Journal of Advanced Zoology

ISSN: 0253-7214

*Volume **43** Issue **1** **Year 2022** Page **1116:1126***

Production and Optimization of Laccase from *Daedaleopsis confragosa* by using Agro-Residual Wastes

[*1]Sudheer I. Manawadi, [1]Sanjotha G, [2]Muhammad Zafar Iqbal Navalgund and [3]Adiveppa B. Vantamuri

[1*]Department of Biotechnology, Government Arts and Science College, Karwar- 581301, Karnataka, India

[2]Department of Zoology, Government Arts and Science College, Karwar- 581301, Karnataka, India

[3]Department of Biotechnology, Karnatak Science College, Dharwad-580001, Karnataka, India

Corresponding Email: *[1]sudheermanawadi@gmail.com

Article History

Received: 12 November 2021
Revised: 10 December 2021
Accepted:19 January 2022

Abstract

A laccase producing fungus Daedaleopsis confragosa was obtained from tree trunk from Karwar coastal region of Karnataka, India. Daedaleopsis confragosa produces laccase by utilizing the agro-wastes. The study revealed that the rice bran was found to be the best supported lignocellulosic substrate for extracellular laccase production under Solid state fermentation and optimum condition for highest laccase activity of pH and temperature were observed at 6.0 and 45 ℃ respectively. Glucose and peptone were the best supported carbon and nitrogen source for the maximum laccase activity. The optimum inoculum size and incubation periods for laccase maximum activity at 6 mm of 5 fungal discs and 10th day, respectively. Solid-state fermentation (SSF) has emerged as a potential technology for the production of microbial products such as feed, fuel, food, industrial chemicals and pharmaceutical products. Its application in bioprocesses such as bioleaching, bio-beneficiation, bioremediation, bio-pulping, etc. has offered several advantages. Utilisation of agro-industrial residues as substrates in SSF processes provides an alternative avenue and value-addition to these otherwise under- or non-utilised residues. The objective of this research work was to determine the most promising fungus, the best solid substrate and the optimal conditions for the production of laccase.

Keywords: *Daedaleopsis confragosa, Laccase, Solid State Fermentation*.

1. Introduction

The enzyme production is a growing field of biotechnology. Annual world sales figures are close to billion dollars (Layman et al., 1990) within increasing number of patents and research articles related to this field. Since the biotechnological applications require large amounts of low-cost enzymes, one of the appropriate approaches for this purpose is to utilize the potential of lignocellulosic wastes, some of which may contain significant concentrations of soluble carbohydrates and inducers of enzyme synthesis ensuring efficient production of ligninolytic enzymes (Reddy et al., 2003, Moldes et al., 2004). Laccases have copper atoms at their catalytic sites and are oxidative enzymes (EC 1.10.3.2) which are widely found in many

species of fungi, where they are involved in lignin degradation, in higher plants where they are involved in biosynthesis of lignin (Mayer and Staples, 2002; Sharma and Kuhad, 2008), in bacteria (Claus, 2003; Liers et al., 2007). Some species of fungi and insects produce laccases as intracellular proteins but most of the laccases are produced as extracellular proteins by all other types of producers (Arora and Sharma, 2010). The importance of laccase in various biotechnological areas underlines the need for expanding the spectrum of laccase-producing organisms and enhancing the potential of their laccase-producing ability. The extracellular laccases obtained by basidiomycete fungi usually have low activities (Bollag & Leonowicz 1984). Their production, however, can be considerably stimulated in the presence of a wide variety of inducing substances. To enhance laccase production, various nutritional supplements, inducers such as veratryl alcohol, 2,5-xylidine, ferulic acid, guaiacol and lignin preparations (indulin, reax, lignosulphonate) have been used (Lee et al. 1999, Arora & Gill 2000).

In recent years, there has been an increasing trend towards efficient utilization of agro-industrial wastes for the production of value-added products such as edible mushrooms, ethanol, enzymes, organic acids etc. (Kirk and Farrell, 1987). Solid state fermentation (SSF) is a technique in which fungi are grown on solid substrate or substrate moistened with a low quantity of mineral salt solution and it has a great potential to produce enzyme especially where the fermented raw materials are used as a source of nutrients for the fungi. The enzymes produced by this method have several applications in several fields including food and fermentation industry. These enzymes are also used to prepare several bioactive compounds. SSF system is much better than the submerged system because a number of reasons. The benefits of SSF over SMF include the high production of the enzyme and fewer effluent generations. Moreover, comparably simple equipment is required for SSF (Pandey, 1994). The present work undertaken to examine the effectiveness of selected agro-wastes in production medium and optimizing the parameters using agro-wastes for maximizing laccase production.

