IN THE NAME OF ALLAH,

THE MOST MERCIFUL AND THE MOST GRACIOUS

Diagnostic potential of indigenously hyperproduced urate oxidase

By

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Declaration

I hereby declare that the contents of the thesis, "**Diagnostic potential of indigenously hyperproduced urate oxidase**" are product of my own research and no part has been copied form any published source (except the references, standard mathematical or genetic models/equations/formulate/ protocols etc). I further declare that this work has not been submitted for award of any other diploma/degree. The University may take action if the information provided is found inaccurate at any stage. (In case of any default the scholar will be proceeded against as per HEC plagiarism policy).

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TO

My Affectionate
Parents & Brothers
My caring Husband
&
My innocent son
Muhammad Fahd Irfan
Muhammad saad irfan

ACKNOWLEDGEMENT

All the praises are attributed to the sole creator of the whole universe the "**ALMIGHTY ALLAH"** who bestowed on me power of vision to unravel the mysterious of universe enabling me to make this material contribution to already existing of knowledge. In invoke **Allah's** blessings and peace for **Hazrat Muhammad (peace be upon him).** The most perfect, whose moral and spiritual teaching enlightened my hearts and mind.

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May Allah grant the above cited personalities with long and happiest life, peace of mind, prosperity, honor and greater heights…….. Aamin

MUNAZZAH MERAJ

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ABSTRACT

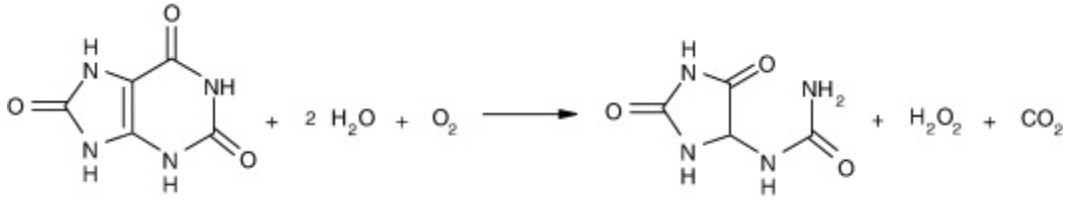
The main purpose of this research work was to optimize the production of urate oxidase through mutagensis of *Bacillus subtilis.* The organism was subjected to ultra violet irradiation and chemical mutagenesis. Ethyle methane sulfonate treated *B. subtilis* (180 minutes) was proved to be the best for optimum production of urate oxidase by 3 log kill/survival curve. Fermentation medium was also optimized, it was found that substrate concentration (0.5%), fermentation period (36 h), pH (8.5), temperature (35 ^{o}C), yeast extract (0.3%) and sucrose (2%) enhanced the activity of the parent and mutant derived enzyme. The enzyme was purified by adopting different techniques i.e ammonium sulfate precipitation, ion exchange and gel filtration chromatography. It was observed that mutated enzyme exhibited 97.56 U/mg specific activity with 256.73 fold improvement. The purified urate oxidase was run on SDS-PAGE which determined a single band with molecular weight of 34 kDa. The purified BSM-2 possessed K_m and V_{max} value 0.067 M and 133.3 IU mg^{-1} min^{-1} respectively. The optimum pH and temperature for catalytic activity were 7.5 and 35^{o}C respectively. The activation energy for formation of ES complex was 43.4 kJ/mol. Enthalpy (ΔH^*), entropy of activation and Gibbs free energy demands for urate oxidase inactivation were 30.26 kJ/mol, -106.27 J mol^{-1} K^{-1}, 62.99 kJ/mol respectively. Barium chloride, potassium cyanide and zinc sulfate decreased the activity of the enzyme. Whereas, sodium chloride (0.6M), potassium chloride (0.4M) and calcium chloride (0.4M) enhanced the enzymatic activity 123%, 117% and 119% respectively.

HAPTER 1

INTRODUCTION

Urate oxidase has a great significance for both diagnosis and treatment of uric acid in body fluids and body tissues. The uric acid diagnostic kits are widely used for the estimation of uric acid in the human body. Unfortunately, much of the diagnostic kits, like uric acid estimation kit, are not available locally and we are totally dependent on their import. The manufacturing of diagnostic kits in our own country is a need of hour. So the present research work was designed to focus the hyperproduction, purification and characterization of the urate oxidase for its multiple usages (formation of uric acid diagnostic kit and treatment of hyperuricemia).

In the diagnostic kit, urate oxidase breaks down uric acid into allantoin, hydrogen peroxide and carbon dioxide. The mechanism of the reaction is as follow.



Uric Acid Allantoin

The solubility of uric acid (11 mg/ dL) is less than the allantoin (147 mg/dL). The solubility of allantoin is high because pyrimidine rings of uric acid losses its identity with the breakdown of C-N bond and the removal of H_2O_2 and CO_2. The remnant (allantoin) becomes water-soluble and easily excretes out of the body through urine. This product is found in mammals (except primates) (Gianfrancesco *et al*., 2004;Kanai 2008), plants (Umamaheswari *et al*., 2007; Chen *et al*., 2008) and microbial cells (Retailleau *et al*., 2005; Li *et al*., 2006; Ramazzina *et al*., 2006; Lotfy 2008).

The synonyms of the said enzyme are urate oxidase, uric acid oxidase, urate: Oxygen oxidoreductase, uricase, and uricase II. The enzyme belongs to the class oxidoreductase, subclass is 7; as it is acting on nitrogen containing compound. Its sub subclass is 3 in which oxygen play a role as an acceptor, and finally the enzyme number is 1.7.3.3. This enzyme,

produced from *Bacillus subtili,s* contains 300-400 amino acids with well conserved sequences (Fig 1.1) (Motojima and Goto 1990; Colloc'h *et al*., 1997; Colloc'h *et al*., 2006) and has molecular weight 34 kDa (Yamamoto *et al*., 1996).

It is a homotetrameric enzyme having four identical active sites with two copper binding sites. These active sites are located at the interfaces among its subunits (Gabison *et al*., 2010). According to previous studies, it was considered that the said enzyme is a copper containing enzyme but later research revealed that the urate oxidase isolated from *Aspergillus flavus* and *Bacillus subtilis* have no copper. So the enzyme obtain from these both resources is having no other transition ion (Kahn and Tipton, 1997; Kahn *et al*., 1999; Imhoff *et al*., 2003). The appearance of urate oxidase has white amorphous powder and its lyophilized form is stable for three weeks at room temperature. Upon reconstitution, this enzyme will be active just for 1 week at 4 ^{o}C (Dittemann *et al*., 1999).

The specificity of urate oxidase towards uric acid (substrate) is high but it is not active towards substituted uric acids such as monomethyl, dimethyl, trimethyl or ethyl derivatives (Saxild *et al*., 1996, Milena *et al*., 2003). Its activity may be monitored at various ultra violet wavelengths (i.e. 293 nm, 340 nm) or colorimetrically by coupled chromogenic response (Gokicke and Gokicke 1973; Kabasakalian *et al*., 1973; Itiaba *et al*., 1975; Kuan *et al*., 1975; Hamada *et al*., 2008; Sanz *et al*., 2008).

The initial enzyme used for the degradation and estimation of uric acid is urate oxidase. Many of the enzymes (allantoinase, allantoicase, ureidoglycolate lyase and urease) used for the degradation of uric acid are mentioned in figure 1.2 (Usuda *et al*., 1994; Vigetti *et al*., 2000; Loughlin *et al*., 2004; Rueichi *et al*., 2006). During the vertebrate evolution, these enzymes have been lost in the primates (humans, gibbons, orangutans, and chimpanzees) and the reason is unknown (Vigetti *et al.*, 2000; Enomoto *et al*., 2003; Gianfrancesco *et al*., 2004; Zhang *et al.,* 2010). These primates have a nonsense codon introduced into the gene of urate oxidase, which results in the synthesis of a short fragment (10 amino acid) devoid of enzymatic activity (Abeles *et al*., 2007; Pillinger *et al*., 2007; Vitart *et al*., 2008). Due to this reason, some amount of uric acid remains in urine and blood of the human all the time, but in some pathological conditions i.e uremia, leukemia and renal insufficiency, the amount of uric acid is abnormally increased in biological fluids (Enomoto *et al*., 2003; Gianfrancesco *et al*., 2004). When it reaches to saturation: it precipitates out of

the solution and deposits into the joints and tissues of the body. It results in inflammatory reactions, pain and permanent damage to connective tissues, joints and kidneys. Similarly, those patients undergoing organ transplant and cancer chemotherapy are at high risk of causing hyperuricemia. In tumor lysis syndrome, the tumor is rapidly destructed and released a large amount of uric acid into the blood, which is demonstrated by renal failure and cause acute uric acid nephropathy (Ravindranath, 2003; Hummel *et al*., 2008).

Lys	Asp	Glu	Thr	Thr	Tyr	Pro	Phe	His	Met
Lys	Asp	Met	Glu	Ile	Pro	His	Ala	Arg	Ser
Tyr	Glu	Ile	Val	Ile	Thr	Ala	Phr	His	Asn
Gly	Glu	Ser	Thr	Thr	His	Trp	Pro	Arg	Gly
Asp	Asn	Asn	Val	Gly	Arg	His	Lys	Lys	Leu
Ils	Lys	Pro	Leu	Ile	Pro	Glu	Gly	Gly	Phe
Gln	Asn	Ile	His	Hhis	Glu	Glu	Ser	Gln	Thr
Gly	Ser	Asn	Cys	Lys	Val	Leu	Ser	Phr	Ser
Lys	Tyr	Val	Gly	Ser	Ser	Asn	Tyr	Leu	Lys
Asn	Ile	Asp	Asp	Val	Thr	Glu	Ile	Trp	Ser
Asp	Ile	Arg	Asp	Ser	Asp	Arg	Gln	Arg	Ala
Phe	Ile	Val	Lys	Ser	Asp	Trp	Val	Trp	Phe
Val	Gln	Ile	Lys	Arg	Thr	Glu	Thr	Thr	Ala
Ile	Asp	Leu	Leu	Trp	Gln	Ala	Lys	Gln	Leu
Thr	Val	Phe	Thr	Ala	Asp	Leu	Val	Ile	Glu
Trp	Gly	Glu	Asp	Leu	Thr	Ser	Gln	Val	Glu
Leu	Lys	Val	Gly	The	Ala	Glu	Thr	Phe	Gln
Thr	Asp	Ser	Lys	Val	Ala	Glu	Tyr	Leu	Asn
Asn	Phe	Pro	Glu	Ser	Ile	His	Gly	Gly	Thr
His	Val	Arg	Asn	Met	Lys	Lys	Tyr	His	Gly
Thr	Ala	Asp	Lys	Leu	His	Arg	Val	Val	Lys
Ala	Glu	Tyr	Gln	Asn	Ala	Lys	Thr	Glu	Asn
Thr	Met	Gln	Pro	Trp	Tyr	Gln	Leu	Ala	Ala
Ala	Arg	Leu	Ser	Ala	Val	Leu	Lys	Ser	Glu
Tyr	Ser	Leu	Lys	Val	Val	Thr	Asp	Lys	Val
Ile	Asp	Val	Arg	Gly	Tyr	Thr	Ala	Phe	Phe
Asn	Ala	Val	Thr	Cys	Ala	Thr	Leu	Glu	Ala
Ser	Asn	Lys	Val	Ser	Ile	Glu	Ile	Val	Lys
Pro	Gln	Ser	Asp	Val	Asn	Gly	Leu	Ile	Ser
Csy	Thr	Val	Gly	Pro	Ser	Ser	Leu	Lys	Arg
Lys	Leu								

Fig 1.1 molecular structure of urate oxidase

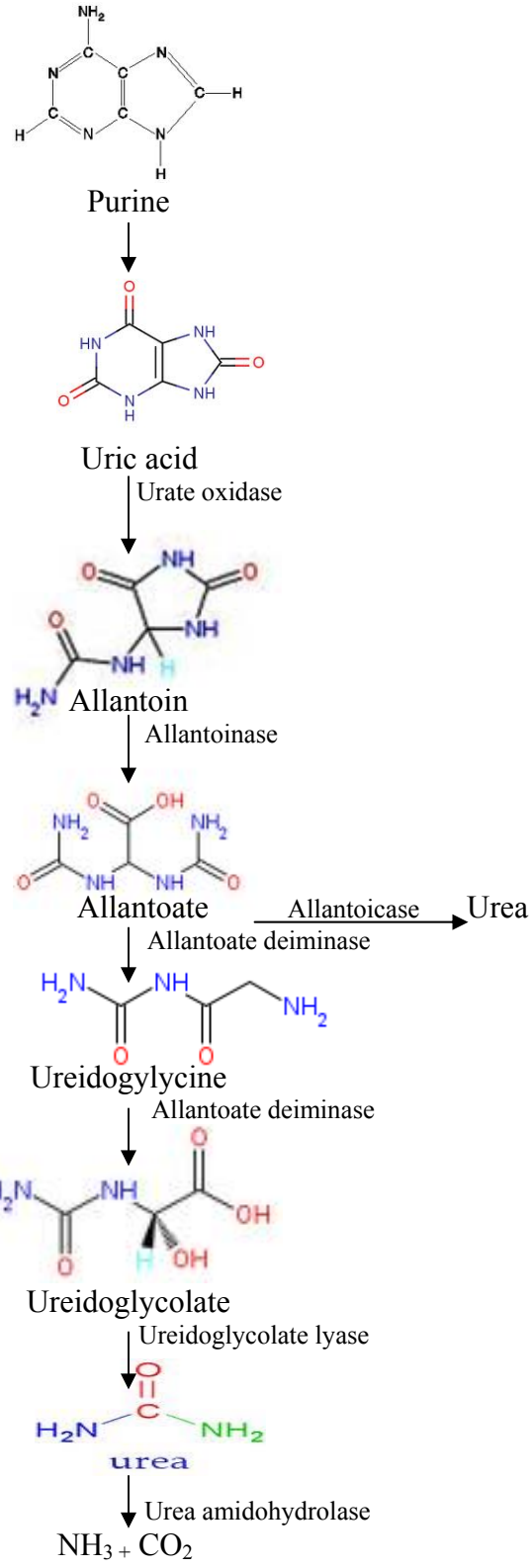


Fig 1.2 Uric acid degrading enzymes

In the human body, uric acid is formed by the degradation of dietary nucleic acids (purine) in the small intestinal lumen (Carl *et al*., 2002; Sochacka *et al*., 2008). Purines are constantly synthesized, degraded (Kim *et al.,* 2007; Korycka *et al*., 2008; Wu *et al.,* 2008) and reutilized by purine salvage pathway (Katahira and Ashihara 2006; Beck and Donovan, 2008). These degraded purines (purine nuleosides and free bases) undergo deamination by the action of specific enzymes (deaminases) to form xanthine and hypoxanthine. These specific enzymes include adenine deaminase, adenosine deaminase, aminohydrolase and guanine deaminase (Fig 1.3). Adenine deaminase is the only enzyme which is present in traces; while, all other enzymes are widely distributed in all animal's tissues (Cristin and Joshua *et al*., 2004; McAnulty *et al*., 2007; Vitart *et al*., 2008; Amaro *et al*., 2008; Duan and Ling, 2008), But, the distribution of urate oxidase very limited distributed in the animals (Nakaminami *et al*., 1999).

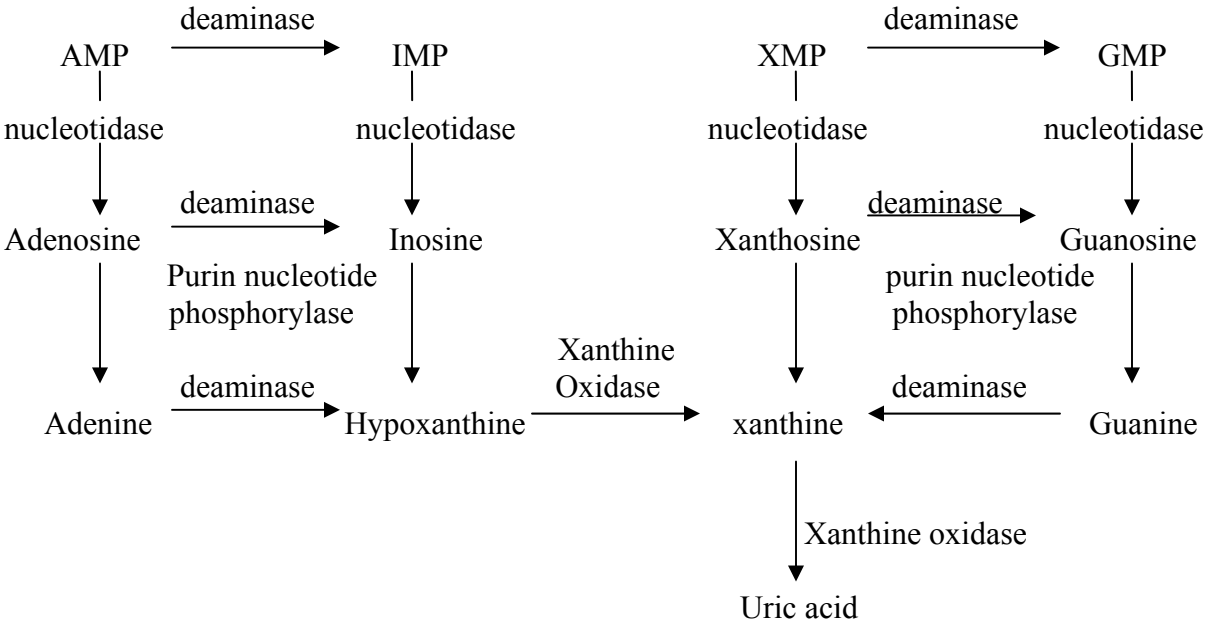


Fig 1.3 Purine degrading pathway

Urate oxidase has substantial importance owing to the following reasons;

(i) It is used in pharmaceutical preparation.

(ii) It has more advantages in the treatment of nephrolithiasis as compared to other drugs.

(iii) It shows better results in the reduction of hyperuricemia of the order of fifty percent in less than 1 day.

(iv) This enzyme protects the kidney against lithiasis better than other drugs such as allopurinol (Torres *et al*., 2007).

(v) The estimation of uric acid in the biological fluid by the enzymatic method (urate oxidase enzyme assay) is preferred over non-enzymatic methods. These non-enzymatic methods give false results due to the interference of many antibiotics: paracetamol, glutathione, aspirin and ascorbic acid (Gokicke and Gokicke. 1973; Kabasakalian *et al*., 1973; Itiaba *et al*., 1975; Kuan *et al*., 1975).

Urate oxidase is usually extracted from internal animal organs, like kidney (Rajoka *et al*., 2006) and liver (Zhu *et al*., 2004) by extraction and fractionation. These processes are complicated and their raw materials are very expensive. So urate oxidase produced by this procedure will have high price. In the near past, the interest was developed for the isolation of urate oxidase by the fermentation of different microbial sources due to its economical benefits (Alamillo *et al*., 1991; Liu *et al*., 1994; Aguilar *et al*., 2002; Wu *et al.,* 2009).

Accordingly, the bacterium used for fermentation in the present work was *Bacillus subtilis*. It was known as *Vibrio subtilis* in 1935 and this name was given by *Christian Gottfried Ehrenberg* but later on, this name was changed to *Bacillus subtilis* by Ferdinand Cohn in 1975 (Nakano and Zuber, 1998). It has also known as *Grass bacillus* or *Hay bacillus*. Owing to its rod shape (bacilli shaped), it is called *Bacillus* (Madigan and Martinko, 2005).

Bacillus subtilis is widely used and has many advantages such as

⇒ It is used as a model organism for laboratory studies

⇒ It has the ability to form protective and tough endospores that tolerate extreme environmental conditions (Madigan and Martinko, 2005).

⇒ It is widely used for the production of many enzymes.

⇒ It has ability to produce many chemicals.

⇒ It produces enzymes as additive in laundry detergents.

⇒ It has natural fungicidal activity.

⇒ It is also used as biological control agent because it has the ability to convert explosives into nontoxic compounds of nitrogen and CO_2 (Madigan and Martinko, 2005; Hartig et *al*., 2006; Miethke *et al*., 2006; Coleman and Setlow, 2009; Wray and Fisher, 2010).

This organism contains approximately 4,100 genes, out of which more than fourteen genes are involved in purine catabolic pathway. For, the proper functions of the urate oxidase pucL and pucM genes are required. It is observed, that pucL is involved in encoding urate oxidase itself, while, the function of pucM is still not clear (Lee *et al*., 2005).

Urate oxidase has been produced and purified from microbes such as *Bacillus subtilis* (Huang and Wu., 2004), other *Bacillus* sp.(Xue *et al*., 2005; Zhao *et al.,* 2006), *Bacillus fastidious* spores (Bongaerts *et al.,* 1978; Salas *et al.,* 1985), *Arthrobacter globiformis* (Suzuki *et al.,* 2004), *Aspergillus flavus* (Retailleau *et al.,* 2005; Li *et al.,* 2006), *Pseudomonas aeruginosa* (Yasser *et al*., 2005, Saeed *et al.,* 2004a and 2004b), *Rhizopus oryzae* (Peter *et al*., 2002), *Candida* sp. (Liu *et al.,* 1994), *Candida utilis* (Han *et al*., 1993; Vladimir *et al*., 1990; Chen *et al.* 2008), *Candida tropicalis* (Tanaka *et al.,* 1977), *Chlamydomonas reinhardtii* (Alamillo *et al.,* 1991) and yeast (Nishimura and Tipton., 1979). Moreover, this enzyme has also been isolated and purified from soybean (Tajima *et al.,* 2004), rabbit (Motojima and Goto 1990), rat liver (Stefano *et al.,* 1992), porcine liver (Wu *et al*., 2009), ox kidney (Farina and Faraone 1979), caprine kidney (Rajoka *et al.,* 2006b) and bovine kidney (Rajoka *et al*., 2006a).

Mutagenesis techniques have been used to improve the production of urate oxidase by several investigators through cloning. However, this technique is very expensive and requires intensive care and lengthy procedure. But on the other hand the techniques, such as ultraviolet radiations or chemical mutagens (N methyl, N-nitro N nitroso guanidine, ethyl methyl sulfate, ethidium bromide etc) to induce mutation are more useful for the productions of many enzymes by various microbes. These techniques were used to induce mutation for hyperproduction of many enzymes by various microorganisms. The advantages to adopt these methods were their simplicity and low cost. Moreover, the screening method used in the present research was very useful; it is simple and did not require any detailed study of physiology and molecular biology of the organism, being manipulated.

AIMS AND OBJECTIVES

Keeping in view the above said discussion, the study was planned to achieve the following objects:

- To develop hyper producing mutants of *Bacillus subtilis* through UV and chemical mutagenesis to enhance the production of urate oxidase.
- To optimize the conditions for the production of urate oxidase
- To get thermal characterization/ kinetics of urate oxidase

CHAPTER 2

REVIEW OF LITERATURE

Urate oxidase is a homotetrameric enzyme with four active sites at which urate anions are occupied. It helps in catalysis of uric acid decomposition into allantoin with the lifetime of about twenty minutes (Ramazzina *et al.,* 2006; Gabison *et al.,* 2010; Tan *et al.,* 2010). In the mammals (primates), the gene encoding this enzyme is inactivated. Consequently, the mammals are grouped into two categories on the bases of purine catabolic end product (Fig 2.1). In one category, the enzyme allantoinase is lost and end product is allantoin, while in other category (homonoids), the enzyme urate oxidase is lost and uric acid is the end product (Keebaugh and Thomas 2009). As a result, some amount of uric acid remains in body fluid of the humans. Meanwhile, in pathological conditions, the concentration of uric acid is exceeded. These conditions are controlled by the use of urate oxidase, which is a potential therapeutically active drug in the treatment and prevention of hyperuricemia, gout, tumor lysis syndrome, lymphoma and leukemia (Colloc'h *et al.,* 1997; Bomalaski *et al.,* 2002; Mulhbacher *et al.,* 2002; Patte *et al.,* 2002; Jeha *et al.,* 2005; Moolenburgh *et al.,* 2006; Tosi *et al.,* 2008; Ishizawa *et al.,* 2009; Feng *et al.,* 2010; Liu *et al.,* 2011). It is also used for the determination of uric acid in biological fluids after isolation and purification from different animal or microbial sources. Immobilized urate oxidase are also used for this purpose (Akyilmaz *et al.,* 2003; Cete *et al.,* 2006; Lio *et al.,* 2006a and b; Wang *et al.,* 2007; Zhang *et al.,* 2007; Arora *et al.,* 2009; Bhambi *et al.,* 2010; Chen *et al.,* 2010). Owing to its importance and applications as mentioned above, there is a great need to extract and purify the enzyme indigenously.

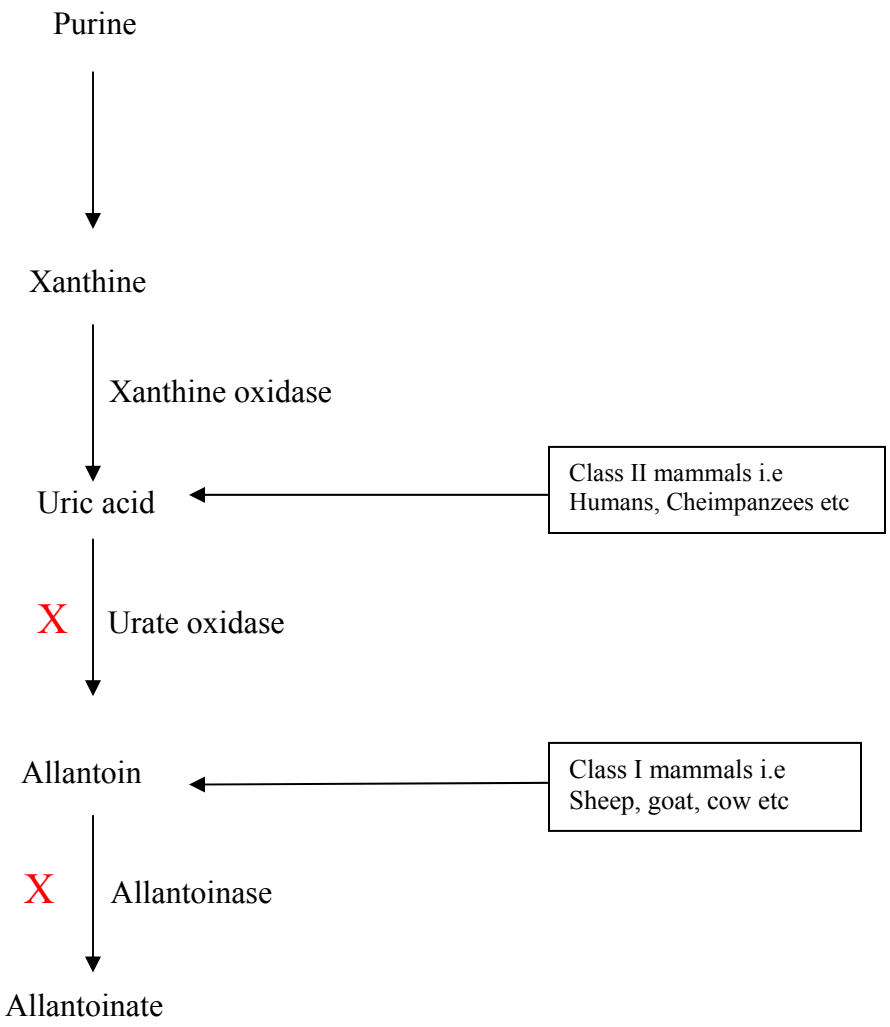


Fig 2.1 Classification of mammals based on purine catabolic end product

2.1 Mechanism of action

Uric acid is decomposed to form allantoin and hydrogen peroxide by the action of enzyme urate oxidase. By the action of another enzyme (peroxidase), hydrogen peroxide is converted to water and oxygen (Fig 2.2) (Zoppi *et al.,* 1980; Sanders, *et al.,* 1980; Ingebretsen *et al.,* 1982; Shigeyuki *et al.,* 1985; Liu *et al.,* 1994; Bhargava *et al.,* 1999; Gholamreza *et al.,* 2006; Zhang *et al.,* 2007; Akyilmaz *et al.,* 2003; Yu *et al.,* 2011). The substrate (urate) tightly binds to one subunit of the enzyme by interaction with arginine (Arg180), leucine (Leu222) and glutamine (Gln223). While the other subunit of the enzyme binds with threonine (Thr67) and aspartate (Asp68) (Juan *et al.,* 2008; Gabison *et al.,* 2010).

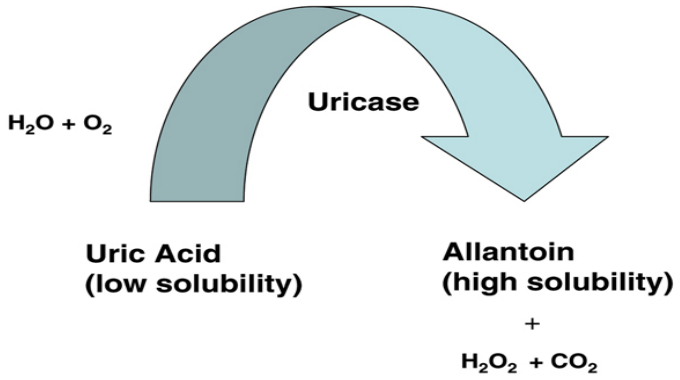


Fig. 2.2. Mechanism of action of urate oxidase (uricase)

This enzyme is used as a therapeutic drug to decrease the concentration of uric acid in human and preferred over the other drugs that are used in the treatment of disorders caused by hyperuricemia. Basically, many drugs are the xanthine oxidase inhibitors that block the formation of uric acid, while, urate oxidase degraded uric acid (purine catabolic product or pre-existing) and formed water soluble metabolites that readily excrete out of the body through kidney (Fig 2.3). It shows better results by reducing 50 % hyperuricemia in less than 24 hours (Cheng *et al.,* 2000. Goth *et al.,* 2008; Juan *et al.,* 2008; Altarsha *et al.,* 2009; Terzuoli *et al.,* 2009; Gibison *et al.,* 2010; Dong *et al.,* 2011; Meotti *et al.,* 2011).

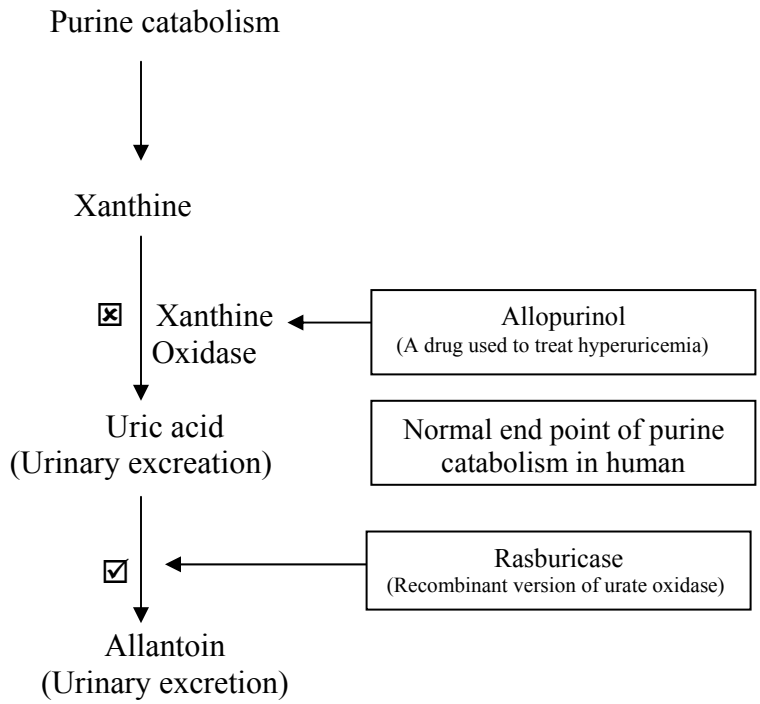


Fig 2.3 Mechanism of action of urate oxidase as therapeutic drug

2.2 History of urate oxidase

Originally, urate oxidase was isolated from plants and used as therapeutic drug but it was observed that, this form of enzyme is linked with allergic reactions. Later on, it was isolated from microbial and animal sources in order to avoid this side effect. Before 1970s, the therapeutic use of urate oxidase was very limited but in earlier 1975, it was commercially available in France under the trade name of uricozyme (recombinant form of urate oxidase from *Aspergillus flavu*s) and in 1980s it was introduced in Italy. Now in Europe and USA this drug has been approved and widely used in the treatment of hyperuricemia (Patte *et al.,* 2001; Annemans *et al.,* 2003; Navolanic *et al.,* 2003; Liu *et al.,* 2011; Giffard *et al.,* 2011; Bose and Qubaiah 2011; Darmon and Guichard 2011; Lin *et al.,* 2011).

Recently, Savient Pharmaceuticals introduced recombinant procine urate oxidase (pegloticase), which has approved by U.S FDA (United State food and drug administration) for marketing in the treatment of gout (Richette *et al.*, 2011).

