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PRODUCTION OF LACCASE BY NEWLY ISOLATED *MARASMIUS* SP. BBKAV79 IN SOLID STATE FERMENTATION AND ITS ANTIPROLIFERATIVE ACTIVITY

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ABSTRACT: The aim of the present study was to investigate the production of laccase by *Marasmius* sp. BBKAV79 in Solid State Fermentation (SSF) and its antiproliferative activity. Experiments were also conducted to determine the variables that could affect activity of laccase. In particular, the effects of the initial moisture content, pH, temperature, and inoculum volume, incubation period in addition to carbon and nitrogen sources were evaluated. Rice bran was found to be the best supported lignocellulosic substrate for extracellular laccase production under SSF. Highest activity of laccase was achieved at pH 6.0 and temperature at 40°C. Inoculum size and incubation period for laccase production at 2000µl culture broth and 10 days respectively. The presence of starch and ammonium sulphate in the growth medium was found to be favourable for the production of the laccase. Such a high activity was obtained without any addition of inducers. Thus, the indigenous isolate is found to be a potential producer of laccase using SSF and can be exploited for further biotechnological applications. The process also promises economical utilization and value addition of agro-residues and it potently suppressed the proliferation of tumor cell lines KB, MCF7 and VERO with an IC50 value of 88.61 µl, 49.5 µl, and ~67603 µl respectively, signifying that it is an anticancer protein.

INTRODUCTION: A number of anthropological activities has resulted in extensive pollution [1] aggravating the need to understand the toxic impacts of xenobiotics in the environment. The deteriorating conditions of water and soil quality has affected a wide range of organisms including fishes [2, 3, 4, 5] and amphibians [6].

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The increasing exploitation has resulted in need of technology for remediation of toxic chemicals and dyes [7]. Enzymatic degradation of these stains and dyes through microbial approach has become an integrated and preferred method of addressing the issues of environmental pollution globally and hence enzyme production is noted to be an emerging field of biotechnology [8].

Annual world sales figures are close to billion dollars [9] with 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 [10, 11]. SSF is defined as any fermentation process performed on a non-soluble material that acts both as physical support and source of nutrients in absence of free flowing liquid [12]. The low moisture content means that fermentation can only be carried out by a limited number of microorganisms, mainly yeasts and fungi, although some bacteria have also been used [13].

Laccase (E.C. 1.10.3.2, p-benzenedial: oxygen oxidoreductases) belongs to oxidoreductase class and is able to catalyse the oxidation of various aromatic compounds (particularly phenol) with the concomitant reduction of oxygen to water [14]. Laccase was first discovered in the sap of the Japanese lacquer tree *Rhusvernicifera*, and its characteristic as a metal containing oxidase was discovered by Bertrand in 1985 [15]. Since then, laccases have also been found in various basidiomycetous and ascomycetous fungi and thus far fungal laccases have accounted for the most important group of multicopper oxidases (MCOs) with respect to number and extent of characterization [15]. The potential use of laccases in biotechnology has stimulated the need to discover suitable enzymes in large quantities. Production of any enzyme including that of lacasses is known to be affected by fermentation factors such as, medium composition, pH, temperature and aeration. There have been reports describing increased production of extracellular laccases in many species of white rot fungi when grown on natural substrates, such as cotton stalk [16], molasses wastewater [17], wheat bran [18] and barley bran [19].

However, the literature support towards optimization is found to be very limited. Utilization of industrial and agricultural wastes for laccase production is an effective way to reduce production costs and also simultaneously utilise these substrates efficiently [20]. On other hand, strategies for effective large scale production of laccases through SSF is found to be limited as well. Hence, the present work is planned to study various physicochemical parameters for optimization of laccase by *Marasmius* sp. BBKAV79 and antiproliferative assay was performed.

MATERIALS AND METHODS:

Microorganism: Organism screening for laccase-producing microbes on potato dextrose agar plates (PDA) containing indicators namely, guaiacol, ABTS, syrindalzine and tannic acid resulted in isolation of 8 fungal strains. Isolates showing positive reaction were maintained on PDA plates at 30°C and stored at 4°C. The best laccase producing isolate was identified by molecular characterization technique at Agharkar Research Institute, Pune, India and identified as *Marasmius* sp. BBKAV79 (NCBI Gen Bank accession number KP455496, KP455497).

