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RESEARCH ARTICLE

An insight into the growth and nutritional requirements for ligninolytic enzymes production by *Rigidoporus vinctus*

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ABSTRACT

Rigidoporus vinctus (Berk. and Ryverdan) is a white-rot fungi that belongs to the Agaricomycete class of division Basidiomycota. It produces the ligninolytic enzymes having many biotechnological applications such as the degradation of xenobiotic compounds and dyes. It is also used in inducing agarwood formation and another application of this fungi is that it produces oil/lipids so it can also be used as biofuel. The study was carried out to examine the in vitro growth and ligninolytic enzymes production by the fungus. The growth of fungi was examined with different physical parameters (pH, temperature and days of incubation), in contrast the growth and ligninolytic enzymes activity was examined with respect to different carbon and nitrogen sources. The optimum growth of fungi was observed with Richard's medium at 32°C and with initial pH of 6.0 on 24th day of incubation. Starch is the best carbon source for growth, whereas LiP and MnP activity both were maximum expressed in maltose and laccase activity was maximum in glucose supplemented media. In the case of inorganic nitrogen compounds, Rigidoporus vinctus attained optimum growth with ammonium sulphate and maximum activity for LiP, MnP and laccase were observed with ammonium chloride, ammonium sulphate and ammonium oxalate supplemented media, respectively. In the case of organic nitrogen sources fungi expressed optimum growth with L-proline, whereas LiP, MnP and laccase activity were maximum in DL-tryptophan, DL-valine and DL-aspartic acid supplemented media, respectively.

Keywords: Agaricomycete, ligninolytic enzymes, *Rigidoporus vinctus*, white rot

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INTRODUCTION

Fungi are heterotrophic organisms that feed on nutrients by secretion of extracellular enzymes followed by absorption of solubilized breakdown products and after colonization of a food source, they form branching tubes called hyphae which together make up mycelium (Webster and Weber, 2007). The phylum Basidiomycota includes fungi that decompose wood, it includes species that cause white and brown rot and play an important role in the global carbon cycle. Fungi produce a wide range of extracellular enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase that are beneficial for their biotechnological applications such as biopulping (Garcia-Torreiro et al., 2018; Martins et al., 2018), degradation of pesticides (Kumari et al., 2022), degradation of polycyclic aromatic hydrocarbons (Imam et al., 2022) and degradation of dyes

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(Brenk and Wosten, 2021). During the secondary metabolic phase, ligninolytic enzymes production is mainly triggered by limitation in concentration of carbon, nitrogen and sulphur (Viswanath et al., 2014). Laccase is a copper-containing glycoprotein with molecular weight ranges from 50-100 kDa. It is produced by some bacteria, insects, higher plants and fungi but laccase produced by white-rot fungi is highly explored (Claus, 2004; Mishra et al., 2011). Some of the common laccases producing basidiomycetous fungi are *Coprinus cinereus, Agaricus bisporus, Phlebia radiata, Pleurotus pulmonarius, Pleurotus ostreatus, Botrytis cinerea, Trametes versicolor, Ganoderma lucidum, Rigidoporus lignosus, Rigidoporus microporous, Schizophyllum commune, Coriolopsis gallica and Cyathus bulleri* (Peralta et al., 2017; Yang et al., 2017). Laccase is widely used in the treatment of dyes and degradation of phenolic contaminants (Voberkova et al., 2018).

Manganese peroxidase (MnP) is a glycosylated haem protein with molecular weight ranges from 38-62.5 kDa, averaging at 45 kDa (Janusz et al., 2013). It is produced by relatively all basidiomycetes that cause white rot and some litter decomposing fungi (Voberkova et al., 2018). The MnP has various biotechnological applications such as bioremediation of organopollutants in soil and water, removing of hazardous wastes, pulping and bleaching of cellulose. It oxidizes Mn2+ to Mn3+ with H2O2. Some white-rot fungi producing MnP are Schizophyllum sp., Trametes sp., Stereumostrea, Phanerochaete chrysosporium, Irpex lacteus, Rhizoctonia sp. and Schizophyllum sp. Crude MnP from Cerrena unicolor decolorizes dyes such as Congo red, Methyl orange, Remazol brilliant blue R, Bromophenol blue and Crystal violet (Zhang et al., 2018).

Lignin peroxidase (LiP) is a haem containing glycoprotein having the lowest molecular weight of 38-42 kDa than laccase and manganese peroxidase. It can cleave phenolic and non-phenolic lignin compounds using H2O2 (hydrogen peroxide). Lignin peroxidase is an effective pollutant degrader with high oxidation and reduction potential and has a great ability to degrade pollutants than MnP and laccase (Voberkova et al., 2018).