2.3 Microbial production of urate oxidase

2.3.1 Microorganism

In many of the studies, for the production of urate oxidase, the researchers have used the variety of microbes such as *Bacillus subtilis*, *Bacillus fastidious* spores, *Arthrobacter globiformis*, *Aspergillus flavus, Pseudomonas aeruginosa*, *Rhizopus oryzae*, *Candida* sp., *Candida utilis*, *Candida tropicalis*, *Chlamydomonas reinhardtii* and yeast (Tanaka *et al.,* 1977; Bongaerts *et al.,* 1978; Nishimura and Tipton.,1979; Salas *et al.,* 1985; Vladimir *et al.,* 1990; Alamillo *et al.,* 1991; Han *et al.,* 1993; Liu *et al.,* 1994; Peter *et al.,* 2002; Suzuki *et al.,* 2004; Saeed *et al.,* 2004a and 2004b; Huang and Wu., 2004; Xue *et al.,* 2005; Yasser *et al.,* 2005, Retailleau *et al.,* 2005; Li *et al.,* 2006; Zhao *et al.* ,2006; Chen *et al.,* 2008; Wray and Fisher, 2010). Among all the above-mentioned microorganisms, *Bacillus subtilis* has reported as best one and preferred over the other microbes because it produces more enzymes. It is also used as a model organism for laboratory studies and has natural fungicidal activity (Yamamoto *et al*., 1998; Nishya *et al.,* 2002; Imhoff *et al*., 2003; Madigan and Martinko, 2005; Lotfy 2008; Gabison *et al.,* 2010). Urate oxidase gene has also expressed in *E. coli* through genetic engineering (Yamamoto *et al*., 1990; Huang *et al*., 2003 and Huang and Wu 2004).

Nishiya *et al.,* (2002) purified urate oxidase from *Bacillus*. They constructed and

expressed gene, encoding urate oxidase with a C-terminal 6-histidine tag. In this process, they removed this tag easily by using protease K, which did not affect the enzymatic properties of urate oxidase. This technique gave purified enzyme from the crud extract with a single step protocol. The isolated enzyme produced a single band on sodium dodecyle sulfate-PAGE, which was fully active. Santha *et al.,* (2002) compared urate oxidase, isolated from three different sources i.e. *Bacillus fastidious*, *Candida utili*, porcine liver and found the significant difference in the suitability of three different enzymes. The results indicate that beside specific activity also the molecular structure of the enzymes must be taken into account by a biosensor application.

Urate oxidase characterized from *Bacillus fastidious* for the estimation of uric acid in the serum by using a kinetic uricase method (Zhao *et al.,* 2006). Lotfy, (2008) identified the sequence of urate oxidase from *Bacillus thermocatenulatus*. The K_m and V_{max} were observed to be 0.25 mM and 0.99 U/mL respectively by using uric acid as a substrate. The enzyme retained 100% activity, when the strain was cultured under optimum conditions for 45 minutes at 75^{o}C.

2.3.2 Strain improvement techniques

Keeping in view the above said importance of urate oxidase, there is a great need to enhance its production. It is necessary to develop some strategies. In this reference, many workers have reported the improvement techniques for hyperproduction of urate oxidase by mutation through genetic engineering. Similarly, the strain improvement techniques by using different mutagens (ultraviolet radiation, gamma radiation and chemical mutagens) have reported for the hyperproduction of different enzymes (Gromada and Fiedurek, 1997; Khattab and Bazaraa, 2005; Lofty *et al.,* 2007), but there is no reported work for hyperproduction of urate oxidase through fermentation by using mutagens. Leplatois *et al.,* (1992) constructed the strains of *Saccharomyces cerevisiae* producing *Aspergillus flavus* urate oxidase and found that this strain was efficient in directing the synthesis of uaZ mRNAs encoding urate oxidase.

It has been observed that urate oxidase was produced industrially from a strain of *Aspergillus flavus*. The production of this enzyme was enhanced by adopting two strategies for the improvement of *Aspergillus*. “Classical mutation selection protocol” was the base of one strategy. According to this protocol, a mutant strain was isolated and subjected to

enzyme assay. By using this strategy, the production of urate oxidase was improved two fold as compare to industrial strain. The other strategy was base on the construction of transformed strains. By adopting this method, the production of urate oxidase was improved twenty-fold (Chevalet *et al.,* 1993).

Urate oxidase gene was cloned by Hongoh *et al.,* (2000) from *Nilaparvata lugens* and expressed in *Escherichia coli*. This gene contained an open reading frame (987 bp) that was divided by a single intron (96 bp) into two fractions. This encoded enzyme was comprised of 296 amino acids that was showed sequence identity (62%) with that of *A. flavus*. This produced enzyme was as active as that enzyme that was isolated from animals and plants, but its activity was less than that urate oxidase that was isolated from fungi.

On the other hand, Imhoff *et al.,* (2003) expressed and cloned the gene that coding urate oxidase from another source *Bacillus subtilis*. In this way, the cloned and expressed enzyme was purified and characterized. That enzyme was observed as it contained an ionizable group at pH of 6.4. They discussed that it must be unprotonated for catalysis by steady state kinetics studies. Thr 69 is hydrogen-bonded to the ligand at the active site, and Lys 9, which does not contact the ligand, is hydrogen bonded to Thr 69. The T69A and K9M mutant enzymes purified by Imhoff and his colleagues had V_{max} 3 % and 0.4 % of wild type respectively.

Mutant urate oxidase was derived from thermophillic bacterium *Bacillus subtilis.* This mutant urate oxidase was found to show higher specific activity such that 13.1 U/mg. Finally, the efficiency of modified colorimetric assay is more than the conventional ones. Therefore, it reduces the assay time from 96 hours to 20 hours (Huang and Wu. 2004).

Saeed et *al*., (2004a) isolated and cloned a fragment of DNA (1.35 kb) that encoded urate oxidase gene from genomic library of *P. aeruginosa*. A cloned DNA fragment when expressed in *E coli,* then it showed the uricolytic activity. The sequences of this manufactured urate oxidase gene from different strains was closely related to the cloned gene, such as 44 % sequence of the cloned gene was close similar to *Cellulomonas flavigena* while only 24 % was similar to *Bacillus* sp. BT-90 and *Candida utilis*. This isolated urate oxidase gene was also exhibited close similarity to urate oxidase from yeast like *Cerataphis fransseni* (24%), *Paecilomyces tenuipes* (27%), *Tolypocladium inflatum* (29%) and *Beauveria bassiana* (35%).

The production of urate oxidase is enhanced by immobilization. Urate oxidase was immobilized on commercial supports, amino ethyl high amylose as well as on carboxymethyl high amylose. The N ethyl 5 phenylisooxazolium 3' sulfonate provides a high binding but it was inhibited the activity of the enzyme. carboxymethyl high amylose gave best results. Immobilized urate oxidase and free enzyme had V_{max} of 16 U/mg and 18 U/mg respectively. Immobilized urate oxidase had K_m of 0.17 mM while free enzyme had K_m value of 0.03 mM. It was studied that immobilized urate oxidase exhibits more resistance towards proteolysis than the free urate oxidase. In pancreatic proteolysis medium, modified enzyme had 25 % of its initial activity after one hour, whereas free enzyme retained 5% of its initial activity. Carboxymethyl high amylose produced best immobilization yield and gave better results as compared to commercial supports based on agaros (Mulhbacher *et al.,* 2002).

Urate oxidase was also immobilized onto polyaniline film by using glutaraldehyde that was exhibited better shelf life. It was found that K_m of immobilized enzyme was $5.1x10^{-6}$ mM mL^{-1} while free enzyme had K_m $3.4x10^{-4}$mM mL^{-1}. These results suggested that the immobilized enzyme had high affinity with substrate as compared to free enzyme. The immobilized urate oxidase retained 95% activity at 4 ^oC for 4-5 months. Cete *et al.,* (2007) immobilized urate oxidase onto polypyrrole film by using glutraldehyde. The Michaelis Menten constant (K_m) and V_{max} of immobilized urate oxidase was found to be 0.44 mM and 5.1×10^{-5} U/mL respectively. While K_m and V_{max} of free enzyme was 0.39 mM and 4.2×10^{-4} u/mL respectively (Arora *et al.,* 2007).

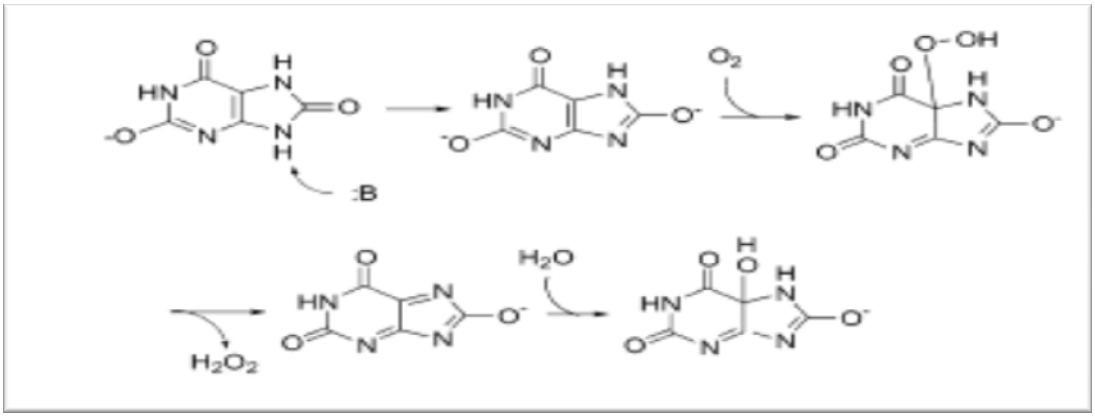
Source	**Nature**	**Specific Activity**	**Refrence**
B. subtilis	Mutant	13.1 U/mg	Huang and Wu, 2003
	Immobilized	16 U/mg	Mulhbacher *et al*., 2002
	Immobilized	5.1×10^{-5} U/mL	Cete *et al*., 2007
	Immobilized	4.2×10^{-4} U/mL	Arora *et al*., 2007

Table 2.1 Summary the activities of urate oxidase by strain improvement techniques

2.4 Substrate for urate oxidase production

Several substrates (uric acid, n-alkane) were used in the fermentation medium to enhance the production of urate oxidase by many workers (Tanaka *et al.,* 1977; Fattah and Hamed. 2002; Wilkinson and Grove *et al.*, 2004; Doll *et al.,* 2005; Gabison *et al.,* 2006; Colloc'h *et al.,* 2008; Juan *et al.,* 2008; Lotfy, 2008; Oksanen *et al.,* 2009; Gabison *et al.,* 2010; Meotti *et al.,* 2011). Greene and Ronald (1955) have observed that the growth of *Neurospora crassa* was stimulated by the addition of uric acid in the fermentation medium and gave hyperproduced yield of urate oxidase.

Kahn and Tipton (1997) isolated urate oxidase from soybean root nodules and determined its kinetics mechanism. They explained that uric acid and oxygen bind to the enzyme sequentially. Xanthine is a competitive and non-competitive inhibitor versus urate and oxygen respectively. In the absence of urate, oxygen does not bind productively to the enzyme, they also reported that 9-methylurate is pH independent and a potential competitive inhibitor of urate, proved that urate is ionized at N9 position is not important for binding. Uric acid reacts with oxygen by the action of urate oxidase to form an intermediate (urate hydroperoxide). Hydrogen peroxide removed from urate hydroperoxide and form another product (5-hydroxyisourate). Therefore, it was observed that uric acid undergoes sequential deprotonation, first from N3 and then from N9, electron density accumulates at C5, the detail of mechanism of action is as under



Aguilar *et al.,* (2002) purified urate oxidase to electrophoretic homogeneity from the rust *Puccinia recondita*. They described that the specific activity was 8.4 U/mg. The results exhibited strong similarity to other plants and fungal urate oxidase. The purified enzyme had

optimum pH (9.0) and temperature (35 ^{o}C) and it was also made clear that Michaelis Menten constant (K_m) of urate oxidase was 35 mM for uric acid whereas xanthine and oxonate inhibited the activity of this enzyme.

When the ammonium chloride was used as a source of nitrogen in minimal medium for the growth of *Rhizopus oryzae* mycelium then there would be no activity of urate oxidase. While uric acid was added in the same medium, the activity of urate oxidase was enhanced 60-78 folds (Peter *et al.,* 2002). Furthermore, when urate oxidase was isolated and purified from *Pseudomonas aeruginosa* and had specific activity 636.36 with the use of urate as a substrate. Suzuki *et al.,* (2004) observed that urate oxidase is produced intracellularly by *Arthrobacter globiformis*. When the uric acid was added in the culture medium, then the yield of *Arthrobacter globiformis* urate oxidase was increased.

Yazdi *et al.,* (2006) isolated intracellular urate oxidase from *Mucor hiemalis* in the fermentation medium containing uric acid. The fermentation medium was optimized, consisted of uric acid (7.0 g), maltose (6.0 g), Vogel stock solution (amounts for 1 L of 50X stock solution: 150g sodium citrate.$2H_2O$, 250 g KH_2PO_4, 100 g NH_4NO_3, 10 g $MgSO_4$,5g $CaCl_2$, 2.5 mL biotin solution, and autoclave the solution) (20 mL) and 0.5 M copper sulfate (1 mL) per liter of distilled water. The activity of the enzyme (1.0 U/ml) was enhanced in the presence of uric acid in the culture medium. It was retained 100 % of its initial activity after heat treatment for 30 minutes at 70 °C (Xue *et al.,* 2005; Lotfy, 2008).

2.5 Techniques for urate oxidase production

Urate oxidase is produced from all animals (except primates), plants and microbes through extraction, cloning and fermentation (Hongoh *et al.,* 2000; Saeed *et al.,* 2004; Suzuki *et al.,* 2004; Li *et al.,* 2006; Pfrimer *et al.,* 2010; Giffard *et al.,* 2011). The techniques used for extraction and fractionation of urate oxidase are very complicated. Moreover, the raw materials, used for the purification of the enzyme, are very expensive. That is why; urate oxidase produced by the above said method will be high priced. This enzyme is also produced from cloning. It is not only a sensitive technique, where a minor mistake in handling may result a major loss, but also it requires high price equipments and chemicals. Eventually, in the last few decades, the interest was developed towards microbial fermentation. This technique is the absolute choice because it is simple, fast and cheaper than all other techniques. (Tanaka *et al.,* 1977; Fattah and Hamed. 2002; Suzuki *et al.,* 2004;

Nakagawa *et al.* ,2006; Chen *et al.*, 2008; Lotfy *et al.*, 2008)

Accordingly, the production of urate oxidase was enhanced by *Pseudomonas aeruginosadium*. The two levels Plackett Burman design was used for the screening of bioprocesses parameters, which significantly influence the activity of the urate oxidase. Because of their significant effect on the activity of the enzyme, fifteen variable tests were selected. Response surface methodology (RSM) was adapted to obtain the best conditions for the process. A polynomial model was created to correlate the relationship between three variables and the urate oxidase activity. The optimum conditions of the medium for the production of urate oxidase were $CuSO_4$ of 10^{-3} M, $FeSO_4$ of 10^{-3} M and pH of 5.5. The optimum activity of the urate oxidase was 7.1 U mL^{-1} min^{-1}, which is 16.5 times more than the activity of the urate oxidase obtained by the basal medium (Yasser *et al.,* 2005).

Chen *et al.,* (2008) expressed urate oxidase of *Candida utilis* in *Hansenula polymorpha* by using *Saccharomyces cerevisiae*. The effect of different parameters ware studied in shaking flask cultures. It was observed that, pH and inoculum size had significant influence on the recombinant urate oxidase production. The optimum extracellular urate oxidase yield (2.6 U mL^{-1}) was obtained in shaking flask culture. High cell-density cultivation technique was used for the production of urate oxidase. After induction for 58 hrs, the production of recombinant uricase reached 52.3 U/mL extracellularly and 60.3 U/mL intracellularly in fed-batch fermentation, which was much higher than those were expressed in other expression systems.

2.6 Factors effecting the production of urate oxidase

Various factors affect the fermentation techniques. These factors are used in balanced concentration to increase the efficiency of the procedure. The control of both of these factors, physical (temperature, pH etc) and chemical factors (nitrogen, phosphorus, carbon, magnesium, trace elements etc) have great influence on microbial growth.

2.6.1 Chemical factors

Various concentrations of carbon sources have also affect the production of urate oxidase. Most reported carbon sources are glucose, fructose, glycerol and maltose for the optimum yield of urate oxidase by different microorganisms. They required carbon sources (as a source of energy) for their growth. Because carbohydrates are easily taken up, metabolized and provide energy to microbes. Glycerol, sucrose and maltose were the best

carbon sources for *A terreus, A. flavus* and *Trichoderma* sp. respectively (Fattah and Hamad, 2000). Xue *et al.,* (2005) investigated that maize milk was the best carbon source. Yasser *et al.,* (2005) isolated urate oxidase from *Pseudomonas aeruginosa*, the activity of of this enzyme was inhibited by the addition of glucose in the fermentation medium. Optimum yield of the *Mucor hiemalis* urate oxidase was obtained by the addition of maltose (6%) in the fermentation medium (Yazdi *et al.,* 2006).

Magnesium (Mg^{++}) has great influence on the production of urate oxidase. In many enzyme reactions, it plays an important role as a cofactor. Magnesium sulfate is an important compound in the synthesis of urate oxidase (Trakya *et al.,* 2000). Other researchers also investigated that the presence of magnesium sulfate (0.5%) in the fermentation medium (uric acid medium and czapek-Dox medium) enhanced the production of urate oxidase (Fattah and Hamed 2002; Huang and Wu 2004).

The production of urate oxidase was enhanced by the addition of nitrogen sources (ammonium chloride, peptone, yeast extract, ammonium sulfate etc) in the fermentation medium. Several investigators had studied that uric acid was the best nitrogen source for the production of urate oxidase (Fattah and Hamad 2002; Peter *et al.,* 2002; Santha *et al.,* 2002; Suzuki *et al.,* 2004; Yazdi *et al.,* 2006, Lotfy 2008). It has also observed that the production of urate oxidase was inhibited by the addition of ammonium compound in the fermentation medium (Peter *et al.,* 2002).

The affect of metallic ion on the activity and stability of urate oxidase was studied by many researchers (Alamillo *et al.,* 1991; Liu *et al.,* 1994; Green and Ronald 1955). Copper has great influence on the activity of urate oxidase because it is involved in the oxidation of uric acid. However, the excessive concentration of copper ions inhibited the activity of the enzyme (Alamillo *et al.,* 1991; Suzuki *et al.,* 2004). Various compound and metallic ions such as cobalt sulfate, zinc sulfate, iron sulfate, silver, mercury, and cyanide was inhibited the activity of the urate oxidase, whereas, calcium chloride and copper sulfate enhanced the uricolytic activity of the enzyme (Alamillo *et al.,* 1991; Saeed *et al.* ,2004b; Yasser *et al.,* 2005, Yazdi *et al.,* 2006; Kai *et al.,* 2008). The extraction of urate oxidase was activated by the addition of sodium chloride (1M) and potassium chloride (1M). While the addition of magnesium chloride (1M) and barium chloride (1M) inhibited the activity of urate oxidase (Tajima *et al.,* 2004).

2.6.2 Physical factors

The production of urate oxidase is also affected by different levels of pH, temperature, agitation etc. Under the following conditions, maximum urate oxidase activity as well as more values of biomass in the mechanically agitated fermentor was obtained: rotation impeller speed 7 s^{-1}, time 50 hours and air flow rate 1.25×10^{-5} m^3s^{-1}. The effect of some general factors, such as temperature and pH were also investigated. Specific activity of the enzyme was optimum at the pH 8.5 and temperature 30 ^oC (Liu *et al.,* 1994).

Han *et al*., (1993) investigated the separation of intracellular urate oxidase from *Candida utilis*. Favorable conditions for extraction of urate oxidase were established. The effects of different factors for instance concentration of surfactant, reducing agent, salt, and pH of the stripping solution were observed. Seeing that, the concentration of surfactant increased. Subsequently, the yield of urate oxidase was also increased. It was also observed that, when the concentration of reducing agent was increased then the yield of protein was also increased.

Urate oxidase was isolated from uricolytic fungi, *Aspergillus flavus*, *A. terreus* and *Trichoderma* sp. The optimum temperature for the production of urate oxidase and biomass yield was observed to be 30 ^oC. Whereas, the time required for optimum growth of this enzyme was six days for *Trichoderma* sp and 4 days for two *Aspergillus* species. It was also investigated that optimum pH for the production of urate oxidase was at 6.6 for both *A. flavus* and *Trichoderma* sp. and 6.4 for *A. terreus.* The medium supplemented with 4% poultry waste produced maximum fungal biomass yield (Fattah and Hamad, 2002).

Urate oxidase was isolated from various sources and compared their properties (optimum pH, affinity and retention of enzyme activity). It was observed that the enzymatic properties of *Candida utilis* were more favorable (John *et al.,* 2002). Kai *et al*., (2008) isolated urate oxidase from *Microbacterium* sp. and characterized it. They also observed optimum temperature (30 ^oC) and pH (8.5) of the purified enzyme. It was also observed that Fe^{+3} enhanced the activity of the enzyme. On the other hand, SDS, o-phenanthroline, silver and mercuric ions inhibited the enzyme activity.

2.7 Techniques for urate oxidase purification

The enzyme was produced either from extraction of animal tissues or from microbial fermentation. The desired enzyme is generally found with various other macromolecules

such as proteins, various enzymes, and other undesirable materials. Various well known methods were adopted for the purification of urate oxidase, such as organic solvent precipitation, ammonium sulfate fractionation, ion exchange chromatography and gel filtration chromatography, xanthine-agarose affinity chromatography, affinity chromatography etc (Larsen 1990; Alamillo *et al.,* 1991; Liu *et al.,* 1994; Zhang *et al.,* 2010; Giffard *et al.,* 2011).

Larsen, (1990) purified urate oxidase from soybean root nodules. He used single affinity chromatography for the purification of urate oxidase on Arginine-Sepharose, which was formed by the combination of L-Arginine and CH-Sepharose 4B. Columns of Arginine-Sepharose were prepared and crude extracts of soyabean root nodules were loaded onto the said small columns. The fractions that had maximum urate oxidase activity were re-run on the same column to obtain highly purified protein.

Vladimir *et al.,* (1990) studied the production of urate oxidase by *Candida utilis*. It was demonstrated that 0.1% hypoxanthine exhibited the most effective inducer of urate oxidase production. Alamillo *et al.,* (1991) purified urate oxidase from the unicellular green alga *Chlamydomonas reinhardtii* by a procedure, in which following main steps were included as ammonium sulfate fractionation, ion exchange chromatography, gel filtration chromatography, and xanthine-agarose affinity chromatography. It was expressed its maximal absorption at 276 nm. Absorption spectrum of urate oxidase (native) showed two transient maxima, identical to those of metal urate complexes, at 392 and 570 nm. In the presence of cyanide, metal-urate complexes were disappeared. Therefore, it was concluded that cyanide and neocuproin inhibited the activity of this enzyme. Although salicylhydroxamic acid was not inhibited the activity of the enzyme. This evidence proposed that, copper is involved in the oxidation of enzymatic urate.

Liu *et al.,* (1994) purified urate oxidase from *Candida* sp. which was carried out by a procedure in which following steps were involved i.e. ammonium sulfate precipitation and sephadex G200. Then the fractions that have maximum activity were further proceeding with DEAE-cellulose (DE52 chromatography). After purification, the specific activity of urate oxidase was increased from 0.05-12 U/mg. it was also observed that the purified enzyme was stable at a pH 8.5-9.5 and temperature less than 35 ^{o}C.

Urate oxidase has been purified by different methods that obtained highest specific

activity than that of previously purified urate oxidase (Greene and Ronald, 1955; Greene and Mitchell, 1957). Montalbini, *et al.,* (1997) purified urate oxidase to electrophoretic homogeneity from leaves of *Triticum aestivum* L.*, Vicia faba* L., and *Cicer arietinum* L. They adopted different purified techniques in which xanthine agarose affinity chromatography was the main step. Recovery ratio of urate oxidase was observed between the ranges of 80-90%. Specific activity of the purified urate oxidase was ranged from 600-800 nkat.mg/protein.

Saeed *et al.,* (2004b) isolated and purified urate oxidase from *Pseudomonas aeruginosa.* Purification was carried out by adopting different techniques such as ammonium sulfate precipitation, ion exchange chromatography and gel filtration chromatography. Kai *et al., (*2008) purified urate oxidase from *Microbacterium* sp. by using different techniques i.e. ammonium sulfate precipitation, hydrophobic and molecular sieve, DEAE-cellulose ion exchange chromatography.

Li *et al*., (2006) purified recombinant urate oxidase from *E coli*. For purification of urate oxidase different techniques were used, such as hydrophobic interactions, gel filtration and anion exchange chromatography. By adopting these techniques >99 % the recombinant urate oxidase was purified. Approximately 27g of pure and biologically active recombinant urate oxidase per Kg of cell paste were obtained from fermentation.

2.8 Kinetics of urate oxidase

Kinetics is the study of enzymes that catalyze the chemical reactions. The rate of reaction and affect of different conditions are measured by enzyme kinetics. Many investigators have calculated the Michaelis Menten constant (K_m) and V_{max} of purified urate oxidase that were isolated from various microorganisms (*Candida sp. B. subtilis, B. fastidious, B. thermocatenulatus, Microbacterium* sp. Strain ZZJ4-1) plants (*Cicer arietinum* L., *Vicia faba* L., *Triticum aestivum* L *Puccinia recondite*) and animals (bovine kidney and caprine kidney) (Liu *et al.,* 1994; Montalbini *et al.,* 1997; Aguilar *et al.,* 2002, Imhoff *et al.,* 2003; Benboubetra *et* al., 2004; Rajoka *et al.,* 2006a, Rajoka *et al.,* 2006b; Zhao *et al.,* 2006, Kai *et al.,* 2008; Lotfy 2008).

Michaelis Menten constant (K_m) of purified urate oxidase was estimated from 5×10^{-6} to 1×10^{-4} mM/mL was estimated. These results suggested that, the integrated rate equation that uses the predictor variable of reaction time was reliable for the estimation of enzyme

kinetic parameters and applicable for the characterization of enzyme inhibitors. Zhao *et al., (*2006) characterized urate oxidase from *Bacillus fastidious* and found that Michaelis-Menten constant (K_m) was 2.04 x 10^{-4} mM/mL and inhibition constant (Ki) for xanthine was 4.1x 10^{-5} mM/mL.

The Michaelis Menten constant and V_{max} of immobilized enzyme was calculated and compared with free enzyme by many workers (Mulhbacher *et al.,* 2002; Arora *et al.,* 2007; Cete *et al.,* 2007). Alamillo *et al., (*1991) calculated the activation energy (53 kJ mol^{-1}) of urate oxidase from unicellular green alga. Rajoka *et al., (*2006b) purified urate oxidase from caprine kidney. It possessed K_m value 6.5 x 10^{-3} mM/mL for the hydrolysis of uric acid while V_{max} values 3512 U/mg protein. For catalytic activity, the optimum pH and temperature of purified enzyme were 8.5 and 40 ^oC respectively. The activation energy of the urate oxidase for the formation of the enzyme substrate complex was 13.6 kJ/mol. Entropy of activation, enthalpy and Gibb's free energy, demand of urate oxidase inactivation were -102 J/mol K, 62.8 kJ/mol and 104.3 kJ/mol respectively.

Rajoka *et al., (*2006a) purified urate oxidase to homogeneity level from bovine kidney. The K_m value of urate oxidase for the urate hydrolysis was 0.125 mM and V_{max} of 102 U/mg protein. The activation energy requirement for urate hydrolysis by urate oxidase was 11.6 kJ/mol and inactivation of enzyme was 14.5 kJ/mol. Lotfy, (2008) identified the sequence of urate oxidase from *Bacillus thermocatenulatus*. The K_m and V_{max} values were observed to be 0.25 mM and 0.99 U/mL respectively

2.9 Determination of molecular mass

A number of investigators have been studied the homogeneity and purity of many enzymes by electrophoresis. Gel filtration and SDS-PAGE techniques have been used for the determination of molecular weight and subunits of the enzyme (Larsen 1990; Alamillo *et al.,* 1991; Liu *et al.,* 1994; Yamamoto *et al.,* 1996; Montalbini *et al.,* 1997, Hogoh *et al.,* 2000; Yamamoto *et al.,* 2002; Saeed *et al.,* 2004b, Rajoka *et al.,* 2006b; Zhao *et al.,* 2006; Kai *et al.,* 2008).

The metal content of recombinant urate oxidase was investigated and it was found that copper ions are present only in trace amount. The molecular mass of the enzyme was determined to be 35,059.8 Da by MALDI-TOF mass spectrometry while calculated molecular mass of the enzyme was 35,052 Da. These results were showed that, in urate

oxidase, no any covalently bound cofactor is present. (Kahn and Tipton, 1997; Pfrimer *et al.,* 2011). Alamillo *et al., (*1991) studied that *Chlamydomonas reinhardtii* urate oxidase has a relative molecular mass of 124,000. The enzyme has four similar size subunits with relative molecular mass of (Mr) 31,000

Larsen, (1990) analyzed purified urate oxidase by SDS-polyacrylamide gel electrophoresis. Two isoforms of urate oxidase were observed by analytical isoelectric focusing, one dominating, had pI 9.0 and the other one minor, had pI 7.0. Yamamoto *et al., (*1996) selected the thermophillic bacterium (*Bacillus s*p. TB-90) for the determination of nucleotide sequence of urate oxidase gene. From the nucleotide sequences the primary structure of the urate oxidase was deduced which contain 332 amino acids 34,000 Da molecular weight by SDS-PAGE. Zhao *et al., (*2006) studied that molecular weight of urate oxidase from *Bacillus fastidious* was 151 kDa by gel filtration (sephadex G-200).

2.10 Determination of uric acid

The enzyme urate oxidase used for the estimation of uric acid is preferred over the other non-enzymatic techniques due to its high accuracy. Non-enzymatic methods are interfered by the presence of ascorbate, glutathione, aspirin and many antibiotics (Gokicke and Gokicke, 1973; Kabasakalian *et al.,* 1973; Itiaba *et al.,* 1975; Kuan *et al.,* 1975; Imhoff *et al.,* 2003; Huang *et al.,* 2004; Huang *et al.,* 2004). Bhargava *et al., (*1999) immobilized commercially available peroxidase and urate oxidase onto arylamine glass and alkylamine glass beads respectively. These immobilized enzymes were used for the determination of uric acid in the serum. By immobilized urate oxidase, H_2O_2 produced from serum uric acid. This H_2O_2 was measured by a color reaction that catalyzed by immobilized peroxidase. By this method, very low concentration of uric acid can be detected in the sample, its minimum detection limit was 8 µg/0.1 mL sample. The coefficient of variation between and within the assay were <10.77% and <6.8% respectively. An apparent healthy adult, the serum uric acid was determined to be 25-55 µg/mL and 32±2.25 (range, mean±S.D.) while in persons suffering from different diseases was 55-200 µg/mL; 52±6.4 (range, mean±S.D.) by this method. A good correlation (r = 0.8170) was achieved between the serum uric acid values by this method and with those achieved by commercial Enzo-kit method.

Urate oxidase isolated from three different sources i.e. *Bacillus fastidious*, *Candida utilis* and porcine liver. A new type of enzymatic uric acid biosensor was used to determine

the concentration of uric acid (Santha *et al.,* 2002). Huang *et al., (*2004) designed a complementary metal oxide semiconductor sensor. Urate oxidase-peroxidase immobilized on this polymeric enzyme biochip. The complementary metal oxide semiconductor sensor response was stronger at a higher temperature range from 20-40 ^{o}C with optimum pH 8.5. Purified uric acid provided a linear calibration curve in the concentration rang from 2.5-12.5 mg/dL.

Huang and Wu (2004) modified colorimetric assay in flexible 96-well microtiter plates. For colorimetric reactions urate oxidase, uric acid, horseradish peroxidase, 4 amino antipyrine 3, 5 dichloro-2 hydroxybenzene sulfonate were used in microtiter plates. Liao *et al., (*2006b) evaluated the kinetic urate oxidase method for the determination of uric acid in the serum. Initial absorbance (A_o) of the reaction mixture before the addition of urate oxidase was measured at 293nm, than the background absorbance (A_b) was predicted. Urate concentration in reaction solution was calculated from absorbance (A_o-A_b). This kinetics urate oxidase method showed CV < 4.3 % and recovery 100 %. Ascorbic acid, bilirubin, hemoglobin, lipids, xanthine and reduced glutathione <0.32 mmol/L in the serum had no any significant effects. Zhao *et al., (*2006) characterized urate oxidase from *Bacillus fastidious* for the estimation of uric acid in the serum by using a kinetic uricase method.

2.11 Therapeutic effect of urate oxidase

Commercially available urate oxidase (rasburicase) was proved to be safe and potentially active drug for the treatment of hyperuricemia (Pui *et al.,* 2001; Bruno, 2005; Li *et al*., 2006; Sundy *et al.,* 2007; Kikuchi, 2009; Eaddy *et al.,* 2010; Hooman and Otukesh 2011). Bayol *et al., (*2002) controlled the concentration of uric acid, in those patients that have received chemotherapy by using urate oxidase. Rasburicase, a recombinant enzyme expressed in *Saccharomyces cerevisiae*, was compared with Uricozyme, a natural enzyme obtained by *Aspergillus flavus*. It was demonstrated that, Rasburicase has higher purity by chromatographic analysis and SDS/PAGE. The specific activity of the Rasburicase was also better as compare to uricozyme. It was observed that uricozymes contained a cystein adduct on Cys103 by MS analysis. In the crystal structure, the cystein residue contained sulfur at position 103 which was oriented to the external surface of the tetramer. While the other two cystein residues (Cys 35, 290) oriented sulfur atoms to the centre of the canal formed by tetramer. By incubation of the rasburicase with cystein the same adduct was produced.

A recombinant urate oxidase (rasburicase) was effective in decreasing concentration of uric acid in those patients with hematologic malignancies. It has also shown that urate oxidase (Rasburicase) prevents the uric acid accumulation in patients who had hyperuricemia and who were at a risk for developing hyperuricemia. In USA rasburicase has been approved pediatric use. Rasburicase is a new alternate of allopurinol and prevention of excess uric acid in the serum. It showed better results as compare to allopurinol because it rapidly reduces the preexisting elevated uric acid level. Patients who used rasburicase immediately may also receive chemotherapy treatment (Yim *et al.,* 2003). Chevalet *et al., (*1993) industrially produced urate oxidase from. It is an important enzyme that used in human therapy. Bayol *et al., (*2002) controlled the concentration of uric acid in patients receiving chemotherapy by using urate oxidase.

