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Non-recombinant Mutagenesis of *Bacillus subtilis* MTCC 2414 for Hyper Production of Laccase

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ABSTRACT

The advent of protein engineering and sophisticated molecular technologies has opened possibilities for screening variants of enzymes and tailor-made proteins from microorganisms with enhanced production yields which may be of interest for specific commercial applications. During this investigation the indigenous strain of *Bacillus subtilis* MTCC 2414 procured from Institute of Microbial Technology (IMTECH) Chandigarh, India was improved for enhanced laccase production by using physical mutagen (ultraviolet rays) and chemical mutagens *viz.*,0.5 mg/ml ethidium bromide and 0.5 mg/ml ethyl methane sulfonate. Mutant GCBR 4 with hyper laccase production (178.8 ± 3.67 U/ml)was obtained after treating wild strain for 80 min with ethidium bromide. The effectiveness of hyper laccase producing mutant GCBR4 indicates its possible applicability in various biotechnological and industrial processes.

INTRODUCTION

Enzymes are delicate protein molecules ubiquitous in occurrence, and are essential for cell growth and differentiation[1, 2].The extracellular enzymes are of commercial value and find multiple applications in various industrial sectors[3]. Although there are many microbial sources available for producing extracellular enzymes, only a few are recognized as commercial producers[4]. Of these, strains of *Bacillus sp*. dominate the industrial sector[5].Laccase(p-diphenol: oxygen oxidoreductase; EC 1.10.3.2; also known as p-diphenol oxidase; *p-DPO;* p-diphenolase) is a copper-containing hydrolase[6], which has an ability to catalyze the oxidation of a wide variety of organic and inorganic compounds by coupling it to the reduction of oxygen to water[7].

There is an increasing demand for laccase in the market for various applications such as biopulping[8], biobleaching[9], denim bleaching[10], organic synthesis[11], decolorization[12], dechlorination of xenobiotic compounds[13], bioremediation [14], plant fiber modification, ethanol production, wine stabilization, baking[15], cosmetic and dermatological preparations [16], biofuel cells *etc*.[17].Many microorganisms like species of *A. lipoferum*[18], *Aquifexaeolicus*[19], *Azospirillumlipoferum*[20], *B. subtilis*[21], *B. sphaericus*[22], *B. halodurans* LBH-1 [23], *Escherichia coli* [24], *Marinomonasmediterranea* [25], *Oceanobacillus iheyensis*[26], *P. maltophila*[27], *P. syringae*[28], *P. fluorescens* GB-1[29], *P. putida* GB-1[30], *P. desmolyticum* NCIM 2112[31], *P. aerophilum*[32], *Streptomycetes sp.*[33], *Thermusthermophilus* TTC1370[34], *Xanthomonascampesteris*[35] have been evaluated for the production of laccase.

Microbes serve as the preferred source of laccase because of their rapidgrowth, the limited space required for their cultivation, and the ease with which they can be genetically manipulated to generate new enzymes with altered properties[2]. However, high cost and low yields of laccase have been the main problems for its industrial production. Therefore, there is a great need to develop new strains with inexpensive mutagens that provides a high laccase yield.

For industrial use enzyme must be produced at low cost and should be reusable and reproducible[2]. To achieve this many techniques has been developed for strain improvement. Strain improvement is usually done by mutating the microorganism that produces the enzyme by techniques such as classical mutagenesis, which involves exposing the microbe to physical mutagens such as X-rays, ?-rays, UV rays, etc., and chemical mutagens such as NTG, EMS, EtBr, etc.[2,36].

There are a great number of literatures reported to use the strain improvement process for producing various industrial enzymes like lipase, chitinase, cellulase, glucoamylase, protease and fibrinolytic protease [2,37-39]**.** But there was no report available on mutation studies of *Bacillus subtilis* MTCC 2414 for laccase production. The present investigation was undertaken to improve the laccase quantitatively from *Bacillus subtilis* MTCC 2414 strain through exposure to physical and chemical mutagens.

MATERIALS AND METHODS

Experimental Chemicals

All chemicals and reagents of analytical grade were used in this research and are mostly purchased from sigma USA and Hi media Mumbai. All the experiments were conducted in triplicate and the mean values are considered.

