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Bioparametric Investigation of Mutant *Bacillus subtilis* MTCC 2414 Extracellular Laccase Production under Solid State Fermentation

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Abstract

This work has been undertaken to investigate the bio parameters such as various substrates, initial moisture level, inoculum size, pH, incubation temperature, incubation period, metal ions and nitrogen sources effect on the production of laccase in solid-state fermentation using mutant *Bacillus subtilis* MTCC 2414. The laccase production was observed with a sesame oil cake (183.32 ± 0.29 U/g), initial moisture level 80% (189.28 ± 0.52 U/g), inoculum size 1.5% (196.12 ± 0.26 U/g), initial pH 8 (215.20 ± 0.48 U/g), incubation temperature 37°C (225.80 ± 0.52 U/g), incubation period 48h (258.80 ± 0.29 U/g), $CuSO_4$ (263.16 ± 0.12 U/g) and yeast extract (268.14 ± 0.16 U/g) in the production medium.

Keywords: Bioparameters, solid-state fermentation, *Bacillus subtilis* MTCC 2414, laccase.

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).

Solid state fermentation (SSF) has been known for centuries and was used successfully in the production of oriental foods (3). It has gained much importance in the production of microbial enzymes due to several economic advantages over conventional submerged fermentation (SmF) (3). Several reports on SSF have been published on the production of fine chemicals (18-22), enzymes (3, 23, 24), antibiotics (25-27), and immune suppressants (28-30). Solid state processes are of special economic interest for countries with an abundance of biomass and agro-industrial residues, as these can be used as cheap raw materials (3). Many microorganisms like species of *A.lipoferum* (31-33), *B.subtilis* (34), *B.sphaericus* (35), *B.halodurans* LBH-1 (36), *E.coli* (37), *M.mediterranea* (38), *O.iheyensis* (39), *P.maltophila* (40), *P.syringae* (41), *P.fluorescens* GB-1 (42), *P.putida* GB-1 (43), *P.desmolyticum*

NCIM 2112 (44), *P.aerophilum* (45), *Streptomycetes sp.* (46), *T.thermophilus* TTC1370 (47), *X.campesteris* (48) have been evaluated for the production of laccase.

High cost and low yields of commercial enzymes have been the main problems for its industrial production. Therefore, there is a great need to develop a new medium with inexpensive substrates that provides a high enzymes yield. Solid state fermentation has generated much interest because it reduces manufacturing cost by utilizing unprocessed or moderately processed raw materials (3, 49). There are a great number of literatures reported on the use of sawdust, coconut flesh, groundnut shell, sugarcane bagasse, banana skin, rice straw, orange bagasse, tea waste, palm oil waste, corn, wheat bran, barley as substrates for the solid state production of laccase (50, 51).

However, utilization of sunflower oil cake, sesame oil cake, cotton seed meal, apple pomace and soybean meal for the production of laccase has not been reported using the SSF. India is the largest producer of apple, cotton, soybean and rice in the world (52, 53). These relatively low priced agro-industrial residues, containing abundant nutrients (hemicellulose, cellulose, proteins and starch), have a great potential to be utilized as alternative substrates for fermentation. In the present investigation, the productivity of extracellular laccase by mutant *Bacillus subtilis* MTCC2414 using solid agro-industrial residues such as sunflower oil cake, sesame oil cake, cotton seed meal, apple pomace and soybean meal was evaluated. In addition, the culture conditions as initial moisture content, initial pH, inoculum size, incubation temperature and incubation time as well as the extra supplementation of metal ions and nitrogen sources were optimized to maximize the extracellular laccase production.

Materials and Methods

Experimental Chemicals : Sesame oil cake, sunflower oil cake, cotton seed meal, soybean meal and apple pomace were obtained from local markets at Bangalore, India. They were dried in an oven at 60°C and ground in a hammer mill (54). The ground material was passed through 30- and 50-mesh sieves (3). The fraction which passed through the 30-mesh sieve, but retained by the 50-mesh sieve was collected and used as basic fermentation media. 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 mean values are considered.

Microorganism and Inoculum preparation : The mutant *Bacillus subtilis* MTCC 2414 strain that produces laccase was employed in the present study. The organism was mutated by UV irradiation, Ethyl methyl sulphonate (EMS) and Ethedium bromide (EtBr) for hyper production of the enzyme (2, 54-63). Stock cultures were maintained in nutrient broth medium with 70% glycerol, cultures were preserved at -20°C (64). The inoculum was prepared by transferring a loopful of stock culture (mutant *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.

