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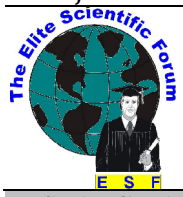

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RESEARCH ARTICLE

Bacillus subtilis Improvement through UV and Chemical Mutagenesis for Indigenously Hyperproduced Urate oxidase

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ABSTRACT
The main purpose of this research was to optimize the production of urate oxidase through mutagenesis of *Bacillus subtilis.* The organism was subjected to ultra violet irradiation and chemical mutagenesis. The strain was chemically mutated by using ethyl methane sulfonate and ethidium bromide. 2- thiouric acid was used as a selective marker to identify the UV and chemically mutated *Bacillus subtilis*. Ethyl methane sulfonate treated *B. subtilis* (180 minutes dose) 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), inoculum size (3%) enhanced the activity (42.77±0.36 U/mL) of mutant derived enzyme.

INTRODUCTION

Urate oxidase has a great significance for diagnosis and treatment of uric acid in body fluids and body tissues respectively. Uric acid breaks down into allantoin, hydrogen peroxide and carbon dioxide by the action of the said enzyme. The mechanism of the reaction is as follow.

Uric acid + 2 H_2O + O_2 ⟶ Allantoin + H_2O_2 + CO_2

The solubility of uric acid is ~ 11 mg/ dL which is considered to be a poor soluble as compare to the allantoin whose solubility is ~ 147 mg/ dL. So, it is readily excreted in urine. These products (Allantoin + H_2O_2 + CO_2) have been found in mammals (except primates) (Kai et al., 2008; Gianfrancesco et al., 2004), plants (Chen et al., 2008; Umamaheswari et al., 2007) and microbial cells (Lotfy, 2008; Ramazzina et al., 2006). During the vertebrate evolution, urate oxidase has been lost in the primates (Gianfrancesco et al., 2004; Zhang et al., 2010). These primates have a nonsense codon in the gene of urate oxidase that devoid of enzymatic activity. Urate oxidase gene is located at chromosome number 1with strand size 32,936 which is started from 84,830,640 and end 84,863,576 with exon count 9 (Abeles et al., 2007; Pillinger et al., 2007). It is a homotetrameric enzyme (34 kDa) having four identical active sites with two copper binding sites. These active sites are located at the interfaces between their subunits (Gabison et al., 2010). According to early studies, it was considered that it is a copper containing enzyme but later research revealed that isolated urate oxidase from *Aspergillus flavus* and *Bacillus subtilis* have no copper, which is proved that *Bacillus subtilis* has no any other transition ion (Imhoff et al., 2003). The said enzyme produced from *Bacillus subtilis* contains 300-400 amino acids with well conserved sequences (Colloc'h et al., 2006; Yamamoto et al., 1996).

The specificity of this enzyme towards uric acid (substrate) is high but it is not active towards substituted uric acids (Milena et al., 2003). Urate oxidase is usually extracted from internal animal organs, like kidney and liver by extraction and fractionation (Rajoka et al., 2006). These processes are complicated and the raw materials are very expensive, so produced enzymes by this procedure will be high

priced. In the last few decades, the interest was developed for the isolation of enzyme by the microbial fermentation (Wu et al., 2009; Aguilar et al., 2002).
Mutagenesis techniques have been used to improve the production of urate oxidase by several investigators through cloning. But this technique is very expensive and requires high technical facilities. While the techniques of ultraviolet radiations or chemical mutagens to induce mutation are very useful for the productions of many enzymes by various microbes. The advantages to adopt these methods, are their simplicity and low cost. Moreover, the screening method was used in the present research was very useful because it is simple and not required any detailed study of physiology and molecular biology of the organism being manipulated.

MATERIALS AND METHODS

All chemicals and reagents of analytical grade were used in this research, and mostly purchased from Sigma (Aldrich-Fluka, UK; Sigma-Aldrich, USA). All the experiments were carried out in triplicate flasks (n = 3).

Strain improvement techniques

The colonies of *Bacillus subtilis* were prepared in nutrient medium (pH: 8) by using 250 mL Erlenmeyer flasks in rotatory shaker at 120 rpm (Sanyo-GallenKemp, UK check the country nme).

Radiation mutagenesis

Mutagenesis by UV lamp

Bacillus subtilis colonies (1×10^7 spores same blunder not spores but CFU/mL) were mutated by using UV (λ_{360}) germicidal lamp of 20W (Phillips) to enhance the production of urate oxidase. The colonies (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.