2. Materials and Methods

Microorganisms

Selected organism was screened for laccase production on potato dextrose agar plates (PDA) containing indicators namely, guaiacol, ABTS, syrindalzine and tannic acid. Isolated organism was showed positive reaction for ligninolytic enzymes (Lac, MnP and Lip) was maintained on PDA plates at 30℃ and stored at 4℃ and identified as *Daedaleopsis confragosa* (Manawadi et al., 2019).

Screening of different lignocellulosic substrates for extracellular laccase production

The mentioned agro-residual wastes were used for the initial screening: rice bran, paddy straw, sugarcane bagasse and saw dust. All of them were locally procured and were sterilized at 121℃ and 15lb pressure for 20 mins and were used for the study (Usha et al., 2020).

Media preparation

According to the Jhadav et al., (2009) the media was prepared by adding 2% of each of the agro-wastes to the Mineral Salt (MS) medium. The media was then sterilizing at 121ºC and 15lb pressure for 20 minutes. This agro-wastes mineral salt (AWMS) media was used for the study. The 250 ml conical flasks with 100 ml of the above AWMS media and were inoculated with well grown fungal discs from PDA plates and the flasks were adjusted at a pH of 6 and incubated at 40℃ for 5 days and the enzyme extraction and assay were done as guaiacol assay method. The best agro waste which supports the highest laccase assay were selected and used for the optimization study.

Laccase harvesting

Followed by Saqib et al., (2015) after some specified days of incubation, laccase was extracted by a simple contact method. For this purpose 100ml of sodium acetate buffer (pH 5.5) was added in the flasks. The flasks were placed on incubator shaker at 150 rpm for 1 hour. Mixture was then filtered with filter paper and the filtrate was centrifuged at 10,000 rpm for 10 minutes at -10℃ to remove all spores and other impurities. The supernatant was collected and subjected to laccase assay.

Effect of initial moisture content on laccase production

To investigate the influence of the initial total moisture content (before autoclaving) of the substrate was carried out under various initial moisture content adjusted with salt solution. Samples containing 5 moisture levels (30%, 45%, 50%, 65% and 70%) were prepared by moistening 5g of studied substrates with salt solution (Niladevi et al., 2007). The optimum initial moisture content of solid substrate achieved by this step was fixed in subsequent experiment. After soaking, the sample was again dried as described above and percent moisture content was calculated as follows, Percent of moisture content (initial) of solid medium = (wt. of the rice bran - dry wt.) x 100 / dry wt (Ellaiah et al., 2002; Ronak S and Chhaya, 2013).

Effect of physical factors on laccase production

The effect of pH on laccase production in rice bran broth was carried out by incubating with different initial pH. The experiments were carried out individually at various pH ranging from 4 to 9. The enzyme assay was carried out individually after 8 days of incubation.

The effect of various temperature ranges on laccase production in rice bran broth was studied by inoculating the *Daedaleopsis confragosa* and the incubating the flasks at different temperatures viz., 30℃, 40℃, 45℃, 50℃ and 55℃. The flasks were incubated for 8 days and assay was carried out.

Effect of incubation period and inoculum size for laccase production:

The effect of incubation period on laccase production in rice bran broth was studied by inoculating with *Daedaleopsis confragosa* and incubated at room temperature for various time intervals. The enzyme was extracted and its activity was determined.

The effect of inoculum size was studied by adding different size of 1, 2, 3, 4, 5 and 6 mm of 6 fungal discs from 5 days old fungal plate to the rice bran broth and incubated at room temperature and assay was done.

Effect of carbon and nitrogen source for laccase production

The effect of different carbon and nitrogen sources on laccase production was studied. Different carbon sources namely glucose, mannose, cellobiose and maltose were tested for laccase production by the *Daedaleopsis confragosa*. Organic and inorganic nitrogen sources like ammonium nitrate, peptone, and urea were amended to the culture medium with the *Daedaleopsis confragosa* for laccase production. The flasks were incubated at 40℃.

Extracellular laccase assay

The Laccase activity was assayed at room temperature by using 10mMGuaiacol in 100 mM sodium acetate buffer (pH 5.0). The reaction mixture contained 3.0 ml acetate buffer, 1.0 ml Guaiacol and 1.0 ml enzyme source. The change in the absorbance of the reaction mixture containing guaiacol was monitored at 470 nm for 10 mins of incubation using UV Spectrophotometer. Enzyme activity is measured in U/ml which is defined as the amount of

enzyme catalysing the production of one micromole of coloured product per min per ml (Adiveppa and Kaliwal, 2016)

$$\text{Calculation: Volume activity (U/ml)} = \frac{\Delta A470nm/min \times 4.0 \times Vt \times \text{dilution factor}}{€ \times Vs}$$

Where, Vt = final volume of reaction mixture (ml) = 5.0 Vs = sample volume (ml) = 1.0 €=extinction co-efficient of guaiacol = 6,740/M/cm 4 = derived from unit definition & principle

3. Results and Discussion

One of the effective approaches to reduce the cost of enzyme production was to replace pure carbohydrates as substrates with relatively cheaper materials namely lignocellulosics. Majority of organic materials available in nature like polysaccharides, proteins and lignin were polymeric in structure.