Screening of different lignocellulosic substrates: The following agro wastes were used for the initial screening: rice bran, rice straw, sugarcane bagasse, saw dust and pigeon pea waste. All of them were locally procured and were sterilized at 121 °C and 15lb pressure for 20 mins and were used for the study.

Media preparation: 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 30 ºC in shaking incubator with 150 rpm for 5 days and the enzyme extraction and assay were done as guaiacol assay method [21]. The best agro waste which supports the maximal laccase assay were selected and used for the optimization study.

Laccase Harvesting: After 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 ºC to remove all spores and other impurities. The supernatant was collected and subjected to laccase assay [22].

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 5 g of studied substrates with salt solution [23]. 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. [24, 25].

Effect of pH for laccase production: The effect of various pH viz., 3, 4, 5, 6, 7, 8 and 9 on laccase production in rice bran broth was done by inoculating the flasks (with the above pH) with *Marasmius* sp. BBKAV79 and the flasks were incubated and assay was done as guaiacol assay method.

Effect of temperature for laccase production: The effect of various temperature ranges on laccase production in rice bran broth was studied by inoculating the *Marasmius* sp. BBKAV79 and the incubating the flasks at different temperatures viz., 37°C, 40°C, 45°C , 50°C and 55°C. The flasks were incubated for 5 days and assay was done as guaiacol assay method.

Effect of inoculums volume for laccase production: The effect of inoculum volume was studied by adding different levels of inoculum (250, 500, 1000 and 2000) from 5 days old fungal broth to the rice bran broth and incubated at room temperature and assay was done as guaiacol assay method.

Effect of incubation period for laccase production: The effect of incubation period on laccase production in rice bran broth was studied by inoculating with *Marasmius* sp. BBKAV79 and incubated at room temperature for various time intervals. The enzyme was extracted and its activity was determined.

Effect of carbon sources for laccase production: Carbon sources as maltose, sucrose, starch, fructose, glucose and arabinose were added to rice bran broth on laccase production was studied by inoculating the *Marasmius* sp. BBKAV79 and the flasks were incubated at room temperature and assay was done as guaiacol assay method.

Effect of nitrogen sources for laccase production: Nitrogen sources as tryptone, peptone, sodium nitrate, ammonium sulphate and sodium carbonate were added to rice bran broth on laccase production was studied by inoculating the *Marasmius*sp.BBKAV79 and the flasks were incubated at room temperature and assay was done as guaiacol assay method.

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 [21, 26, 27, 28].

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

Assay of Antiproliferative Activity: Antiproliferative activity has been reported for many mushroom proteins. Performed cytotoxicity by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cell lines were cultured in DMEM medium (Invitrogen) which was supplemented with 10% FBS (Gibco, Invitrogen) and 1% Antibiotic–Antimycotic 100X solution (Thermo fisher Scientific).

The cells were seeded at a density of approximately 5×10^3 cells/well in a 96-well flat-bottom micro plate (NEST-Biotechnology) and maintained at 37ºC in 95% humidity and 5% CO_2 for overnight. Different concentration (100, 50, 25, 12.5, 6.5, 3.125 µg/300 mL) of purified laccase were treated. The cells were incubated for another 48 hours. The cells in well were washed twice with phosphate buffer solution, and 20 µL of the MTT staining solution (5mg/ml in phosphate buffer solution) was added to each well and plate was incubated at 37ºC. After 4h, 100 µL of di- methyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals, and absorbance was recorded with a 570 nm using micro plate reader.