CHAPTER 3

MATERIALS AND METHODS

The research work was designed to obtain optimum yield of urate oxidase. The strain improvement techniques (chemical and UV irradiations) were used to induce mutagenesis in *Bacillus subtilis* for hyperproduction of urate oxidase. To acquire the desired purpose the present work was completed through adopting the following steps.

3.1 Chemicals, reagents and enzymes

All chemicals and reagents of analytical grade were used in this research, and mostly purchased from Merck and Sigma.

3.2 Microorganism procurement and maintenance

The pure characterized culture of gram-positive bacteria, *Bacillus subtilis* was obtained from Department of Veterinary Microbiology, University of Agriculture, Faisalabad, Pakistan. The stock culture was maintained on nutrient agar medium in a refrigerator at 4 °C.

3.2.1 Sporulation medium

Sporulation medium was prepared (table 3.1) and pH was adjusted with the HCl (1M)/ NaOH (1M). The medium was transferred to test tubes and sterilized for 15 minutes at 121^{o}C. The test tubes were set to the slant position and allowed the medium to be solidified. These test tubes (slants) were contained sporulation medium and kept in the incubator at 30 ^{o}C for 48 hours as to check the contamination. The bacterial culture was raised by streaking on solidified slants aseptically with the help of a wire loop. These slants were kept in an incubator at 30 ^{o}C for 2 days (Xue *et al*., 2005; Zhao *et al*., 2006).

Table: 3.1 Preparation of Nutrient agar medium

Sr. No.	Ingredients	Quantity g/100mL
1	Peptone	0.5
2	Yeast extract	0.3
3	Agar	1.5
4	NaCl	0.5
5	Distilled water	Upto 100 mL

3.2.2 Inoculum preparation

For the prepration of inoculum, the pH (8) of the medium (table 3.2) was adjusted with HCl (1 M)/ NaOH (1 M) at pH 8, then autoclaved for 15 minutes at 121^{o}C. The *Bacillus subtilis* spores were transferred aseptically with the help of a wire loop in the inoculum medium. This medium was incubated (30^{o}C) for 48 hours on the orbital shaker (120 rpm) (Xue *et al,* 2005).

Table: 3.2 Preparation of inoculum medium for *Bacillus subtilis*

Serial No	Ingredients	Quantity g/100mL
1	Peptone	0.5
2	Yeast extract	0.3
3	NaCl	0.5
4	Distilled water	Upto 100 mL

3.2.3 Spores Counting

Numbers of spores were counted with the help of haemocytometer by the method of Kolmer, (1959) in the homogenous spore suspension. The concentration of the spores was adjusted at 10^{7}-10^{8} spores/mL (Kolmer, 1959; Zia *et al*., 2010).

3.3 Techniques to improve strain

The spores of *Bacillus subtilis* were prepared in nutrient medium (pH 8) by using 250 mL Erlenmeyer flasks in rotatory shaker (120 rpm).

3.3.1 Radiation mutagenesis

3.3.1.1 Mutagenesis by UV lamp

Bacillus subtilis spores (1×10^7 spores/mL) were mutated by using UV germicidal lamp of 20W (Phillips) to enhance the production of urate oxidase. The spores (10 mL) were transferred in sterilized petri plates and exposed to UV light at specific time interval, started from 30 minutes to 240 minutes. The sample of 1 mL was withdrawn after every 30 minutes. The exposure of uv light was carried out from the center of the germicidal lamp to the petri plates at a distance of 20 cm. Kill/survival curve was prepared to select the optimum dose. The time of exposure giving 3 log kill was selected for *Bacillus subtilis* mutation which yields highest production of urate oxidase (Gromada and Fiedurek, 1997; Khattab and Bazaraa, 2005).

3.3.2 Chemical mutagenesis

Ethyl methane sulfate (EMS) and ethidium bromide (EB) (Gromada and Fiedurek, 1997; Khattab and Bazaraa, 2005) were used to induce mutagenesis in *Bacillus subtilis* for hyperproduction of urate oxidase.

3.3.2.1 Mutagenesis by EMS and EB

The stock solutions of the chemicals (EMS and EB) were prepared separately by adding 0.15 mg chemical compound in 1 mL of buffer saline. Various time intervals were selected for chemical mutagenesis. The stock solution of each chemical were added in 9 mL of nutrient broth medium which contained the spores of *Bacillus subtilis* (1×10^7 spores mL^{-1}) and then kept it at 37°C in water bath. After a specific time interval of 30 minutes (30-210 minutes) the sample (1 mL) was withdrawn and was centrifuged thrice to remove the mutagen from the spore suspension at 10,000 rpm for 1 minute. A dose of EMS after 180 minutes producing 83 % kill, which was proved to be the best by kill/survival curve. While a dose of EB after 150 minutes producing 70% kill, which was proved to be the best by kill/survival curve.

3.4 Mutant selection

The specific mutant that yields highest production of urate oxidase was selected by adopting the following steps.

3.4.1 Colony restrictor selection

Triton X-100 was used to inhibit the growth of other bacterial colonies in the nutrient agar medium (Belavin *et al.,* 1988; Khattab and Bazaraa, 2005).

3.4.2. 3 Log kill mutant dose selection by kill/survival curve

The spores of *B. subtilis* were treated with three different mutagens and prepared hundred fold serial dilution of each treated mutagen spore to obtain approximately thirty colonies or less than thirty per plate. In the dark room, 0.1 mL of these spore dilutions were spread on nutrient agar plates that contain 0.1 % triton X-100 as a colony restrictor (Belavin *et al*., 1988). As a control non treated spores (parental/wild spores) were also plated on nutrient agar medium. All the processes were carried out in laminar air flow under strict aseptic conditions. Aluminum foil was used to cover the plates, and then kept these plates in an incubator at 30^{o}C for 48 hours. For the selection of each mutant more than thousands colonies were screened and few mutants were isolated on the nutrient agar plates to determine the activity of the enzyme. From a number of mutants the best one which showed highest activity of urate oxidase was selected (Petruccioli *et al.*, 1999; Khattab and Bazaraa, 2005).

3.4.2.1 Calculation of colony forming units (C.F.U. mL^{-1})

The colony forming units were calculated as follows:

$$\text{CFU mL}^{-1} = \frac{\text{Number of colonies on agar plate x 1}}{\text{Amount plated (0.1mL) x Dilution Factor}}$$

3.4.3 Screening Methods

3.4.3.1 Plate screening procedure

The nutrient agar medium was used as the basal medium to select the mutant which was supplemented with 0.7 % uric acid as a nitrogen source and 0.1 % triton X-100 as a colony restrictor. After the incubation (30^{o}C) of plates in the dark room at 48 hours, it was shown clear zones. These colonies were further sub-cultured which showed bigger zones. Few of the colonies were selected which showed bigger zones than parental/wild type (Petruccioli *et al.*, 1999; Khattab and Bazaraa, 2005).

3.4.3.2 Mutants isolation of selective marker

For the selection of the colonies which showed resistance to the catabolite repression, 2- thiouric acid was used. It was inhibited the formation of wild type strain. The mutant

spores were allowed to grow on the nutrient agar plates for 48 hours at 30^{o}C. Those colonies which showed background growth were isolated and subjected to preliminary urate oxidase identification (Bergmann *et al*., 1962; Philippides and Scazzocchio, 1981; Diallinas *et al*., 1995; Glatigny and Scazzocchio , 1995).

3.4.4 Mutant identification

Following two tests were performed for the identification of specific *Bacillus subtilis* mutant.

3.4.4.1 Enzyme diffusion zone analysis

Urate oxidase positive strains were recognized on nutrient agar plates containing 0.1 g/ L o-dianisidine and purified horseradish peroxidase (310 U/mg). Enzymatic reaction was occurred when urate oxidase was formed and gave rise a brown color (El-Enshasy, 1998; Petruccioli *et al.,* 1999; Khattab and Bazaraa, 2005). Those strains which exhibited the biggest diffusion areas (mm) were further examined.

3.4.4.2 Analytical examination

The strains which produced larger zone were scratched, dissolved and homogenized in buffer and then filtered. The activity of the urate oxidase was determined by using spectrophotometer.

3.5 Urate oxidase production

3.5.1 Inoculum preparation

The above described criteria were used for the selection of mutated strain. The mutated strain was cultivated in basal medium that contained uric acid as a nitrogen source and pH (8) was maintained before sterilization to examine the strain more accurately. The basal medium was autoclaved at 15 lbs. pressure for 15 minutes at (121°C) temperature. Then inoculated the basal medium with 2% spore suspension ($2x10^{7}$ spores mL^{-1}). In the orbital shaker (120 rpm), the inoculated flasks were incubated (30°C) for 48 hours (Gromada and Fiedurek, 1997).

3.5.2 Urate oxidase production by liquid-state fermentation

Liquid state fermentation was the method of choice for the growth of selected mutant in order to analyze the activity of urate oxidase. Furthermore, the parental *Bacillus subtilis* was also used for the production of urate oxidase. The comparison between parental and mutated strains was recorded (Fiedurek *et al*., 1998).

Triplicate flasks with 0.7g substrate were plugged with cotton. The basal medium containing substrate (0.7 %), peptone (0.5 %) and yeast extract (0.3 %). The pH (8) and temperature of the medium was adjusted. Then the flasks were autoclaved at 15 lbs. pressure for 15 minutes at 121^{o}C. After cooling, the spore suspension (inoculum) aseptically was added to each flask by using sterilized pipette in the laminar air flow. Then the flasks were incubated at 120 rpm for 48 hours at 30^{o}C in the shaker.

3.5.3 Optimization conditions for urate oxidase production

Various parameters were optimized to obtain the highest production of urate oxidase with mutant as well as parental strains. At optimized conditions, the results were highly significant.

Growth medium was fermented with *Bacillus subtilis* with varying concentration of substrate, at different fermentation periods, pH, temperature, inoculum size, nitrogen and carbon sources in triplicate flasks. The experiments were performed in such a way that the parameters optimized in one experiment were continued in the next studied experiment (Zia *et al.*, 2007; Zia *et al*., 2010).

3.5.3.1 Substrate level

Different levels of the substrate viz. 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 g were tested to obtain the highest yield of enzyme. In the triplicate flasks, different concentrations of substrate were added and autoclaved (121 ^{o}C) for 15 minutes (Table 3.3). All the flasks were inoculated aseptically in the laminar air flow with the help of sterilized wire loop. The maximum production of urate oxidase was obtained with 0.5 % substrate level from parental and mutant derived strains.

Table 3.3 Composition of growth medium with varying substrate level (parental and mutant derived strain)

Ingredient	**Treatments**								
	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9
Uric acid (%)	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0

3.5.3.2 Fermentation period

Triplicate flasks with 0.5 g of uric acid were autoclaved then 5 mL of inoculum was added aseptically into each flask. In shaker the inoculated flasks were incubated (30^{o}C) in the

shaker (120rpm). After a specific time period i.e 24, 36, 48, 60, and 72 hours, the samples were harvested from liquid state fermentation (Table 3.4).

Table 3.4 Composition of growth medium with varying fermentation period (parental and mutant derived strain)

Ingredient	Treatments				
	T_1	T_2	T_3	T_4	T_5
Uric acid (%)	0.5	0.5	0.5	0.5	0.5
Fermentation period (h)	24	36	48	60	72

3.5.3.3 pH

Triplicate flasks with optimum substrate level (0.5%) and fermentation period (36 h) were prepared for parental and mutant strains. The flasks were adjusted at different pH values viz., 7, 7.5, 8, 8.5, 9, 9.5 and 10 by using HCl (1 M) / NaOH (1 M). All the sterilized flasks were inoculated with parental and mutated *Bacillus subtilis* spores suspension. These flaks were incubated at 30 ^{o}C for 36 hours (Table 3.5). The optimum pH of the enzyme was determined to be 8.5 for parental as well as mutant derived strains.

Table: 3.5 Composition of growth medium with different pH values (parental and mutant derived strain)

Ingredient	Treatments									
	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}
Uric acid (%)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Fermentation period (h)	36	36	36	36	36	36	36	36	36	36
pH	5.5	6	6.5	7	7.5	8	8.5	9	9.5	10

3.5.3.4 Temperature

Different flasks were prepared with preoptimized fermentation medium. All the flasks were autoclaved for 15 minutes at 121 ^{o}C then cool it and inoculated with 5 mL spore suspension (parent/wild and mutant derived *B. subtilis*). The effect of incubation temperature on urate oxidase production by *Bacillus subtilis* was checked. The flasks were incubated at

different temperatures viz 30, 35, 40, 45 and 50 ^{o}C (table3.6). The optimum temperature for parental as well as mutant strains was found to be 35^{o}C.

Table: 3.6 Composition of growth medium with different temperature (parental and mutant derived strain)

Ingredient	Treatments				
	T_1	T_2	T_3	T_4	T_5
Uric acid (%)	0.5	0.5	0.5	0.5	0.5
Fermentation period (h)	36	36	36	36	36
pH	8.5	8.5	8.5	8.5	8.5
Temperature ^{o}C	30	35	40	45	50

3.5.3.5 Inoculum size

The flasks were containing with preoptimized fermentation medium. Different concentrations (i.e 1, 2, 3, 4, and 5 mL) of inoculum (wild and mutant derived) were used to inoculate the flasks for the determination of the most effective concentration of inoculum which yield highest production of enzyme (Table 3.7). The optimum inoculum size was estimated to be 3 mL for parent and mutant derived urate oxidase.

Table: 3.7 Composition of growth medium with different inoculum size (parental and mutant derived strain)

Ingredient	Treatments				
	T_1	T_2	T_3	T_4	T_5
Uric acid (%)	0.5	0.5	0.5	0.5	0.5
Fermentation period (h)	36	36	36	36	36
pH	8.5	8.5	8.5	8.5	8.5
Temperature ^{o}C	35	35	35	35	35
Inoculum (mL)	1	2	3	4	5

3.5.3.6 Effect of nitrogen sources

Effect of different nitrogen sources (peptone, yeast extract, ammonium chloride, and sodium nitrate) on the production of urate oxidase with varying levels i.e 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 % were studied to determine the most effective nitrogen (Table 3.8). It was found that yeast extract (0.3) was the best nitrogen source.

Table: 3.8 Effect of various concentrations of different nitrogen sources (parental and mutant derived strain)

Nitrogen sources (%)	Treatments					
	T_1	T_2	T_3	T_4	T_5	T_6
peptone	0.1	0.2	0.3	0.4	0.5	0.6
Yeast extract	0.1	0.2	0.3	0.4	0.5	0.6
Ammonium chloride	0.1	0.2	0.3	0.4	0.5	0.6
sodium nitrate	0.1	0.2	0.3	0.4	0.5	0.6

3.5.3.7 Effect of carbon sources

Various concentrations (1-6%) of different carbon sources (i.e maltose, sucrose, glucose, and galactose) were added in different flasks containing preoptimized fermentation medium. All the flasks were autoclaved for 15 minutes at 121^{o}C and inoculated with 3 mL spore suspension (optimized inoculum size) and incubated for 36 hours to check the activity of the enzyme. It was determined that maximum production of urate oxidase was obtained with sucrose (2 %) from parental and mutant derived *B. subtilis* (Table 3.9).

Table 3.9 Effect of various concentrations of different carbon sources (parental and mutant derived strain)

Carbon sources (%)	Concentrations (%)					
	T_1	T_2	T_3	T_4	T_5	T_6
Maltose	1	2	3	4	5	6
Sucrose	1	2	3	4	5	6
Glucose	1	2	3	4	5	6
Galactose	1	2	3	4	5	6

3.5.4 Sample harvesting

The material was centrifuged for 10 minutes at 10,000 rpm. The filtrate was used as crud enzyme and subjected to enzyme assay.

3.6 Enzyme assay

The velocity of the reaction was determined by measuring the absorbance that decrease with time at 293 nm resulting from the oxidation of urate to allantoin. One unit of

urate oxidase oxidizes one µmole of urate per minute under the specified conditions at (35 °C and pH 8.5).

The assay for urate oxidase was performed by following the procedure of Worthington, 1988. The reagents and steps were followed as under.

3.6.1 Preparation of borate buffer (0.1 M)

Borax (sodium tetraborate) 38.13 g and boric acid 6.18 g was dissolved in distilled water and volume was made upto one liter. The pH (8.5) of the buffer was adjusted with HCl (1 M)/NaOH (1M).

3.6.2 HCl (1 M)

Hydrochloric acid solution (1 M) was prepared by adding 83 mL of pure hydrochloric acid (37%) in the distilled water and volume was prepared until 1000 mL.

3.6.3 NaOH (1 M)

For the preparation of one molar sodium hydroxide solution, NaOH (40.0 g) was dissolved in distilled water and volume was made upto 1000 mL.

3.6.4 Uric acid (0.12 mM)

Lithium carbonate 60 mg was added in 15 mL water and filtered. Uric acid (100 mg) was dissolved in filtrate to prepare the fresh solution. The solution was heated at 60°C in the water bath, cool and the volume was made upto 100 mL with distilled water.

3.6.5 Procedure

The following reagents were pipette in milliliters into suitable cuvettes

	Test	blank
Borate buffer (0.1 M)	3.00	3.00
Uric acid (0.12 mM)	0.10	0.10

The reagents were mixed by swirling. To get the equilibration, the reagents were incubated for 5 minutes. Then add

	Test	blank
Enzyme solution	0.02	……
Borate buffer (0.1 M)	……	0.02

The decrease in absorbance was recorded by setting the spectrophotometer at 293 nm wavelength after inserting the blank and test solutions in cuvettes.

3.6.6 Uric acid Analysis

The concentration of uric acid was determined by following the method of Ramesh *et al*., 1978.

3.6.6.1 Preparation of uric acid standard curve

A standard curve was formed by plotting change in absorbance at the wavelength of 293 nm ($\Delta A = A_{20s} - A_{300s}$) vs different concentration of uric acid (Fig. 3.1). Uric acid standard solution was prepared for this purpose. For the preparation of uric acid standard solution, lithium carbonate (60 mg) was added in 20 mL water and filtered. Uric acid (100 mg) was dissolved in filtrate to prepare the fresh solution. The solution was heated at 60°C in the water bath, cool and the volume was made upto 100 mL with distilled water. This stock solution of uric acid was diluted by adding distilled water which gave 30, 60, 90,120 and 150 mg/L solution. The concentration of the uric acid in the sample was calculated by following the method of Ramesh *et al*., (1978).

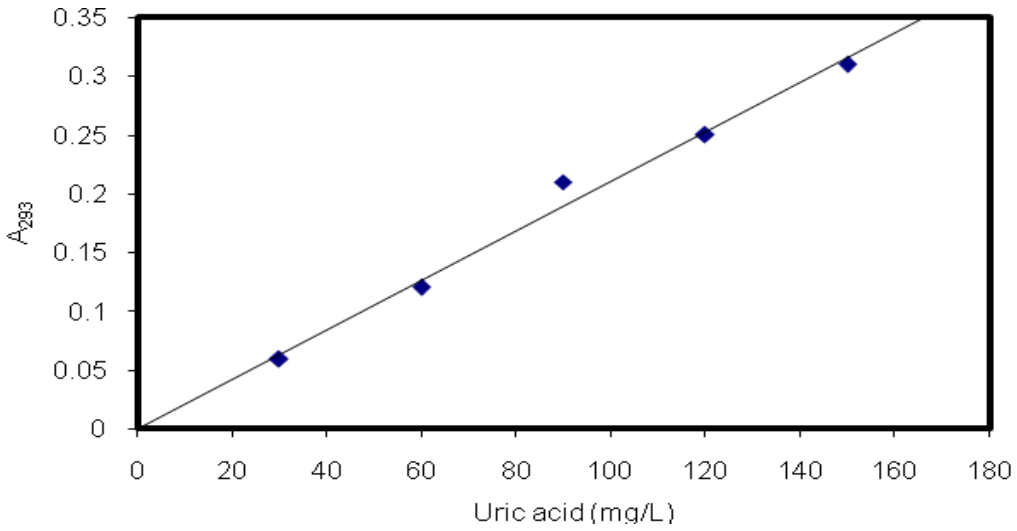


Fig 3.1 Standard curve of uric acid

3.6.7 Determination of protein contents

Protein contents were determined by using Biuret method (Gornall *et al*., 1949).

3.6.7.1 Preparation of biuret reagent

I. Sodium potassium tartrate tetrahydrate (12 g) and copper sulfate pentahydrate (3 g) were added to the distilled water and the volume was made upto 500 mL.

II. 10% NaOH solution (300mL) was added in the above solution and poured distilled water upto the final concentration of 1000 mL (This solution was stored at 4°C in dark bottle because of its sensitivity towards light).

3.6.7.2 Preparation of protein standard curve

Different concentrations of bovine serum albumin (0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL) were used to prepare the standard curve. Biuret reagent (1 mL) was mixed with one mL of each standard dilution. It was incubated for fifteen minutes in an incubator. Spectrophotometer was used to record its optical density at the wavelength of 540 nm. The standard curve was plotted between concentration (mg/mL) and absorbance. For test the solutions the above procedure was repeated and then calculated the protein contents (Fig. 3.2).

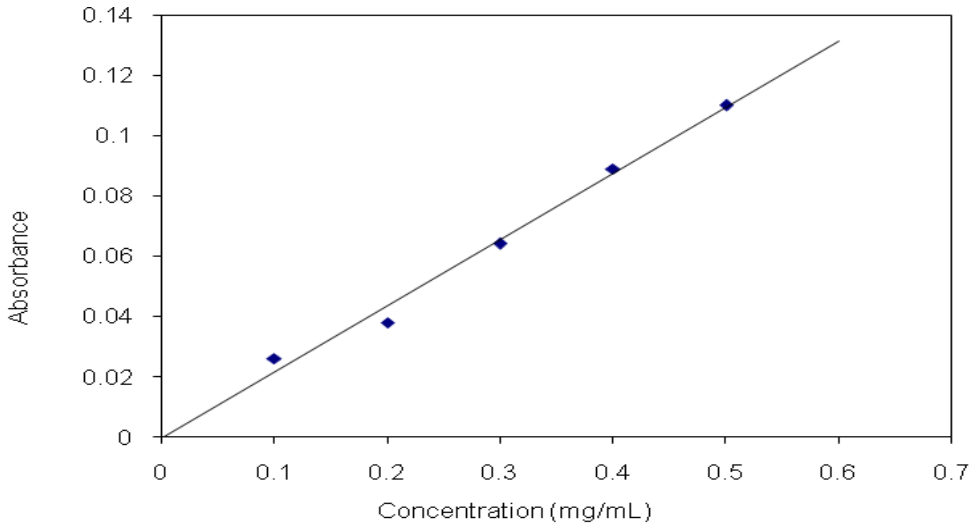


Fig 3.2 Standard curve of bovine serum albumin for protein estimation by biuret method

3.7 Purification of urate oxidase

Urate oxidase was purified to homogeneity by using different techniques such as Ammonium sulfate precipitation, gel filtration and ion exchange chromatography.

3.7.1 Partial purification by $(NH_4)_2SO_4$ precipitation

Crude enzyme was subjected to ammonium sulfate precipitation technique by the method of Meraj (2004). The crude extracts (parental and mutant) were precipitated with ammonium sulfate at 80% level of saturation (56.1 g) (Huang *et al*., 2004, Alamillo *et al*, 1991). Measured quantity of ammonium sulfate was added periodically in the crud extract

and stirrer it continuously for 2 hours. The precipitated crud extract was centrifuged at 4 ^{o}C for 10 minutes at 10,000 rpm. The supernatants were stored in sterilized bottles at 4 ^{o}C.

3.7.1.1 Dialysis

Supernatants were dialyzed after preparing the cellulose bags. The bags were washed for 4 hours in running water to remove the glycine then it was treated with 0.3 % (w/v) sodium sulfate solution for 1 minute at 80 ^{o}C to remove the sulfur compounds from the cellulose bags. Again the bags were washed for 2 minutes in the hot water (60 ^{o}C) followed by acidification with 0.2 % (v/v) sulphuric acid. At the end, the bags were rinsed with hot water for the removal of acid.

The enzyme mixture was poured in the dialysis bag and dialyzed at room temperature for 20 hours against 0.1 M borate buffer (pH 8.5) until the fluid portion becomes clear and the brown precipitates were settled out from the dialysis bag. The dialyzed portion was centrifuged (5,000 rpm). The colorless, clear phosphate buffer was obtained and again centrifuged room temperature for 10 minutes at 10,000 rpm. The resulting extract was preserved which was slightly opalescent. The extract was subjected with borate buffer (0.1 M) to enzyme assay (Section 3.5).

3.7.2 Partial purification by gel filtration

The dialyzed sample was subjected to further purification by gel filtration chromatography (Alamillo *et al*., 1991, Li *et al*, 2006, Rajoka *et al*, 2006a, b). Various steps were involved in this technique, which are as under.

3.7.2.1 Column preparation

A column of Sephadex G-200 was prepared by adopting the method of Meraj (2004).

3.7.2.2 Swelling of resin

Sephadex G-200 (2 g) was suspended into 30 mL of distilled water and heated it for 5 hours in water bath at 95 ^{o}C. The gel should not be dried through out the heating process.

3.7.2.3 Filling the column

The column was washed with detergent, distilled water, organic solvent and at the end again washed with distilled water. After drying, the column was mounted vertically on a vibration free and stable table. Closed the outlet of the column then gel slurry was poured in order to fill the column of 4 cm width and 21 cm length. It was left undisturbed over night until a distinguished layer of gel and water was established.

3.7.2.4 Equilibration of the column

It was achieved by washing the column with borate buffer (pH 8.5) for appropriate time.

3.7.2.5 Application of the sample

The outlet of the column was opened to remove the excessive water present in the column, and then closed it when there was a small layer of water on the top of the gel remains. The desalted sample (2 mL) was applied on the top of the column by using a fine pipette and opened the outlet. The sample was allowed to penetrate into the gel pores.

3.7.2.6 Elution

The sample was eluted with 0.1 M borate buffer (pH 8.5) and 50 fractions (containing 2 mL) were collected at a constant drop rate. These fractions were subjected to enzyme assay (Section 3.5) and protein estimation (Section 3.5.7).

3.7.3 Purification by ion exchange chromatography

The fractions from gel filtration that had maximum specific activity were subjected to further purification by ion exchange chromatography (Alamillo *et al*., 1991; Li *et al*, 2006; Rajoka *et al*, 2006a, b). The steps involved in this procedure are as follow:

3.7.3.1 Preparation of NaOH (4 %)

Dissolved NaOH (4g) in distilled water and volume was prepared until 100 mL

3.7.3.2 Preparation of HCl (4%)

Added 3.65 mL of hydrochloric acid into distilled water and volume was made up to 100 mL.

3.7.3.3 Preparation of column

A column of DEAE cellulose was prepared by following the method of Meraj (2004) and Zia *et al*., (2008) to purify the urate oxidase. For the preparation of slurry, the resin was added to the phosphate buffer then it was heated for 5 hours at 95 ^{o}C. Carefully the column was filled with slurry. Then the column was left undisturbed overnight.

3.7.3.4 Washing of column with base

The buffer was removed by opening the outlet then 50 mL of NaOH (4 %) solution was used for washing the column. The base was allowed to flow throughout the DEAE cellulose column. Then the distilled water was poured in it until the pH (7) of the column was adjusted.

3.7.3.5 Washing of column with acid

The distilled water was removed from the column then washed with 4 % HCl (50 mL) solution and allowed it to flow through the column. Again the distilled water was poured in the column until the pH (7) of the column was adjusted.

3.7.3.6 Equilibration of the column

Equilibration of the column was achieved by washing the column with borate buffer (8.5) for appropriate time. Equilibration is a process in which electrical conductivity of the buffer is equal before and after passing through the column.

3.7.3.7 Application of sample

The outlet tubing of the column was opened to remove the excessive water from the column, until the small amount of the water remains on the top of the column. Then applied 2 mL sample on the top of the column with the help of the fine pipette and allowed the sample to penetrate into the column bed.

3.7.3.8 Elution

The sample was eluted in stepwise fashion with 0.1 M borate buffer (pH 8.5). A total of 50 fractions of (2 mL) were collected at a constant drop rate. Then these fractions were subjected to enzyme assay (Section 3.5) and protein estimation (section 3.5.7).

3.8 Molecular mass determination

3.8.1 Electrophoresis

To analyze the homogeneity and purity of the urate oxidase, different urate oxidase preparation of sodium dodecyle sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 10%) was done as studied by Laemmli (1970).

3.8.1.1 Stock solutions

1. 30% (w/v) acryl amide + 0.8% (w/v) bis-acrylamide
2. 1.5 M Tris/HCl pH 8.8 + 0.3% (w/v) SDS
3. 0.5 M Tris/HCl pH 6.8 + 0.4% (w/v) SDS

3.8.1.2 Resolving gel preparation

All the following reagents were added together in a Buckner flask (250 mL) and degassed by a vacuum pump for five minutes. .

1. Stock solution-1 = 13.3 mL
2. Stock solution-2 = 10.0 mL

3. Distilled water = 16.7 mL

The following reagents were mixed after degassing to initiate polymerization.

4. 2% (w/v) aqueous ammonium persulfate freshly prepared = 133 µL

5. TEMED = 27 µL

The above mentioned mixture was poured into the gel apparatus for preparing the resolving gel which was assembled by sandwich between two glass plates (10 cm x 8 cm x 1.5 cm). After polymerization 1 butanol was layered on the gel surface. Then it (1 butanol) was removed by washing the gel surface many times with distilled water.

3.8.1.3 Stacking gel preparation

All the following reagents were added together In a Buckner flask (250 mL) and degassed for five minutes.

1. Stock solution-1 = 1.5 mL
2. Stock solution-3 = 2.5 mL
3. Distilled water = 6.0 mL
4. 2% (w/v) aqueous ammonium persulfate (APS) freshly prepared = 100 µL
5. TEMED = 10 µL

The above mentioned mixture of the stacking gel was poured on the top of the polymerized resolving gel. The well maker (comb) was inserted immediately in the stacking gel and allowed it to polymerize.

3.8.1.4 Sample buffer

The sample buffer was prepared by adding the following reagents together.

1. 0.75 M Tris/HCl buffer = 200 µL
2. Distilled H_2O = 6.3 mL
3. Glycerol = 2.5 mL
4. Aqueous SDS (10% w/v) = 1 mL
5. Bromophenol blue = 2.5 mg
6. β-mercaptoethanol = 5% (v/v)

In the presence of sodium dodecyle sulfate the subunits of protein (non-covalently attached) are dissociated into its monomers. β-mercaptoethanol was added into the above buffer to break down the S-S bridge if present between the subunits.

3.8.1.5 Stock electrode buffer

The stock electrode buffer was prepared by adding following reagents together. Just before the use, the stock solution was diluted by adding distilled water (10 folds).

1.	Tris base	=	30 g
2.	Glycine	=	144 g
3.	SDS	=	10 g
4.	Distilled water	=	1 L

3.8.1.6 For SDS-PAGE prepare urate oxidase

Urate oxidase (10mg/mL) was added in the sodium dodecyle sulfate buffer (4X) and boiled it for 5 minutes.

3.8.1.7 For SDS-PAGE prepare protein markers ladder

As standard, Protein markers ladder was used for sodium dodecyle sulfate polyacrylamide gel electrophoresis. This protein ladder was contained four bands which ranging from 45-200 kDa. This ladder was mixed in gel loading buffer (2% SDS, 30 mM NaCl, 50% glycerol, 62.5 mM Tris/HCl pH 7, 50 mM DTT, 1 mM NaN_3 and 0.01% bromophenol blue) and applied directly after slight warming on SDS-PAGE.

3.8.1.8 Running of PAGE

Sodium dodecyle sulfate gel was run at constant voltage of 100 volts when the tracking dye was reached at the bottom of gel, the polyacrylamide gel electrophoresis was stopped.

3.8.1.9 Protein staining of sodium dodecyle sulfate-polyacrylamide gel

The gel was treated with isopropyl alcohol (20% v/v) in 50 mM sodium acetate buffer at (pH 5) to remove sodium dodecyle sulfate for 15 minutes. The gel was dipped in sodium acetate buffer (50 mM, pH 5) to remove isopropyl alcohol by washing thrice in forty minutes and three changes of 40 minutes each were given.

Coomassie brilliant blue R-250 was used to stained the polyacrylamide gel (Kelley and Reddy, 1986). The protein bands were clearly showed after dipping the gel in the stain

for ten minutes. To improve the visualization of the bands, the gel was washed with distilled water after staining.

3.9 Kinetic and thermodynamic studies

3.9.1 Optimum pH

Urate oxidase (parent and mutant derived) were assayed (section 3.5) at various pH i.e 2, 3, 4, 5, 6, 7, 8, 9, and 10 to determined the optimum pH (Sukhacheva *et al*., 2004, Rajoka *et al*., 2006a).

3.9.2 Optimum temperature

Parent and mutant derived urate oxidase were assayed at various temperatures (20-80 ^{o}C) to determine the optimum temperature of the enzyme (Sukhacheva *et al*., 2004).

3.9.3 Activation energy (E_a)

Urate oxidase was subjected to enzyme assay at different temperatures (20-80 ^{o}C) for the determination of activation energy (Siddiqui *et al.*, 1997; Sukhacheva *et al*., 2004). The results were plotted according to Arrhenius as described by Rashid and Siddiqui (1998) and Rajoka *et al*., (2006a).

