Hyperproduction and Comparison of Wild and Mutated Urate Oxidase from *Bacillus Subtilis*

Munazzah Meraj, Khalil-Ur-Rehman, Sadia Javed, Rao Irfan, and Nazish Jahan

***Abstract*—** Urate oxidase has substantial importance; it is used in pharmaceutical preparation and in the treatment of nephrolithiasis. It is also used for the determination of uric acid in biological fluids after isolation and purification from different animal or microbial sources. Owing to its importance and applications as mentioned above, there is a great need to extract and hyperproduced this enzyme. So, this research work was focused on the hyperproduction of urate oxidase through mutagenesis of *Bacillus subtilis*. The strain was subjected to chemical mutagenesis. Ethyl methane sulfonate treated *Bacillus subtilis* (BEM-2) at 180 minutes dose rate was showed the best for optimum yield of urate oxidase. Fermentation medium for both parental and mutated strain was optimized. It was observed that 0.5% substrate concentration, 36 hours fermentation period, 8.5 pH, 35 ^{o}C temperature, 0.3% yeast extract and 2% sucrose, enhanced the activity of the parent (19.48±1.26U/mg) and mutant (68.05±3.50U/mg) derived enzyme. The nutritional requirements of mutant *Bacillus subtilis* observed to be the same as those of wild/parent strain.

Keywords*—Bacillus subtilis*, Ethyl methane sulfonate, fermentation medium. mutagenesis, urate oxidase

I. Introduction

URATE oxidase has significance for diagnosis of uric acid in the body fluids. This enzyme breaks down uric acid into allantoin, carbon dioxide and hydrogen peroxide. It is a homotetrameric, having four identical active sites [1]. The substrate (urate) tightly binds to the one subunit of the enzyme by interaction with arginine (Arg180), leucine (Leu222) and glutamine (Gln223) while to the other subunit with threonine (Thr67) and aspartate (Asp68) [1] , [2]. The solubility of uric acid in the body is considered to be poor as compare to the allantoin [3] , [4]. According to previous studies, it was considered that urate oxidase has copper at its active site but later on it was proved that when this enzyme was isolated from *Bacillus subtilis* and *Aspergillus flavus*, contain no copper [5]. In the primates (mammals) the gene encoding urate oxidase is inactivated. Consequently, the mammals are grouped into two categories on the bases of purine catabolic end product. In one category the enzyme allantoinase is lost and end product is allantoin, while in second category, the enzyme urate oxidase is lost and uric acid is the end product [6]. Owing to this reason, some amount of uric acid remains in the body fluid of the humans. Urate oxidase has substantial importance; it is used in pharmaceutical preparation and in the treatment of nephrolithiasis. It is also used for the determination of uric acid in biological fluids after isolation and purification from different animal or microbial sources [7] , [8]. Owing to its importance and applications as mentioned above, there is a great need to extract and hyperproduced this enzyme indigenously.

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II. Materials and Methods

The research work was designed to obtain optimum yield of urate oxidase. The strain improvement technique was used to induce mutagenesis in *Bacillus subtilis* for hyperproduction of urate oxidase. All chemicals and reagents of analytical grade were used in this research, and mostly purchased Sigma-Aldrich-Fluka, Germany.

A. Microorganism Procurement

The pure characterized culture of gram-positive bacteria, Bacillus subtilis, was obtained from Department of Veterinary Microbiology, University of Agriculture, Faisalabad, Pakistan.

B. Inoculum Preparation

For the preparation of inoculum, the pH (8) of the medium was adjusted then autoclaved. 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 at 120 rpm on the orbital shaker (Sanyo-GallenKemp, UK) [9].

C. Mutagenesis by Ethyl Methane Sulfonate

The stock solution of the ethyl methane sulfonate (Sigma-Aldrich, USA) was prepared by adding 0.15 mg chemical compound in 1 mL of buffer saline. Different time intervals were selected for this mutagenesis. Then, this stock solution was added in nutrient broth medium (9 mL) which contained the *Bacillus subtilis* spores (1×10^{7} spores mL^{-1}) and then kept it in water bath at 37°C. After specific time interval of 30 minutes (30-210 minutes) 1 mL sample was withdrawn and centrifuged (10,000 rpm) thrice for one minute to remove the mutagen from the spore suspension.

D. Mutant Selection

The specific mutant that yields highest production of urate oxides was selected. Triton X-100 was used to inhibit the growth of other bacterial colonies in the nutrient agar medium [10]. The colonies, that produced urate oxidase, were recognized on agar plates which contain purified horseradish peroxidase (310 U/mg) and 0.1 g/ L o-dianisidine. When urate oxidase was produced then a reaction was done which produced a brown color [10] , [12]. The colonies which produced bigger zone were selected, dissolved and homogenized in buffer saline and then filtered. Then checked the activity of the enzyme by spectrophotometer (T-60, PG Instruments, UK).