Formula:

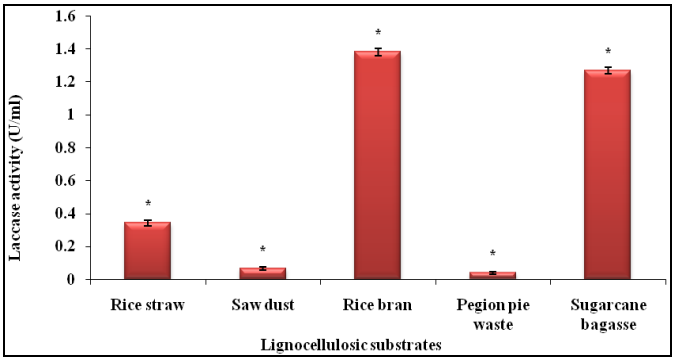
Surviving cells (%) = Mean OD of test compound /Mean OD of Negative control ×100

Inhibiting cells (%) =100- Surviving cells

Statistical analysis: Data were analysed by one-way analysis of variance (ANOVA) followed by Tukey's Highest Significant Difference (HSD) post hoc test. Readings were considered significant when *P* was ≤0.05.

RESULTS AND DISCUSSION

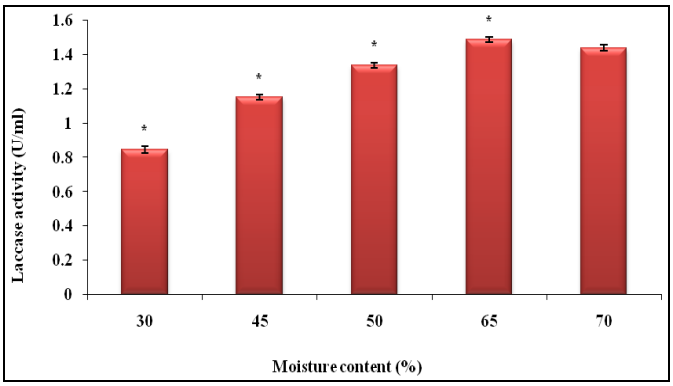
Screening of lignocellulosic substrates: Different lignocellulosic substrates like rice bran, rice straw, sugarcane bagasse, saw dust and pigeon pea waste were screened for laccase production. But these substrates showed marked variation with respect to the laccase production. Rice bran was supported maximum laccase production followed by other substrates. The laccase production varied significantly between rice bran and all other substrates (**Graph 1**).



GRAPH 1: SCREENING OF LIGNOCELLULOSIC SUBSTRATES FOR LACCASE PRODUCTION BY *MARASMIUS* SP. BBKAV79

The selection of a substrate for SSF processes depends upon several factors mainly related with cost and availability and thus may involve screening of several agro-industrial residues. Moreover, the utilization of this type of supports helps to solve the pollution problems caused by their disposal. According to these results, rice bran was selected to be used for improving laccase production as solid substrate. Rice bran was the best for laccase productions in our result, which may be due to it contain protein and carbohydrates enhance fungal growth and increase its metabolic activity. Laccase synthesis was encouraged by phenolic compounds containing in rice bran, leading to increasing of laccase production. This induction mechanism may help to humiliate lignin or aromatic compounds in rice bran to supply further nutrients especially carbon and nitrogen [29]. As previously reported similar results that the rice bran was best substrate for maximum laccase production from *Streptomyces chartreusis* and *Pleurotusostreatus*, respectively [30, 31] and Hendro *et al.*,[32] have evaluated rice straw and rice husk for the production of laccase by white rot fungi.

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 (**Graph 2**).



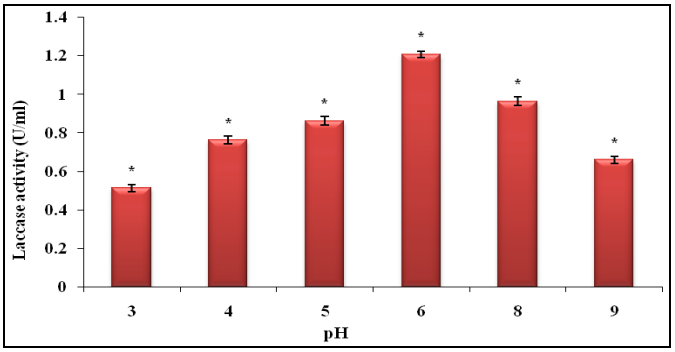
GRAPH 2: EFFECT OF INITIAL MOISTURE CONTENT FOR LACCASE PRODUCTION FROM *MARASMIUS* SP. BBKAV79

Moisture is another key parameter to control the growth of microorganisms and metabolites production in solid state fermentation [23].