Solid state fermentation : Ten grams of sesame oil cake, sunflower oil cake, cotton seed meal, soybean meal and apple pomace were taken into separate 250 ml Erlenmeyer flasks and 2 ml of salt solution [0.2% K_2HPO_4, 0.04% $CaC1_2$, 0.02% $MgSO_4$, 0.0002% $FeSO_4$, 0.001% $ZnSO_4$] was added. Distilled water was added in such a way that the final substrate moisture content was 50%. After sterilization by autoclaving, the flasks were cooled to room temperature and inoculated with 1% (v/w) inoculum of 24h culture and incubated at 37°C for 48h.

Estimation of different moisture contents : The moisture content of the substrates was estimated by drying 10 g of substrates to constant weight at 105°C and the dry weight was recorded. To fix the initial moisture content of the solid medium, the substrates were soaked with desired quantity of additional water (3, 65). After soaking, samples were dried again as described above and percent of moisture content was calculated as follows,

Percent of moisture content (initial) of solid medium =

$$\frac{(\text{Wt. of the Substrate - Dry wt. of Substrate})}{\text{Dry wt. of Substrate}} \times 100$$

Measurement of pH : The pH was determined using 1g of fermented material in 10ml of distilled water, and then the mixture was agitated. After 10min, the pH was measured in the supernatant using a pH meter (3).

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 µl of 1 mM guaiacol, 300 µl of culture filtrate, and 300 µl of 0.2 M sodium phosphate buffer (pH 6). One unit of the enzyme activity was defined as the amount of enzyme that oxidized 1 µmol of guaiacol per minute. The enzyme activity was expressed in U/ml (66).

Bioparametric Investigation on Extracellular Laccase Production under Solid State Fermentation

Effect of different solid substrates : In SSF, the solid substrate not only serves as an anchor, but also supplies nutrients to the organism. The chemical composition of the substrate is of critical importance. The ideal solid substrate is the one that provides maximum nutrients to the microorganism for its optimal growth and metabolic function. The solid substrates, sunflower oil cake, sesame oil cake, apple pomace, cotton seed meal and soybean meal have been identified as ideal substrates and used to study their effect on the production of extracellular laccase. Each substrate (10g) was taken into a separate 250 ml Erlenmeyer flask and 2 ml of salt solution [0.2% K_2HPO_4, 0.04% $CaC1_2$, 0.02% $MgSO_4$, 0.0002% $FeSO_4$, 0.001% $ZnSO_4$] was added. Distilled water was added in such a way that the final substrate moisture content was 50%. After sterilization by autoclaving, the flasks were cooled to room temperature and inoculated with 1% (v/w) inoculum of 24h culture and incubated at 37°C for 48h (3). The samples were assayed as per above mentioned protocol (66). The best solid substrate achieved by this step for the maximum production of laccase was fixed for the subsequent experiments.

Effect of different initial moisture contents : To investigate the influence of the initial total moisture content of the substrate (sesame oil cake), the fermentation was carried out under various initial moisture contents (20, 30, 40, 50, 60, 70, 80, 90 and 100%) of substrate, which was adjusted with distilled water (3). The other fermentation conditions were sesame oil cake as substrate, 1% inoculum level, 2ml salt solution and the fermentation was carried out for 48h at 37°C. The samples were assayed as per above mentioned protocol (66). The optimum initial moisture content of solid substrate achieved by this step for maximum laccase production was fixed in subsequent experiments.

Effect of different inoculum levels : Various inoculum levels were tried (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5%) to study their effect on laccase production (3). The fermentation was carried out using sesame oil cake as substrate. The other conditions were moisture content 80%, salt solution, 2ml, incubation temperature 37°C and incubation period 48h. The samples were assayed as described earlier (66). The optimum inoculum level achieved by this step was fixed in subsequent experiments.

Effect of different initial pH : pH varying from 3.0 to 11.0 (adjusted with 1N HCl or 1N NaOH) was used to optimize initial pH of the basal media (3). The fermentation was carried out at 37°C to study their effect on laccase production. The other conditions were sesame oil cake as substrate, 1.5% inoculum level, moisture content of 80%, salt solution 2ml, incubation temperature 37°C and the fermentation time of 48h. The samples were assayed as described earlier (66). The optimum pH achieved by this step was fixed in subsequent experiments.

Effect of different incubation temperatures : The fermentation was carried out at various temperatures such as 20, 25, 30, 37, 40, 45, 50°C (3) to study their effect on laccase production. The fermentation was carried out using the above optimized conditions viz., sesame oil cake as substrate, moisture content 80%, salt solution 2ml, pH8 and inoculum level 1.5% for 48h. The samples were assayed as described earlier (66). The optimal incubation temperature obtained in this step was used in the subsequent experiments.

