Lignin peroxidase: Optimization of media for higher productivity by single factor optimization method

Shukla Shruti* and Padhiar Anjali
Department of Biotechnology, Kadi Sarva Vishwa Vidyalaya, Gandhinagar, Gujarat, INDIA
*shru.shukla@yahoo.com

Abstract

Lignin peroxidase belongs to ligninolytic enzyme group and is one of the industrial important enzymes as it has wide applications in different sectors. Lignin peroxidase is produced by submerged fermentation process which requires optimization of physical and chemical parameters to achieve higher activity and make the process cost effective. The present study aimed at the optimization of physical as well chemical parameters of production medium. The optimization includes physical parameter such as incubation time, inoculum size, temperature, pH, RPM (Rotation per minute) while chemical parameters include carbon source, nitrogen source and different mineral elements.

Form the optimization study, it was observed that highest lignin peroxidase production was achieved after 72 hours of incubation at temperature 30^{0}C, pH 6 and RPM 120. Optimization of chemical parameters reveals that incorporation of sodium nitrite (9g/L) in the media gave significant increase in enzyme activity. It was found that the maximum productivity achieved after optimization was 2214 U/ml which was four times higher than process without optimized parameters.

Keywords: Lignin peroxidase, pH, temperature, fructose, sodium nitrite.

Introduction

Lignin is large size aromatic compound found in plant cell wall and it is link between cellulose and hemicellulose; main function of lignin is to provide protection against invading pathogens[8]. In nature, lignin is degraded by microorganism which can produce ligninolytic enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP) and dye-decolorizing peroxidase (DyP)[4].

Moreover, lignin adds to major carbon source but due to its large structure, it shows resistant towards its degradation. For 20 years, researchers are working on ligninolytic enzymes produce by microorganism to achieve fast recycling of lignin, as it has several industrial applications in various fields[3].

In the present study, out of different ligninolytic enzymes, lignin peroxidase producing microorganism is studied. Lignin peroxidase is heme containing protein which degrades lignin in presence of hydrogen peroxide[16]. In environment, both fungal as well as bacterial strain are capable of producing lignin peroxidase such as *Phanerochaete chrysosporium, Streptomyces viridosporous, Trametes trogii, Fusarium proliferatem, Pseudomonas, Norcadia* and *Arthobacter*[2].

Microorganisms require optimum production condition and nutrient to achieve maximum productivity of enzyme. Moreover, media optimization should be carried out to get a specific media and condition which is cost effective as submerged fermentation is expensive[13].

In the present study, media optimization was carried out using one parameter at a time to achieve higher productivity of enzyme by lignin peroxidase producing fungal strain FSV 3.

Material and Methods

Fungal culture: FSV 3 fungal culture was isolated from Andaman and Nicobar soil after screening on different dye and ligninolytic model. The 72 hours culture was preserved on PDA agar at 4^{0}C[12].

Lignin peroxidase production in submerged fermentation

Seed culture: 1 X 10^5 spores of culture were inoculated in 100 ml seed medium containing potato dextrose broth having 1% kraft lignin prepared in 250 ml flask. The flask was incubated for 24 hours on rotatory shaker (120 rpm) at 30^{0}C.

Production condition: 2% culture inoculum from seed was inoculated in production medium having composition 2% rice bran as ligninolytic source, $NaNO_3$ 6g, $MgSO_4.7H_2O$ 0.52g and KH_2PO_4 0.82g. Production flasks were incubated at 30^{0}C in rotatory shaker having 120 RPM for 144 hours. At every 24 hours, lignin peroxidase activity was checked.

Lignin peroxidase assay: Enzyme assay was carried out using 10mM veratryl alcohol as a substrate[9]. The reaction mixture contains 1ml of sodium tartrate buffer (pH 3), 0.5 ml substrate, 0.5 ml culture supernatant and reaction was started using 0.5 ml 5mM H_2O_2. The oxidation of veratryl alcohol (ε= 9300 x 10 6 m^{-1} cm^{-1}) was monitored by measuring the change in absorption at 310 nm for 1 min. One unit of enzyme activity was defined as amount of enzyme that oxidizes 1µm of veratryl alcohol per min.