The optimum moisture level in SSF is governed by the water holding capacity of the substrate, the type of the end product, and water requirements of the fungus [33, 34]. Higher and lower water contents adversely affect the primary metabolic activities of microbes, leading to secretion of lower activities of ligninases in secondary growth [35, 36]. Low moisture contents in SSF have also been reported to decrease the enzyme formation and metabolic activities of fungi due to reduced solubility of nutrients, low substrate swelling, and higher water tension [37].

This result is consistent with previously reported moisture content 65%, 60%, 66%, 60% and 60 % by *Streptomyces psammoticus*, *Schyzophylum commune*, *Pleurotusostreatus*, *Pleurotusostreatus* HP-1 and *Pleurotusostreatus* PVCRSP-7, respectively [23, 38, 39, 40, 41]. The moisture level in SSF has a great impact on the physical properties of the substrate. Solid substrates used in SSF are insoluble in water therefore; water will have to be absorbed on to the substrate particles, which could be used by the microorganisms for growth and metabolic activity. Thus the degree of hydration of the substrate plays an important role on the growth of the fungi and subsequently the enzyme production [12].

Influence of pH for laccase production: The results showed that the *Marasmius* sp. BBKAV79 was able to produce maximum laccase production at pH 6. The laccase production was found to be significantly high at pH 6 as compared with all other pH conditions (**Graph 3**).



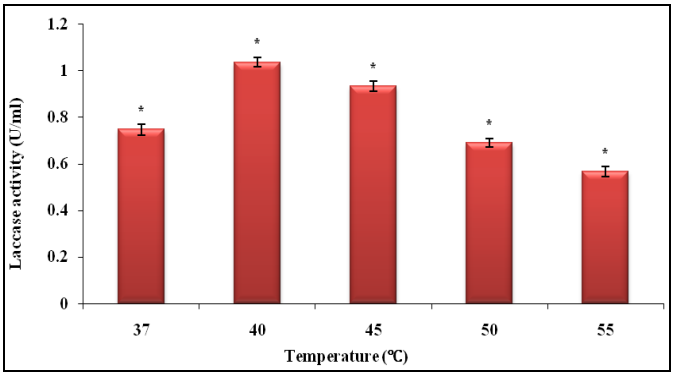
GRAPH 3: EFFECT OF pH ON LACCASE PRODUCTION BY *MARASMIUS* SP. BBKAV79

As previously reported similar results that the highest laccase production observed at pH 6 from *Pleurotuseous*, *Rigidoporus* sp., *Pleurotusostreatus* POXA1 and *Rigidoporuslignosus*, respectively [42, 43, 44, 45].

Most reports indicated that the initial pH between 4.5 and 6.0 was suitable for enzyme production [14, 46]. Soden*et al.,*[47] have reported that the laccase showed maximum activity at pH 6.5 by *Pleurotussajor-caju*Lac4.Ronak and Modi,[30] have suggested that the *Streptomyces chartreusis* showed maximum laccase production at pH 8.

pH is important parameters that determine the growth rate of fungi and significantly affect the level of laccase produced. The production of the enzyme strongly depends upon the initial pH of the culture medium as it may influences many enzymatic processes and transport of nutrients across the cell membrane [42]. At higher alkaline pH, the enzyme activity decreased gradually, due to the difference in redox potential between a reducing substrate and the type 1 copper in the active site of the enzyme and the inhibition of type 3 copper by the hydroxyl ion at higher pH [48]. Laccase was proved to be active pH in the range of 3.3 to 6.0 and in consensus with date reported by others [49, 50, 51, 52, 53].

Influence of temperature for laccase production: The results indicate that the *Marasmius* sp. BBKAV79 strain was able to produce high laccase production at temperature 40ºC. The laccase production was found to be significantly high at 40ºC as compared with other temperatures except for 45ºC which was found to be insignificant when compared with 40 ºC (**Graph 4**).