Effect of different incubation periods : Different incubation periods (48, 72, 96, 120, 144, 168, 192, 216 and 240h) were employed to study their effect on laccase production (3). The fermentation was run using the above optimized conditions viz., substrate sesame oil cake, moisture content 80%, salt solution 2ml, inoculum level 1.5%, pH8 and incubation temperature of 37°C. The samples were assayed as described earlier (66). The optimum incubation period achieved by this step was fixed in subsequent experiments.

Effect of different metal ions and inorganic nitrogen sources : Different metal ions and inorganic nitrogen sources (0.5%) K_2HPO_4, $CaC1_2$, $MgSO_4$, $FeSO_4$, $ZnSO_4$, KNO_3, NH_4Cl, $(NH_4)_2HPO_4$, NH_4NO_3, $CuSO_4$ and $(NH_4)_2SO_4$) were employed to study their effect on laccase production (3). The fermentation was carried out using the above optimized conditions viz., substrate sesame oil cake, moisture content 80%, salt solution 2ml, inoculum level 1.5%, pH8, incubation period 48h and incubation temperature of 37°C. The samples were assayed as described earlier (66). The optimum enzyme production was achieved by this step was fixed in subsequent experiments.

Effect of different organic nitrogen sources : Different organic nitrogen sources (Casein, Yeast extract, Peptone, Serine, Histidine and Aspartate 1%) were employed to study their effect on laccase production (3). The fermentation was carried out using the above optimized conditions viz., substrate sesame oil cake, moisture content 80%, salt solution 2ml, inoculum level 1.5%, $CuSO_4$ (0.5%), incubation period 48h, pH8 and incubation temperature of 37°C. The samples were assayed as described earlier (66). The optimum enzyme production was achieved by this step was fixed in subsequent experiments.

Results and Discussion

Evaluation of different agro-industrial material for extracellular laccase production : The fermentation profile of laccase production in SSF varied with the type of agro material used. Highest laccase production (183.32 ± 0.29 U/g substrate) was observed with a sesame oil cake and the least laccase production (170 ± 0.32 U/g substrate) was observed with sunflower oil cake (Fig. 1). Variation was noticed with these materials. This could be attributed to solid materials dual role-supply of nutrients and anchor to the growing microbial culture which influence the microbial growth and subsequent metabolite production. Such substrate dependent microbial product yield variations were also reported in the literature. These results depict that the selection of an ideal agro biotech source for laccase production depends primarily on the availability of carbon and nitrogen source and thus screening of several agro-industrial residues are essential.

However, in the present study, among all studied materials, sunflower oil cake supported least production of laccase. This may be attributed to the fact that the strains used may vary in their

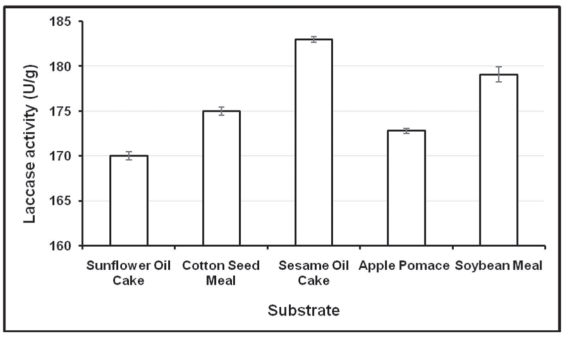

Fig: 1. Effect of various substrates on laccase production by *Bacillus subtilis* MTCC 2414 under SSF metabolic pattern compared to *Bacillus subtilis* MTCC 2414 used in the present study or carbon source material associated with sunflower oil may not be utilized by the *Bacillus subtilis* MTCC 2414. To evaluate the same, the hemicellulose and cellulose hydrolysis ability of the strain was investigated. This data further confirmed that high laccase associated with sesame oil cake were due to the maximum production of hemicellulose and cellulose hydrolyzing enzyme by the strain and as strain *Bacillus subtilis* MTCC 2414 is hemicellulase positive hence could utilize hemicellulose as carbon source. Hence, it could be concluded that the selected strain requires substrates that provide hemicellulose as its enzymatic machinery that hydrolyzes the polysaccharides present in substrates.

Effect of moisture level : The moisture level in the solid-state fermentation critically affects the process due to its interference in the physical properties of the solid particle. Increased moisture is believed to reduce the porosity of substrate, thus limiting the oxygen transfer. The decreased moisture content causes lower availability of media nutrients to the *Bacillus subtilis* MTCC 2414 resulting into the lower extent of production. The effect of total moisture content on laccase production for 20, 30, 40, 50, 60, 70, 80, 90 and 100% moisture was investigated. The result indicated that 80% moisture gave the higher laccase production during fermentation compared to other treatments. The maximum yield of laccase production (189.28 ± 0.52 U/g substrate) was obtained from 80% moisture (Fig. 2). The results from the previous study stated that the reduction in enzyme yield could occur with low and to higher moisture level.