Selection of lignocellulosic substrates

The choice of a suitable agro-residual waste for a fermentation process is an important factor for the microbial growth and enzymes secretion (Sathish et al., 2008). Agro-residual wastes like rice bran, rice straw, sugarcane bagasse and saw dust waste were screened for laccase production. In the present study, total 4 substrates as stated in the materials and methods, were tested for the suitability of maximum laccase production by isolated *Daedaleopsis confragosa***.** Among all studied substrates rice bran has yielded the higher amount of laccase at 5th day of incubation (Fig 1). Paddy straw also best substrate for laccase production next to the rice bran. As per industrial point of view incubation time is also very important, so less incubation and higher amount yielding rice bran was consider for the further studies. Tan et al., (1997) found high laccase yield reported on rubber tree sawdust by *P. sajor-caju*. These results indicate that the production of lignolytic enzymes by SSF is dependent on the substrate material as well as the microbial strain employed for the production.

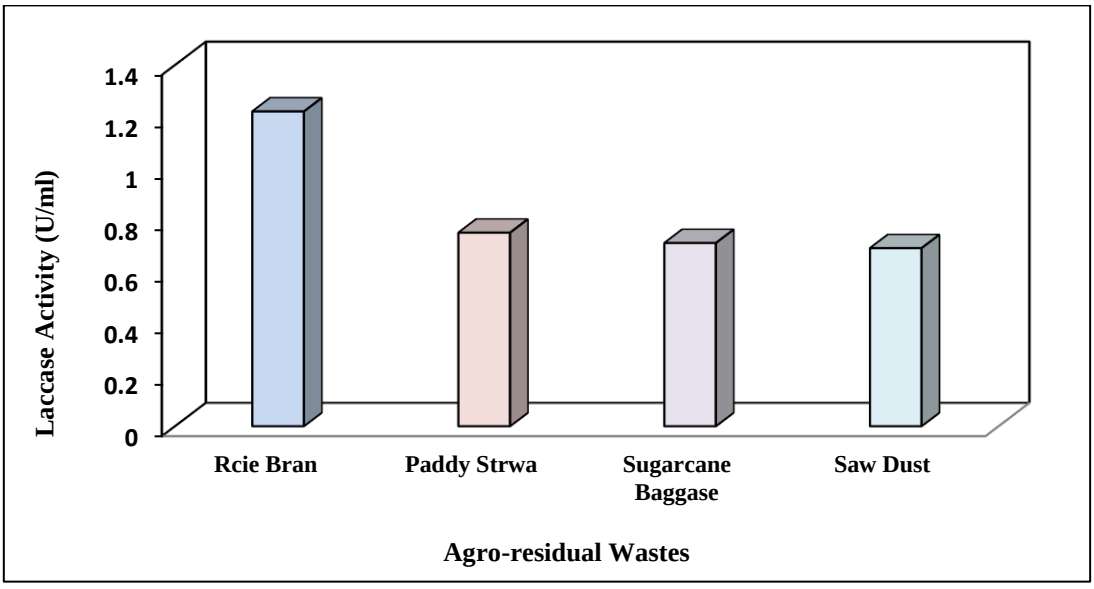

Fig 1. Screening of lignocellulosic substrates for laccase production by *Daedaleopsis confragosa*

Influence of initial moisture content on laccase production

In the present study an initial moisture content of 65% was found optimum for laccase production. The laccase production varied significantly between 65% and all other percentages of moisture content with an exception being at 70% of moisture content. When the moisture content less than 60% reduced laccase production was observed. This might be due to at lower moisture contents result in decreased solubility of nutrients, lower substrates welling and higher water tension (Lonsane et al., 1998). Patel et al. (2009) reported that *P. ostreatus* HP-1 at 60% moisture content is suitable for higher amounts of laccase secretion during growth on wheat straw. Aslam and Asgherm, (2011) stated that at 66% IMC is best suitable for maximum laccase production by *P.ostreatus* under SSF conditions using wheat straw as a substrate (Fig 2).