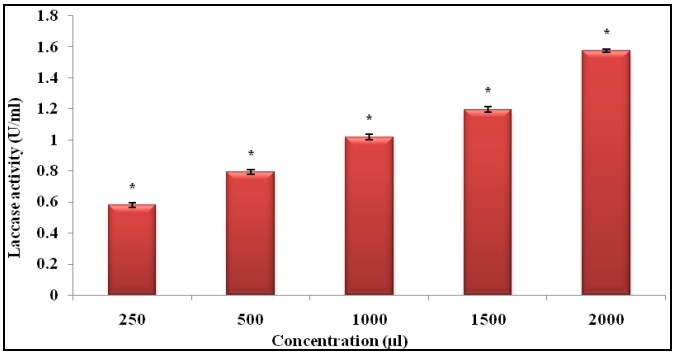


GRAPH 4: EFFECT OF TEMPERATURE ON LACCASE PRODUCTION BY *MARASMIUS* SP. BBKAV79

Temperature is influencing the growth in SSF, the production enzyme and their metabolites [54]. Temperature is much significance in the SSF systems because during fermentation there is a general increase in the temperature of the fermenting mass due to respiration [55].

Even though the impact of temperature is more prominent in the scale up processes it remains an inevitable factor in all fermentation systems due to its impact on microbial growth and metabolite production [46]. Earlier reported similar results that the highest laccase production was observed at 40ºC from *Trametes hirsute* [56]. Narayanan *et al.,*[57] have reported that the temperature profile showed differences in the enzyme activity between rice bran and wheat bran the maximum laccase activity was observed at 30ºC for rice bran when compared to wheat bran at 40ºC by *Bacillus subtilis*. A significant influence of incubation temperature on ligninolytic enzymes of *Pleurotus* sp. and *Dichomitussqualen* and other white rot fungi has been reported, and temperatures ranging from 25 to 30ºC were found optimum for laccase production [58, 59, 61, 62]. Shanmugam *et al.,* [42] have reported the *Pleurotuseous* showed highest laccase production at temperature 50 ºC. Abrahão *et al.,* [62] have reported a class of isolated Basidiomycetes with an optimum laccase activity at 70ºC.

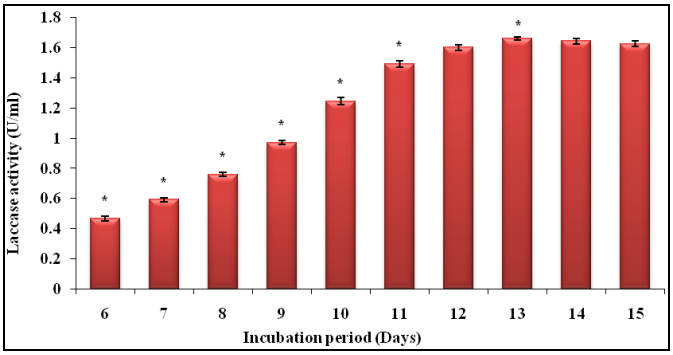
Influence of inoculum volume for laccase production: The results showed that the highest laccase production was obtained when using 2000 µl of culture broth. The laccase production was found to be significantly high at 2000 µl as compared with all other volumes of inoculum considered for present study (**Graph 5**).



GRAPH 5: EFFECT OF INOCULUM SIZE ON LACCASE PRODUCTION BY *MARASMIUS* SP. BBKAV79

Inoculum size plays a significant role in enzyme production in solid state fermentation. A lower level of inoculum may not be sufficient to initiate the growth, whereas a higher level may cause competitive inhibition [63]. Thus, determination of optimum inoculum size becomes a crucial step in SSF. In previous studies different inoculums size have been used for laccase production, four *Pleurotuspulmonarius* mycelial plugs measuring 10 mm in diameter were used for inoculation of 5 g of wheat bran [10]. Moleds *et al.,* [64] have observed that the highest specific activity for laccase was obtained when 20 *Trametes hirsute* agar plugs measuring 3 mm in diameter for inoculation 2.5 gm barley bran or 5 g grape seed was used. Ronak and Modi, [31] have reported that the maximum production of laccase was observed when using 1.5 x 10^4Colony formation unit (CFU). Patel *et al.,* [40] have observed that the highest activity of laccase was obtained when five *Plerotusostreatus* agar plugs measuring 8 mm in diameter for inoculation. Hafiz *et al.,* [65] have showed the maximum ligninase activity by *Trametesversicolor* at 15 ml of inoculum. The maximum production of laccase was observed by *Streptomyces psammoticus* when using 1.5 x 10^7CFU [23].

