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# USE OF RICE HUSK SUPPLEMENTED WITH WATERMELON RESIDUE FOR THE PRODUCTION OF LIGNOCELLULOLYTIC ENZYMES BY *PLEUROTUS OSTREATUS*

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**ABSTRACT**

The post - harvest residue of rice (*Oryza sativa*) and watermelon (*Citrullus lanatus*) in the state of Tocantins, mainly in the region of Formoso do Araguaia, where the highest production of these products in the state is concentrated, may serve as a substrate for obtaining hydrolytic enzymes and oxidative stresses involved in the degradation of lignocellulosic materials by *Pleurotus ostreatus* grown in solid state. The main component of agricultural, forest and urban waste consists mainly of three types of polymers: cellulose, hemicellulose and lignin, such as laccase, manganese peroxidase and xylanase. Some microorganisms, especially fungi, produce enzymes capable of degrading lignocellulose by making available and breaking the constituent polymer chains, allowing the subsequent conversion of the molecules originating in materials of interest (bioconversion). The use of the basidiomycete *P. ostreatus* showed great potential for the enzymatic degradation of these residues, at different moisture contents and without the need for any supplementation. The medium based on watermelon peel and rice yielded high activity of enzymes with great potential for industrial use, especially laccase (48.0 ± 0.35 UL $^{-1}$ dry substrate after 25 days of culture) and manganese peroxidase (8.00 ± 0.23 Ug $^{-1}$ substrate after 25 days of culture).

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## INTRODUCTION

The use of several agro-industrial wastes has aroused a growing interest among producers, since the difficulty for disposal without affecting the environment (Pandey *et al*., 1999, Rosales *et al*., 2005, Raol, Raol, Prajapatiand Bhavsar, 2015). The development of bioprocesses that use these materials as substrates to obtain several molecules with high added value, such as: microbial proteins, organic acids, ethanol, enzymes and biologically active secondary metabolites have been widely studied by the industries. The use of agricultural residues as substrates, besides being economically viable, helps to solve the environmental problems arising from their accumulation in nature.

Watermelon is among the most produced and consumed fruits in the world, according to FAO data, in 2006, the world production of watermelon was about 95.2 million tons, with the largest producers being China, Turkey, Iran, the United States and Egypt that together account for 82% of world production (FAO, 2007). The cultivation of watermelon is one of the most developed agricultural activities in Brazil, the South and Northeast macro-regions with respectively 34% and 30% of the national production of this fruit (IBGE, 2007).In Brazil, the consumer market takes into account the size and shape of the fruit, color of the pulp, content of soluble solids, presence or absence of seeds and price. It is observed that in the majority of the planted areas, the predominance is of big fruits, with average weight above 6 kg. However, in some

producing regions, which are not close to trading centers, wholesalers even classify fruits under 8 kg as 'refuse', reducing their remuneration. In the Company of Warehouses and General Warehouses of São Paulo (CEAGESP), the compensation for large fruits (≥9 kg) of the Crimson Sweet variety and hybrids with the same external pattern was higher by approximately 44% in relation to the small fruits of the mentioned in 2009. Probably, the lower remuneration of these small fruits stems from the high frequency of immature fruits, and, therefore, of lower quality. However, recently, the emergence of new types of watermelons, the so-called mini-watermelons, between 1 kg and 2 kg. This is mainly due to the market demand for alternative products, where the consumer opts for smaller, seedless and quality fruits (CEAGESP, 2009).The main use of watermelon residues is as soil fertilizer and only a small part and used as a complement to the animal feed, with good acceptance by cattle and goats (Silva, 2003; Lousada *et al*., 2005). Some limitations mean that these residues have a restricted use, among them the large amount of water and sugar they contain, which causes collection, transportation and storage problems. Due to the high cost of drying, companies are interested in developing markets for wet bagasse. This interest is greater, particularly for those small farmers who do not intend to spend the high investment necessary in their crops to dry the watermelon bark.

