

The results obtained from pH influence on uricase enzyme activity showed the maximum activity (100%) at pH 8.0 (Figure 18b). It is followed by pH 7.0 (96%) and pH 6.0 (84%). The uricase enzyme activity was highly affected at the lowest (5.0) and highest pH (9.0 & 10). The stability of purified uricase was found in a extensive span of pH is from 6.0 to 9.0.

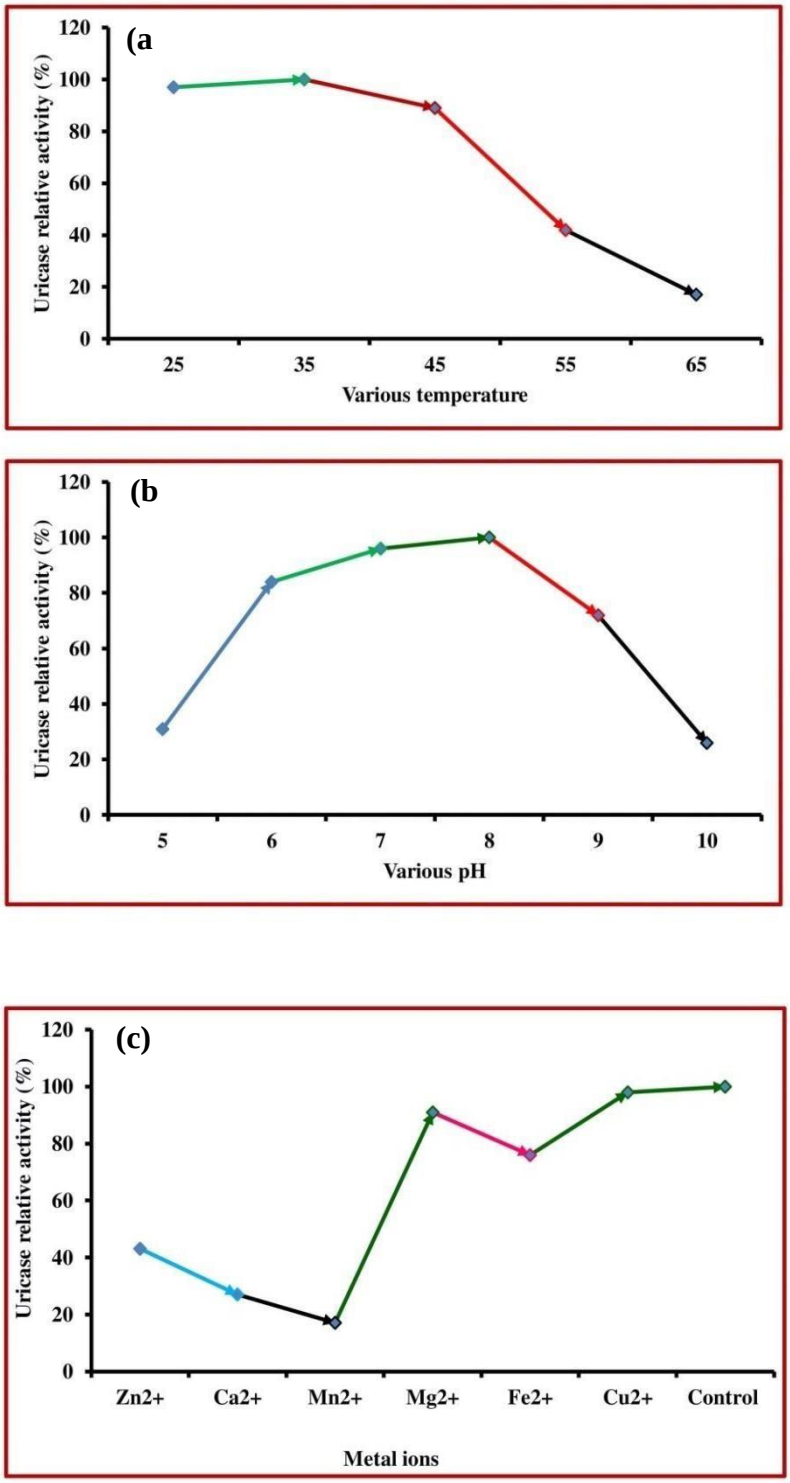
This property increases the commercial application possibilities and value. From slight acid to slight alkaline pH facilitate the uricase activity to metabolize the uric acid.

4.8.3 Metal ions effect in the activity and stability of the uricase enzyme

Metal ions that are appropriate can effectively chelate or enhance the enzyme activity. In this regard, discovering appropriate metal ions for increased uricase activity is essential to boost the enzyme's commercial use potential. As a result, the effects of several metal ions on uricase enzyme activity and stability remained found to be most effective with Cu_{2+} (98 %), followed by Mg_{2+} (91 %), and Fe_{2+} (76 %) (Figure 18c). Thus, this purified uricase enzyme can be used to metabolize or breakdown the uric acid with co- factor or chelator or support of Cu_{2+}.

Figure 18. Physical and chemical characterization of uricase enzyme.

(a) Temperature (b) pH (c) Metal ions



4.9 MTT assay

The MTT assay was employed to verify the inhibitory effect of uricase on RAW 264.7 murine macrophage cells (Figure 19). The cells were handled through uricase at doses ranging from 10 to 100 µg/ml for 24 hours. Uricase decreased cell proliferation in a dose- dependent manner, indicating that it suppresses RAW 264.7 cell growth. With 54.26% inhibition, the LC50 was determined to be 50 µg/ml concentrations.

4.9.1 Trypan Blue Exclusion Assay

The most frequent and first method for determining cell viability was the Trypan blue exclusion experiment. Cells that have been weakened by uricase treatment become permeable, and it attaches to intracellular proteins, turning them bluish in colour, resulting in cell death. The proportion of surviving cells in RAW 264.7 cells treated with uricase at varied concentrations (10 to 100 µg/ml) were measured after a 24 hours incubation period. Cell mortality was dose-dependent, and cell viability reduced as concentrations increased (Figure 20).

Figure 19: Cytotoxic effect of uricase on RAW 264.7 cells. Different concentrations of uricase (10-100 µg/ml) were used for cell treatment.

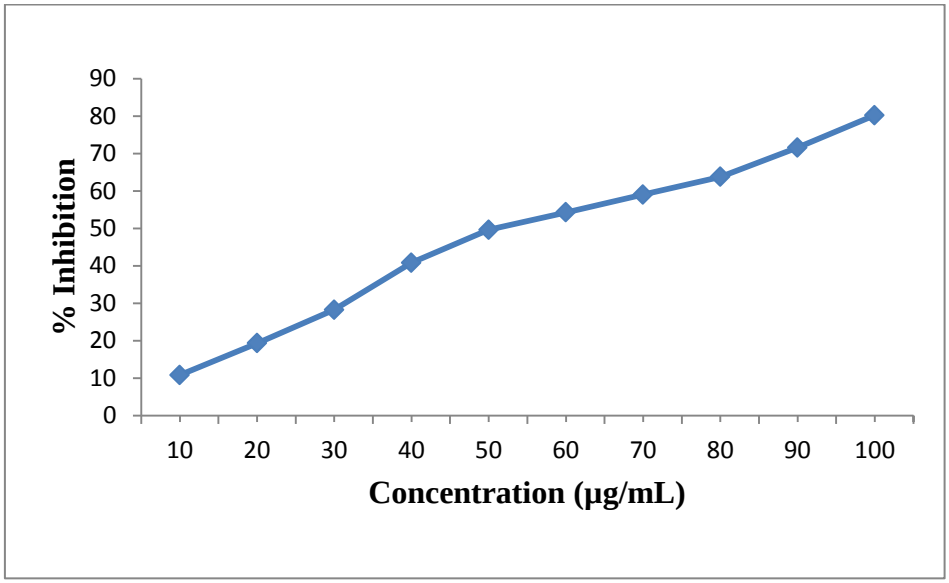
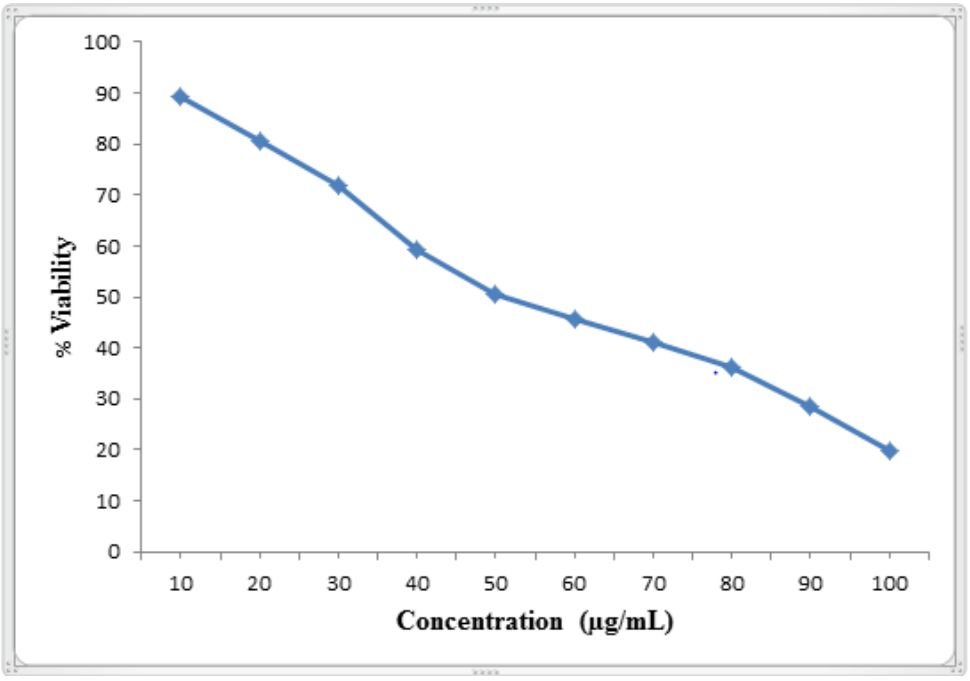


Figure 20: Effect of uricase on cell viability of RAW 264.7 cell line - The cell death rate was determined using trypan blue assay. RAW 264.7 cells were treated with Uricase (10 – 100 µg/ml) for 24 hours. Uricase caused cell death in a dose-dependent manner.



4.9.2 Morphological changes by phase contrast inverted microscope

Under uricase therapy, changes in cellular shape were detected at various doses (Figure 21). When compared to the control, the cells shrank and then detached in a dose- dependent manner, similar to the cell proliferation and cell inhibition studies. RAW 264.7 cells that had not been treated looked to be normal (circular shaped).

4.9.3 Xanthine oxidase assay

The suppression of xanthine oxidase enzyme by three different concentrations of uricase (30 µg/ml, 50 µg/ml and 60 µg /ml), as compared to LPS generated RAW 26.47 cells and control (untreated cells) as shown in (Figure 22). XO inhibitory action was identical at all three uricase doses. One-way ANOVA was used to analyse the data (Tukey multiple comparison tests).

4.9.4 Uric acid estimation

The in *vitro* effects of uricase on uric acid concentration in cells (Figure 23). After pretreatment with LPS, uricase significantly reduced uric acid concentration in all three concentrations (30 g/ml, 50 g/ml, and 60 g/ml) compared to the non-treated LPS induced group ($p < 0.0001$).

4.9.5 Gene expression analysis

The roles of pro-inflammatory cytokine (TNF-α) and anti-inflammatory cytokines (IL-10) were investigated. Down-regulated gene expression levels were observed for TNF- α in uricase treated cells and an upregulated expression was observed for IL- 10 gene, in a dose-dependent manner (Figure 24). IL-10 is a potent inhibitor of TNF-α and uricase enzyme at a defined concentration can help to reduce the pro- inflammatory cytokine TNF-α, thereby reducing cell damage.

Figure 21: Effect of uricase on RAW 264.7 - Photomicrograph (20 X) images were captured by phase contrast microscope.

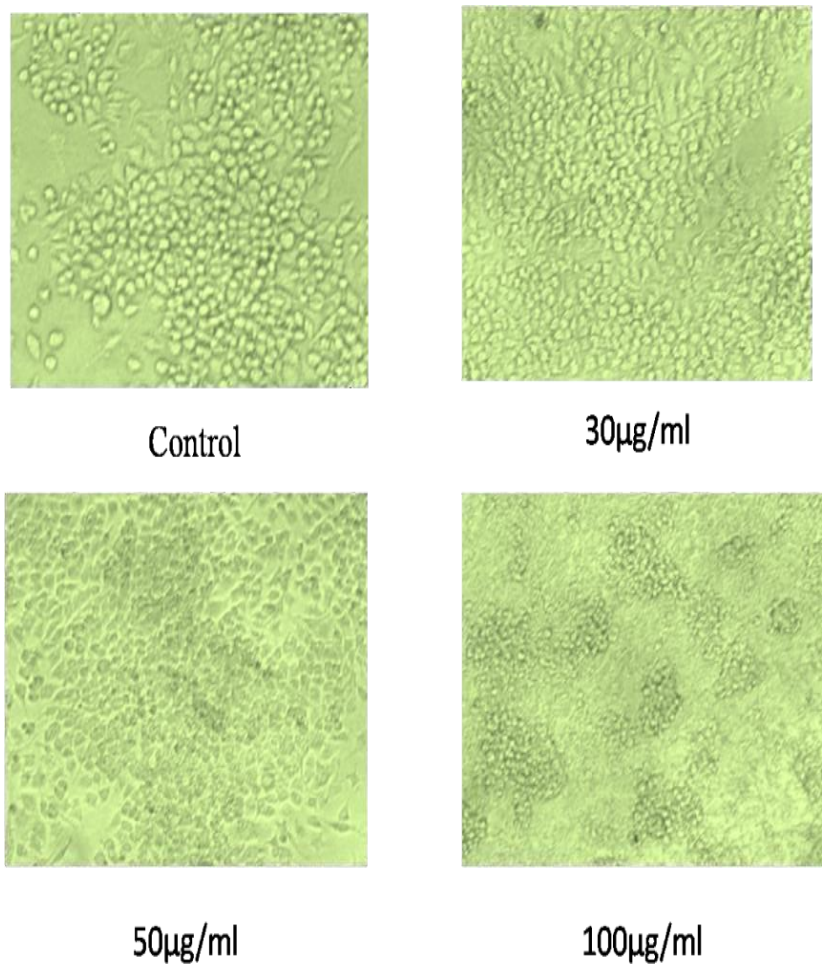


Figure 22: Xanthine oxidase assay

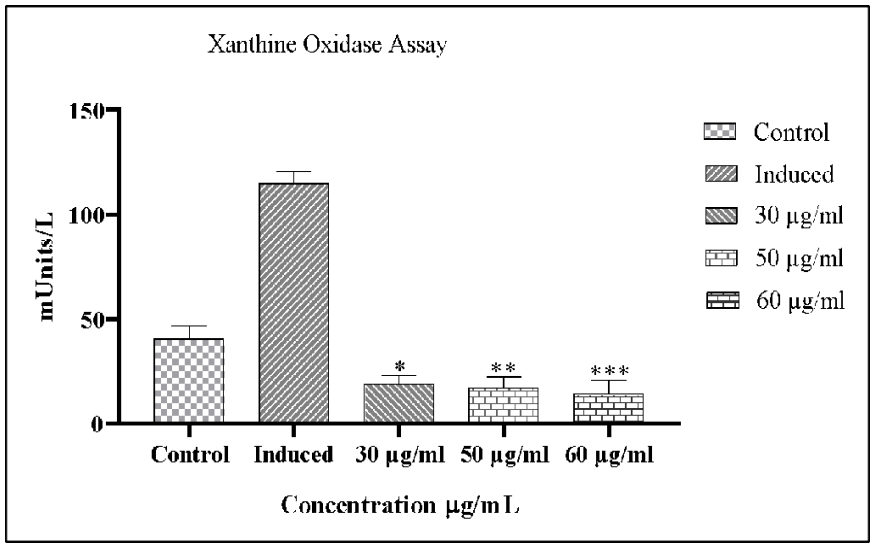


Figure 23: Uric acid production

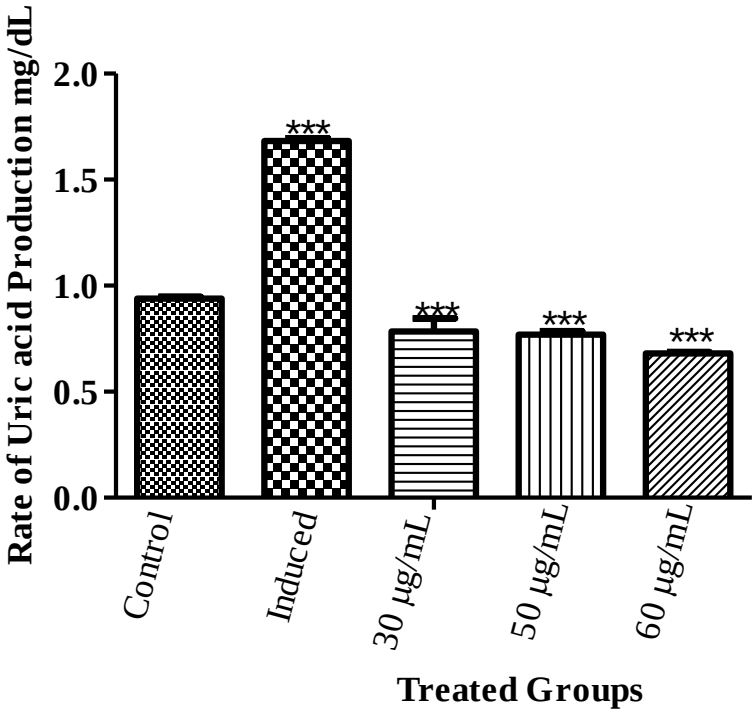
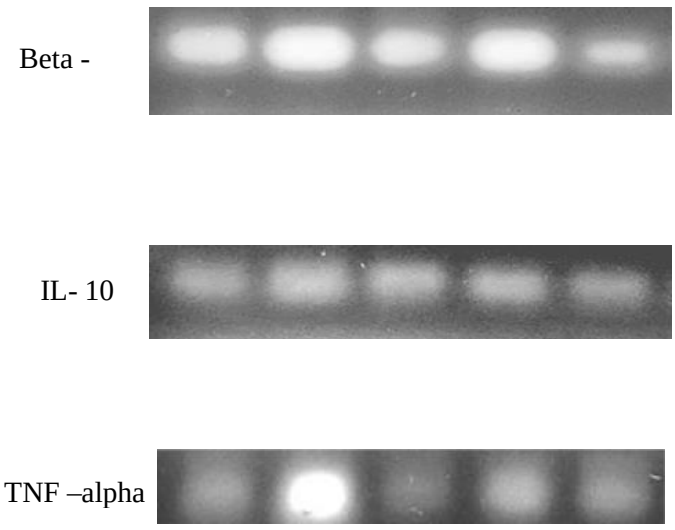


Figure 24: Gene expression analysis of TNF-α and IL-10



Lane 1 - Control

Lane 2 – Induced

Lane 3 – Sample (30 µl)

Lane 4 – Sample (50 µl)

Lane 5 – Sample (60 µl)

Chapter – 5

DISCUSSION

CHAPTER – 5

DISCUSSION

Significance of uricase and the prevalence of uricase producers

Enzymes are biological catalysts that are actually needed for the different biochemical reactions required for life to exist. They speed up all energy metabolism reactions in the body and perform a specific function. Enzymes are extremely efficient, increasing reaction levels by 100 million to 10 billion fold faster than any other type of chemical reaction. This catalysts have emerged as an essential molecule which has been commonly used in various industrial and pharmaceutical purposes as a result of advancements in biopharmaceuticals and bioengineering. Because of the rapid evolution of enzyme technology, enzymes derived from microbial sources are receiving increased attention due to their multi-potential properties.

Microbial enzymes have always been favored owing to economic suitability, better productivity, uniformity, comfort of improving existing products and optimization, sustained demand due to lack of seasonal variations, fast growth of microbes on low-cost media, reliability, and higher catalytic performance. Microbial enzymes are important for the diagnosis, therapies, biochemical examination, and supervising of a variety of serious infectious diseases.

Several microbial enzymes such as proteases, keratinases, amylases, xylanase, uricase, laccase or ligninase, cellulose, and so on have been investigated by several researchers. Among, various enzymes, the uricase is very significant enzyme that have been explored very extensively in the recent years and are extremely important in a variety of industries, including the pharmaceutical and food industries

Gurung *et al.*, (2013). Microbial enzymes are renowned due to their ability and significant activity under adverse environments, mostly notable temperature and pH. Microbial enzymes can thus be categorized as thermophilic, alkalophilic, or acidophilic.

Microbes with heat stable metabolic enzymes which can perform at greater than average reaction temperatures might decrease the potential of microbiological contamination in huge industrial reactions that last for a long time. The value of thermo-stability in enzymatic reactions encourages the break - down and metabolism of materials; additionally, a higher activation temperature improves enzyme penetration. Comprehensive saccharification as well as hydroxylation of carbohydrates comprising crop wastes demands a prolonged reaction time, which is closely correlated with extended duration contamination risks. Thus, hydrolytic enzymes are in great demand because they are ideal at elevated temperatures and uphold steadiness over a significant duration of handling a several of temperature range.

High thermal enzymes also aid in increasing mass transport and decreasing substrate surface tension during hydrolysis of substances in various industrial processes. Uricase is a hepatic enzyme, which directly convert uric acid/urate into allantoin, a water-soluble compound which is more conveniently eliminated from the body by the renal. Throughout evolutionary history in human, a missense as well as frame shift mutation tends to result in an inactivated gene producing uricase. Thus, blood serum urate concentrations are near towards the solubilization. Whenever this threshold is exceeded, urate precipitates in soft tissue structures can establish as sodium urate crystals. Unless no urate-lowering therapy is used, these uric acid deposits can ultimately cause medical symptoms similar to gout.

The organisms of choice for uricase production are microbes, especially bacteria. Because of its simplicity, cost-effectiveness, and reliability, enzyme production via bacterial isolate is frequently preferred by investigators. However, current uricase production capacity is insufficient to meet future demand, necessitating the identification of new bacterial strains as soon as possible **Daisley *et al.*, (2020).** *Bacillus subtilis, B. fastidious, Microbacterium sp., Pseudomonas aeruginosa, Arthrobacter globiformis,* and *Nocardia farcinica* were isolated from soil, agriculture wastes, bird faeces, and other sources **Dabbagh *et al.*, (2012)**. Aside from this, the uricase enzyme has been found in fungi, animals, and plants. In this study, the soil samples were taken from a variety of locations, including pigeon, turkey, parrot, and chicken farms, which were contaminated with bird faeces. Around 13 uricase producing bacterial isolates were enumerated from 4 faeces contaminated soil samples after three days of incubation at 37^{o}C in uric acid enriched medium.

This was confirmed by occurrence of zone of clearance around the bacterial colonies, since uricase metabolize the urate/uric acid in the media resulting disappearance of color/clear zone around the colonies. Interestingly, ten bacterial isolates such as MM01, PM16, SC21, EC04, MM20, KS17, EC07, MM08, MM13, and SC14 were identified as considerable uricase producer (based on huge size of zone of clearance around the colonies) out of thirteen bacterial isolates. Subsequently, the isolate MM13 chosen (pigeon droppings contaminated soil) from among the ten because of its wide zone formation. The morphological, cultural, and biochemical characteristics study disclosed that the bacterial isolate MM13 was identified as a *Bacillus* species. The zone of clearance formed around the colony is the result of

uricase converting uric acid into 5-hydroxyisourate with the help of O_2 and H_2O, which produced H_2O_2 and was then reduced as allantoin.

The size of the uric acid clearance zone surrounding the colony was thought to be an important predictor of a bacterial isolate's uricase producing capacity. *Bacillus cereus* DL3 isolated from chicken farms was also discovered to be highly capable of synthesizing uricase. To determine this, the zone of clearance around the colonies on uric acid medium was measured. Pigeon faeces may contain more uric acid than faeces from other birds. As a result, the bacteria isolated (MM13) may be the most capable of releasing uricase enzyme and consuming uric acid in pigeon faeces. *Clostridia* sp., which produces uricase, was isolated from the guts of several termites using a zone of clearance has been established around the colonies..

The MM13 isolate was genotypically characterized using 16S rRNA sequencing. The sequence-based MM13 16S rRNA gene sequence have been deposited to GenBank under the NCBI and received accession number (MM13) *Bacillus subtilis* as MK503710. To determine phylogenetic similarity, the *Bacillus subtilis* MM13 isolate's sequence was evaluated by comparing to test sequences of *Bacillus* sp. from the BLAST database using the neighbour joining tree technique. The *Bacillus subtilis* MM13 isolate had an identical 680bp sequence in every way analysis and contained 56 and 44% of GC and AT bases respectively. *Bacillus subtilis* MM13 was represented in the phylogenetic tree as a single clade segment, with bars corresponding to an evolutionary distance of 0.0006. Furthermore, this 680bp partial sequence of *Bacillus subtilis* MM13.

The majority of research on uricase synthesis used avian faecal contaminated soil samples as a source for microorganism isolation. Similar findings were found in this study, and several bacteria were isolated from bird faeces contaminated soil samples. According to one report, *Alcaligenes faecalis* **Sambandam *et al.*, (2021)**, *Pseudomonas aerogionosa* **Selvaraj and Thirumalaivasan (2017),** and *Bacillus subtilis* **Jagathy *et al.*, (2016)** were most familiar bacterial species capable of producing uricase and recovered from poultry faecal contaminated soil samples. Ravichandran et al. isolated an uricase-producing bacterial isolate from duck farm waste-contaminated soil and identified it as *Sphingobacterium thalpophilum* VITPCB5 via molecular characterization **Ravichandran *et al.*, (2015).**

Other uricase producers were identified from deep muck and fowl dropping from a poultry farm, including *Comamonas testosteroni* ATCC 11996, *Bacillus flexus* IFO 15715, *Staphylococcus capitis* SD, *Bacillus aryabhattai* B8W22, *Comamonas testosteroni* ATCC 11996, *Ochrobactrum anthropic* **Atty, *et al.*, (2016)**, *Bacillus subtilis* RNZ **Kotb, (2016),** *Pseudomonas putida* **Poovizh, *et al.*, (2014)**, *Bacillus cereus* strain DL3 **Nanda, P. *et al.*, (2013)** were identified as possible uricase producer and they characterized and identified through molecular techniques **Lekshmy, *et al.*, (2016).** Interestingly, **Shi, et al, (2019) Lotfy, (2008) Zhou, *et al.*, (2005)** have isolated substantial hyperthermostable and thermostable uricase producers from contaminated Egyptian soil samples and those strains were identified as *Arthrobacter globiformis, Bacillus thermocatenulatus,* and *Microbacterium* ZZJ4-1 through molecular characterization techniques **Shi, *et al.*, (2019); Lotfy, (2008); Zhou, *et al.*, (2005).**

Similarly, **Pustake *et al.*, (2019)** enumerated the uricase- producing *Bacillus subtilis* SP6 from poultry waste through 16S rRNA sequencing. Another report stated that the chicken farm possessed a significant number of Bacillus cereus DL3 strain and that the strain possessed exceptional uricase synthesizing potential, and that this strain was identified and characterized using molecular characterization techniques **Khade & Srivastava, (2017)**. Some other report stated that the uricase producing bacteria were isolated from some other sources. For example *Streptomyces exfoliatus* UR10 is halophilic bacteria isolated from Iranian salt lakes possess considerable level of uricase producing activity and used the molecular characterization techniques to identified this strain **Honarbakhsh *et al.*, (2021).**

Similarly, the uricase producing *Bacillus* sp. was enumerated from agriculture waste and noted that effective uricase producer and characterized by 16S rRNA sequencing **Tork *et al.*, (2020**). Apart from birds droppings contaminated soil samples, the uricase producing bacterial isolates have been also reported from some other sites. According to this**,** isolated uricase producing *Pseudomonas otitidis* strain SN4 from hot spring sites of Malaysia **Lee, *et al.*, (2015)** and similarly, **Amirthanathan, *et al.*, (2012)** enumerated the *Bacillus cereus* from marine sediments of mangrove environments and identified as it possess significant uricase producing potential.

