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ORIGINAL ARTICLE

Extracellular Ligninolytic Enzymes in *Bjerkandera adusta* and *Lentinus squarrosulus*

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Abstract Extracellular ligninolytic enzyme activities were determined in two white-rot fungi, *Bjerkandera adusta* and *Lentinus squarrosulus*. To investigate the activity of extracellular enzymes, cultures were incubated over a period of 20 days in nutrient rich medium (NRM) and nutrient poor medium under static and shaking conditions. Enzymatic activity was varied with media and their incubation conditions. The highest level of Aryl alcohol oxidase (AAO) was detected under shaking condition of both medium while Manganese peroxidase (MnP) activity was best in NRM under both conditions. AAO is the main oxidases enzyme in *B. adusta* while laccase plays important role in *L. squarrosulus*. MnP is the main peroxidase enzyme in both varieties.

Keywords White-rot fungi · Ligninolytic enzymes · Nutrient rich medium · Nutrient poor medium

Introduction

Mushrooms are cultured world wide for their taste, nutritional attributes and potential applications in industries [1]. In addition, they have many medicinal uses and are good agents of bioremediation [2, 3]. Mushrooms are highly nutritious containing protein (19–35%), low fat content (1.3–2%), relatively large amounts of carbohydrate (51–88%) and fiber (4–20%) in dry mushroom fruit bodies [4]. From an eco-physiological point of view, mushrooms can be broadly classified into wood-decaying, mycorrhiza-forming, and litter-decomposing fungi. The most important and potent wood-destroying organisms are white- and brown-rot fungi, which attack various components of the wood cell wall [5]. White-rot fungi (WRF) play an essential role in the decomposition of dead trees, especially in the degradation of lignin. The only organisms reported to degrade lignin efficiently are the WRF that under natural conditions mostly colonize dead or living wood [6]. To be able to degrade the complex lignin molecule, WRF use various extracellular enzymes with low specificity and strong oxidative activity [7]. The main extracellular enzymes participating in lignin degradation are heme-containing lignin peroxidase (LiP, EC 1.11.1.14), MnP (EC 1.11.1.13) and Cu-containing laccase (EC 1.10.3.2) [8]. In addition, enzyme involved in hydrogen peroxide production such as aryl alcohol oxidase (AAO, EC 1.1.3.7) is considered to belong to the ligninolytic system [8].

We have selected two mushroom strains, *Bjerkandera adusta* and *Lentinus squarrosulus* for their extracellular enzyme studies. The interest of *B. adusta* arises from their ability to degrade many xenobiotic compounds as well as lignin and other components of wood. However, little is known about ligninolytic enzymes produced by *B. adusta*. *L. squarrosulus* is edible mushroom and cultivated on saw dust. This is also a wood degrading WRF and produced ligninolytic and cellulolytic enzymes [9].

Materials and Methods

Cultures

Pure mycelial cultures of *B. adusta* and *L. squarrosulus* were selected from the "Mushroom Gene Bank" of

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Chemicals

Analytical grade 2,2′-azinobis(3-ethylbenzthiazoline-6-sulphonate) (ABTS, MW 548.7 g/mol) was obtained from Sigma and veratryl alcohol (VA, MW 168.19 g/mol) was purchased from Aldrich. All other chemicals used were of analytical grade.

Screening of Ligninolytic Enzymes Through Spot Test

Laccase Spot Test

Laccase spot test was performed in an agar medium containing the ABTS, a very sensitive substrate that gave a rapid reaction of extracellular oxidative enzymes by a colour zone. Spot test was performed in Petri dishes (90 mm diameter) with 20 ml of the modified Kirk medium containing: 10 g of glucose, 2 g of KH_2PO_4, 0.5 g of $MgSO_4{\cdot}7H_2O$, 0.1 g of $CaCl_2$, 2.2 g of 2,2-dimethylsuccinate, 0.5 g of ammonium tartrate, 0.2 g of yeast extract, 0.2 g of ABTS and 20 g of agar–agar, per liter of medium. The pH was adjusted to five before autoclaving at 15 psi for 20 min [10].