Chemical mutagenesis

Ethyl methane sulfonate (Sigma-Aldrich, USA) and ethidium bromide (Sigma-Aldrich, UK Same company but different countries) were used to induce mutagenesis in *Bacillus subtilis* for hyperproduction of urate oxidase.

Mutagenesis by EMS and EB

The stock solutions of the chemicals (EMS and EB) were prepared separately buffer saline (0.5 mg/mL). Various time intervals (i.e 30, 60, 90, 120, 180 and 210 minutes) were selected for chemical mutagenesis. The stock solution of each chemical were added in 9 mL of nutrient broth medium which contained the colonies of *Bacillus subtilis* (1×10^7 see unit spores per mL) and then kept it at 37°C in water bath. After time interval of 30 minutes (30-210 minutes) the sample (1 mL) was withdrawn and was centrifuged thrice by using centrifuge machine (Mikro 20 Hettich) to remove the mutagen from the spore suspension at 10,000 rpm for 1 minute.

3Log kill mutant dose selection by kill/survival curve

The colonies 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 was 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 colonies (parental/wild colonies) were also plated on nutrient agar medium. All the processes were carried out in laminar air flow (Dalton, Japan) under strict aseptic conditions. Aluminum foil was used to cover the plates, and then kept these plates in an incubator at 30°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 (Khattab and Bazaraa, 2005; Petruccioli et al., 1999).
The colony forming units were calculated as follows:

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

Screening Methods

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°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 (≥2mm) than parental/wild type (2 mm zone size).

Mutant's isolation of selective marker

For the selection of the colonies which showed resistance to the catabolite repression, 2- thiouric acid was used as a selective marker. Those colonies which showed background growth were isolated and subjected to preliminary urate oxidase identification.

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. The strains which produced larger zone were picked, dissolved and homogenized in buffer and then filtered. The activity of

the urate oxidase was determined by using spectrophotometer (T-60, PG Instruments, UK) at λ_{293}.

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. 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 (30°C) of the medium was adjusted. Then the flasks were autoclaved. After cooling, the spore suspension (inoculum) was added aseptically 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°C in the shaker (Sanyo-GallenKemp, UK).

Optimization conditions for urate oxidase production

Various parameters (i.e substrate concentration, fermentation period, pH, temperature and inoculum size) were optimized to obtain the optimum production of urate oxidas. At optimized conditions the results were highly significant.

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. The assay for urate oxidase was performed by following the procedure of Worthington (1988).

RESULTS

Mutation induced by UV radiation

Different doses of UV radiation were compared after preparing kill/ survival curve to optimize the treatment (Fig. 1). A dose after 210 minutes was produced 82% killing ($0.5x10^{-2}$ CFU/mL) in the presence of selective marker where it produced 3 log kill as optimum dose (Fig. 2). It was observed that higher doses of UV radiations reduced the number of colonies and frequency of positive mutation. Five colonies that produced larger and darker zones of brown colored were picked and the activity of the urate oxidase was determined spectrophotometrically.

Mutation induced by chemicals

Chemicals (Ethyl methane sulfonate and ethidium bromide) were 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 (BSM-180) was produced 83% killing ($0.4x10^{-2}$ CFU/mL), which was proved to be the best (having ability to hyperproduced urate oxidase) by kill survival curve (Fig. 3, 4).

It has been proved that ethidium bromide is a strong mutagen. For hyperproduction of urate oxidase, *B. subtilis* was treated with EB for 150 minutes (BSE-150) which was producing 70% killing / 30% survival ($1.1x10^{-3}$ CFU/mL) as shown in figure 5 and 6. Five colonies after each chemical treatment that produced bigger and darker zones of brown colored were used to check the activity of the urate oxidase.