The uricase producing *Pseudomonas aeroginosa Ps*43 were isolated from various clinical samples and characterized by 16S rRNA sequencing **Abdullah and Flayyih, (2015)**. **ElAdawy *et al.*, (2012)** isolated the uricase producing *Ochrobactrum anthropi* from poultry droppings contaminated soil and they were characterized through 16S rRNA gene sequencing. Based on 16s rRNA sequencing, *Comamonas* sp.

was identified as the most common uricase- producing bacterium isolated from poultry soil **Tork *et al.,* (2020).** Interestingly, the uricase-producing Actinobacteria species *Streptomyces graminofaciens* enumerated from poultry faeces contaminated soil **Magda, *et al.,* (2013**).

In addition to bacterial species, numerous fungal species *Aspergillus carenus, A. flavus A. welwitschiae,* and *Emericella quadrilineata* were reported to be the most active uricase producing fungal strains, while *A. peyronelii* and *A. terrus* produced only a small amount of uricase. **El-Weshy, *et al.,* (2018).** *Gliomastix gueg* **Atalla, *et al.,* (2009)** and *Mucor hiemalis* was another fungal species identified as uricase producer **Yazdi, *et al.,* (2006).** However limited number of uricase producing yeast isolates was reported yet as *Kluveromyces lactis*, *Candida utilis,* and *Hansenula polymorpha* **Dmytruk, *et al.,* (2011).**

With all of these available sources, bacterial sources seem to be the most beneficial and resilient due to their low growth requirements, rapid development rates, and regular purification methods. With all these practical advantages allow for the uricase production with lesser time **Jagathy *et al.,* (2016)**. Other sources, on the other hand, have slow rate of growth and a complicated system that necessitates expensive purifying operations, making them unsuitable for large-scale production **Jhample *et al.,* (2015).** Despite the fact that the uricase enzyme has been extracted from a variety of species, there is always room to discover new sources of uricases which are both cost effective and have higher selectivity **Eichmann *et al.,* (2019).**

Optimization of growth and enzyme production

Recently, there has been an increase in the usage for uricase enzymes, particularly in the medical sector, to cure hyperuricemia and associated illnesses **Jin *et al.*, (2012**). The bacterial based uricase production can be determined by supplementation of suitable physical (pH and temperature), chemical (carbon and nitrogen sources), and other suitable growth supplements facilitate the outstanding metabolic activity of bacterial cell and yielded considerable volume of uricase in a short duration of time. Furthermore, the cost of nutritional materials used for uricase production must be considered before using this *Bacillus subtilis* MM13 for commercial uricase enzyme production. As a result, optimizing the growth parameters for uricase production is critical.

pH and temperature influence on uricase activity

Enzymes work best at specific temperatures and pH levels, and terrible living conditions can end up causing an enzymatic activity to lose its ability to adhere to a substrate. In general, raising the temperature of a reaction speeds it up, whereas the lowering the temperature slows it down. High temperatures, on the other side, could indeed induce an enzymatic activity to end up losing its shape and stop working. Correspondingly, each enzyme does have a preferred pH range, and trying to adjust the pH outside of this range slows enzyme activity. When enzymes are exposed to high pH levels, they can denature. As long as there is a component to bind to, gradually increasing enzyme concentration may increase the rate of reaction.

Throughout this investigation, the most uricase enzyme activity was found to be 1.85 and 1.25 U/ml at pH 7.0 and 40°C, respectively, after 48 hours of submerged

fermentation. Furthermore, the uricase enzyme activity was significantly reduced at pH 5, 6, 8, and 9 and at temperatures of 30, 50, 60, and 70°C, respectively. Interestingly, these results were partially correlated with the findings of Kotb (2016) reported the optimal pH and temperature for *Bacillus subtilis* RNZ-79 isolated from birds droppings contaminated soil as pH 8.0 and at 40°C. At this optimal concentrations, the uricase producing efficiency of *Bacillus subtilis* RNZ-79 was significantly increased **Kotb, (2016).** Increased temperature (increasing kinetic energy) and optimal pH can accelerate the reaction; nevertheless, elevated temperature and pH (breaking bonds) immediately reduce enzyme and metabolic activity **Rollin et al., (2015)**. The optimal pH and temperature thresholds for enzyme activity have been revealed as 4.0-9.0 and 30-50°C, respectively, so these ranges might well be associated to the different strains and substrates used for enzyme production **Fu *et al.*, (2016)**. Accordingly, the optimal pH and temperature for *Saccharopolyspora* sp. PNR11 uricase enzyme activity were reported as 7.0 and 30°C, respectively **Khucharoenphaisan & Sinma (2011)**. **Azab, *et al.*, (2005)** found and reported that the *Streptomyces graminofaciens* and *S. albidoflavus,* isolated from crops cultivated soil of Egypt were possess the optimized pH and temperature as 7.0 and 28ºC.

Suitable carbon and nitrogen sources

Bacterial enzymatic and metabolic activities, as well as growth rate, are influenced by the supply of suitable carbon and nitrogen sources. Carbon and nitrogen sources, as well as their concentrations, have always caught the interest of industry research teams on low-cost media composition. This is also recognized that perhaps the expense of microbes growing medium is approximated to be 30–40% of the expense of commercial enzyme production. As a result, optimizing the circumstances for cost-

effective enzyme production is critical. However, research into the effects of carbon and nitrogen components revealed that not every carbon and nitrogen would act as just an enhancer for the concurrently producers of such microbial enzymes in a single fermentation process. Apart from in single-enzyme fabrication, nutrients play a significant role in multi-enzyme manufacturing because few essential nutritional additives improve simultaneous fabrication of all microbial enzymes in a single fermenter **Juwon and Emmanuel, (2012**).

Various carbon sources, including glucose, maltose, lactose, sucrose, and fructose, were investigated in the present study to determine the best carbon source for *Bacillus subtilis* MM13 to secrete uricase enzymatic activity. According to the results, the test isolate preferred sucrose as a major carbon source over other sugars and demonstrated the highest uricase enzyme activity of 2.54 U/ml. Similarly, the *Bacillus subtilis* MM13 strain used yeast extract as the preferred nitrogen source and produced 2.37 U/ml of uricase enzyme activity after 48 hours of incubation. Other N sources, such as peptone, ammonium nitrate, ammonium chloride, and casein, significantly reduced uricase activity. Like the present study, *Alcaligenes faecalis* isolated from poultry waste showed maximum uricase activity of 36 U/ml/min, with sucrose as a carbon source. But coming to the nitrogen source the organism produced maximum uricase production of 31 U/ml/min with uric acid as a nitrogen source **Thillainayagi *et al.*, (2021).**

Similarly in one more study, the isolate *Gliomastix gueg* used sucrose and uric acid as the best carbon and nitrogen sources for uricase production **Atalla *et al.*, (2009)**. *Comamonas testosteroni* strain from poultry waste observed maximum uricase activity with 0.15U/ml with cellulose and 0.058U/ml with yeast extract as the ideal best

carbon and nitrogen sources **Lekshmy *et al.*, (2016).** *Bacillus cereus* DL3 isolated from poultry was found to preferentially use Carboxymethyl cellulose and asparagine as best carbon and nitrogen sources, yielding approximately 15.43 U/ml of uricase activity and it was significantly around 1.61 times higher than the other best carbon and nitrogen sources **Nanda & Babu, (2014)**. Another study found that *Bacillus subtilis* SP6 isolated from poultry waste had the highest uricase enzyme activity (1.2 to 15.87 U/ml) when lactose and soya peptone were used as the preferred carbon and nitrogen sources, respectively **Pustake *et al.*, (2019).**

Optimal uric acid concentration for uricase production

Uric acid is the end product of purine metabolism in humans, as well as an alternate biological substrate for the uricase enzyme. Such an enzyme based uric acid oxidation produces urate free radicals as well as uric acid crystals hydroperoxide, a strong oxidant as well as feasibly antibacterial agent. Uric acid is a kind of weak acid with a maximum solubility of 7 mg dL-1 in the blood plasma. The normal range for plasma urate levels is 3.3 to 6.9 mg/dl. As previously stated, uricase degrades uric acid to allantoin. Uric acid is reduced by urate by uricase to allantoin as characterized formerly. At 25°C, allantoin seems to have a much better bioavailability of 400 mg/dl in extracellular fluid as well as water. The phrase "Zone of clearance" might have been coined to describe the clear zone that formed as a result of urate decomposition owing to the existence of uricase as in agar well **Carvalho *et al.*, (2018**).

Uric acid was the main inducer of uricase activity in the bacteria. In this study, the ideal uric acid concentration for *Bacillus subtilis* MM13 uricase enzyme activity seemed to be 0.32%. In such a 48 hours incubation time, it produced approximately

1.17 U/ml of uricase enzyme activity. Numerous different *Bacillus subtilis* strains, which including RNZ- 79, efficiently reported highest uricase enzymatic activity in such a short amount of time using only 0.4% uric acid **Kotb, (2016).** Correspondingly, another one *Bacillus subtilis* SP6 strain demonstrated highest uricase activity at such a uric acid concentration of 2.55 g L-1 **Pustake *et al.*, (2019**). According to another study, *Streptomyces exfoliatus* UR10 isolated from poultry exhibited highest uricase enzymatic activity at 0.2% of uric acid **Aly *et al.*, (2013**). Such an uricase enzyme derived from bacteria converts uric acid into H_2O-dissoluble allantoin through a 5-hydroxyisourate as an intermediate as well as released H_2O_2 as a byproduct **RoyChoudhury *et al.*, (2018).**

Surprisingly, **Dwivedi *et al.*, (2012)** revealed that the presence of uric acid across the medium may be thought to influence uricase yield in the appearance of 0.1 % w/v uric acid happening the medium. Uric acid has indeed been characterized by lactoperoxidase interrupting the establishment of hypothiocyanite as well as the H_2O_2, thiocyanate mechanism killing activity against *Pseudomonas aeruginosa*.

The dehydrated and sterilized finely ground form of uric acid fortified wheat straw, chick peas, broccoli, and pigeon fecal were investigated, and surprisingly, the *Bacillus subtilis* MM13 managed effectively the bird fecal matter and demonstrated significant uricase enzymatic activity as 0.7 U/ml, which was substantially lower than that of the pre made pattern of urate (1.17 U/ml). This result indicates that perhaps the normal urate supplemented contributors tested had no measurable influence on enzyme production in *Bacillus subtilis* MM13. This could be due to inadequate uric acid substrate in these sustainable materials.

Uricase synthesize under optimized conditions

The preliminary results of the optimization research indicate that glucose along with yeast extract at pH 7.0 and 40°C seemed to be appropriate carbon and nitrogen factors for outstanding uricase enzymatic activity in *Bacillus subtilis* MM13. As a result, the optimal concentrations of these two power sources for *Bacillus subtilis* MM13 to reach high uricase enzyme activity were determined to be 1% sucrose and 0.2% yeast extract, yielding 2.54 and 2.37 U/ml, respectively. According to another report, *Bacillus subtilis* SP6 extracted and purified from chicken manure was discovered to be a uricase manufacturer and addressed and resolved uricase activity employing lactose as both a carbon source as well as soya peptone as a source of nitrogen at concentrations of 12.2 and 12.79 g L^{-1}, respectively **Pustake *et al.*, (2019).**.

The optimized carbon and nitogen source concentration can sustain cell growth and metabolic activity, which increases enzyme synthesize activity and chelates the various metabolic process **Jacob *et al.*, (2018**). *Proteus vulgaris* isolated from soil samples prefers 15% glucose and 0.5 % ammonium phosphate as carbon and nirogen sources respectively and generates huge uricase enzymatic activity in a short period of time.

Similarly, **Jagathy *et al.*, (2016)** isolated the *Bacillus subtilis* strains from poultry wastes from Tamil Nadu and identified that possess remarkable uricase producing activities. Furthermore, they also optimized the suitable growth conditions for this *Bacillus subtilis* strains to yield maximum uricase activity as pH 7.0, 30°C, 1% sucrose, 0.2% of peptone and 24 hours of incubation time. Under this optimized conditions the *Bacillus subtilis* strains yielded about 32 U/ml^{-1} of uricase. Interestingly

after a partial purification of attained uricase enzyme its activity is significantly increased up to 60.0 U/ ml^{-1}.

Extraction, purification, and characterization of uricase

Uricase plays an important role in nitrogen metabolism and can be used as a diagnostic representative in medical science. The uricase enzyme has been discovered in vertebrate creatures **Nelapati *et al.*, (2021).** Moreover, this information has recently been obtained from a variety of microorganisms, which include bacteria. The enzyme was discovered to be available in every case **Murea and Tucker, (2019).** The presence of uriate or even another stimulant with in reaction volume is needed for such enzymatic activity **Nanda and Babu, (2014)**. Multiple microbes can use urate as a predominant nitrogen basis or meet their carbon and nitrogen requirements by generating uricase, and then this reaction was identified through as a clear zone that aided the microbial growth on agar media.

Similarly, fungal or bacterial growth has no effect on uricase production in uric acid-containing media **Murea and Tucker, (2019)**. In certain cases, the uricase enzyme can really be retrieved from certain bacterial species without causing any harm to the cell. *B. fastidiosus* and *P. aeruginosa*, for example, can produce extracellular uricase. As a result, no need for cell disruption **Nanda and Babu, (2014)**.

Molecular weight analysis

In the present study, the molecular weight of the cell was found as 48 kDa through SDS-PAGE analysis. This result was exactly correlated with the findings of they conveyed that the uricase enzyme molecular weight of extracted from *Bacillus subtilis* strains isolated from poultry wastes was reported as 48kDa through SDS-PAGE

analysis **Jagathy *et al.*, (2016).** In an another study, the purified uricase enzyme showed an individual protein group with a molecular weight (MW) of 44.0 kDa through SDS-PAGE analysis **El-Naggar *et al.*, (2016).** Uricase derived from various causes might have varying MW as well as amino acid sequence data **Fan *et al.*, (2021)**. In an investigation, uricase's molecular weights were estimated to be 34.0 to 54.0 kDa **Ghosh and Sarkar, (2014).** The purified thermotolerant enzyme (uricase) that can be applied in many clinical applications in which thermostability is significant **Hafez *et al.*, (2017**). The ideal temperature and pH for ultimate uricase activity seem to have been 45 °C and pH 8.5, respectively **Hu *et al.*, (2018).**

Optimal cofactors required for better uricase activity

To operate effectively some enzymes may require the addition of some other non-protein moiety **Peters *et al.*, (2013).** Those are all widely recognized as cofactors, and without these cofactors the enzymes are inactive "apoenzyme" forms persist. When the cofactor is introduced, the enzyme transforms into an active "holoenzyme" **Vignais and Colbeau, (2004).** Co - factors can sometimes be ions like zinc as well as metal salts or organic molecules like vitamins or vitamin-derived substances. Much of these co - factors might well bind adjacent the substrate binding location toensure the substrate attach towards the enzyme **Rumpf and Jentsch, (2006)**. Co-factors have been classified as "prosthetic groups" or "co-enzymes" depending about how firmly they are bonded towards the enzyme; co-enzymes attach quite weakly to the enzyme and are therefore altered during in the enzymatic activity, whereas prosthetic groups attach more firmly and are therefore not altered **Bhatia and Bhatia,(2018**).

It can also be ions like Zn^{2+} ions or Fe^{2+} ions in enzymes. Substances like tryptophan tryptophylquinone (TTQ) play a role as a catalytic subunit in methylamine dehydrogenase-catalyzed reactions **Anbu *et al.*, (2014).** Some other compound, flavin adenine dinucleotide (FAD), can always be recreated in during catalyzed activity and can therefore be assumed a prosthetic band because its overall concentration remains constant **Johnson *et al.*, (2021).**

In the existing investigation, the purified uricase enzyme showed outstanding activity at 35°C and pH 8 in the presence of Cu^{2+}. These enhance the uricase enzyme activity on uric acid metabolism. Because some uricases necessitate covalently bonded metallic ions or co-factors, which are components of uricase **Nanda and Jagadeesh Babu, (2014)**, such elements are critical for sustaining the highest catalytic performance. Chelating reactant (20 mM EDTA) and the ions such as Li^{+}, Ag^{+}, and Hg^{+} strongly suppressed the activity of enzymes **Sherman *et al.*, (2008).** In some instances, uricase might emerge as tetramer of repeating units, each with two Cu binding spots; this trait differed between bacterial species **Sunny *et al.*, (2007)**. The Cu^{2+}, Fe^{3+}, $Ag^{+,}$ and Zn^{2+} were also acting as moderate uricase inhibitors; though, they never affect the activity of uricase derived from some bacterial species, such as uricase derived from *Bacillus subtilis*. Finally, the uricase derived from *Bacillus subtilis* might be considerable to produce clean uricase, which can reduce uric acid quantity and test uric acid in urine and blood samples.

Hyperuricemia and its associated diseases can be treated by agents that upholds urate concentrations from precipitating and eliminate prevailing urate crystals. Uric acid-lowering agents (ULTs) classified into three main classes: xanthine oxidase

inhibitors that reduce uric acid synthesis, uricosurics that inhibit URAT1 thereby increasing uric acid excretion, urolytics/ uricase that enhance metabolic hydrolysis of uric acid **Benn *et al.*, (2018)** have been used conventionally in the treatment of hyperuricemia ***et al.*, (2016).** Among these three classes, Uricase are said to be effective in treating diverse forms of hyperuricemia and removing pre-existing urate crystals in joints and believed to have negligible drug-drug interaction, however, available approved uricase formulations have been reported unsuitable for continuous treatment **Yang *et al.*, (2012**).

Myeloperoxidase and other peroxidases oxidise uric acid, producing the unstable urate free radical, which responds with superoxide at a diffusion-controlled rate to form urate hydroperoxide **Carvalho *et al.*, (2018)**. This process is especially important in the phagosome, where superoxide, hydrogen peroxide, and myeloperoxidase are abundant **Winterbourn *et al.*, (2016).** Urate hydroperoxide is a powerful oxidant that has the potential to be a bactericide. As a result, the oxidation of uric acid and the generation of urate hydroperoxide in neutrophils may contribute to their bactericidal impact. Owing to this, uricase from microbial sources can be represented as an alternative approach that can be more effective than the modified uricase formulations **Tripathi *et al.*, (2020).** In arthritic gout and renal disease, microbial uricase has emerged as a potential source of therapy for hyperuricemia **Chiu *et al.*, (2021).** Fermented extracts from microbes with substantial XOD inhibition activity and high uricase activity have also been reported **Chen *et al.*, (2017).**

Though various bacteria such as *Bacillus* species **Tan *et al.*, (2010**), *Microbacterium* sp **Zhou *et al.*, (2005)** and *Streptomyces* sp **Nanda *et al.*, (2014),** have

been reported as uricase producers, not all bacterial uricase are thermostable and their biocompatibility and anti-inflammatory and the anti-hyperuricemic activityare unknown. Uricase enzymes with these properties will have the advantage for mass production, transportation, storage and clinical application. Among the other bacteria's, urate oxidase from thermophilic *Bacillus* sp. TB-90 has been reported to be used for diagnostic purposes since it reveals high activity and thermostability in an extensive range of pH **Asano *et al.*, (1971)**. Based on this theory, *Bacillus subtilis* was isolated from soil samples in and around Hosur area, Krishnagiri District, Tamil Nadu and an isolate that produced the highest uricase activity was identified, designated as MM13 **Manimekalai**, **(2019)** and its efficacy was evaluated. The primary aim of this study is to review the anti-inflammatory and the anti-hyperuricemic activity of the potent local uricase produced by MM13 was evaluated in LPS-induced RAW 264.7 cells.

Application of uricase

The most frequent inflammatory arthritis, characterized by prevalence higher than 1% in both occidental and oriental countries is gout **Dehlin *et al.*, (2020)**. A disease that should be considered seriously, due to its association with various other complications including hypertension, cardiovascular risk, chronic kidney disorder, obesity, and metabolic syndrome **Choi *et al.*, (2005)**, **Daoudi *et al.*, (2020).**

Though a variety of treatment strategies including are followed, their adverse effects limit the effective clinical applications. Despite advances in therapy, the management of gout is still a challenge and a search for new and safe alternative agents were necessitated **Fields, (2019)**. Uricase, a therapeutic enzyme has examined to play a crucial role in gout treatment. Majority bacteria comprising animals, plants, and

microbes can produce uricase, amongst them microbial sources have an advantage because of their faster growth rates, simple medium adjustment, and low-cost bioprocessing **Nanda and Jagadeesh Babu, (2014)**. The current study evaluates the anti-inflammatory and anti-hyperuricemic potential of one such uricase produced by locally available *Bacillus* species (designated as MM13).

MTT and trypan blue assay revealed that MM13 uricase had strong inhibitory activity against LPS induced RAW 264.7 cells in a dose-dependent manner. At 100 µg/ml concentration more than 80% of cell growth was inhibited. The IC_{50} was determined to be 50 µg/ml by the MTT assay which was further confirmed by trypan blue assay. Hence, a concentration one below and one above the IC_{50} was selected for further studies along with the concentration which exhibited the IC_{50} range.

Morphological evaluation on the concentrations 30 µg/ml, 50 µg/ml and 60 µg/ml were in line with the MTT and trypan blue assay results. Changes indicative of cell inhibition, where LPS induced RAW 264.7 cells deteriorated, in a dose-dependent manner and parallel morphological changes including shrinkage, blabbing and distorted cell membrane followed by cellular detachment were observed in response to MM13 uricase treatment.

Xanthine oxidase plays the role of housekeeping and the rate-limiting enzyme during purine catabolism. In this study, the inhibition of XO activity was found in all uricase treated concentrations (30 µg/ml, 50 µg/ml and 60 µg/ml). LPS induced RAW cells showed higher activity compared to treated and control groups ($p \leq 0.01$). Despite the fact that there were substantial differences between the control (untreated) and treated groups. No significant difference was observed between the three

concentrations. All uricase concentrations showed inhibition of more than 50%. Xanthine oxidase inhibitors(XOI) are considered the first-line drugs in ULT for the gout treatement **Bove *et al.*, (2017).** Furthermore, studies on hyperuricemic subjects revealed that XOIs are more effective in secondary inhibition, considerably lowering the happening of major adverse cardiovascular events in people who had earlier experienced transient ischemic attacks, stroke, unstable angina, or myocardial infarction **Bredemeier *et al.*, (2018)**. In the current investigation, all uricase-treated RAW cells were inhibited by more than 50%, demonstrating uricase's anti-hyperuricemic action.

Prevention and maintaining uric acid at normal levels may reduce gout, hyperuricemia and its related chronic diseases. Therefore, we also evaluated the urate-lowering effects of MM13 uricase at different concentrations on LPS induced RAW 264.7 cells. Uricase at all three concentrations (30, 50 and 60 µg/ml) was found to be effective in reducing uric acid, which was consistent with the XO inhibition results that suggested that the results of the hypouricemic action paralleled the reduction in XOI activities. As a preliminary evaluation, this study first confirmed that MM13 uricase reduced the uric acid levels suggesting that this study should be furthered in vivo to be confirmed as a therapeutic model.

We further elucidated the molecular mechanism of MM13 uricase by Gene expression analysis. The release of cytokines with pro-inflammatory properties contribute a major role in the commencement of the gout disease. In fact, the crystals of monosodium urate provide a dose-dependent stimulus that leads to the activation of monocyte and then releases of TNF. Studies have proposed that stimulation of IL- I

and TNF production by monosodium crystals maybe a crucial link and the inhibition of these could be a promising strategy **Di Giovine *et al.*, (1991), Pierce, (1990); Erwig and Rees, (1999) Wadleigh *et al.*, (2000)**.

In the present investigation, gene expression studies with uricase from MM13 indicated a dose-dependent suppression of TNF-α. TNF-α was strongly down-regulated by a higher dose of uricase whereas a milder suppression was observable in a low dose of uricase. To further evaluate if uricase triggers cytokine suppression in LPS induced RAW 264.7 cells, we estimated the expression of the IL-10 gene, a major anti-inflammatory cytokine. From our results, it is evident that IL-10 reduces the effect of TNF-α indicating that the uricase from MM13 has significant anti-inflammatory activity. A report on IL-10 suggests that reducing the effect of TNF-α significantly decrease the secretion of PGE2, COX-2, and PLA2 **Wojdasiewicz *et al.*, (2014)**.The reduction in the levels of TNF- α in the treated cells indicates that the uricase from MM13 has significant anti-inflammatory activity.

Anti-hyperuricemia effects

An enzyme medication, including such uricase usage, denotes the much more especially effective approach than conventional chemotherapeutics. Globally, the prevalence of hyperuricaemia and associated illnesses is rapidly increasing **Chen *et al.*, (2016)**. Allopurinol and febuxostat, which are frequently used, seem to be inefficient at disintegrating established uric particles, as well as their lengthy use may disrupt purine metabolism **Wu *et al.*, (2021).** Uricase means converting uric acid into much more dissolved allantoin to dissolve current uric acid. Since over four decades, uricase is being used in the early diagnosis of urate as well as the therapies of hyperuricemia **Rees *et al.*, (2014)**. It is often used to diagnose and reduce tumour lysis symptoms

Zhou *et al.*, (2016). A Phase I research of uricase for the treatment of acute and chronic graft-versus-host illness following myeloablative allogeneic bone marrow transplantation was recently completed. Moreover, wider use of uricase has really been impacted by major drawbacks attributed to innate enzyme characteristics, including such fairly low enzymatic performance and therapeutic efficacy under physical illnesses **Yan *et al.*, (2019)**. As a result, outstanding uricase dosage forms with improved stability and high catalytic activities while prescribed in vivo are urgently needed. Several attempts have been devoted to enhance uricase, such as: establishing novel uricase and chimeric uricase to greater activity via mutation; immobilizing in polyethylene polypyrrole-polyvinyl sulphonate film to acquire new biomarkers; conjugated verbs to polyethylene glycol to attain sustainable benefits include improved enzymes durability; and enveloping in alkaline enzymosomes (Nano sized liposomes) **Zhou *et al.*, (2016).**

Three approaches are being continued to pursue to provide such a favourable micro - environment for uricase in order to finally enhance its own catalytic efficiency: through using right kinds of barrier to comprise the distillation process **Schmidt *et al.*, (2020).** Where the uricase tries to play the pivotal role; enclosing the uricase within a fatty acid vesicular membrane, which again is primarily a partially permeable membrane; as well as encapsulated CAT as well as uricase inside the same cell wall to provide further substances for uricase (i.e., oxygen) **Rees *et al.*, (2014)**. The uricase system functions as a systemic circulation bioreactor, consisting primarily of a high - molecular - weight uricase, a lipoprotein enzymosome cell wall, as well as an inner liquid micro - environment whereby the catalyzed reactions occur **Pierigè *et al.*, (2017).**

The lipoprotein enzymosomal membrane is typically thought about as a different rates membrane pores, comparable to a biomembrane, capable of locating and isolating uricase of large molecular mass (140 kD) within the fermenter and isolating the uricase from the various external blood system **Carvalho *et al.*, (2018a)**. In contrast, uricase's low - molecular weight substrates can pass through the membrane, and also the eventually results catalysates (allantoin and H_2O_2) can exit the fermenter **Johnson *et al.*, 2021**.