Characterization of crude lignin peroxidase production: The cell free extract of the fungus was taken as the crude LiP preparation. While studying each parameter, the other reaction conditions were kept constant.

Optimization of physical parameters

Effect of inoculum size: Different concentrations of seed inoculum were inoculated in production medium to check the optimum concentration of culture to achieve higher production. Different inoculation sizes ranging from 1%, 2 % to 5% were used in respective production flasks and productivity was checked after every 24 hours using veratryl alcohol assay.

Effect of incubation time: It has been observed that the culture shows maximum activity at particular time of incubation. The production flask was incubated up to drop in activity and daily lignin peroxidase activity was measured.

Effect of Temperature: Temperature plays a critical role in production of enzyme as culture shows maximum efficacy for enzyme production at particular temperature. In the present study, production flask was incubated at various temperatures ranging from 25 to 45^0C at the interval of 5^0C.

Effect of pH: Production medium was prepared with varying pH (5-8) in the interval of 0.5. The flask was incubated for optimized time and at optimized temperature. After every 24 hours, activity was observed.

Effect of RPM: RPM in submerged fermentation maintains the rheology of medium which is necessary to provide proper dissolved oxygen and even distribution of medium for optimum enzyme production. Production flasks were incubated at different RPM ranging from 80 RPM to 160 RPM at the interval of 40 RPM and enzyme activity was measured after every 24 hours.

Optimization of chemical parameters

Effect of Concentration of rice bran: In the production medium, rice bran was used as lignin source; varying concentration of rice bran from 0%-5% was used to check the enzyme production.

Effect of Nitrogen source and Carbon source in production medium: In the basal medium, nitrogen source used was sodium nitrate which was replaced by other nitrogen sources which include sodium nitrite, yeast extract, peptone and ammonium sulphate and for different carbon sources curd source, rice bran were replaced by glucose, fructose, sucrose, lactose and dextrin.

Effect of different concentration of Nitrogen source and Carbon source in production medium: The optimum concentration of nitrogen and carbon is necessary in production medium to achieve optimum growth and productivity. In the present study, varying concentrations of nitrogen from range 0.3 to 1.5 gm/L and carbon 1% to 5% were optimized.

Effect of mineral salt concentration: Production media contain two mineral salts KH_2PO_4 and $MgSO_4$. Different concentration of mineral salts [0-1.6 gm/L (interval of 0.4gm/L) KH_2PO_4, 0-0.1 (interval of 0.25 gm/L) $MgSO_4$] were optimized in respective production media and at every 24 hours lignin peroxidase activity is measured.

Results and Discussion

FSV culture: After primary screening, 35 bacterial strains and 10 fungal stains were isolated. In secondary screening, FSV 3 fungal strain gave 131.18 U/ml maximum LiP activity among other cultures based on which FSV 3 fungal strain (Figure 1) was selected for future work.

Optimization of physical parameters

Effect of incubation time: Study of incubation time is necessary for any experiment as it gives an idea of length of entire fermentation process, moreover it gave an idea of actual time when culture is producing maximum enzyme. In the present submerged fermentation, FSV 3 shows maximum productivity of lignin peroxidase of 134.62 Unit/ml at 72 hours (Graph 1).

Effect of inoculum size: In the study conducted by Zarinum et al[17], production of lignin peroxidase by *Pycnoporus spp.* shows linear increase in productivity with increase in inoculum size. In the present study, production medium inoculated with 2 % inoculum shows maximum productivity at 72 hours, above 2% inoculum activity starts decreasing as rheology of media is not maintained due to overgrowth of culture. Moreover, over 2% inoculum growth of culture turns into filamentous growth which hinders proper aeration in flask (Graph 2).

Effect of Temperature: In the present experiment, productivity of lignin peroxidase was checked in range of temperature between 25^0C-45^0C at the interval of 5^0C. FSV shows maximum production at 30^0C, similar result was observed when white rot fungus shows maximum activity at 30^0C[13]. Temperature not only affects the productivity of enzymes but culture morphology also varies as temperature varies. Similar observation was recorded by Vyas et al[14] who worked with *Phanerochaete chrysosporium*. They observed decrease in culture growth when temperature increases.[14]. Thus, from the findings, it can be said that fungal strain producing lignin peroxidase shows the maximum activity in between 30^0C-40^0C depending upon the strain.