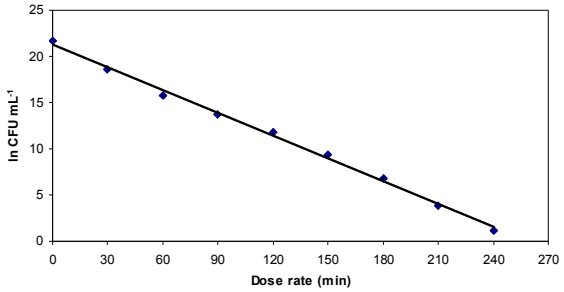

Fig. 1: Kill/survival curve for UV treated *Bacillus subtilis*

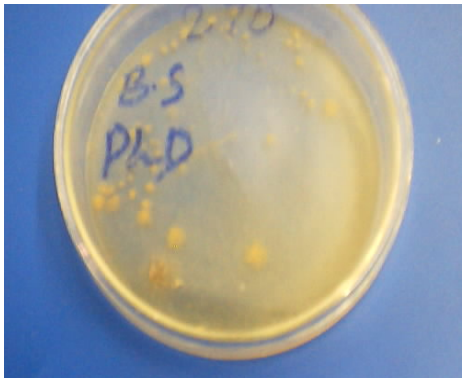

Fig. 2: Screening of urate oxidase hyperproduced by UV radiations induced mutants resistant to 2-thiouric acid

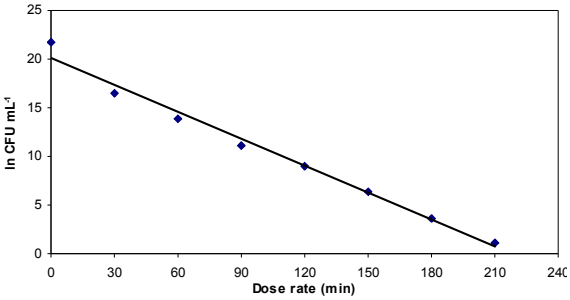

Fig. 3: kill/survival curve for ethyl methane sulfonate treated *Bacillus subtilis*

Enzyme Production in shake flask

Five selected mutants after treatment of UV and chemical mutagenesis of *Bacillus subtilis* were used for hyperproduction of urate oxidase. BSM-2 mutant producing 17.54 U/mL activity after 48 hours. It was also observed that all the selected mutants exhibited better mutation in the incoming gene mutant. The effect

of the medium composition on the activity of mutant derived urate oxidase was determined by performing a series of preliminary experiments in a sequential order.

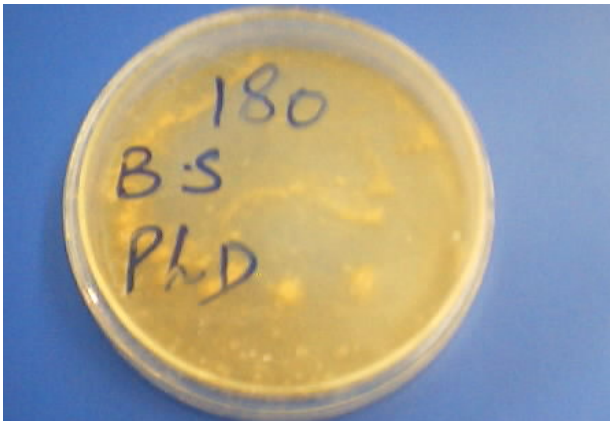

Fig. 4: Screening of urate oxidase hyperproduced by ethyl methane sulfonate treated mutants resistant to 2-thiouric acid

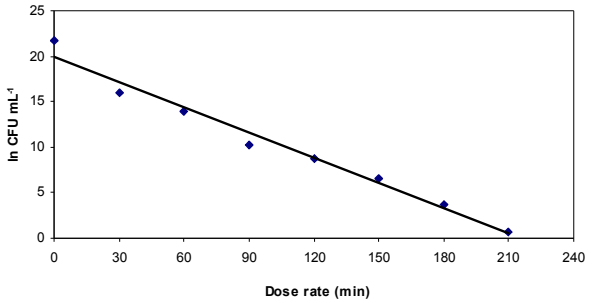

Fig. 5: kill / survival curve for ethidium bromide treated *Bacillus subtilis*

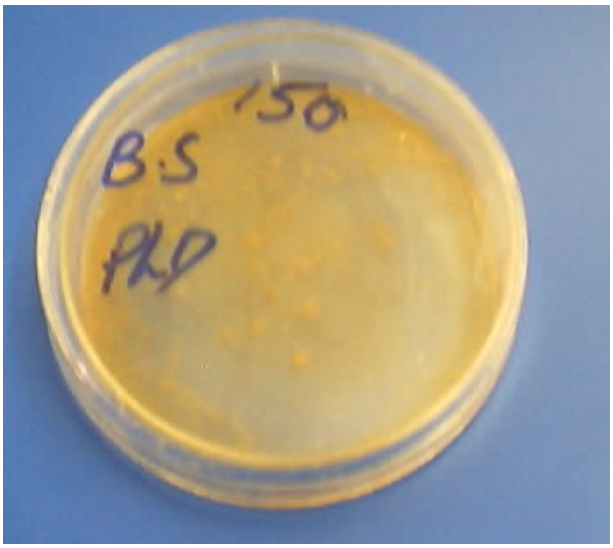

Fig. 6: Screening of urate oxidase hyperproduced by ethidium bromide treated mutants resistant to 2-thiouric acid