Effect of inoculum size : The optimum inoculum size for laccase production (196.12 ± 0.26 U/g substrate) by *Bacillus subtilis* MTCC 2414 was 1.5 $\times 10^6$ CFU/g initial dry substrate (Fig. 3). Adequate inoculum can initiate fast growth and product formation, thereby reducing the growth of contaminants. A decrease in enzyme production was observed when the inoculums size was increased beyond the optimum level. Enzyme production attains its peak when sufficient nutrients are available for the biomass. Conditions with a misbalance between nutrients and proliferating biomass result in decreased laccase synthesis.

Effect of initial pH of the medium on laccase production : The initial pH of the fermentation media may change during fermentation because the substrates employed in SSF usually have the least buffering. Some samples from the fermented mass were aseptically withdrawn, homogenized and pH was checked. The pH of the medium during fermentation was found to be between 3.0 and 11.0, i.e. around acidic to alkaline conditions. The initial pH is another important factor which affects the growth and enzyme production during solid-state fermentation. The substrate was adjusted to different initial pH using 1N HCl and 1N NaOH prior

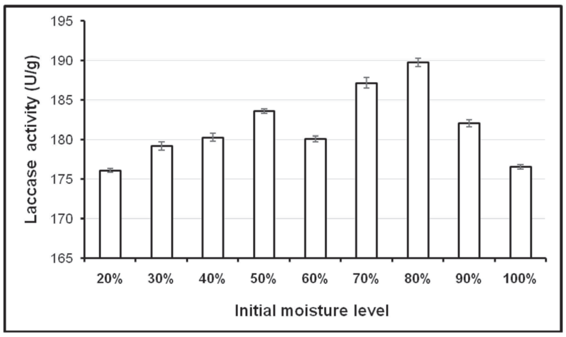

Fig. 2: Effect of various moisture level (%) on laccase production by *Bacillus subtilis* MTCC 2414under SSF

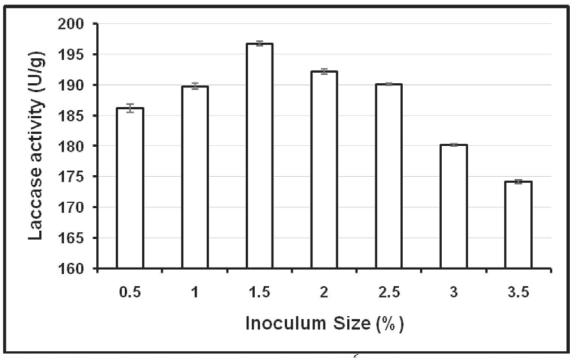

Fig. 3: Effect of various inoculum sizes ($\times 10^6$) on laccase production by *Bacillus subtilis* MTCC 2414 under SSF

to inoculation. The maximum yield of laccase production (215.20 ± 0.48 U/g substrate) was observed in an initial pH of 8.0 (Fig. 4).

Effect of temperature : The maintenance of an optimal process temperature is one of the major factors in the economics of a process. Temperature affects microbial cellular growth, spore formation, germination and microbial physiology, thus affecting product formation in turn. 37°C was found to be the optimum temperature in this case. The maximum yield of laccase production (225.80 ± 0.52 U/g substrate) was observed at 37°C (Fig. 5).

Effect of incubation period : Solid-state process was performed for various incubation periods. Remarkably maximal level (258.80 ± 0.29 U/g substrate) of laccase was achieved after 48h of fermentation (Fig. 6). Important rise in laccase yield with increased biomass was observed during 48 - 72h of fermentation cycle. Significant variation in laccase production was observed during different fermentation periods.

Effect of metal ions and nitrogen sources on laccase production : Generally, the high concentration of metal ions and nitrogen sources in media is effective in enhancing the production of laccase by *Bacillus subtilis* MTCC 2414. The protein content in sesame oil cake is very low so that the nitrogen levels as well as the commercial value decrease greatly. Hence, the exogenous addition of various metal ions, inorganic and organic nitrogen levels to the solid medium was studied.