3.9.4 Determination of Michealis-Menten constants

Different concentrations of uric acid ranging from 0.1-1% (w/v) were used for the determination of Michaelis-Menten kinetic constants (K_m and V_{max}) (Witt *et al.,* 1998; Siddiqui *et al.*, 1997; Rajoka *et al*., 2006b). The activity of the urate oxidase was estimated at each level of uric acid by keeping the concentration of enzyme constant.

3.9.5 Irreversible thermal denaturation

For the determination of irreversible thermal denaturation of urate oxidase, the enzyme was incubated at different temperatures (i.e 40, 45, 50, 55, 60, and 65 ^{o}C). After specific time, one of the samples was withdrawn, cooled for 30 minutes in the ice and then subjected to enzyme assay at specific temperature (Rajoka *et al*., 2006a, 2006b). This process was repeated at six various temperatures (40, 45, 50, 55, 60, and 65 ^{o}C). The data was fitted to 1st order plot and analyzed as described Montes *et al.* (1995); Witt *et al.,* (1998) and Rajoka *et al*., (2006b).

3.9.6 Activation energy of thermal denaturation

The 1st order rate constant for irreversible thermal denaturation of urate oxidase were studied and Arrhenius plot was applied to analyze the activation energy for denaturation (E_a).

3.9.7 Thermodynamics of irreversible thermal inactivation

By rearranging the Eyring's absolute rate equation, the thermodynamic parameters for thermostability were calculated from transition state theory (Eyring and Stearn, 1939; Stearn, 1949; Tanford, 1968) as studied by Siddiqui *et al.* (1997).

$K_d = (k_B/h)\, e^{(-\Delta H^*/RT)} .e^{(\Delta S^*/R)}$ ……. (1)

Where,

h = Planck's constant = 6.63×10^{-34} Js

k_B = Boltzman's constant (R/N) = 1.38×10^{-23} JK^{-1}

R = Gas constant = 8.314 JK^{-1} mol^{-1}

N = Avogadro's No. = 6.02×10^{23} mol^{-1}

T = Absolute temperature

$\Delta H^* = E_a - RT$ ……...(2)

Where,

ΔH^* = Enthalpy of activation of denaturation

E_a = Activation energy for denaturation

$\Delta G^* = -RT \ln(K_d, h/k_B.T)$……..……..(3)

Where,

ΔG^* = Free energy of activation of denaturation

$\Delta S^* = (\Delta H^* - \Delta G^*)/T$……..……..(4)

Where,

ΔS^* = Entropy of activation of denaturation

3.10 Stability determination

The stability of urate oxidase from EMS treated *B. subtilis* was determined against different agents like potassium cyanide, zinc sulfate, sodium chloride, calcium chloride, barium chloride, and potassium chloride. (Tietz 1995 and Liao *et al*., 2006b)

3.11 Uric acid estimation

Uric acid in the biological fluid was estimated by following the method of Fossati *et al.,* 1980.

3.11.1 Comparison with standard kit

The self prepared kit was compared with two standard uric acid estimation kits upon the serum of volunteers.

CHAPTER 4

RESULT AND DISCUSSION

Several microorganisms have been reported for the production of urate oxidase; however, *Bacillus subtilis* is the main microorganism that is used for industrial production of this enzyme. Improvement, screening and evolution of new hyperproducing urate oxidase strain play an important role in improving the economics and efficiency of industrial process. The use of *B. subtilis* improving techniques (classical screening, genetic engineering and optimization of cultivation conditions) have been used by many workers for hyperproduction of urate oxidase (Rowlands, 1984; Khattab and Bazaraa 2005).

4.1 Production of mutants

In the present research, *B. subtilis* was mutated for hyperproduction of urate oxidase. The type and dose of mutagen was also optimized. .

4.1.1 Mutation induced by UV radiation

UV radiation was used to enhance the activity of urate oxidase from *B. subtilis*. Various doses of UV radiation were compared to optimize the treatment. A dose after 210 minutes was produced 82% killing (50 CFU/ mL) where it produced 3 log kill as optimum dose and detailed findings have been arranged in figure 4.1. It was observed that, higher doses of UV radiations reduced the number of colonies and frequency of positive mutation. Khattab and Bazaraa, (2005) isolated 11 out of 200 mutant colonies that were resistant to 2 deoxy D-glucose from UV treated *A. niger*. When the time of exposure was increased then the resistant colonies were decreased. It was further observed that only two colonies did not produce enzyme. Our results are resembled to the above said refrence in this agreement that, the number of colonies were decreased by increasing the the time of exposure of uv radiations. Park *et al*., (2000) also treated *S. cervevisiae* that contain *Aspergilus niger* gene to UV mutagenesis and found that the activity (460 U/mL) of the enzyme was increased 80% as compared to original untreated strain.

4.1.2 Mutation induced by chemicals

Chemicals (Ethyl methane sulfonate and ethidium bromide) were also used, for the induction of mutation in *B. subtilis*, to enhance the production of urate oxidase.

Ethyl methane sulfonate treated *B. subtilis* at 180 minutes dose rate produced 83% killing (40 CFU/mL), which was proved to be the best (having ability to hyperproduced urate oxidase) by kill survival curve (Fig. 4.2). Lino and Teresa (1998) also induced chemical mutation (ethyl methane sulfonate and MNNG) in *Cellulomonase*, for achieving hyperxylanotic mutant. The mutated derived strain produced 2.5 times higher production of enzyme as compared to parent strain. Khattab and Bazaraa (2005) treated *A. niger* with ethyl methane sulfonate for hyperproduction of glucose oxidase. Out of 200 EMS treated colonies, only 21 were resistant to 2 deoxy D glucose after screening. It was also observed that when the time of exposure was increased then the number of resistant colonies decreased. Mutant derived strain was produced higher concentration of glucose oxidase (332.1%) as compared to parent strain. Whereas, only one colony did not produce glucose oxidase and twelve mutants produced enzyme in less amount as compared to parent strain. High dose of chemical mutagen was requisited for getting instant mutants.

It has been proved that ethidium bromide (EB) is a strong mutagen. EB treated mutants having the ability to hyperproduce many enzymes and chemicals (Witteveen *et al.*, 1990; Lomkatsi *et al*., 1990). For hyperproduction of urate oxidase, *B. subtilis* was treated with EB for 150 minutes, which was producing 70% killing / 30% survival (1.1×10^3 CFU/mL) as shown in figure 4.3. Witteveen *et al.*, (1990) isolated EB treated *A. niger* that yielded higher production of glucose oxidase, with survival ranged 23-77%. Lomkatsi *et al*., (1990) used *P. canescene* mutant for hyperproduction of glycosidase, with survival ranged between 35-65%.

4.2 Selection and evaluation of mutant

After mutagenesis, the suspension was diluted in serial in such a way that on nutrient medium 0.1 mL suspension was plated. The growth of colonies was restricted to 25 or less than this number.

4.2.1 Colony restriction

The selection of colonies can be observed clearly by using colony restrictor. Triton X-100 (0.1%) was used in basal medium and was found to be very effective as colony restrictor. All the further studies, for the selection of colonies, were based on this concentration of Triton X-100.

Belavin *et al*., (1988) studied that bacterial colonies were restricted to small size by using triton X-100 (0.1%). Khattab and Bazarra (2005) used Triton X-100 (0.1%) to the isolation of mutated *A. niger*. Zia *et al.,* (2010) also used Triton X-100 for the isolation of mutated *A. niger* as a colony restrictor, for enhanced the production of glucose oxidase. The results of present work are inaccordance with the above reported investigators. On the other hand, triton X-100 (0.01%) was used to restrict the size of colony, to facilitate the screening and isolation of mutated *Trichoderma reesei* for enhanced the production of cellulase by Gadgil *et al*., (1955). Brown *et al*., (1987), used ox gall (0.2%) as colony restrictor for the isolation of mutated *Penecillium pinophilum.* Kumar *et al*., (1995), used sodium tauroglycocholate (0.1%) as a colony restrictor for the growth of hyper-xylanolytic mutant of *F. oxysporum.* Zia *et al.,* (2010) also used Triton X-100 for the isolation of mutated *A. niger* as a colony restrictor for enhanced the production of glucose oxidase.

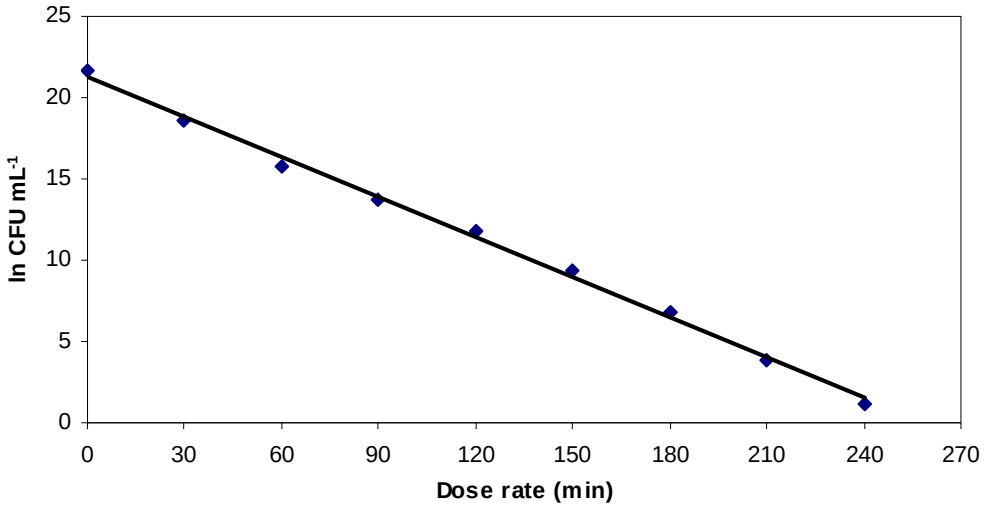


Fig. 4.1 kill/survival curve for UV treated *Bacillus subtilis*

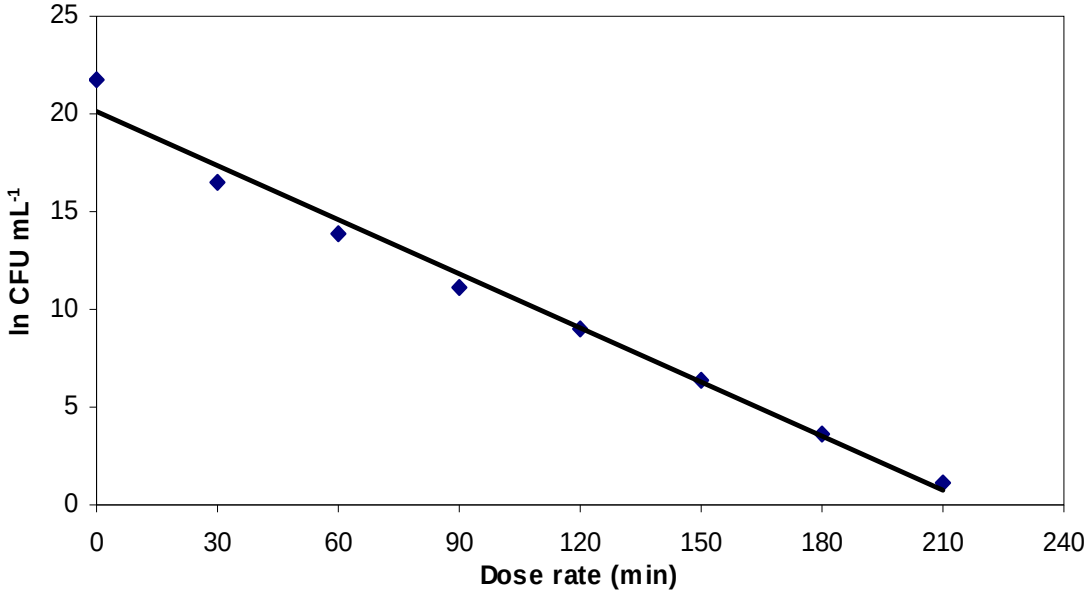


Fig. 4.2 kill/survival curve for EMS treated *Bacillus subtilis*

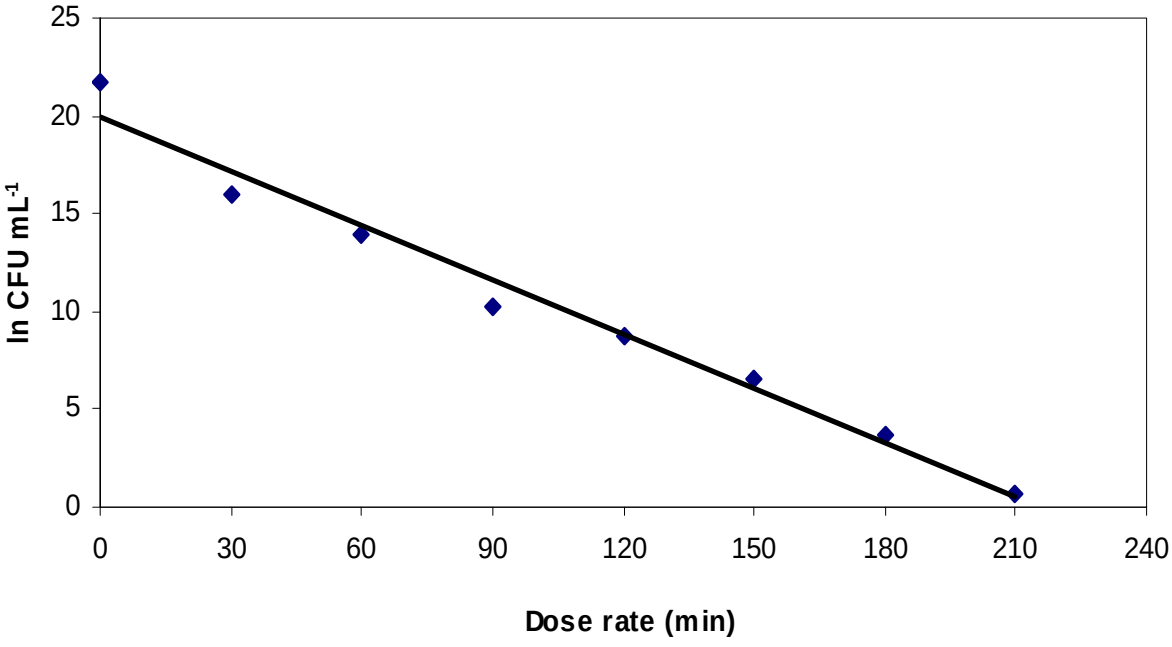


Fig. 4.3 kill/survival curve for EB treated *Bacillus subtilis*

4.2.2 Selection of specific mutants by using 2-thiouric acid

For the selection of mutated *B. subtilis,* several workers (Pybus *et al*., 2010; Maria *et al*., 2008) have adopted a number of schemes. The literature revealed that resistance to 2-thiouric acid (toxic urate analogue) has been commonly used to select the mutants (Tamta *et al*., 2007, Diallinas *et al*., 1995, Glatigny and Scazzocchio 1995). These mutants enhanced the production of urate oxidase. Selective isolation medium was prepared by the addition of 2-thiouric acid (50 µg/mL). Those mutants were isolated that showed resistant to 2- thiouric acid (Fig 4.4-4.6). These resistant colonies were subjected to enzyme diffusion zone analysis for the selection of best colony that produced maximum yield of urate oxidase. Philippides and Scazzocchio (1981) used 2-thiouric acid for the isolation of mutated *Aspergillus nidulans*. Darlington and Scazzocchio (1967) isolated *Aspergillus nidulans* mutants that were resistant to many purine analogues such as 2-thiouric acid, 2-thioxanthine and 8-azaguanine.

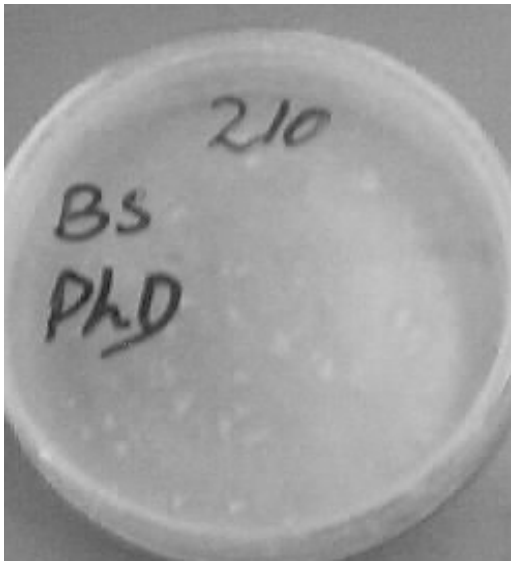


Fig 4.4 (a) Colonies of UV treated (210 min) *Bacillus subtilis* mutants on agar plate containing 2-thiouric acid as colony restrictor

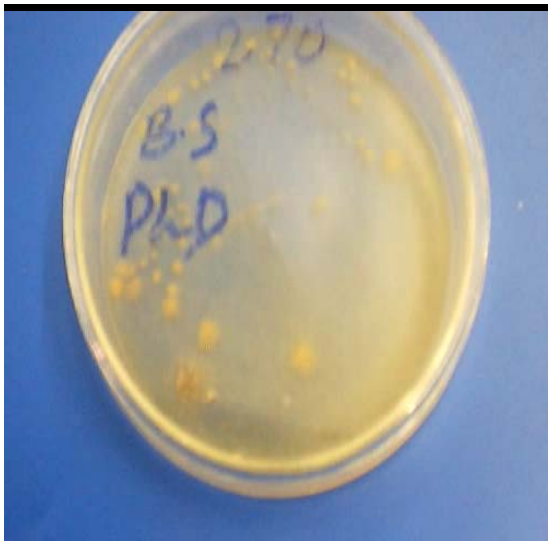


Fig 4.4 (b) Colonies of UV treated (210 min) *Bacillus subtilis* mutants on agar plate containing 2-thiouric acid as colony restrictor

Fig: 4.5 (a) Colonies of EMS treated (180 min) *Bacillus subtilis* mutants on agar plate containing 2-thiouric acid as colony restrictor

Fig: 4.5 (b) Colonies of EMS treated (180 min) *Bacillus subtilis* mutants on agar plate containing 2-thiouric acid as colony restrictor

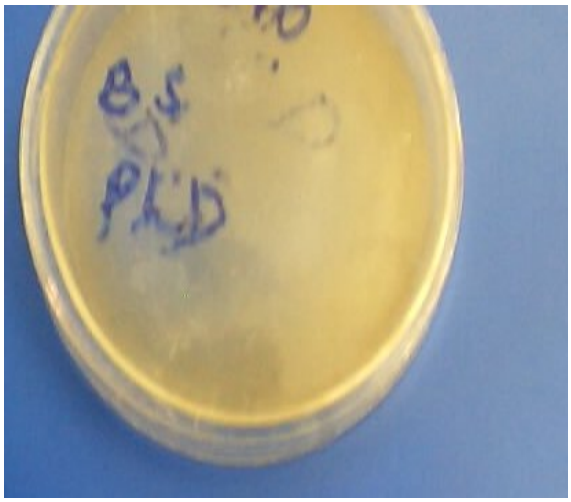


Fig: 4.6 (a) Colonies of EB treated (150 min) *Bacillus subtilis* mutants on agar plate containing 2-thiouric acid as colony restrictor

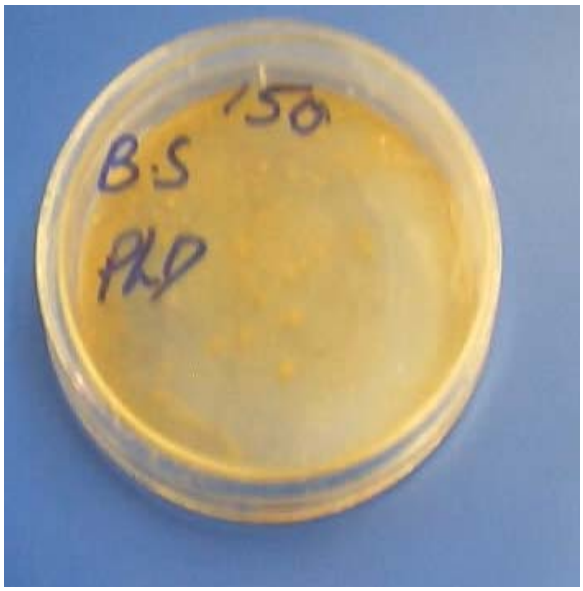


Fig: 4.6 (b) Colonies of EB treated (150 min) *Bacillus subtilis* mutants on agar plate containing 2-thiouric acid as colony restrictor

4.2.3 Enzyme diffusion zone

It is a specific procedure based on the enzymatic reaction on the agar plate, and was used for the screening and identifying the specific mutant. The agar plate was used to identify urate oxidase producing positive strains containing purified horseradish peroxidase (310 U/mg) and 100 mg o-dianisidine. The enzymatic reactions take place as under, giving rise a brown color.

$$\text{Uric acid} + O_2 \xrightarrow{\text{urate oxidase}} \text{Allantoin} + H_2O_2 + CO_2$$

$$\text{o-dianisidine}_{red} + H_2O_2 \xrightarrow{\text{Peroxidase}} \text{o-dianisidine}_{ox} + 2\ H_2O$$

The results exhibited that EMS treated mutant (BSM-2), obtained at 180 minutes dose, formed 16 mm diffusion zone as compared to wild type (2mm) with 763% increased in activity (Fig 4.7c).

Moreover, UV treated mutant (BSU-2) at 210 minutes produced 6 mm diffusion zone and ethidium bromide treated mutant (BSE-3) at150 minutes produced 7mm diffusion zone with 235% and 298% increased in activity respectively (Fig 4.7 and Table 4.1). Mutant induced by EMS (BSM-2), that obtained at 180 minutes dosage was used to enhance the production of urate oxidase in the fermentation processes. However, another test "zone analysis" was also done to get the selective colonies.

4.2.4 Analytical test

The strains that produced larger and darker zones (in section 4.2.3) were scratched and dissolved in the 0.1 M borate buffer (pH 8.5), filtered and homogenized it. Finally the activity of the urate oxidase was determined spectrophotometrically and arranged the results in table 4.1. Depending upon these results it was recommended that the potential mutant for the highest production of urate oxidase was EMS treated *Bacillus subtilis* (BSM-2).

Fig. 4.7 (a) Selection of urate oxidase hyperproducing colonies by enzyme diffusion zone (control)

Fig. 4.7 (b) Selection of urate oxidase hyperproducing mutants by enzyme diffusion zone (BSU-2)

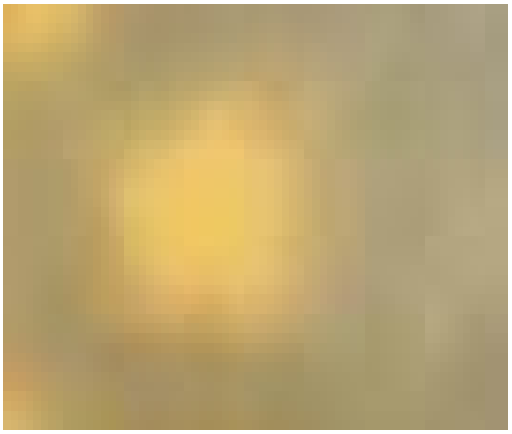


Fig. 4.7 (c) Selection of urate oxidase hyperproducing mutants by enzyme diffusion zone (BSM-2)

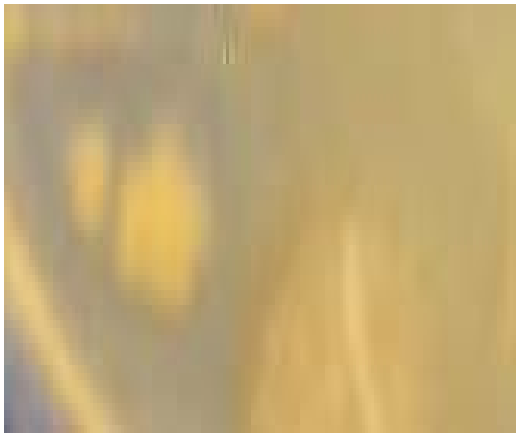


Fig. 4.7 (d) Selection of urate oxidase hyperproducing mutants by enzyme diffusion zone (BSM-3)

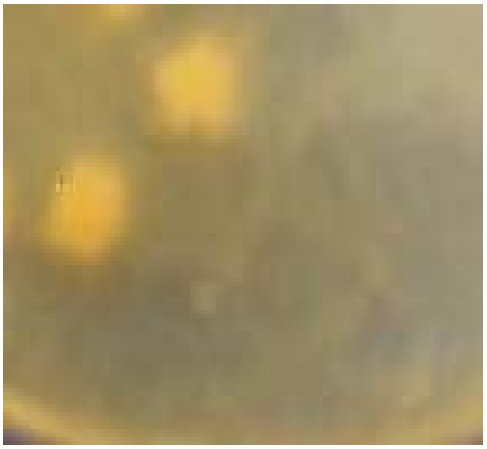


Fig. 4.7 (e) Selection of urate oxidase hyperproducing mutants by enzyme diffusion zone (BSE-3)

Table: 4.1 Activity of urate oxidase by various mutants for analytical test

B. subtilis mutant strain	Zone size (mm)	Activity U/mL	Activity (U/mm of colony diameter)
Wild type/control	2	2.33	1.16
BSU-1 (UV-210 min)	3	3.68	1.22
BSU- 2	6	5.40	0.9
BSU-3	3	3.97	1.32
BSU-4	2	2.78	1.39
BSU-5	4	4.34	1.08
BSM-1 (EMS-180 min)	13	12.88	0.99
BSM-2	16	19.54	1.22
BSM-3	12	10.41	0.867
BSM-4	10	7.98	0.79
BSM-5	11	9.24	0.84
BSE-1 (EB: 150 min)	4	4.64	1.16
BSE-2	6	5.77	0.96
BSE-3	7	6.85	0.97
BSE-4	6	5.91	0.98
BSE-5	4	3.38	0.84

BSU = UV treated *Bacillus subtilis* at 210 min dose rate
BSM = EMS treated *Bacillus subtilis* at 180 min dose rate
BSE = EB treated *Bacillus subtilis* at 150 min dose rate

4.3 Optimization of fermentation parameters by *Bacillus subtilis*

The genotype of the mutant strain may be changed as compared to its wild type strain. So, it is necessary to optimize the culture conditions that was exhibited the real potential of the mutant and wild strains. The effect of the medium composition on the activity of the wild as well as mutant derived urate oxidase was determined by performing a series of preliminary experiments in a sequential order.

4.3.1 Effect of substrate

To select a suitable concentration of substrate for the optimal microbial growth and hyperproduction of enzyme is a critical factor. In the present study, uric acid was used as a

substrate for the production of urate oxidase by mutated as well as wild type strains. Different concentrations of uric acid (substrate) were tested for optimum yield of the urate oxidase. Optimum activity of the urate oxidase by the mutated and wild type strain was achieved when the fermentation medium containing 0.5% substrate at 30 ^{o}C, inoculated with 5 % spore suspension after fermentation period of 48 hours. The results exhibited that optimum production of enzyme activity was obtained by mutated ***B. subtilis*** as compared to wild type. The activity of mutant and wild derived urate oxidase was obtained 20.87 ± 0.06 U/mL and 3.33 ± 0.19 U/mL respectively. The results showed that, the activity of mutant derived urate oxidase was increased seven folds as compared to the wild strain (Fig 4.8).

Green and Ronald (1955) studied the effect of uric acid on the growth of *Neurospora crassa* and on the production of urate oxidase. Fattah and Hamad (2002) revealed that uric acid as a substrate was the best nitrogen source for production of urate oxidase. It was also observed that optimized fermentation medium (uric acid medium) contained 0.1% uric acid, enhanced the production of urate oxidase. Peter *et al.,* (2002) examined that the activity of the urate oxidase was increased by using uric acid as a substrate. Saeed *et al*., (2004b) isolated and purified urate oxidase from *Pseudomonas aeruginosa* and revealed that when uric acid was used as a substrate, the specific activity of the purified enzyme was found to be 636.36 U/mg. Here, the difference in specific activity may be due to the use of different strains. Xue *et al*., (2005) isolated urate oxidase producing bacterium from soil with a medium containing uric acid and revealed that uric acid acts as an inducer for urate oxidase production. Yazdi *et al*., (2006) studied that *Mucor hiemalis* produced urate oxidase in the medium that contained uric acid as a substrate. Lotfy (2008) optimized the concentration of substrate and observed that the growth medium containing 0.7% uric acid was found to be the best for optimum yield of urate oxidase. The results of present work are correlated to several investigators that used uric acid as a substrate for hyperproduction of urate oxidase.

The data was subjected to statistical analysis by using CRD. The results exhibited a significant difference ($P \leq 0.01$) in the production of parent and mutant derived urate oxidase at varying concentration of substrate. Comparison the means of parent-derived enzyme by DMR test revealed a significant difference among all treatment except at the level of 0.4% and 0.6% of uric acid while mutant derived urate oxidase (BSM-2) exhibited a significant difference among all the treatments as shown in table 4.2 and 4.3.

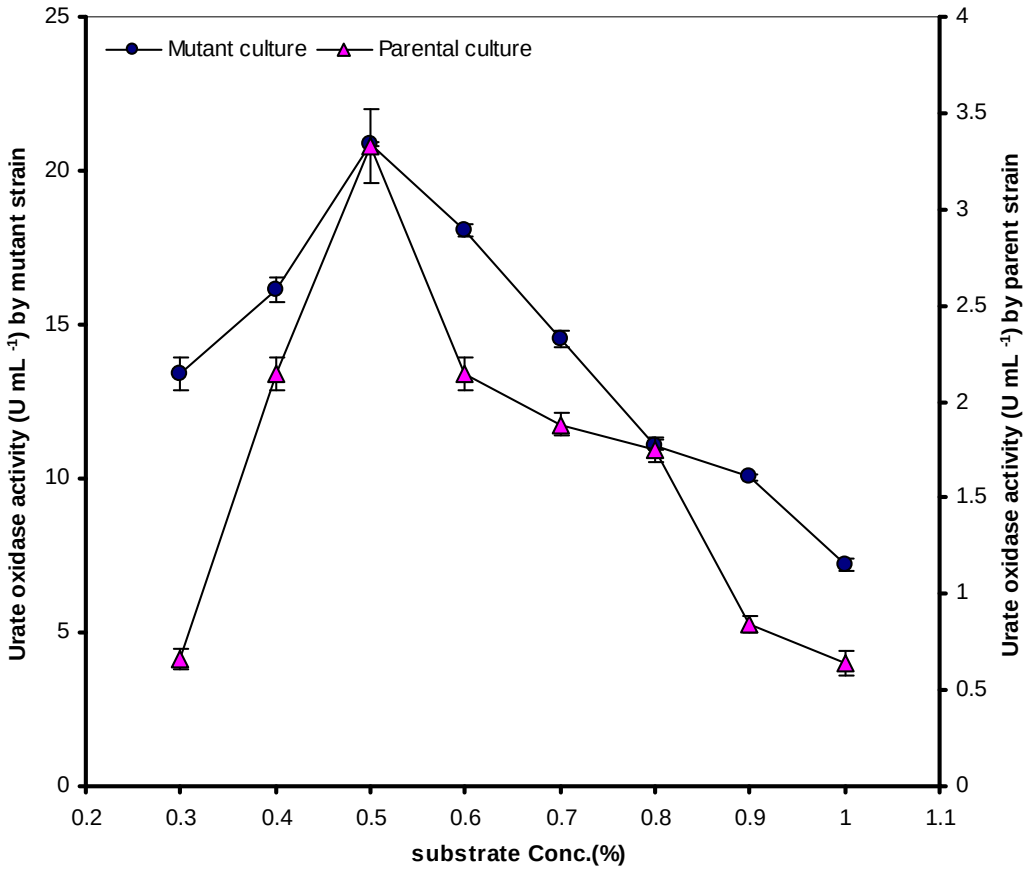


Fig. 4.8 Effect of substrate concentrations on the production of urate oxidase by parent and mutant culture

Table: 4.2 Analysis of variance (ANOVA) for urate oxidase production by mutant and parental Bacillus subtilis with varying substrate levels

Source of variation	Degrees of freedom	Mean squares	
		Mutant	Parental
Treatment	7	59.4532**	2.5769**
Error	16	0.2382	0.0251

(P<0.01)

Table: 4.3 Production of urate oxidase by parent and mutant *Bacillus subtilis* at various substrate level

Substrate concentration (%)	Enzyme activity U/mL (M)	Enzyme activity U/mL (P)
0.3	13.40±0.51 E	0.66±0.05 D
0.4	16.14±0.42 C	2.14±0.08 B
0.5	20.87±0.06 A	3.33±0.19 A
0.6	18.07±0.19 B	2.14±0.08 B
0.7	14.54±0.28 D	1.88±0.06 BC
0.8	11.10±0.17 F	1.75±0.06 C
0.9	10.05±0.11 G	0.84±0.05 D
1.0	7.23±0.20 H	0.64±0.06 D

Means sharing similar letter in a column are statistically non-significant (P>0.05).

4.3.2 Effect of fermentation period

The results showed that the liquid state fermentation with 0.5 % uric acid (optimum substrate level) produced maximum yield of urate oxidase i.e 4.16 ± 0.11 U/mL by parent strain after 36 hours. The mutant strain under same conditions showed 26.72 ± 0.26 U/mL activity of the urate oxidase. All the details of the results are shown in figure 4.9.

Xue *et al*., (2005) isolated urate oxidase producing bacterium from soil that yields highest activity of enzyme when the strain was incubated for 36 hour. Lotfy (2008) identified that urate oxidase activity was peaked when *Bacillus thermocatenulatus* was cultured for 30-36 hours. Trakya *et al.,* (2000) optimized the fermentation medium for the production of urate oxidase by *Aspergillus niger* and obtained highest yield after 72 hours. Fattah and Hamad (2002) studied that the time required for maximum production of urate oxidase was 96 hours for *Aspergillus terreus* and *Aspergillus flavus* while 144 hours for *Trichoderma* sp. These values that reported earlier are different to my results that may be due to different conditions or different organisms, etc.