Studies revealed that many nutritional and physical parameters can influence the synthesis of ligninolytic enzymes, including medium composition, carbon and nitrogen sources, inducer additions, pH of culture media, incubation temperature, and aeration rate (Schneider et al., 2018). The nutritional and physicochemical requirements are extensively studied for the enhancement of ligninolytic enzymes for Pleurotus spp. (Bellettini et al., 2016), Stereum ostrea (Usha et al., 2014), Pleurotus citrinopileatus and Irpex lacteus (Zerva et al., 2017), Tricholoma giganteum (Patel and Gupte, 2016), Cerrena unicolor (Zhang et al., 2018), Morchellacrassipes (Kanwal and Reddy, 2011), Aspergillus niger (Dhakar et al., 2015), Phlebia floridensis (Arora and Gill, 2005), Phlebia fascicularia, Dichomitus squalens, and Phlebia floridensis (Arora and Gill, 2000), Phlebia brevispora, Daedalea flavida, Phlebia brevispora, Polyporus sanguineus and Phlebia radiate (Arora and Gill, 2001), Pleurotus ostreatus (Zhu et al., 2016), Trametes maxima-Paecilomyces carneus (Chan cupul et al., 2014), Pleurotus sajor-caju (Patrick et al., 2011), Coriolopsis gallica strain (Elisashvili et al., 2017), Fomes fomentarius (Neifar et al., 2009) and Trametes velutina (Yang et al., 2013). Because the enzymes released by basidiomycetes are depends on the strain and culture factors, so more white rot fungi must be assessed for their capacity to degrade xenobiotics (Levin et al., 2010). As per our knowledge, there is lack of such data concerning indigenous strains, so the study was conducted to know the physiological behaviour of agaricomycetous fungi, *Rigidoporus vinctus* in relation to physical factors (temperature, H-ion concentration and days of incubation) and the effect of carbon and nitrogen sources on growth and enzyme production. *Rigidoporus vinctus* is a white-rot fungus that produces ligninolytic enzymes, LiP, MnP and laccase. It is also used for inducing agarwood formation (Chen et al., 2018) and due to the presence of gloeoplerous hyphae that is hyphae containing oil/lipids (Webster and Weber, 2007), this fungi can also be used as biofuel. This study will help the other researchers in optimizing the production of ligninolytic enzymes by other fungi under in vitro conditions for commercial purposes.

MATERIALS AND METHODS

Physiological studies

Fungus was studied in vitro on liquid basal media to determine the optimum physical factors and nutritional requirements for growth and ligninolytic enzymes activity. For experiments, the inoculum was prepared by culturing Rigidoporus vinctus on MEA for 7 days at 24°C. In 100 ml flask 25ml media was taken and subjected to autoclave at 15lbs for 20 minutes, and after cooling, a mycelial disc (dry weight of 2.5mg) was inoculated aseptically in each flask. The flasks were incubated for 10 days (taken tentatively) at 24°C. After optimizing the basal medium, temperature, pH and days of incubation, further experiments were conducted by incubating the test fungi under the optimized physical conditions. Three replicates were kept for each treatment in each experiment, along with the control (wherever required). The experiments were conducted in a completely randomized design. At the end of each experiment the microscopic and cultural characteristics were observed. The mycelial dry weight was noted down for each replicate using pre weighed Whatman filter paper No. 1 using an electronic balance (Sartorius Analytical BL 210S). The pH of each replicate's culture filtrate was recorded using Microprocessor pH meter ME 63. The cultural charctersitics, including the type of growth such as superficial (aerial growth) or submerged growth of mycelium, colour, and growth rate, were observed under naked eyes and recorded. The growth rate was recorded as complete growth if mycelium growth is reaching up to the margin of the conical flask and incomplete if the mycelium growth is less if mycelium does not touches the margin of the flask. After each treatment, mycelium slides were prepared using 2% congo red and were examined under a microscope (Matrix VRS-2F) for morphological studies at 100X magnifications.

Basal media

The fungus was grown in twelve different liquid basal media viz., Coon's, Raulin's, Richard's, Glucose peptone, Dox's, Glucose nitrate, Czapek's-I, Czapek's-II, Brown's-I, Brown's-II, Elliot's and Asthana and Hawker's.

Temperature

The fungus was cultured in a basal medium that supported the optimum growth for a period of 10 days at various temperatures ranging from 16 to 32°C with a 4°C difference to determine the temperature required for optimum growth.

pH

The impact of pH was measured by adjusting the pH range varying from 3.0 to 9.0 with a unit difference by using 1N HCL and 1N KOH and data was recorded on Microprocessor pH meter ME 63.

Days of incubation

The fungi was grown for a period of 30 days at optimal temperature and pH to determine the optimal duration required for growth and enzyme production. On every alternate day, three flasks were taken to examine the growth and ligninolytic enzymes activity. Further experiments were conducted under optimized physical parameters.

Impact of carbon sources

The effects of the carbon sources (xylose, sucrose, sorbose, lactose, maltose, fructose, pectin, glucose raffinose and starch) on mycelial growth and ligninolytic enzyme production were evaluated after optimum days of incubation at optimum pH and temperature. The sucrose of Richard's medium was replaced singly by each of the carbon compounds to provide 1.75g/L of carbon (a substituent of sucrose i.e., 50.0g/L).

Impact of organic and inorganic nitrogen sources

The impact of inorganic and organic nitrogen sources were assessed by replacing the potassium nitrate with amino acids and inorganic nitrogen compounds. The organic nitrogen compounds include: DL-alanine, DL-aspartic Acid, DL-phenylalanine, L-histidine HCl, L-leucine, DL-methionine, L-proline, DL-tryptophan, L-α amino-n butyric acid, L-cysteine HCl, L-glutamic acid, L-leucine, L-ornithine HCl, L-tyrosine, L-arginine HCl, L-cystine, glycine, lysine HCl, DL-threonine, DL-valine, DL-serine HCl, L-asparagine, L-ornithine HCl and L-proline. The inorganic nitrogen compounds employed include: ammonium acetate, ammonium chloride, ammonium nitrate, ammonium oxalate, ammonium phosphate, ammonium sulphate, potassium nitrate, sodium nitrate and sodium nitrite. The potassium nitrate of Richard's medium was replaced by the equivalent inorganic and organic nitrogen compounds to provide 0.0989g/L nitrogen (a substituent of potassium nitrate i.e. 10.0g/L in basal medium).