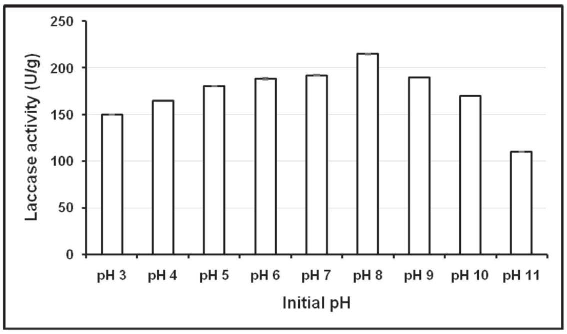

Fig. 4: Effect of various initial pH on laccase production by *Bacillus subtilis* MTCC 2414 under SSF

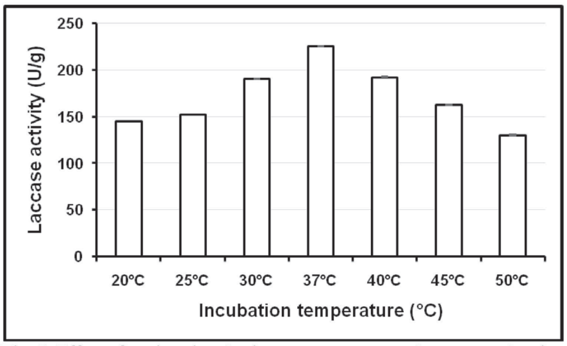

Fig. 5: Effect of various incubation temperatures on laccase production by *Bacillus subtilis* MTCC 2414 under SSF

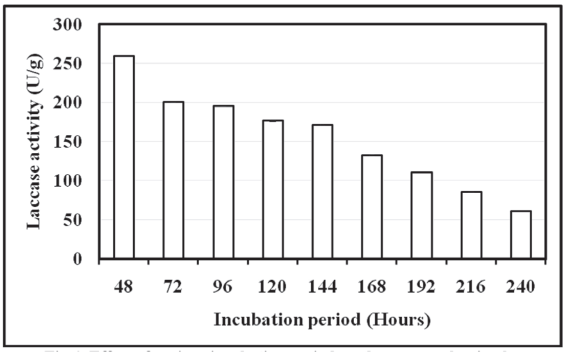

Fig.6: Effect of various incubation periods on laccase production by *Bacillus subtilis* MTCC 2414 under SSF

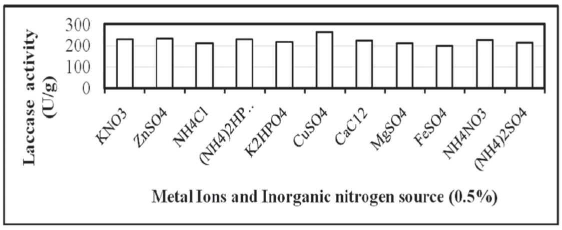

Fig. 7: Effect of various metal ions and inorganic nitrogen sources (0.5 %w/v) on laccase production by *Bacillus subtilis* MTCC 2414 under SSF

$CuSO_4$, $CaC1_2$, $MgSO_4$, $FeSO_4$, $ZnSO_4$, KNO_3 and K_2HPO_4 as metal ions, NH_4Cl, $(NH_4)_2HPO_4$, NH_4NO_3 and $(NH_4)_2SO_4$ as inorganic nitrogen sources and casein, yeast extract, peptone, serine, histidine and aspartate were used as complex organic nitrogen sources. Based on the results it was found that $CuSO_4$ was the best metal ion source (263.16

± 0.12 U/g substrate) and yeast extract was the best organic nitrogen source (268.14 ± 0.16 U/g substrate) its supplementation led to further increase in laccase production (Fig. 7 and 8).

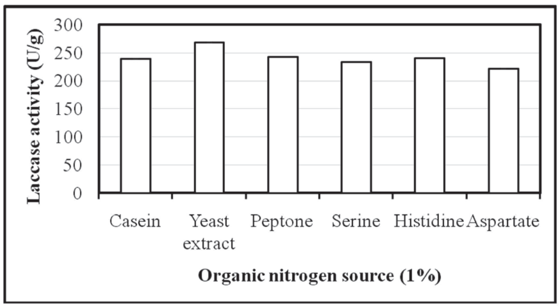

Fig. 8: Effect of various organic nitrogen sources (1 %w/v) on laccase production by *Bacillus subtilis* MTCC 2414 under SSF

Conclusion

The role of agro wastes in laccase production was identified and production parameters were determined. The sesame oil cake can be the least expensive alternative active substrates in the production of laccase. The optimal conditions for laccase production using SSF for sesame oil cake were initial pH (8), initial moisture level (80%), inoculum size (1.5 x 10^6 CFU/g), incubation temperature (37°C), incubation period (48h), $CuSO_4$ (0.5% w/v), yeast extract (1% w/v) respectively. Such processes would not only help in reducing the cost of production, but also pave the way in effective solid waste management. With the above encouraging leads, it will be interesting to study the sesame oil cake as substrate for the production of other enzymes from different microbes.