Micro organism and Inoculum Preparation

The *Bacillus subtilis* MTCC 2414 strain that produces laccase was employed in the present study. The organism was procured from IMTECH, Chandigarh, India. Stock cultures were maintained in nutrient broth medium with 70% glycerol, cultures were preserved at -20°C[40]. The inoculum was prepared by transferring a loopful of stock culture (*Bacillus subtilis* MTCC 2414) to a certain volume (100 ml) of sterile nutrient broth, stock medium, then incubated it overnight at 37°C on a rotary shaker 200 rpm, before being used for inoculation[2]. A stock suspension was prepared and adjusted to 7×10^3 cells/ml.

Mutation and Selection

Mutagenesis by UV Irradiation

Four ml of the spore suspension containing 1×10^7 spores/ml was pipetted aseptically into sterile petridish of 80 mm diameter having a flat bottom. The exposure of spore suspension to UV light was carried at a distance of 30 cm away from the UV lamp (15 W, 2537Å). The exposure times were 30 to 90 min [2,41-46].Each UV exposed spore suspension was stored in the dark overnight to avoid photo reactivation, then was serially diluted in saline and plated in agar medium using 0.04% guaiacol. The plates were incubated for 24h at 37°C and the numbers of colonies on each plate was counted. Each colony was assumed to be formed from a single spore. Mutants for hyper production of laccase were detected visually by the intensity of zone sand were further selected based on their capacity of enzyme production in the liquid medium.

Mutagenesis by Ethidium bromide (EtBr)

Four ml of the UV mutated spore suspension containing 1×10^7 spores/ml was pipetted aseptically into 15 ml of (0.5 mg/ml) Ethidium bromide (EtBr) solution and incubated at 37°C. The sample (2 ml) of this solution was taken at intervals of 30 to 270 min and centrifuged immediately at 10,000 rpm for 5 min at 37°C [2,43,47-50]. The supernatant was decanted and the cell pellet obtained was resuspended in 5 ml saline to stop the reaction. The washed cell suspension was serially diluted in saline and plated on agar medium using 0.04% guaiacol. The plates were incubated for 24h at 37°C and the number of colonies on each plate was counted. Each colony was assumed to be formed from a single spore. Mutants for hyper production of laccase were detected visually by the intensity of zones and were further selected based on their capacity of enzyme production in the liquid medium.

Ethyl Methane Sulfonate (EMS) Mutagenesis

Four ml of the EtBr mutated spore suspension containing 1×10^7 spores/ml was pipetted aseptically into 15 ml of (0.5 mg/ml) ethyl methane sulfonate (EMS) solution and incubated at 37°C. The sample (2 ml) of this solution was taken at intervals of 30 to 270 min and centrifuged immediately at 10,000 rpm for 5 min at 37°C [2,43,44,49-51]. The supernatant was decanted and the cell pellet obtained was re suspended in 5 ml saline to stop the reaction. The washed cell suspension was serially diluted in saline and plated on agar medium using 0.04% guaiacol. The plates were incubated for 24h at 37°C and the number of colonies in each plate