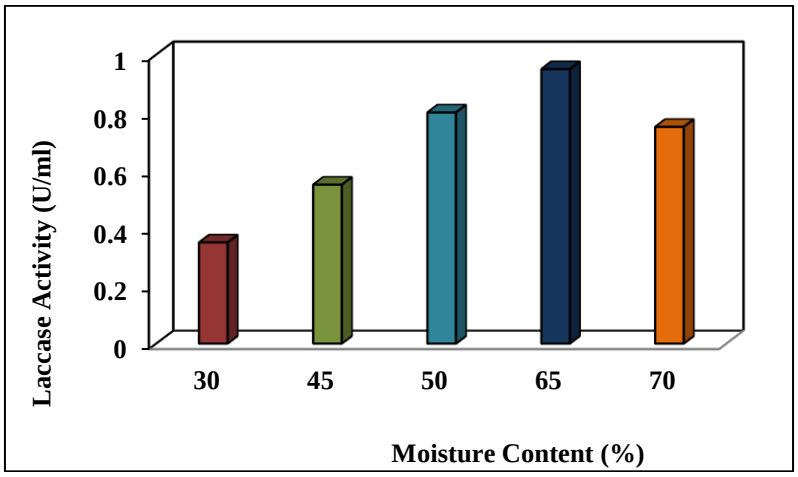

Fig 2. Effect of initial moisture content for laccase production from *Daedaleopsis confragosa*

Optimum pH and temperature for laccase

In order to investigate the effect of the initial medium pH on laccase production by the *Daedaleopsis confragosa*, the production medium was adjusted to different pH values ranged between pH 4 and pH 8. The result showed that the maximum laccase production was obtained when the pH value of the production medium was adjusted to 6.0. Thurston, (1994) reports indicated that the initial pH between 4.5 and 6.0 was suitable for enzyme production. The pH variation during fermentation depended highly on the nature of microorganism used. With *Aspergillus* sp., *Penicillium* sp., and *Rhizopus* sp. a rapid drop in pH below 3.0 was reported due to the possibility of secretion of organic acids (Fig 3). In the case of *Trichoderma* sp., *Sporotrichum* sp. and *Pleurotus* sp. the pH was more stable between 4 and 5 during fermentation (Raimbault, 1998).

Different incubation temperatures (35, 40, 45, 50 and 55℃) were used to determine the optimum temperature for laccase production by *Daedaleopsis confragosa*. The result showed that laccase specific activity is increased with temperature at 45 ℃ and decreased at higher temperatures. Kuntal Kalra and Mohd. Shavez have reported that the laccase of *Trametes hirsuta* showed maximum activity at 40℃ (Fig 4). The optimum temperatures previously reported for other fungal laccases, ranging from 40℃ to 50℃ (Nagai, Shin and Lee, Xiao).