E. Urate oxidase production by liquid-state fermentation

Liquid state fermentation was used for the growth of selected mutant. Furthermore, the parental *Bacillus subtilis* was also used for the production of urate oxidase. The comparison between parental and mutated strains was recorded [13].

F. 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. 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 [14].

G. 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. It was found that yeast extract (0.3%) was the best nitrogen source.

H. 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. It was determined that maximum production of urate oxidase was obtained with sucrose (2 %) from parental and mutant derived *B. subtilis*.

I. Sample harvesting and enzyme assay

The material was centrifuged for 10 minutes at 10,000 rpm. The filtrate was used as crud enzyme and subjected to enzyme assay by following the procedure of reference [15].

III. Result and Discussions

A. Mutation induced by ethyl methane sulfonate

Ethyl methane sulfonate (EMS) was used for the induction of mutation in *B. subtilis* to enhance the production of urate oxidase. EMS treated *B. subtilis* at 180 minutes dose rate was produced 40 CFU/mL, which was proved that it has ability to hyperproduced the enzyme.

B. Enzyme Production

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.

C. Effect of substrate

To select a suitable concentration of substrate for the microbial growth and hyperproduction of enzyme is a critical factor. The substrate (uric acid) was used for the production of urate oxidase by mutated as well as wild type strain. Various levels of uric acid were tested for optimum production of this enzyme. Optimum activity of the enzyme by the mutated and wild type strain was obtained when the fermentation medium containing substrate (0.5%) at 30^{o}C 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 obtained by mutant and wild derived urate oxidase was 20.87 ± 1.06 U/mg and 3.33 ± 1.19 U/mg respectively. The results showed that the activity of mutant derived urate oxidase was increased seven folds as compared to the wild strain (Table 1).

D. Effect of fermentation period

The results showed that the liquid state fermentation with 0.5 % uric acid (optimum substrate level) was produced maximum yield of urate oxidase i.e 4.16±0.91 U/mg and 26.72±1.26 U/mg by parent and mutant strains respectively after 36 hours. All the details of the results are shown in table 2.

E. Effect of pH

The pH of medium was optimized to obtain optimum production of urate oxidase. The parental and mutant strains were achieved maximum yield of the enzyme 7.38±0.75 U/mg and 31.87±1.12 U/mg respectively at pH 8.5 (Table 1).

F. Effect of temperature

When the fermentation medium was incubated at pH 8.5 for 36 hours at 35 ^{o}C then urate oxidase was obtained maximum activity 9.53±1.28 U/mg and 38.18±1.51 U/mg from wild and mutant strain respectively (table 2).

G. Effect of Inoculum Size

Different concentrations of inoculum were observed to enhance the growth of *the* strain for hyperproduction of enzyme. It was studied that inoculum (3%) was used for the optimum production of parent and mutant derived urate oxidase. Parent and mutant strains were exhibited 13.67±1.29 U/mg and 42.77±2.36 U/mg activity of enzyme respectively

TABLE I
PRODUCTION OF PARENT AND MUTANT DERIVED URATE OXIDASE AT DIFFERENT SUBSTRATE LEVEL AND PH

Sub. conc. (%)	Enzyme activity U/mg		pH	Enzyme activity U/mg	
	P	M		P	M
0.1	0.31±0.16 F	7.03±1.12 H	5.5	0.88±0.34 H	5.09±1.21 I
0.2	0.43±0.21 E	11.64±1.09 G	6.0	1.47±0.57 G	7.12±1.70 H
0.3	0.66±0.15 D	13.40±1.51 E	6.5	2.00±0.72 F	9.10±1.23 G
0.4	2.14±1.08 B	16.14±1.42 C	7.0	4.43±0.95 D	15.66±1.34 F
0.5	3.33±1.19 A	20.87±1.06 A	7.5	5.74±0.22 C	22.02±1.46 D
0.6	2.14±1.08 B	18.07±1.19 B	8.0	6.05±0.55 BC	29.11±1.24 B
0.7	1.88±1.06 BC	14.54±1.28 D	8.5	7.38±0.75 A	31.87±1.12 A
0.8	1.75±0.86 C	11.10±1.17 F	9	6.32±0.80 B.	28.12±1.14 C
0.9	0.84±0.45 D	10.05±1.11 G	9.5	4.07±0.78 D	18.92±1.46 E
1.0	0.64±0.36 D	7.23±0120 H	10	3.01±0.93 E	7.69±1.21 H

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

H. 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. All the results are described as follow.

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 (15.97±1.46 U/mg) and mutant derived urate oxidase (50.93±2.41 U/mg). When peptone (0.4%) was added in the medium it gives yield of 14.06±2.05 U/mg parent derived urate oxidase and 45.11±1.52 U/mg mutant derived urate oxidase. It was also investigated that 0.5 % of sodium nitrate produced optimum yield of urate oxidase 12.90±1.49 U/mg and 38.35±1.77 U/mg from parent and mutant derived strains respectively. It was studied that the production of urate oxidase was inhibited by adding ammonium chloride in the fermentation medium (Fig 1 and 2).