Determination of the pH of culture filtrate

Electronic pH meter ME 63 was used to determine the pH value of the culture filtrates. The standardised pH metre electrode was put into the isolate's crude filtrate. The values were promptly read and recorded on the metre record. The ligninolytic enzymes activity were estimated in the experiment on days of incubation, carbon and nitrogen compound for the growth of the fungi as follows.

Laccase enzyme assay

The reaction mixture composed of 0.5ml distilled water, 1ml 50mM sodium acetate buffer (pH 4.5), 0.5ml 46mM guaiacol, and 0.5ml culture filtrate. The enzyme activity was determined by measuring the optical density of the reaction mixture at 440 nm with a 30 second time interval for up to 90 seconds using an Evolution 201 UV visible spectrophotometer (Coll et al., 1993).

Lignin peroxidase enzyme assay

The reaction mixture composed of 600µl of 0.3M citrate/0.4M phosphate buffer (pH 4.5), 300µl of 8mM veratryl alcohol, 1890µl distilled water and 60µl of culture filtrate. The oxidation of veratryl alcohol to veratryl aldehyde (ε310, = 9300M-1cm-1) was determined spectrophotometrically at 310nm. The reaction mixture was then incubated at 30°C for 2 minutes. The reaction was initiated by the addition of 150µl of H_2O_2 (5mM). The absorbance of the solution was measured immediately in the 1 minute interval after the addition of H_2O_2 at 310nm (Takamiya et al., 2008, modified by Atalla et al., 2010).

Manganese peroxidase enzyme assay

The reaction mixture contained 300µl of 0.5M sodium succinate buffer (pH 4.5 at 27°C), 300µl guaiacol (4mM), 600µl $MnSO_4$ (1mM), 300µl culture filtrate and 1200µl distilled water and guaiacol was used as a substrate. The reaction mixture was incubated at 30°C for 2 minutes and the reaction was initiated by addition of 300µl of H_2O_2 (1mM). The absorbance of the solution due to oxidation of guaiacol (ε465 = 12,100M-1cm-1) was measured at 465nm in the 1 minute intervals after the addition of hydrogen peroxide (Atalla et al., 2010).

Statistical analyses

All the experiments were performed in triplicates and obtained data were tested in a one way ANOVA at P=0.05 using PASW Statistics 18 software and Tukey's test was used to evaluate differences between treatments.

RESULTS AND DISCUSSION

Impact of basal media, temperature and pH

Fungi exhibited complete, superficial and cottony growth of white colored mycelium with all basal media except Raulin's. In Elliot's medium, both submerged and superficial fungal growth was observed. The optimum growth was observed in Richard's media, whereas nil growth was observed in Raulin's media, Figure 1(a). Richards media constitutes all the essential macro and micro nutrients that fungi are able to utilize for their growth and development, such as sucrose, potassium dihydrogen phosphate, potassium nitrate, potassium chloride, magnesium sulphate and ferric chloride hexahydrate. Other researchers also observed the optimum growth of fungi with Richard's media (Sharma and Sharma, 2011; Koley and Mahapatra, 2015). Some of the environmental or ecological parameters that influence the fungal growth includes temperature, pH, and substrate nature. Different fungus species, and even isolates of the same species, prefers vastly different environment for their growth. As a result, describing a single set of ideal circumstances for fungal growth is challenging. Environmental conditions also impact many physiological processes that are crucial to fungal survival and competition (Mannaa and Kim, 2017). Microorganisms ability to detect and respond to changes in the environment is critical to their existence. Temperature is a crucial abiotic component that affects intracellular metabolism and modulates fungal growth (Qiu et al., 2018). So there is a need to find the temperature that favours the optimum growth of fungi. At high temperature, there are the chances of break down of hydrogen bond and hydrophobic interactions that further result in denaturation of proteins, nucleic acids and enzymes formation and at high temperature fatty acid synthesis and membrane fluidity is also affected. Due to the decline of cell viability, death of fungal cells occurs (Irshad Asgher, 2011; Walker and White, 2017). The test fungus is able to grow over a wide range of temperatures, varying from 16°C to 32°C and optimum growth is observed at 32°C, Figure 1(b). The mycelial growth was less and white coloured in the form of superficial and submerged at 16°C, whereas mycelial growth was complete, white coloured and superficial at 20, 24, 28 and 32°C. The finding of this study are correlated with other researchers (Hoa and Wang 2015, De leon et al; 2017; Min et al., 2020; Landingin et al; 2020). The final H-ion concentration of the basal media changed significantly with the growth of Rigidoporus vinctus at temperatures 20, 24, 28 and 32°C.

The initial pH of the culture media influences the growth of fungi. Micro-organisms have the ability to regulate pH and modify the pH of their environment by secreting acids or alkali (Vylkova S. 2017). The optimum pH is directly related to an enzymatic system, essential vitamins entry in the cell, surface metabolic reactions and mineral capture (Barros L. et al., 2006, Islam and Ohga, 2013). The test fungi can grow at a wide range of pH value from 3.0 to 9.0 and obtained the optimum growth at pH 6.0, Figure 1(c). Other researchers also recorded the growth of fungi with different pH such as Dhakar and Pandey (2013) reported optimum growth of Trametes hirsuta with pH range of 5 to 7.0, Dhakar et al., (2014) reported optimum growth of Penicillium pinohylum at pH 7.5, Prasher and Chauhan (2015) reported optimum growth of Dictyoarthrinium synnematicum at pH 5.0, Chauhan (2016) reported optimum growth of T. versicolor at pH 6.0, De Leon et al., (2017) reported optimum growth of L. Sajor caju at pH 5.0. Due to the uptake of anions or cations present in the medium, the pH of the culture media changed to the acidic range. This variance in the pH range of culture filtrate is in the accordance with prior studies indicating fungi are acidophilic in general (Juwon A.D. and Emmanuel O.F., 2012).