According to the statistical analysis of the data by ANOVA under CRD, a significant difference ($P \leq 0.01$) was found in the production of urate oxidase at varying level of fermentation period. Comparison of the means of parent derived urate oxidase by DMR test showed a significant difference among all treatments except at 24 and 48 hours fermentation period which sharing the same letters. While, EMS treated *B. subtilis* derived urate oxidase exhibited the significant difference among all the treatments (Table 4.4, 4.5).

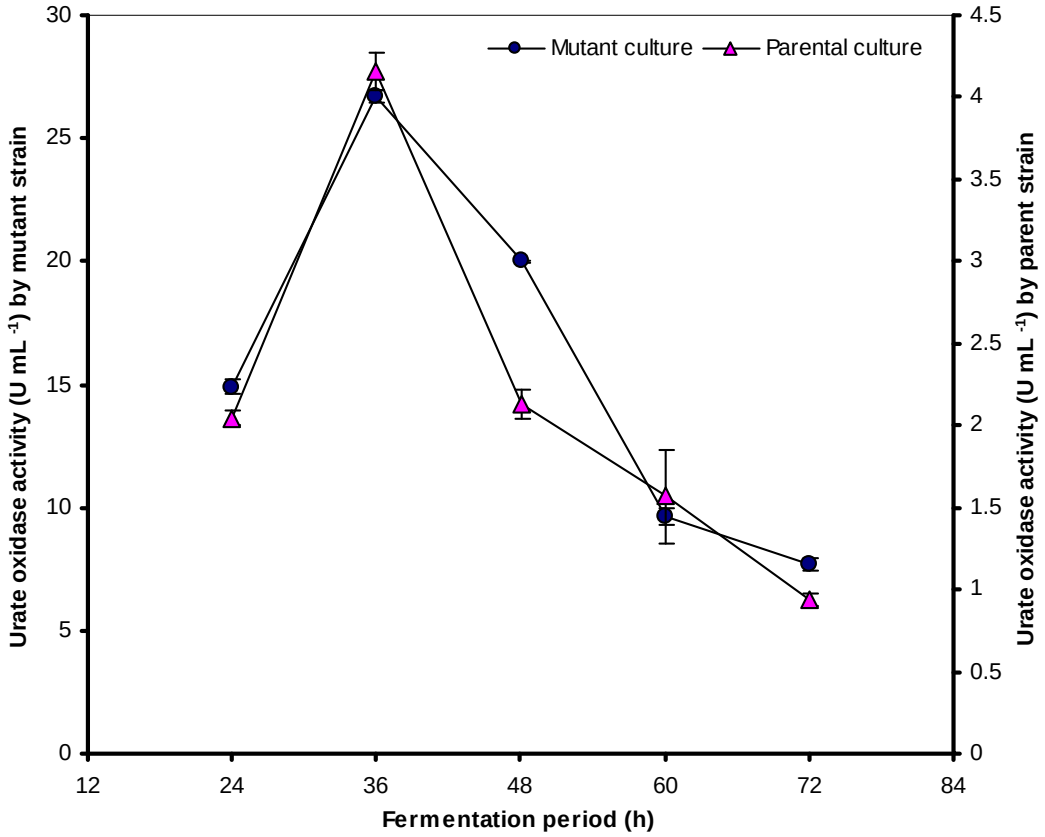


Fig. 4.9 Effect of fermentation periods on the production of urate oxidase by parent and mutant culture

Table: 4.4 Analysis of variance (ANOVA) for effect of fermentation period on production of urate oxidase by mutant and parental cultures.

Source of variation	Degrees of freedom	Mean squares	
		Mutant	Parental
Treatment	4	181.046**	4.3810**
Error	10	0.214	0.0646

(P<0.01)

Table: 4.5 Production of urate oxidase by parent and mutant *Bacillus subtilis* at various fermentation periods

Fermentation Period (h)	Enzyme activity U/mL (M)	Enzyme activity U/mL (P)
24	14.90±0.31 C	2.04±0.05 B
36	26.72±0.26 A	4.16±0.11 A
48	20.02±0.05 B	2.13±0.09 B
60	9.63±0.35 D	1.57±0.29 C
72	7.71±0.26 E	0.94±0.04 D

Means sharing similar letter in a column are statistically non-significant (P>0.05).

4.3.3 Effect of pH

The pH of medium was optimized to achieve the highest yield of urate oxidase after screening the suitable substrate concentration (0.5%) and fermentation period (36 h). The parental and mutant strains were achieved maximum production of urate oxidase 7.38 ± 0.25 U/mL and 31.87 ± 0.12 U/mL respectively at pH 8.5.

Optimum pH plays an important role in the growth of microbes and their metabolic activities. The metabolic activities of microbes are very sensitive to change in pH. The optimum pH for the production of urate oxidase by *B. subtilis* was found to be 8.5 as shown in figure 4.10.

The results proved a good coincidence with the values that reported by various research workers. Alamillo *et al*., (1991) and Huang *et al*., (2004) revealed that urate oxidase from *Chlamydomonas reinhardtii* and *Bacillus subtilis* showed highest activity at pH 8.5. Moreover, Kai *et al*., (2008) and Liu *et al*., (1994) also observed that highest activity of urate oxidase was obtained at pH 8.5. Saeed *et al*., (2004b) observed that urate oxidase from *Pseudomonas aeruginosa* exhibited the peak activity at pH 9. Yasser *et al*., (2005) studied that maximum activity of the urate oxidase was obtained when *Pseudomonas aeruginosa* was cultured at pH 5.5. Chen *et al*., (2008) studied that the production of recombinant urate oxidase was considerably enhanced when *Candida utilis* was cultured at pH ranging from 5.5-6.5. Lotfy, (2008) studied that optimum activity of urate oxidase was obtained when *Bacillus thermocatenulatus* was cultured at pH 7.0. Here the difference in finding may be due to different conditions and different organisms etc.

These findings were also supported by statistical analysis. According to ANOVA by using CRD a significant difference (P≤0.01) was found in the production of urate oxidase at varying levels of pH. Comparison of means of parent derived urate oxidase by DMR test was

exhibited a significant difference while mutant derived urate oxidase (BSM-2) showed a significant difference among all the treatments except the means sharing similar letter e.g at pH 6 and 10 (Table 4.6 and 4.7).

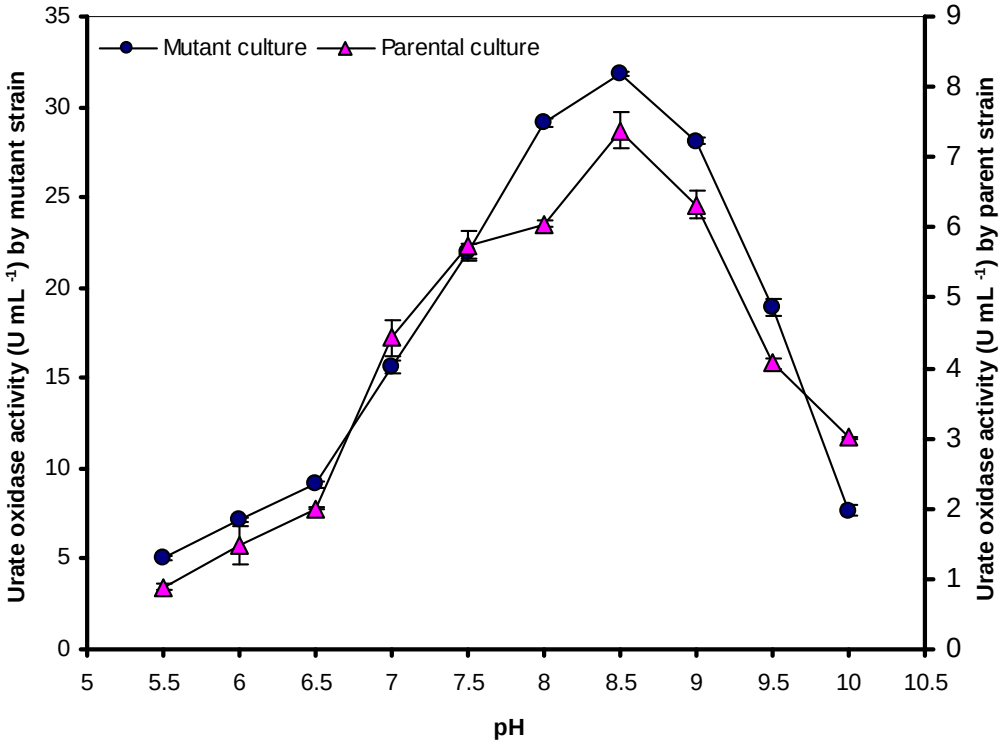


Fig. 4.10 Effect of pH on the production of urate oxidase by parent and mutant culture

Table: 4.6 Analysis of variance (ANOVA) for effect of pH on production of urate oxidase by mutant and parental cultures.

Source of variation	Degrees of freedom	Mean squares	
		Mutant	Parental
Treatment	9	302.768**	15.0437**
Error	20	0.240	0.0886

(P<0.01)

Table 4.7 Production of urate oxidase by parent and mutant *Bacillus subtilis* at various pH

pH	Enzyme activity U/mL (M)	Enzyme activity U/mL (P)
5.5	5.09±0.11 I	0.88±0.04 H
6.0	7.12±0.10 H	1.47±0.27 G
6.5	9.10±0.23 G	2.00±0.02 F
7.0	15.66±0.34 F	4.43±0.25 D
7.5	22.02±0.46 D	5.74±0.22 C
8.0	29.11±0.24 B	6.05±0.05 BC
8.5	31.87±0.12 A	7.38±0.25 A
9.0	28.12±0.14 C	6.32±0.20 B
9.5	18.92±0.46 E	4.07±0.08 D
10.0	7.69±0.31 H	3.01±0.03 E

Means sharing similar letter in a column are statistically non-significant (P>0.05).

4.3.4 Effect of temperature

When the fermentation medium was incubated at 35 ^{o}C for 36 hours at pH 8.5, then urate oxidase attained maximum activity 9.53 ± 0.28 U/mL and 38.18 ± 0.51 U/mL from wild and mutant strain respectively (Fig. 4.11).

Alamillo *et al*., (1991) isolated urate oxidase from *Chlamydomonas reinhardtii* which produced maximum yield 40 ^{o}C. Liu *et al*., (1994) revealed that optimum urate oxidase activity was obtained when the *Candida* sp. was cultured at 30^{o}C. Trakya *et al.,* (2000) studied that urate oxidase activity was peaked when *Aspergillus niger* was subjected at 30^{o}C. Fattah and Hamad (2002) revealed that optimum production of urate oxidase by *Aspergillus terreu, Aspergillus flavus* and *Trichoderma* sp. obtained when the culture was worked at 30^{o}C temperature. Yazdi *et al*., (2006) optimized the medium and obtained the maximum yield of urate oxidase when the medium was autoclaved at 30 ^{o}C. Lotfy (2008) observed that maximum urate oxidase activity was obtained when *Bacillus thermocatenulatus* was cultured at 30^{o}C. Our findings differ from the values that reported earlier that may be due to change in environmental conditions and different organisms etc.

The results were subjected to statistical analysis through CRD that exhibited a significant difference (P≤0.01) in the production of urate oxidase from parent and EMS treated strain at different levels of temperature. Comparison of means of parent derived urate oxidase by DMR test showed a significant difference (P≤0.05) among all treatment of parent except at 30 and 40 ^{o}C. While, mutant derived enzyme (EMS-2) showed a significant

difference among all the treatments and the detailed results are arranged in Tables 4.8 and Table 4.9.

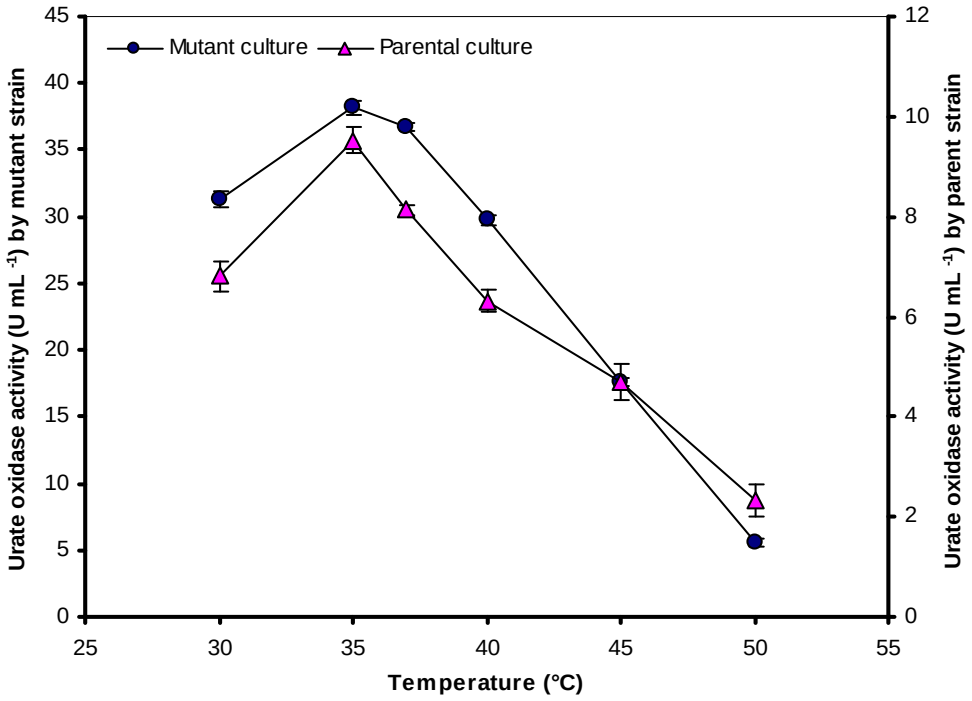


Fig. 4.11 Effect of temperature on the production of urate oxidase by Parent and mutant culture

Table: 4.8 Analysis of variance (ANOVA) for effect of temperature on production of urate oxidase by mutant and parental cultures.

Source of variation	Degrees of freedom	Mean squares	
		Mutant	Parental
Treatment	5	474.567**	19.462**
Error	12	0.484	0.231

(P<0.01)

Table: 4.9 Production of urate oxidase by parent and mutant *Bacillus subtilis* at various temperature

Temperature (°C)	Enzyme activity U/mL (M)	Enzyme activity U/mL (P)
30	31.31±0.58 B	6.80±0.30 B
35	38.18±0.51 A	9.53±0.28 A
40	29.73±0.32 C	6.32±0.23 B
45	17.62±0.29 D	4.70±0.36 C
50	5.56±0.31 E	2.32±0.32 D

Means sharing similar letter in a column are statistically non-significant (P>0.05).

4.3.5 Effect of Inoculum Size

Different levels of inoculum were tested to increase the growth of *B.subtilis* for hyperproduction of urate oxidase. It was observed that when 3% inoculum was used then maximum yield of parent and mutant derived urate oxidase was obtained. Parent and mutant strains exhibited 13.67±0.29 U/mL and 42.77±0.36 U/mL activity of enzyme respectively. It was observed that when the concentration of the inoculum was increased the production of the enzyme was also increased till 3 mL of the spore suspension. Beyond 3 mL, when the concentration of sporulation was increased it reduced the biosynthesis of the enzyme, as shown in figure 4.12. Chen *et al*., (2008) optimized the parameters in the shaking flask culture and revealed that inoculum size had great influence on the production of urate oxidase. The data was subjected to statistical analysis which supported the results that was obtained through ANOVA.

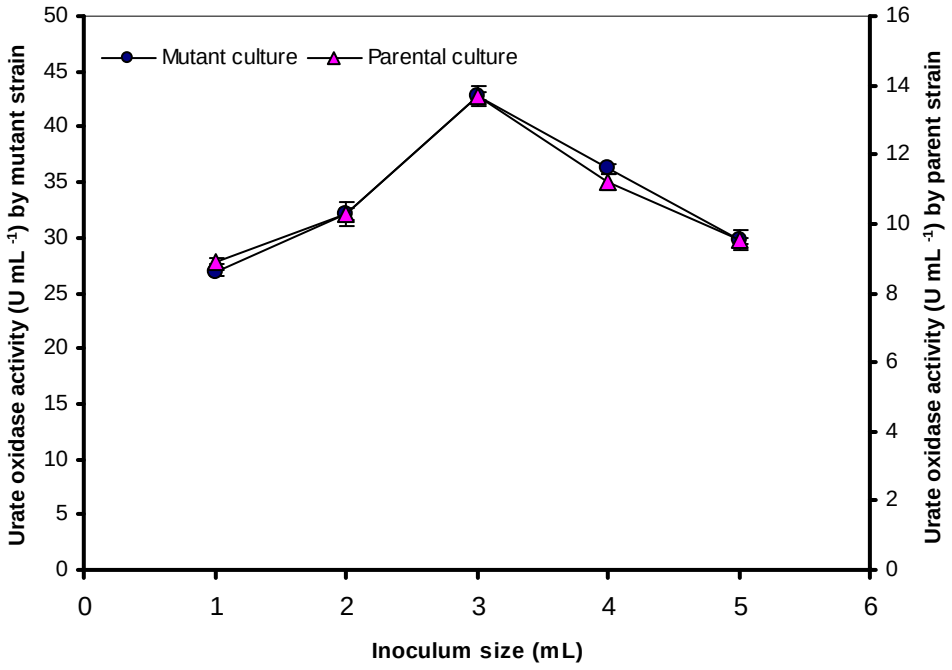


Fig. 4.12 Effect of inoculum size on the production of urate oxidase by parent and mutant culture

Table: 4.10 Analysis of variance (ANOVA) for effect of inoculum size on production of urate oxidase by mutant and parental cultures.

Source of variation	Degrees of freedom	Mean squares	
		Mutant	Parental
Treatment	4	114.572**	10.316**
Error	10	0.512	0.207

(P<0.01)

Table: 4.11 Production of urate oxidase by parent and mutant *Bacillus subtilis* at various inoculum size

Inoculum size (ml)	Enzyme activity U/mL (M)	Enzyme activity U/mL (P)
1	26.93±0.38 E	8.92±0.07 D
2	32.10±0.49 C	10.30±0.35 C
3	42.77±0.36 A	13.67±0.29 A
4	36.24±0.49 B	11.22±0.22 B
5	29.73±0.32 D	9.55±0.29 CD

Means sharing similar letter in a column are statistically non-significant (P>0.05).

4.3.6 Effect of nitrogen source and concentration

The effect of various nitrogen sources (peptone, yeast extract, ammonium chloride, and sodium nitrate) and its concentration (0.1-0.6%) were also checked for enhanced the biosynthesis of urate oxidase. The results are described below under the heading of each nitrogen source.

4.3.6.1 Effect of peptone

Different concentrations of peptone (0.1-0.6 %) were added in the production medium as an additional nitrogen source. The effect of peptone on the biosynthesis of wild and mutant derived urate oxidase was studied. It was observed that the production of urate oxidase was enhanced 14.06 ± 0.05 U/mL and 45.11 ± 0.52 U/mL from parent and mutant derived strains respectively by the addition of 0.4% peptone as shown in figure 4.13.

According to statistical analysis 0.4% peptone gave optimum production of urate oxidase. The data was subjected to ANOVA under CRD that exhibited a significant (P≤0.01) difference in the production of urate oxidase by varying concentration of peptone, which is used as nitrogen source and required for microorganism growth. The means of parent derived urate oxidase by DMR test were compared that revealed a significant difference among all treatments except at 0.3 and 0.5% concentration of peptone. Similarly, mutant derived

enzyme (BSM-2) was also exhibited a significant difference among all the treatments except at 0.3 and 0.5% concentration of peptone (Table 4.12 and Table 4.13).

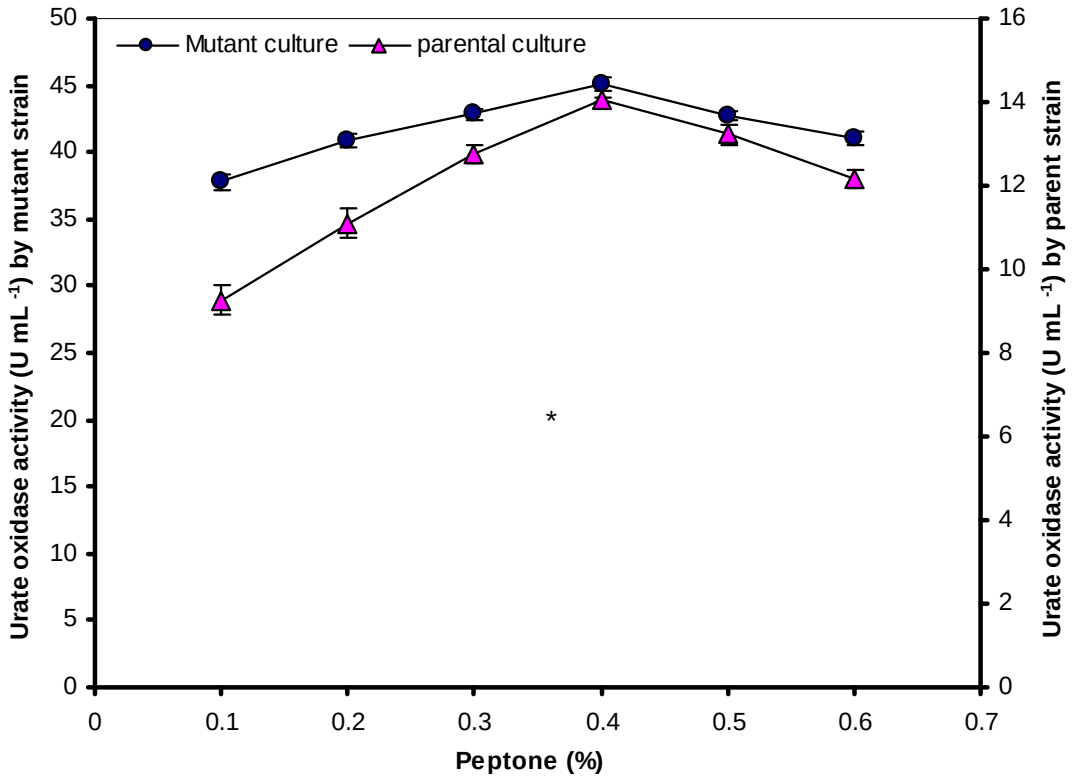


Fig. 4.13 Effect of peptone on the production of urate oxidase by parent and mutant culture

Table: 4.12 Analysis of variance (ANOVA) for effect of peptone on the production of urate oxidase.

Source of variation	Degrees of freedom	Mean squares	
		Wild	Mutant
Treatment	5	8.7486**	18.1747
Error	12	0.1996	0.7052

(P<0.01)

Table: 4.13 Production of urate oxidase by parent and mutant *Bacillus subtilis* at various peptone concentrations

Peptone (%)	Enzyme activity U/mL (P)	Enzyme activity U/mL (M)
0.1	9.27±0.33 E	37.82±0.61 D
0.2	11.10±0.35 D	40.86±0.46 C
0.3	12.75±0.24 BC	42.87±0.44 B
0.4	14.06±0.05 A	45.11±0.52 A
0.5	13.24±0.25 B	42.76±0.31 B
0.6	12.17±0.23 C	41.07±0.52 C

Means sharing similar letter in a column are statistically non-significant (P>0.05).

4.3.6.2 Effect of Yeast Extract

Six different levels (0.1-0.6%) of yeast extracts were tested in triplicate flasks at optimum pH and temperature. The most effective yeast extract level was screened for hyperproduction of parent and mutant derived urate oxidase. It was studied that the production of urate oxidase was enhanced by adding yeast extract in the fermentation medium. Yeast extract at the rate of 0.3% concentration exhibited the optimum yield of urate oxidase 15.97 ± 0.46 U/mL and 50.93 ± 0.41 U/mL correspondingly from parent and mutant derived strains (Fig. 4.14).

Statistical results are presented in table 4.14 and 4.15. According to statistical analysis (ANOVA) a significant difference (P≤0.01) was observed in the production of urate oxidase from parent and mutant derived strains at varying concentration of yeast extract. The means of parent derived urate oxidase by DMR test were compared that showed a significant difference among all treatments except at the level of 0.2 and 0.4% yeast extracts. Similarly, the means of mutant derived urate oxidase were compared and found significant difference among all the treatments except at the concentration of 0.2 and 0.4% yeast extract (Table 4.14 and Table 4.15).

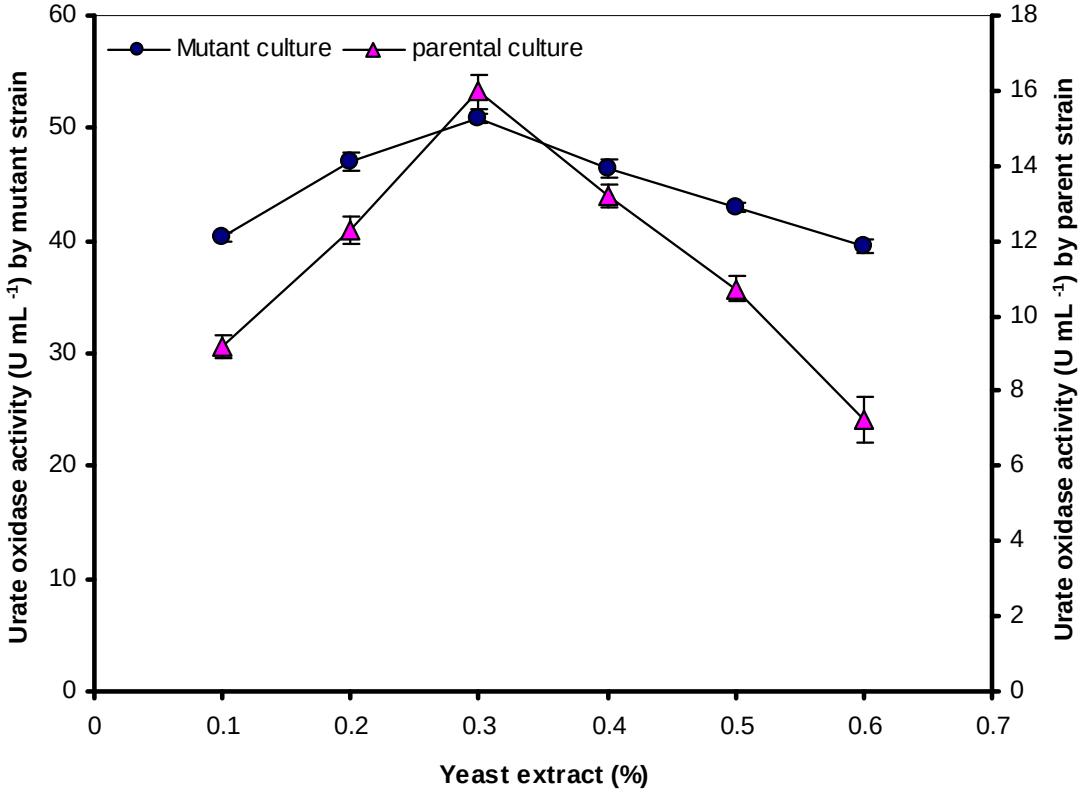


Fig. 4.14 Effect of yeast extract on the production of urate oxidase by parent and mutant culture

Table: 4.14 Analysis of variance (ANOVA) for effect of yeast extract on the production of urate oxidase.

Source of variation	Degrees of freedom	Mean squares	
		Wild	Mutant
Treatment	5	28.4969**	57.6700**
Error	12	0.5201	1.1346

(P<0.01)

Table: 4.15 Production of urate oxidase by parent and mutant *Bacillus subtilis* at various yeast extract levels

Yeast extract (%)	Enzyme activity U/mL (P)	Enzyme activity U/mL (M)
0.1	9.16±0.31 D	40.28±0.40 D
0.2	12.29±0.37 B	47.04±0.81 B
0.3	15.97±0.46 A	50.93±0.41 A
0.4	13.20±0.32 B	46.41±0.87 B
0.5	10.72±0.35 C	42.92±0.44 C
0.6	7.26±0.62 E	39.56±0.58 D

Means sharing similar letter in a column are statistically non-significant (P>0.05).

4.3.6.3 Effect of Ammonium Chloride

Different levels of ammonium chloride (0.1-0.6%) were added in the fermentation medium to determine its effect on the production of urate oxidase by wild and mutant *B. subtilis*. It was investigated that the production of urate oxidase was inhibited by adding ammonium chloride in the fermentation medium (Fig 4.15).

The data was subjected to statistical analysis and the detailed results are arranged in table 4.16 and 4.17).

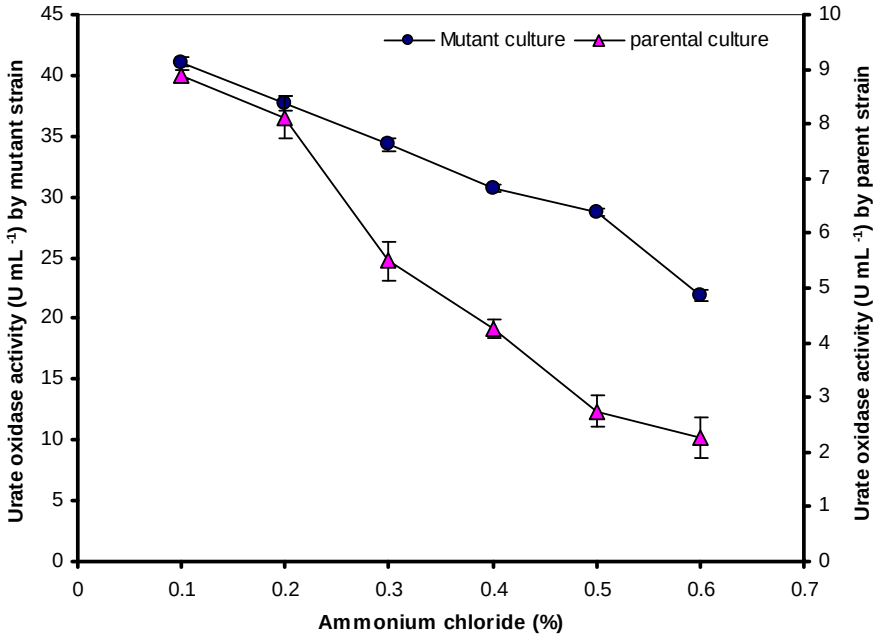


Fig. 4.15 Effect of ammonium chloride on the production of urate oxidase by parent and mutant culture

Table: 4.16 Analysis of variance (ANOVA) for effect of ammonium chloride for the production of urate oxidase.

Source of variation	Degrees of freedom	Mean squares	
		Wild	Mutant
Treatment	5	22.663**	139.758**
Error	12	0.262	0.607

(P<0.01)

Table: 4.17 Production of urate oxidase by parent and mutant *Bacillus subtilis* at various ammonium chloride concentration

Ammonium chloride (%)	Enzyme activity U/mL (P)	Enzyme activity U/mL (M)
0.1	8.89±0.08 A	41.11±0.36 A
0.2	8.12±0.38 A	37.65±0.60 B
0.3	5.49±0.35 B	34.29±0.59 C
0.4	4.25±0.17 C	30.78±0.30 D
0.5	2.75±0.30 D	28.71±0.29 E
0.6	2.26±0.37 D	21.92±0.45 F

Means sharing similar letter in a column are statistically non-significant (P>0.05).

4.3.6.4 Effect of Sodium Nitrate

Effect of sodium nitrate on the production of urate oxidase was studied by adding its different concentrations in the production medium. It was investigated that 0.5 % of sodium nitrate produced optimum yield of urate oxidase 12.90 ± 0.49 U/mL and 38.35 ± 0.77 U/mL from parent and mutant derived strains respectively. The details are shown in figure 4.16.

According to ANOVA with the help of CRD, the production of urate oxidase at different concentration of sodium nitrate, a significant difference (P≤0.01) was observed in all the treatments. Comparison the means of parent/wild and mutant derived enzyme by DMR test showed a significant difference among all treatments except 0.4 and 0.5% of sodium nitrate from mutant derived urate oxidase (BSM-2) (Table 4.18 and 4.19).

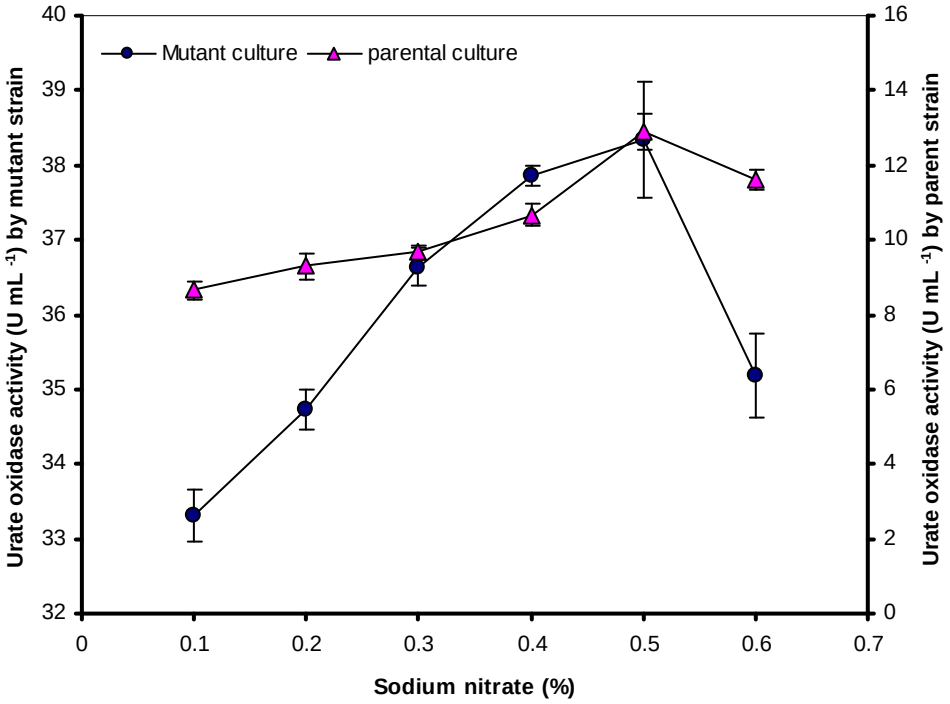


Fig. 4.16 Effect of sodium nitrate on the production of urate oxidase by parent and mutant culture

Table: 4.18 Analysis of variance (ANOVA) for effect of sodium nitrate on the production of urate oxidase.