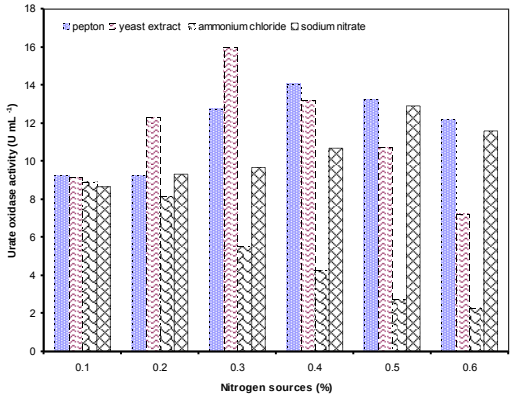

Fig. 1 comparison of the effect of different levels of nitrogen sources on the production of urate oxidase by parent culture

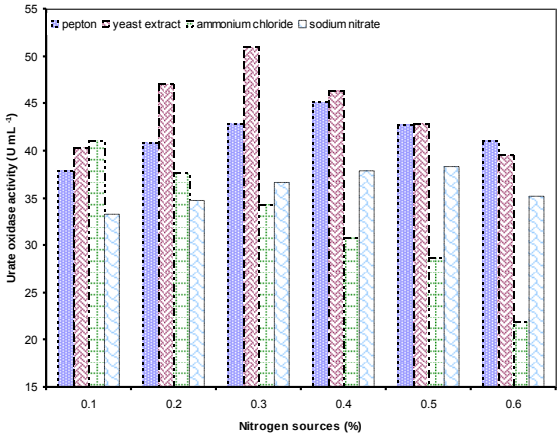

Fig. 2 comparison of the effect of different levels of nitrogen sources on the production of urate oxidase by mutant culture

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Comparison of different carbon sources

The effect of various carbon sources were compared and observed that sucrose (2%) was the best carbon source As the sucrose was added, the activity of parent and mutant derived urate oxidase was enhanced 19.48±1.26 U/mg and 68.05±3.50 U/mg respectively. It was studied that the activity of urate oxidase was maximum 15.43±1.38 U/mg and 49.00 ± 1.12 U/mg from parent and mutant derived strain respectively when 2.5 % maltose was added in the production medium. (Fig 3 and 4). At 3% glucose produced optimum yield of parent and mutant derived urate oxidase 15.24±1.34 U/mg and 45.69 ± 2.30 U/mg respectively but above this level the activity was decreased. Galactose (3%) was shown 42.83±1.32 U/mg and 14.46±1.21 U/mg activity of urate oxidase by mutant and parent strains respectively

In short, the nutritional requirements of mutant *Bacillus subtilis* observed to be the same as those of wild/parent strain. Zia *et al.* (2010) optimized the strains (parent and mutant) for

the hyperproduction of glucose oxidase and found that nutritional requirements of mutated and parental *Aspergillus niger* were the same. The present research conclusions are correlated to the above reported results. The detailed of the results are shown in figure 3 and 4.

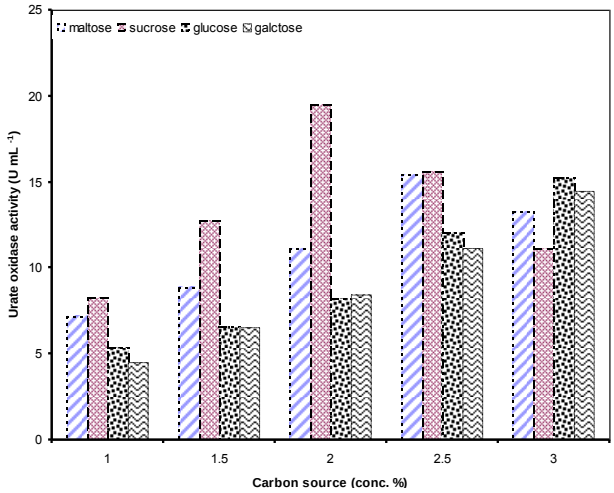

Fig. 3 Comparison the effect of different levels of carbon sources on the production of urate oxidase by parent culture