Effect of pH: FSV 3 culture shows 7.5 pH as an optimum pH for lignin peroxidase enzyme production. Under acidic pH range from 5-6, negligible activity of LiP was observed as growth of fungal culture stunted, while from pH 6.5, LiP activity keeps on increasing up to 7.5. Beyond 7.5 pH, morphology of culture changes from pellet to mycelial into decrease in productivity. Similar result was obtained for *Apergillus niger* which shows maximum activity at 7.5 while activity decreased under acidic pH.[5]

Effect of RPM: Present study shows linear increase in productivity of lignin peroxidase with increasing RPM upto

120 RPM. On further increase of RPM, LiP activity is affected as morphology of culture pellet changes. Similar observation was reported by Zanirun et al[17], they studied range of RPM from 50 to 110 and observed linear increase in productivity.

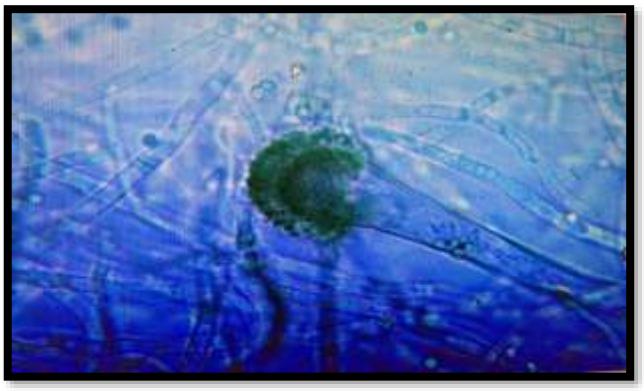
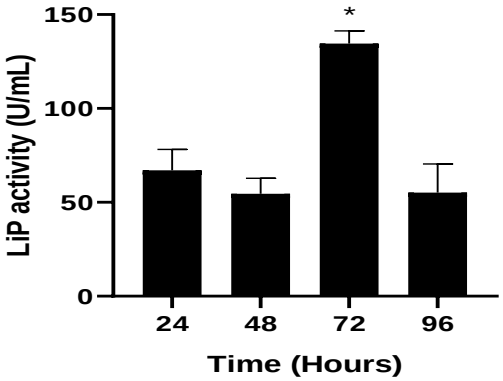
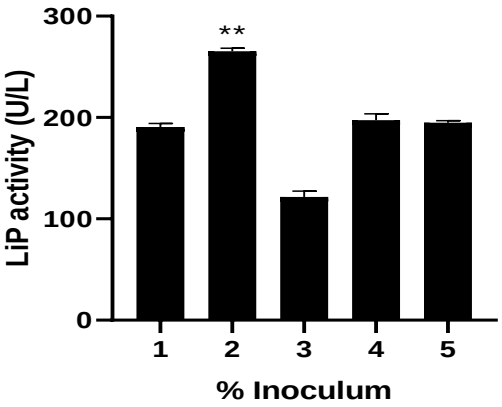


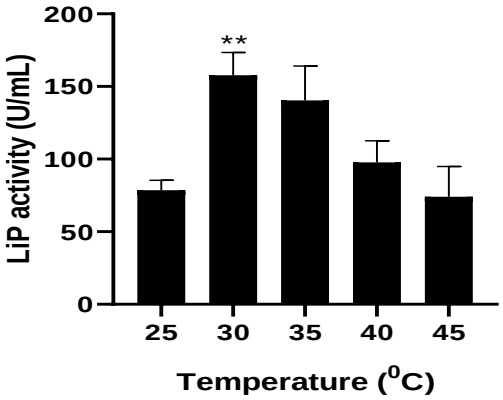
Figure 1: Microscopic observation of FSV 3 strain



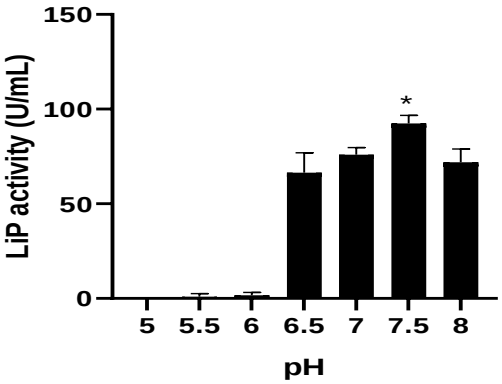
Graph 1: Effect of incubation time: one sample t and Wilcoxon test show the study carried out as significant. p < 0.05. '*' suggest significant parameter.