Effect of substrate

In the present study, uric acid was used as a substrate for the production of urate oxidase by mutated strain. Different concentrations of uric acid (substrate) were tested for optimum yield of the urate oxidase. It was observed that the mutated strain was achieved optimum activity when the fermentation medium containing 0.5% substrate at 30 ^{o}C, inoculated with 5 % spore suspension after fermentation period of 48 hours. The activity obtained by mutant derived urate oxidase was 20.87 ± 0.06 U/mL. The results showed that the activity of mutant derived urate oxidase was increased seven folds (Fig 7).

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 26.72 ± 0.26 U/mL by BSM-2 after 36 hours (Figure 8).

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 BSM-2 achieved optimum production of urate oxidase 31.87 ± 0.12 U/mL 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 (Figure 9).

Effect of temperature

When the fermentation medium was incubated at 35 ^{o}C for 36 hours at pH 8.5 then urate oxidase was achieved optimum activity 38.18 ± 0.51 U/mL from BSM-2 (Fig. 10).

Effect of Inoculum Size

Different levels of inocula were tested to increase the growth of B. subtilis for hyperproduction of urate oxidase. It was observed that 3 % inoculum was used for the maximum yield of mutant derived urate oxidase (BSM-2). Mutant strain was exhibited 42.77±0.36 U/mL activity of enzyme. 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 but after 3 mL when the concentration was increased it was reduced the biosynthesis of the enzyme (Fig. 11). Summary of urate oxidase production by BSM-2 is described in table 1.

Table 1: Summary of urate oxidase production by BSM-2

Parameters	Enzyme activity (U/mL)
Substrate (0.5%)	20.87±0.06
Fermentation period (36 h)	26.72±0.26
pH (8.5)	31.87±0.12
Temperature (35 ^{o}C)	38.18±0.51
Inoculum size (3%)	42.77±0.36

(n=3)

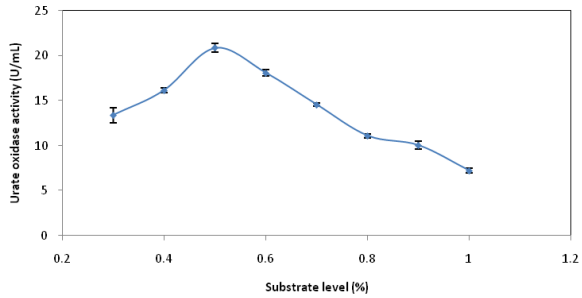

Figure 7: Effect of substrate level on the production of urate oxidase by *Bacillus subtilis* under optimum fermentation conditions (n = 3)

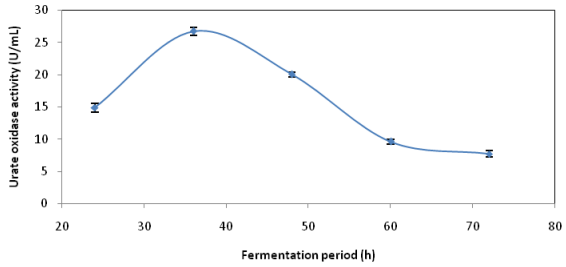

Figure 8: Effect of fermentation period on the production of urate oxidase by *Bacillus subtilis* under optimum fermentation conditions (n = 3)

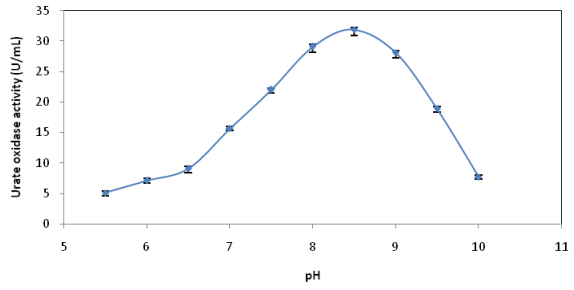

Figure 9: Effect of pH on the production of urate oxidase by Bacillus subtilis under optimum fermentation conditions (n = 3)