Several studies have proposed other uses for watermelon residues, including obtaining organic fertilizers, pectin, essential oils (Lousada Junior *et al*., 2006), Despite all these possibilities, the residues of the watermelon crops remain largely unused. The estimated production of paddy rice in the state of Tocantins for the year 2017 is 348 thousand tons According to the Brazilian Institute of Geography and Statistics - IBGE. Only in the region of Formoso do Araguaia, is estimated a production of 100 thousand tons for the same period. Rice cultivation annually generates 2 million tons of waste, which can take an average of 5 years to decompose when disposed of in landfills. Within this scenario, the reuse of these wastes is undoubtedly the most interesting option from an environmental, economic and, often, social point of view. Waste recycling represents an undeniable benefit: the minimization of the environmental problem that represents its inappropriate disposal (IPEA, 2012). Pleurotus spp. (Jacq: Fr) Kumm. (Pleurotaceae, Upper Basidiomycetes) is a group of mushrooms with high nutritional value, possessing several therapeutic properties and biotechnological applications (Cohen *et al*., 2002). They are called fungi that cause white wood rot, by efficiently degrading lignin, a recalcitrant phenolic polymer found in plants (Eriksson *et al*., 1990). This ability is due to the fact that they produce several lignocellulolytic enzymes, mainly laccases, Mn peroxidase and versatile peroxidase, which have numerous industrial applications (M.DI Fusco *et al*., 2010). The laccases have been applied in different processes in the beverage and food industry, textile industries, paper mills and pharmaceutical industry, as well as in bioremediation processes.Solid state culture is defined as the type of culture in which a microorganism grows in a mixture of solid (water-insoluble) material, in the absence or presence of limited amount of free water (Gervais and Molin, 2003; Moo-Young *et al*. al., 1983). It mimics the natural environment of basidiomycetes and, in general, enables high enzyme activity, including ligninolytic enzymes (Pandey *et al*., 1999). Microorganism growth may occur on the surface or on any substrate, depending on porosidase and substrate moisture (Gervais and Molin, 2003). The choice of a specific substrate for solid-state cultivation takes into account a number of factors, mainly related to cost and viability. Cultivation in lignocellulosic substrates makes it possible to provide elements to fungal nutrition, similar to what occurs in natural habitats. Due to the wide availability of watermelon residues generated in the region and with the purpose of adding value to such residues and minimizing the environmental impact caused by the accumulation of these residues, in this work we propose the use of watermelon residues as substrate to obtain hydrolytic enzymes and oxidative enzymes involved in the degradation of lignocellulosic materials such as laccase, manganese peroxidase, xylanase and glucanase by *P. ostreatus* grown in state solid.

## MATERIALS AND METHODS

### Microorganism

*P. ostreatus* was obtained from the Collection of Cultures of the Laboratory of Biotechnological Processes of the Federal University of Tocantins. In the laboratory, the species is maintained by periodic repechages in potato dextrose agar (BDA). The inoculum were obtained from fully colonized plaques, up to two weeks old and consisted of 10 mm diameter discs.

### Preparation of watermelon and rice husk

Watermelon and rice residues were obtained from the irrigated irrigation projects in the southern region of Tocantins, in the municipality of Formoso do Araguaia, and did not receive any treatment other than drying and milling. The drying was carried out in a forced ventilation oven, at a temperature of 35°C up to constant weight. After drying, the residues were mixed in the proportion of 30 and 70% respectively and ground into particles with an average diameter of 4 mm.

### Culture conditions

Four grams of dried watermelon peel added with rice husk were placed in Erlenmeyer flasks with a capacity of 250 mL. Mineral solution was added to the substrate to obtain initial moisture ranging from 50 to 90% ($p.v^{-1}$). The media were autoclaved for 15 minutes at 121 ° C. Four 10 mm diameter discs obtained from the cultures of *P. ostreatus* in BDA were aseptically transferred to the flasks. Cultures were maintained at varying temperatures of 20 to 35 ° C for up to 30 days in the presence or absence of light. Cultures were discontinued every five days and where possible the mycelial masses obtained were carefully removed with a spatula, washed with distilled water twice and oven dried at 60 ° C until constant weight for biomass determination (Souza *et al*., 2006).

### Extraction of enzymes

After removal of the biomass, the enzymes were extracted by adding 20 mL of distilled water to the remaining culture substrate and they were then held at 8 ° C under 120-minute stirring. The solids were separated by gauze filtration and the filtrates were centrifuged for 10 minutes at 10,000 g. Clear supernatants were used as sources of the enzymes.