It is worth noting that such uricase catalysate (H_2O_2) seems to be the the only starting material of CAT (always a pharmacotherapy enzyme), while the eventually results CAT catalysate (oxygen) is among uricase's substrates **Ming *et al.*, (2021)**. When both uricase as well as CAT were being encapsulated inside the same enzymosome at the same time, this should have been a more advantageous delivery mechanism than uricase on its own **Carvalho *et al.*, (2018).** Furthermore, the alkaline buffer seems to be important and essential as it is the only component of such an uricase distribution system's functional micro - environment, as well as the impacts of natural pH on enzymatic activity seems to be hugely important **Liu *et al.*, (2021)**. Since the configurations of enzymes in various buffer solutions are alterable, the functions of such an enzyme can be impacted by the buffer typeA most commonly utilized uricase buffer seems to be borate buffer; nevertheless, this should not imply that this was the best alternative. Obviously, more research is needed so that we can determine whether the buffer is best for an ESU or ESUC **Hacker *et al.*, (2014).**

The development of efficient nanostructure pharmacotherapy enzymosomes is just a beneficial strategy in such an efficient enzymatic treatment that really is distinct

from conventional chemotherapeutics as well as the emerging new genetic manipulation **Bomalaski *et al.*,(2002)**. The study of enzymosome micro-environmental as well as rearrangements has led to a better understanding of such an efficient enzymatic therapy strategy for illness remedy **Chen *et al.*, (2016).** The improved properties of ESUC-D were caused by favorable micro-environmental as well as rearrangements in uricase, which were achieved through three techniques: selecting a bicine buffer for uricase, entrapping uricase into such a phospholipid bilayer, as well as abducting CAT alongside uricase **Nie *et al.*, (2020).**

Photoluminescence spectroscopy, spherical dichroism, born and bred gel electrophoresis, as well as conductivity were used to examine the favourable micro-environment as well as conformational changes of ESUC-D **Johnson *et al.*, (2021**). Enhance the final electrophoresis was used to examine the molecular as well as chemical changes of uricase. ESUC-D could be a beneficial uricase range of potential applications, as well as complicated alkaline enzymosomes could be a great option for such a pharmacotherapy enzyme **Ming *et al.*, (2021).** There is still much progress to be made until we can successfully use ESUC in health centers. More research is needed to assess ESUC's prolonged storage consistency and rearrangements, as well as its immunogenicity as well as enzymatic resistance after concurrent administration **Nie *et al.*, (2020).**

Uricase mimic enzyme applications

Since of their chemical stability, cost of nanozymes were such an innovative research area that could substitute normal enzymes. Moreover, most nanozyme study has concentrated on peroxidase, with such little attention paid to nanozymes to oxidase-

like action, particularly those that simulated oxidase of molecules associated with human biology **Liu *et al.,* (2021).** Hyperuricemia and gout can be associated with high uric hormones in the blood. Since it could oxidize urea, organic uricase healed this illness **Ming *et al.,* (2021)**. The oxidase-like operation of variable oxidation state metal organic structures with cerium (MVSM) would have previously been investigated and reported.

A wide range of analytical techniques were used to investigate the catalytic procedure of urate with MVSM, which would have been close to the normal uricase with the exception of additional oxidation of H_2O_2 **Saeid *et al.,* (2018).** Since of their chemical stability, cost of nanozymes were such an innovative research area that could substitute normal enzymes **Johnson *et al.,* (2021).** Moreover, most nanozyme study has concentrated on peroxidase, with such little attention paid to nanozymes to oxidase-like action, particularly those that simulated oxidase of molecules associated with human biology **Huang *et al.,* (2021).** Hyperuricemia and gout can be associated with high uric hormones in the blood. Since it could oxidise urea, organic uricase healed this illness **Chen *et al.,* (2016).**

The oxidase-like operation of variable oxidation state metal organic structures with cerium (MVSM) would have previously been investigated and reported. A wide range of analytical techniques were used to investigate the catalytic procedure of urate with MVSM, which would have been close to the normal uricase with the exception of additional oxidation of H_2O_2 **Dmytruk *et al.,* (2011)**. The Michaelis-Menten equation to determine the electro catalytic constant values of MVSM. In both in vitro and in vivo models experimentations, MVSM catalyzed urate better. It really was possible to

develop an enzyme-free assessment resemble uricase for urate. All of the experiments showed that MVSM used to have a great chance of replacing organic uricase **Liu *et al.*, (2021).**

Uricase formulated polyethylene glycol

People have such a nonsense base pairs incorporated into the 5 prime end of a transcription start site of uric acid oxidase, resulting as in expression of such a proteolytic enzymes inactivated fragment of such a protease; as a result, they seem unable to convert urate into allantoin and thus are susceptible to hyperuricemia as well as gout **Hancks and Kazazian, (2016).** Numerous urate oxidases from mammalian species and microbes have been given to hyperuricemia and gout patients. Despite their achievement in reducing plasma urate, such therapeutic approaches have seen constrained use due to the unwanted chemical characteristics of the enzymes utilized, the summary circulatory half-life, as well as the innate immunogenicity of such preparations **Liu *et al.*, (2021**). **Bomalaski et *al.*, (2002)** used urate oxidase obtained from Candida utilis seemed to have greater enzymatic characteristics, as well as PEG of 20,000 MW (nicknamed uricase-PEG 20) used to have significantly lower immunogenicity and a longer circulatory half-life than any of those described earlier. Uricase-PEG 20 is expected to be useful as a therapies for hyperuricemia as well as gout **Bomalaski et al., (2002)**.

Microbial urate biosensor

Numerous biosensors used for urate persistence in serum and urine demand previously been exposed. Countless amperometric urate based sensors predicated on uricase as well as uricase combined with peroxidase have already been explained, with

differences in electrode type, immobilization methodology, capacity as well as kind of of reductant mediator(s), superconductors, and so on **Kanaparthi *et al.*, (2019)**.

Rendered immobile uricase on to some of gold nanoparticles as well as carbon nano-tubes resulted in the creation among many innovative amperometric urate biosensors**.** Such uric acid crystals biosensors employ costly commonlyavailable uricases extract obtained from *B. fastidiosus, A. globiformis,* or *C. utilis* **Zhang, (2013)**. As a result, producing low-cost urate-selective amperometric biosensors remains a difficult task. As a bioreceptor, microbes of such chimeric heat tolerant methylotrophic yeast *Hansenula polymorpha* were used to create a uric acid crystals microbiological biosensor **Andreu and Li del Olmo, (2018).** The development of uricase yielding yeast was being characterized by over-expression of such uricase gene of *H. polymorpha*. Having followed an initial evaluation of transgene with enhanced uricase activity in coating system yeast cells, the ideal cultivation circumstances for maximum uricase yield, thus a 40-fold rise in uricase activity, were identified **Koleva *et al.*, (2020)**. The uricase producing microbes were physically entrapped behind such a dialysis bag and immobilized on graphite by coupling to peroxidase . A elevated urate specificity was discovered, with such a limit of detection of around 8 μM **Dmytruk *et al.*, (2011)**.

Engineered uricase for oral treatment

Hyperuricemia, characterized as a serum uric acid concentrations higher than 7.0 mg/dl, affecting nearly 20% of American adults as well as facilitates the development of hyperuricemia (gout), that has a 4% occurrence in the United States **Borghi *et al.*, (2015).** Tissue filled with monosodium uric acid crystals in gout

encourage both associated with inflammatory arthritis. Furthermore, urolithiasis is widespread in gout and therefore is caused by reduced urate solubilization with in acidic condition of such urine as well as elevated urinary uric acid density **Borghi *et al.*, (2018)**. Gout is linked to lower wellbeing standard of living and higher health-care utilization. Furthermore, hyperuricemia has already been suggested as a promising contributing to the pathophysiology of multiple health conditions connected with gout as well as symptomless hyperuricemia **Chalès, (2019).** High blood pressure, kidney disease, fatness, metabolic disease, type 2 diabetes, as well as coronary heart disease are examples. Even though some hyperuricemia patients have hereditary diseases that make urate increased production, a most frequent condition of gout is inadequate kidney excretion of urate **Ragab *et al.*, (2017)**. Approximately two-thirds of urate efflux through normal persons is renal. Uric acid is also naturally produced into the intestinal tract, in which it is deteriorated by gut microbes (small intestine uricolysis) or excreted **Stiburkova *et al.*, (2019)**. Through people with normal urate metabolic activities, bowel secretion responsible for roughly one-third (200 mg/day) of everyday uric acid in the blood eradication, with both the power to enhance urate eradication to 300 mg/day **Skolarikos *et al.*,(2015).**

For those with Renal dysfunction, the portion of urate completely eradicated via the intestine may significantly raise to 50 to 70%, overtaking renal eradication **Liu *et al.*, (2021)**. The assessment that sevelamer, a non-absorbed phosphate binding agent which is also a semi urate adhesive molecule, greatly reduces serum uric acid concentration in hyperuricemic patients supports the significance of bowel urate removal in CKD patients **Grinfeld *et al.*, (2010).** The significance of the sevelamer uric acid-lowering consequences, nevertheless, was really only reasonable,

with such a mean serum urate reduction of 0.6–0.7 mg/dl achieved in a randomized clinical trial, with the greatest serum urate decrease witnessed in subject areas with its most serious hyperuricemia **Viggiano *et al.*, (2018).** Urate transit and urate homeostatic mechanisms are maintained by a complicated web of carriers situated in the renal tubules and the small bowel. SLC22A12, SLC2A9, as well as the urate secretory transporter ABCG2 seem to be significant carriers associated in renal urate disposal which are encrypted by gout-risk genes (ATP-binding cassette) **Lai *et al.*, (2017).**

ABCG2 can be demonstrated in the small bowel, in which it plays a significant role in reducing serum urate, unlike the most of kidney urate carriers. Furthermore, as kidney function starts to decline, the direct effect of intestinal ABCG2 on urate homeostasis tends to increase. It is also demonstrated in animal modeling techniques, where normal serum urate levels were retained despite continued bowel ABCG2 interpretation in normal rats undergoing subtotal surgical excision. Similarly, knocking out the ABCG2 carrier led to reduced bowel urate removal as well as hyperuricemia advancement in null mice **Hoque *et al.*, (2020).** Specific corrupt and inefficient ABCG2 variations in living beings are linked to hyperuricemia as well as an elevated chance of hyperuricemia, such as early symptoms of hyperuricemia illness as well as appearance of the serious tophaceous hyperuricemia trait **MacKenzie, (2015).** Of that kind variations include the widespread pervasiveness ABCG2 Q141K, that has an especially high genotype frequencies (up to 30%) in numerous Asian communities and, not surprisingly, an even greater allele frequency (up to 50%) throughout patients with gout from such populations **Ieiri, (2011).** Hyperuricemia as well as gout are caused by extreme disorder of ABCG2 activity, which is affiliated to extreme renal urate filtration **Toyoda *et al.*, (2021).**

Gout as well as hyperuricemia are more common in CKD patients than for those to maintained kidney function, with such an approximate population of gout in patients with primary 3 CKD with in United States ranging from 20 or 30 % **Vargas-Santosand Neogi, (2017).** This poses a great challenge to healthcare professionals desiring efficient treatment to the patients to Renal dysfunction, because both renal impairment as well as nephrolithiasis restrict the use of established urate-lowering treatments, such as uricosurics, which significantly raise uric acid efflux, as well as xanthine oxidase inhibition, due to the increased risk of toxic potential just at optimized therapeutic dose. Considering the current limitations treatments as well as the rigorous efflux of urate into small bowel, a fresh uricase, ALLN-346, must have been developed as just a possibly safer alternative treatment which is not soaked up into bloodstream and has the ability to degrade the urate naturally produced and/or founded in the small bowel, thus further lowering the systemic urate hardship **Hyndman *et al.*, (2016)**.

The protein engineering based modification in *Candida utilis* and produced uricase to confer proteolytic consistency while maintaining particular activity as compared towards the majestic enzyme employing specialized Protein based GPS equipment **Hyndman *et al.*, (2016).** The proteolytic consistency of an uricase in the gastrointestinal system was improved through with a computer vision molecular transformation process, allowing it to deteriorate urate. **Pierzynowska *et al.*, (2020).**

Chapter – 6

SUMMARY

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The focus of present study was to obtain uricase-producing bacteria from fecal contaminated soil from bird farms (pigeon, turkey, parrot, and chicken farms). Based on the size of the zone of measurement (37mm) around the colonies on uric acid agar medium, one among ten isolates was identified as a probable uricase synthesizing bacterial isolate (from pigeon faecal contaminated soil). The chosen bacterial isolate was labeled as MM13 strain and identified as *Bacillus* based on morphological and biochemical characterization studies. Furthermore, using 16S rRNA sequencing, the isolate MM13 was recognised as *Bacillus subtilis*, and the resulting sequence was submitted to NCBI-GenBank, accession number MK503710. BLAST analysis of the MM13 *Bacillus subtilis* MK503710 sequence revealed that the isolated bacterium seemed to have the closest homology (99%) with *Bacillus subtilis*.

The optimal essential growth factors required to obtain maximum uricase enzyme activity from *Bacillus subtilis* MM13 were found as pH 7.0, 40°C, 1% sucrose, 0.2% yeast extract, and 0.32% of uric acid. Under above condition , yield of uricase ranged from 1.25 to 2.54 U/ml submerged fermentation process under optimized conditions for 48 hours.

In this study, the uricase enzyme was extracted from *Bacillus subtilis*, and it was initially precipitated and purified with 80% ammonium sulphate and membrane dialysis. It was followed by DEAE cellulose with uricase 12.55 fold, and DEAE sephadex A-50 column with 15.08 fold with overall yield of yield of 23.15%.

The molecular weight of this purified uricase enzyme was 48 kDa determined by SDS PAGE. This purified uricase activity was seen as 1352 U/mg and the final specific pure protein quantity as 1.6 mg/g. The optimal temperature, pH, and metal ions required to express the ultimate activity of the uricase enzyme was 35°C, pH 8.0, and Cu_{2+}.The temperature and pH stability of this *Bacillus subtilis* produced uricase enzyme ranged from 25ºC to 45ºC and 6.0 to 9.0.

Gout, the most common inflammatory arthritis is associated with a persistently raised plasma uric acid concentration. When these levels exceed the solubility limit, crystal deposition in joints occurs, as does acute inflammation with intense pain. Injections of non-human uricase, as well as anti-hyperuricemic and anti-inflammatory medicines, are used to lower uric acid levels and inflammation in severe gout. Although various microbiological sources of uricase have been hypothesised, few have been identified and tested as prospective anti-hyperuricemic and anti-inflammatory medicines. Uricase isolated from *Bacillus subtilis* MM13 can be used as therapeutic agent to reduce the uric acid and also used as diagnostic agent for urine and blood sample analysis. In the contemporary study, the anti-inflammatory and the anti-hyperuricemic activity of the uricase produced by *Bacillus* (designated as MM13), isolated from soil was evaluated in LPS-induced RAW 264.7 cells. The anti-inflammatory and anti-hyperuricemic activities of MM13 was assessed in lipopolysaccharide (LPS)-induced RAW 264.7 cells. To measure the effects of MM13, MTT assay, trypan blue assay, xanthine oxidase activity assay, uric acid production and gene expression analysis were performed. MTT and trypan blue assay demonstrated that uricase suppressed cell proliferation in a dose-dependent manner. The *in vitro* anti-

hyperuricemic effect of uricase was identified by inhibition of xanthine oxidase in LPS-induced RAW 264.7 cells. Further, gene expression studies confirmed uricase efficacy to conquer the expression of inflammatory cytokine, tumour necrosis factor α (TNF- α), and activate production of IL-10, a potent inhibitor of TNF- α. Considering the appreciable *in vitro* anti-hyperuricemic and anti-inflammatory activities, the current study proposed that this preparation can be further studied for safety and efficacy for therapeutic application of gout.

Chapter – 7

CONCLUSION AND SCOPE OF FUTURE

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The current study indicated that both the isolation of a powerful uricase-producing bacteria and the optimization of uricase production were effective. On the basis of 16s rDNA sequencing, this newly isolated pigeon faecal contaminated soil bacteria was identified as MM13 *Bacillus subtilis* MK503710. The flask approach identified 1%sucrose, 0.2%yeast extract, and 0.32 % uric acid as key parameters, which were further improved using submerged fermentation process. When compared to the initial production medium, the yield of uricase was increased by 15.08 fold, with a yield of 23.15% in the refined medium. *Bacillus subtilis* MM13 MK503710 is the most prolific uricase-producing bacterium known to date, producing 2.54 U/ml of uricase enzyme, which is more than any previously recorded bacterium and equivalent to many bacterial uricase synthesizers. The enzyme may be industrially important for in vitro clinical applications in uric acid detection due to its pH stability, thermostability, extended half-life, and storage stability. Furthermore, no uric acid analogues were shown to be competitive enzyme inhibitors, showing that uricase specificity is strong. Finally, gene expression analysis revealed that uricase reduced the expression of the inflammatory cytokine tumour necrosis factor (TNF-) while increasing the production of IL-10, a strong inhibitor of TNF-. Given the significant anti- hyperuricemic and anti-inflammatory actions observed in vitro, we suggest that this formulation be tested in vivo for safety and effectiveness before it is recommended for use in the treatment of gout.

REFERENCES

REFERENCES

Abbas, A.A. (2016). Extraction, optimization of uricase from *Aspergillus niger*. *Int. J.Adv.Res.*, 4(3): 1865-1872.

Abdel-Fattah, YR, Saeed, HM, Gohar, YM & El-Baz, MA. (2005). Improved production of *Pseudomonas aeruginosa* uricase by optimization of process parameters through statistical experimental designs. *Process Biochemistry*, 40, (5), pp:1707-1714.

Abdullah SK, Flayyih MT. (2015). Evaluation the Uricase Produced from Different Clinical isolates of *Pseudomonas aeruginosa* by Plate Assay Methods. *World J Exp Biosci* 3: 26-29.

Abdullah, S.K. and M.T. Sneha Sneha Flayyih.(2015). Study the effect of purified uricase from *P.aeruginosa* on Hyperuricemia in Vivo. *Int. J. Biol.Pharma*. Res., 6(6): 469-472.

Adamek, V., B.Kralova, M.Suchova, O.Valentova and K.Demnerova. (1989). Purification of microbial uricase. *J. Chromatograph*., 497: 268- 275.

Akhtar, N., A. Shoaib, S. Munir, A. Ali and S. Khurshid. (2014). Isolation, identification and enzyme production profile of *A. niger*. J. *Anim. Plant Sci*., 24(5): 1438-1443.

Alderman, M.H., H. Cohen, S. Madhavean and S. Kivlighn. (1999). Serum uric acid and cardiovascular events in successfully treated hypertensive patients. *Hyperten*., 34:144–150.

Aly M., Tork S., Al-Garni S., Allam R. (2013). Production and characterization of uricase from *Streptomyces exfoliatus* UR10 isolated from farm wastes, *Turkish Journal of Biology*, 37 (2013) 520-529.

Amirthanathan, A., and Subramaniyan, A. (2012). Studies on uricase production by marine *Bacillus cereus* and its optimum conditions. *Int J Med Biosci*,1:05-12.

Ammar, M. S., S. H. Elwan and E. M. El-Desouky. (1988). Purification and some properties of uricase from *Aspergillus flavus* S-97. *Egypt. J. Microbiol*.,23: 83.

An, Y., Wang, Y., Liang, X., Yi, H., Zuo, Z., Xu, X., Zhang, D., Yu, C., Han, X. (2017). Purification and partial characterization of M1-UVs300, a novel bacteriocin produced by *Lactobacillus plantarum* isolated from fermented sausage, *Food Control*, 81: 211- 217.

Anbu, S., Ravishankaran, R., Guedes da Silva, M.F.C., Karande, A.A., Pombeiro, A.J (2014). Differentially selective chemosensor with fluorescence off–on responses on Cu^{2+} and Zn^{2+} ions in aqueous media and applications in pyrophosphate sensing, live cell imaging, and cytotoxicity. *Inorganic chemistry*, 53(13): 6655-6664.

Anderson, A & Vijayakumar, S. (2011). Purification and optimization of uricase enzyme produced by *Pseudomonas aeruginosa*, *Journal of Experimental Sciences*, 2, (11): 5-8.

Andreu, C., Li del Olmo, M. (2018). Yeast arming systems: pros and cons of different protein anchors and other elements required for display. *Applied microbiology and biotechnology*, 102 (6):2543-2561.

Asano, S., K. Yamamoto, S. Teshima, T. Kikuchi, Y. Kawamura. (1971). Study of a liquid type reagent for determination of uric acid by using thermostable recombinant uricase. *Rinsho Kagaku*. 23: 214–220.

Atalla, M. M., Farag, M. M., Eman, R. H., Abd-El-Lataif, M. S. and Nehad E. A. (2009). Optimum conditions for uricase enzyme production by Gliomastix gueg. *Malaysian Journal of Microbiology*, 5: 45-50.

Atty, F. K and Joseph, J. (2016). Isolation and identification of uric acid degrading bacteria, optimization of uricase production and purification of uricase enzyme. *International Journal of Advanced Research* 4: 2732–2742.

Azab, A.E., Magda, M.A., and Mervat, F.F. (2005). Studies on Uricase induction in certain bacteria. Egyptian Journal of Biology 7: 44-54.

Benn, C. L., Dua, P., Gurrell, R., Loudon, P., Pike, A., Storer, R. I., & Vangjeli, C. (2018). Physiology of hyperuricemia and urate-lowering treatments. *Frontiers in Medicine*, *5*:160.

Bergey, D. H., Krieg, N. R., & Holt, J. G. (2000). *Bergey's Manual of systematic bacteriology*. Williams & Wilkins.

Bhatia, S., Bhatia, S. (2018). Introduction to enzymes and their applications. Introduction to Pharmaceutical Biotechnology*; IOP Publishing Ltd.: Bristol, UK*, 2. *Bioengg*., 19:599–603.

Bomalaski, J.S., Holtsberg, F.W., Ensor, C.M., Clark, M.A. (2002). Uricase formulated with polyethylene glycol (uricase-PEG 20): biochemical rationale and preclinical studies. *J Rheumatol*, 29(9), 1942-9.

Bongaerts, G. P., & Vogels, G. D. (1976). Uric acid degradation by *Bacillus fastidiosus* strains. *Journal of Bacteriology*, 125(2):689-697.

Bordelon, T., S.P.Wilkinson, A.Grove and M.E. Newcomer, (2006). The crystal structure of the transcriptional regulator HucR from *Deinococcus radiodurans* reveals a repressor preconfigured for DNA binding. *J. Mol. Biol*., 30; 360(1): 168-77.

Borghi, C., Rosei, E.A., Bardin, T., Dawson, J., Dominiczak, A., Kielstein, J.T., Manolis, A.J., Perez-Ruiz, F., Mancia, G. (2015). Serum uric acid and the risk of cardiovascular and renal disease. *Journal of hypertension*, 33(9), 1729-1741.

Borghi, C., Tykarski, A., Widecka, K., Filipiak, K.J., Domienik-Karłowicz, J., Kostka-Jeziorny, K., Varga, A., Jaguszewski, M., Narkiewicz, K., Mancia, G. (2018). Expert consensus for the diagnosis and treatment of patient with hyperuricemia and high cardiovascular risk. *Cardiology journal*, 25(5), 545-564.

Bove, M., Cicero, A. F. G., Veronesi, M., & Borghi, C. (2017). An evidence-based review on urate-lowering treatments: implications for optimal treatment of chronic hyperuricemia. *Vascular Health and Risk Management*, 13, 23.

Bredemeier, M., Lopes, L. M., Eisenreich, M. A., Hickmann, S., Bongiorno, G. K., d'Avila, R., Morsch, A. L. B., da Silva Stein, F., & Campos, G. G. D. (2018). Xanthine oxidase inhibitors for prevention of cardiovascular events: a systematic review and meta-analysis of randomized controlled trials. *BMC Cardiovascular Disorders*, *18*(1): 1–11.

Carvalho, L.A., Lopes, J.P., Kaihami, G.H., Silva, R.P., Bruni-Cardoso, A., Baldini, R.L., Meotti, F.C. (2018). Uric acid disrupts hypochlorous acid production and the bactericidal activity of HL-60 cells. *Redox biology*, 16:179-188.

Chalès, G, (2019). How should we manage asymptomatic hyperuricemia? Joint Bone Spine, 86(4):437-443.

Chen, C., Lü, J.-M., Yao, Q. (2016). Hyperuricemia-related diseases and xanthine oxidoreductase (XOR) inhibitors: an overview. *Medical science monitor: international medical journal of experimental and clinical research*, 22:2501.

Chen, C., Lü, J.-M., Yao, Q. (2016). Hyperuricemia-related diseases and xanthine oxidoreductase (XOR) inhibitors: an overview. *Medical science monitor: international medical journal of experimental and clinical research*, 22:2501.

Chen, R.-J., Chen, M.-H., Chen, Y.-L., Hsiao, C.-M., Chen, H.-M., Chen, S.-J., Wu, M.-D., Yech, Y.-J., Yuan, G.-F., & Wang, Y.-J. (2017). Evaluating the urate-lowering effects of different microbial fermented extracts in hyperuricemic models accompanied with a safety study. *Journal of Food and Drug Analysis*, *25*(3):597–606.

Cheng, X., Yang, B., Liu, D., He, L, J, and Chen G. (2012) Genetic Engineering of Bacteria that can Produce Urate Oxidase. *Intern Med*.2:114.

Cheristians, S & Kaltwasser, H. (1986). Nickel-content of urease from *Bacillus pasteurii*, *Archives of Microbiology*, 145:51-55.

Chiu., Hsu, T.-S., Huang, C.-Y., & Hsu, C.-H. (2021). Structural and biochemical insights into a hyperthermostable urate oxidase from *Thermobispora bispora* for hyperuricemia and gout therapy. *International Journal of Biological Macromolecules*, 188:914–923.

Chohan, S. and M.A. Becker. (2009). Update on emerging urate-lowering therapies. *Curr. Opin. Rheumatol*, 21:143–149.

Choi, H. K., Atkinson, K., Karlson, E. W., & Curhan, G. (2005). Obesity, weight change, hypertension, diuretic use, and risk of gout in men: the health professionals follow-up study. *Archives of Internal Medicine, 165*(7):742–748.

Christen, P., W.C. Peackock, A.E. Christen and W.E. Wacker. (1971). Urate oxidase in primate phylogenesis. *Eur. J. Biochem*., 12:3–5.

Crowley, L. C., Marfell, B. J., Christensen, M. E., & Waterhouse, N. J. (2016). Measuring cell death by trypan blue uptake and light microscopy. *Cold Spring Harbor Protocols*, (7):087155.

D. da Silva Freitas, P.J. Spencer, R.C. Vassão, J. Abrahão-Neto, (2010). Biochemical and biopharmaceutical properties of PEGylated uricase, *International journal of pharmaceutics* 387:215-222.

Dabbagh, F., Moradpour, Z., Ghasemian, A., Ghasemi, Y. (2012). Phylogeny of urate oxidase producing bacteria: on the basis of gene sequences of 16S rRNA and uricase protein. *Iranian Journal of Pharmaceutical Sciences*, 8(2):99-102.

Daisley, B.A., Pitek, A.P., Chmiel, J.A., Al, K.F., Chernyshova, A.M., Faragalla, K.M., Burton, J.P., Thompson, G.J., Reid, G. (2020). Novel probiotic approach to counter *Paenibacillus larvae* infection in honey bees. *The ISME journal*, 14(2):476-491.

Daoudi, N. E., Bouhrim, M., Ouassou, H., & Bnouham, M. (2020). Medicinal Plants as a Drug Alternative Source for the Antigout Therapy in Morocco. *Scientifica*, :2020.

Dehlin, M., Jacobsson, L., & Roddy, E. (2020). Global epidemiology of gout: prevalence, incidence, treatment patterns and risk factors. *Nature Reviews Rheumatology*, 16(7):380–390

Di Giovine, F. S., Malawista, S. E., Thornton, E., & Duff, G. W. (1991). Urate crystals stimulate production of tumor necrosis factor alpha from human blood monocytes and synovial cells. Cytokine mRNA and protein kinetics, and cellular distribution. *The Journal of Clinical Investigation, 87*(4), 1375–1381.