Source of variation	Degrees of freedom	Mean squares	
		Wild	Mutant
Concentration	5	7.5284**	11.3477**
Error	12	0.3163	0.5998

(P<0.01)

Table: 4.19 Production of urate oxidase by parent and mutant *Bacillus subtilis* at various sodium nitrate levels

Concentration (%)	Enzyme activity U/mL (P)	Enzyme activity U/mL (M)
0.1	8.66±0.25 E	33.31±0.35 D
0.2	9.29±0.36 DE	34.72±0.27 C
0.3	9.68±0.19 CD	36.64±0.26 B
0.4	10.66±0.30 BC	37.86±0.13 AB
0.5	12.90±0.49 A	38.35±0.77 A
0.6	11.61±0.28 B	35.19±0.57 C

Means sharing similar letter in a column are statistically non-significant (P>0.05).

4.3.6.5 Comparison of different nitrogen sources

The effect of various nitrogen sources were observed to obtain maximum yield of urate oxidase. It was studied that yeast extract (0.3%) was the best nitrogen source for hyperproduction of parent and mutant derived urate oxidase. With the addition of 0.2% peptone and 0.5% sodium nitrate in the medium yield maximum production (Fig 4.17a and 4.17b).

It was also observed that the activity of urate oxidase was decreased when ammonium chloride was added in the medium considering as a nitrogen source. The findings of present study are in agreement with Peter *et al*., (2002) who determined that no activity of urate oxidase was detected from R. oryzae when ammonium chloride was added in the basal medium. Trakya *et al*., (2000) investigated that the activity of urate oxidase from *A. flavus* was inhibited when ammonium compound was added in the production medium. Devis *et al*., (1966) observed that the production of urate oxidase was enhanced by adding 2.0% yeast extract in the fermentation medium (yeast extract sucrose medium).

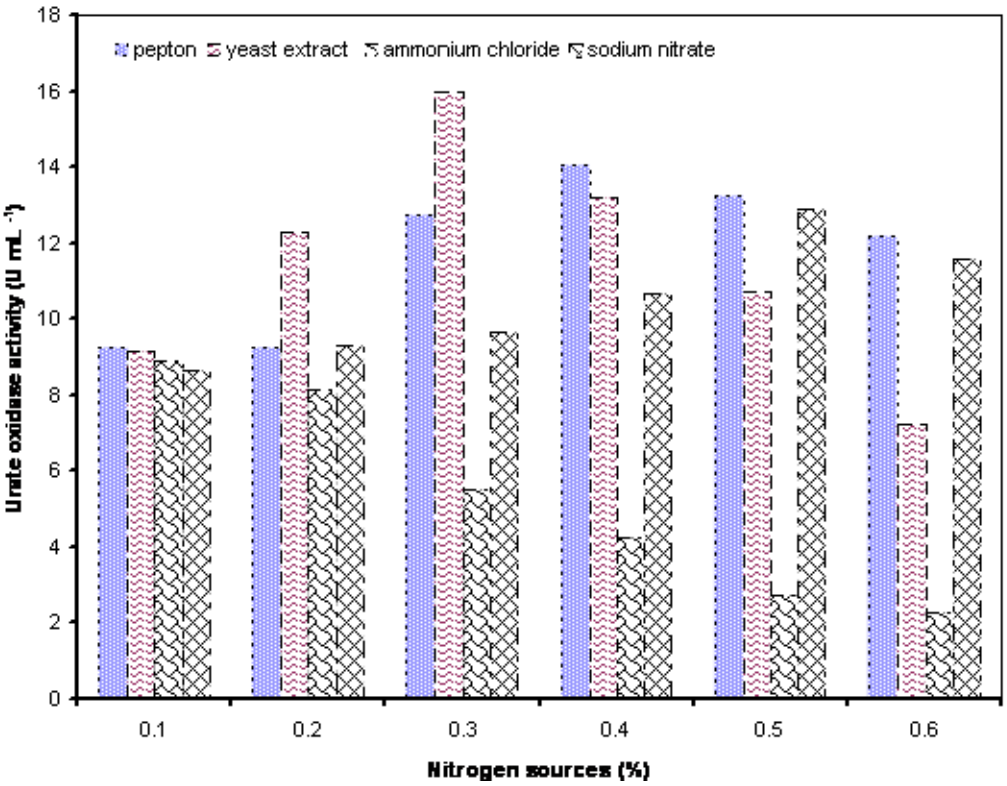


Fig 4.17a Comparison of the effect of different levels of nitrogen sources on the production of urate oxidase by parent culture

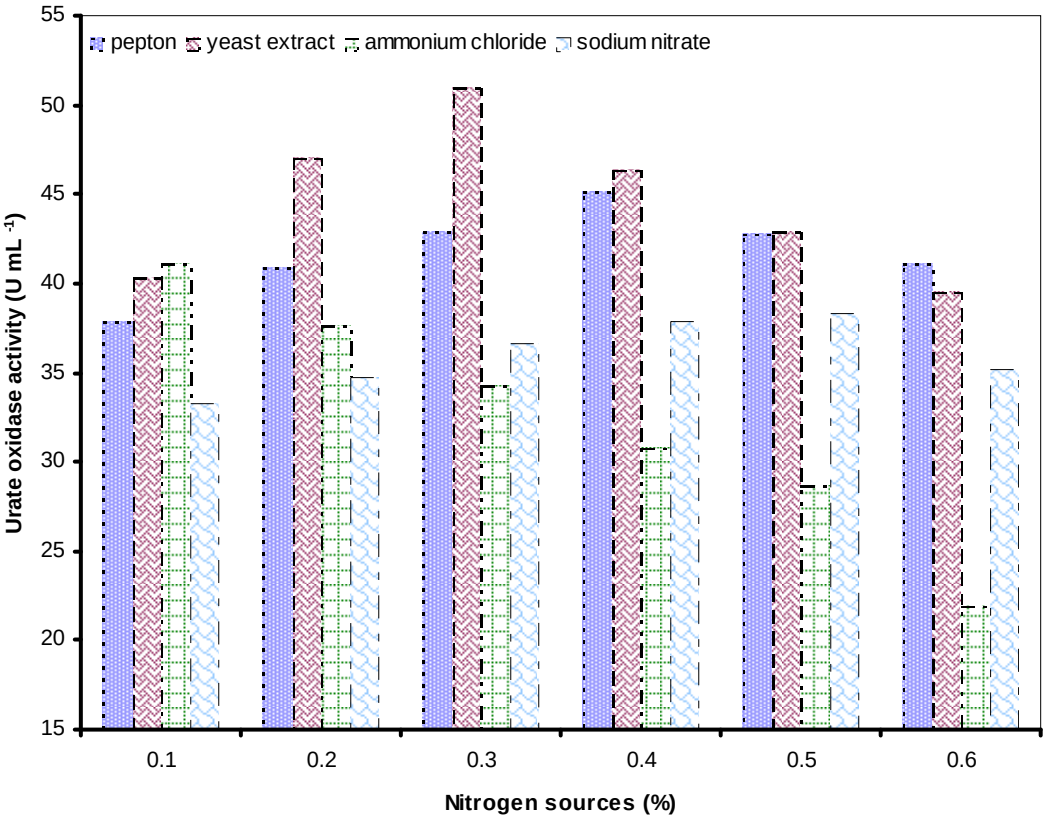


Fig 4.17b Comparison of the effect of different levels of nitrogen sources on the production of urate oxidase by mutant culture

4.3.7 Effect of carbon source

The effect of different carbon sources i.e maltose, sucrose, glucose and galactose with different concentrations were optimized for the production of urate oxidase in the fermentation medium.

4.3.7.1 Effect of maltose

Different levels of maltose were optimized for the hyperproduction of urate oxidase by wild and mutant strains in triplicate flasks. It was studied that the activity of urate oxidase was maximum 15.43 ± 0.38 U/mL and 49.00 ± 0.12 U/mL from parent and mutant derived strain respectively when 2.5 % maltose was added in the production medium (Fig 4.18). Yazdi *et al*., (2006) optimized the composition of fermentation medium and observed that the activity of urate oxidase was increased by using (6%) maltose. The findings of present work are differ from the above reported value due to difference in environmental conditions.

The data was obtained from above findings was subjected to statistical analysis; According to ANOVA, a significant difference ($P \leq 0.01$) was observed in the production of urate oxidase at varying concentration of maltose. Comparison the means of parent and mutant derived urate oxidase by DMR test showed a significant difference among all treatment (Table 4.20 and Table 4.21).

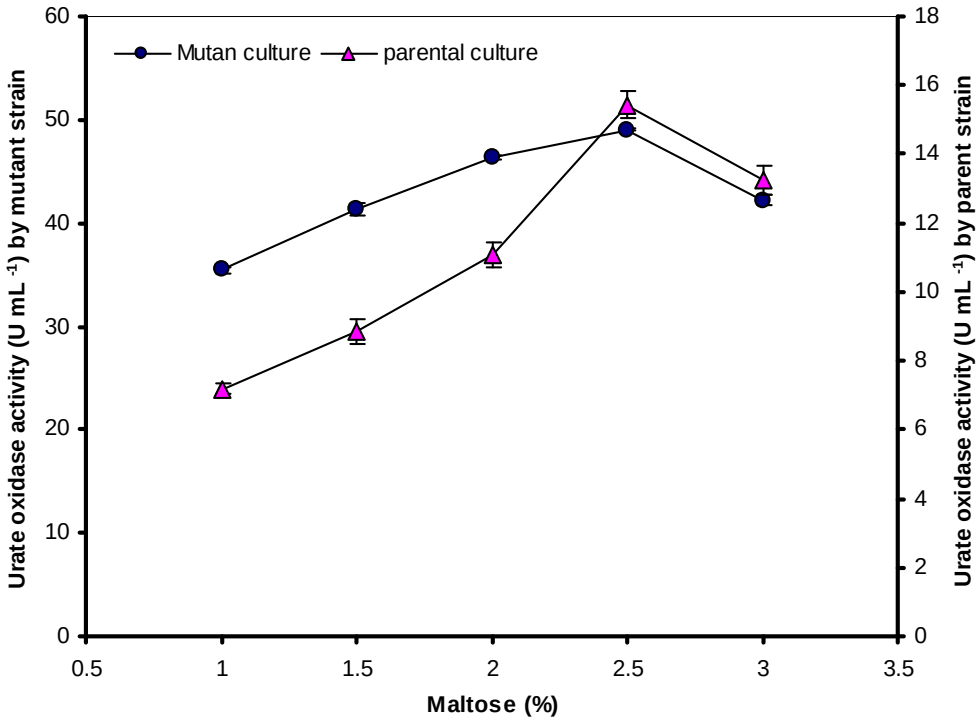


Fig. 4.18 Effect of maltose on the production of urate oxidase by parent and mutant culture

Table: 4.20 Analysis of variance (ANOVA) for effect of maltose on the production of urate oxidase.

Source of variation	Degrees of freedom	Mean squares	
		Mutant	Parental
Treatment	4	80.3405**	32.7287**
Error	10	0.4572	0.3573

(P<0.01)

Table: 4.21 Production of urate oxidase by parent and mutant *Bacillus subtilis* at various maltose levels

Carbon source (%)	Enzyme activity U/mL (P)	Enzyme activity U/mL (M)
1.0	7.19±0.14 E	35.47±0.29 D
1.5	8.87±0.36 D	41.32±0.57 C
2.0	11.09±0.36 C	46.33±0.23 B
2.5	15.43±0.38 A	49.00±0.12 A
3.0	13.25±0.42 B	42.23±0.54 C

Means sharing similar letter in a column are statistically non-significant (P>0.05).

4.3.7.2 Effect of sucrose

Effect of different levels of sucrose i.e 1-3 % was studied for the highest yield of urate oxidase. It was observed that 2 % sucrose provided optimum yield of urate oxidase. As the sucrose was added, the activity of parent and mutant derived urate oxidase was enhanced correspondingly upto 19.48±0.26 U/mL and 68.05±0.50 U/mL (Fig 4.19).

According to ANOVA with the help of CRD, a significant (P≤0.01) difference was observed in the production of urate oxidase by varying concentration of sucrose. The comparison of means (parent and mutant derived urate oxidase) by DMR test showed a significant difference among all treatments (Table 4.22 and Table 4.23).

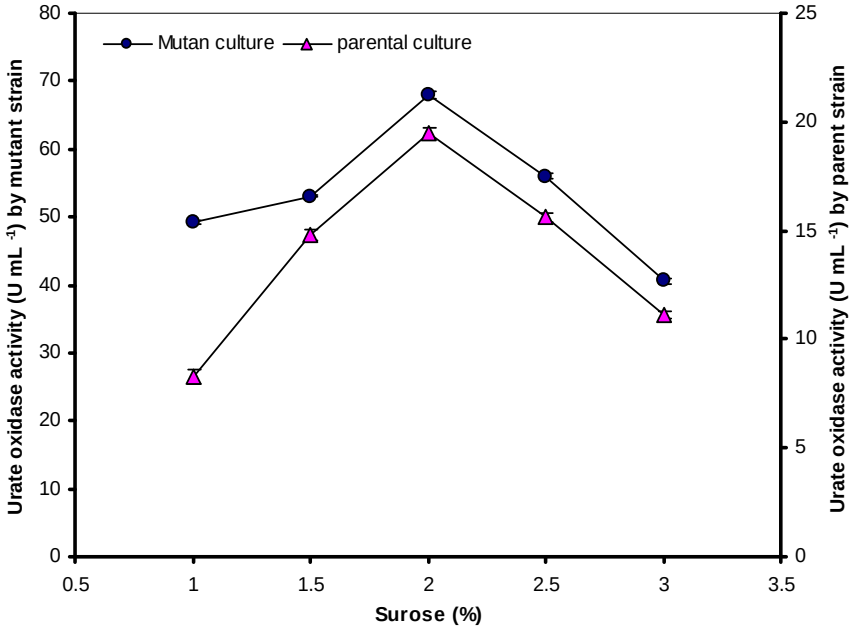


Fig. 4.19 Effect of sucrose on the production of urate oxidase by parent and mutant culture

Table: 4.22 Analysis of variance (ANOVA) for effect of sucrose on the production of urate oxidase.

Source of variation	Degrees of freedom	Mean squares	
		Mutant	Parental
Treatment	4	67.8263**	34.7403**
Error	10	0.3975	0.2571

(P<0.01)

Table: 4.23 Production of urate oxidase by parent and mutant *Bacillus subtilis* at various sucrose under optimum conditions

Carbon source (%)	Enzyme activity U/mL (P)	Enzyme activity U/mL (M)
1.0	8.28±0.33 E	49.19±0.29 D
1.5	14.77±0.25 C	53.08±0.12 C
2.0	19.48±0.26 A	68.05±0.50 A
2.5	15.61±0.22 B	55.97±0.36 B
3.0	11.12±0.13 D	40.59±0.44 E

Means sharing similar letter in a column are statistically non-significant (P>0.05).

4.3.7.3 Effect of glucose

Seven different levels of glucose (1-4%) were tested for the production of maximum yield of urate oxidase. It was determined that when the concentration of glucose (1-3%) was added in the production medium the activity of urate oxidase was enhanced. At the concentration of 3% glucose optimum yield of parent and mutant derived urate oxidase 15.24 ± 0.34 U/mL and 45.69 ± 0.30 U/mL respectively was prodced. Above this level of glucose, the activity of urate oxidase was decreased (Fig 4.20). The findings of present work are differed from Yasser *et al*., (2005) who studied that glucose supplementations to the fermentation medium inhibited the production of urate oxidase when purified from *Psudomonase aeuroginosa*. This difference may be due to the use of different organism.

Statistical analysis of data exhibited that glucose (3%) showed optimum production of urate oxidase. The results by ANOVA under CRD showed a significant difference (P≤0.01) in the production of urate oxidase by varying levels of glucose. Comparison of means (parent and mutant derived strains) by DMR test showed a significant difference among all treatment except at 2.5 and 3.5 glucose level of parent and mutant derived strains (Table 4.24 and Table 4.25).

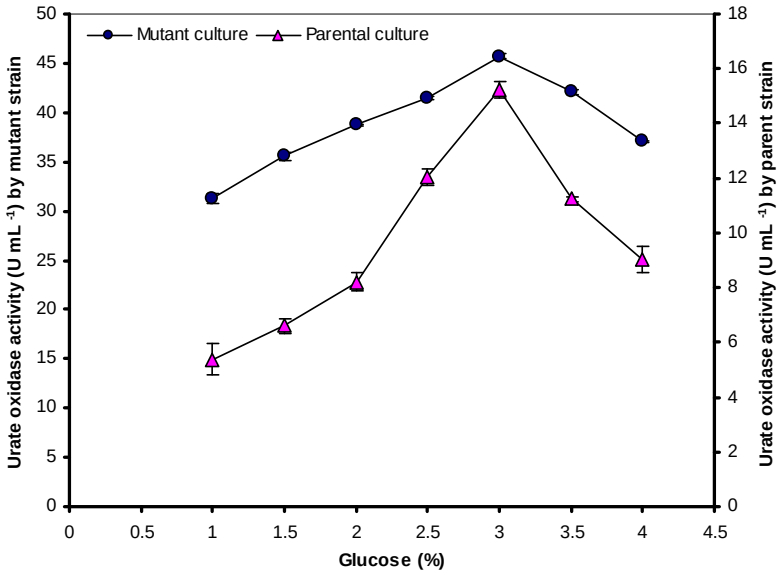


Fig. 4.20 Effect of glucose on the production of urate oxidase by parent and mutant culture

Table: 4.24 Analysis of variance (ANOVA) for effect of glucose on the production of urate oxidase.

Source of variation	Degrees of freedom	Mean squares	
		Mutant	Parental
Treatment	6	67.8263**	34.7403**
Error	14	0.3975	0.2571

(P<0.01)

Table: 4.25 Production of urate oxidase by parent and mutant *Bacillus subtilis* at various glucose levels

Carbon source	Enzyme activity U/mL (M)	Enzyme activity U/mL (P)
1.0	31.23±0.58 F	5.38±0.16 E
1.5	35.61±0.28 E	6.59±0.22 D
2.0	38.76±0.31 C	8.21±0.20 C
2.5	41.45±0.31 B	12.04±0.12 B
3.0	45.69±0.30 A	15.24±0.34 A
3.5	42.06±0.07 B	11.24±0.35 B
4.0	37.06±0.48 D	9.05±0.49 C

Means sharing similar letter in a column are statistically non-significant (P>0.05).

4.3.7.4 Effect of galactose

Different concentrations of galactose were optimized to obtain the maximum production of urate oxidase in preoptimized fermentation medium. It was determined that when galactose was added in the fermentation medium there was observed a slight increase in the production of urate oxidase. Addition of galactose (3%) was shown 42.83±0.32 U mL^{-1} and 11.11±0.46 U/mL activity of urate oxidase by mutant and parent strains respectively (Fig 4.21).

Statistical analysis of data by CRD, exhibited a significant difference ($P \leq 0.01$) in the production of urate oxidase by varying concentration of galactose. Comparison of the means of parent urate oxidase by DMR test showed a significant difference among all the treatments and mutant derived enzyme was also showed a significant difference among all the treatments except at 2.5 and 3.5% of glucose (Table 4.26 and 4.27).

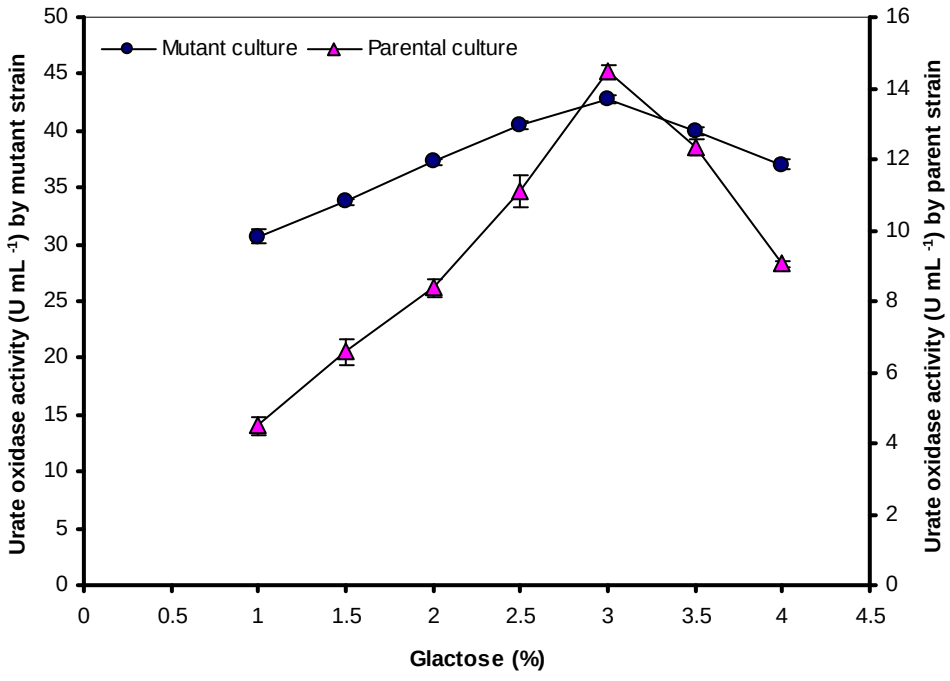


Fig. 4.21 Effect of glactose on the production of urate oxidase by parent and mutant culture

Table: 4.26 Analysis of variance (ANOVA) for effect of galactose on the production of urate oxidase.

Source of variation	Degrees of freedom	Mean squares	
		Mutant	Parental
Treatment	6	51.9270**	35.2009**
Error	14	0.4346	0.2436

(P<0.01)

Table: 4.27 Production of urate oxidase by parent and mutant *Bacillus subtilis* at various galactose

Carbon source	Enzyme activity U/mL (M)	Enzyme activity U/mL (P)
1.0	30.70±0.58 E	4.48±0.25 F
1.5	33.75±0.32 D	6.58±0.35 E
2.0	37.28±0.29 C	8.38±0.26 D
2.5	40.47±0.35 B	11.11±0.46 C
3.0	42.83±0.32 A	14.46±0.21 A
3.5	39.98±0.31 B	12.33±0.23 B
4.0	37.03±0.42 C	9.05±0.09 D

Means sharing similar letter in a column are statistically non-significant (P>0.05).

4.3.7.5 Comparison the effect of carbon sources

The effect of various carbon sources were compared and observed that sucrose (2%) was the best carbon source. It produced optimum yield (19.48±0.26 and 68.05±0.50 U/mL) of the urate oxidase by parent and mutant derived strain (Fig 4.22a and 4.22b). Devis *et al.*, (1966) optimized the fermentation medium (yeast extract sucrose medium) for the production of urate oxidase by adding 15% yeast extract in the medium. Huang and Wu (2004) dissolved 0.2 % sucrose in the czapek Dox medium for the optimum yield of urate oxidase. Fattah and Hamed (2002) optimized the fermentation medium (uric acid medium) with 2% sucrose was found to be best for hyperproduction so urate oxidase. Fattah and Hamad (2002) investigated that for the production of urate oxidase the best carbon sources were maltose, sucrose and glycerol by *Trichoderma* sp., *A. flavus*, and *A. terrus* respectively. Yazdi *et al*., (2006) determined that maltose was the best carbon source for the production of urate oxidase from *Mucor hiemalis*. Yasser *et al*., (2005) investigated that glucose supplementation in the fermentation medium inhibited the yield of urate oxidase by *P. aeruginosa*. Xue *et al*., (2005) studied that maize milk was the best carbon and nitrogen source for the production of urate oxidase from soil bacterium. These differences in the

results of present research and the values reported by above investigators may be due to the use of different microbes for the isolation of enzyme.

The nutritional requirements of mutant *Bacillus subtilis* were observed same as those of wild/parent strain. Zia *et al.*, (2010) optimized the conditions for the hyperproduction of glucose oxidase (parent and mutant) and found that nutritional requirements of mutated and parental *Aspergillus niger* were the same. The present research conclusions are similar to the above reported results. The detail of the results are shown in table 4.28.

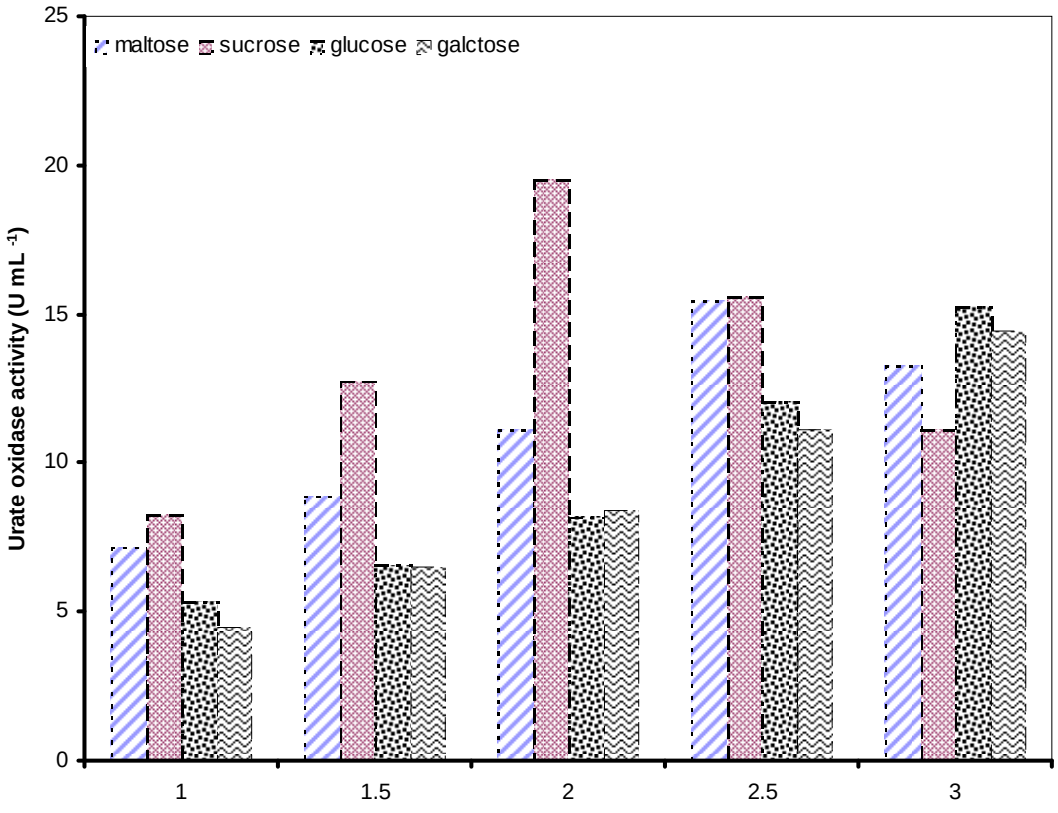


Fig. 4.22a Comparison the effect of different levels of carbon sources on the production of urate oxidase by parent culture

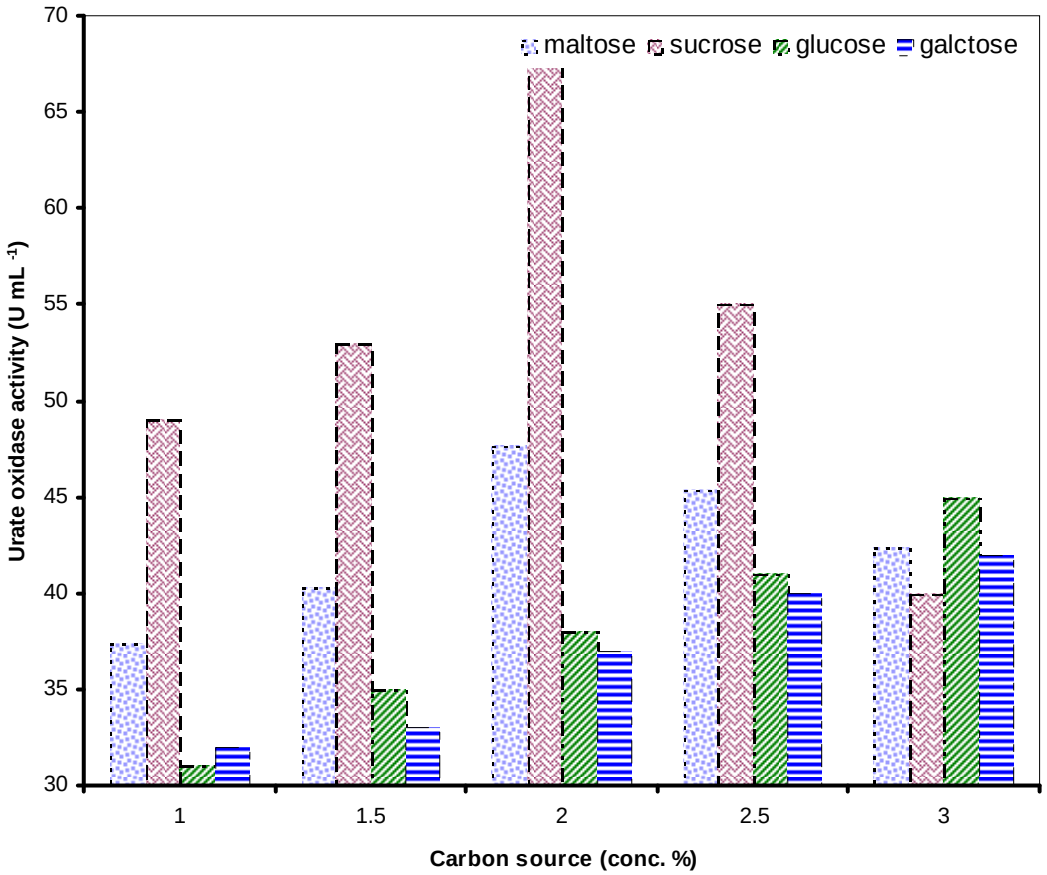


Fig. 4.22b Comparison the effect of different levels of carbon sources on the production of urate oxidase by mutant culture

Table 4.28 Summary of urate oxidase production and dry cell mass by parent (P) and EMS treated (M) strain

Parameters	Enzyme activity U/mL (P)	Dry cell mass (P) (g)	Enzyme activity U/mL (M)	Dry cell mass (M) (g)
Substrate (5%)	3.33 ± 0.19	0.053	20.87 ± 0.06	0.087
Fermentation period (36 h)	4.16 ± 0.11	0.086	26.72 ± 0.26	0.097
pH (8.5)	7.38 ± 0.25	0.091	31.87 ± 0.12	0.162
Temperature (35 ^{o}C)	9.53 ± 0.28	0.097	38.18 ± 0.51	0.197
Inoculum size (3%)	13.67 ± 0.29	0.26	42.77 ± 0.36	0.317
Yeast extract (0.3%)	15.97 ± 0.46	0.54	50.93 ± 0.41	0.871
Sucrose (2%)	19.48 ± 0.26	0.86	68.05 ± 0.50	1.023

4.4 Purification of urate oxidase

Different techniques were used for the purification of the urate oxidase which are discussed as under.

4.4.1 Purification with ammonium sulfate precipitation

The enzyme produced from parental as well as mutant strain was subjected to ammonium sulfate precipitation after optimized the culture conditions. This reagent is used most commonly because of its higher solubility for salting out the protein (Voet *et al*. 1999).

The supernatant of parental and mutant derived urate oxidase exhibited the activity 2.19 and 28.63 U/mL correspondingly, while it was 7.4 and 60.39 U/mL in sediment of wild and mutant derived enzyme correspondingly. After desalting process, wild and mutant derived enzyme was increased 11.98 and 89.83 U/mL respectively that showed the potential purification of urate oxidase (Fig. 4.23 and 4.24). Enzyme from parental and mutant derived strains attained 8.06 and 24.5 fold purification respectively. The detailed results are shown in table 4.29 and 4.30. Addition of ammonium sulfate in exact amount can selectively precipitate the desired protein, while other proteins remain soluble (Saeed *et al*., 2004; Liu *et al*., 1994). Desalting is a process to remove the contaminating salt which is present in sediments. As a result, the activity of the enzyme was enhanced (Zia *et al*., 2010).

Aguilar *et al*., (2002) subjected urate oxidase (isolated from *Puccinia recondita*) to ammonium sulfate precipitation and obtained 120% yield and found 0.028 U mg^{-1} specific activity with 9.5 folds purity. Farina and Faraone (1979) subjected ox kidney urate oxidase to 35 and 27% yield saturation and found 0.49 U mg^{-1} specific activity with 38 fold purification. Many other research workers have purified microbial urate oxidase (from *Microbacterium* sp., *Pseudomonas aeruginosa*, *Candida* sp., and *Chlamydomonas reinhardtii*) by ammonium sulfate precipitation (Alamillo *et al*., 1991; Liu *et al*., 1994; Saeed *et al*., 2004b; Kai *et al*., 2008).