### Determination of enzymatic activities

The laccase activity was measured using as substrate syringaldazine ($\varepsilon 525 = 65{,}000\ M^{-1}.cm^{-1}$). The reaction mixture

consisted of 1.5 ml of phosphate buffer (100 mM, pH 6.5), 0.2 ml syringaldazine (0.5 mM ethanolic solution) and 0.1 ml culture filtrate. Oxidation of the substrate after 5 minutes at 30°C was estimated at 525 nm (Leonowicz and Grzywnowitz, 1981). Mn peroxidase activity was measured by oxidation of 10 mM MnSO 4 at 30 ° C in 50 mM sodium malonate, pH 4.5 and in the presence of 0.5 mM $H_2O_2$. The Mn 3+ manganic ion complex forms malonate, which absorbs 270 nm (ε270 = 11.590 $M^{-1}.cm^{-1}$) (Wariishi *et al*., 1992). The activities of xylanase, amylase, pectinase and endoglucanase were determined by the appearance of reducing sugars from the hydrolysis of the xylan, starch, pectin and carboxymethylcellulose substrates (1% in 50 mM citrate buffer and pH 5.5), respectively, by the method of 3 (Miller *et al*., 1959). B-glycosidase and β-xylosidase activities were determined using the synthetic substrates p-nitrophenyl-β-glucopyranoside and p-nitrophenyl-β-xylopyranoside, respectively (Lenartovicz *et al*., 2003). A unit of enzyme activity (U) was defined as the amount of enzyme required to produce 1 µmol product / minute at 30 ° C. The enzymatic activities were expressed as international units per liter of dry substrate ($UL^{-1}$).

### Other analytical methods

Total soluble carbohydrates were estimated by the sulfuric phenol method using glucose as standard (Dubois *et al*., 1956). The amount of reducing sugars was estimated by the 3.5 dinitroxalicylic acid method (Miller, 1959), using glucose as standard.

### Statistical analyzes

The data were submitted to ANOVA and compared by the Tukey test ($p < 0.05$) using program R.

## RESULTS

Effect of temperature on growth and production of enzymes by *P. ostreatus.* At all temperatures tested (20-35 ° C), no difference was observed in fungus growth by the temperature variation within the tolerance range defined in the literature (Furlan *et al*., 1997, Bononi *et al*. al., 1999). Visual analysis allowed to conclude that the fungus grew better at temperatures between 25 and 30 ° C. In view of these results, the cultures were maintained at 28 ° C in 25 days of incubation (Table 1).

**Table 1. *Pleurotus ostreatus* radial mycelial growth (mm) in 3% malte-agar culture media and in dry substrate of watermelon peel and rice with temperature variation after 25 days of incubation (1)**

| Culture Mediun | Temperatures (° C) | | | |
|---|---|---|---|---|
| | 20 | 25 | 30 | 35 |
| Substrate | 4.91Ac | 8.96Ac | 7.20Ab | 2.80Ad |
| Malt-ágar 3% | 5.86Bb | 15.42Ba | 4.60Bb | 2.60Ac |

[1]Types followed by equal letters, uppercase in the columns and lowercase in the lines, do not differ by Tukey test, 5% probability.

The development of fungal biomass was strongly affected by temperature and humidity. Visual analyzes of the cultures allowed us to conclude that the development and growth of the fungus were earlier when the initial moisture content was higher than 65%. The initial humidities were considered adequate, allowing complete colonization of the substrates until the tenth day of culture, at 28 ° C. For the cultures in which the initial moisture varied between 70 and 85%, the fungus grew homogeneously in the particles of the solid substrate. In cultures where the initial moisture content was higher than 70%, the growth was characterized by the formation of a thick mycelial mass, above the substrate. Such a mycelial mass was easily removed from the residual substrate, allowing the determination of the produced fungal biomass (Figure 1).