Distasio, JA, Niederman, RA, Kafkewitz, D & Goodman, D. (1976). Purification and characterization of L-asparaginase with anti- lymphoma activity from Vibrio succinogenes, *Journal of Biological Chemistry*, 251, (22)6929-6933.

Dmytruk, K.V., Smutok, O.V., Dmytruk, O.V., Schuhmann, W., Sibirny, A.A. (2011). Construction of uricase-overproducing strains of *Hansenula polymorpha* and its application as biological recognition element in microbial urate biosensor. *BMC Biotechnology*, 11(1), 58. repeated

Du, H., S.Kim, K.H.Nam, M.S.Lee, O.Son,S.H.Lee and C.I. Cheon. (2010). Identification of uricase as a potential target of plant thioredoxin: Implication in the regulation of nodule development. *Biochem. Biophys. Res. Commun*., 397:22–26.

Dwivedi., H., K. Agrawal and S.A. Saraf. (2012). Screening of uricase producing microorganisms and uricase estimation: a simple and novel approach. *Int. J. Pharm. Pharm. Sci.*, 4(1): 422-424.

Eichmann, J., Oberpaul, M., Weidner, T., Gerlach, D., Czermak, P. (2019). Selection of high producers from combinatorial libraries for the production of recombinant proteins in *Escherichia coli* and *Vibrio natriegens*. *Frontiers in bioengineering and biotechnology*, **7**:254.

ElAdawy, H., Hotzel, H., Tomaso, H., Neubauer, H., Hafez, H.M. (2012). Isolation and characterization of *Ochrobactrum anthropi* and *Ochrobactrum pecoris* from caecal content of commercial turkeys. *Veterinary microbiology*, 155(2-4), 349-354.

El-Dein and El-Fallal. (1996), Screening of some fungi for Uricolytic activity, *Qatar Univ. Sci. J.* 16(1): 71-76

El-Desouky, E. M. (1981) Microbiological-Biochemical studies on certain uric acid-decomposing microorganisms. Ph. D. Thesis, Department of Botany, Faculty of Science, Al-Azhar University, Cairo, Egypt.

El-Naggar, N.E.-A., Deraz, S.F., Soliman, H.M., El-Deeb, N.M., El-Ewasy, S.M. (2016). Purification, characterization, cytotoxicity and anticancer activities of L-asparaginase, anti-colon cancer protein, from the newly isolated alkaliphilic *Streptomyces fradiae* NEAE-82. *Scientific reports*, 6(1):1-16.

El-Weshy, E. M., El-Naggar, N. E., Haroun, S. A., and Sherief, A. A. (2018). Screening of some Fungal Isolates for their Potentialities for Uricase Production and Optimization of the Factors Affecting the Production. *Journal of Plant Production* 9:919-922.

Ertan, F. and E. Aksz, (2000). *Aspergillus niger* Õden .rikaz Enziminin. retilmesi ve Aktiviteye Etkili BazÝ Fakt.rlerin Belirlenmesi. *Turk J.Biol.*, 24:11-23.

Erwig, L.-P., & Rees, A. J. (1999). Macrophage activation and programming and its role for macrophage function in glomerular inflammation. *Kidney and Blood Pressure Research*, 22(1–2):21–25.

Fan, N., Yu, Y., Li, L., Xia, H., Dong, X., Li, Y., Chen, H., Duan, W. (2021). Uricase deficiency causes mild and multiple organ injuries in rats. *Plos one*, 16(8):e0256594.

Farley, P. and S. Santosa. (2002). Regulation of expression of the *Rhizopus oryzae* uricase and urease enzymes. Can. *J. Microbiol*., 48(12): 1104-8.

Fattah, Hamed GM & Abo-Hamed NA. (2002). Bioconversion of poultry wastes. I--Factors influencing the assay and productivity of crude uricase by three uricolytic filamentous fungi. *Acta Microbiol Immunol Hung*. 49(4):445-54.

Fazel, R., N. Zarei, N. Ghaemi, M. M. Namvaran, S. Enayati, E. M. Ardakani, M. Aziziand V. Khalaj.(2014). Cloning and expression of *Aspergillus flavus* urate oxidase in *Pichia pastoris*. *SpringerPlus*, 3(395): 1-7.

Fields, T. R. (2019). The challenges of approaching and managing gout. *Rheumatic Disease Clinics*, 45(1):145–157.

Fitzpatrick, D.A., O. Fitzgerald and K.F. McGeeney. (1975). Immuno enzymology of purified urate oxidase. *Biochem. J.*, 125: 114.

Friedman, T.B., G.E.Polanco, J.C. Appold and J.E.Maylet. (1985). On the loss of uricolytic activity during primate evolution-I. Silencing of urate oxidase in a hominoid ancestor. *Comp. Biochem. Physiol.*, 81(3): 653-9.

Fu, X., Yan, Q., Wang, J., Yang, S., Jiang, Z. (2016). Purification and biochemical characterization of novel acidic chitinase from *Paenicibacillus barengoltzii*. *International journal of biological macromolecules*, 91:973-979.

Geweely, N. S. and L. S. Nawar. (2011). Production, optimization, purification and properties of uricase isolated from some fungal flora in Saudi Arabian soil. Screening, 5(10): 220-230.

Ghasemi, Mohammad & Bakhtiari, Mohammad Reza & Fallahpour, Masoud & Noohi, Ashrafossadat & Moazami, Nasrin & Z. (2004). Screening of Urease Production by *Aspergillus niger*. *Iranian Biomedical Journal*. 8: 47-50.

Ghosh, T., Sarkar, P. (2014). Isolation of a novel uric-acid-degrading microbe *Comamonas* sp. BT UA and rapid biosensing of uric acid from extracted uricase enzyme, *Journal of biosciences* 39(5): 805-819.

Gochman, N. and M. J. Schmitz. (1971). Automated determination of uric acid, with use of a urease-peroxidase system. *Clin. Chem.*, 17: 1154-1159.

Gorfinkiel, L., Diallinas, G., & Scazzocchio, C. (1993). Sequence and regulation of the uapA gene encoding a uric acid-xanthine permease in the fungus *Aspergillus nidulans*. *Journal of Biological Chemistry*, 268(31):23376-23381.

Grinfeld, J., Inaba, A., Hutchison, A.J. (2010). Update and critical appraisal of sevelamer in the management of chronic renal failure. *Open access journal of urology*, 2, 161.

Gurung, N., Ray, S., Bose, S., Rai, V. (2013). A broader view: microbial enzymes and their relevance in industries, medicine, and beyond. *Biomed Res Int*, 329121.

Hacker, J.S., Huber, G.A., Nichols, A., Rehm, P., Schlesinger, M., Valletta, R., Craig, S. (2014). The economic security index: A new measure for research and policy analysis. *Review of Income and Wealth*, 60, S5-S32.

Hafez, R.M., Abdel-Rahman, T.M., Naguib, R.M. (2017). Uric acid in plants and microorganisms: Biological applications and genetics-A review. *Journal of advanced research*, 8(5):475-486.

Hancks, D.C., Kazazian, H.H. (2016). Roles for retrotransposon insertions in human disease. *Mobile DNA*, 7(1):1-28.

Harper, H.A., (edt.), (1977). Review of physiological chemistry, 16th eds., Lange Medical Publication, *San Francisco, CA*:406pp.

Hatijah, S.M. and W. R. W. Ruhayu. (2013). Preliminary study on factors that enhanced the production of uricase by *Aspergillus* flavus. *Int. J. Biosci. Biochem. Bioinfo.,* 3(5): 513-515.

Hesham, MS, Yasser, R, Abdel-Fattah, Y, Gohar, M & Mohammed, AE. (2004). Purification and characterization of extracellular *Pseudomonas aeruginosa* urate oxidase enzyme, *Polish Journal of Microbiology,* 53, (1):45-52.

Hink, H.U., N.Santanam, S.Dikalov, L.McCann, A.D.Nguyen, S.Parthasarathy, D.G.Harrison and T. Fukai, (2002). Peroxidase properties of the extracellular superoxide dismutase: role of uric acid in modulating in vivo activity. Arterioscler Thromb. *Vasc. Biol.*, 22: 1402–1408.

Hochberg, M.C., J. Thomas, D.J. Tomas, L. Mead, D.M. Levine and M.J. Klag, (1995). Racial differences in the incidence of gout. *Arthritis Rheum.,* 38:628–632.

Honarbakhsh, F., Abolmaali, S., Amoozegar, M.A. (2021). Uricase Activity of Halophilic Bacteria from Iranian Salt Lakes. *Iranian Journal of Science and Technology, Transactions A: Science,* 45(5):1597-1606.

Hoque, K.M., Dixon, E.E., Lewis, R.M., Allan, J., Gamble, G.D., Phipps-Green, A.J., Kuhns, V.L.H., Horne, A.M., Stamp, L.K., Merriman, T.R. (2020). The ABCG2 Q141K hyperuricemia and gout associated variant illuminates the physiology of human urate excretion. *Nature communications*, 11(1):1-15.

Hosoya, T. and I.Ohno, (2011). Metabolic disease, hyperuricemia and chronic kidney disease. *Nihon Naika Gakkai Zasshi*. 100: 1206-1212.

Hu, X., Wang, Y., Liu, C., Jin, Z., Tian, Y. (2018). Dextrin-uricase conjugate: Preparation, characterization, and enzymatic properties. *International journal of biological macromolecules*, 111, 28-32.

Huang, S., Y.Shih,, C. Wu and C. Yuan, (2004) .Detection of serum uric acid using the optical polymeric enzyme biochip system. *Syst.*, 19: 1627-1633.

Huang, X., Zhang, S., Tang, Y., Zhang, X., Bai, Y., Pang, H. (2021). Advances in metal– organic framework-based nanozymes and their applications. *Coordination Chemistry Reviews*, 449:214216.

Hyndman, D., Liu, S., Miner, J.N. (2016). Urate handling in the human body. *Current rheumatology reports*, 18(6), 34.

Ieiri, I. (2011). Functional significance of genetic polymorphisms in P-glycoprotein (MDR1, ABCB1) and breast cancer resistance protein (BCRP, ABCG2). *Drug metabolism and pharmacokinetics*, 1111220274:1111220274.

Itiaba, K., M.Hsiung and J.Crawhall. (1975). Uric Acid Estimation: A Comparison of the manual uricase-uv and the phosphotungstate auto-analyzer methods. *Circulation*, 8: 316.

Izuhara M., M. Ito and Y. Takagi. (1995). Transcription of the rat liver uricase-encoding gene is regulated via a cis-acting element responsive to cAMP Gene167:267-272.

Jacob, J.M., Karthik, C., Saratale, R.G., Kumar, S.S., Prabakar, D., Kadirvelu, K., Pugazhendhi, A. (2018). Biological approaches to tackle heavy metal pollution: a survey of literature. *Journal of environmental management*, 217:56-70.

Jagathy, K., Pushparaj, A., Ronald, J. (2016). Uricase production from *Bacillus subtilis* isolated from poultry waste. *Int J Adv Res Biol Sci*, 3(6), 255-262.

Jhample, Sowmya B.; Bhagwat, Prashant K.; Dandge, Padma B. (2015). Statistical media optimization for enhanced production of fibrinolytic enzyme from newly isolated *Proteus penneri* SP-20. *Biocatalysis and Agricultural Biotechnology*: S1878818115000729

Jin, M., Yang, F., Yang, I., Yin, Y., Luo, J.J., Wang, H., Yang, X.-F. (2012). Uric acid, hyperuricemia and vascular diseases. *Frontiers in bioscience: a journal and virtual library*, 17: 656.

Jinqing, K., Shaolin, M and Jianbing, Z. (1992). Bioelectrochemical responses of polyaniline uricase electrode, *J. Electroanal. Chem*. 334:121–132.

Johnson, K.M., Su, D., Zhang, D. (2021). Characteristics of Major Drug Metabolizing Cytochrome P450 Enzymes. in: *Cytochrome P450, Springer* : 27-54.

Johnson, R.J., J. H. Acosta, G.F. Schreiner and B. R. Iturbe. (2002). Subtle acquired renal injury as a mechanism of salt-sensitive hypertension. *Eng. J. Med.*, 346:913–923.

Johnson, R.J., S.D. Kivlighn, Y.G. Kim, S. Suga and A. Fogo. (1999). Reappraisal of the pathogenesis and consequences of hyperuricemia in hypertension, cardiovascular disease, and renal disease. *Am. J. Kid. Dis.*, 33:225–234.

Juwon, A.D., Emmanuel, O.F. (2012). Experimental Investigations on the Effects of Carbon and Nitrogen Sources on Concomitant Amylase and Polygalacturonase Production by *Trichoderma viride* BITRS-1001 in Submerged Fermentation., Biotechnology Research International:904763.

Kai.L, X.-H. Ma, X.-L. Zhou, X.-M. Jia, X. Li, K.-P. Guo. (2008). Purification and characterization of a thermostable uricase from *Microbacterium* sp. strain ZZJ4-1, *World Journal of Microbiology and Biotechnology*, 24(3): 401-406.

Kaltwasser, H. (1968).Inductive formation of particle-bound uricase in *Hydrogenomonas* H16 and other aerobic bacteria, *Archives of Microbiology*, 60:160-171.

Kanaparthi, S., Supraja, P., Singh, S.G. (2019). Smart, portable, and noninvasive diagnostic biosensors for healthcare. in: Advanced biosensors for health care applications, *Elsevier*:09-226.

Kean, R.B., S.V.Spitsin andT.Mikheeva,(2000). The peroxynitrite scavenger uric acid prevents inflammatory cell invasion into the central nervous system in experimental allergic encephalomyelitis through maintenance of blood-central nervous system barrier integrity. *J. Immunol.*, 165:6511–6518.

Khade, S., Srivastava, S. (2017). Effect of surfactants and inducers on increased uricase production under submerged fermentations by *Bacillus cereus*. *Preparative Biochemistry and Biotechnology*, 47(1):81-85.

Khucharoenphaisan, K. and K.Sinma, (2011). Production and partial characterization of uric acid degrading enzyme from new source *Saccharopolyspora* sp.PNR11. Pak, *J. Biol. Sci.*, 14(3):226-231.

Kida, J. and M. Kunihisa. (1966). Studies on bacterial uricase (I) isolation of uricase producing bacteria and some cultural conditions for production. *J. Ferment. Technol.*, 44: 789- 796.

Klose, S., M. Stoltz, E. Munz and R. Portenhauser. (1978). Determination of uric acid on continuous-flow (AutoAnalyzer II and SMA) systems with a uricase /phenol /4- aminophenazone color test. *Clin. Chem*., 24:250–255.

Koleva, L., Bovt, E., Ataullakhanov, F., Sinauridze, E. (2020). Erythrocytes as carriers: from drug delivery to biosensors. *Pharmaceutics,* 12(3):276.

Kotb, E., (2016). Improvement of uricase production from *Bacillus subtilis* RNZ-79 by solid state fermentation of shrimp shell wastes. *Biological* 71: 229-238

Koyama, Y., T.Ichikawa and E.Nakano, (1996).Cloning, sequence analysis, and expression in *Escherichia coli* of the gene encoding the *Candida utilis* urate oxidase (uricase). *J. Biochem*.,120: 969–973.

Laemmli, U.K., (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nat.,* 227:680–685.

Lai, J.-H., Luo, S.-F., Hung, L.-F., Huang, C.-Y., Lien, S.-B., Lin, L.-C., Liu, F.-C., Yen, B.L., Ho, L.-J. (2017). Physiological concentrations of soluble uric acid are chondroprotective and anti-inflammatory. Scientific reports, 7(1):1-12.

Lee, N. S. I. S., Khosravi, H. M., Ibrahim, N., and Shahir, S. (2015). Isolation, partial purification and characterization of thermophilic uricase from *Pseudomonas otitidis* strain SN4. *Malaysian Journal of Microbiology*, 11: 352-357.

Lekshmy, P.V.V. and Raval, K . (2016). Isolation and production studies of Uricase producing bacterial strains from poultry waste. *International Journal of Advanced Research Trends in Engineering and Technology,* 3: 152-157.

Leplatois, P., B. Le Douarin and G. Loison. (1992). High-level production of a peroxisomal enzyme: *Aspergillus flavus* uricase accumulates intracellularly and is active in *Saccharomyces cerevisiae*. Gene, 122:139-145.

Li et al., 2006 Li J, Chen Z, Hou L, Fan H, Weng S, Xu C, Ren J, Li B, Chen W. (2006). High-level expression, purification, and characterization of non-tagged *Aspergillus flavus* urate oxidase in *Escherichia coli*. *Protein Exp. Purif.* 49: 55-59.

Liming Cai., Li, Q., Deng, Y., Liu, X., Du, W., & Jiang, X. (2020). Construction and expression of recombinant uricase‐expressing genetically engineered bacteria and its application in rat model of hyperuricemia. *International journal of molecular medicine*, 45(5):1488–1500.

Lin, C.S., W.L.Lee, Y.L.Hung, D.Y.Lee, K.F.Chen, W.C.Chi and S.C.Chang, (2012).Prevalence of hyperuricemia and its association with antihypertensive treatment in hypertensive patients in Taiwan. *Int .J .Cardiol.*,156 (1):41–46.

Liu, D., Yang, P., Wang, F., Wang, C., Chen, L., Ye, S., Dramou, P., Chen, J., He, H. (2021). Study on performance of mimic uricase and its application in enzyme-free analysis. *Analytical and Bioanalytical Chemistry*, 413(26):6571-6580.

Lotfy, WA. (2008). Production of a thermostable uricase by a novel Bacillus *Thermocatenulatus* strain, *Bioresource Technology*, 99, (4):699-702.

Lowry, OH, Rosebrough, NJ, Farr, A L and Randall, RJ. (1951). *J. Biolchem.* 193-265.

MacKenzie, C.R. (2015). Gout and hyperuricemia: an historical perspective. *Current Treatment Options in Rheumatology*, 1(2):119-130.

Magda ALY, Tork, Sanna, Garni, S.A and Allam, R. (2013). Production and Characterization of uricase from *Streptomyces exfoliates* UR10 isolated from farm wastes, *Turk J Biol* 37: 520-529.

Mansour, F.A., M.M.Nour- El- Dein, A.A.El- Fallel and M.I.M.Abou-Do- Bara. (1996). Purification and general properties of uricase from *Streptomyces aureomonopodiales. Acta Microbiologica Polonica*, 45:45-53.

Marinello, E., L.Arezzini, M.Pizzichini, B. Frosi, B.Porcelli and L.Terzuoli. (2002). Purine nucleotide catabolism in rat liver: labelling of uric acid and allantoin after administration of various labelled precursors. *Life Sci.*, 70(24):2931-41.

Massey, V., H.Komai, G.Palmer and G.B.Eliob. (1970).On the mechanism of inactivation of xanthine oxidase by allopurinol and other pyrazolo [3,4d] pyrimidines. *J. Biol. Chem.*, 245: 837-2844.

Mazzali, M., J. Hughes, Y.G. Kim, J.A. Jefferson, D.H. Kang, K.L. Gordon, H.Y. Lan, S. Kivlighn and R.J. Johnson. (2001). Elevated uric acid increases blood pressure in the rat by a novel crystal-independent mechanism. *Hyperten.*, 38:1101–1106.

McCarthy, M. and D.B. Johnson. 1977. Urate oxidase immobilized on elastin. *Biotech. Bioengg.*, 19:599–603.

Ming, J., Zhu, T., Li, J., Ye, Z., Shi, C., Guo, Z., Wang, J., Chen, X., Zheng, N. (2021). A Novel Cascade Nanoreactor Integrating Two-Dimensional Pd-Ru Nanozyme, Uricase and Red Blood Cell Membrane for Highly Efficient Hyperuricemia Treatment. *Small*, 17(46):2103645.

Mona I. Shaaban, Eman Abdelmegeed, and Youssif M.Ali. (2015). Cloning, Expression, and Purification of Recombinant Uricase Enzyme from *Pseudomonas aeruginosa* Ps43 Using Escherichia coli. *J. Microbial. Biotechnol* 25. (6), 887-892.

Montalbini, P., J.Redondo, J.L. Caballero, J.Cardenas and M.Pineda, (1997). Uricase from leaves: its purificación and characterization from three different higher plants. *Planta Heidelberg*, 202: 277-283.

Moongkarndi, P., Kosem, N., Kaslungka, S., Luanratana, O., Pongpan, N., & Neungton,N. (2004). Antiproliferation, antioxidation and induction of apoptosis by *Garcinia mangostana* (mangosteen) on SKBR3 human breast cancer cell line. *Journal of Ethnopharmacology*, 90(1):161–166.

Murea, M., Tucker, B.M. (2019). The physiology of uric acid and the impact of end-stage kidney disease and dialysis. Seminars in dialysis. *Wiley Online Library*:47-57.

Nakagawa, S, Ishino, S & Teshiba, S. (1996).Construction of catalase deficient *Escherichia coli* strains of the production of uricase, *Bioscience Biotechnology and Biochemistry*, 60:415-420.

Nanda, P. and P.E. J. Babu, (2012). Study on Pegylation of therapuetic enzyme uricase and its physio-chemical properties for improving its pharmaceutical characteristics. *Int. J. Pharmacol. Pharmaceu. Technol.*, 1(1): 58-62

Nanda, P., & Jagadeesh Babu, P. E. (2014). Isolation, screening and production studies of uricase producing bacteria from poultry sources. *Preparative Biochemistry and Biotechnology*, 44 : 811-821.

Neda Motamedi, Barani M, Lohrasbi-Nejad A, Mortazavi M, Riahi-Medvar A, Varma RS, Torkzadeh-Mahani M. (2021). Enhancement of Thermostability of *Aspergillus flavus* Urate Oxidase by Immobilization on the Ni-Based Magnetic Metal-Organic Framework. *Nanomaterials (Basel).* 6,11(7):1759.

Nelapati, A.K., Meena, S., Singh, A.K., Bhakta, N., Ponnan Ettiyappan, J. (2021). In silico structural and functional analysis of *Bacillus* uricases. *Current Proteomics*, 18(2):124- 142.

Neveen, S., G. Lubna and S. Nawar, (2011). Production, optimization, purification and properties of uricase isolated from some fungal flora in Saudi Arabian soil. *Aust. J. Basic Appl. Sci.,* 5(10): 220-230.

Nie, Y.-M., Li, S.-H., Lin, M.-Y., Yan, J. (2020). A micro-environment tuning approach for enhancing the catalytic capabilities of lanthanide containing polyoxometalate in the cyanosilylation of ketones. *Chemical Communications*, 56(26), 3809-3812.

O'Neill, R.D. and J.P. Lowry, (1995). On the significance of brain extracellular uric acid detected with *in-vivo* monitoring techniques: a review. Behav. *Brain Res.*, 71:33–49.

Pan, X., S. Zhou, C. Chen and J. Kan, (2006).Preparation and properties of an uricase biosensor based on copolymer of o-aminophenol-aniline, *Sens. Actuators B*, 113:329- 334.

Peters, J.W., Boyd, E.S., D'Adamo, S., Mulder, D.W., Therien, J., Posewitz, M.C. (2013). Hydrogenases, nitrogenases, anoxia, and H_2 production in water-oxidizing phototrophs. in: Algae for biofuels and energy, *Springer* :37-75.

Pierce, G. F. (1990). Macrophages: important physiologic and pathologic sources of polypeptide growth factors. *Am J Respir Cell Mol Biol*, 2(3):233–234.

Pierigè, F., Bigini, N., Rossi, L., Magnani, M. (2017). Reengineering red blood cells for cellular therapeutics and diagnostics. Wiley Interdisciplinary Reviews: *Nanomedicine and Nanobiotechnology*, 9(5):e1454.

Pierzynowska, K., Deshpande, A., Mosiichuk, N., Terkeltaub, R., Szczurek, P., Salido, E., Pierzynowski, S., Grujic, D. (2020). Oral Treatment with an Engineered Uricase, ALLN-346, Reduces Hyperuricemia, and Uricosuria in Urate Oxidase-Deficient Mice. *Frontiers in Medicine*, 7(726).

Pooja Nanda, & Jagadeesh Babu, P. E. (2014). Isolation, screening and production studies of uricase producing bacteria from poultry sources. *Preparative Biochemistry and Biotechnology*, 44(8):811–821.

Poovizh, T., Gajalakshmi, P., and Jayalakshmi, S. (2014). Production of uricase a therapeutic enzyme from *Pseudomonas putida* isolated from poultry waste. *Int J adv Res*, 2 : 34-40.

Pui, C.H, M.V. Relling, F. Lascombes, P.L. Harrison, A. Struxiano, J.M. Mondesir, R.C. Ribeiro, J.T. Sandlund, G.K. Rivera, W.E. Evans and H.H. Mahmoud. (1997). Urate oxidase in prevention and treatment of hyperuricemia associated with lymphoid malignancies. *Leukemia*, 11: 1813–1816.

Pustake, S. O., Bhagwat, P. K., and Dandge, P. B. (2019). Statistical media optimization for the production of clinical uricase from *Bacillus subtilis* strain SP6. *Heliyon*, 5: e01756.

Ragab, G., Elshahaly, M., Bardin, T. (2017). Gout: An old disease in new perspective–A review. *Journal of advanced research*, 8(5):495-511.

Rao, G.N., M.A. Corson and B.C. Berk. (1991). Uric acid stimulates vascular smooth muscle cell proliferation by increasing platelet-derived growth factor A-chain expression. *J. Biol. Chem., 2*66:8604–8608.

Ravichandran, R., Hemaasri, S., Cameotra, Swaranjit Singh and Jayaprakash, N.S. (2015). Purification and characterization of an extracellular uricase from a new isolate of *Sphingobacterium thalpophilum* (VITPCB5*). Protein Expression and Purification*, 114:136–142.

Rees, F., Hui, M., Doherty, M. (2014). Optimizing current treatment of gout. *Nature Reviews Rheumatology*, 10(5), 271-283.

Rollin, J.A., del Campo, J.M., Myung, S., Sun, F., You, C., Bakovic, A., Castro, R., Chandrayan, S.K., Wu, C.-H., Adams, M.W. (2015). High-yield hydrogen production from biomass by in vitro metabolic engineering: mixed sugars coutilization and kinetic modeling. *Proceedings of the National Academy of Sciences*, 112(16):4964-4969.

Rouf, M.A. and T.F. Lomprey. (1968). Degradation of uric acid by certain aerobic bacteria. *J. Bacteriol.,* 96: 617-622.

RoyChoudhury, S., Umasankar, Y., Hutcheson, J.D., Lev-Tov, H.A., Kirsner, R.S., Bhansali, S. (2018). Uricase based enzymatic biosensor for non-invasive detection of uric acid by entrapment in PVA-SbQ polymer matrix. *Electroanalysis*, 30(10): 2374-2385.

Rumpf, S., Jentsch, S. (2006). Functional division of substrate processing cofactors of the ubiquitin-selective Cdc48 chaperone. *Molecular cell*, 21(2):261-269.

Saeed, M.H., Y.Abdel-Fatah, M.Gohar and A.M.Elbaz, (2004). Purification and characterization of extracellular *Pseudomonas aeruginosa* urate oxidase enzyme. *Polish J. Microbiol.,* 53 (1): 45-52.

Saeid, S., Tolvanen, P., Kumar, N., Eränen, K., Peltonen, J., Peurla, M., Mikkola, J.-P., Franz, A., Salmi, T. (2018). Advanced oxidation process for the removal of

ibuprofen from aqueous solution: a non-catalytic and catalytic ozonation study in a semi-batch reactor. *Applied Catalysis B: Environmental*, 230:77-90.