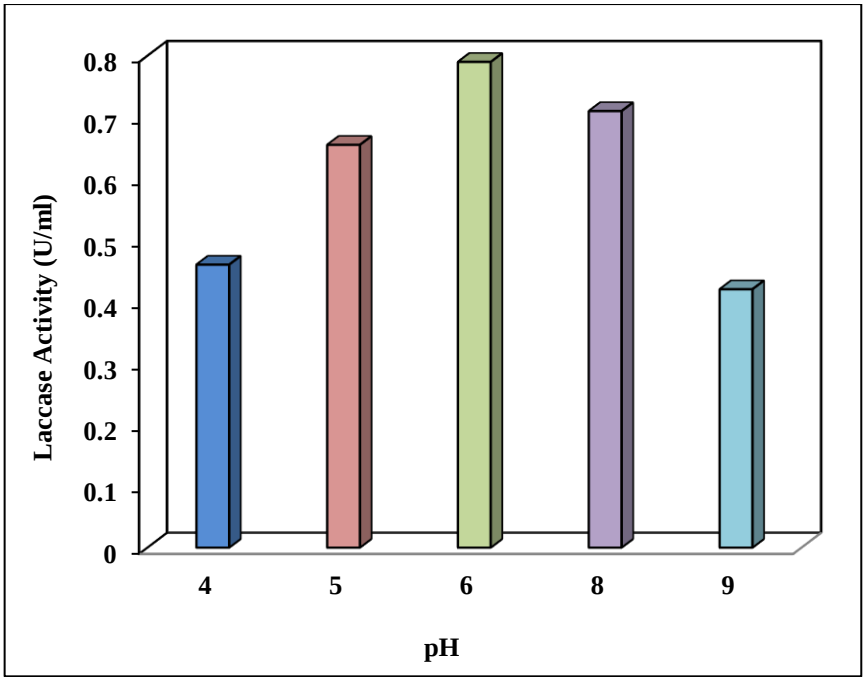

Fig 3. Effect of pH on laccase production by *Daedaleopsis confragosa*

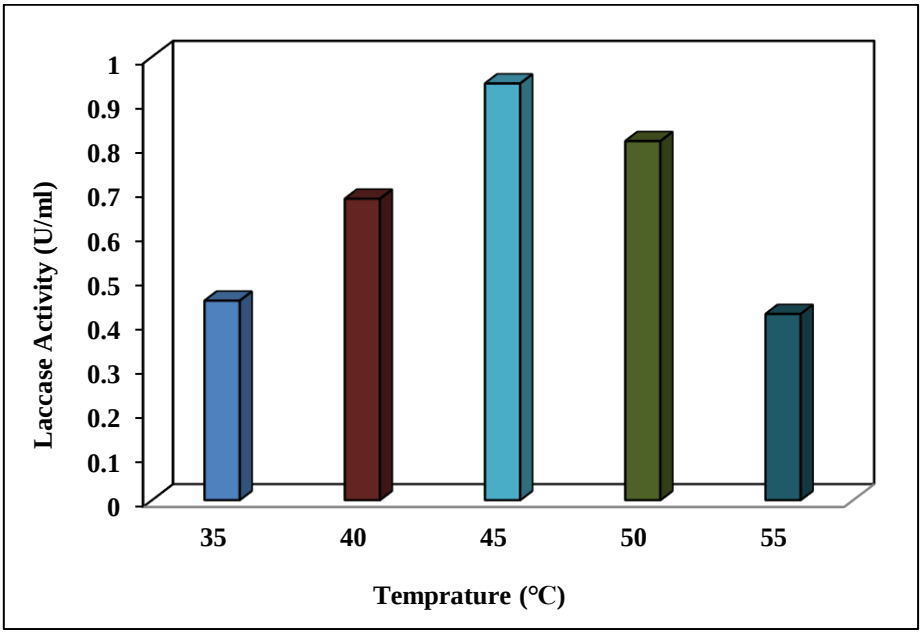

Fig 4. Effect of temperatures on laccase production by *Daedaleopsis confragosa*

Effect of incubation period on laccase production

The present experiment aims to determine the optimal incubation period for laccase production by *Daedaleopsis confragosa*. The results indicate that the 8th day of incubation showed maximum laccase production and below or above this incubation value a considerable (Fig 5). After 10th days of incubation the enzyme might be inactivated by the secretion of proteases by the fungus or due to the denaturation of the enzyme protein (Kashyap et al., 2002). Chiranjeevi et al., (2014) have reported that the maximum level of laccase production was observed at 18th days of incubation period by *Pleurotus ostreatus.*

Nadeem and Sheikh, (2014) have reported that the maximum level of laccase production was observed at 16th days of incubation period by *Pleurotus ostreatus.*

Effect of inoculum size on laccase production

In the present study, an attempt was made to investigate the influence of 6 different mycelia discs (6 mm) from 7 days old *Daedaleopsis confragosa* of on the fermentation efficiency. The flasks were incubated for 10 days at static condition. The result showed that the higher yield of laccase was noticed at 6 mm of 5 mycelia discs of inoculums size. Above 6 mm and below of 5 mm of 6 mycelia discs of inoculum size decreased laccase production was observed (Fig 6).

In SSF process, the inoculum density is of great importance. Production of enzyme in sufficient amount required optimum inoculums size of cells; lowering inoculum size required longer time for cells to multiply for sufficient number and produce enzyme. On the other hand, an increase in the number of the inoculum would ensure a rapid proliferation and biomass synthesis, after a certain time, enzyme production could be decreased because of the depletion in the nutrients which may result in decreased in metabolic activity (Kashyap et al., 2002).