MnP Spot Test

The basic medium used for MnP spot test was Kirk medium, consisted of 10 g of glucose, 2 g of KH_2PO_4, 0.5 g of $MgSO_4.7H_2O$, 0.1 g of $CaCl_2$, 2.2 g of 2,2-dimethylsuccinate, 0.5 g of ammonium tartrate, 0.1 g of $MnCl_2.4H_2O$, 20 g of agar–agar, per liter of medium. The pH was adjusted to five before autoclaving at 15 psi and 120°C for 20 min [11].

Fungal growth was estimated in terms of diameter of fungal colony. Extracellular enzymatic activity was measured by the colour intensity of the medium. Plates were observed once a day for three consecutive weeks.

Extracellular Ligninolytic Enzyme Essay

The production of extracellular enzymes were carried out in the NRM containing 2 g of ammonium tartrate, 10 g of glucose, 1 g of KH_2PO_4, 1 g of yeast extract, 0.5 g of $MgSO_4.7H_2O$, 5 g of KCl, 1 ml of solution containing trace elements per liter of medium and the NPM containing 10 g of glucose, 2 g of KH_2PO_4, 0.2 g of yeast extract, 0.1 g of peptone, 1 ml of solution containing trace elements per liter of medium. Solution of trace elements containing 10 mg of $Na_2B_4O_7{\cdot}10H_2O$, 7 mg of $ZnSO_4{\cdot}7H_2O$, 5 mg of $FeSO_4.7H_2O$, 1 mg of $CuSO_4{\cdot}5H_2O$, 1 mg of $(NH_4)^6Mo_7O_{24}{\cdot}4H_2O$, 1 mg of $MnSO_4$ dissolved in 100 ml of H_2O [12]. The pH was adjusted to seven before autoclaving at 15 psi and 120°C for 20 min. 150 ml flasks containing 50 ml liquid medium were inoculated with 5–8 days old five mycelium bits (5 mm in diameter). Three replicates of flaks of both medium were incubated in static condition in BOD incubator and shaking condition in a rotatory shaker at 150 rev/min at 30°C.

The cultures were harvested at the 5th, 10th, 15th and 20th day of incubation. Each sample was centrifuged (10,000×g for 10 min) at 4°C. The supernatant of liquid culture was used for enzyme assay. The enzymatic reactions were carried out in triplicate and determined using a double beam Perkin Elmer Lambda 12 UV/VIS spectrophotometer. All the enzyme activities were measured at room temperature (20 ± 2°C). The enzymatic activity was expressed as international units (U) defined as the amount of enzyme required to produce 1 μmol product min^{-1} and expressed as Ul^{-1}.

Protein concentration was determined following Bradford method [13]. Protein content in the sample was determined from standard curve and the amount of protein μg ml^{-1} was calculated. Laccase activity was measured following the oxidation of ABTS (ε_{420}, 36,000 M^{-1} cm^{-1}). The assay mixture contained 100 mM sodium acetate buffer pH 5 and 5 mM ABTS [14]. AAO activity was assayed as the oxidation of Veratryl alcohol to veratraldehyde at 310 nm (9,300 M^{-1} cm^{-1}) and activity was measured with 5 mM VA in 100 mM sodium phosphate buffer at pH 6 [15]. LiP activity was measured by the oxidation of 2 mM VA to veratraldehyde (ε_{310}, 9,300 M^{-1} cm^{-1}) in 100 mM sodium tartrate buffer (pH 3) in the presence of 0.4 mM H_2O_2 (30%) [16]. MnP activity was determined by the production of a Mn^{3+} tartrate complex (ε_{238}, 6,500 M^{-1} cm^{-1}) from 0.1 mM $MnSO_4$ in 100 mM sodium tartrate buffer pH 4.5 with 0.1 mM H_2O_2 (30%) [17].