Sambandam Thillainayagi, and Harikrishnan Sekar and Jayalakshmi Singaram. (2021).Screening, Optimization and Production of Uricase from *Alcaligenes faecalis* Isolated from Poultry Farm Litter. *International Journal of Scientific Research and Growth*, 9: 2455-6211.

Sankari, N., M.Vijayalakshmi and M.Deecaraman. (2015). Isolation and screening of microorganism producing urate oxidase from poultry waste soil. *Int. J. Pharm. Tech Res.*,8(4): 531-534.

Schiavon, O., P.Calicati, P.Ferruti and F.M.Veronese. (2000).Therapeutic proteins: a comparison of chemical and biological properties of uricase conjugated to linear or branched poly (ethylene glycol) and poly (N-acryloylmorpholine). *Il Farmaco*, 55:264– 269.

Schmidt, C.K., Medina-Sánchez, M., Edmondson, R.J., Schmidt, O.G. (2020). Engineering microrobots for targeted cancer therapies from a medical perspective. *Nature Communications*, 11(1):1-18.

Selvaraj, C and Thirumalaivasan, P, (2017). Screening, production and optimization of Uricase from *P. aeruginosa*. *Europ J Biotechnol Biosci*, 5: 57-61.

Shaaban, MI, Abdelmegeed, E & Ali, YM (2015), Cloning, expression, and purification of recombinant uricase enzyme from *Pseudomonas aeruginosa* Ps43 using *Escherichia coli*, *Journal of Microbiology and Biotechnology*, 25,(6):887-892.

Sherman, M.R., Saifer, M.G., Perez-Ruiz, F. (2008). PEG-uricase in the management of treatment-resistant gout and hyperuricemia. *Advanced drug delivery reviews*, 60(1), 59- 68.

Shi, Y., Wang, T., Zhou, X. E., Liu, Q. F., Jiang, Y., & Xu, H. E. (2019). Structure-based design of a hyperthermostable AgUricase for hyperuricemia and gout therapy. *Acta Pharmacologica Sinica* 40 :1364-1372.

Shweta Pawar, Rathod & Virendra. (2017). Optimization of novel and greener approach for the co-production of uricase and alkaline protease in *Bacillus licheniformis* by Box Behnken Model. *Preparative Biochemistry and Biotechnology*: 48.

Skolarikos, A., Straub, M., Knoll, T., Sarica, K., Seitz, C., Petřík, A., Türk, C. (2015). Metabolic evaluation and recurrence prevention for urinary stone patients: EAU guidelines. *European urology*, 67(4):750-763.

Stiburkova, B., Pavelcova, K., Pavlikova, M., Ješina, P., Pavelka, K. (2019). The impact of dysfunctional variants of ABCG2 on hyperuricemia and gout in pediatric-onset patients. *Arthritis research & therapy*, 21(1), 1-10.

Sunny, N., Owens, S., Baldwin, R.t., El-Kadi, S., Kohn, R., Bequette, B. (2007). Salvage of blood urea nitrogen in sheep is highly dependent on plasma urea concentration and the efficiency of capture within the digestive tract. *Journal of animal science,* 85(4):1006-1013.

Suzuki, H. and P.P. Verma. (1991). Soybean nodule-specific uricase (Nodulin-35) is expressed and assembled into a functional tetrameric holoenzyme in *Escherichia coli*. *Plant Physiol.*, 95: 384-389.

Sylvester, P. W. (2011). Optimization of the tetrazolium dye (MTT) colorimetric assay for cellular growth and viability in Drug design and discovery, Springer: 157–168.

Tan, Q., N.Wang, H.Yang, L.K. Zhang and S. Liu, (2010). Characterization, stabilization and activity of uricase loaded in lipid vesicles. *Int. J. Pharama.*, 384:(1)-2.

Tanaka, A., M. Yamamura, S. Kawamoto and S. Fukui, 1977. Production of uricase by *Candida tropicalis* using nalkane as substrate. *Appl. Environ. Microbiol.*, 34: 342- 346.

Thillainayagi Sambandam, and Harikrishnan Sekar and Jayalakshmi Singaram. (2021).Screening, Optimization and Production of Uricase from *Alcaligenes faecalis* Isolated from Poultry Farm Litter. *International Journal of Scientific Research and Growth*, 9: 2455-6211.

Tork, S.E. M.M. Aly and S.Q. Al-Fattani. (2018). A new uricase from *Bacillus cereus* SKIII: Characterization, gene identification and genetic improvement, *International Journal of Biological*. 2020 (10):183.

Toyoda, Y., Pavelcová, K., Bohatá, J., Ješina, P., Kubota, Y., Suzuki, H., Takada, T., Stiburkova, B. (2021). Identification of two dysfunctional variants in the ABCG2 urate transporter associated with pediatric-onset of familial hyperuricemia and early-onset gout. *International Journal of Molecular Sciences*, 22(4):1935.

Tripathi, S., Parmar, J., Kumar, A. (2020). Structure-Based Immunogenicity Prediction of Uricase from Fungal (*Aspergillus flavus*), Bacterial (*Bacillus subtillis*) and Mammalian Sources Using Immunoinformatic Approach. *The protein journal*, 39(2):133-144.

V.Manimekalai, b A. (2019). Poultry soil derived bacterial flora from hosur, tamilnadu and their uricase activities. *Journal of applied science and computations*, 6(6): 3756–3762.

Vargas-Santos, A.B., Neogi, T. (2017). Management of gout and hyperuricemia in CKD. *American Journal of Kidney Diseases*, 70(3), 422-439.

Viggiano, D., Gigliotti, G., Vallone, G., Giammarino, A., Nigro, M., Capasso, G. (2018).Urate-lowering agents in asymptomatic hyperuricemia: role of urine sediment analysis and musculoskeletal ultrasound. *Kidney and Blood Pressure Research*, 43(2):606-615.

Vignais, P., Colbeau, A. (2004). Molecular biology of microbial hydrogenases. *Current issues in molecular biology*, 6(2):159-188.

Wacker, W.E. (1970). Man: sapient but gouty. *Engl. J. Med.*, 283:151–152.

Wadleigh, D. J., Reddy, S. T., Kopp, E., Ghosh, S., & Herschman, H. R. (2000). Transcriptional activation of the cyclooxygenase-2 gene in endotoxin-treated RAW 264.7 macrophages. *Journal of Biological Chemistry*, 275(9):6259–6266.

Wakamiya, W. X. M., S.Vaishnav, R.Geske, C.Montgomery, P.Jones, A. Bradley and T.Caskey, (1994). Hyperuricemia and urate nephropathy in urate oxidase deficient mice. *Proc Natl. Acad. Sci., USA*, 91: 742- 746.

Watanabe, Y. and J. Fukumoto, (1970). Studies on the formation of uricase by *Streptomyces*. Part II. The induced formation of uricase by resting cells. *Agri. Biol. Chem.*, 34: 1625- 1632.

Watanabe, Y., M. Yano and J. Fukumoto, (1969). Studies on the formation of uricase by *Streptomyces*. I. *The effect of purine bases on the induced formation of uricase by the cultured cells. Agri. Biol. Chem.*, 33: 1282- 1290.

Werner, M., J.M. Robert, J.R. Carol, M. Enzo and C. Stefano. (1979). Immobilized enzymes in continuous flow analysis. *Clin. Chem*., 25:20–23.

Whu, Y.-C., Hsu, T.-S., Huang, C.-Y., & Hsu, C.-H. (2021). Structural and biochemical insights into a hyperthermostable urate oxidase from *Thermobispora bispora* for hyperuricemia and gout therapy. *International Journal of Biological Macromolecules*, 188:914–923.

Wilkinson, S.P. and A. Grove, (2004).HucR, a novel uric acid-responsive member of the MarR family of transcriptional regulators from *Deinococcus radiodurans*. *J. Biol. Chem.,* 279:51442–51450.

Winterbourn, C.C., Kettle, A.J., Hampton, M.B. (2016). Reactive oxygen species and neutrophil function. *Annual review of biochemistry,* 85:765-792.

Wojdasiewicz, P., Poniatowski, Ł. A., & Szukiewicz, D. (2014). The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of osteoarthritis. Mediators of Inflammation, 2014:1-19.

Wright, MN., Adelskov,J., Greene, AC . (2017). Bacterial DNA Extraction Using Individual Enzymes and Phenol/Chloroform Separation. *J Microbial Biol Educ.*,18(2):1-3.

Wu, X., D.M. Muzny, C.C. Lee and C.T. Caskey, (1992). Two independent mutational events in the loss of urate oxidase during hominoid evolution. *J. Mol. Evol.*, 34:78–84.

Wu, X.W., C.C.Lee, D.M.Muzny and C.T.Caskey,(1989). Urate oxidase: primary structure and evolutionary implications. *Proc Natl. Acad. Sci., USA.*, 86: 9412-9416.

Wu, Y., Ye, Z., Feng, P., Li, R., Chen, X., Tian, X., Han, R., Kakade, A., Liu, P., Li, X. 2021. *Limosilactobacillus fermentum* JL-3 isolated from "Jiangshui" ameliorates hyperuricemia by degrading uric acid. *Gut microbes*, 13(1):1-18.

Xue-lai Zhou, Xiao-hang Ma, Gui-qin Sun, Xia Li, Kang-ping Guo. (2005). Isolation of a thermostable uricase-producing bacterium and study on its enzyme production conditions. *Process Biochemistry*, 40:3749–3753.

Yan, L., Zhao, F., Wang, J., Zu, Y., Gu, Z., Zhao, Y. (2019). A Safe-by-Design strategy towards safer nanomaterials in nanomedicines. *Advanced Materials*, 31(45): 1805391.

Yang, P., Liu, D., Wang, F., Wang, C., Chen, L., Ye, S., Dramou, P., Chen, J., He, H. (2021). Study on performance of mimic uricase and its application in enzyme-free analysis. *Analytical and Bioanalytical Chemistry,* 413(26):6571-6580.

Yasuda, Y., T. Fusita, Y. Takakura, M. Hashida and H. Sezaki. (1990). Biochemical and biopharmaceutical properties of macromolecular conjugates of uricase with dextran and polyethylene glycol. *Chem. Pharm. Bullet.,* 30:2053–2066.

Yazdi, M. T., G.Zarrini, E.Mohit, M.A.Faramarzi, N.Setayesh and F.A.Mohseni, (2006). *Mucor hiemalis*: a new source for uricase production. *World J.Microbiol. Biotechnol*.,22:325- 330.

Yutaka ,A., I.Hiroshi, N. Hiroomi, A.Tsugutoshi and Y.Mitsutatk. (1992). Effects of serum bilirubin on determination of uric acid by the uricase-peroxidase coupled reaction. *Clin. Chem.,* 38:1350–1352.

Zahide Esra Durak, Bahadur Ozturk. (2014). A modified xanthine oxidase activity method based on uric acid absorption. *ChemXpres,* 6(1):09–13.

Zhang, G. (2013). Functional gold nanoparticles for sensing applications. *Nanotechnology Reviews*, 2(3):269-288.

Zhang, Y. H. P. and L. R. Lynd, (2004). Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems. *Biotechnol. Bioeng.*, 88(7): 797–824.

Zhang, Y.Q., W. D. Shen, R. A. Gu, J. Zhu, and R. Y. Xue. (1998). Amperometric biosensor for uric acid based on uricase immobilized silk fibroin membrane. *Analytica. Chimica. Acta.*, 369(1-2): 123–128.

Zhou, X. L., Ma, X. H., Sun, G. Q., Li, X., & Guo, K. P. (2005). Isolation of a thermostable uricase-producing bacterium and study on its enzyme production conditions. *Process Biochemistry* 40:3749-3753.

Zhou, X., Ma, X., Sun, G., Li, X., & Guo, K. (2005). Isolation of a thermostable uricase-producing bacterium and study on its enzyme production conditions. *Process Biochemistry*, 40(12):3749–3753.

Zhou, Y., Zhang, M., He, D., Hu, X., Xiong, H., Wu, J., Zhu, B., Zhang, J. (2016).Uricase alkaline enzymosomes with enhanced stabilities and anti-hyperuricemia effects induced by favorable micro environmental changes. *Scientific Reports*, 6(1):20136.

Zhu, X.J., J.G., Liu and G.X.Li, (2001). Cloning and expression of urate oxidase and its application in serum uric acid analysis. *Chinese J. Biotechnol.*, 6: 68-72.

LIST OF PUBLICATION

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1. **Manimekalai V and Anandharaj, B. (2022)**. Anti-inflammatory and anti-hyperuricemic effect of uricase from *Bacillus subtilis* MM13 as a potential therapeutic agent for redemption from gout isolated from birds fecal contaminated soil. *International Journal of Biology, Pharmacy and Allied Sciences*, 11(1):203-215.
2. **Manimekalai V and Anandharaj, B. (2020).** Optimization of Growth parameters for Uricase enzyme activity of *Bacillus subtilis* MM13 enumerated from birds fecal contaminated soil. *Bulletin of Environment, Pharmacology and Life Sciences,* 10(9):77-84.
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ANTI-INFLAMMATORY AND ANTI- HYPERURICEMIC EFFECT OF URICASE FROM *Bacillus subtilis* MM13 AS A POTENTIAL THERAPEUTIC AGENT FOR REDEMPTION FROM GOUT ISOLATED FROM BIRDS FECAL CONTAMINATED SOIL

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ABSTRACT

Gout, the most common inflammatory arthritis is associated with a persistently raised plasma uric acid concentration. When these levels rise above the solubility limit it results in crystal deposition in joints, acute inflammation in response to those crystals causes severe pain. Treatment for severe gout includes injection of non-human uricase and anti-hyperuricemic and anti-inflammatory agents to reduce uric acid levels and inflammation. In the present study, the anti-inflammatory and the anti-hyperuricemic activity of the uricase produced by *Bacillus subtilis* MM13, isolated from poultry soil was evaluated in LPS-induced RAW 264.7 cells. To measure the effects of the uricase produced by *Bacillus subtilis* MM13, MTT assay, Trypan blue assay, xanthine oxidase activity assay, uric acid production and Gene expression analysis were performed. MTT and trypan blue assay demonstrated that uricase suppressed cell proliferation in a dose-dependent manner. The *in vitro* anti-hyperuricemic effect of uricase was identified due to the inhibition of Xanthine oxidase in LPS-induced RAW cells. Gene expression studies showed that Uricase suppressed the expression of inflammatory cytokine,

tumour necrosis factor α (TNF- α), and increased production of IL-10, a potent inhibitor of TNF- α. Considering the appreciable *in vitro* anti-hyperuricemic and anti-inflammatory activities, it is proposed that this preparation can be further studied for safety and efficacy under *in vivo* conditions which can be subsequently used for the prophylactic treatment of gout.

Keywords: Uricase, hyperuricemic, MTT assay, Trypan blue assay, prophylaxis

INTRODUCTION

Gout, the most common inflammatory arthritis with a prevalence ranging from 1–4% worldwide is associated with a persistently raised uric acid concentration. In India, approximately 0.12-0.19% population is affected by gout and is been reported as a disproportionate burden of disease in men, the elderly and racial/ethnic minorities **[1]**. When the serum uric acid levels rise above 7.0 mg/L, hyperuricemia occurs where the tissues are supersaturated with uric acid; subsequently, monosodium urate crystals accumulate in and around the joints where they are phagocytosed by neutrophils, macrophages, mast cells and dendritic cells within the synovium, which release inflammatory cytokines such as tumour necrosis factor (TNF-α) and can cause severe pain **[2]** affecting the individual's normal life routine.

Developed countries have seen a surge in the prevalence of gout and hyperuricemia over the past two decades, necessitating more intensive research **[3]**. It has been reported that about 80–90% of people with gout are hyperuricemic, and 10% of the patients with hyperuricemias develop gout **[4]**. Chronic hyperuricemia may lead to symptoms of gout, chronic kidney disease, hypertension, and cardiovascular diseases **[5, 4]**. Hyperuricemia and its associated diseases can be treated by an agent that upholds urate concentrations from precipitating and eliminate prevailing urate crystals. Uric acid-lowering agents (ULTs) classified into three main classes: xanthine oxidase inhibitors that reduce uric acid synthesis, uricosurics that inhibit URAT1 thereby increasing uric acid excretion, urolytics/ uricase that enhance metabolic hydrolysis of uric acid **[3]** have been used conventionally in the treatment of hyperuricemia **[6]**.

Owing to this, uricase from microbial sources can be represented as an alternative approach that can be more effective than the modified uricase formulations. In arthritic gout and renal disease, microbial uricase has emerged as a potential source of therapy for hyperuricemia **[7]**. Fermented extracts from microbes with substantial XOD inhibition activity and high uricase activity have also been

reported **[6]**.

Though various bacteria such as *Bacillus* species **[8]**, *Microbacterium* sp. **[9]** and *Streptomyces* **[10]**, have been reported as uricase producers, not all bacterial uricase are thermos table and their biocompatibility and anti-inflammatory and the anti-hyperuricemic activity are unknown. Uricase enzymes with these properties will have the advantage for mass production, transportation, storage and clinical application. Among the other bacteria's, urate oxidase from thermophilic *Bacillus* sp. TB-90 has been reported to be used for diagnostic purposes since it reveals high activity and thermo stability in an extensive range of pH **[11]**. Based on this theory, *Bacillus subtilis* was isolated from soil samples in and around Hosur area, Krishnagiri District, Tamil Nadu and an isolate that produced the highest uricase activity was identified, designated as *Bacillus subtilis* MM13 **[12]** and its efficacy was evaluated. The main objective of this study is to evaluate the anti-inflammatory and the anti-hyperuricemic activity of the potent local uricase produced by *Bacillus subtilis* MM13 and it was evaluated in LPS-induced RAW 264.7 cells.

MATERIALS AND METHODS

Chemicals and Reagents

Chemicals and reagents used in the present study were procured from standard chemical manufacturers and distributors (Sigma Aldrich Chemicals Pvt. Ltd).

Cell culture

RAW 264.7 cells were procured from the Cell repository of ATCC. These cell lines are cultured in 1X Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PenStrep), and maintained in a humidified atmosphere of 5% CO_2 at 37 °C. When confluent (80% confluence), cells were passaged and used for further experiments in an exponential growth stage.

Cytotoxicity Evaluation

Effects of uricase was evaluated using a modified MTT assay **[13]**, a mitochondrial-dependent reduction of 3-(4,5-dimethylthizaol-2yl)2,5-diphenyl tetrazolium bromide (MTT) to formazan. The plates were kept on a shaker for 10 min at room temperature and then the absorbance was measured using a microplate reader at a wavelength of 490 nm and 630 nm. The control group consisting of untreated cells was considered as 100% of viable cells after carrying out the assay, the inhibition rate was calculated using the following formula:.

% Inhibition Rate = {1- [A_{490} - A_{630} (Treated) / A_{490} – A_{630} (control)] x 100

Trypan Blue Exclusion Assay

Based on the principle that living cells possess

intact cell membranes, the dye exclusion test is used to determine the number of viable cells present in a cell suspension **[14]**. Viable (unstained) and nonviable (blue-stained) cells were counted using a hemocytometer and calculated as given below.

The average number of cells per field = Sum of cells per field /Number of fields

Total number of cells per mL ($x* 10^4$/mL) = Average number of cells per field × dilution factor.

(The dilution factor is 2 because the cells were with equal volumes of trypan blue)

%Viability = Number of colourless cells/Total number of cells × 100

Morphological Analysis - Phase Contrast Microscopy

LPS induced RAW264.7 Cells (5×10^5 cells/well) were incubated for 24 h with selected four concentrations of uricase (0, 30, 50, and 100 μg/ml) in tissue culture dishes (60 mm diameter). After incubation, the medium was discarded and cells were washed once with Phosphate Buffer Saline (1X PBS). The morphological changes observed were viewed at 200x magnification **[15]**.

Xanthine oxidase (XO) activity assay

The Xanthine oxidase activity was carried out by **[16]**. Xanthine oxidase levels were measured, an increase in the absorbance indicates the formation of uric acid from xanthine. The treated cell pellets were mixed with assay buffer and the supernatants were isolated and pre-incubated at 37 °C for 40 min. Blanks contained the same reaction mixture without xanthine. A volume of 0.1 mL of 100% (w/v) trichloroacetic acid (TCA) was added to the working solution and the mixture was centrifuged at 5000 x g for 10 min and a second absorbance (A2) was measured again at 293 nm. The absorbance change (A) is calculated as follows:

A = [(A2) assay - (A2) blank] – [(A1) assay - (A1) blank]

Note: A/min =A/30

Uric acid Estimation

Uric acid was estimated using a commercial Uric Acid Kit (Uricase - PAP method, *Tulip Diagnostics*) according to the manufacturer's instruction. Briefly, RAW264.7 cells were plated at 5×10^5 cells/well. The contents were mixed well and incubated for 5 min at 37 °C and the absorbance was measured at 520 nm within 30 min after incubation and calculated as follows:

Uric acid in mg/dL = (Absorbance at T / Absorbance at S) X 8

Gene Expression Analysis

RAW264.7 cells were seeded in culture flasks induced with 500 ng/mL LPS for 4 h and treated with 30, 50, and 60 µg/mL of uricase for 24 h. DNA was isolated using a Nucleospin

DNA isolation kit according to the manufacturer's instruction and PCR was performed with the below-given primers:

β-Actin

5' TCAAGGTGGGTGTCTTTCCTG 3' (F)

5' ATTTGCGGTGGACGATGGAG 3' (R)

TNF- alpha

5'ACACAGAAGACACTCAGGGA 3' (F)

5' CCGGTACTTAACCCTACCCCC 3'(R)

IL10

5'AATCACGGCTCAGTTCTCCC 3'(F)

5' GGTCACACCATTTGCTGGGT 3'(R)

The amplification sequence protocol was conducted for 35 cycles: Initial denaturation - 1 cycle at 95 °C for 2 min, DNA denaturation - 95 °C for 1 min, Annealing Temperatures - 54 °C (β-Actin), 54.6 °C (IL-10) and 55.4 °C (TNF- alpha) for 30 s and Extension - 72 °C for 1 min, Final extension - 72 °C for 5 min.

RESULTS:

MTT ASSAY

The inhibitory effect of uricase was assessed on RAW264.7 mouse macrophage cells using the MTT assay in **Figure 1**. The cells were treated with uricase at concentrations of 10 - 100 µg/ml for

24 h. Uricase mediated cell proliferation in a dose-dependent manner suggests that uricase inhibits the growth of RAW264.7 cells. The LC50 was determined to be 50 µg/ml concentrations with 54.26% inhibition.

Trypan Blue Exclusion Assay

Trypan blue exclusion assay, the most common and earliest method was used for cell viability measurement. Cells that are compromised by uricase treatment become permeable and it binds to the intracellular proteins and render them bluish colour and the cell death rate was determined. Dose-dependent cell death was observed, cell viability decreased with the increasing concentration shown in **Figure 2**.

Morphological changes by Phase Contrast Inverted Microscope

Changes in the cellular morphology under uricase treatment were observed in different concentrations in **Figure 3**. Similar to the cell proliferation and cell inhibition assays the cells shrank and subsequently detached in a dose-dependent manner when compared with the control. Untreated RAW264.7 cells appeared normal (circular shaped).

Xanthine Oxidase assay.

Figure 4 illustrates the effect of three different concentrations of uricase (30 µg/mL, 50 µg/mL and 60 µg/mL) as compared to LPS induced RAW cells and control (untreated cells), in inhibition of xanthine oxidase enzyme. All three concentrations of uricase showed similar XO inhibitory activity. Results were subjected to one-way ANOVA (Tukey multiple comparison tests).

Uric acid Estimation

In vitro effects of uricase, uric acid concentration in the cells are illustrated in **Figure 5**. The uricacid concentration was significantly reduced by uricase in all three concentrations (30 µg/mL, 50 µg/mL and 60 µg/mL) after pretreatment with LPS compared with the non-treated LPS induced group ($p < 0.0001$).

Gene Expression Analysis

The roles of pro-inflammatory cytokine (TNF-α) and anti-inflammatory cytokines (IL-10) were investigated. Down-regulated gene expression levels were observed for TNF- α in uricase treated cells and an upregulated expression was observed for IL- 10 gene, in a dose-dependent manner in **Figure 6**. IL-10 is a potent inhibitor of TNF-α and administration of uricase enzyme at a defined concentration can help to reduce the pro-inflammatory cytokine TNF-α, thereby reducing cell damage.

DISCUSSION

The most frequent inflammatory arthritis, characterized by prevalence was higher than 1% in both occidental and oriental countries and was found to be gout **[17]**. It should be viewed seriously, due to its manifestation with various other complications including hypertension, cardiovascular risk, chronic kidney disorder, obesity, and metabolic syndrome **[18, 19]**.

MTT and trypan blue assay revealed that *Bacillus subtilis* MM13 uricase had strong inhibitory activity against LPS induced RAW cells in a dose-dependent manner (Fig. 1). At 100 µg/mL concentration more than 80% of cell growth was inhibited. The IC50 was determined to be 50 µg/ml by the MTT assay which was further confirmed by trypan blue assay. Hence, a concentration one below and one above the IC50 was selected for further studies along with the concentration which exhibited the IC50 range. Morphological evaluation on the concentrations 30 µg/mL, 50 µg/mL and 60 µg/mL were in line with the MTT and trypan blue assay results. Changes indicative of cell inhibition, where LPS induced RAW cells deteriorated, in a dose-dependent manner and parallel morphological changes including shrinkage, blebbing and distorted cell membrane followed by cellular detachment were observed in response to *Bacillus subtilis* MM13 uricase treatment (Fig. 3).

Xanthine oxidase plays the role of housekeeping and the rate-limiting enzyme during purine catabolism. In the present study, the inhibition of XO activity was found in all uricase treated concentrations (30 µg/mL, 50 µg/mL and 60 µg/mL). LPS induced RAW cells showed higher activity compared to treated and control groups ($p \leq 0.01$). All uricase concentrations showed inhibition of

more than 50%. Xanthine oxidase inhibitors(XOI) are considered the first-line drugs in ULT for gout **[20]**. Further **[21]** reported that hyperuricemic subjects showed that XOIs are more effective in secondary prevention, significantly reducing the occurrence of major adverse cardiovascular events in individuals with previous transient ischemic attacks, stroke, unstable angina, or myocardial infarction. In the present study, all uricase treated RAW cells showed inhibition of more than 50% revealing the anti-hyperuricemic effect of uricase. In the present investigation urate-lowering effects of *Bacillus subtilis* MM13 uricase was evaluated at different concentrations on LPS induced RAW cells in **Figure 5**. Uricase at all three concentrations (30, 50 and 60 µg/mL) was found to be effective in reducing uric acid, which was consistent with the XO inhibition results that suggested that the results of the hypouricemic action paralleled the reduction in XOI activities. As a preliminary evaluation, this study first confirmed that *Bacillus subtilis* MM13 uricase reduced the uric acid levels suggesting that this study shall be further performed under *in vivo* conditions to be confirmed as a therapeutic model.

Further elucidated the molecular mechanism of *Bacillus subtilis* MM13 uricase Gene expression analysis was carried out. Studies have proposed that stimulation of IL- I and TNF production by monosodium crystals maybe a crucial link and the inhibition of these could be a promising strategy **[22-25]**. In the present investigation, gene expression studies with uricase from *Bacillus subtilis* MM13 indicated a dose-dependent suppression of TNF-α. TNF-α was strongly down-regulated by a higher dose of uricase whereas a milder suppression was observable in a low dose of uricase. To further evaluate if uricase triggers cytokine suppression in LPS induced RAW cells, we estimated the expression of the IL-10 gene, a major anti-inflammatory cytokine. From our results, it is evident that IL-10 reduces the effect of TNF-α indicating that the uricase from *Bacillus subtilis* MM13 has significant anti-inflammatory activity. A report on IL-10 suggests that reducing the effect of TNF-α significantly decrease the secretion of PGE2, COX-2, and PLA2 **[26]**. The reduction in the levels of TNF- α in the treated cells indicates that the uricase from *Bacillus subtilis* MM13 has significant anti-inflammatory activity.