Impact of days of incubation

To figure out how many days are required for optimum growth and production of ligninolytic enzymes, the period of 30 days was selected. During the study, it was observed that the growth of fungi increased with the incubation period. The optimum growth of Rigidoporus vinctus was obtained on the 24th day of incubation and started decreasing after the 24th day of incubation, Figure 2(a). This decrease in growth rate could be attributed to inhibited cellular processes and depletion of nutritional elements in the growth media (Chauhan et al; 2013). The final pH of the culture filtrate was also decreased with days of incubation; this decrease in pH added more H+ ions into the nutrient media that might further inhibit the growth of fungi (Schneider et al., 2018). Fungi expressed activity for all the three ligninolytic enzymes, LiP, MnP and laccase and optimum activity was observed on 10th (188U/ml), 6th (359U/ml) and 16th (514U/ml) day of the incubation for LiP, MnP and laccase, respectively. The laccase activity was predominant compared to LiP and MnP, Figure 2(b). Other researchers also recorded the activity of enzymes with various fungi, such as Sparassis latifolia showed optimum activity for LiP, laccase and MnP at a 7, 21 and 28th day of incubation, respectively (Sou et al., 2017), Stereum ostrea expressed optimum MnP activity on 6th day of incubation, while LiP and laccase activity were maximum at 4th day of incubation (Parveen et al., 2011), mushroom expressed optimum growth at 21st day of incubation (Illuri et al; 2021).

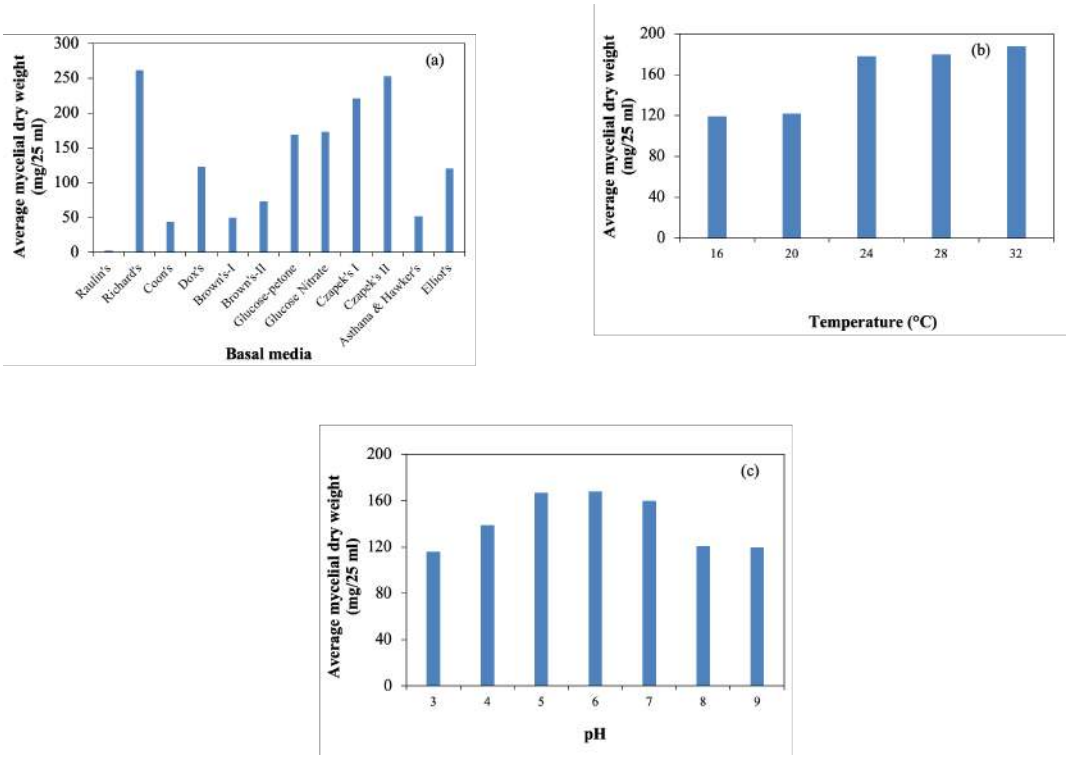

Figure 1: Growth of Rigidoporus vinctus with selected (a) basal media, (b) temperature and (c) pH after 10 days of incubation.