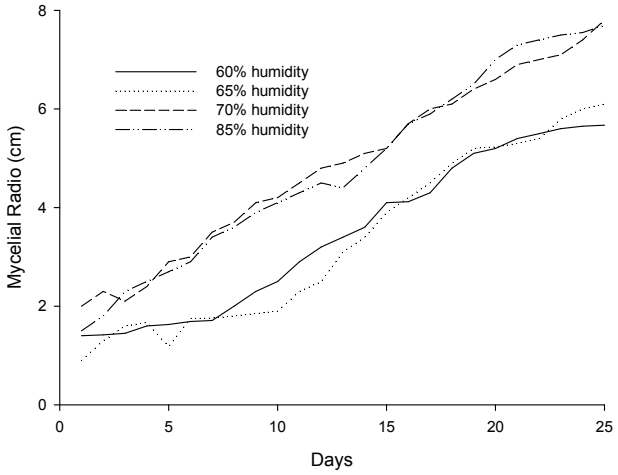


**Figure 1. Mycelial growth relative humidity**

The production of several oxidative and hydrolytic enzymes by P. ostreatus was determined after 10 days of cultivation at six different initial humidities. The main oxidative enzymes found in the culture filtrates were laccase and Mn peroxidase. Laccase production was positively affected by the increase in initial moisture ($p < 0.05$), and the highest activity was found in cultures with initial moisture of 80% (48.0 ± 0.35 $UL^{-1}$ substrate). The production of Mn peroxidase seems not to have been affected by the variation of the humidity of the cultures, presenting an average activity of 8.00 ± 0.23 $UL^{-1}$ substrate (Figure 2). The same occurred with the production of the hydrolytic enzymes xylanase, endoglucanase, β-xylosidase and β-glycosidase ($p > 0.05$) (Figure 3). Low levels of xylanase were detected. These results are consistent with the general observation that Pleurotus spp exhibits low xylanolytic and cellulolytic activities during the anamorphic stage of development (Munoz *et al*., 1997; Tan and Wahab, 1997).

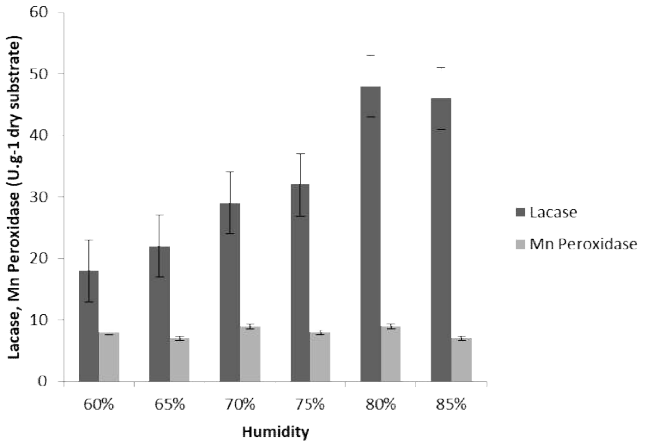


**Figure 2. Effect of oxidative enzymes in the initial moisture by *P. ostreatus* paddy rice and watermelon**

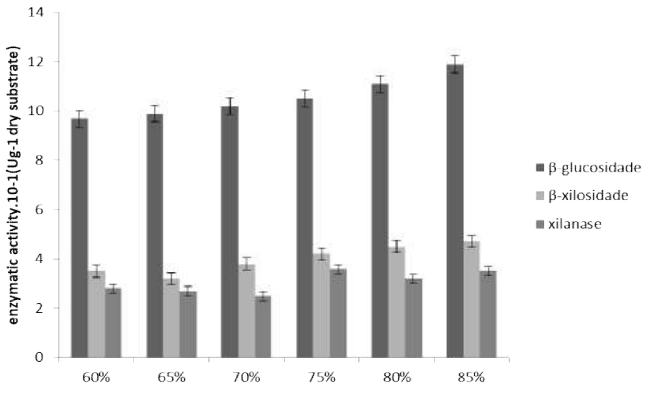


**Figure 3. Production of hydrolytic enzymes by *P. ostreatus* under the effect of moisture variation in temperature of 28 ± 2 ° C.**

### Production of biomass and enzymes in cultures with 85% initial moisture

The characteristic of superficial mycelial growth in substrate with initial humidity of 85% allowed the separation of the biomass of the residue of watermelon and remnant rice husk. The maximum fungal bio¬ mass was obtained after 20 days of cultivation (75 ± 4.3 mg.g $^{-1}$ dry substrate), with a mean value of 43 ± 4.8 mg.g $^{-1}$ of dry substrate. We can observe that the fungus actively uses the reducing sugars as carbon source in the first five days, but after that period, the amount of reducing sugars remains constant, suggesting that they are being produced from the hydrolysis of the polysaccharides present in the material. The watermelon shell is rich in soluble polysaccharides, mainly pectin (18% dry weight). When the humidity values used were higher than 80%, there was difficulty of mycelial growth toward the lower layers of the solid substrate, forcing the fungus to develop in the more superficial layers of the substrate. In this way, such cultivation is more properly considered as surface culture. The concentration of fungal biomass on the surface of the crops allowed them to be separated from the substrate, and growth can be evaluated by the direct determination of the fungal biomass produced (Figure 4).