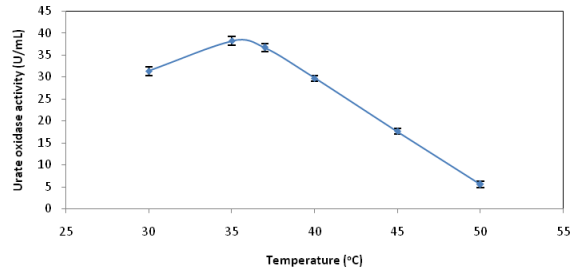

Figure 10: Effect of temperature on the production of urate oxidase by *Bacillus subtilis* under optimum fermentation conditions (n = 3)

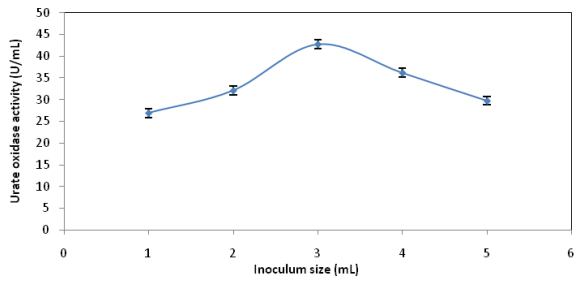

Figure 11: Effect of inoculum size on the production of urate oxidase by *Bacillus subtilis* under optimum fermentation conditions (n = 3)

DISCUSSION

The genotype of the mutant strain may be changed after induced mutation as compared to its parental strain. So, it is necessary to optimize the culture conditions that was exhibited the real potential of the mutant and parental/wild strains. It was observed that all selected mutants exhibited significantly ($P \leq 0.05$) better results as compared to the wild strain.

To select a suitable concentration of substrate (uric acid) for the microbial growth and hyperproduction of enzyme is a critical factor. The results exhibited that optimum production of enzyme activity was obtained with 5% uric acid by mutated *B. subtilis* (BSM-2) (20.87±0.06 U/mL) as compared to wild type (3.33±0.19 U/mL). Saeed et al. (2004) isolated and purified urate oxidase from Pseudomonas aeruginosa and revealed that when uric acid is used as a substrate, the specific activity of the purified enzyme was found to be 636.36 U/mg. Peter et al. (2002) studied that urate oxidase activity was enhanced by using 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. Our results are coincident to several investigators in that manner that they used uric acid as a substrate for hyperproduction of urate oxidase (Yazdi et al., 2006; Xue et al., 2005; Fattah and Hamad, 2002).

The results showed that the liquid state fermentation with 0.5 % uric acid (optimum substrate level) was produced maximum yield 26.72±0.26 U/mL by BSM-2 after 36 hours. 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. Our findings are good coincident to the above reported values. Fattah and Hamad (2002) studied that the time required for optimum production of urate oxidase was 96 hours from Aspergillus terreus and Aspergillus flavus while 144 hours for Trichoderma sp. These values that reported earlier are different to our results may be due to the use of different organisms, etc.

The metabolic activities of microbes are very sensitive to change in pH. The optimum pH for the production of BSM-2 (31.87±0.12 U/mL) was found to be 8.5. The results proved a good coincidence with the values that reported references. Kai et al. (2008) and Liu et al. (1994) observed that highest activity of urate oxidase was obtained at pH 8.5. 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 as the utilization of organisms etc.

When the fermentation medium was incubated at 35 ^{o}C then urate oxidase was achieved maximum activity 38.18±0.51 U/mL from mutant strain. 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 may be due to the difference in environmental conditions, organisms etc.

Different levels of inocula were tested to increase the growth of B.subtilis for hyperproduction of urate oxidase. It was observed that 3 % inoculum was used for the maximum yield (42.77±0.36 U/mL) of mutant derived urate oxidase. Chen et al. (2008) optimized the parameters in the shaking flask culture revealed that inoculum size had great influence on the production of urate oxidase.

Conclusion

Ethyle methane sulfonate treated *Bacillus subtilis* (BSM-2) was proved to be the best mutant among all the different mutants for hyperproduction of urate oxidase. With the help of selected mutant, the production of urate oxidase was further enhanced by optimization of fermentation medium. It was observed that when the fermentation medium was incubated at 35 $^{\circ}$C for 36 hours at pH 8.5 with 3% inoculum size then urate oxidase achieved optimum activity.

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