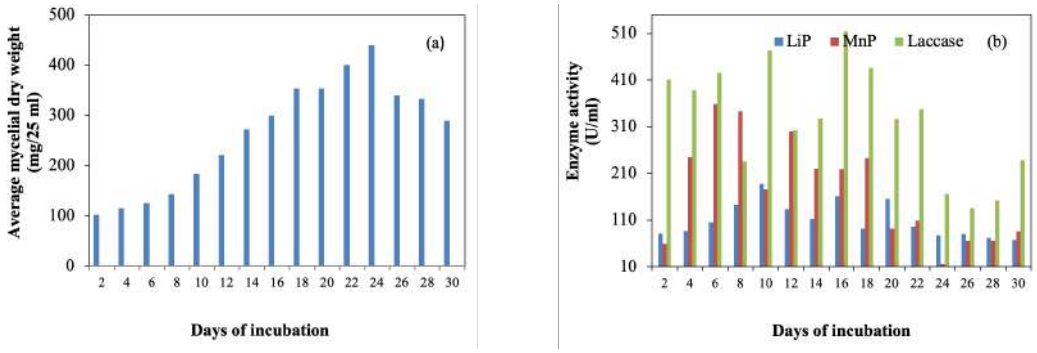

Figure 2: (a) Growth and (b) enzyme activity in relation to days of incubation.

Impact of carbon sources

The elemental concentration of carbon in fungal biomass varied from 38 to 57 % (Zhang and Elser 2017). Rigidoporus vinctus showed growth with all the carbon compounds and maximum growth was observed in media supplemented with starch, followed by pectin, maltose, sorbose, glucose, lactose, sucrose, xylose, and fructose, Figure 3(a). The mycelial growth was white coloured in the form of superficial and submerged with all carbon sources. Starch is the most abundant carbon source present in the environment. In higher plants, starch is a major reserve carbohydrate and fungi can efficiently produce amylase, which can breakdown starch. This might be the reason that fungi prefer to grow on starch supplemented media (Juwon A.D. and Emmanuel O.F., 2012). Other fungi also expressed optimum biomass yield with starch supplemented media, such as Trichoderma viride (Juwon and Emmanuel O.F., 2012), Agaricus blazei (Hamedi et al., 2012), Fusarium oxysporum (de Moraes Catarino et al., 2018). Pleurotus ostreatus expressed optimum mycelium growth with glucose, sucrose, and molasses, while glucose, sucrose, and dextrose favoured the mycelium growth of oyster mushroom Pleurotus cystidiosus (Hoa and Wang, 2015).

Fungi isolated from epiphytic orchids, Sparassis latifolia also expressed high growth with starch (Sou et al; 2017), Diaporthe phaseolorum showed optimum growth with sucrose (Kumar and Prasher, 2021). In control (media without carbon) no mycelial growth was observed. The final pH of culture filtrate shifted towards the acidic region with all selected carbon sources from the initial pH (pH 6.0). Extensive research has been carried on the production of ligninolytic enzymes using various defined media and basidiomycete respond differently to carbon source and their concentration in nutrient media (Elisashvili and Kachlishvili 2009). In this experiment, it is observed that MnP and laccase activity was high as compared to LiP. The optimum activity of LiP (467U/ml) and MnP (724U/ml) was observed in maltose containing media, in contrast laccase activity (695U/ml) was optimum in media supplemented with glucose followed by sucrose (608U/ml), Figure 3(b). Other researchers also observed the highest laccase activity in glucose supplemented media (Galhaup et al., 2002; Mikiashvili et al., 2005; Stajic et al., 2006; Schneider et al., 2018). Other researchers observed the activity of ligninolytic enzymes with different carbon sources such as, Diaporthe phaseolorum expressed optimum activity for LiP, MnP and laccase with fructose, glucose and pectin, respectively (Kumar and Prasher, 2021).

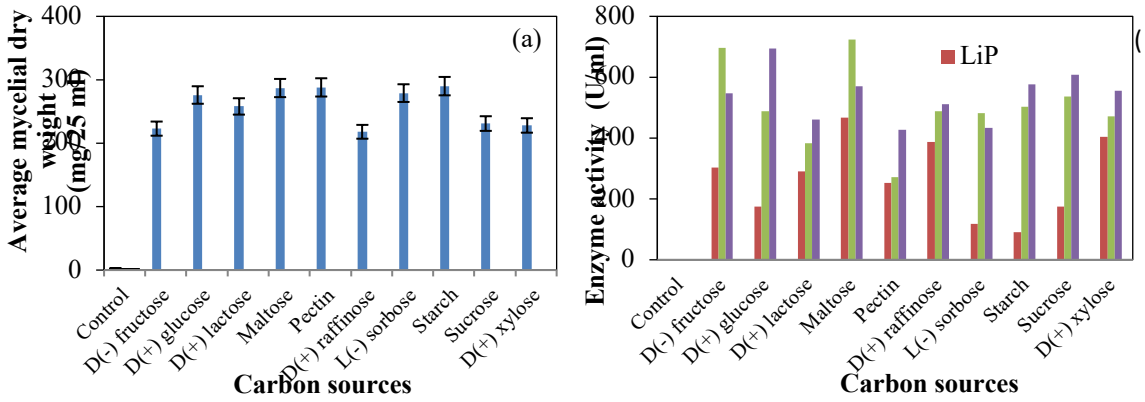

Figure 3: (a) Growth and (b) ligninolytic enzyme activity with different carbon sources.