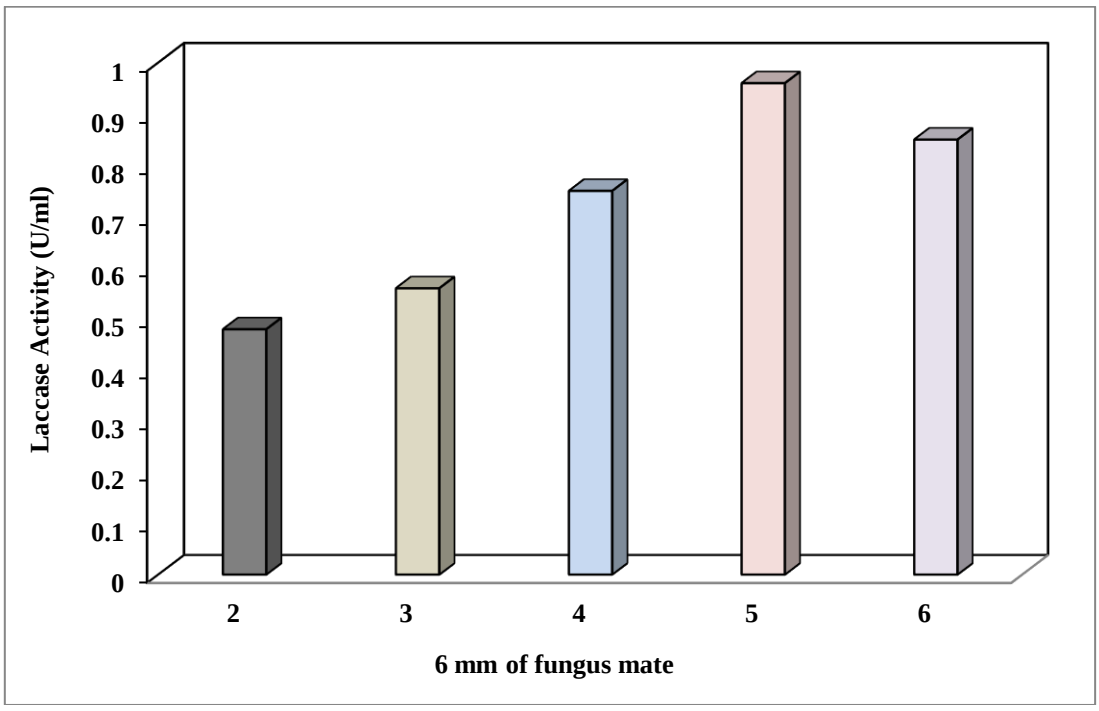

Fig 5. Effect of inoculum size on laccase production by *Daedaleopsis confragosa*

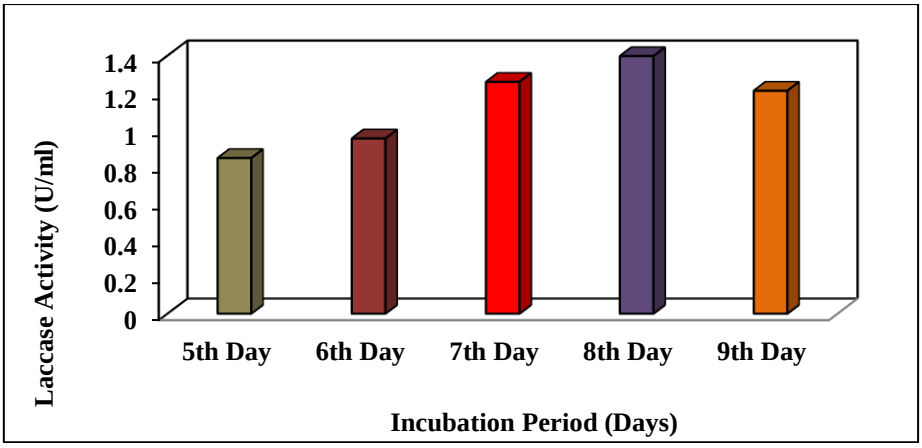

Fig 6. Effect of incubation period on laccase production by *Daedaleopsis confragosa*

Effect of carbon and nitrogen sources on laccase production

The carbon source is powerful nutrition regulation factors for producing the lignolytic enzymes (Patel et al., 2009). Glucose was the most enhancing carbon supplement; it produced maximum laccase production (Fig 7. Chiranjeevi et al., (2014) have reported that the glucose supported maximum of laccase production by *P. ostreatus*. Patel et al (2009) 20 observed addition of glucose to the wheat straw raises the laccase production by *P.ostreatus* HP-1.

The results showed that the peptone was supported the maximum laccase production. These results are in accordance with the findings of Chiranjeevi et al., (2014) where the authors observed an enhanced laccase production was with peptone followed by urea supplementation (Fig 8).

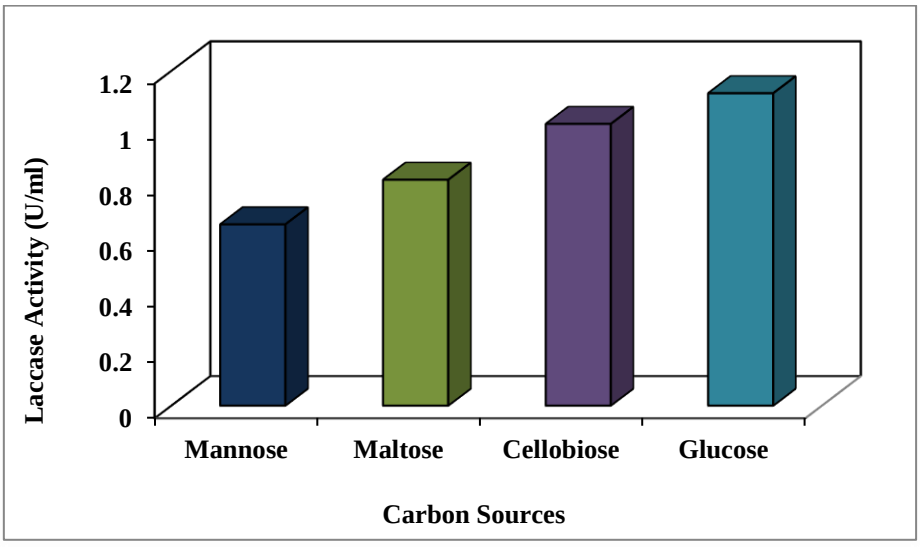

Fig 7. Effect of carbon source on laccase production by *Daedaleopsis confragosa*