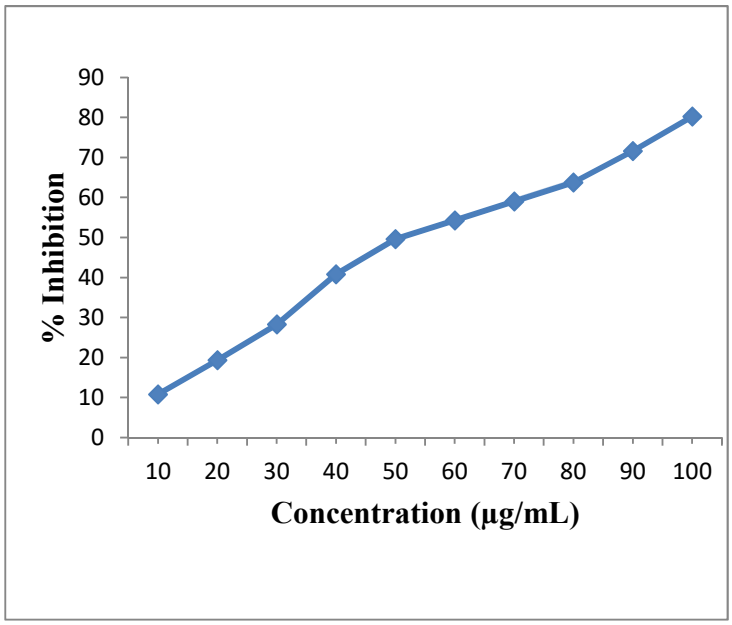

Figure 1: Cytotoxic effect of uricase on Raw 264.7 cells. Different concentrations of uricase (10-100 µg/mL) were used for cell treatment

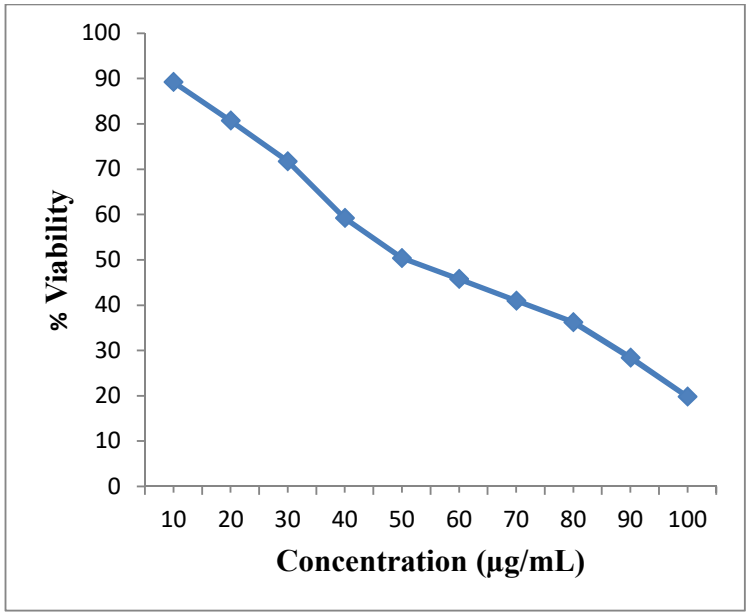

Figure 2: Effect of uricase on cell viability of RAW264.7 cell line - The cell death rate was determined using trypan blue assay. RAW264.7 cells were treated with Uricase (10 – 100 µg/mL) for 24 h. Uricase caused cell death in a dose-dependent manner

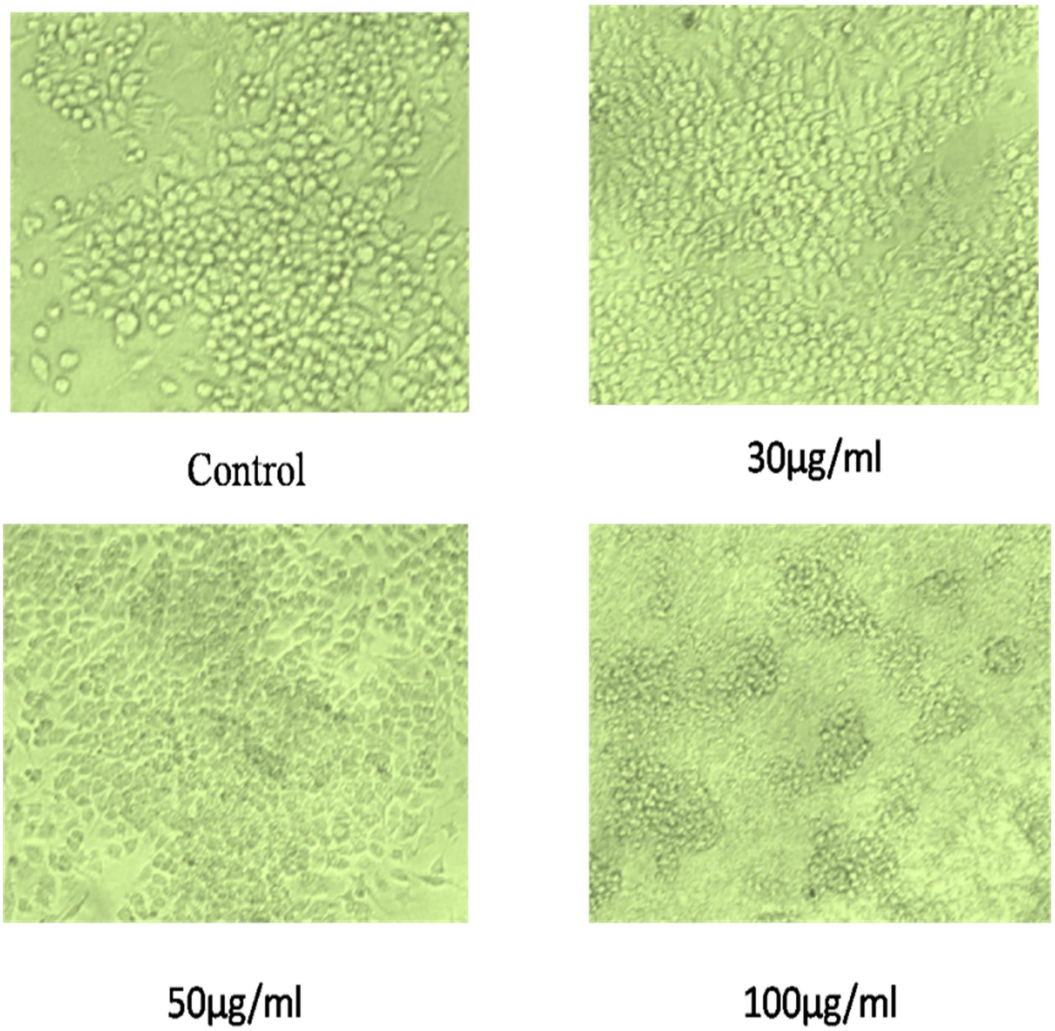

Figure 3. Effect of uricase on RAW 264.7 - Photomicrograph (20 x) represents morphological changes with Shrinkage, detachment, membrane blebbing and distorted shape induced by uricase treatment (30, 50 and 100 µg/ml for 24 h) as compared with control. Control showed normal intact cell morphology and their images were captured by phase contrast microscope.

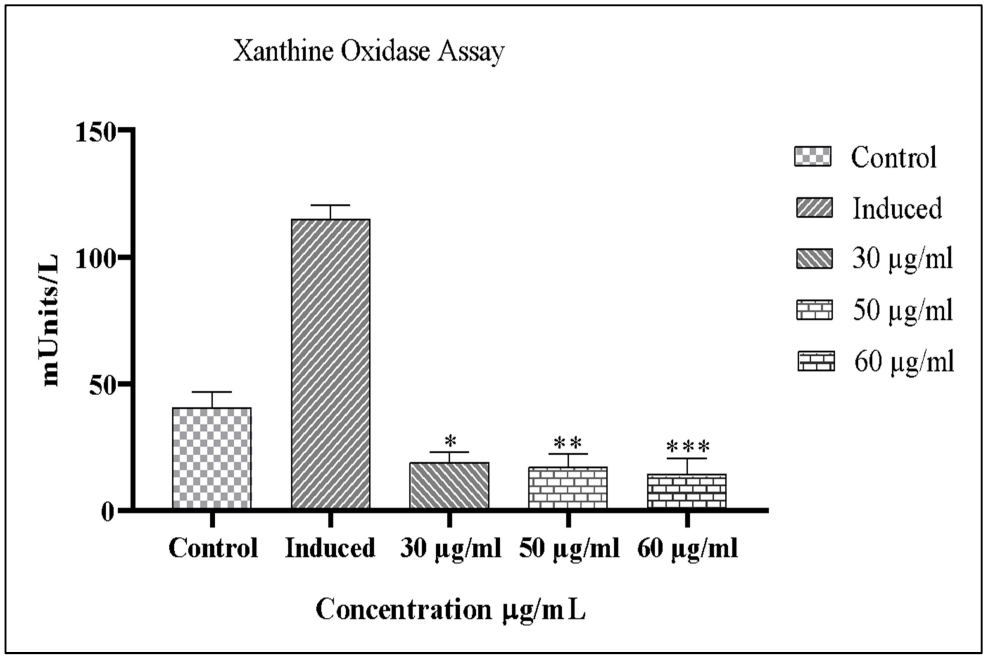

Figure 4: Xanthine oxidase assay - Xanthine Oxidase production of treated groups was significantly decreased when compared to induced group. Results are expressed as mean ± SD. Treated groups showed high significance compared to control (P< 0.001)

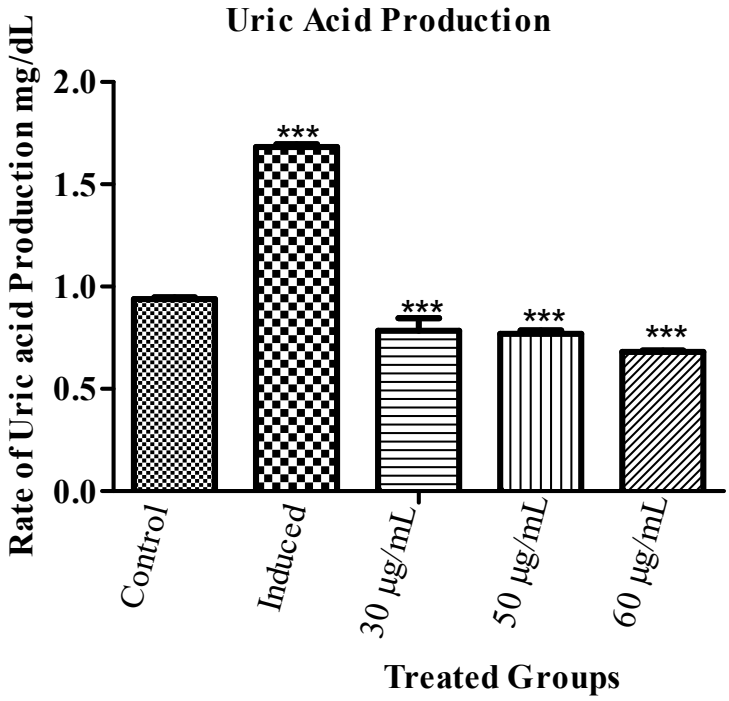

Figure 5: Estimation of Uric acid production - Uric acid production of treated groups was significantly decreased when compared to induced group. Results are expressed as mean ± SD. Treated groups showed high significance compared to control (P< 0.0001)

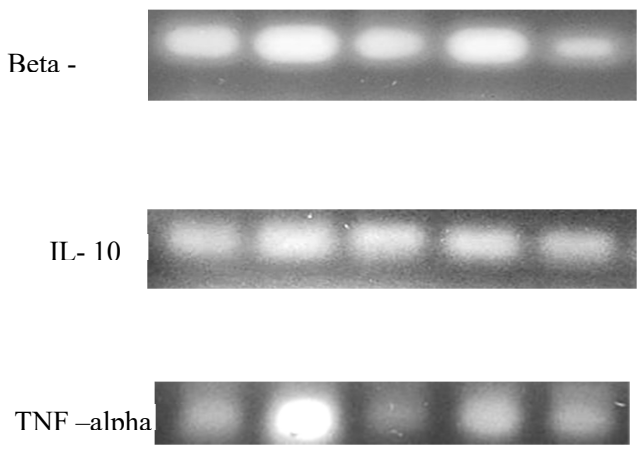

Figure 6: Gene expression analysis of TNF-α and IL-10. Decreased gene expression levels were observed for TNF- alpha in uricase treated cells and increased expression level was observed for IL- 10 gene, with increase in uricase concentration. Housekeeping gene (β – Actin)

CONCLUSION

This study provided conclusive evidence that uricase isolated from *Bacillus subtilis* MM13 has shown anti-inflammatory and anti-hyperuricemic effects under *in vitro*. One of the important properties of uricase is its very high specificity. Considering the appreciable specific anti-hyperuricemic and anti-inflammatory activities, this preparation can be taken further for safety and efficacy testing in vivo and subsequently can be recommended for the prophylactic treatment of gout.

REFERENCE

[1] Singh, J. A., & Gaffo, A. Gout epidemiology and comorbidities. *Seminars in Arthritis and Rheumatism*, 2020, 50(3), S11–S16.

[2] Derek G. Waller, A. S. Medical Pharmacology and Therapeutics (five). *Elsevier Limited.* 2018

[3] Benn, C. L., Dua, P., Gurrell, R., Loudon, P., Pike, A., Storer, R. I., & Vangjeli, C. Physiology of hyperuricemia and urate-lowering treatments. *Frontiers in Medicine*, 2018, 5, 160.

[4] Smith, E. U. R., Díaz-Torné, C., Perez-Ruiz, F., & March, L. M. Epidemiology of gout: An update. *Best Practice and Research: Clinical Rheumatology*, 2010, 24(6), 811–827.

[5] Janssens, H., Van de Lisdonk, E. H., Bor, H., Van den Hoogen, H. J. M., & Janssen, M. Gout, just a nasty event or a cardiovascular signal? A study from primary care. *Family Practice*, 2003, 20(4), 413–416.

[6] Chen, R.-J., Chen, M.-H., Chen, Y.-L., Hsiao, C.-M., Chen, H.-M., Chen, S.-J., Wu, M.-D., Yech, Y.-J., Yuan, G.-F., &

Wang, Y.-J. Evaluating the urate-lowering effects of different microbial fermented extracts in hyperuricemic models accompanied with a safety study. *Journal of Food and Drug Analysis,* 2017,25(3), 597–606.

[7] Chiu, Y.-C., Hsu, T.-S., Huang, C.-Y., & Hsu, C.-H. Structural and biochemical insights into a hyperthermostable urate oxidase from *Thermobispora bispora* for hyperuricemia and gout therapy. *International Journal of Biological Macromolecules,* 2021,188, 914–923.

[8] Tan, Q., Zhang, J., Wang, N., Li, X., Xiong, H., Teng, Y., He, D., Wu, J., Zhao, C., & Yin, H.Uricase from *Bacillus fastidious* loaded in alkaline enzymosomes: enhanced biochemical and pharmacological characteristics in hypouricemic rats. *European Journal of Pharmaceutics and Biopharmaceutics,* 2012, 82(1), 43–48.

[9] Zhou, X., Ma, X., Sun, G., Li, X., & Guo, K. Isolation of a thermostable uricase-producing bacterium and study on its enzyme production conditions. *Process Biochemistry,* 2005, 40(12), 3749–3753.

[10] Nanda, Pooja, & Jagadeesh Babu, P. E. (2014). Isolation, screening and production studies of uricase producing bacteria from poultry sources. *Preparative Biochemistry and Biotechnology*, 2014, 44(8), 811–821.

[11] Yamamoto, K., Kojima, Y., Kikuchi, T., Shigyo, T., Sugihara, K., Takashio, M., & Emi, S. Nucleotide sequence of the uricase gene from *Bacillus* sp. TB-90. *The Journal of Biochemistry*, 1996, 119(1), 80–84.

[12] V.Manimekalai, B. Anandharaj Poultry soil derived bacterial flora from Hosur, Tamilnadu and their uricase activities. *Journal of Applied Science and Computations*, 2019, 6, 6, 3756–3762.

[13] Sylvester, P. W. (Optimization of the tetrazolium dye (MTT) colorimetric assay for cellular growth and viability. *In Drug design and discovery* 2011, 157–168)

[14] Crowley, L. C., Marfell, B. J., Christensen, M. E., & Waterhouse, N. J. Measuring cell death by trypan blue uptake and light microscopy. *Cold Spring Harbor Protocols,* 2016,(7), pdb-prot087155.

[15] Moongkarndi, P., Kosem, N., Kaslungka, S., Luanratana, O., Pongpan, N., & Neungton, N. Antiproliferation, antioxidation and induction of apoptosis by *Garcinia mangostana* (mangosteen) on SKBR3 human breast cancer cell line. *Journal of Ethnopharmacology,* 2004, 90(1), 161–166.

[16] Zahide Esra Durak, Bahadur Ozturk..A modified xanthine oxidase activity

method based on uric acid absorption. *ChemXpres*, 2014, 6(1), 09–13.

[17] Dehlin, M., Jacobsson, L., & Roddy, E. Global epidemiology of gout: prevalence, incidence, treatment patterns and risk factors. *Nature Reviews Rheumatology*, 16(7), 2020, 380–390.

[18] Choi, H. K., Atkinson, K., Karlson, E. W., & Curhan, G. Obesity, weight change, hypertension, diuretic use, and risk of gout in men: the health professionals follow-up study. *Archives of Internal Medicine*, 165(7), 2025,742–748.

[19] Daoudi, N. E., Bouhrim, M., Ouassou, H., & Bnouham, M. Medicinal Plants as a Drug Alternative Source for the Antigout Therapy in Morocco. *Scientifica*, 2020.

[20] Bove, M., Cicero, A. F. G., Veronesi, M., & Borghi, C. An evidence-based review on urate-lowering treatments: implications for optimal treatment of chronic hyperuricemia. *Vascular Health and Risk Management*, 2017, 13, 23.

[21] Bredemeier, M., Lopes, L. M., Eisenreich, M. A., Hickmann, S., Bongiorno, G. K., d'Avila, R., Morsch, A. L. B., da Silva Stein, F., & Campos, G. G. D. Xanthine oxidase inhibitors for prevention of cardiovascular events: a systematic review and meta-analysis of randomized controlled trials. *BMC Cardiovascular Disorders*, 2018,18(1), 1–11.

[22] Di Giovine, F. S., Malawista, S. E., Thornton, E., & Duff, G. W. Urate crystals stimulate production of tumor necrosis factor alpha from human blood monocytes and synovial cells. Cytokine mRNA and protein kinetics, and cellular distribution. *The Journal of Clinical Investigation*, 1991, 87(4), 1375–1381.

[23] Pierce, G. F. Macrophages: important physiologic and pathologic sources of polypeptide growth factors. *Am J Respir Cell Mol Biol,* 1990,2(3), 233–234.

[24] Erwig, L.-P., & Rees, A. J. Macrophage activation and programming and its role for macrophage function in glomerular inflammation. *Kidney and Blood Pressure Research,* 1999,22(1–2), 21–25.

[25] Wadleigh, D. J., Reddy, S. T., Kopp, E., Ghosh, S., & Herschman, H. R. Transcriptional activation of the cyclooxygenase-2 gene in endotoxin-treated RAW 264.7 macrophages. *Journal of Biological Chemistry,* 200, 275(9), 6259–6266.

[26] Wojdasiewicz, P.,Poniatowsi, Ł.A., & Szukiewicz, D. The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of osteoarthritis. *Mediators of Inflammation*, 2014.

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ORIGINAL ARTICLE **OPEN ACCESS**

Optimization of Growth parameters for Uricase enzyme activity of *Bacillus subtilis* MM13 enumerated from birds fecal contaminated soil

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ABSTRACT
This research was designed to enumerate the uricase producing bacterial isolate from bird's fecal contaminated soil and optimize the growth parameters suitable for uricase enzymatic activity. Isolation done by dilution plate method using selective medium. In this study, a potent uricase producing organism was isolated by a thorough screening and identified as Bacillus subtilis strain by using 16s rDNA sequencing. Optimization of various factors influencing maximum enzyme coproduction by Bacillus sp is performed. The statistical experimental design method was further applied to obtain optimal concentration of significant parameters such as pH, temperature, uric acid concentration, urea concentration, carbon, nitrogen, substrate are tested. Totally ten isolates were recovered and one uricase producing bacterial isolate selected for optimal studies and characterized by 16SrDNA sequencing. The optimal essential growth factors required to obtain maximum uricase enzyme activity by the tested isolate were found as pH 7.0, 40°C, 1% glucose, 0.2% yeast extract, and 0.32% of uric acid. The B. subtilis MM13 yielded uricase ranged from 1.25 to 2.54 U/ml in a 48 h submerged fermentation process under these optimized conditions. None of the tested carbon and inorganic nitrogen sources had significant stimulatory effect on uricase productivity except Sucrose and yeast extract This predominant uricase producing bacterial isolate Bacillus subtilis MM13 sequence was submitted to NCBI-GenBank and received accession number as MK503710. Use of statistical optimization upsurges uricase yield from 1.25 U/ml to 15.87 U/ml enhancing the overall production by 13.23 fold; which confirms that the model is effective for process optimization. These results conclude that the B. subtilis MM13 enumerated from pigeon fecal contaminated soil may be considered for further purification and biomedical applications related in-vitro and in-vivo studies.
Keywords: *Fecal matter; endospore; Uricase; Optimization; Submerged fermentation*

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INTRODUCTION

Hyperuricemia is a condition that is characterized by an increased quantity of serum urate that usually precedes the emergence of monosodium crystals [1-2]. This monosodium crystal accumulates in distal tendons and peripheral tissues, causing gout, an inflammatory arthritic disease[3-4]. Overconsumption of fructose-enriched corn syrup, purine-rich foods, red meat, seafood, organ meat, beans, and excessive alcohol consumption may contribute to hyperuricemia development [5-6]. Uric acid is the major byproduct of purine metabolism. Hyperuricemia can occur as a result of uric acid excess production or poor excretion in humans. According to a recent report, hyperuricemia affects approximately 14.4% of adolescents (aged 35 to 49) worldwide [7-8]. According to human evolutionary theory, elevated uric acid quantities might well have offered a survival benefit during periods of starvation in the previous era [9-10]. Even though evidence that hyperuricemia can be both an effective protection and a causative factor in non-communicable disorders that encompasses cardiovascular, neurological disorder, gout, leukemia, toxemia of pregnancy, severe renal impairment, and idiopathic calcium urate nephrolithiasis[11-12]. The main levels of serum uric acid (SUA) have been observed in recent years, and hyperuricemia seems prominent in developed and emerging nations[13-14]. The global prevalence of non-communicable diseases is rising as a result of industrialization, urbanization, and ageing [15-17]. The uricase is a hepatocellular and urate oxidase enzyme that reduces uric acid to allantoin, a water-soluble molecule that is more rapidly eliminated by kidneys [18-19].

In some cases, though, mutations in missense and frame shift after evolution resulted in an inactive

uricase gene [20-22]. Hence the uricase enzyme has been used to treat hyperuricemia and related disorders[23]. Many species, encompassing higher plants and microbes, may synthesize uricase, influenced by culture medium composition. All these organisms' capability to break down uric acid and utilize this for development is an inducible feature [24]. Although this enzyme is found in abundance in most animals, it is not found in humans. Humans as well as other higher mammals are unable to generate uricase and are hence prone to uric acid-related illnesses [25-26]. Among various organisms, the microbes, especially bacteria are preferably used for uricase fabrication, and bacterial species such as *Bacillus pasteurii*, *Proteus mirabilis*, and *Escherichia coli* produce intracellular enzyme and while *Streptomyces albosriseolus*, *Microbacterium* sp., *Bacillus thermocatenulatus*, *Candida tropicalis*, and *Pseudomonas aeruginosa* [27]. However, the existing uricase production is unable to meet the global requirement, thus finding potential bacterial strains are timely needed [28]. Urate oxidase in most species converts uric acid to 5-hydroxyisourate. Depending on species, 5-hydroxyisourate is degraded further and eliminated from the body as allantoin and ammonia[29]. Uricase is just a non - soluble crystalloid that comprised with peroxisomes in vertebrate animals [30]. In most microbes and aquatic vertebrates, uricases are dissolvable as well as present in either the cytosol (bacteria) or the peroxisome (yeast). In general the researchers prefer the enzymes production from bacterial culture due to simple, cost-effective and reliable production. Bacterial enzyme production is primarily determined by growth factors such as pH, temperature, nutritional sources such as carbon and nitrogen sources, and so on[31]. As a result, optimizing the growth parameters is the most important factor in determining a bacterial strain's uricase producing maximum potential. This is the first report about enumerating uricase producing predominant bacterial species from rose garden soil and optimized the suitable growth conditions for the uricase producing bacterial strain. Since, under the optimal growth conditions, the cell growth, metabolic activity, and reproduction rate will be significantly increased and yielded considerable enzymes. Hence, this research was designed to isolate the uricase producing predominant bacterial strain from various birds' fecal contaminated soil and optimize the suitable growth conditions for uricase producing bacteria to produce maximum yield.

MATERIAL AND METHODS

Collection and processing of fecal contaminated soil sample

The fecal contaminated soil samples were collected from pigeons, turkey, parrot, and poultry farms/nests in Rayakota, Berigai, Perandapalli, and Kumudepalli. The samples were collected in sterile zip-lock cover and immediately transferred to the laboratory, and subjected to a standard serial dilution process for each sample individually.

Enumeration and confirmation of uricase producing predominant bacterial isolates

From the standard serial dilution, 0.1 mL of 10^{-6} dilution of each sample was individually inoculated on sterilized uric acid agar medium containing plates by spread plate method. The inoculated plates were incubated at 37˚C for 24 h. After incubation, the clearance zone formed around the colonies was measured, and colonies that showed maximum clearance zone were enumerated. The uricase secreting potential of enumerated bacterial isolates was confirmed by performing uric acid or uricase assay using Amplex™ uricase assay kit (Thermo Fisher Scientific: Cat. log. No: A22181) and standard Lowry's method.

Preliminary growth parameters optimization[32]

To evaluate the basic optimal growth parameters required for the better growth and synthesis of uricase were studied as follows . The one-factor-at-a-time method was applied for each factor such as pH 5-9, temperature 30-70˚C under static submerged condition. 5 mL (%) of 24 h old *B. subtilis* is inoculated on 100 ml of uric acid broth medium and incubated in a shaker incubator with 150 rpm for 48 h. The standard uricase assay activity was performed after incubation of each growth parameter

Secondary growth parameter optimization[33]

Iinfluence of various carbon and nitrogen sources on growth and uricase production was assessed with 2% of carbon source: glucose, sucrose, maltose, fructose, and lactose, 1% of nitrogen source: yeast extract, peptone, ammonium nitrate, ammonium chloride, and casein. 100 ml of uric acid broth medium enriched with above carbon and nitrogen independently and autoclaved. the various concentration of uric acid (0.12, 0.22, 0.32, 0.42, and 0.52%), and 5% of natural uric acid enriched with dried and sterilized powdered form of wheat bran, beans, cauliflower, and pigeon fecal with standard growth conditions in 100 mL of uric acid broth medium containing 250 mL conical flask individually. About 5 mL (%) of 24 h old *B. subtilis* MM13 (OD_{600}: 1.5) was inoculated on each flask and incubated in a shaker incubator with 150 rpm for 48 h. The standard uricase assay activity was performed after incubation of each growth parameter

Effect of Sucrose and yeast extract

Production of enzyme at different concentrations of sucrose (0.25, 0.5, 0.75, 1.0, and 1.5%) and yeast extract (0.2, 0.3, 0.4, 0.5, and 0.6%) required for attaining maximum yield from *B. subtilis* MM13 were optimized through submerged fermentation process one-factor-at-a-time approach in 100 mL of uric acid broth in 250 mL of the conical flask. The culture inoculated flask was incubated in a shaker incubator at 150 rpm for 48 h, and a uricase assay was performed to calculate the uricase activity

Uricase activity assay [34]

Uric acid at a concentration of10µg/ml was dissolved in 2 ml of 200mM borate buffer (pH 8.5), mixed with 0.8 ml water and 0.1 ml of crude enzyme (CFCS). The mixture was incubated at 35°C for 10 min and then the reaction was stopped by adding 0.2 ml of 100 mM potassium cyanide solution (PCS). The PCS added to the mixture before the CFCS addition was served as the reference. The absorbance was measured at 293 nm. The difference in the absorbance between the test and the reference is equivalent to the decrease in uric acid during the enzyme reaction. One unit of uricase enzyme was equivalent to the amount of enzyme that converts 1µmol of uric acid to allantoin per min.