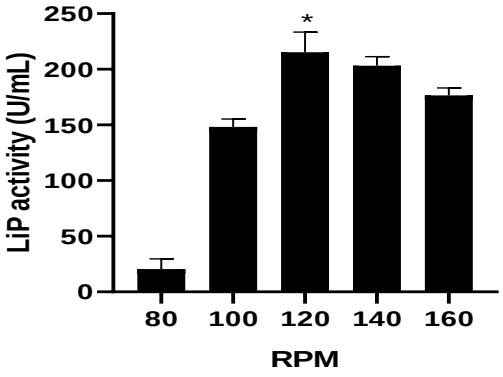
Graph 2: Effect of inoculum size: one sample t and Wilcoxon test show the study carried out as significant. p < 0.05. '*' suggest high significant parameter.



Graph 3: Effect of Temperature: one sample t and Wilcoxon test show the study carried out as significant. p < 0.05. '' suggest high significant parameter.**



Graph 4: Effect of pH: one sample t and Wilcoxon test show the study carried out as significant. p < 0.05. '*' suggest significant parameter.



Graph 5: Effect of RPM: one sample t and Wilcoxon test show the study carried out as significant. p < 0.05. '*' suggest significant parameter.

Optimization of chemical parameter

Effect of rice bran: Ligno cellulosic waste such as corn stover/cob, sugarcane bagasse, wheat straw, rice husks were utilized to generate an industrial useful product of which rice bran is used as lignin source for production of LiP[6,15]. Roushdy et al[10] reported that *Cunninghamella elegans* gave 1.5 U/ml enzyme productivity *using* rice husk[10]. In the present study, different concentrations of rice bran were studied ranging from 0 to 2.5% in liquid production medium. The result shows that production medium without rice bran shows the maximum productivity (125.41 U/ml) compared to in presence of rice bran (Graph 6).

From the experiment, it can also be concluded that rice bran hinders the fluidity of medium which may decrease dissolved oxygen level in medium as increase in rice concentration shows decrease in productivity.

Effect of different nitrogen source and carbon source in production medium: In present study with FSV 3, it was observed that sodium nitrite showed significant effect on productivity of enzyme as in presence of sodium nitrite, productivity increased by 50% (1062 U/ml) compared to control. While among different carbon sources fructose gave 595.65 U/ml activity to that of control while remaining other carbon sources do not show any significant increase in enzyme activity (Graph 7 and 8).

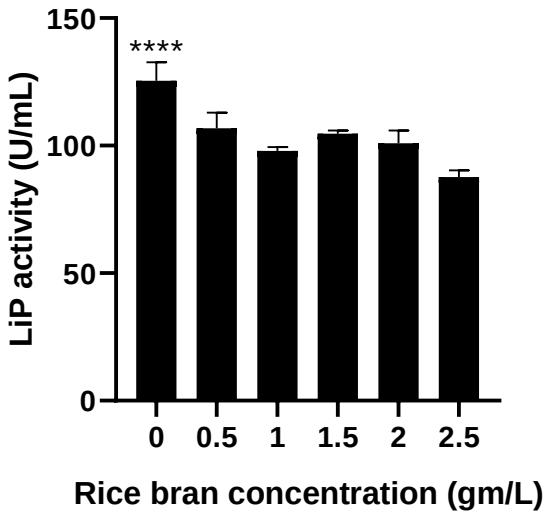
According to study by Ahammed and Parukuttyamma[1], *Aspergillus* sp gave higher productivity of LiP in medium containing glucose along with corn pith compared to other pure and raw carbon sources and in case of nitrogen, ammonium sulphate gives highest productivity while use of peptone in production medium shows no LiP production[1]. Similarly, it was reported that use of corn oil as sole carbon source can bring drastic increase in productivity of LiP[7].