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References

1. Sharma, S., Aneja, M.K., Mayer, J., Scholter, M. and Munch, J.C. (2004). RNA fingerprinting of microbial community in the rhizosphere soil of grain legumes. Fems Microbiol Lett, 240: 181-186.
2. Venkatanagaraju, E. and Divakar, G. (2013a). *Bacillus Cereus* GD 55 Strain Improvementby Physical and Chemical Mutagenesis forEnhanced Production of Fibrinolytic Protease.International Journal of Pharma Sciences and Research, 4(5): 81-93.
3. Venkatanagaraju, E. and Divakar, G. (2013b). Optimization and Production of Fibrinolytic Protease (GD kinase) from Different Agro Industrial Wastes in Solid State Fermentation. Current Trends in Biotechnology and Pharmacy, 7(3): 763-772.
4. Gupta, R., Beeg, Q., Khan, S. and Chauhan, B. (2002a). An overview on fermentation, downstream processing and properties of microbial alkaline proteases. Appl Microbiol Biotechnol, 60(4): 381-395.
5. Gupta, R., Beeg, Q. and Loranz, P. (2002b). Bacterial alkaline protease: molecular approaches and industrial applications. Appl Microbiol Biotechnol, 59(1): 15-32.
6. Yoshida, H. (1883). Chemistry of lacquer (Urushi).Journal of the Chemical Society of London,43: 472-486.
7. Dwivedi, U.N., Singh, P., Pandey, V.P. and Kumar, A. (2011). Laccases production. Journal of Mol Catal, 68: 117-128.
8. Call, H.P. and Mucke, I. (1997). History, overview and applications of mediated lignolytic systems, especially laccase-mediator systems (Lignozym® process). J Biotechnol, 53: 163.
9. Balakshin, M., Capanema, E. and Chen, C.L. (2001). Biobleaching of pulp with dioxygen in the laccase mediator system–reaction mechanisms for degradation of residual lignin. J Mol Catal, 13: 1.
10. Pazarlýoglu, N.K., Sariiþik, M. and Telefoncu, A. (2005). Laccase: production by Trametes versicolor and application to denim washing.Process Biochem, 40: 1673.

11. Aktas, N. and Tanyolaç, A. (2003). Reaction conditions for laccase catalyzed polymerization of catechol. Bioresour Technol, 87: 209.

12. Soares, G.M.B., Amorim, M.T.P. and Hrdina, R. (2002). Studies on the biotransformation of novel disazo dyes by laccase. Process Biochem, 37: 581.

13. Ullah, M.A., Bedford, C.T. and Evans, C.S. (2000). Reactions of pentachlorophenol with laccase from *Coriolus versicolor*. Appl Microbiol Biotechnol, 53: 230.

14. Pointing, S.B. (2001). Feasibility of bioremediation by white-rot fungi. Appl Microbiol Biotechnol, 57: 20.

15. Selinheimo, E., Kruus, K. and Buchert, J. (2006). Effects of laccase, xylanase and their combination on the rheological properties of wheat doughs. J Cereal Sci, 43: 152.

16. Golzberner, K., Walzel, B. and Zastrow, L. (2004). Cosmetic and dermatological preparation containing copper binding proteins for skin lightening. Int Pat Appl, WO2004017931.

17. Alcalde, M. (2007). Lacases: Biological Functions, Molecular Structure and Industrial Applications. J Mac Cabe, 4: 461-476.

18. Vandenberghe, L.P.S., Soccol, C.R. and Lebeault, J. M. (2000). Solid-state fermentation for the synthesis of citric acid by *Aspergillusniger*. BioresTechnol, 74: 175-178.

19. Roukas, T. (1999). Citric acid production from carob pod by solid-state fermentation, Enzyme Microb Tech,24: 54-59.

20. Leangon, S., Maddox, I.S. and Brooks, J.D. (2000). A proposed biochemical mechanism for citric acidaccumulation by *Aspergillusniger* growing in solid state fermentation. World J of Microbiol and Biotech, 16: 271–275.

21. Kar, B., Benerjee, R. and Bhattacharya, B.C. (1998).Biotransformation of tannic acid to gallic acid bysolid state fermentation. In Advances in Biotechnology, 1998; 2: 67-74.

22. Soccol, C.R., Marin, B., Raimbault, M. and Lebeault, J.M. (1994). Potential of solid state fermentation forproduction of L (+) - lactic acid by *Rhizopusoryzae*. ApplMicrobiolBiotechnot, 41:286-90.

23. Pandey, A., Soccol, C.R. and Mitchell, D. (2000). New developments in solid-state fermentationbioprocesses and products. Process Biochem, 35: 1153-1169.

24. Suresh, P.V. and Chandrasekaran, M. (1999). Impact of process parameters on chitinase production byan alkalophilic marine *Beauveriabassiana*in solid state fermentation. Process Biochem, 34: 257-267.

25. Kota, K.P. and Sridhar, P. (1999). Solid state cultivation of *Streptomyces clavuligerus*for CephamycinC production. Process Biochem, 34: 325–328.