Molecular characterization of test isolates [35-36]

The preliminary screening (based on clear zone formation) and uricase assay results declared that 1 isolate out of 10 has an outstanding uricase enzyme activity, and that isolate was termed as MM13. The molecular characterization (16S rRNA sequencing) study was performed to identify the genus and species of test isolate MM13. The bacterial total genomic DNA extraction kit (gDNA extraction kit, from ThermoFisher Scientific). The thermo cycler (ProFlex – Thermo Fisher Scientific) was used to perform the 16S rRNA sequencing amplification process using forward: 5′-CCAGTAGCCAAGAATGGCCAGC-3′ (EN1F) and reverse:5′-GGAATAATCGCCGCTTTGTGC-3′ (EN1R). The standard operating conditions (denaturation, annealing, and extension) were followed, with 25 cycles of amplification were performed. The amplified PCR product was purified using a readymade PCR product purification kit (GenElute™ PCR clean up kit (NA1020)-Sigma -Aldrich), and 518F/800R sequencing system was performed and submitted at NCBI-GenBank and obtained accession number (MK503710). To confirm the genus and species of test isolate. The MEGA X was applied to construct the phylogenetic tree and circular sequence analyses to understand the evolutionary relationship and possible restriction sites and GC percentile analyses.

RESULT AND DISCUSSION

The demand for uricase enzymes has been increasing recently, especially in the medical sector, to treat hyperuricemia and related diseases. Hence, finding a suitable and potential uricase producing bacterial isolate promptly is required to meet global demand. A total of 10 bacterial isolates enumerated from a pigeon (5 nos.), turkey (2 nos.), parrot (2 nos.), and poultry fecal (1 no.) contaminated soil was found as possessing uricase activity. One isolate out of 10 isolates named MM13 enumerated from pigeon fecal contaminated soil showed outstanding uricase enzyme activity (30 mm). It was determined by the zone of clearance around the colony in millimeters. The zone of clearance formed around the colony is due to the conversion of uric acid into 5-hydroxyisourate by uricase with the aid of O_2 and H_2O and yielded H_2O_2 and subsequently reduced as allantoin. The size of zone of clearance formed around the colony from uric acid was considered a key factor in determining the uricase secreting potential of bacterial isolate [38].

Similarly, the *Bacillus cereus* DL3 enumerated from poultry farms had been reported to possess excellent uricase secreting activity. It was determined by measuring the zone of clearance around the colonies on uric acid media. The pigeon fecal matter may contain a significant quantity of uric acid than other bird's fecal matter. Thus the bacteria isolated (MM13) could possess the maximum potential to secrete uricase enzyme and utilize the uric acid in the pigeon fecal matter[37]. Thong et al. [38] enumerated uricase producing *Clostridia* sp. isolated from the gut region of various termites and identified by a zone of clearance around the colonies**.**

The optimal essential growth parameters are required to obtain the maximum uricase enzyme activity of *B. subtilis* MM13. The metabolic activity and growth rate of bacteria can be influenced by essential growth factors such as pH, temperature, carbon and nitrogen sources, etc. Thus table 1 represents the concept of the research to optimize the growth parameters like pH, temperature, carbon , nitrogen and uric acid for uricase production by *Bacillus* sp.

The optimal physical factors such as pH and temperature are required for producing maximum uricase enzyme activity. The obtained results showed that the maximum uricase enzyme activity was 1.85 and 1.25 U/ml at pH 7.0 and 40°C respectively at 48 h of the submerged fermentation process, and these were statistically significant at $P<0.05$ (Fig. 1a and Fig. 1b). Furthermore, the uricase enzyme activity was significantly reduced at pH 5, 6, 8, and 9 and at 30, 50, 60, and 70°C, respectively. The increased temperature (increasing kinetic energy) and optimal pH can speed up the reaction, however, increased

temperature and pH (breaking bonds) instantly reducing the enzyme and metabolic activities[39]. The optimum pH and temperature ranges for enzyme activity were reported as 4.0-9.0 and 30-50°C, respectively, and these ranges may be related to various strains and substrates using for enzyme production. Similarly, the optimal pH and temperature for uricase enzyme activity of *Saccharopolyspora* sp. PNR11 was reported as 7.0 and 30°C, respectively[40].

The availability of suitable carbon and nitrogen sources determines the enzymatic and metabolic activities and the growth rate of bacteria. In this study, various carbon sources such as glucose, maltose, lactose, sucrose, and fructose were subjected to identify the suitable carbon source for *B. subtilis* MM13 to secrete uricase enzyme activity. The obtained results state that the test isolate preferably used glucose as a major carbon source than other sugars and showed maximum uricase enzyme activity 2.54 U/ml. This value was statistically significant at $P<0.05$ (Fig. 1c). Similarly, the *B. subtilis* MM13 strain effectively utilized the yeast extract as the preferred nitrogen source and yielded the uricase enzyme activity as 2.37 U/ml in a 48 h period of incubation. This was statistically significant at $P<0.05$ (Fig. 1d). The uricase activity was considerably reduced in other nitrogen sources such as peptone, ammonium nitrate, ammonium chloride, and casein. The bacteria *Bacillus cereus* DL3 isolated from the poultry source was preferably utilizing the Carboxymethylcellulose and asparagine as a suitable carbon and nitrogen source and yielded around 15.43 U/mL of uricase activity. Another report stated that the *Bacillus subtilis* SP6 isolated from poultry wastes was showed maximum uricase enzyme activity (1.2 to 15.87 U/ml) using lactose and soya peptone as preferred carbon and nitrogen sources, respectively . The *Bacillus subtilis* RNZ-79 effectively utilize raw carbon source such as rice bran and uric acid as preferable carbon and nitrogen sources and yielded a significant volume of uricase activity .

The uric acid served as a primary inducer for uricase activity in bacteria. The optimal concentration of uric acid for the uricase enzyme activity of *B. subtilis* MM13 was 0.32%. It yielded about 1.17 U/ml of uricase enzyme in a 48 h of incubation period and this value was statistical significance at $P<0.05$ (Fig. 1e). Other strains of *B. subtilis*, such as RNZ-79, effectively produced maximum uricase enzyme activity using 0.4% of uric acid in a short period . Similarly, the other strain *Bacillus subtil**is*** SP6 showed maximum uricase activity using 2.55 g/L concentration of uric acid . Another report stated the *Streptomyces exfoliatus* UR10 enumerated from poultry form effectively showed maximum uricase enzyme activity at 0.2% uric acid. This uricase enzyme converts the uric acid into water soluble allantoin (Fig. 2) with the intermediate of 5-hydroxyisourate and byproduct of H_2O_2 . The dried and sterilized powdered form of uric acid enriched wheat bran, beans, cauliflower, and pigeon fecal were studied and interestingly, the *B. subtilis* MM13 effectively utilized the pigeon fecal matter and showed considerable uricase enzyme activity as 0.7 U/ml, however this was significantly lower than the readymade form uric acid (1.17 U/ml). This result suggests that the tested natural uric acid enriched sources have not significant influence on enzymatic activity in *B. subtilis* MM13. This might be due to the insufficient quantity of uric acid content in these natural sources[41].

The initial optimization study results revealed that the sucrose and yeast extract were found as suitable carbon and nitrogen sources for excellent uricase enzyme activity in *B. subtilis* MM13. Hence, the suitable concentration for these two energy sources for *B. subtilis* MM13 to produce maximum uricase enzyme activity were found as 1% for sucrose and 0.2% for yeast extract and yielded 2.54 and 2.37 U/ml, respectively. These values were statistically significant at $P<0.05$ (Fig. 2a and 2b). A report stated that the *Bacillus subtilis* SP6 isolated from poultry waste was found as a uricase producer and effectively showed outstanding uricase activity using lactose as carbon source and soya peptone as nitrogen source at the concentration of 12.2 and 12.79 g/L, respectively . The optimal concentration of carbon and nitrogen source can support the cell proliferation and cell metabolism process that enhances the enzyme secretion activity and chelate the metabolism process[42]. *Proteus vulgaris* enumerated from soil sample use 15 of glucose and 0.5% of ammonium phosphate as the preferable concentration of carbon and nitrogen source and produce significant uricase enzyme in short duration of time[43].

Molecular characterization of bacterial test isolates

The 16S rRNA sequencing and phylogenetic tree analysis results revealed that the uricase producing predominant test bacterial isolate MM13 was identified as *Bacillus subtilis* MM13 and obtained sequence was submitted to GenBank and received accession number for the sequence as MK503710. The sequence of *Bacillus subtilis* MM13was 96% similarity matched with *Bacillus subtilis* 3691 strain (Fig. 3). The circular sequence analysis by BioLab software revealed the GC and AT content of genomic DNA and possible restriction sites. The results state that the Bacillus subtilis MM13 contains about 56% of GC and 44% AT (Fig. 3). Pustake et al. enumerated the uricase producing *Bacillus subtilis* SP6 from poultry wastes, and it was characterized and confirmed using 16*S* rRNA sequencing[44]. Another bacterial strain isolated from poultry form, which possesses uricase producing potential, was identified as Bacillus cereus DL3 using molecular characterization (16*S* rRNA sequencing). Apart from *Bacillus* sp., the *Streptomyces*

exfoliatus UR10 enumerated from farm wastes was recognized as uricase producer and characterized by 16S rRNA molecular sequencing.

Table 1 Enzyme activity on different substrate

Carbon	**U/mL**	**Nitrogen**	**U/mL**	**% Uric acid**	**U/mL**
Glucose	0.45	**Yeast extract**	**0.72**	0.12	0.14
Sucrose	**0.69**	Peptone	0.49	0.22	0.26
Maltose	0.27	Ammonium Nitrate	0.34	**0.32**	**0.63**
Fructose	0.19	Ammonium Chloride	0.27	0.42	0.36
Lactose	0.49	Casein	0.16	0.52	0.33

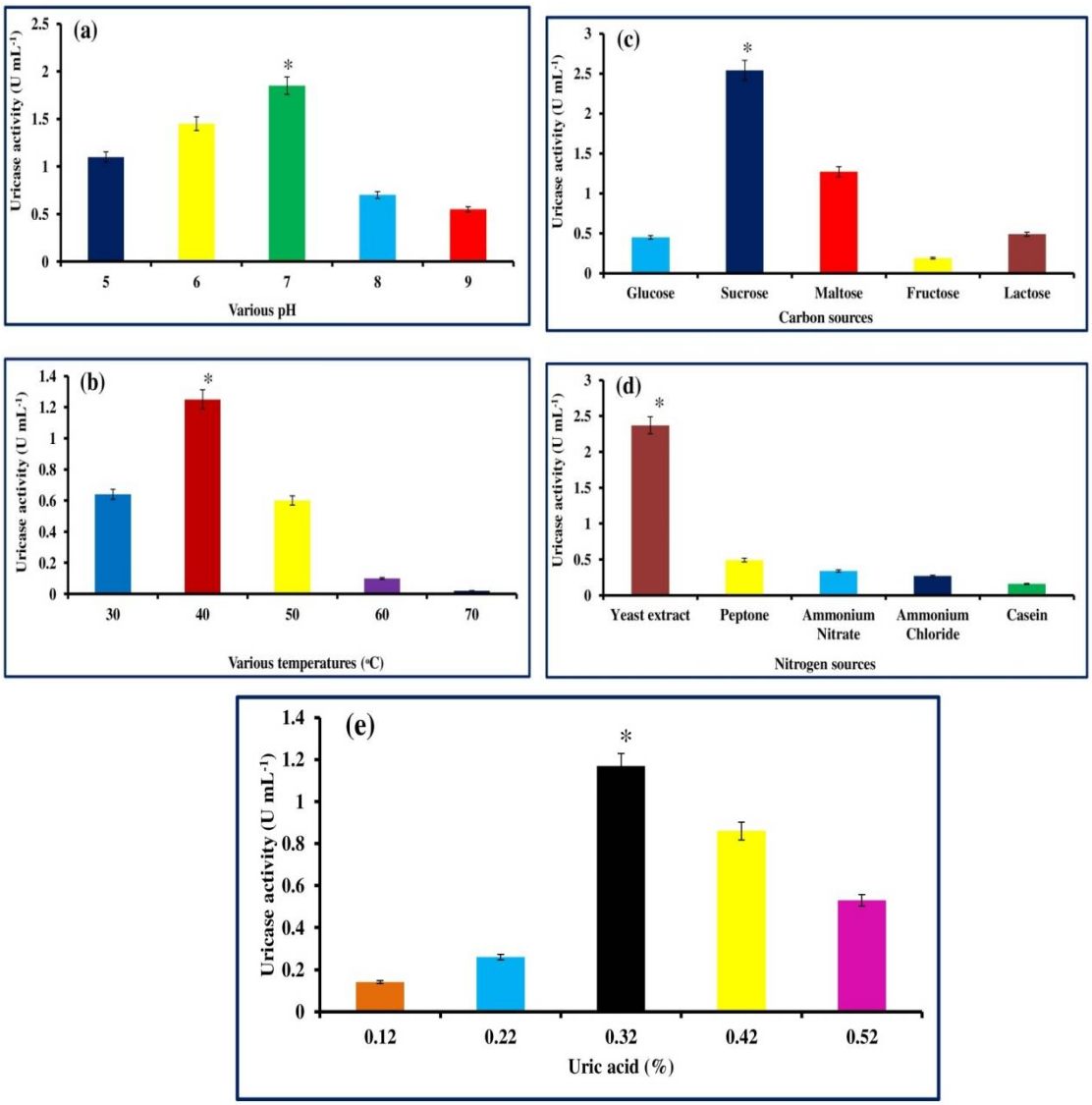


Figure 1. Optimization of growth parameters for uricase enzyme activity in *B. subtilis* MM13. **(a)** various pH **(b)** various temperature **(c)** various carbon sources **(d)** various nitrogen sources **(e)** various concentration of uric acid * indicates statistical significance at P<0.05

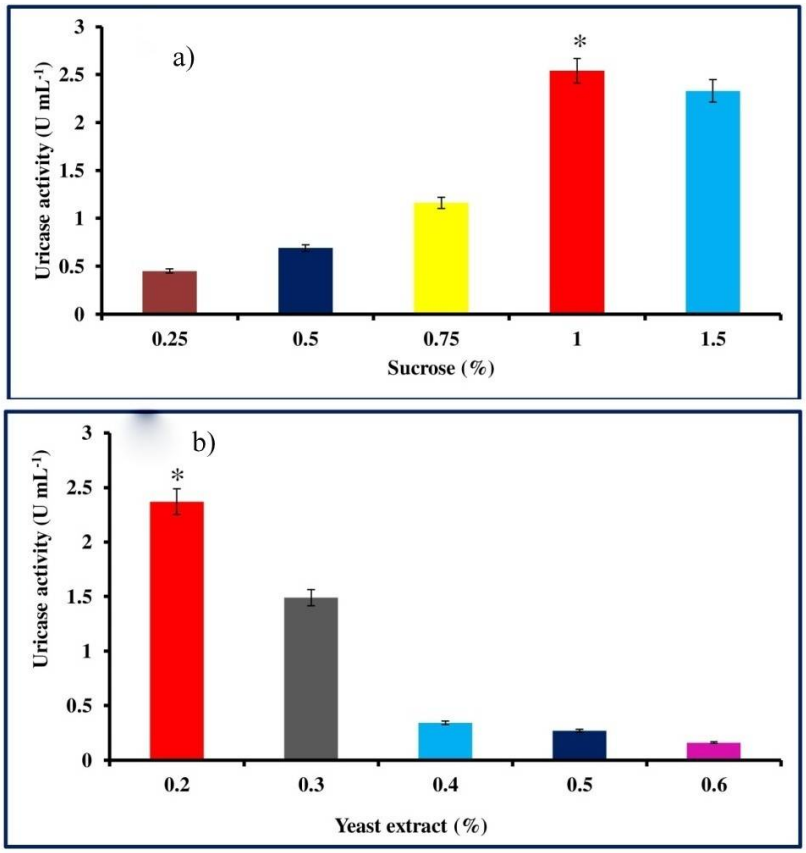
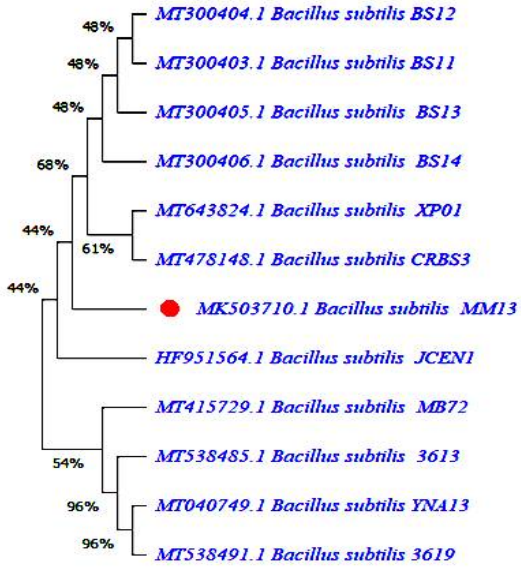


Figure 2(a) various concentration of sucrose **(b)** various concentration of yeast extract.

Figure.2 Phylogenetic tree analysis of *Bacillus subtilis* MM13



CONCLUSION

Among various bird's fecal contaminated soil, the pigeon fecal contaminated soil possesses excellent uricase producing bacterial isolate than other birds fecal contaminated soil. This bacterial isolate was identified as *Bacillus subtilis* MM13 through molecular characterization. The growth parameters required for this strain to produce uricase enzyme were optimized as pH 7.0, 40°C, 1% glucose, 0.2% yeast extract, and 0.32% of uric acid. Under these optimized conditions, the uricase enzyme activity was ranged from 1.25 to 2.54 U/ml in 48 h of the submerged fermentation process. These results suggest that the *B. subtilis* MM13 isolated from pigeon fecal contaminated soil can produce a significant quantity of uricase enzyme

under optimal conditions. The purification and application related studies are needed to understand their maximum biomedical potential and commercialization possibilities.

REFERENCES

1. Butler F, Alghubayshi A, Roman Y (2021) The Epidemiology and Genetics of Hyperuricemia and Gout across Major Racial Groups: A Literature Review and Population Genetics Secondary Database Analysis, Journal of Personalized Medicine. 11: 231.
2. Mohanasundari C, Anbalagan S, Srinivasan K, Chinnathambi A, Salmen S.H, Meganathan V, Narayanan M (2021) Evaluation of antibacterial efficacy of various solvent extracts of Evolvulus alsinoides and Mucuna pruriens against multidrug resistant (MDR) pathogenic bacteria, Applied Nanoscience.5: 1-11.
3. Erickson T.D, Assylbekova B, Chong A.C (2021) Multiple Subcutaneous Gouty Tophi Even with Appropriate Medical Treatment: Case Report and Review of Literature, Kansas Journal of Medicine. 14: 12.
4. Pugazhendhi A, Vasantharaj S, Sathiyavimal S, Raja R.K, Karuppusamy I, Narayanan M, Kandasamy S, Brindhadevi K. (2021) Organic and inorganic nanomaterial coatings for the prevention of microbial growth and infections on biotic and abiotic surfaces, Surface and Coatings Technology. 127739.
5. Jamnik J, Rehman S, Mejia S.B, de Souza R.J, Khan T.A, Leiter L.A, Wolever T.M, Kendall C.W, Jenkins D.J, Sievenpiper J.L (2016) Fructose intake and risk of gout and hyperuricemia: a systematic review and meta-analysis of prospective cohort studies, BMJ open. 6: e013191.
6. Narayanan M, Deepika M, Ma Y, Nasif O, Alharbi S.A, Srinivasan R, Natarajan D (2021) Phyto-fabrication, characterization, and biomedical activity of silver nanoparticles mediated from an epiphytic plant Luisia tenuifolia Blume, Applied Nanoscience. 1-11.
7. Chen L, Han S, Liu F, Chen S, Chen X, Chen H. Global prevalence of hyperuricemia in adolescents from 2000 to 2019: A meta-analysis, in, Research Square. 2020.
8. Subramaniam S, Kumarasamy S, Narayanan M, Ranganathan M, Rathinavel T, Chinnathambi A, Alahmadi T.A, Karuppusamy I, Pugazhendhi A, Whangchai K (2021) Spectral and structure characterization of Ferula assafoetida fabricated silver nanoparticles and evaluation of its cytotoxic, and photocatalytic competence, Environmental Research. 111987.
9. Johnson R.J, Stenvinkel P, Martin S.L, Jani A, Sánchez-Lozada L.G, Hill J.O, Lanaspa M.A (2013) Redefining metabolic syndrome as a fat storage condition based on studies of comparative physiology, Obesity. 21: 659-664.
10. Narayanan M, Jayashree T, Kandasamy S, Natarajan D, Liu G, Elesawy B.H, Elfasakhany A, Pugazhendhi A. (2021) An in vitro investigation of the antidermatophytic, antioxidant, and nephroprotective activity of Solanum surattense, Process Biochemistry. 109: 178-185.
11. Jin M, Yang F, Yang I, Yin Y, Luo J.J, Wang H, Yang X-F (2012) Uric acid, hyperuricemia and vascular diseases, Frontiers in bioscience: a journal and virtual library. 17: 656.
12. Narayanan M, Krishnan L, Natarajan D, Kandasamy S, El Askary A, Elfasakhany A, Pugazhendhi A. (2021) Evaluation of antibacterial, antioxidant, and nephroprotective proficiency of methanol extract of Aerva lanata, Process Biochemistry. 109: 98-103.
13. Sun D.-Q, Wu S.-J, Liu W.-Y, Lu Q.-D, Zhu G.-Q, Shi K.-Q, Braddock M, Song D, Zheng M.-H (2016) Serum uric acid: a new therapeutic target for nonalcoholic fatty liver disease, Expert opinion on therapeutic targets. 20: 375-387.
14. Anusha P, Narayanan M, Natarajan D, Kandasamy S, Chinnathambi A, Alharbi S.A, Brindhadevi K. (2021) Assessment of hexavalent chromium (VI) biosorption competence of indigenous Aspergillus tubingensis AF3 isolated from bauxite mine tailing, Chemosphere. 131055.
15. Khorrami Z, Rezapour M, Etemad K, Yarahmadi S, Khodakarim S, Hezaveh A.M, Kameli M, Khanjani N (2020) The patterns of non-communicable disease multimorbidity in Iran: a multilevel analysis, Scientific reports. 10: 1-11.
16. Egbuna C, Awuchi C.G, Kushwaha G, Rudrapal M, Patrick-Iwuanyanwu K.C, Singh O, Odoh U.E, Khan J, Jeevanandam J, Kumarasamy S. (2021) Bioactive Compounds Effective Against Type 2 Diabetes Mellitus: A Systematic Review, Current Topics in Medicinal Chemistry..
17. Kandasamy S, Narayanan M, He Z, Liu G, Ramakrishnan M, Thangavel P, Pugazhendhi A, Raja R, Carvalho I.S. (2021) Current strategies and prospects in algae for remediation and biofuels: An overview, Biocatalysis and Agricultural Biotechnology. 102045.
18. Chen C, Lü J.-M, Yao Q. (2016) Hyperuricemia-related diseases and xanthine oxidoreductase (XOR) inhibitors: an overview, Medical science monitor: international medical journal of experimental and clinical research. 22: 2501.
19. Narayanan M, Vigneshwari P, Natarajan D, Kandasamy S, Alsehli M, Elfasakhany A, Pugazhendhi A. (2021) Synthesis and characterization of TiO2 NPs by aqueous leaf extract of Coleus aromaticus and assess their antibacterial, larvicidal, and anticancer potential, Environmental Research. 111335.
20. Marchetti M, Liuzzi A, Fermi B, Corsini R, Folli C, Speranzini V, Gandolfi F, Bettati S, Ronda L, Cendron L. (2016) Catalysis and structure of zebrafish urate oxidase provide insights into the origin of hyperuricemia in hominoids, Scientific reports. 6: 1-13.
21. Shivakumar S, Ramkumar G (2021) Eco-friendly approach to control mosquitos (A. stephensi, C. quinquefasciatus, and A. aegypti) using silver nanoparticle, Journal of Environmental Treatment Techniques. 9: 203-210.
22. Whangchai K, Van Hung T, Al-Rashed S, Narayanan M, Kandasamy S, Pugazhendhi A (2021). Biodegradation competence of Streptomyces toxytricini D2 isolated from leaves surface of the hybrid cotton crop against β cypermethrin, Chemosphere. 276: 130152.