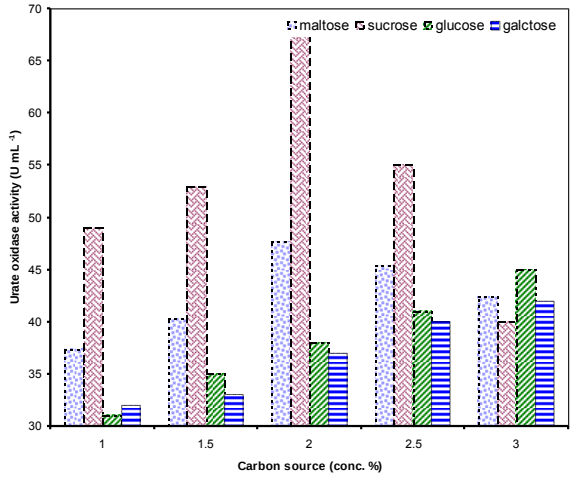

Fig. 4 comparison the effect of different levels of carbon sources on the production of urate oxidase by mutant culture

IV Discussion

Various workers induced chemical mutagenesis for hyperproduction of different enzymes and chemicals. Reference [16]; also induced chemical mutagenesis (ethyl methane sulfonate and MNNG) of *Cellulomonase* for achieving hyperxylanotic mutant. The mutated derived strain produced 2.5 times higher production of enzyme as compared to parent strain. It is necessary to optimize the culture conditions that was exhibited the real potential of the mutant and wild strains.

The results showed that optimum production of mutant derived urate oxidase was obtained as compared to parental type. The activity obtained by mutant and parent derived enzyme was 20.87 ± 1.06 U/mg and 3.33 ± 1.19 U/mg respectively. Reference [19] studied that urate oxidase activity was enhanced by using uric acid as a substrate. Reference [17] studied that *Mucor hiemalis* was produced urate oxidase in the medium containing uric acid as a substrate. Reference [18] optimized the substrate concentration and observed that uric acid (0.7%) increased the yield of urate oxidase. Our findings are coincident to several workers that used uric acid as a substrate for hyperproduction of the urate oxidase. Fermentation period also effect the activity of urate oxidase. Optimum production of parent derived urate oxidase (4.16±0.91 U/mg) and mutant derived enzyme (26.72±1.26 U/mg) was obtained after 36 hours. Reference [9] isolated urate oxidase producing bacterium from soil that yields highest activity of enzyme when the strain was incubated for 36 hour. Reference [18] identified that urate oxidase activity was peaked when *Bacillus thermocatenulatus* was cultured for 30-36 hours. Our findings are good coincident to the above reported values.

The parental and mutant strains were showed maximum production of the enzyme at pH 8.5. The results proved a good coincidence with the values that reported by various research workers that showed highest activity at pH 8.5 [19]-[21], [26]. When the fermentation medium was incubated at 35 ^{o}C then urate oxidase was obtained maximum activity from wild and mutant strain. Various investigators observed that optimum production of urate oxidase was obtained when the culture was worked at 30 ^{o}C [17], [18], [22]. Our findings differ from the values that reported earlier due to difference in environmental conditions and choice of organisms.

It was studied that 3% inoculum was used for the optimum production of parent and mutant derived enzyme. Reference [3] optimized the parameters and showed that inoculum size had great influence on the production of urate oxidase.

The effect of various nitrogen sources were observed to obtain maximum yield of urate oxidase. It was studied that the production of urate oxidase was increased from parent and mutant derived strains by the addition of peptone (0.4%), yeast extract (0.3%) and sodium nitrate (0.5%). It was studied that urate oxidase yield was inhibited by the addition of ammonium chloride in the growth medium (table 3). The findings of present study are in agreement with reference [19]; who determined that no activity of urate oxidase was detected from *R. oryzae* when ammonium chloride was added in the basal medium.

The effect of different carbon sources were also studied individually to obtain optimum production of parent and mutant derived urate oxidase. It was observed that urate oxidase production was enhanced by the addition of maltose (2.5%), sucrose (2%), glucose (3%) and galactose (3%). Reference [17] optimized the composition of fermentation medium and observed the activity of urate oxidase was increased by using (6%) maltose. The findings of present work are differing from the above reported value due to difference in environmental conditions. Reference [23]; who

was 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. Reference [21] dissolved 0.2 % sucrose in the czapek Dox medium for the optimum yield of urate oxidase. Reference [22] 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. Reference [9] 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.

In short, the nutritional requirements of mutant *Bacillus subtilis* observed to be the same as those of wild/parent strain. Reference [14] optimized the strains (parent and mutant) for the hyperproduction of glucose oxidase and found that nutritional requirements of mutated and parental *Aspergillus niger* were the same. The present research conclusions are correlated to the above reported results.

IV. Conclusion

Mutated *Bacillus subtilis* yield highest production of urate oxidase as compared to wild type strain. While, it was observed that the nutritional requirement for both the strains were same.

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