Statistical Analysis

All the experimental analysis was carried out in triplicates. The results are expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test using SAV v.9.1.3 program. Differences at $P < 0.05$ were considered to be significant.

Results and Discussion

Spot Test of Extracellular Enzymes

With the agar plate screening, selected strains of wood colonizing WRF were tested for spot test. In comparison to the growth on MEA, strains grew noticeably slower on the

selected agar media. In particular ABTS had inhibitory effects on fungal growth.

Laccase Spot Test

The extracellular ABTS-oxidizing activity of the both fungal strains into the modified Kirk medium is showed in Fig. 1. Whereas, *L. squarrosulus* showed high ABTS-oxidizing activity however, *B. adusta* showed very low ABTS-oxidizing activity. The absence of extracellular ABTS-oxidizing activity does not necessarily imply the lack of capacity to produce these oxidative enzymes but could reflect a possible inhibition of their expression; the oxidative enzyme system is not homogeneous; its production and properties depend on the conditions and culture media. Fungal strains oxidized ABTS to the dark green ABTS cation radicals ($ABTS^{+}$) indicating the production of extracellular oxidoreductases [11]. *L. squarrosulus* gave positive reaction immediately after inoculation, formed dark green zone around the mycelial bit and *B. adusta* showed very light green colour after 5 days of inoculation. In the case of ABTS agar plates, the positive reaction (dark green ring around the mycelia) appeared often immediately after inoculation or during the first day of incubation, the green colour preceded the fungal mycelium clearly demonstrating that extracellular mechanisms were responsible for the oxidation [11].

MnP Spot Test

Both varieties showed positive reaction of extracellular MnP and formed brownish colour in medium plate (Fig. 2). Mn plates were evaluated after 5 weeks of incubation for the formation of brownish flecks of manganese oxide (MnO_2) caused by the action of MnP. MnP was thought to play a crucial role during the primary attack on lignin, because it generates highly reactive Mn^{3+} which acts as a low molecular mass redox mediator and forms water soluble lignin fragments. Moreover, there are indications that MnP is even capable of mineralizing lignin up to carbon dioxide [11].

Extracellular Ligninolytic Enzymes

Quantitative Protein Determination

The Bradford assay is a protein determination method that involves the binding of Coomassie brilliant blue G-250 dye to proteins [13]. When the dye binds to protein, it is