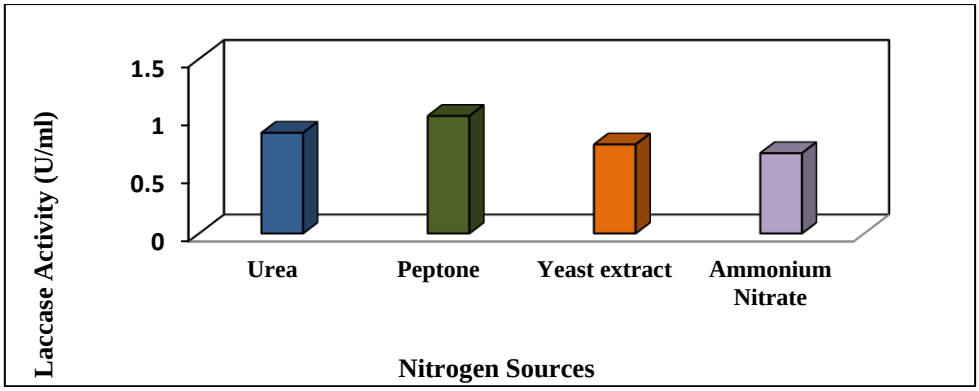

Fig 8. Effect of nitrogen source on laccase production by *Daedaleopsis confragosa*

4. Conclusion

From the results obtained, it can be concluded *Daedaleopsis confragosa*is capable of utilizing the rice bran and produce the laccase under SSF conditions. In optimizing SSF process the factors such as the carbon source and the levels of the nitrogen could influence the growth and production of the metabolites. It followed the trend of the rice bran which has more amount of total carbohydrates and nitrogen content gave the more yield of laccase by the *Daedaleopsis confragosa*. After optimization of the fermentation conditions like pH, temperature, inoculum size, incubation period 100% improvement was noticed. These promising results suggest the application of the system to industrial-scale operation in order to produce laccase enzyme economically challenging.

Acknowledgement: Authors are grateful to the University Grant Commission New Delhi, India for providing the financial support under UGC MRP NO. F.MRP/12th plan/14-15/KAKA088/UGC-SWRO/(10/12/14) and authors are also acknowledged to the Department of Biotechnology, Government Arts and Science College, Karwar for providing laboratory and technical support.

Conflicts of Interest: The authors hereby declare no conflicts of interest.

5. References

1. Arora D.S. and Gill P.K. Effect of various media and supplements on laccase production by some white-rot fungi. Bioresour Technol. 2000, 77: 89–91.
2. Arora D.S. and Sharma R.K. Ligninolytic fungal laccases and their biotechnological applications. Appl Biochem Biotechnol., 2010, 160: 1760–1788.
3. Aslam S. and Asgher M. Partial purification and characterization of ligninolytic enzymes produced by *Pleurotus ostreatus* during solid state fermentation. Afr J Biotechnol., 2011, 10: 17875-17883.
4. Baig N.S. and Sheikh N. Mycotechnological Production of Laccase by *Pleurotus ostreatus* and its Inhibition Study. The J Anim Plant Sci., 2014, 24(2): 492-502
5. Bollag J.M. and Leonowicz A. Comparative studies of extracellular fungal laccases. Appl Environ Microbiol. 1984, 48: 849–854.
6. Chiranjeevi P.V., Sathish T. and Pandian M.R. Harmonizing various Culture Conditions and Inducers for Hyper Laccase Production by *Pleurotus ostreatus* PVCRSP-7 in Solid State Fermentation. J Pharm Res., 2014, 8(4): 526-532
7. Ellaiah P., Adinarayana K., Bhavani Y., Padmaja P. and Srinivasulu B. Optimization of process parameters for glucoamylase production under solid state fermentation by a newly isolated Aspergillus species. Process Biochem. 2002; 38(4): 615-620.