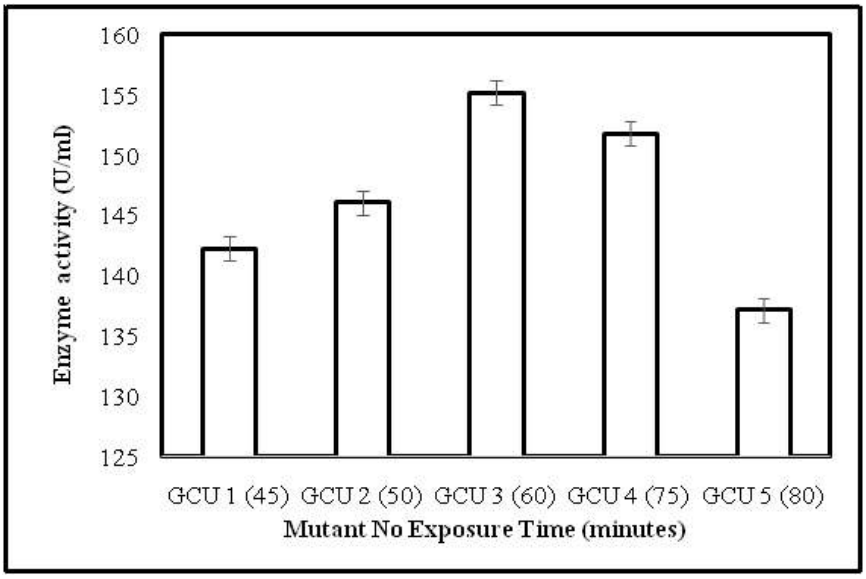

Fig 1. : Mutants after the treatment with UV radiations

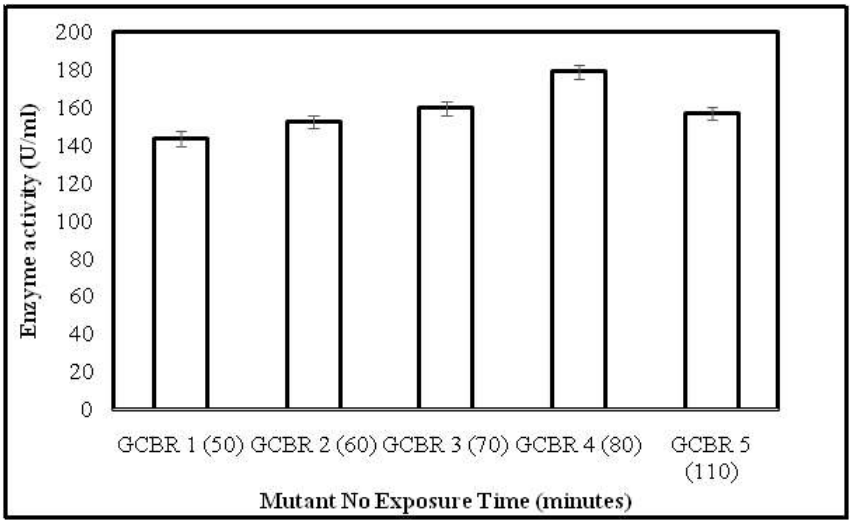

Fig 2. : Mutants after the treatment with EtBr

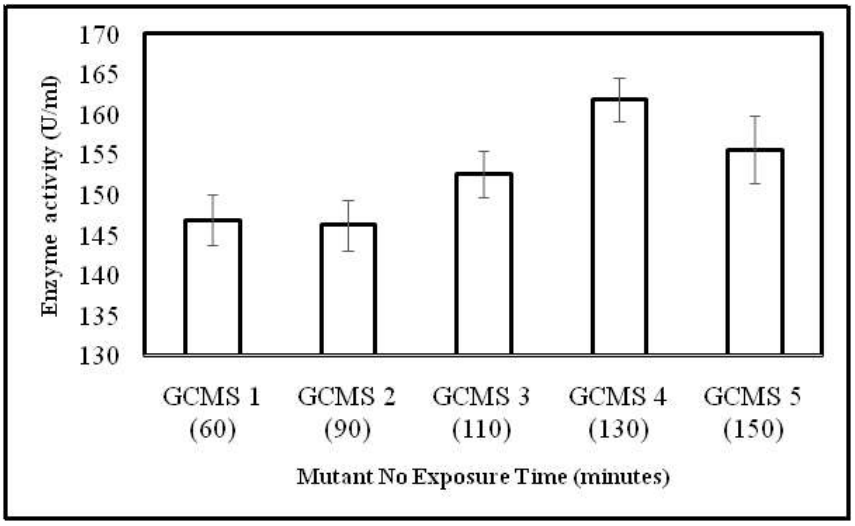

Fig 2. : Mutants after the treatment with EMS

were counted. Each colony was assumed to be formed from a single spore. Mutants for hyper production of laccase were detected visually by the intensity of zones and were further selected based on their capacity of enzyme production in the liquid medium.

Production of Laccase by Shake Flask Fermentation

Hyper laccase producing mutant strains of ultra-violet rays, ethyl methane sulfonate and ethidiumbromide were inoculated separately in the fermentation medium. The medium used for the production of laccase contains (g/l)1.0 glucose, 1.0 peptone, 0.2 K_2HPO_4, 0.04 $CaC1_2$, 0.02 $MgSO_4$, 0.0002 $FeSO_4$, 0.001 $ZnSO_4$ and 0.02 guaiacol (Hi media, Mumbai, India) as inducer for laccase production. The pH 6 of the medium was adjusted with 1N HC1/NaOH. Two percent (v/v) of 24h old inoculum suspension was transferred to 50 ml of growth medium in 250 ml Erlenmeyer flasks. These flasks were then placed in the rotary incubator shaker (Lab top) rotating at 200rpm for 24h at 37°C. After the completion of fermentation the whole fermentation broth was centrifuged at 10,000 rpm at 4°C for 10 min and the clear supernatant (crude enzyme) was used for the estimation of laccase. All the experiments were run parallel in triplicate.