Impact of inorganic nitrogen sources

In fungal cells, nitrogen is required to synthesize various macromolecules, including proteins, nucleic acids, and chitin (Hayer et al., 2014). The tested fungus can utilize both ammonium and nitrate salts and expressed growth with both ammonium and nitrate salts. Rigidoporus vinctus attained optimum growth in media supplemented with ammonium sulphate followed by ammonium chloride, ammonium acetate, potassium nitrate, ammonium oxalate, ammonium phosphate and sodium nitrate, Figure 4(a). The mycelial growth was superficial and white in color with all nitrogen compounds. Other researchers also observed the optimum growth of fungi with ammonium sulphate and ammonium chloride supplemented media such as, Pleurotus cystidiosus and Pleurotus ostreatus (Hoa and Wang 2015), Cryphonectria parasitica (Cheng et al., 2013), Pleurotus florida and Pleurotus ostreatus (Neelam et al., 2013) and Villosiclava virens (Fu et al., 2013). Because nitrate ions have been linked to the inhibitory action of some basidiomycetes, fungi prefers to grow with salts of chorides and sulphate rather than nitrates, which may be difficult to move across the fungal membrane where it might encourage growth (Neelam et al., 2013). Fungus showed nil growth in medium supplemented with sodium nitrite might be because the genome of fungi does not contain genes encoding for nitrite transporters (Zhao et al., 2021). Other inorganic nitrogen sources also supported the optimum mycelial growth such as Grammothele fuligo expressed optimum growth with ammonium oxalate (Chauhan, 2019) and Diaporthe phaseolorum expressed optimum growth with potassium nitrate supplemented media (Kumar and Prasher, 2021).

The final pH of the culture filtrate of all the nitrogen sources changed significantly towards an acidic range of pH. The growth and production of ligninolytic enzymes are both influenced by nitrogen sources. Fungi showed activity for LiP, MnP and laccase with all nitrogen sources except sodium nitrite supplemented media, and laccase activity was predominant, Figure 4(b). The LiP (112U/ml) and MnP activity (176U/ml) were maximum in media supplemented with ammonium chloride and ammonium sulphate, respectively. Other researchers also observed maximum LiP activity in ammonium chloride supplemented media (Varshney et al., 2013; Chauhan, 2019). Laccase activity was maximum in media supplemented with ammonium oxalate 301U/ml, followed by ammonium phosphate (297U/ml) and ammonium nitrate (240U/ml). Grammothele fuligo expressed optimum LiP and MnP activity with ammonium chloride and ammonium acetate supplemented media, respectively (Chauhan, 2019), Diaporthe phaseolorum expressed optimum LiP, MnP and laccase activity in ammonium acetate, ammonium phosphate and ammonium nitrate supplemented media, respectively (Kumar and Prasher, 2021).

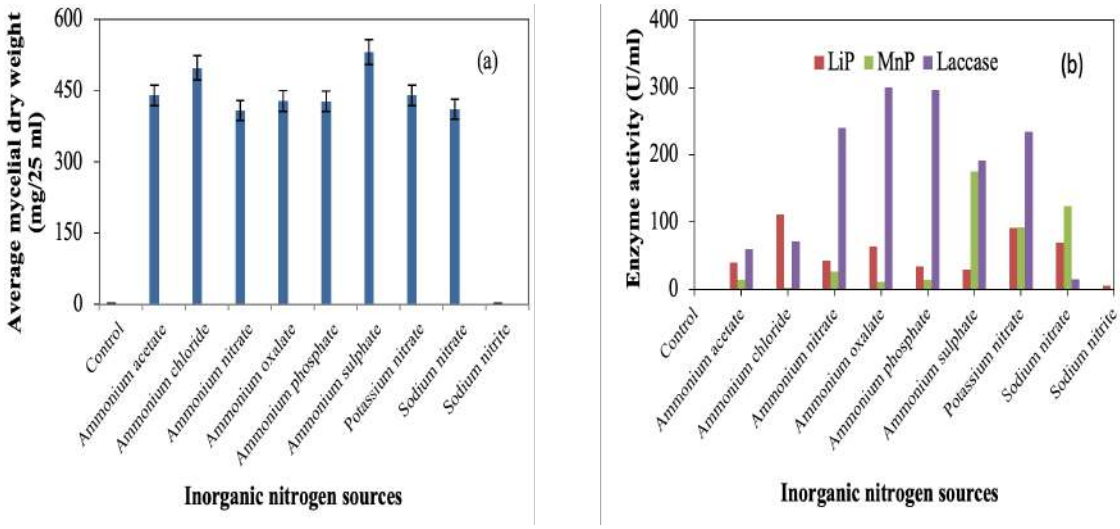

Figure 4: (a) Growth and (b) ligninolytic enzyme activity with different inorganic nitrogen sources.

Impact of organic nitrogen sources

Some fungi prefers amino acids for their growth and exhibit better growth in the presence of organic nitrogen sources compared to inorganic nitrogen sources (Levin et al., 2010; Leonowicz et al., 1991). The test fungi exhibited white coloured superficial mycelial growth with all organic nitrogen compounds. Rigidoporus vinctus attained maximum growth in media supplemented with L-proline followed by DL-valine, DL-methionine, Lysine HCl, L-leucine, L-arginine, glycine, L-α-amino-n butyric acid, DL-serine HCl, DL-tryptophan, L-ornithine and L-cysteine and minimum growth was attained in media supplemented with L-tyrosine, Figure 5(a). Other researchers also observed optimum growth of fungi with different amino acids, such as Porostereum spadiceum showed optimum growth in DL-valine supplemented media (Prasher and Manju, 2018), Grammothele fuligo showed optimum growth with DL-alanine supplemented media (Chauhan, 2019), Diaporthe phaseolorum showed optimum growth in L-asparagine (Kumar and Prasher, 2021).