26. Yang, S.S. and Swei, W.J. (1996). Cultural condition and Oxy-tetracycline production by *Streptomycesrimosus*in solid state fermentation of corn cob. World J of Microbiol and Biotech, 12: 43- 46.

27. Ohno, A., Ano, T. and Shoda, M. (1996). Production of Lipopeptide antibiotic, surfactin by recombinant*Bacillus subtilis* in solid state fermentation. BiotechnolBioeng,47: 209-214.

28. Murthy, M.V.R., Mohan, E.V.S. and Sadhukhan, A.K. (1999). Cyclosporine A production by*Tolypocladiuminflatum*using solid state fermentation. Process Biochem, 34: 269-280.

29. Sekhar, C. and Balaraman, K. (1998). Optimization studies on the production of cyclosporine A bysolid state fermentation. Bioprocess Eng, 18: 293-296.

30. Sekhar, C., Rajasekar, V.W. and Balaraman, K. (1997). Production of cyclosporine A by solid statefermentation. Bioprocess Eng, 17: 257-259.

31. Diamantidis, G., Effosse, A., Potier, P. and Bally, R. (2000). Purification and characterization of the first bacterial laccase in the rhizospheric bacterium *Azospirillumlipoferum*. Soil Biol Biochem, 32: 919-927.

32. Deckert, G., Warren, P.V., Gaasterland, W.G. and Young, A.L. (1998). The complete genome of the hyperthermophilic bacterium *Aquifexaeolicus*. Nature, 393: 353-358.

33. Givaudan, A., Effosse, A., Faure, D., Potier, P., Bouillant, M.L. and Bally, R. (1993). Polyphenol oxidase in *Azospirillum lipoferum* isolated from rice rhizosphere: Evidence for laccase activity in nonmotile strains of *Azospirillum lipoferum*. FEMS Microbiol Lett, 108: 205-210.

34. Narayanan, P.M., Beenie, J., Antony, J. and Murugan, S. (2015). Production of Extracellular Laccase from *Bacillus subtilis* MTCC 2414 Using Agroresidues as a Potential Substrate. Biochemistry Research International, doi.org/10.1155/2015/765190.

35. Claus, H. and Filip, Z. (1997). The evidence of a laccase like activity in a *Bacillus sphaericus* strain. Microbiol Res, 152: 209-2015.

36. Ruijssenaars, H.J. and Hartmans, S. (2004). A cloned *Bacillus halodurans* multicopper oxidase exhibiting alkaline laccase activity. Applied Microbiol Biotechnol, 65: 177-182.

37. Kim, C., Lorentz, W.W., Hoopes, J.T. and Dean, F.F. (2001). Oxidation of phenolate siderophores by the multicopper oxidase encoded by the *Escherichia coli* yacK gene. J Bacteriol, 183: 4866-4875.

38. Kalme, S., Parshetti, G., Jadhav, S. and Govindwar, S. (2007). Biodegradation of benzidine based dye Direct Blue-6 by *Pseudomonas desmolyticum* NCIM 2112. Bioresour Technol, 98: 1405-1410.

39. Takami, H., Takaki, Y. and Uchiyama, I. (2002). Genome sequence of *Oceanobacillus iheyensis* isolated from the Iheya Ridge and its unexpected adaptive capabilities to extreme environments.Nucl Acids Res, 30: 3927-3935.

40. Isono, Y. and Hoshino, M. (1989). Laccase like activity of nucleoside oxidase in the presence of nucleosides. Agri Biol Chem, 53: 2197-2203.

41. Cha, J.S. and Cooksey, D.A. (1991). Copper resistance in *Pseudomonas syringae*by periplasmic and outer membrane proteins. Proc Natl Acad Sci USA, 88: 8915-8919.

42. Okazaki, M., Sugita, T., Shimizu, M., Ohode, Y. and Iwamoto, K. (1997). Partial purification and characterization of manganese oxidizing factors of *Pseudomonas fluorescens*GB-1. Applied Environ Microbiol, 63: 4793-4799.

43. Brouwers, G.J., Devring, J.P.M. and Cornelis, P. (1999). *CumA*, a gene encoding a multicopper oxidase, is involved in Mn^{2+} oxidation in *Pseudomonas putida*GB-1. Applied Environ Microbiol, 65: 1762-1768.

44. Solano, F., Lucaselio, P., Lopezserrano, D., Fernandez, E. and Sanchezamat, A. (2001). Dimethoxyphenol oxidase activity of different microbial blue multi copper proteins. FEMS Microbiol Lett, 204: 175-181.

45. Fitzgibbon, S.T., Ladner, H., Kim, U.J., Stetter, K.O., Simon, M.I. and Miller, J.H. (2002). Genome sequence of the hyper thermophilic archaeon *Pyrobaculum aerophilum*. Proc Natl Acad Sci, 99: 984-989.