Determination of Laccase Activity

Laccase activity was measured by monitoring the oxidation of 1mM guaiacol (Hi media, Mumbai, India) buffered with 0.2 M sodium phosphate buffer (pH 6) at 420 nm for 1 min. The reaction mixture (900 µl) contained 300 l of 1 mM guaiacol, culture filtrate, and 0.2 M sodium phosphate buffer (pH 6). One unit of the enzyme activity was defined as the amount of enzyme that oxidized 1 mol of guaiacol per minute. The enzyme activity was expressed in U/ml [52].

RESULTS

Improvement of Strain by Physical (UV Radiation) Mutagenesis

Data of Fig: 1shows the production of laccase by UV treated strains of *Bacillus subtilis* MTCC 2414. The parental strains of *Bacillus subtilis* MTCC 2414 was subjected to UV treatment for different time intervals i.e. from 30 to 90 min. Of all the isolates investigated, maximum enzyme production (155.2 ± 1.19 U/ml/min) was obtained by GCU 3 which was selected after 60

min of UV treatment.

Improvement of Strain by Ethidium Bromide (EtBr) Mutagenesis

The mutant strains of *Bacillus subtilis* MTCC 2414 was selected after the treatment of the parental strain with EtBr (Fig: 2). The GCU 3 strain of *Bacillus subtilis* MTCC 2414 was subjected to EtBr treatment for different time intervals i.e. 30 to 270 min. Of all the isolates investigated, maximum enzyme production (178.8 ± 3.67 U/ml/min) was obtained by GCBR 4.

Improvement of Strain by Ethyl Methane Sulfonate (EMS) Mutagenesis

The strains of *Bacillus subtilis* MTCC 2414 were screened after the chemical treatment with EMS (Fig: 3). The GCBR 4 strains of *Bacillus subtilis* MTCC 2414 was subjected to EMS treatment for 30 to 270 min. Of all the isolates investigated, maximum enzyme production (161.8 ± 2.76 U/ml/min) was obtained by GCMS 4.

DISCUSSION

Five UV treated mutant strains of *Bacillus subtilis* MTCC 2414 were isolated on the basis of a bigger zone of guaiacol oxidation in the petri plates and named as GCU isolates. These strains were screened for laccase production under submerged fermentation. Of all the isolates investigated, maximum enzyme production (155.2 ± 1.19 U/ml/min) was obtained by GCU 3 which was selected after 60 min of UV treatment. The production of enzyme following the growth of the organism was found to be highly significant than other mutant derivatives. But this mutant was not stable. It may be due to the reason that the mutant produced by UV irradiations had undergone back mutations when they were exposed to light [41]. Five EtBr treated mutant strains of *Bacillus subtilis* MTCC 2414 was selected on the basis of a bigger zone of guaiacol oxidation in the petriplates and named as GCBR isolates. Further screening of the strains for laccase production under submerged fermentation. Of all the isolates investigated, maximum enzyme production (178.8 ± 3.67 U/ml/min) was obtained by GCBR 4. Five EMS treated mutant strains of *Bacillus subtilis* MTCC 2414 was isolated on the basis of a bigger zone of guaiacol oxidation in the petriplates and named as GCMS isolates. This mutant showed improvement in the production of the enzyme. Of all the isolates investigated, maximum enzyme production (161.8 ± 2.76 U/ml/min) was obtained by GCMS 4.

CONCLUSION

The search for promising strains of laccase producers is a continuous process. In this study the wild strain of *Bacillus subtilis* MTCC 2414 was improved for enhanced laccase production by using physical and chemical mutagens.GCBR 4 with hyper laccase production (178.8 ± 3.67U/ml/min) was obtained after 80 min treating wild strain with Ethidium bromide. Finally, from the above results it was concluded that *Bacillus subtilis* MTCC 2414 mutant strain was developed by using EtBr treatment remained as a stable mutant after multiple culture cycles.GCBR 4 mutant showed 2 fold higher laccase production than the wild strain in flask fermentation. Thus the selected mutant has potential in minimizing the cost of enzyme for its biotechnological applications.

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