The final pH of the media changed significantly towards acidic in the course of the investigation. During the study it was observed that the growth of fungi was higher when nitrogen was supplied in an organic form than in an inorganic form. Rigidoporus vinctus exhibited the activity for all three enzymes and LiP activity was maximum in media supplemented with DL-tryptophan (1056U/ml). The highest activity for MnP and laccase was obtained in media supplemented with DL-valine (750U/ml) and DL-aspartic acid (610U/ml), respectively, Figure 5(b). Other researchers also observed ligninolytic enzymes activity with different amino acids, Grammothele fuligo expressed highest activity for MnP and laccase in DL-tryptophan (Chauhan, 2019), Diaporthe phaseolorum expressed the highest activity for LiP, MnP and laccase with DL-tryptophan, L-tyrosine and L-asparagine, respectively (Kumar and Prasher, 2021). The study revealed that nutritional factors as well as culture conditions affect the growth and activity of ligninolytic enzymes. Similar to previous findings by other researchers this study also revealed that the most biomass yielding media does not mean it can sustain significant enzyme activity (Xavier et al., 2001). It can be explained on the basis that due to enhanced biomass synthesis, there is a depletion of nutrients which would result in decreased of metabolic activity (Vaithanomsat et al; 2010). The other reason is that fungi have a strategy to

survive under extreme conditions by limiting their growth and producing secondary metabolites constantly (Dhakar et al., 2014).

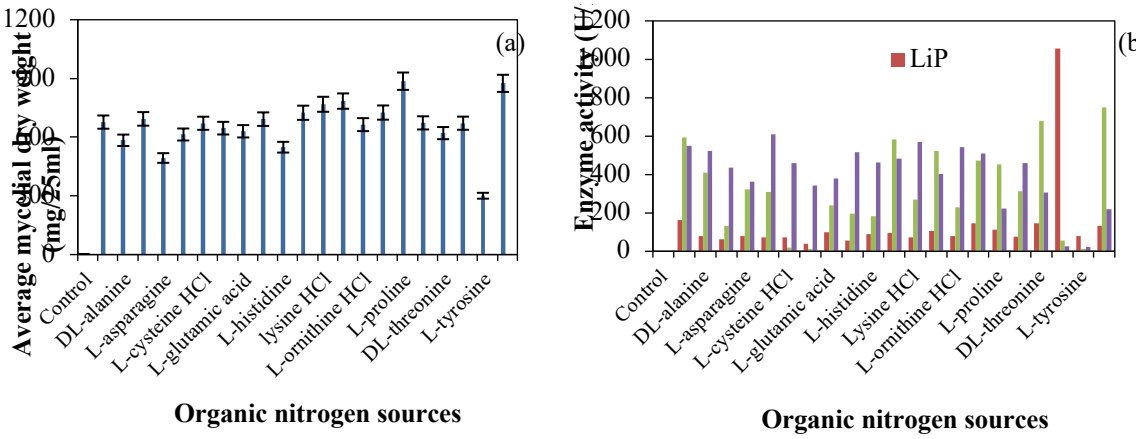

Figure 5: (a) Growth and (b) ligninolytic enzyme activity with different organic nitrogen sources.

Morphological study of Rigidoporus vinctus with different physical and nutritional parameters

Fungi have acquired the ability to detect and respond to a variety of environmental cues to improve their fitness, as they live in a variety of niches with varying environmental limits on their growth, reproduction, and survival. Fungi use a variety of strategies to deal with abiotic stress, including changing their form (Francisco et al., 2019). Chlamydospores are one of the specific structure either present in the middle of hyphae (intercalary) or at the top of hyphae with condensed cytoplasm. They are produced in harsh environments such as unfavorable pH, temperature and nutrient deficiency (carbon and nitrogen starvation) (Liu et al., 2020). As they are resistant to adverse conditions so they help in increasing the survival rate of fungi. Both terminal and intercalary chlamydospore were present in fungi grown with carbon and nitrogen sources. Even though fungi expressed good growth with all nitrogen (organic and inorganic) and carbon sources, but the production of chlamydospores might be due to the deficiency of nutrients from culture media after certain days. In the case of experiment on the growth of fungi with days, the chlamydospores were formed after 15th of incubation and with different carbon sources such as fructose, lactose, starch, and different nitrogen sources such as L-cystine, L-histidine, ammonium acetate, ammonium sulphate and ammonium chloride, Figure 6(a,b). The gloeoplerous hyphae i.e., hyphae with oil/ lipids, are present in R. vinctus (Webster and Weber, 2007; Prasher, 2015). Oleaginous microorganisms can synthesize and accumulate lipids above 20% of their dry cell mass. Oleaginous microorganisms continuously produce acetyl coenzyme A (acetyl-CoA) and nicotinamide adenine dinucleotide phosphate (NADPH) under nitrogen limitation and these enzymes are responsible for the synthesis of fatty acids through reversed oxidation (Ochsenreither et al., 2016). The ability of microorganisms to accumulate lipids is mainly governed by their genetic composition, which can vary significantly between species or strains within a species (Mhlongo et al., 2021). Gloeoplerous hyphae were observed in fungi grown in glucose peptone media and with different carbon and nitrogen sources supplemented in the media, Figure 6 (c,d). The septum is the wall present in hyphae to divide the hyphae into various compartments to translocate of nutrients from one part of the fungus thallus to another (Bracker and Butler, 1963). Their presence means fungi can use the nutrients for their growth. Septum were observed in fungus grown in glucose peptone,

glucose, raffinose, L-tyrosine and DL-tryptophan, Figure 6 (e,f). In the dikaryotic state of the life cycle of some basidiomycetous fungi, there are two nuclei in dikaryotic hyphae, one from each parent cell, share a single cytoplasm for short time without undergoing nuclear fusion. There are two processes that are taking place simultaneously first is nuclear division and the second is the formation of special hook like projections called as clamp connections; they are formed close to the position of the future septum formation. During mitosis occuring in two different cell compartments, one nucleus enters and divides in the developing clamp cell and the other divide in the main hypha. These mechanisms occur in the G2 phase, which must be sufficiently extended (Perez-Martin and Sena Tomas, 2011). The clamp connections were formed in fungi throughout the study depicting sexual reproduction, Figure 6 (g,h). The skeletal hyphae were also observed in fungi during experimentation, depicting the vegetative growth, shown in Figure 6 (i,j).