8. Hadri S.H., Asad M.J., Gulfraz M., Asghar M., Minhas N.H. and Mahmood R.T. Solid State Fermentation for the production of Laccase by *Neurospora sitophila* using agro-wastes and its partial purification. Int. J. Adv. Res. Biol.Sci. 2015; 1(9): 33-44.
9. Jhadav A., Vamsi K.K., Khairnar Y., Boraste A., Gupta N., Trivedi S., Patil P., Gupta G., Gupta M., Mujapara A.K., Joshi B. and Mishra D. Optimization of production and partial purification of laccase by *Phanerochaete chrysosporium* using submerged fermentation. Int J Microbiol Res., 2009; 1(2): 9-12.
10. Kalra K. and Shavez M. Production of Laccase from Banana Skin by *Trametes hirsute* in Solid State and Submerged Fermentation. Asian J Biochem Pharmaceut Res., 2014, 4(2): 01-10.
11. Kashyap P., Sabu A., Pandey A., Szakacs G. and Soccol C.R., Extra-cellular L-glutaminase production by *Zygosaccharomyces rouxii* under solid-state fermentation. Process Biochem, 2002, 38: 307-312.
12. Kirk T.K. and Farrell R.L. Enzymatic combustion: the microbial degradation of lignin. Annu Rev Microbiol 1987, 41: 465–505.
13. Lee I.Y., Jung K.H., Lee C.H. and Park Y.H. Enhanced production of laccase in Trametesversicolor by the addition of ethanol. Biotechnol Lett., 1999, 21: 965–968.
14. Liers C., Ullrich R., Pecyna M., Schlosser D., Hofrichter M. Production, purification and partial enzymatic and molecular characterization of a laccase from the wood rotting ascomycete *Xylaria polymorpha*. Enz Microb Technol., 2007, 41: 785–793.
15. Lonsane B.K., Castaneda G.S., Raimbault M., Roussos S., Gonzalez G.V., Ghildyal N.P., Ramakrishna M. and Krishnaiah M.M. Scale up strategies for solid state fermentation systems. Process Biochem., 1992, 27: 259- 273.
16. Mayer AM, Staples RC. Laccase: new functions for an old enzyme. Phytochem. 60: 551–565, 2002.
17. Moldes D., Lorenzo M. and Sanroman M.A. Di Verent proportions of laccase isoenzymes produced by submerged cultures of *Trametes versicolor* grown on lignocellulosic wastes. Biotechnol Lett., 2004, 26: 327-330.
18. Nagai M., Sato T., Watanabe H., Saito K., Kawata M. and Enei E. Purification and characterization of an extracellular laccase from the edible mushroom *Lentinula edodes*, and decolorization of chemically different dyes. Appl Microbiol Biotechnol., 2002, 60(3): 327-335.
19. Pandey A. Solid State Fermentation. Pandey A (Ed). Wiley Eastern Publishers, New Delhi. 1994, 3 – 10.
20. Patel H., Gupte A. and Gupte S. Effect of different cultural conditions and inducers on production of Laccase by a basidiomycete fungal isolate Pleurotus ostreatus HP-1 under solid state fermentation. BioRes., 2009, 4: 268-284.
21. Raimbault M. General and microbiological aspects of solid substrate fermentation. Elect J Biotechnol., 1-15 (1998).
22. Reddy G.V., Babu P.R., Komaraiah P., Roy K.R.R.M. and Kothari I.L. Utilization of banana waste for the production of lignolytic and cellulolytic enzymes by solid substrate fermentation using two Pleurotus species (*P. ostreaus* and *P. sajor-caju*). Process Bio Chem., 2003, 38: 1457–1462.
23. Ronak S. and Chhaya: Ph.D thesis. Studies on Laccase from Streptomyces Sp. 2013.
24. Sathish T., Laxmi G.S., Rao C.S., Brahmaiah P. and Prakasham R.S. Mixture design as first step for improved glutaminase production in solid-state fermentation by isolated Bacillus. Let Appl Microbiol., 2008, 47: 256-262.
25. Sharma K.K, and Kuhad R.C. Laccase: enzyme revisited and function redefined. Ind J Microbiol. 2008, 48: 309–316.

26. Shin K.S. and Lee Y.J. Purification and characterization of a new member of the laccase family from the white-rot basidiomycete *Coriolus hirsutus*. Arch Biochem Biophys., 2000, 384(1):109-115.
27. Sudheer I. Manawadi, Adiveppa B. Vantamuri and Sanjotha K. Guruvin. Characterization of ligninolytic enzymes and decolourization of selected textile dyes from the blushing bracket mushroom, *Daedaleopsis confragosa*. Int J Pharm Sci Res., 2019; 10(12): 1000-07.
28. Tan Y.H. and Wahab M.N. Extracellular enzyme production during anamorphic growth in the edible mushroom, *Pleurotus sajor-caju*. World J Microbiol Biotechnol., 1997, 13: 613- 617.
29. Thurston C.F. The structure and function of fungal laccases. Microbiol., 1994, 140(1): 19-26.
30. Usha K.Y., Patil S.J., Dileep K., Shanti B., Reddy R.B. and Kalva P.K. Standardization of methodology for extracting ligninolytic enzymes from solid state fermentation. Res J Biotechnol., 2020, 15(12): 174-181.
31. Vantamuri A.B, and Kaliwal B.B. Purification and characterization of laccase from Marasmius species BBKAV79 and effective decolorization of selected textile dyes. 3 Biotech., 2016; 6: 189.
32. Xiao Y.Z., Tu X.M. Wang J., Zhang M., Cheng Q., Zeng W.Y. Purification, molecular characterization and reactivity with aromatic compounds of a laccase from basidiomycete Trametes sp. strain AH28-2. Appl Microbiol Biotechnol.,2003, 60(6): 700-707.