Effect of different concentrations of nitrogen source and carbon source in production medium: Sodium nitrite and fructose which were showing higher LiP activity, were incorporated in production medium. Different concentrations of sodium nitrite and fructose were studied and it was found that 9gm/L sodium nitrite gave maximum productivity of 2214 gm/L which was almost double in compared to control having 6gm/L while fructose does not show any significant role in production of LiP (Graph 9 and 10).

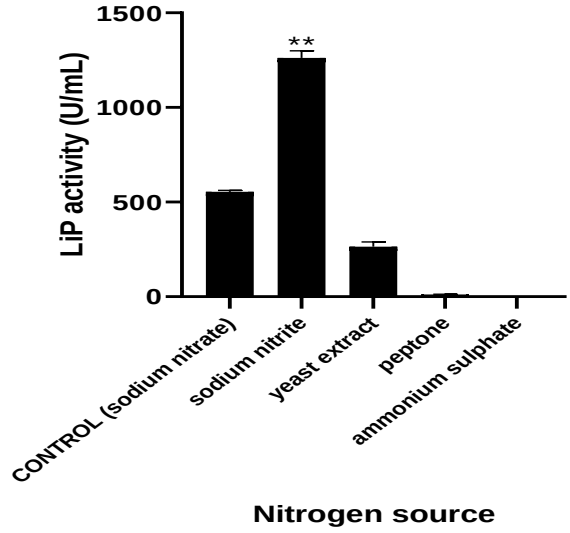
Effect of mineral salt concentration: Present medium contains two mineral salts KH_2PO_4 and $MgSO_4$. Mineral salts played an indirect role in production of enzyme. From the result, it was found that 0.8 gm/L KH_2PO_4 and 0.5 gm/L $MgSO_4$ are optimum concentrations in the medium because study of KH_2PO_4 has shown no increase in productivity, even nil concentration has given similar activity but morphology of culture changes to loosen pellet growth. While for $MgSO_4$, maximum activity observed was at 0.5 gm/L concentration 9 (Graph 11 and 12).

Conclusion

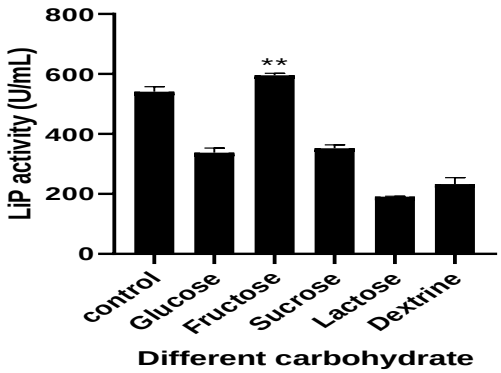
FSV 3 has shown 4 times increase in productivity of lignin peroxidase after optimizing physical and chemical parameters. After optimization of physical parameters, FSV 3 gave maximum Lip production using 2% inoculum at 30^0C temperature and 7.5 pH after 72 hours of incubation in submerged condition with 120 RPM.



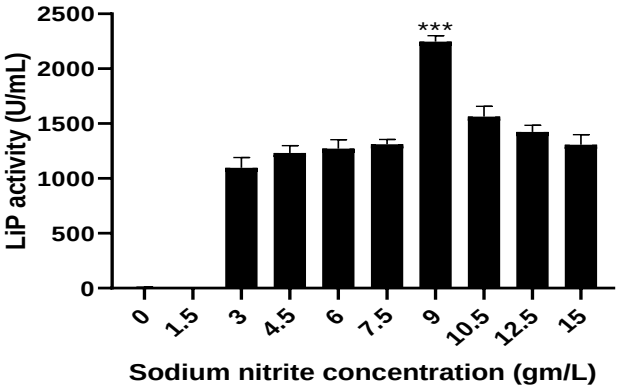
Graph 6*:* Effect of different concentrations of rice bran: one sample t and Wilcoxon test show the study carried out as significant. p < 0.05. '**' suggest highly significant parameter**