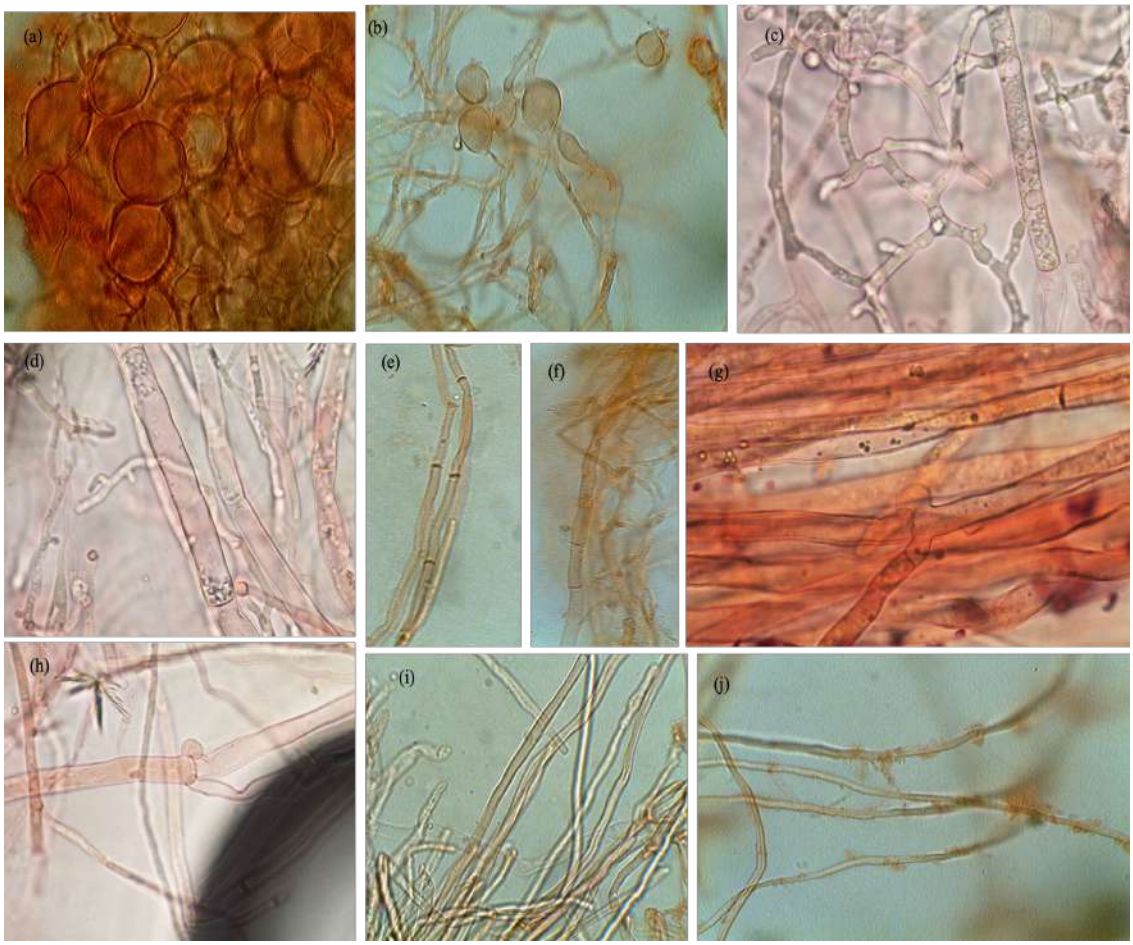

Figure 6: (a,b) Thick-walled chlamydospores were formed with D(-) fructose, D(+) lactose, starch, L-cystine and L-histidine, ammonium acetate, ammonium sulphate and ammonium chloride. (c,d) Hyphae containing oil globules were observed with glucose peptone medium and different carbon and nitrogen sources. (e,f) Hyphae with septa were observed with glucose peptone medium and with glucose, raffinose, tyrosine and tryptophan. (g,h) Thin and thick-walled hyphae with clamp connections were formed with different carbon, inorganic and organic nitrogen sources. (i,j) Skeletal hyphae were observed at all temperatures, pH, Asthana and Hawker's and Brown's-II media.

CONCLUSION

This study provided valuable information about fungus which is not yet explored enough. The results obtained demonstrated that fungi can tolerate a wide range of abiotic factors. The addition of carbon and nitrogen sources into the medium generally favoured fungi growth and enzymatic activity. The fungal biomass was more in media supplemented with various organic nitrogen sources than inorganic nitrogen sources. Fungi produced all three ligninolytic enzymes, i.e., LiP, MnP and laccase, with different carbon and nitrogen sources under investigation. The laccase production was predominant as compared to LiP and MnP. The production of enzymes makes this fungus to be used as potential source for bioremediation of xenobiotic compounds. The production of oil containing hyphae makes this fungus to be used for biofuel production.

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