Influence of incubation time for laccase production: The results indicate that the 13 days of incubation showed excellent laccase production. The laccase production varied significantly between 13 day of incubation as compared with other durations with exceptions being 12, 14 and 15 day of incubation which were found to insignificantly vary as compared to 13 day (**Graph 6**).

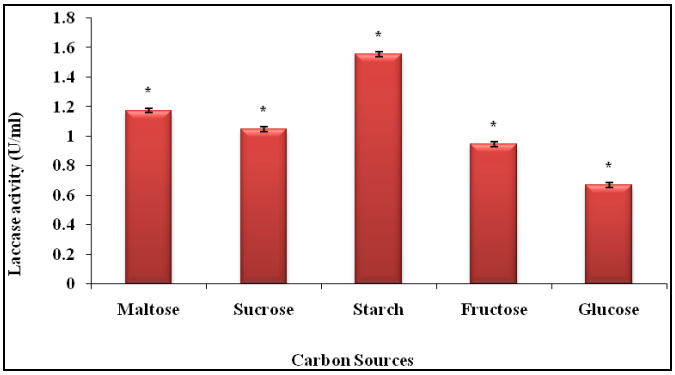


GRAPH 6: EFFECT OF INCUBATION PERIOD ON LACCASE PRODUCTION BY *MARASMIUS* SP. BBKAV79

In SSF process, the inoculum density is of great importance. Too low a density may give insufficient biomass and permit the growth of undesirable contaminants, while for some processes high inoculum densities may produce too much biomass and deplete the carbohydrates and nutrients necessary for product formation [54]. Previously reported the optimum laccase production by *Pleurotusostreatus* was observed after 7 days of incubation in solid state

fermentation medium contain wheat straw as substrate [39]. Masutti *et al.*, [66] have reported the highest level of laccase production from *Pleurotusostreatus* was occurred after 28 days by solid state fermentation. *Marasmius* sp. and a solid substrate of rice straw demonstrated the highest laccase activity on day 10. Abdulkareem, [46] has reported that the maximum level of laccase production was observed at 15 days of incubation period by *Pleurotusostreatus*.

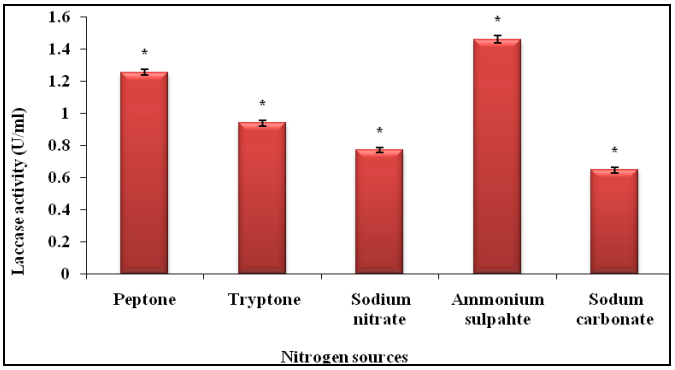
Influence of carbon sources for laccase production: The results showed that the starch supported the maximum laccase activity. The laccase production varied significantly between Starch and all other carbon sources conditions (**Graph 7).**



GRAPH 7: EFFECT OF CARBON SOURCES ON LACCASE PRODUCTION BY *MARASMIUS* SP. BBKAV79

The carbon source is powerful nutrition regulation factors for producing the ligninolytic enzymes. Nature and type of carbon sources are among the most important factors for any fermentation process [55]. The effect of different carbon sources on laccase production has been established in the case of fungal strains [67]. Earlier reported the glucose as a carbon source supported the extreme laccase production from *Streptomyces psammoticus, Pleurotusostreatus* PVCRSP-7, *Coriolusversico*lor, *Trameteshirsuta* and *Pleurotusostreatus* LIG 19, respectively [23, 41, 55, 68, 69].

Influence of nitrogen sources for laccase production: The results showed that the ammonium sulphate supported the maximum laccase activity. The laccase production varied significantly between Ammonium sulphate and all other nitrogen sources (**Graph 8**).