46. Arias, M.E., Arenas, M., Rodriguez, J., Soliveri, J., Ball, A.S. and Hernandez, M. (2003). Kraft pulp biobleaching and mediated oxidation of a nonphenolic substrate by laccase from *Streptomyces cyaneus* CECT 3335. Applied Environ Microbiol, 69: 1953-1958.

47. Miyazaki, K. (2005). A hyper thermophili-claccase from *Thermus thermophiles* HB27. Extremophiles, 9: 415-425.

48. Lee, Y.A., Hendson, M., Panopoulos, N.J. and Schroth, M.N. (1994). Molecular cloning, chromosomal mapping and sequence analysis of copper resistance genes from *Xanthomonascampestris*pv. Juglandis: Homology with small copper proteins and multi copper oxidases. J Bacteriol, 176: 173-188.

49. Barriosgonzalez, J., Fernandez, F.J., Tomasini, A, and Mejia, A. (2005). Secondary metabolism by solid state fermentation. Malaysian j of microbial, 1: 1-6.

50. Shraddha, R., Shekher, S. and Sehgal, M, and Ajay, K. (2011). Laccase: Microbial sources, production, purification, and potential biotechnological applications. Enzyme Research, Article ID 217861, 1-11, doi:10.4061/2011/217861.

51. Narayanan, P., Muthu, K. and Sevanan, M. (2014). Production, purification and application of bacterial laccase A Review. Biotechnology, 13(5): 196-205.

52. Amit, A. and David. L. (2012). India oil seeds and products annual. Global Agricultural Information report, 1-33.

53. Bradshaw, R. (2011). Vermont apple industry survey report, 1-8.

54. Haq, I., Khurshid, S., Ali, S., Ashraf, M.A. and Rajoka, M.I (2001). Mutation of *Aspergillus niger*for hyper production of citric acid from black strap molasses. World J Microbiol Biotechnol, 17: 35-37.

55. Munazzah, M., Ibraheem, M. and Sadia, J. (2012). *Bacillus subtilis* improvement through UV and chemical mutagenesis for indigenously hyper produced urate oxidase. Pak J Life Soc Sci, 10: 123-129.

56. Ambika, V., Karuna, D. and Poonam, S. (2016). Hyper-Production of Laccase by *Pseudomonas putida* LUA15.1 through Mutagenesis. J Microbiol Exp, 3(1): 3-8.

57. Mahmoud, M.G., Rifaat, H.M., El Sayed, O.H., El Beih, F.M. and Selim, M.S. (2013). Effect of inducers and process parameters on laccase production by locally isolated marine *Streptomyces lydicus*from Red Sea, Egypt. International Journal of Chem Tech Research, 5(1): 15-23.

58. Rasheeda, K. and Gyana, R. (2014). Strain improvement of white rot fungi *Pycnoporuscinnabarinus*with the influence of physical and chemical mutagens for enhancing laccase production. Journal of Scientific and Industrial Research, 73: 331-337.

59. Adebayo, E., Adegoke, O., Julius, K., Achana, Y. and Tarun, B. (2012). Improvement of Laccase Production in *Pluerotuspulmonarius*-LAU 09 by Mutation.Journal of Microbiology Research, 2(1): 11-17.

60. Michaelis, G., Douglass, S., Tsai, M. and Criddle, R.S. (1971). Mitochondrial DNA and supressiveness of petite mutants in *Saccharomyces*. Biochem Genetics, 5: 487-495.

61. Mohsinjaved, M.D., Ikram, U.L. and Irfana, M.R. (2011). Multistep mutagenesis for the over expression of cellulose in *Humicolainsolens*. Pak J Bot, 43: 669-677.

62. Malik, S.H., Tehreema, I. and Ikramul, H.A.Q. (2011). Enhanced Amyloglucosidase biosynthesis through mutagenesis using *Asperigillus niger*. Pak J Bot, 43: 111-119.

63. Munazzah, M., Ibraheem, M. and Sadia, J. (2012). *Bacillus subtilis* improvement through UV and chemical mutagenesis for indigenously hyper produced urate oxidase. Pak J Life Soc Sci, 10: 123-129.

64. Richard, R.B. and Murray, P.D. (2009). Guide to protein purification, 2nd ed Academic Press is an Imprint of Elsevier. 463: 14-15.

65. Willian, H. (2000). Official methods of analysis international. Association of official Analytical Chemists, 2: 1-3.

66. Das, N., Sengupta, S. and Mukherjee, M. (1997). Importance of laccase in vegetative growth of *Pleurotus florida*.Applied andEnvironmental Microbiology, 63(10): 4120-4122.