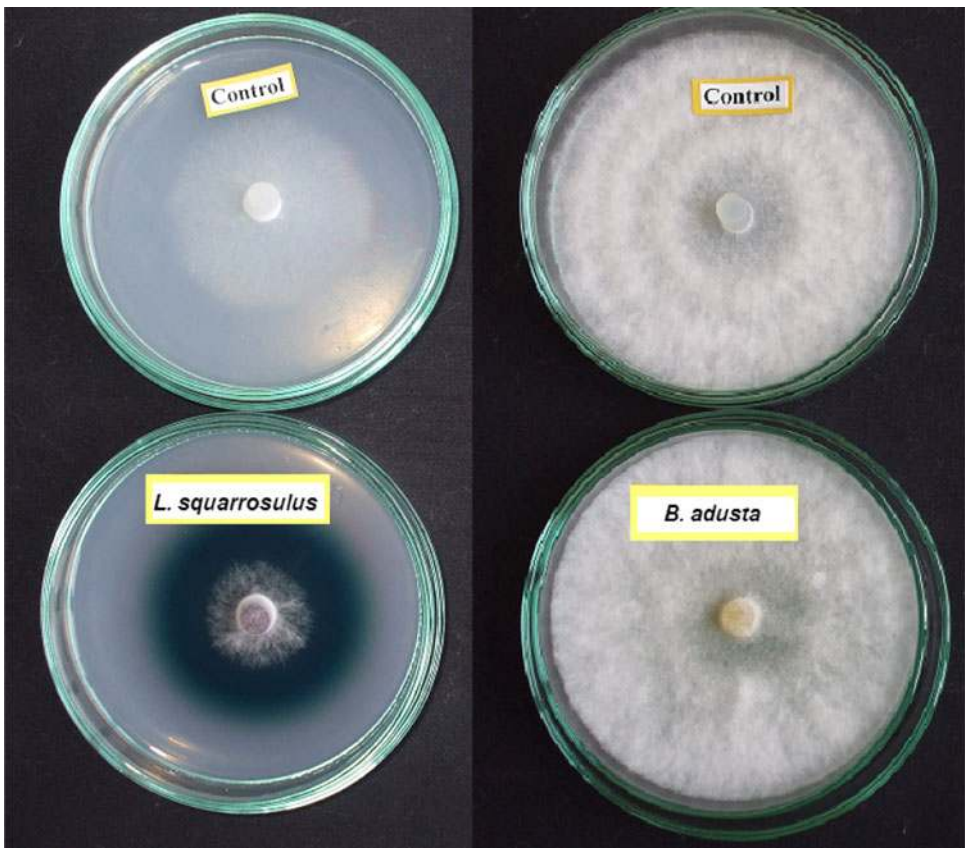

Fig. 1 Extracellular ABTS-oxidizing activity on agar plate

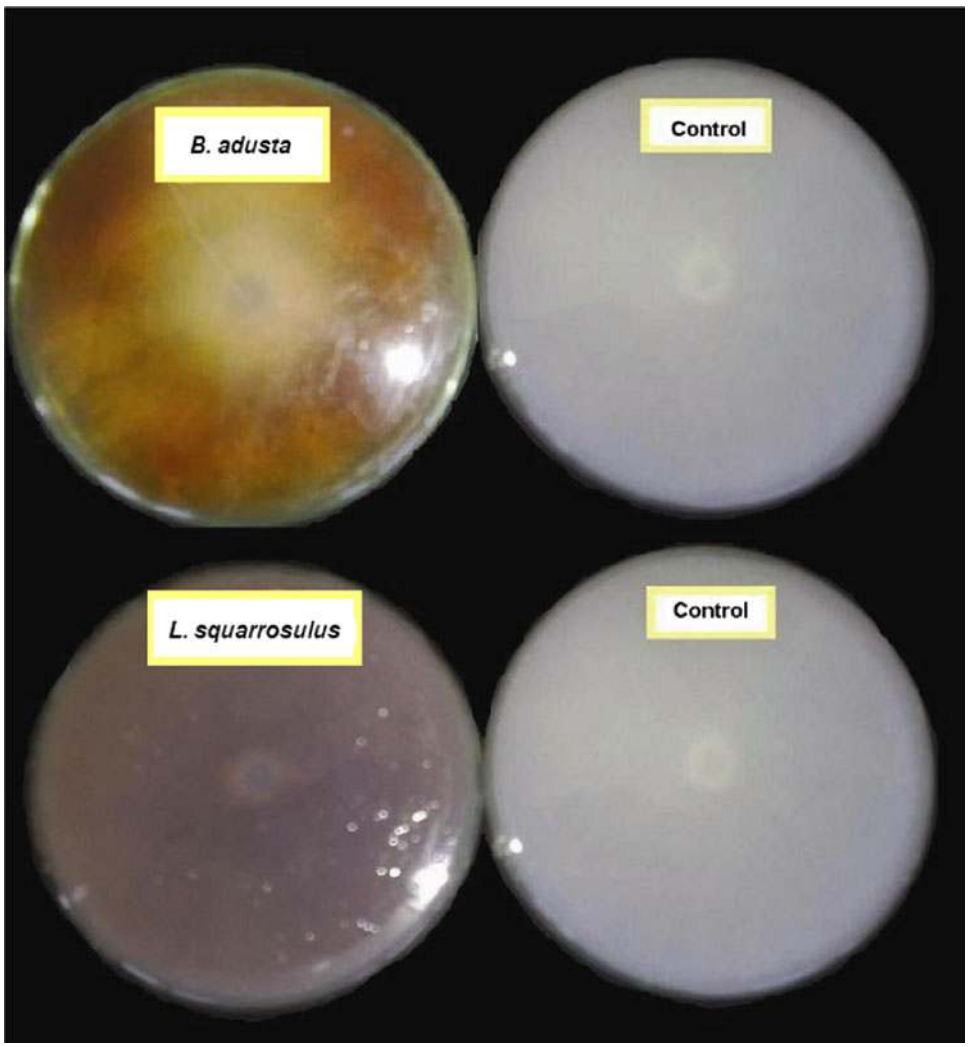

Fig. 2 Extracellular MnP activity on agar plate

converted to a stable unprotonated blue form [18]. This is anionic form of dye.

In *B. adusta*, extracellular protein was not observed on 5th and 10th day in NRM shaking condition and NPM both static and shaking conditions but on 20th day, this medium and condition gave highest protein, 34.5, 30.9, 31.9 μg ml^{-1}, respectively. In *L. squarrosulus*, static condition gave better protein in comparison to shaking condition of both media. The extracellular protein was obtained from both the strains but the amount of protein was higher in *B. adusta* in comparison to *L. squarrosulus*. The crude protein content of *L. squarrosulus* was comparable to *L. tigrinus* [19].

Laccase

Laccase was first detected in the Japanese lac tree *Toxicodendron verniciflua*. Later, it was found in certain other plants and fungi, but is also found in molds, black yeasts and some bacteria [20]. Laccase has been identified as one of the enzyme that plays a major role in lignin degradation. Laccase only attacks phenolic subunits of lignin, but its substrate range can be extended to non-phenolic subunits by the inclusion of a mediator [21]. In *B. adusta* very less laccase activity was found and observed only in NRM static condition. Some studies have been reported that laccase is not present in *B. adusta* [22, 23]. In other WRF like *P. chrysosporium* is not produced laccase under ligninolytic conditions but it is secreted when cellulose is present as a carbon source [24]. In *L. squarrosulus* laccase activity