GRAPH 8: EFFECT OF NITROGEN SOURCES ON LACCASE PRODUCTION BY *MARASMIUS* SP. BBKAV79

The ligninolytic enzymes have been seen to be regulated by the usable concentration nitrogen in the medium. The low nitrogen level can stimulate the ligninoyltic enzyme production [40]. Previously reported similar results that the peptone as a nitrogen source supported maximum laccase production from *Lentinusedodes* and *Pleurotusostreatus, Pleurotusostreatus, Streptomyces psammoticus, Bacillus subtilis* MTCC 2414, *Pleurotusostreatus* PVCRSP-7, *Trametes hirsute*, respectively [70, 71, 23, 57, 41, 56]. Ronak and Modi, [30] have showed that the yeast extract supported maximum laccase production by S*treptomyces chartreusis*. Elisashvili *et al.*,[72] have shown that medium with ammonium sulphate has given highest levels of laccase activity in *Cerrena unicolor*. Zahida *et al.*,[68] have reported that the greatest production of laccase, chick pea powder was used as nitrogen source in solid state fermentation.

Antiproliferative Activity: It is remarkable that some of mushroom components including laccases, lectins, polysaccharopeptides, and antifungal proteins exhibit inhibitory activities towards tumor cells. In the present study, the purified laccase demonstrates antiproliferative activity towards tumor cell lines KB and MCF7 and normal cell line VERO IC50 value of 88.61 µl, 49.5 µl, and ~67603 µl respectively (**Fig.1**) and this compound was not toxic to the normal cell line. It concludes that the laccaseis acting as anticancer protein. On the other hand, the present laccase is purified from Solid State Fermentation, which means that the protein is very easy to obtain. It is noteworthy that it possesses further applications of agents for cancer therapy.

Li *et al.*, [73] have reported that the laccase from *Tricholomamongolicum* exerting antiproliferative activity toward tumor cells. Laccase from *Abortiporusbiennis* demonstrated anti-proliferative activity against Hep G2 and MCF-7 cells with IC50 values of 12.5 μM and 6.7 μM, respectively [74].

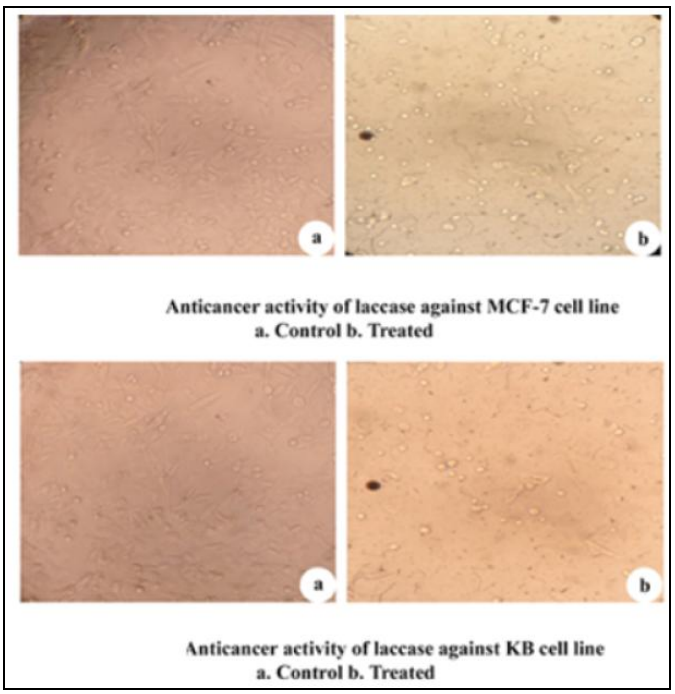


FIG.1: LACCASE WITH ANTIPROLIFERATIVE ACTIVITY FROM *MARASMIUS* SP. BBKAV79

CONCLUSION: The present isolate *Marasmius* sp. BBKAV79 is found to be a very efficient producer of laccase under SSF. The present investigation has confirmed and evaluated the use of rice bran as an inexpensive and easily available agro industrial waste for laccase production under solid state fermentation. The production process can be commercialized after optimization for enzyme production and laccase act as anti-cancer protein.

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