23. Gliozzi M, Malara N, Muscoli S, Mollace V (2016). The treatment of hyperuricemia, International journal of cardiology. 213: 23-27.
24. Londoño-Hernández L, Ramírez-Toro C, Ruiz H.A, Ascacio-Valdés J.A, Aguilar-Gonzalez M.A, Rodríguez-Herrera R, Aguilar C.N (2017). Rhizopus oryzae–ancient microbial resource with importance in modern food industry, International journal of food microbiology. 257: 110-127.
25. Liu N, Xu H, Sun Q, Yu X, Chen W, Wei H, Jiang J, Xu Y, Lu W. (2021) The Role of Oxidative Stress in Hyperuricemia and Xanthine Oxidoreductase (XOR) Inhibitors, Oxidative Medicine and Cellular Longevity. 2021.
26. Saranyadevi S, Suresh K, Mathiyazhagan N, Muthusamy R, Thirumalaisamy R. (2021) Silver nanoparticles Synthesized using Asafoetida resin, Characterization of their broad Spectrum and Larvicidal Activity, Annals of the Romanian Society for Cell Biology.; 15035-15049.
27. Hafez R.M, Abdel-Rahman T.M, Naguib R.M. (2017) Uric acid in plants and microorganisms: Biological applications and genetics - A review, Journal of advanced research. 8; 475-486.
28. Daisley B.A, Pitek A.P, Chmiel J.A, Al K.F, Chernyshova A.M, Faragalla K.M, Burton J.P, Thompson G.J, Reid G (2020). Novel probiotic approach to counter Paenibacillus larvae infection in honey bees, The ISME journal. 14L 476-491.
29. Iwadate Y, Kato J.-i. (2019)Identification of a formate-dependent uric acid degradation pathway in Escherichia coli, Journal of bacteriology. 201: e00573-00518.
30. Hafez R.M, Abdel-Rahman T.M, Naguib R.M (2017). Uric acid in plants and microorganisms: Biological applications and genetics-A review, Journal of advanced research. 8; 475-486.
31. Aly M, Tork S, Al-Garni S, Allam R. (2013) Production and characterization of uricase from Streptomyces exfoliatus UR10 isolated from farm wastes, Turkish Journal of Biology. 37; 520-529.
32. Fu X, Yan Q, Wang J, Yang S, Jiang Z. (2016) Purification and biochemical characterization of novel acidic chitinase from Paenicibacillus barengoltzii, International journal of biological macromolecules. 91; 973-979.
33. Kotb E. (2016) Improvement of uricase production from Bacillus subtilis RNZ-79 by solid state fermentation of shrimp shell wastes, Biologia. 71 229-238.
34. Nanda P, Babu P.E. (2014) Isolation, screening and production studies of uricase producing bacteria from poultry sources, Preparative biochemistry & biotechnology. 44; 811-821.
35. Spennemann D.H, Watson M.J (2017) Dietary habits of urban pigeons (Columba livia) and implications of excreta pH–a review, European Journal of Ecology. 3: 27-41.
36. Ashe S, Maji U.J, Sen R, Mohanty S, Maiti N.K. Specific oligonucleotide primers for detection of endoglucanase positive Bacillus subtilis by PCR, 3 Biotech. 4 (2014); 461-465.
37. Lorenz R, Bernhart S.H, Höner zu Siederdissen C, Tafer H, Flamm C, Stadler P.F, Hofacker I.L. ViennaRNA Package 2.0, Algorithms for Molecular Biology, 6 (2011); 26
38. Thong-On A, Suzuki K, Noda S, Inoue J.-i, Kajiwara S, Ohkuma M. Isolation and characterization of anaerobic bacteria for symbiotic recycling of uric acid nitrogen in the gut of various termites, Microbes Environ. 27 (2012); 186-192.
39. Rollin J.A, del Campo J.M, Myung S, Sun F, You C, Bakovic A, Castro R, Chandrayan S.K, Wu C.-H, Adams M.W (2015) High-yield hydrogen production from biomass by in vitro metabolic engineering: mixed sugars coutilization and kinetic modeling, Proceedings of the National Academy of Sciences. 112: 4964-4969.
40. Khucharoenphaisan K, Sinma K (2011).Production and partial characterization of uric acid degrading enzyme from new source Saccharopolyspora sp. PNR11, Pakistan journal of biological sciences: PJBS. 14: 226-231.
41. RoyChoudhury S, Umasankar Y, Hutcheson J.D, Lev-Tov H.A, Kirsner R.S, Bhansali S. Uricase based enzymatic biosensor for non-invasive detection of uric acid by entrapment in PVA-SbQ polymer matrix, Electroanalysis. 30 (2018); 2374-2385.
42. Jacob J.M, Karthik C, Saratale R.G, Kumar S.S, Prabakar D, Kadirvelu K, Pugazhendhi (2018) A. Biological approaches to tackle heavy metal pollution: a survey of literature, Journal of environmental management. 217: 56-70.
43. Azab E.A, Ali M.M, Fareed M.F. (2005) Studies on uricase induction in certain bacteria, Egyptian Journal of Biology. 7.
44. Pustake S.O, Bhagwat P.K, Dandge P.B (2019) Statistical media optimization for the production of clinical uricase from Bacillus subtilis strain SP6, Heliyon. 5; e01756-e01756.

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Molecular Characterization of Uricase Producing *Bacillus sp* from Poultry Soil Samples

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Abstract

The uricase producing ***Bacillus sp*** from soil was characterized as the species which belongs to the genus ***Bacillus sp*** by means of morphological and several biochemical characterizations. Further, the identity of the strain was confirmed by PCR reaction of 16s rRNA primers, sequenced by automated sager method (accession number was obtained as MM13 *Bacillus subtilis* MK503710). The sequence of MM13 *Bacillus subtilis* MK503710 BLAST analysed and was found that the isolate bacteria had the highest homology (99%) with *Bacillus subtilis*.

Keywords

Uricase, PCR, 16s rRNA, Primers, BLAST, Phylogeny

Introduction

The activities of uricase have been reported from various bacterial, fungal and algal species such as *Bacillus fastidiosus* (Saeed *et al*, 2004), *Streptomyces cynogenus* (Ohe and Wantable, 1981), *Enterobacter cloacae* (Machida and Nakanishi, 1980), *Arthrobacter pascens* (Nose and Arima, 1968), *Arthrobacter globiformis* (Suzuki *et al*, 2004). *Candida utilis* (Liu and Li, 1989), *Neurospora crassa* (Nahm *et al*., 1987), *Candida tropicalis* (Tanaka *et al*., 1977), *Puccinia recondite* (Aguilar *et al*, 2002), *Aspergillus flavus* (Retailleau *et al*., 2005), *Chlamydomonas reinhardtii* (Alamillo *et al*., 1991). Urate oxidase and its association

with peroxisomes have been detected in a protozoa *Acanthamoeba* (Muller and Moller, 1969).

Similarly animal sources of uricase was detected in invertebrates like crustacean, vertebrates like amphibians, reptiles and mammals (Noguchi *et al*., 1979) and in Amphibians like frog liver and kidney (Usuda *et al*,1994)

Further the enzyme has been shown in variety of plants such as *Pirns monophylla* (Theimer and Beevers, 1971), *Phaseolus vulgaris* (Sanchez *et al*, 1987) nodules of cowpea (Rainbird and Atkins, 1981), *Phaseolus coccineus* (Theimer *et al*, 1974), *Triticum aestivum*, *Cicer arietinum*, *Vicia faba majo* (Montalbini *et al*., 1999). Urate oxidase from thermophilic *Bacillus* sp. TB-90 has been significantly studied for diagnostic purposes since it reveals high activity and thermostability in a extensive range of pH (Yamamoto *et al*.,1996).

In the contemporary study, uricase producing bacteria were isolated from soil samples and the bacterial species was identified by various physical, biochemical and molecular characterizations.

Material and Methods

Isolation and screening of potential uricase bacterial strain

Soil samples gathered from various places were used as a source to screen for isolates with uricase activity. The test soil samples were serially diluted and plated on uric acid medium as to detect any zone of clearance indicating uricase activity. The isolates with higher diameter of zone of clearance due to uricase activity were short listed for advanced studies. One such isolate was designated as MM13 and was identified as *Bacillus* species based on morphological and various biochemical characterizations.

Molecular identification of *Bacillus sp*

The DNA from *Bacillus sp* isolate was isolated as recommended by (Wright et al., 2017) was subjected to PCR so as to amplify the fragment region of the DNA.

PCR amplification

During PCR, in a 0.5 ml PCR tube, 10 µl of each downstream primer (5"-AGAGTTTGATCCTGGCTCAG 3"), upstream primer (5"-AGGGCTACCTTGTTACGACTT 3"), 10 µl 10X PCR buffer, 25mM $MgCl_2$ 8 µl, dNTP mix 6 µl, template DNA (50 ng), 5 µl and *Taq* DNA polymerase (3U/µl), sterile distilled deionized water 49 µl was taken. The total 100 µl mixture in a tube was gently spun for 10 seconds and allowed to settle the contents. The total mixture was kept in PCR thermal cycler.

The amplification was done in 35 cycles, (denaturation for 60 seconds at 92°C, primer annealing for 60 seconds at 54°C and polymerization for 90 seconds at 72°C), after polymerization 10 µl of PCR products with 2 µl of loading dye was mixed and loaded on a 1.5% agarose gel and analysed electrophoretically at 60 volts for 45 minutes. The gel was visualized on UV - transilluminator and analysed with 1kb DNA ladder. In a 1% agarose gel the PCR products along with 5 µl of DNA molecular weight marker was electrophoresed at 40 mA and were eluted.

NCBI-BLAST for sequences

Then the PCR product was sent to sequencing by automated sanger sequence method using the PCR primers of the study. The same primers as reported above were used for sequencing. Further, NCBI-BLAST www.ncbi.nlm.nih.gov/ blast was used to compare the sequence similarity of the isolated *Bacillus* strain with the reference strain of the study. The 16S rRNA sequence of the test *Bacillus* was deposited in NCBI with sequences accession

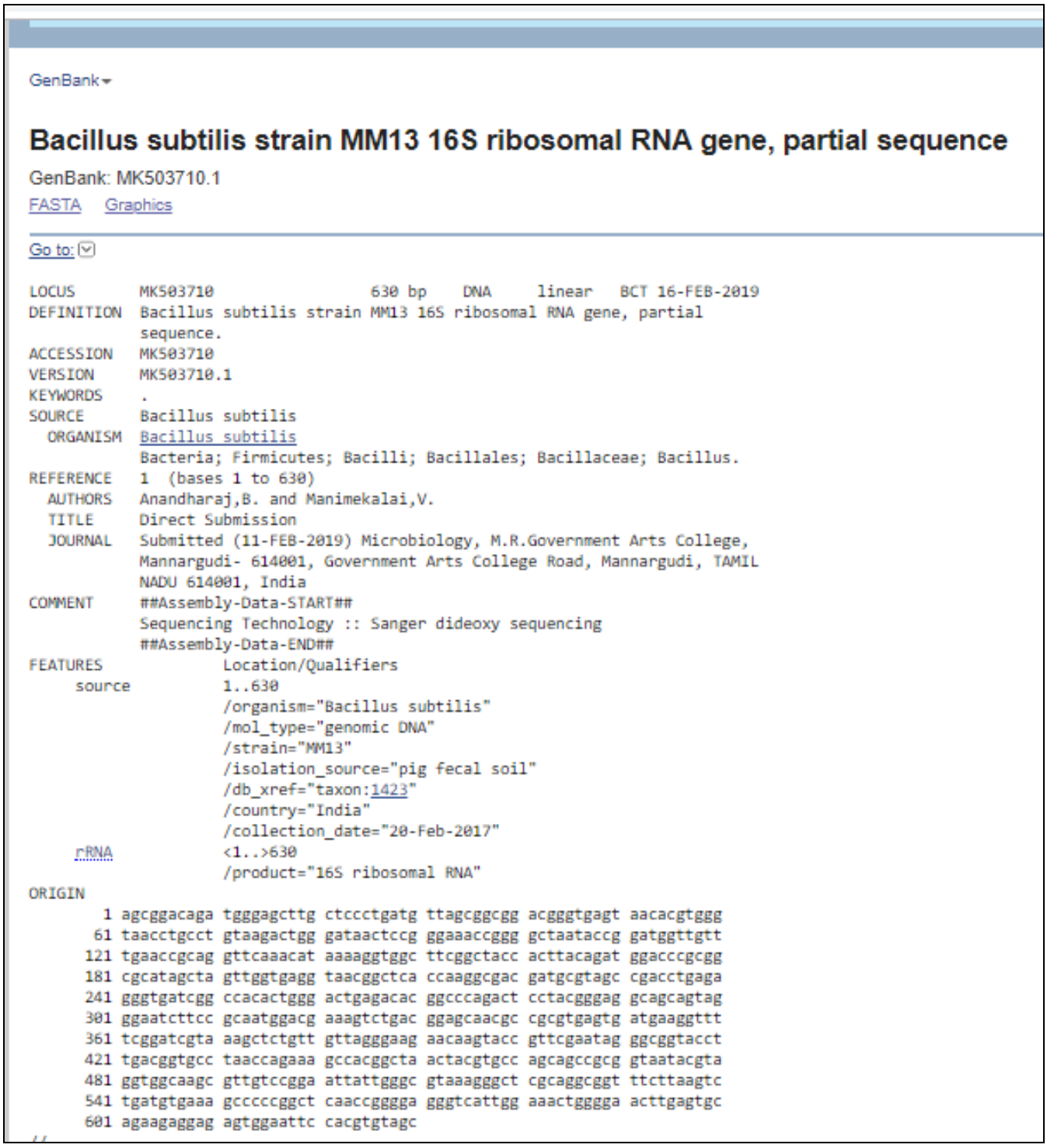
number. In this study, the bootstrapped data set was utilized to build the phylogenetic tree by using the NCBI BLAST.

Result and discussion

Genotypic characterization of isolate MM13

The MM13 isolate was genotypically characterized using 16S rRNA analyses the genomic DNA of the isolate was subjected to PCR amplification. The sequence based MM13 16S rRNA genes was added in genbank **http://www.ncbi.nlm.nih.gov/genbank** with an NCBI accession number MM13 *Bacillus subtilis* (MK503710) (Figure 1 & 2). The sequence of the *Bacillus subtilis* MK503710 isolate was compared with test sequences of *Bacillus* sp. from BLAST database to determine the phylogenetic relatedness using neighbour joining tree method (Figure 3).

The 680bp sequence of the isolate *Bacillus subtilis* (MK503710) had **100%** similarity. In the phylogenetic tree, *Bacillus subtilis* (MK503710) was clustered together as one clade segments corresponding to an evolutionary distance of 0.0006 were shown with bars. Numbers above branches are bootstrap values showing greater than 99 % (Figure 4).



GenBank

Bacillus subtilis strain MM13 16S ribosomal RNA gene, partial sequence

GenBank: MK503710.1

FASTA Graphics

Go to:

```
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DEFINITION  Bacillus subtilis strain MM13 16S ribosomal RNA gene, partial
            sequence.
ACCESSION   MK503710
VERSION     MK503710.1
KEYWORDS    .
SOURCE      Bacillus subtilis
  ORGANISM  Bacillus subtilis
            Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1  (bases 1 to 630)
  AUTHORS   Anandharaj,B. and Manimekalai,V.
  TITLE     Direct Submission
  JOURNAL   Submitted (11-FEB-2019) Microbiology, M.R.Government Arts College,
            Mannargudi- 614001, Government Arts College Road, Mannargudi, TAMIL
            NADU 614001, India
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      121 tgaaccgcag gttcaaacat aaaaggtggc ttcggctacc acttacagat ggacccgcgg
      181 cgcatagcta gttggtgagg taacggctca ccaaggcgac gatgcgtagc cgacctgaga
      241 gggtgatcgg ccacactggg actgagacac ggcccagact cctacgggag gcagcagtag
      301 ggaatcttcc gcaatggacg aaagtctgac ggagcaacgc cgcgtgagtg atgaaggttt
      361 tcggatcgta aagctctgtt gttagggaag aacaagtacc gttcgaatag ggcggtacct
      421 tgacggtgcc taaccagaaa gccacggcta actacgtgcc agcagccgcg gtaatacgta
      481 ggtggcaagc gttgtccgga attattgggc gtaaagggct cgcaggcggt ttcttaagtc
      541 tgatgtgaaa gccccggct caaccggggga gggtcattgg aaactgggga acttgagtgc
      601 agaagaggag agtggaattc cacgtgtagc
//
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Figure 1. 16s rRNA partial sequence submission on NCBI *Bacillus subtilis* (MK503710)

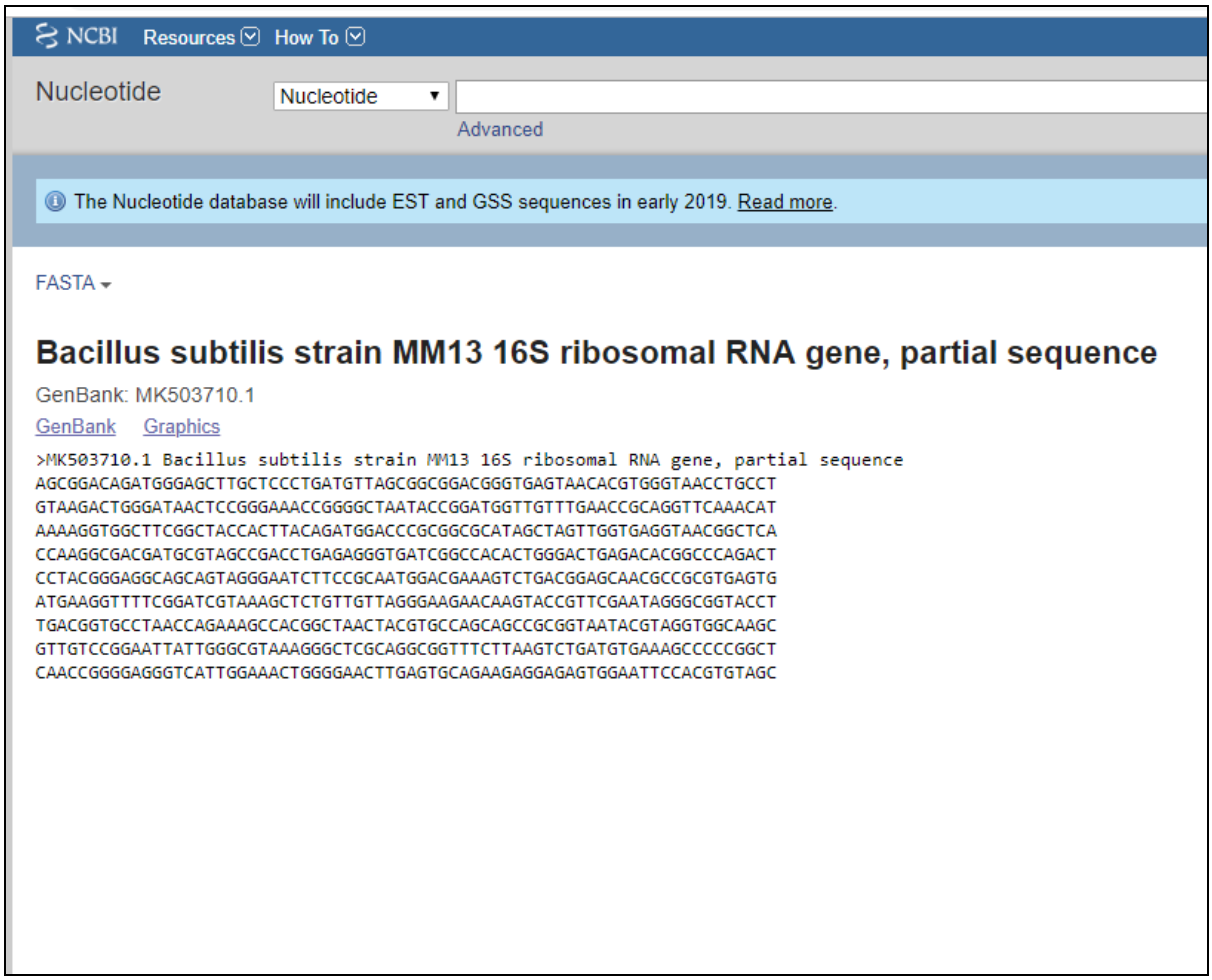

Figure 2. FASTA sequence on NCBI *Bacillus subtilis* (MK503710)

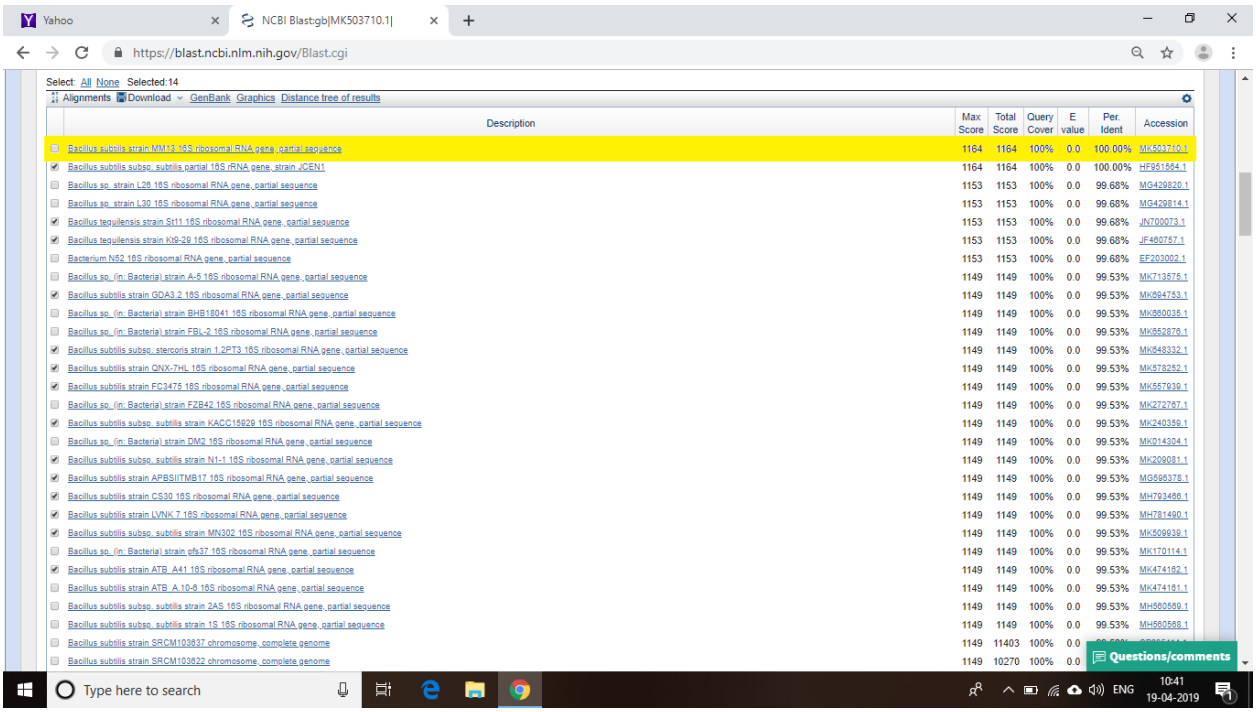

Figure 3. BLAST database of the *Bacillus subtilis* (MK503710)

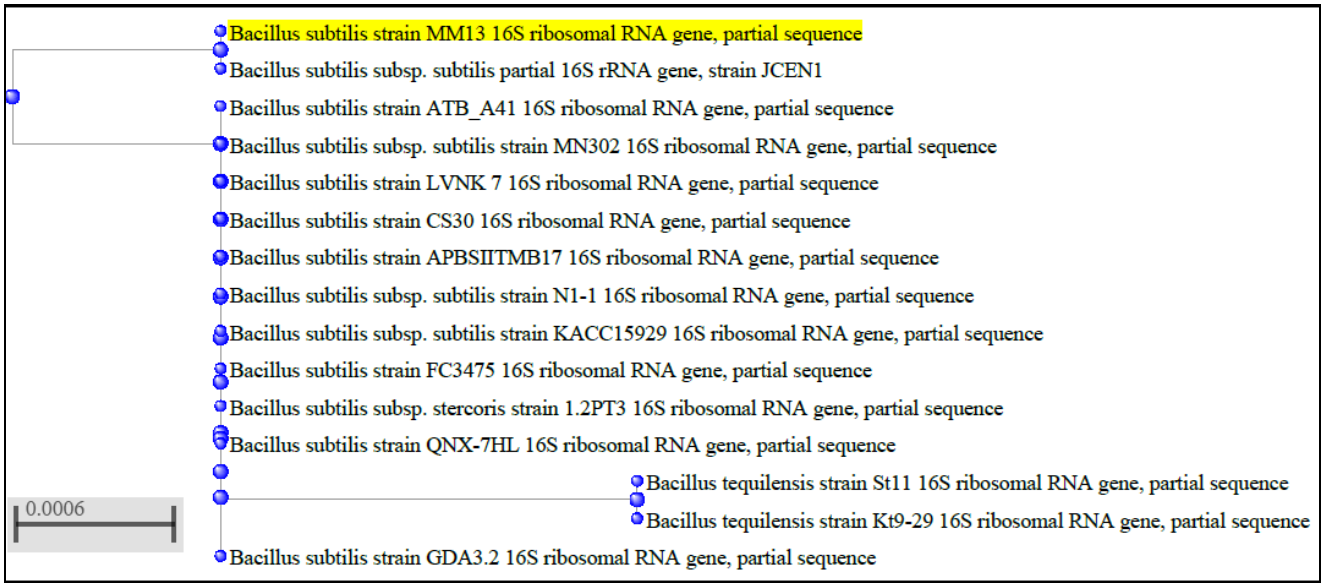

Figure 4.Phylogenetic tree showing phylogenetic position of the *Bacillus subtilis* (MK503710) **neighbour joining method**

References

1. Aguilar,M., Montalbini,P., and Pineda,M. (2002) Urate oxidase from the rust *Puccinia recondita* is a heterotetramer with two different-sized monomers. *Curr Microbiol* 44: 257-261.
2. Alamillo,J.M., Cardenas,J., and Pineda,M. (1991) Purification and molecular properties of urate oxidase from *Chlamydomonas reinhardtii. Biochim Biophys Acta* **1076:** 203 - 208. Muller and Moller, 1969
3. Davidson,J.N. (1938) The purification of uricase. *Biochem J 32:* 1386-1388.
4. Farina,B. and Faraone, M.R. (1973) Studies on bovine kidney uricase. *Boll Soc Ital Biol Sper* **49:** 1079-1084.
5. Liu JG and Li GX. (1989) Culture conditions for uricase formation of *Candida utilis Wei Sheng WuXue Bao.* 29: 45-50. Nahm *et al*., 1987),
6. Mahler,H.R., Hubscher,G., and Baum,R. (1955) Studies on uricase. I. Preparation, purification, and properties of a cuproprotein. *J Biol Chem* **216:** 625-641.
7. Montalbini, K., Aguilar, M., Pineda M. (1999) Isolation and characterization of uricase from bean leaves and its comparison with uredospore enzymes. *Plant science* 147:139-147.
8. Noguchi,T., Takada,Y., and Fujiwara,S. (1979) Degradation of uric acid to urea and glyoxylate in peroxisomes. *JBiol Chem* 254: 5272-5275.
9. Nose,K. and Arima,K. (1968) Studies on bacterial urate:oxygen oxidoreductase. II. Observations concerning the properties and components of the active site. *Biochim Biophys Acta* **151:** 63-69. Suzuki *et al*, 2004).

10. Ohe, T., and Wanatabe, Y.(1981) Purification and properties of urate oxidase from *Streptomyces cyanogenus J Biochem.* **89:** 1769-1776. (Machida and Nakanishi, 1980),
11. Rainbird,R.M. and Atkins,C.A. (1981) Purification and some properties of urate oxidase from nitrogen-fixing nodules of cowpea. *Biochim Biophys Acta* 659: 132-140.
12. Retailleau,P., Colloc'h,N., Vivares,D., Bonnete,F., Castro, B., E1,H.M., and Prange,T. (2005) Urate oxidase from Aspergillus flavus: new crystal-packing contacts in relation to the content of the active site. *Acta Crystallogr D Biol Crystallogr* 61: 218-229.
13. Saeed,H.M., bdel-Fattah,Y.R., Gohar,Y.M., and Elbaz,M.A. (2004) Purification and characterization of extracellular Pseudomonas aeruginosa urate oxidase enzyme. *Pol J Microbiol* 53: 45-52.
14. Sanchez,F., Campos,F., Padilla,J., Bonneville,J.M., Enriquez,C., and Caput,D. (1987) Purification, cDNA Cloning, and Developmental Expression of the Nodule-Specific Uricase from Phaseolus vulgaris L. *Plant Physiol* 84: 1143-1147.
15. Simkin,P.A., Healey,L.A., Jr., and Smuckler,E. (1970) Monkey uricase. *N Engl J Med* 283: 823-824.
16. Tanaka, A., Yamamura,M., Kawamoto,S., and Fukui,S. (1977) Production of uricase by *Candida tropicalis* using n-alkane as a substrate. *Appl Environ Microbiol* 34: 342-346.
17. Theimer R.R.,Heidinger P.Z. (1974) Control of particulate urate oxidase activity in bean roots by external nitrogen supply. *Pflanzenphysiol* **73:** 360-770
18. Theimer,R.R. and Beevers,H. (1971) Uricase and allantoinase in glyoxysomes. *Plant Physiol* **47:** 246-251.

19. Usuda N, Hayashi S, Fujiwara S, Noguchi T, Nagata T, Rao MS, Alvares K, Reddy JK, Yeldandi AV. (1994) Uric acid degrading enzymes, urate oxidase and allantoinase, are associated with different subcellular organelles in frog liver and kidney. *Cell Sci.* 107:1073-81.

20. Wright, MN., Adelskov,J., Greene, AC . Bacterial DNA Extraction Using Individual Enzymes and Phenol/Chloroform Separation. *J Microbial Biol Educ.* 2017,18(2):1-3. doi: 10.1128/jmbe.v18i2.1348