was best in NPM static condition on 10th, 15th and 20th day, respectively (45, 57.2, 64 Ul^{-1}). Laccase activity in *B. adusta* observed only in NRM static condition and it indicates that laccase activity is inducible in *B. adusta* and it requires nutrient rich condition for its secretion. However, in *L. squarrosulus* activity was best in NPM static condition. Supplementation and incubation conditions were affected the enzymatic activity. Some WRF like *Volvariella volvacea* gave good laccase activity under high nitrogen condition and medium was supplemented with Cu but in the absence of Cu activity was not found and under low nitrogen condition laccase activity was very

poor [25]. Laccase is particularly widespread in ligninolytic basidiomycetes (*Phanerochaete*, *Trametes*, *Pycnoporus*, *Nematoloma*, *Sporotrichum*, *Ganoderma lucidum*, *Stropharia* etc.) and more than 125 different basidiomycetous laccase genes have been described [20, 26].

AAO

The lignin degradation system of WRF is mainly composed of laccase, LiP and MnP. It is considered that, these lignin-degrading enzymes do not functions but mutually interact with each other as well as with other oxidases, such as AAO. AAO activity was described for the first time in the fungus *Polystictus versicolor* (a synonym of *Trametes versicolor*) in 1960 [27]. Since then, AAO has been detected and characterized in other white-rot basidiomycetes including *Pleurotus* species, *Bjerkandera adusta* and some ascomycetous fungi [28]. AAO activity in *B.* adusta was better under shaking condition of both media and NRM shaking condition gave best activity on 15th and 20th day respectively (111.7, 163.1 Ul^{-1}). Activity was