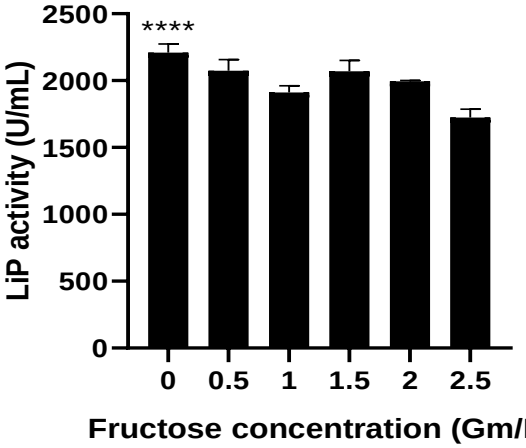
Graph 7*:* Effect of different nitrogen sources: one sample t and Wilcoxon test show the study carried out as significant. p < 0.05. '' suggest high significant parameter**



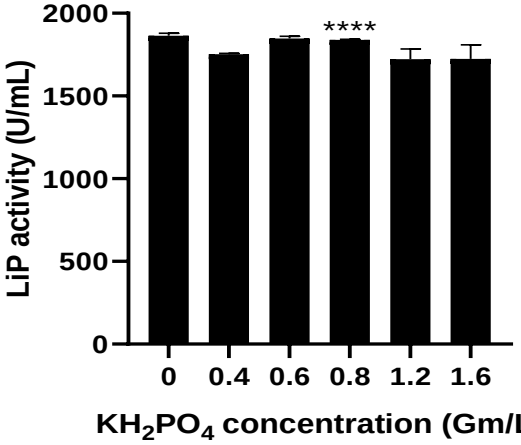
Graph 8: Effect of different carbon sources: one sample t and Wilcoxon test show the study carried out as significant. p < 0.05. '' suggest significant parameter.**



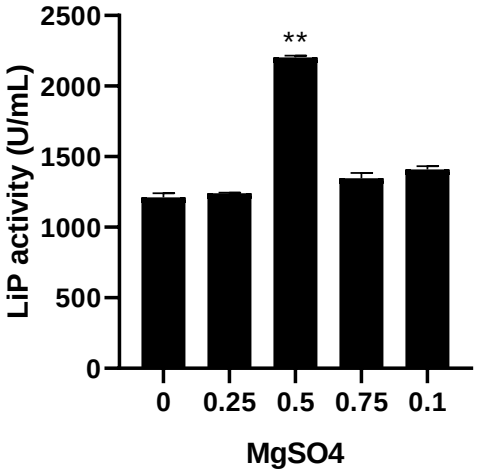
Graph 9: Effect of different sodium nitrite concentrations: one sample t and Wilcoxon test show the study carried out as significant. p < 0.05. '*' suggest highly significant parameter.**



Graph 10: Effect of different concentrations of Fructose: one sample t and Wilcoxon test show the study carried out as significant. p < 0.05. '*' suggest highly significant parameter.



Graph 11: Effect of different concentrations of KH_2PO_4: one sample t and Wilcoxon test show the study carried out as significant. p < 0.05. '*' suggest highly significant parameter.



.. **Graph 12: Effect of different concentrations of $MGSO_4$:one sample t and Wilcoxon test show the study carried out as significant. p < 0.05. '*' suggest high significant parameter.**

Sodium nitrite, KH_2PO_4 and $MgSO_4$ maximize production of enzyme at the concentrations of 9gm/L, 0.8 gm/L and 0.5 gm/L respectively. Optimized parameter can be used to set up production of lignin peroxidase at pilot or large scale production for FSV 3 culture.

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References

1. Ahammed Shamla and Parukuttyamma Prema, Influence of Media Nutrients on Synthesis of Lignin Peroxidase from *Aspergillus* Sp., *Applied Biochemistry and Biotechnology*, **102–103**, 327–36 **(2002)**

2. Bholay A.D., Borkhataria Bhavna V., Jadhav Priyanka U., Palekar Kaveri S., Dhalkari Mayuri V. and Nalawade P.M., Bacterial Lignin Peroxidase: A Tool for Biobleaching and Biodegradation of Industrial Effluents, *Universal Journal of Environmental Research and Technology,* **2(1)**, 58-64 **(2012)**