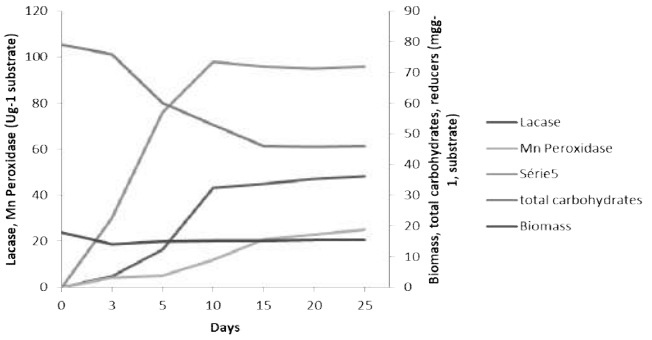


**Figure 4 - Production of enzymes and growth in medium P. ostreatus using watermelon rinds with an initial humidity of 85%**

The effect of the culture time on the watermelon shell in the production of oxidative enzymes by *P. ostreatus* is also presented in Figure 4. The production of laccase and Mn peroxidase seems to be dependent on fungus growth. Maximum laccase activity (4.3 U.g$^{-1}$ dry substrate) was obtained after 10 days of cultivation, while maximum Mn peroxidase activity (4.8 U.g $^{-1}$ dry substrate) was achieved on the 25th day of culture. The production of the xylanase and endoglucanase enzymes by the fungus, in the middle of the watermelon shell and rice, at high initial moisture was higher on the fifteenth day of culture, 75.0 ± 0.03 and 48.0 ± 0.03 U .mg $^{-1}$ of substrate, respectively. The production of other hydrolytic enzymes, such as: amylase and pectinase, was low (values less than 0.2 U.g $^{-1}$ dry substrate). However, the reduction in the amount of total carbohydrates soluble in the culture media (Figure 4), suggests that pectin is being degraded, although our results do not show an effective production of pectinases. Pectin is a complex heteropolysaccharide and several enzymes that act on its structure (Naidu and Panda, 1998). Not all of them produce reducing sugars, such as pectin-lyase and pectin-esterases. Therefore, a possible pectinolytic activity may not have been detected due to the technique used in this work to determine the pectinase activity. The traditional substrates for the cultivation of white wood rot fungi include: bran and wheat straw, oat straw, rice straw, sugarcane bagasse and some other lignocellulosic residues (Cohen *et al*., 2002, Vilas-Boas *et al*., 2002, Zadrazil, 1998). More recently, a number of new substrates have been proposed, many of which are the inedible parts of foods, such as: peels, bagasse, seeds and vegetables (Kalmis and Sargin, 2004; Gomez *et al*., 2005; Morais *et al*., 2005). The low cost of watermelon peel, associated with the growth capacity of the microorganism without the need of any supplementation, supports the use of watermelon residue as a substrate for growth and production of enzymes by *P. ostreatus*.

### Conclusion

Several strategies have been developed to utilize the vast quantities of lignocellulosic residues generated by agricultural activities and food processing industries. Watermelon residues generated by the agricultural crop show humidity above 85%, making it difficult to transport, store and use as animal feed. The data obtained in this work support the use of watermelon residue as a suitable substrate for the cultivation of *P. ostreatus* and the production of enzymes lacase and Mn peroxidase, both with great potential of use in different industrial processes, since the residue of watermelon and of the rice provided the necessary nutritional conditions for fungus growth, and no additional addition of carbon or nitrogen source was required, and high enzyme activities were produced in relatively short periods. Higher levels of Mn peroxidase were detected in longer cultures, and further experiments should be performed to verify if a higher yield could be obtained in cultures with incubation times greater than 25 days. The use of the watermelon crop residues with high initial moisture and without the need for previous drying would reduce the production costs of these important enzymes even further.

### Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

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