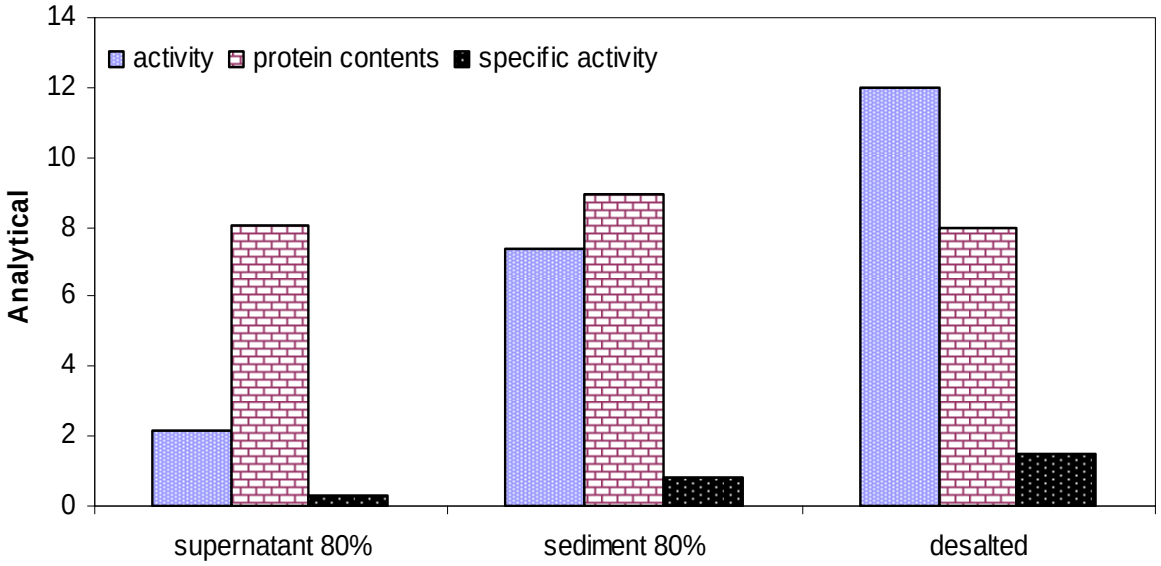


Fig. 4.23 Isolation of urate oxidase by ammonium sulfate precipitation from parent strain

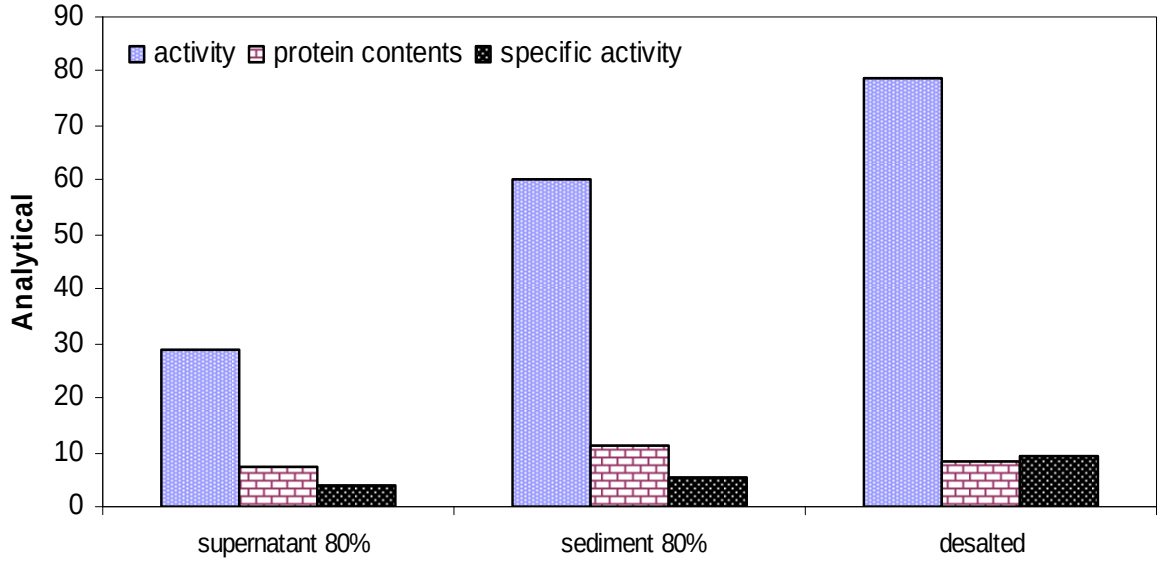


Fig. 4.24 Isolation of urate oxidase by ammonium sulfate precipitation from mutant strain

4.4.2 Ion exchange chromatography

DEAE-cellulose is most commonly used cellulosic anion exchanger. It preferred over a number of other anion exchangers i.e carboxyl methyl cellulose, DEAE-Sephadex etc, because of its higher density of micro granular cellulose and fine particle size. So it provides a high resolution by forming a more compact adsorbent bed (Jakoby, 1971).

For ion exchange chromatography, the dialyzed urate oxidase was applied to DEAE cellulose column. The adsorption of proteins to DEAE cellulose column (ion exchange resin) involves the formation of ionic bonds. These bonds are formed between charged groups which are present on the protein and opposite charged groups present on the adsorbent. The separation of proteins through chromatography depends upon the differential elution of the adsorbed protein. The proteins are eluted by different methods which based upon the change in pH or by using agents that have ability to compete with the adsorbed protein on the absorbent for the charged sites (Jakoby 1971).

It was observed that the 41 fraction of parent derived urate oxidase was obtained 2.05 Umg^{-1} specific activity with 39.67 folde4 purification (Fig 4.25). Whereas urate oxidase from mutated strain contained the maximum activity 46.42 U mL^{-1} and 23.21 U mg^{-1} specific activity with 2 mg mL^{-1} protein contents in 36^{th} fraction (Fig 4.26). Here the enzyme was 61.07 fold purified.

Farina and Faraone (1979) reported that after DEAE-Sephadex treatment, urate oxidase was 330 fold purified with 4.29 U mg^{-1} specific activity. Federico *et al*., (1987) reported that when urate oxidase was subjected to DEAE-cellulose column, result that the protein contents (2.5 mg mL^{-1}) were decreased with increasing specific activity (6.40 U mg^{-1}). So, the enzyme was purified 48 fold with 32% recovery. Aguilar *et al*., (2002) applied urate oxidase isolated from *Puccinia recondita* to DEAE-sephadex column. It was found that urate oxidase exhibited protein contents (54 mg/mL) and specific activity 0.015 U/mg with 5 fold purification and 106% yield. These findings showed a fine correlation with the previous literature in that decrease in protein contents and increase the specific activity after treated by ion exchange chromatography.

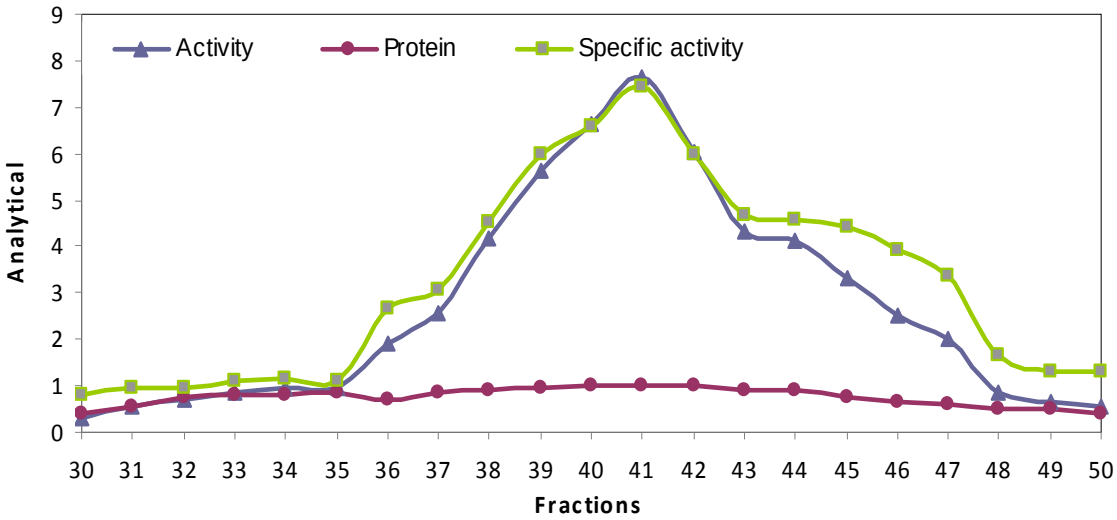


Fig. 4.25 Ion exchange chromatography of urate oxidase by parental *B. subtilis*

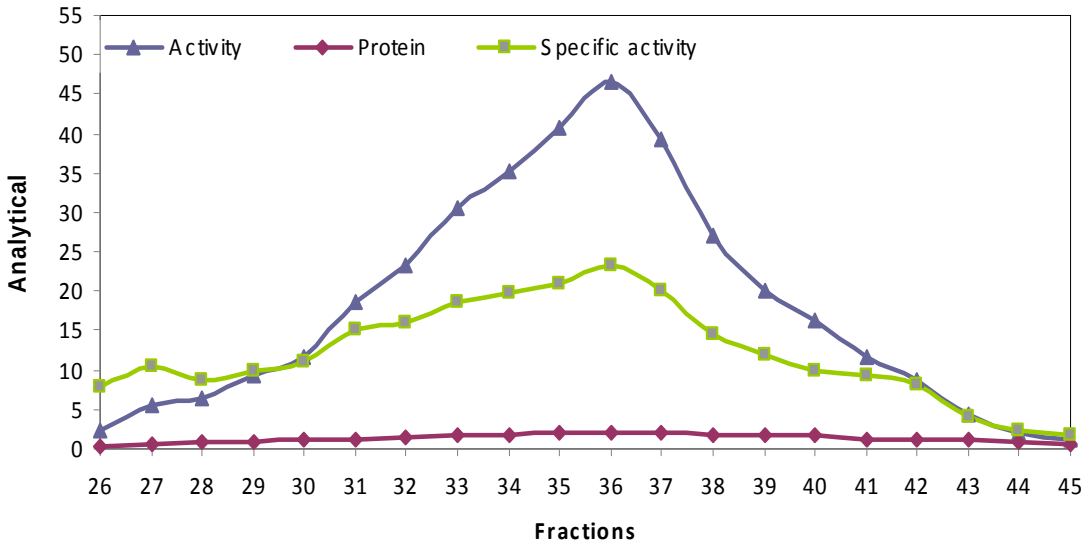


Fig. 4.26 Ion exchange chromatography of urate oxidase by mutant *B. subtilis*

4.4.3 Gel filtration chromatography

For gel filtration chromatography, Sephadex G-200 which has a cross linked polymer with selected pores size was used. In the Sephadex column larger proteins migrated faster because they take a direct path through this column, while the smaller proteins migrated slower because they enter the pores of the gel, so take a labyrinthine route through the column.

The fractions recovered from ion exchange treatment (41 fraction of parent derived and 36th of mutant derived enzyme) were applied to Sephadex G-200 column for gel filtration chromatography. That is a method to separate the proteins according to difference in their molecular size. The separation is obtained by the sample which is percolated through a bed of uncharged porous gel particles. Gel filtration chromatography is widely accepted because of its rapidity, simplicity and economy of the technique. This technique can be used whenever among the sample substances, more molecular size differences exist, providing highly reproducible results. Larger volume of the sample is easily accomplished and the recovery of the solute is approached 100 %. Gel filtration chromatography is rarely caused denaturation of liable substances because this method is extremely gentle.

Maximum specific activity (25.125 U mg^{-1}) along with 134.35 folds purity was attained in 31st fraction by parent derived urate oxidase (Fig 4.27). It was also observed that maximum activity of the enzyme of 31st fraction was 6.03 UmL^{-1} with 0.24 mg mL^{-1} protein contents. The optimum specific activity, activity and protein contents obtained by mutant derived urate oxidase were 97.56 U mg^{-1}, 42.93 U mL^{-1} and 0.44 mg mL^{-1} respectively. Now, the enzyme achieved was 256.73 fold purified in 33rd fraction (Fig 4. 28). During purification the specific activity was increased which proved that most of the contaminated proteins were removed. Further, it was also concluded that mutant derived urate oxidase from *Bacillus subtilis* yield more activity and purification as compared to parent derived urate oxidase.

According to Farina and Faraone (1979) 50 U mg^{-1} specific activity and 0.26 mg mL^{-1} protein contents of ox kidney urate oxidase was obtain through Sephadex G-200. Aguilar *et al*., (2002) applied *Puccinia recondita* urate oxidase fractions from DEAE cellulose to xanthine-agarose column. They exhibited 278.9 fold purification, 103% yield and 8.366 U mg^{-1} specific activity. Saeed *et al*., (2004b) pooled *Pseudomonas aeruginosa* urate oxidase fractions from DEAE- cellulose to Sephadex G-200. It was found that purified urate oxidase

showed 636.36 U mg^{-1} specific activity. Li *et al*., (2006) applied *Aspergillus flavus* urate oxidase to sephadex G-200 show >99% purity. Zhao *et al*., (2006) applied *Bacillus fastidious* urate oxidase to sephadex G-200 show 0.03 UmL^{-1} activity. Liu *et al*., (1994) pooled *Candida* sp. urate oxidase fractions from DEAE cellulose DE52 to Sephadex G-200. They observed 240 folds purification with 12 U mg^{-1} specific activity.

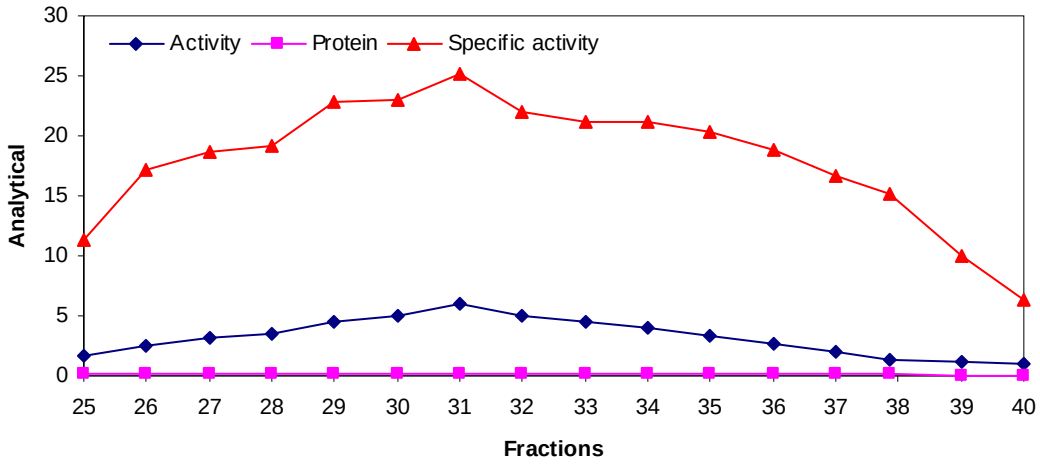


Fig 4.27 Gel filteration chromatography of urate oxidase by parental *B. subtilis*

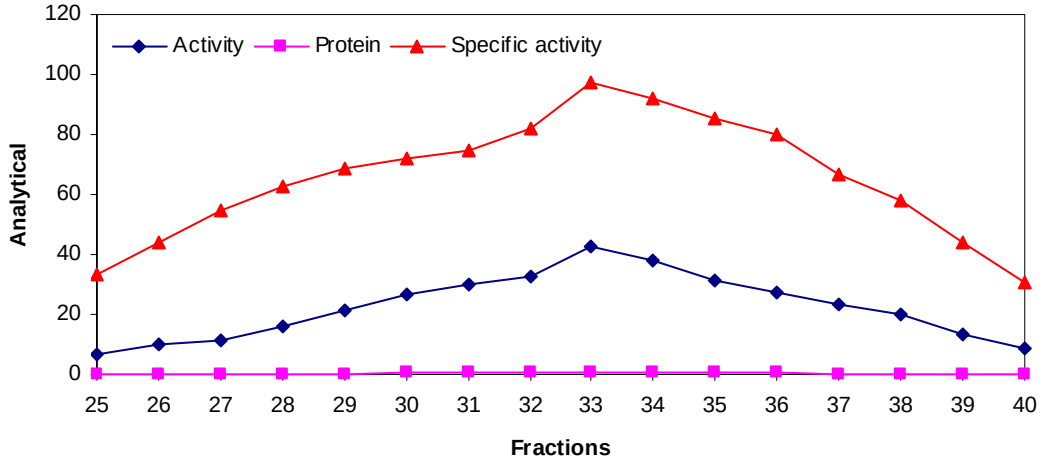


Fig 4.28 Gel filteration chromatography of urate oxidase by mutant *B. subtilis*

Table: 4.29 Purification summary of urate oxidase produced by parental strain

Purification stage	Activity (UmL^{-1})	Protein contents ($mg\ mL^{-1}$)	Specific activity ($U\ mg^{-1}$)	Fold purification
Crude	25.54	136.57	0.187	1
$(NH_4)_2SO_4$ 80% desalted	11.98	7.94	1.508	8.06
DEAE-Cellulose	7.65	1.03	7.42	39.67
Sephadex G-200	6.03	0.24	25.125	134.35

Table: 4.30 Purification summary of urate oxidase produced by mutated strain

Purification stage	Activity (UmL^{-1})	Protein contents ($mg\ mL^{-1}$)	Specific activity ($U\ mg^{-1}$)	Fold purification
Crude	89.83	236.39	0.38	1
$(NH_4)_2SO_4$ 80% desalted	78.55	8.44	9.31	24.5
DEAE-Cellulose	46.42	2	23.21	61.07
Sephadex G-200	42.93	0.44	97.56	256.73

4.4.4 SDS-PAGE

Purified urate oxidase was run on SDS-PAGE (10%) with mercaptoethanol determined a single band with a mobility revealed a molecular weight of 34 kDa.

4.5 Molecular mass determination

The subunit molecular masses of urate oxidase from *Bacillus subtilis,* have been observed by SDS-PAGE (10%). A single band (34 kDa) was observed on SDS-PAGE. This indicated that the urate oxidase is composed of single or identical subunits.

The result of present research work was shown a fine correlation with Yamamoto *et al*., (1996) who was determined the molecular mass of purified *Bacillus* sp. urate oxidase subunit was 34 KDa by SDS-PAGE. Nishiya *et al*., (2002) purified *Bacillus* urate oxidase; the enzyme on the SDS-PAGE yielded a single band which was fully active form.

Larsen, (1990) purified urate oxidase from soybean root nodules exhibited 2 protein bands by SDS-PAGE. Alamillo *et al*., (1991) isolated urate oxidase from *Chlamydomonas reinhardtii* revealed that the native enzyme has 124 kDa molecular mass and contained four identical subunits of 31 kDa. Liu *et al*., (1994) purified urate oxidase from *Candida* sp. and determined the molecular weight was 70-76 kDa by gel filtration technique. Kahn and Tipton, (1997) estimated molecular mass of urate oxidase isolated from soybean root nodules was found to be 35,059.8 Da by MALDI-TOF mass spectrometery. Montalbini, *et al.,* (1997) determined molecular mass of urate oxidase purified from *Cicer arietinum* L., *Vicia faba* L., and *Triticum aestivum* L. by SDS-PAGE was observed to be 120-130 KDa and to have four identical sized subunits 32-34 KDa.

Aguilar *et al*., (2002) purified urate oxidase to electrophoretic homogeneity from *Puccinia recondite* and determined the molecular mass by SDS-PAGE analysis that the enzyme contained 2 different sized subunits. Zhao *et al*., (2006) estimated the molecular mass of urate oxidase purified from *Bacillus fastidious* by gel filtration (sephadex G-200) was found to be 151 KDa.

4.6 Kinetic and thermodynamic studies

4.6.1 Optimum pH

The ionization of active site (essential residues) of the protein is affected by the alteration in hydrogen ions concentration. The substrates are bound to the active site of the protein and ultimately degraded to form product. At the periphery of the active site, some ionizable residues are saturated, which are commonly called as non-essential residues. When these residues are ionized then it may cause distortion in essential residue cleft and thus indirectly affect the activity of the enzyme. When pH and temperature were performed on purified protein, then the differences could very well reflect amino acid sequence differences.

Parent and mutant derived urate oxidase were assayed at different pH (ranging from 2-12) to seek the optimum pH. Hence, parental derive enzyme showed optimum activity at pH range 7-9 as indicated in Fig 4.29. However, it was observed that urate oxidase from mutated *Bacillus subtilis* was active within the pH range of 7-10 with maximum activity (40.97 U mL^{-1}) at pH 8 (Fig 4.30).

The present results are good coincident with Kai *et al*., (2008) who was studied that a thermostable urate oxidase produced from microbacterium sp. was stable between pH 7-10.

Huang and Wu (2004) observed that wild and mutant derived urate oxidase showed highest activity at pH ranging from 6-10. Liu *et al*., (1994) purified urate oxidase from *Candida* sp. which was stable at pH ranges from 8.5-9.5. Zhao *et al*., (2006) characterized urate oxidase from *Bacillus fastidious* was stable between the ranges of 9.0-10.5. Here the difference with the above reported results may be either, due to the treatment of different organisms or environmental conditions.

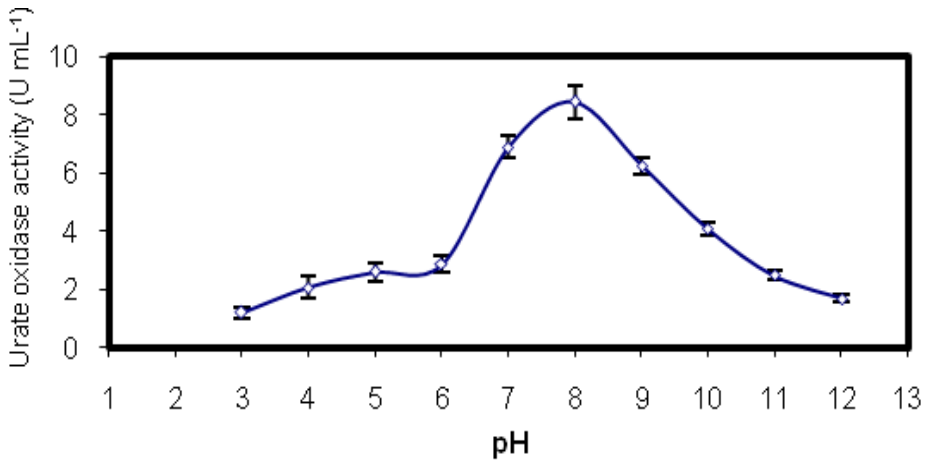


Fig 4.29 Effect of pH on parent derived urate oxidase

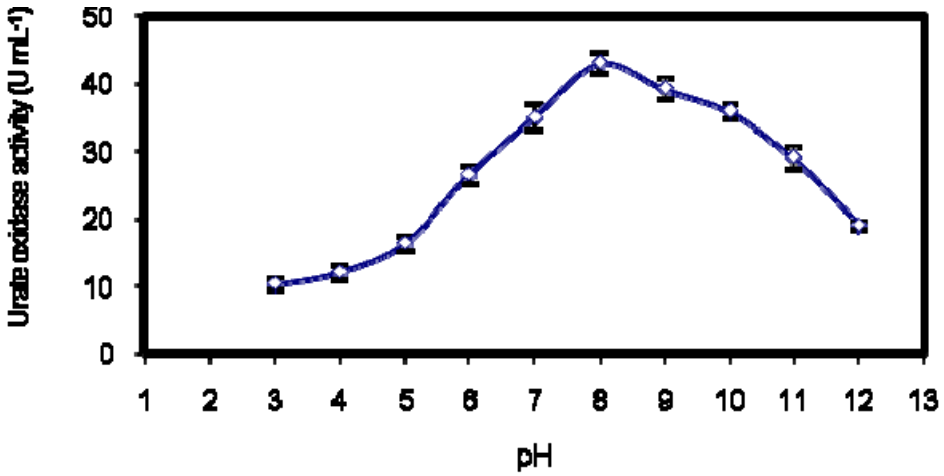


Fig 4.30 Effect of pH on mutant derived urate oxidase

4.6.2 Optimum temperature

Parental and mutant derived urate oxidase was assayed at different temperatures ranging from 20-80^{o}C at pH 8 (optimum pH). Parent derived enzyme exhibited maximum activity 7.34 U mL^{-1} at 35 ^{o}C (Fig 4.31) and activation energy 43.4.kJ mol^{-1}. Optimum temperature and activation energy (E_a) of mutated *Bacillus subtilis* derived urate oxidase was found to be 35^{o}C (44.34 U mL^{-1}) and 32.8 k J mol^{-1} respectively as shown in Fig 4.32. It was obvious from Arrhenius plot (Fig 4.33) that the enzyme had a single conformation up to transition temperature (35^{o}C) and then it showed a decline.

Alamillo *et al.*, (1991) purified urate oxidase from unicellular green alga and observed that the purified enzyme exhibited its maximum activity at temperature 40 ^{o}C with 53 kJ/mol activation energy. Liu *et a*l., (1994) purified urate oxidase from *Candida* sp. which was stable below the temperature 35 ^{o}C. Huang *et al*., (2004) immobilized urate oxidase which responded more active at highest temperature ranging from 20-40 ^{o}C. Huang and Wu (2004) studied the thermal stability of urate oxidase (wild and mutant) and revealed that the activity of the wild and mutant derived urate oxidase was inhibited at 75 and 80 ^{o}C respectively. It was also observed that at 70 ^{o}C the activity of both wild and mutant derived urate oxidase was decreased 45-60%. Xue *et al*., (2005) purified urate oxidase from soil bacterium, which was retained 100% of initial activity after heat treatment at 70 ^{o}C for 30 minutes. Rajoka *et al.*, (2006a) extracted and purified urate oxidase from caprine kidney and studied that purified enzyme showed its maximum activity at 40 ^{o}C with activation energy 13.6 kJ/mol. Rajoka *et al.*, (2006b) purified urate oxidase from bovine kidney and found that the activation energy required for hydrolysis of uric acid was 11.6 kJ/mol. Cete *et al*., (2007) studied that immobilized urate oxidase showed its maximum activity at temperature 55 ^{o}C. The findings of the present work are differ from the earlier reported literature. This may be due to the difference in environmental factors, organisms etc.

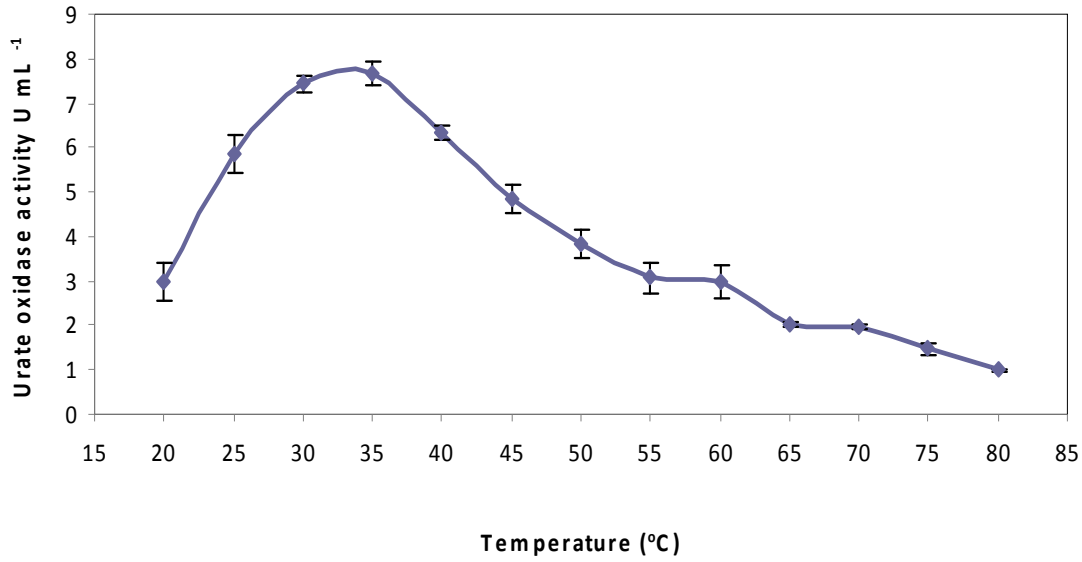


Fig. 4. 31 Effect of Temperature on parent derived urate oxidase

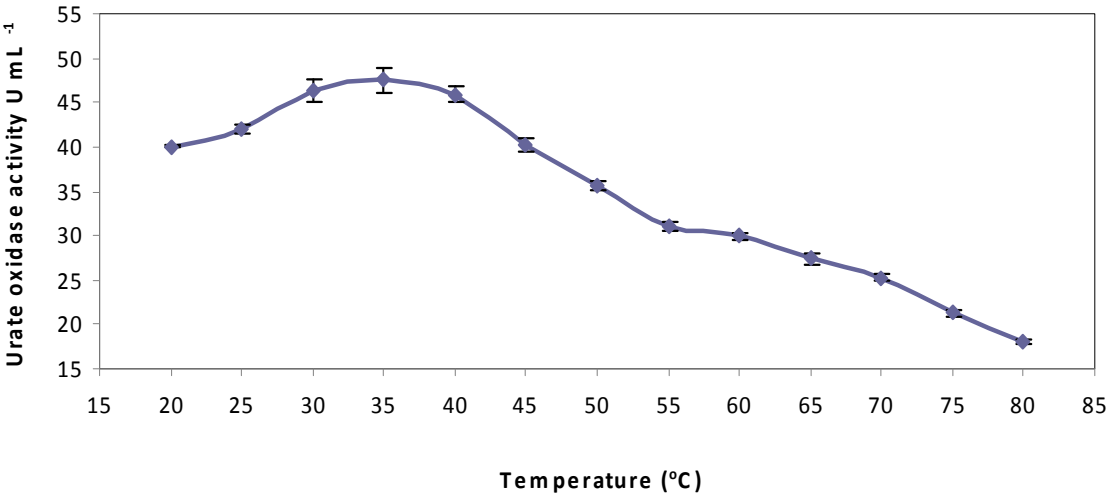


Fig. 4. 32 Effect of Temperature on mutant derived urate oxidase

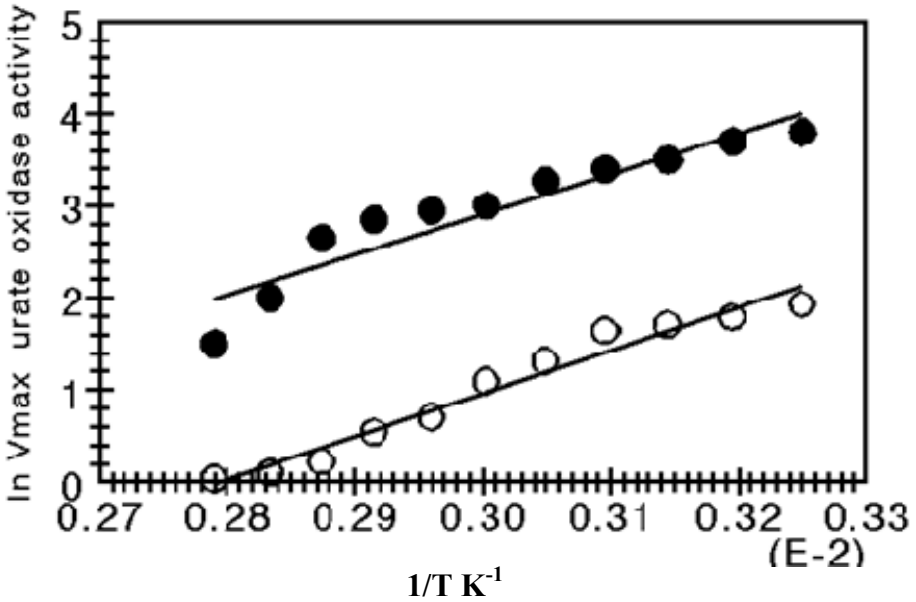


Fig. 4.33 Arrhenius plot to calculate activation energy of substrate oxidation for parent and mutant strain.

Parental urate oxidase (○)
Mutant derived urate oxidase (●)

4.6.3. Michaelis-Menten constant determination

The different concentrations of uric acid were used (0.1-1.0% w/v) for the determination of Michaelis-Menten constant (K_m). The activity of urate oxidase was determined at different uric acid level by keeping the enzyme concentration constant (Saddiqui *et al*., 1997; Witt *et al*., 1998; Rajoka *et al*., 2006a and 2006b). Urate oxidase of parent and mutant *B. subtilis* has K_m value 800 mM and 67 mM for uric acid respectively (Fig 4.34). Purified parent derived urate oxidase showed V_{max} 83.3 ± 2.4 U/mL while the enzyme isolated from mutated *B. subtilis* (BSM-2) exhibited V_{max} 133.3 ± 4.5 U/mL (Table 4.32).

Lotfy, (2008) determined the kinetics constant for *Bacillus thermocatenulatus* urate oxidase with uric acid (as a substrate) was found to be V_{max} 0.99 U/mL and K_m of 0.25 mM. Kai *et al*., (2008) purified urate oxidase from *Microbacterium* sp. and estimated the value of K_m and K_{cat}, which were 0.31 mM and 3.01 S^{-1} respectively and K_{cat}/ K_m value 9.71 $S^{-1}mM^{-1}$. Cete *et al*., (2007) determined the K_m and V_{max} values of immobilized urate oxidase which were 0.44 mM and 0.23 U/mL respectively. While, free enzyme showed K_m and V_{max} 0.39

mM and 0.198 U/mL respectively. Zhao *et al*., (2006) determined the V_{max} and K_m of *Bacillus fastidious* urate oxidase and it was found to be 0.034 U/mL and 4.1 mM respectively. Rajoka *et al*., (2006a) purified urate oxidase from caprine kidney and determined K_m and V_{max} values as 6.54 x 10^{-3} mM/mL and 3512 U/mg protein for hydrolysis of uric acid. Rajoka *et al*., (2006b) purified urate oxidase from bovine kidney and determined K_m and V_{max} with uric acid as a substrate was found to be 0.125 mM and 1021 U/mg protein respectively. Mulhbacher *et al*., (2002) determined V_{max} of immobilized and free enzyme that was 16 U/mg and 18 U/mg respectively while K_m of immobilized and free enzyme was 0.17 mM and 0.03 mM respectively. Aguilar *et al*., (2002) determined K_m 0.035 mM of *Puccinia recondite* urate oxidase. The value of the K_m of *Candida* sp. urate oxidase was found 5.26 x 10^{-6} mM/mL as reported by Liu *et al*., (1994).