3. Conesa Ana, Hondel Cees A.M.J.J. van den and Punt Peter J., Studies on the Production of Fungal Peroxidases InAspergillus Niger, *Applied and Environmental Microbiology*, **66(7)**, 3016–23 **(2000)**

4. Datta Rahul, Kelkar Aditi, Baraniya Divyashri, Ali Molaei, Amitava Moulick, Ram Meena and Pavel Formanek, Enzymatic Degradation of Lignin in Soil: A Review, *Sustainability*, **9(7)**, 1163 **(2017)**

5. Dhakar Kusum, Kooliyottil Rinu, Joshi Archana and Pandey Anita, Simultaneous Production of Ligninolytic Enzymes by a Temperature and PH Tolerant Strain of Aspergillus Niger under Different Cultural Conditions, *Indian J Biotechnol*, **7**, 81-86 **(2015)**

6. Falade Ayodeji O., Nwodo Uchechukwu U., Iweriebor Benson C., Ezekiel Green, Mabinya Leonard V. and Okoh Anthony I., Lignin Peroxidase Functionalities and Prospective Applications, *Microbiology Open*, **6(1)**, 1-14 **(2017)**

7. Gottschalk Leda M.F., Jacyara M.B. Macedo and Elba P.S. Bon, Lignin Peroxidase Production by Streptomyces Viridosporus T7A: Use of Corn Oil as a Carbon Source, *Applied Biochemistry and Biotechnology*, **79**, 771–78 **(1999)**

8. Hariharan Sudha and Padma Nambisan, Optimization of Lignin Peroxidase, Manganese Peroxidase and Lac Production from Ganoderma Lucidum Under Solid State Fermentation of Pineapple Leaf, *Bio Resources*, **8(1)**, 250–71 **(2012)**

9. Ming Tien and Kent Kirk, Lignin-degrading enzyme from Phanerochaete chrysosporium: purification, characterization and catalytic properties of auniqueh202-requiring oxygenase, *Biochemistry*, **81**, 2280-2284 **(1984)**

10. Roushdy M.M., Abdel-Shakour E.H. and El-Agamy E.I., Biotechnological Approach for Lignin Peroxidase (LiP) Production from Agricultural Wastes (Rice Husk) by *Cunninghamella elegans*, *Journal of American Science*, **7(5),** 6-13 **(2011)**

11. Sharma Anuja, Aggarwal Neeraj K. and Yadav Anita, First Report of Lignin Peroxidase Production from Alternaria Alternata ANF238 Isolated from Rotten Wood Sample, *Bioengineering and Bioscience*, **4(5)**, 76-87 **(2016)**

12. Shukla Shruti and Padhiar Anjali, Isolation and screening of lignin peroxides producing microorganism from different geographical location of Andaman and Nicobar Island, *International Journal of Basic and Applied Research,* **9(6)**, 1432-1438 **(2019)**

13. Thammaiah Vandana, Rao Ramya G., Samanta Ashish Kumar, Swaraj Senani and Manpal Sridhar, Enhancing Production of Lignin Peroxidase from White Rot Fungi Employing Statistical Optimization and Evaluation of Its Potential in Delignification of Crop Residues, *International Journal of Current Microbiology and Applied Sciences*, **7(1)**, 2599–2621 **(2018)**

14. Vyas B.R.M., Volc J. and Sasek V., Effect of temperature on the production of manganese peroxidase and lignin peroxidase by *Phanerochaete chrysporium*, *Folia Microbiology*, **39(1)**, 19-22 **(1994)**

15. Usha Y., Praveen K. and Rajasekhar Reddy B., Enhanced Production of Ligninolytic Enzymes by a Mushroom *Stereum Ostrea*, *Biotechnology Research International*, **2014,** 1–9 **(2014)**

16. Yadav Meera, Yadav Pratibha and Yadav Kapil Deo Singh, Purification and Characterization of Lignin Peroxidase from *Loweporus Lividus* MTCC-1178, *Engineering in Life Sciences*, **9(2)**, 124–29 **(2009)**

17. Zanirun Z., Abd-Aziz S., Ling F.H. and Hassan M., Optimization of lignin peroxidase production using Locally isolated *Pycnoporous* sp. through factorial design, *Biotechnology*, **8(3)**, 296-305 **(2009)**.

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