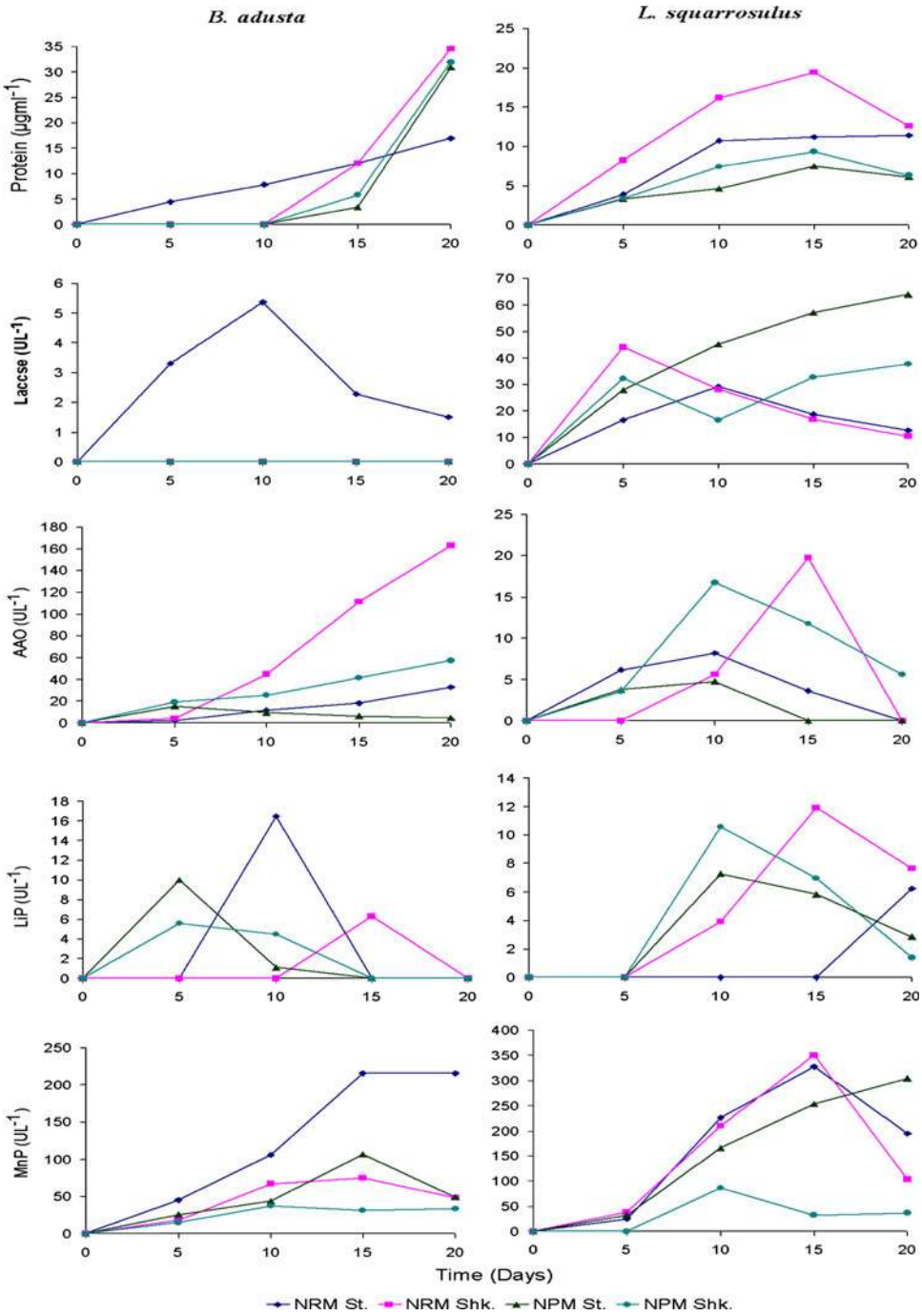

Fig. 3 Extracellular protein (µg ml^{-1}) and ligninolytic enzymes (Ul^{-1}) in *B. adusta* and *L. squarrosulus*