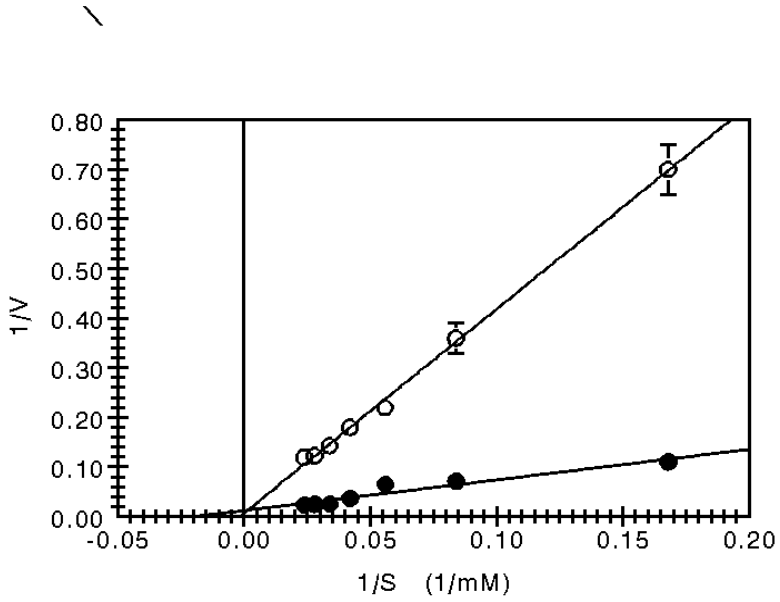


Fig.4.34 Lineweaver-Burk plot to calculate K_m and V_{max} values of urate oxidase derived by the parental (○) and mutant organism (●).

4.6.4 Irreversible thermal denaturation

The temperature at which half activity of the enzyme is lost is known as melting temperature or melting point (T_m). The melting temperature of parent derived enzyme was 66^oC while mutant derived enzyme showed 71^oC (Fig 4.35). Parent derived urate oxidase showed

thermal stability at 40°C with 69.3 minutes half life. While, this half life was decreased to 21 minutes at 65°C under same conditions. EMS treated *B. subtilis* (BSM-2) derived urate oxidase was thermally stable at 40°C with half life of 85.6 minutes. However it was less stable at 65°C and exhibited half life of 36.47 minutes under same conditions.

The isolation of stable enzymes is very important for their application (i.e. biocatalyst). The improvement of techniques (to enhance the stability of enzymes) is another important object in enzyme technology. Like other proteins, catalytic proteins are slightly stable due to the subtle balance of stabilizing and destabilizing interactions (Jaenicke 1991). Detailed explanation of the mechanism, responsible for stabilization and destabilization of enzymes particularly at high temperatures, are very important for both commercial and scientific point of view (Kristjansson and Kinsella, 1991)

The interest is developed for the isolation of thermostble enzyme, because industrial enzyme techniques were carried out in uncertain physiological conditions, i.e. extreme values of pH, high temperature and pressure etc. Among all the environmental conditions, temperature has more importance in controlling the activities of microorganisms (Ward and Mooyoung, 1988). Thermophilic enzymes have more advantages in industrial development such as the rate of reaction increased at high temperature. Rajoka *et al*., (2006) purified urate oxidase from bovine kidney and found inactivation energy of the enzyme 14.5 kJ/mol.

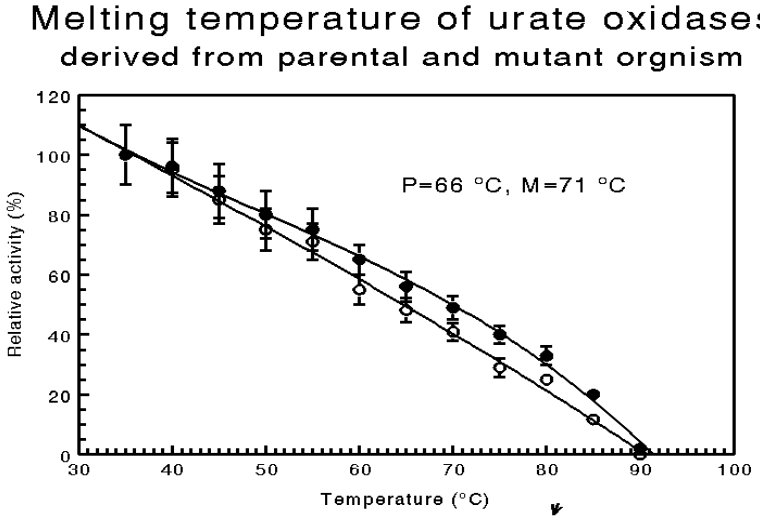


Fig 4.35. Determination of melting point of urate oxidase derived from the parental (○) and mutant organism (●).

4.6.5 Thermodynamics of irreversible thermal inactivation

Thermostability is the quality of an enzyme to resist irreversible change in its chemical or physical structure at a high relative temperature. (Georis *et al*., 2000). Enthalpy of denaturation (ΔH*) of wild and mutant strain urate oxidase was found to be 40.84 kJ/mol and 30.26 kJ/mol at 35^{o}C respectively, detailed results are shown in table 4.32. The value of free energy of thermal denaturation (ΔG*) of parental and mutant derived urate oxidase were found 64.19 and 62.99 kJ/mol respectively. It was observed that free energy of thermal denaturation (ΔG*) obtained from parent derived urate oxidase was within the range of 99.42-104.22 kJ/mol at 40-65 ^{o}C, while the values of ΔG* from mutant derived enzyme was within the range of 99.97-105.77 kJ/mol. The entropy of inactivation (ΔS*) was calculated at different temperatures (313-338K), it gave negative values, indicating that the enzyme is thermodynamically stable. Parental and mutated derived urate oxidase showed ΔS* -75.81 and -106.27 J mol^{-1}K^{-1} at 35^{o}C (308K) respectively. ΔS* of the parent derived enzyme was in the range of -187.22 to -188.28 J mol^{-1} K^{-1} at 40-65^{o}C (313-338K). Mutant derived enzyme showed ΔS* values in the range of -222.91 to -224.29 J mol^{-1} K^{-1} at 40-65^{o}C (313-338K). All the details of the results are arranged in Table 4.31 - 4.32 and Fig 4.36-4.37.

Urate oxidase purified from *Bacillus subtilis* BSM-2 strain was thermally stable and could be used for analytical and other industrial application. The negative entropy of deactivation for urate oxidase observed that there was negligible disorderness. A high value of free energy of thermal denaturation at 65^{o}C indicated that the urate oxidase showed the resistance against thermal unfolding at higher temperatures.

According to Rajoka *et al*., (2006a), the activation energy of the urate oxidase for the formation of the enzyme substrate complex was found 13.6 kJ/mol. Entropy of activation, enthalpy and Gibb's free energy demand of caprine kidney urate oxidase inactivation were observed to be -102 J/mol K , 62.8 kJ/mol and 104.3 kJ/mol respectively. According to Rajoka *et al*., (2006b) activation energy requirement for urate hydrolysis by urate oxidase was found 11.6 kJ/mol and inactivation of energy was observed 14.5 kJ/mol.

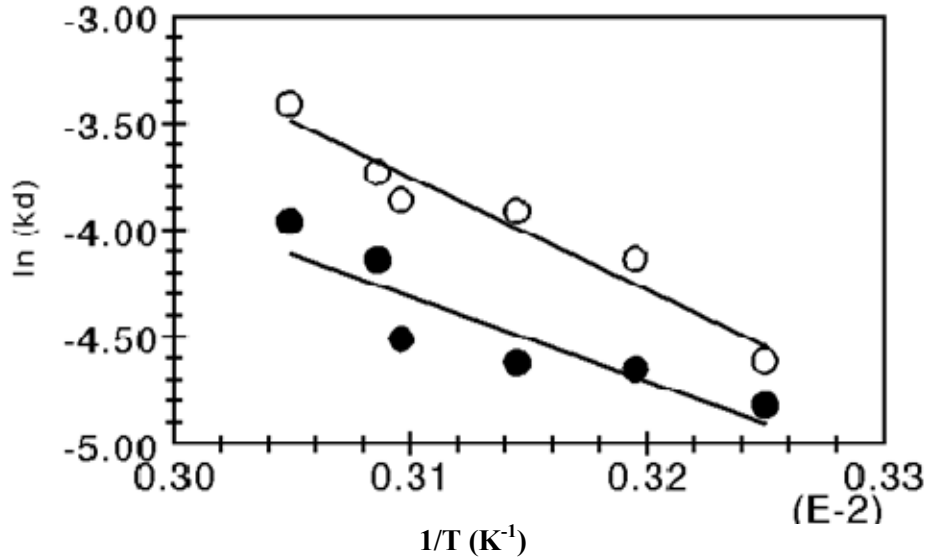


Fig. 4.36 Arrhenius plot to determine the activation energy for deactivation of urate oxidase derived from the parental and mutant derived strain.

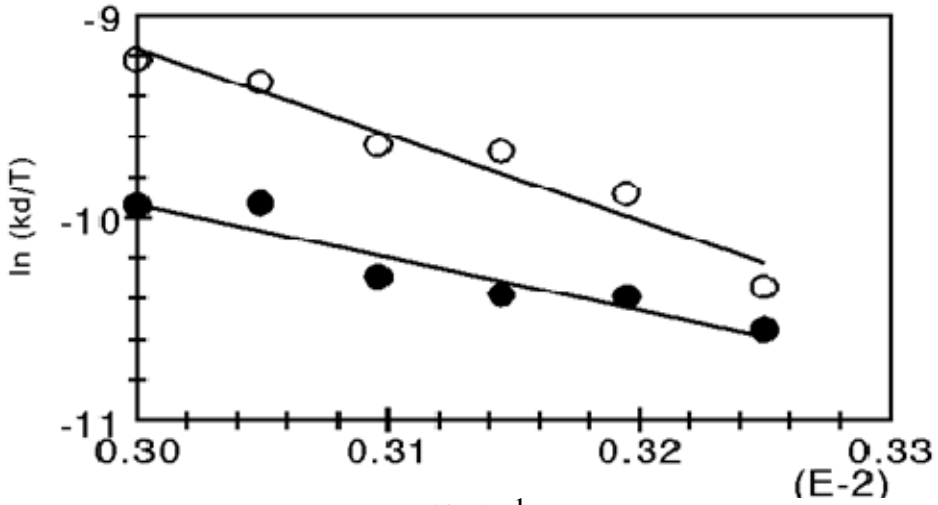


Fig. 4.37. Arrhenius plot to determine the enthalpy and entropy need for deactivation of urate oxidase derived from the parental (○) and mutant (●) organisms.

Table 4.31 Thermodynamic parameters for denaturation of urate oxidase by parental (P) and mutant (M) derived urate oxidase

Temperature (K)		k_d x 10^{-4} (sec^{-1})	Half life (min)	ΔH* (kJ mol^{-1})	ΔG* (kJ mol^{-1})	ΔS* (J $mol^{-1}K^{-1}$)
313	P	1.67	69.3	40.82	99.42	-187.22
	M	1.35	85.6	30.20	99.97	-222.91
318	P	2.67	43.3	40.6	99.81	-186.19
	M	1.60	72.19	30.16	101.16	-223.27
323	P	3.33	34.65	40.73	100.82	-186-04
	M	1.65	70.0	30.11	102.71	-224.77
328	P	3.5	33.0	40.69	102.29	-187.80
	M	1.83	63.0	30.11	104.06	-225.46
333	P	4.83	23.9	40.65	103.00	-187.24
	M	2.67	43.3	30.03	104.64	-224.05
338	P	5.5	21.0	40.58	104.22	-188.28
	M	3.17	36.47	29.96	105.77	-224.29

ΔH* (kJmol^{-1}) = E_{ad} (43.42 and 32.8 kJmol^{-1} for wild type organism and mutant organism respectively) -RT where E_{ad} is activation energy for denaturation,

ΔG* (kJmol^{-1}) = -RT ln (k_d.h)/k_B.T), ΔS* is entropy of irreversible inactivation and was calculated from $\Delta S^* = (\Delta H^* - \Delta G^*)/T$

Table 4.32 Kinetic and thermodynamic properties of urate oxidase derived from the parent (P) and mutant derivative (M) of *Bacillus subtilis* for uric acid hydrolysis at 35 °C

Kinetic parameters/	urate oxidase of strains	
	P	M
V_{max} (IU/.mg.min)	83.3± 2.4	133.3± 4.5
K_m (M)	0.80 ± 0.018	0.067±0.004
E_a (kJ/mol)	43.4	32.8
Temperature optima (°C)	35	35
T_m	66°C	71°C
pH optima	7.3	7.5
ΔG* (kJ/mol)	64.19	62.99
ΔH*(kJ/mol)	40.84	30.26
ΔS* (J/mol.K)	-75.81	-106.27
ΔG^*_{E-T} (kJ/mol)[b]	5.79	-1.76
ΔG^*_{E-S} (kJ/ mol)[c]	17.12	10.77

[b] ΔG^*_{E-T} (free energy of transition state binding) = -RT ln Vmax/K_m

[c] ΔG_{E-S} (free energy of substrate binding) = -RT In K_a, where $K_a = 1/K_m$

4.7 Determination of Stability

The stability of mutant derived urate oxidase was studied against different agents like potassium cyanide, sodium chloride, barium chloride, zinc sulfate, calcium chloride and potassium chloride. It was observed that, when the concentration of barium chloride, potassium cyanide and zinc sulfate increased then the activity of the enzyme was decreased (Fig 4.38-4.40). Whereas, the sodium chloride (0.6 M), potassium chloride (0.4 M) and calcium chloride (0.4 M) enhanced the activity of the enzyme 123%, 117% and 119% respectively (Fig 4.41-4.43).

Saeed *et al* (2004b) studied that purified urate oxidase from *Pseudomonas aeruginosa* was inhibited by sulfate forms of Zn^{++}, Co^{++}, Fe^{++} and potassium cyanide while sodium chloride and calcium chloride enhanced the activity of the enzyme. Tajima *et al*., (2004) revealed that the activity of the enzyme was inhibited by barium chloride (1M) and magnesium chloride (1 M). The findings of present work are in accordance with the results of above reported literature.

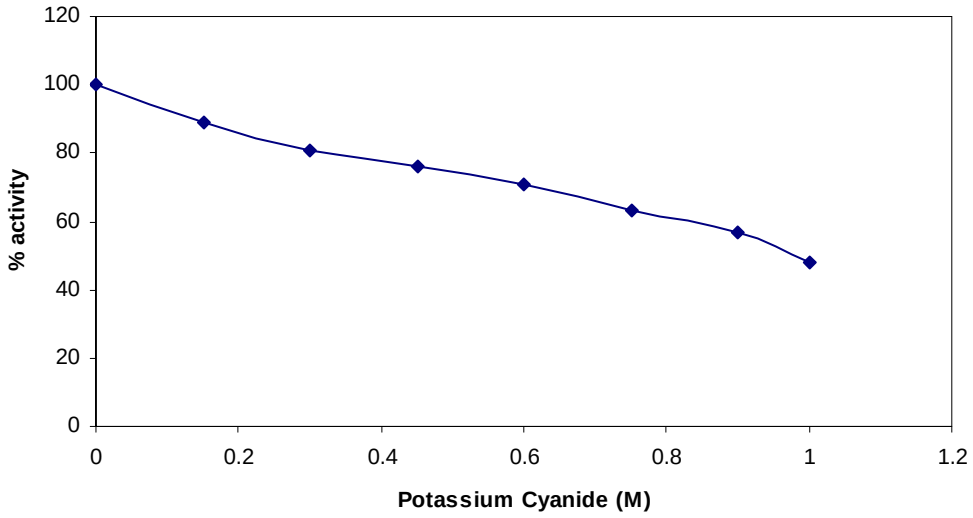


Fig. 4.38 Effect of potassium cyanide on the stability of mutant derived enzyme

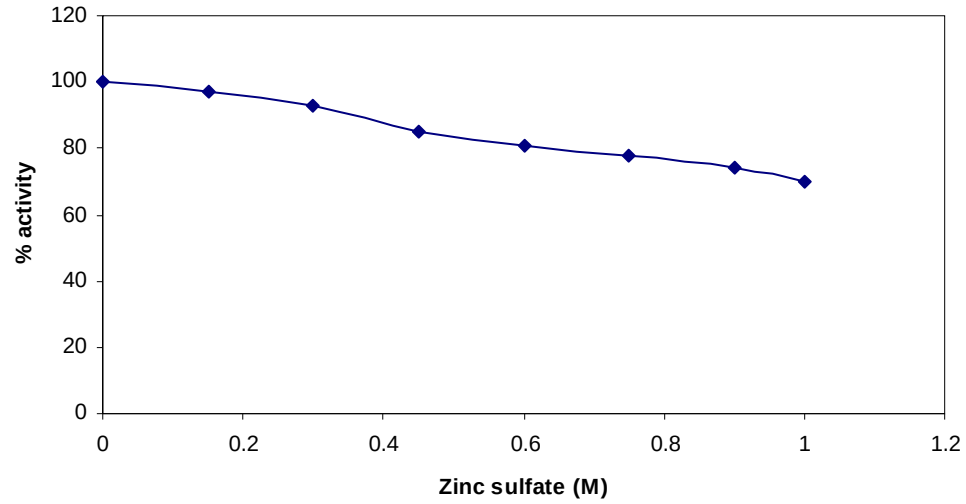


Fig. 4.39 Effect of zinc sulfate on the stability of mutant derived enzyme

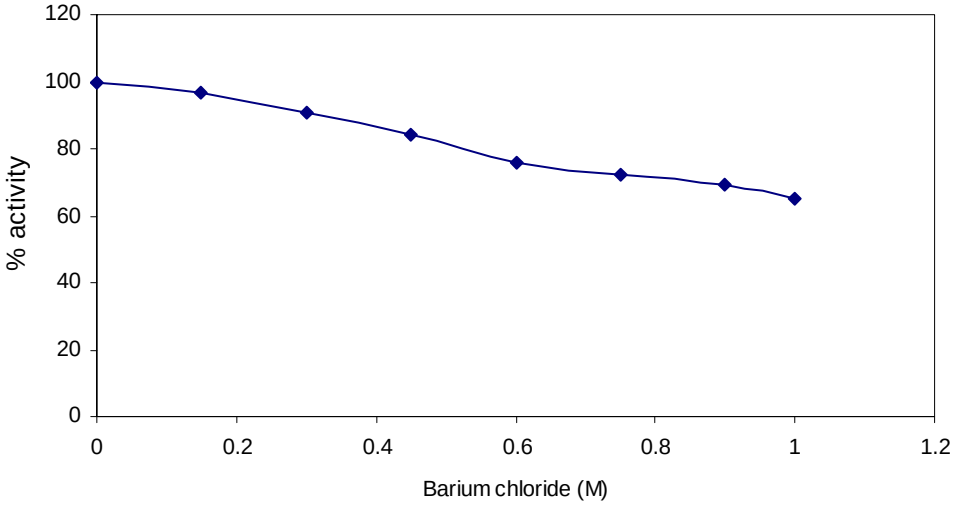


Fig. 4.40 Effect of barium chloride on the stability of mutant derived enzyme stability

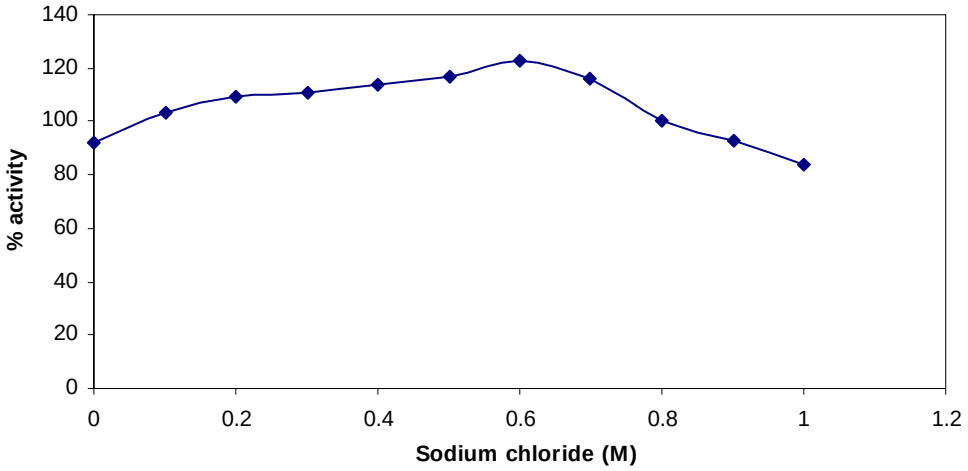


Fig. 4.41 Effect of sodium chloride on the stability of mutant derived enzyme

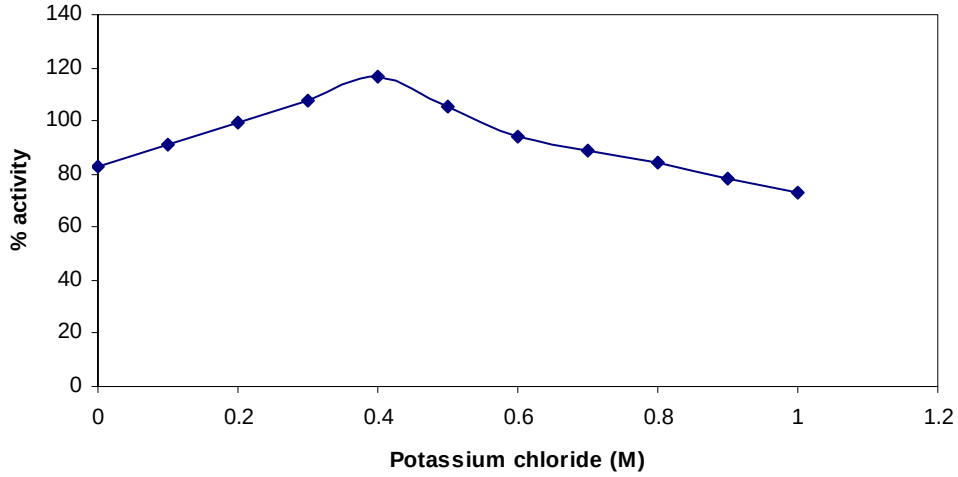


Fig. 4.42 Effect of potassium chloride on the stability of mutant derived enzyme

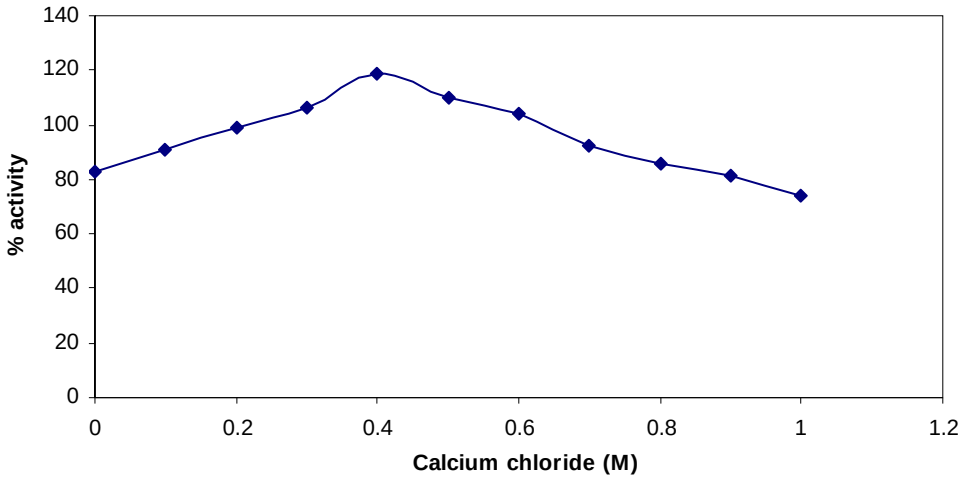


Fig. 4.43 Effect of calcium chloride on the stability of mutant derived enzyme

4.8 Uric acid estimation kit

The present method for the estimation of uric acid is significantly more specific, simple, and quick. Moreover, there is no need of any expensive instrument. This method also eliminates the non-specificity of colorimetric methods and diminishes the use of carcinogenic or unstable dyes (Kroll and Elin, 1994; Liao *et al*., 2005a; Lio *et al*., 2006a and 2006b).

In this method, uric acid is determined in biological fluids (serum and urine) by following the procedure of Fossati *et al.,* 1980. Uric acid is degraded to allantoin and H_2O_2 by the action of urate oxidase. Hydrogen peroxide acts as a substrate for peroxidase and reacts with 4-aminoantipyrine (4AAP) and 3,5-dichloro-2-hydroxybenzenesulfonic acid (DCHBS) and forms quinoneimine, a red compound that is detected by an increase in absorbance at 552 nm. It was observed that, the rate of formation of colored compound (quinoneimine) is equal to the formation of H_2O_2, which in turn equal to the concentration of uric acid. Overall reaction is as follow

$$\text{Uric acid} + 2H_2O + O_2 \xrightarrow{\text{urate oxidase}} \text{Allantoin} + H_2O_2$$

$$H_2O_2 + 4\text{AAP} + \text{DCHBS} \xrightarrow{\text{peroxidase}} \text{Quinoneimine dye}$$

Uric acid is determined in clinical laboratories by various methods (enzymatic method and colorimetric method). Colorimetric methods have been used for the determination of uric acid but it has some limitations. A popular colorimetric method proposed by Tietz (1995), described that uric acid reduced the phototungstic acid to tungsten blue which is absorbed at 700 nm. But, there are some interfering substances that act as reducing agent (i.e. glutathoin, glucose, ergothioneine and ascorbic acid) and thus increased the value falsely. The enzymatic method was increased the specificity. Uric acid degraded enzymatically to allantoin, which results to decrease in absorbance at the wavelength of 293 nm, is proportional to the presence of uric acid. While, allantoin do not absorb light at this wavelength. The kinetic uricase method used for uric acid assay by fitting the Michaelis Menten rate equation to the urate oxidase reaction curve (Liao *et al*., 2005a and 2005b; Lio *et al*., 2006a and 2006b). But this method has many disadvantages such as the measurement of uric acid at ultra violet wavelength is beyond the range of many spectrophotometers that are used in laboratories as well as the cuvets (quartz) required for this purpose are very expensive (Praetorius and Poulson 1953; Ramesh *et al*., 1978; Liao *et al*., 2006b).

The direct uricase method or enzymatic method is improved by coupling the urate oxidase reaction with peroxidase. The uric acid degraded enzymatically to form hydrogen peroxide (H_2O_2). In the presence of peroxidase, H_2O_2 reacts with o-dianisidine that produced a colored product. Unfortunately, the dye (o-dianisidine) used in this method is carcinogenic, thus it is undesirable to use it in clinical laboratories at regular basis (Henry 1976). Moreover, this method is very sensitive to hemoglobine, ascorbic acid, lipids, and bilirubin (Aoki *et al*., 1992; Kroll and Elin, 1994; Liao *et al*., 2006b). Another enzymatic method proposed by Kagayama (1971) revealed that hydrogen peroxide (produce by urate oxidase action) reduced the methanol in the presence of catalase to form formaldehyde. Acetylaceton reacted with this aldehyde to form 3,5-diacetyl-1,4-dihydrolutidine which produced a color. However, the developing of color is a time consuming procedure (~ 70minutes). The procedure of coupling the urate oxidase reaction with peroxidase was adopted for the determination of uric acid in this study. Many other investigators (Fossati *et al*., 1980; Huang and Wu, 2004; Pfrimer *et al*., 2010) have already followed this procedure.

4-aminoantipyrine (4AAP) with 3,5-dichloro-2-hydroxybenzenesulfonic acid (DCHBS) were used as chromogen. Many other chromogens were also reported for the

estimation of uric acid such as Guaiacol, benzidine, o-tolidine and o-dianisidine. However, these reagents have potent mutagenic and carcinogenic properties. Fossati *et al*., (1980) utilized 3,5-dichloro-2-hydroxybenzenesulfonic acid/4-aminophenazone as chromogenic system in the presence of *A. flavus* urate oxidase and peroxidase. Huang and Wu (2004) used 4-AAP (1 mM) and DCHBS (4mM) as a chromogen for the estimation of uric acid in the presence of wild and mutant derived urate oxidase and peroxidase. Henry (1976) proposed a method for uric acid assay by using urate oxidase, peroxidase. H_2O_2 reacts with o-dianisidine that produced a colored product but the dye (o-dianisidine) was carcinogenic.

Uric acid was determined in biological fluid by using buffer, enzymes (urate oxidase and peroxidase) and chromogen (4-aminoantipyrine, 3,5-dichloro-2-hydroxybenzenesulfonic acid) in the ratio of 1.5:0.5:0.02 as described by Fossati *et al.,* 1980. Liao *et al*., (2006b) used 5 µL urate oxidase solution for the determination of uric acid by kinetic uricase method.

Serum sample was used for the estimation of uric acid as described by Fossati *et al.,* 1980.. Liao *et al.,* (2006b) evaluated kinetic uricase method for uric acid assy that contained 15 µL serum sample. Liao *et al.,* (2005a) and Zhao *et al*., (2006) studied that the final reaction mixture contained 15 µL serum sample, 1.18 mL buffer and 5 µL uricase solution for uric acid assay by using kinetic uricase method. Ducan *et al.,* (1982) adopted direct equilibrium method for estimation of uric acid by using 17 µL serum sample in 406 µL reaction solution. Fossati *et al*., (1980) studied that the reaction mixture contained 50 µL sample, uricase peroxidase, ascorbate oxidase and chromogen reagent for the estimation of uric acid.

3.8.1. Comparison with standard kit

The two standard kits (Human, Bio Med) and self-prepared kit was compared. However, there was found a slight difference between the results of self-prepared kit and standard kit. This difference may be due to the presence of ascorbate in the biological fluid. This difference may be eliminated by using ascorbate oxidase (Table 4.33).

Table: 4.33 Comparison of uric acid estimation through self-prepared with standard (Human, Bio med) kits

Sr. No.	Uric acid level (mg dL^{-1})	Uric acid level (mg dL^{-1})	
	Self prepared kit	Standard kit1	Standard kit2
1	17.38	16.98	16.92
2	6.80	6.65	6.69
3	30.31	30.12	31.24
4	7.98	7.90	7.09
5	6.43	5.48	6.01
6	16.78	16.35	15.98
7	15.90	15.10	14.76
8	18.38	17.96	17.96
9	19.27	18.29	18.67
10	25.21	24.16	23.99

CHAPTER 5

SUMMARY

The main purpose of this research work was to optimize the production of urate oxidase through mutagenesis (ultra violet irradiation and chemical mutagenesis) of *B. subtilis*. Ethyl methane sulfonate treated *B. subtilis* (180 minutes) was proved the best one for optimum production of urate oxidase by 3-log kill/survival curve. Mutated and parental/wild strains were used for urate oxidase production in the flasks. It was found that substrate concentration (0.5%), fermentation period (36 h), pH (8.5), temperature (35 ^{o}C), yeast extract (0.3%) and sucrose (2%) enhanced the activity of the parent and mutant derived enzyme. From the experiments, it was concluded that nutritional requirement of mutated strain was exactly the same as those of parental strain.

The specific activity of crude enzyme from parent and mutant derived enzyme was 0.187 U/mg and 0.38 U/mg respectively. The enzyme was subjected to ammonium sulfate precipitation that showed specific activity 9.31 U/mg from mutated urate oxidase. The purification of urate oxidase (BSM-2) by ion exchange chromatography (DEAE cellulose) exhibited 23.21 U/mg specific activity. From ion exchange treatment, 36th fraction was applied for gel filtration chromatography (Sephadex G-200). It was observed that mutated enzyme exhibited specific activity 97.56 U/mg.

The purified urate oxidase was run on SDS-PAGE (10%) which determined a single band with molecular weight of 34 kDa. Urate oxidase from *B. subtilis* (BSM-2) was active at the pH ranging from 7-10 while optimum activity was found at pH 7.5. The optimum temperature and activation energy was observed to be 35^{o}C and 43.4 kJ mol^{-1} respectively. The Michaelis Menten constant (K_m) was found to be 67 mM and V_{max} 133.3 U mg^{-1} min^{-1}.

Melting temperature of the urate oxidase (BSM-2) was 71 ^{o}C. While, it was less stable at 65 ^{o}C with 63.47 minutes half life. The enthalpy denaturation (ΔH^*) of mutant derived enzyme was 30.26 kJ/mol at 35^{o}C and the value of free energy of thermal denaturation (ΔG^*) was 62.99 kJ/mol. The entropy of inactivation (ΔS^*) was calculated at

each temperature that gave negative values. These results showed that the enzyme is thermodynamically stable. The mutated derived enzyme exhibited -106.27 J mol^{-1} K^{-1} at 35^{o}C. The stability of urate oxidase from mutated *B. subtilis* (BSM-2) was studied against different agents, which showed that barium chloride, potassium cyanide and zinc sulfate decreased the activity of the enzyme. Whereas, sodium chloride (1M), potassium chloride (0.7M) and calcium chloride (0.6M) enhanced the enzymatic activity of the enzyme 123%, 117% and 119% respectively.

Urate oxidase was used for the determination of uric acid; this enzymatic method was improved by coupling the urate oxidase reaction with peroxidase. Finally, the self-prepared kit was compared with two standard kits and it was found that there was a slight difference between the results of the self-prepared kit and standard kits.

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