increased up to 20 day in NRM both conditions and NPM shaking condition. It indicates that AAO activity is directly related to nutritional and incubation conditions. *Bjerkandera* spp. biosynthesize veratryl alcohol and veratraldehyde de novo from glucose, together with anisaldehyde, 3-chloro-anisaldehyde and 3,5-dichloro-anisaldehyde and their respective aryl alcohols [29]. It is interesting to note that the AAO of *Bjerkandera* spp. has a much lower affinity for VA than the AAO of the *Pleurotus* spp. Thus, VA is well protected against unwanted AAO mediated oxidation [29]. In comparison to *B. adusta*, *L. squarrosulus* gave very poor AAO activity. Significant activity was observed on 10th day (16.7 Ul^{-1}) in NPM and 15th day (19.8 Ul^{-1}) in NRM shaking condition. AAO is the main oxidases enzyme in *B. adusta* while laccase plays important role in *L. squarrosulus*. In some WRF both enzymes were observed during lignin degradation like *P. ostreatus* has shown that the concerted action of laccase and AAO, produces significant reduction in the molecular mass of soluble lignosulphonates [30]. AAO is H_2O_2-generating enzyme, in *P. sajor-caju*, *P. ostreatus*, *P. eryngii*, *B. adusta* and *P. chrysosporium* which has been purified and their properties characterized [31].

LiP

One of the best known ligninolytic enzymes is LiP, which was discovered a little earlier than MnP [32]. LiP is a glycoprotein that contains one mole of iron protoporphyrin IX as a prosthetic group. LiP catalyzes the oxidation of non-phenolic aromatic compounds like veratryl alcohol. LiP activity was not significant in *B. adusta* and some times LiP activity could not be detected in the culture supernatant, but it appeared during enzyme purification [21]. In *L. squarrosulus* activity was better than *B. adusta*. The occurrence of LiP is less common; however, the fungi found to produce LiP are efficient lignin degraders, for example the *Phanerochaete chrysosporium*, *Phlebia radiata* and *Phlebia tremellosa* as well as the polypore *Trametes versicolor* [33]. The best studied WRF are *P. chrysosporium* and *T. versicolor* for LiP production. LiP is a true peroxidase and the kinetic of enzyme intermediates have been studied in detail and a heme peroxidase which is secreted extracellularly at the onset of secondary metabolism, triggered by nitrogen limitation, in *P. chrysosporium*, but it can also be produced in nitrogen-sufficient conditions by other WRF [34].

MnP

MnP is a second group of extracellular enzymes secreted by WRF. MnP operate by oxidizing Mn^{2+} to chelated Mn^{3+}, which acts as a diffusible oxidant at locations remote from the enzyme active site. MnP is widely distributed in WRF, including *P. chrysosporium*, *P. radiata*, *Nematoloma frowardi*, *P. eryngii* and *B. adusta* [35] and this peroxidase described for the first time in *B. adusta* [36]. MnP activity in *B. adusta* was higher in NRM static condition on 15th day (215.9 Ul^{-1}). However, activity was constant on 20th day (215.7 Ul^{-1}). In NPM shaking condition activity was almost same up to 20 days. In *L. squarrosulus* activity was better in comparison to *B. adusta*. Best activity was observed in NRM under both conditions on 10th and 15th day. MnP has been found from most of the lignin-degrading wood and litter inhabiting fungi studied so far [33]. Purified or crude MnP has been used in cell-free systems (in vitro) and shown to oxidize not only lignin, chlorolignins and synthetic lignin compounds, but also Humic substances (HS) from brown coal, and HS synthesized from catechol, nylon, PAH, chlorophenols, nitroaromatic compounds and arsenic-containing warfare agents [33].

Extracellular enzyme activities of both varieties were varied with medium and their incubation conditions. Among the four ligninolytic enzymes, AAO in *B. adusta*, laccase in *L. squarrosulus* and MnP in both varieties, seemed to be main extracellular enzymes (Fig. 3).

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