Full Length Research Paper

Optimization of *Agaricus blazei* laccase production by submerged cultivation with sugarcane molasses

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Accepted 5 February, 2014

Laccases are copper polyphenol oxidases that are interesting for several applications such as in the food industry, sewage treatment and decolorization. The use of agro-industrial byproducts allows bioprocesses development for the production of large quantities at viable cost enzymes. In this study, the laccase production of *Agaricus blazei* was optimized in submerged cultivation (SmC). First, the following agro-industrial substrates were evaluated: sugarcane molasses, soybean molasses, coffee husks, soybean husks and pellet citrus pulp; and then these nitrogen sources: urea, ammonium sulfate, yeast extract. For the optimization of laccase production, a Plackett-Burman and 2⁴⁻¹ incomplete factorial designs were used to evaluate the effect of KH₂PO₄, MgSO₄, KCl, FeSO₄, ZnSO₄ and the ethanol on laccase activity, and for the optimization of sugarcane molasses, urea, MgSO₄ and ethanol concentrations. Finally, laccase production kinetics was determined. The best substrate for laccase production by *A. blazei* was sugarcane molasses. After optimization, it was found that laccase activity of 9635 U/L was obtained in medium with sugarcane molasses (6 g/L), urea (1.5 g/L), MgSO₄ (12 mM), ethanol (1.2 mM) at 28°C and pH 8.0 during 10 days of cultivation. The results indicate that sugarcane molasses is a promising substrate for *A. blazei* laccase production.

Key words: *Agaricus brasiliensis*, *Agaricus subrufescens*, laccase, manganese peroxidase, sugarcane molasses.

INTRODUCTION

Laccases are copper containing polyphenol oxidases where copper atoms are distributed among three different highly-conserved binding sites, each one with an important role in the catalytic mechanism of the enzyme (Soden and Dobson, 2001; Couto and Toca-Herrera, 2006). Its presence has been described in plants, bacteria and insects (Morozova et al., 2007), but especially found in ligninolytic basidiomycete fungi (Mikolasch and Shauer, 2009). In basidiomycetes, laccases have different physiological functions such as delignification, defense against oxidative stress produced by lignin degradation and morphogenesis (Giardina et al., 2010).

The delignification process by laccase involves the transfer of electrons from phenolic substrates to molecular oxygen, reducing it to water while oxidizing phenolic substrates to semiquinone radicals (Zhou et al., 2013). One of its most striking features is the low specificity of their substrates. The number and diversity of substrates susceptible to oxidation by laccase vary greatly from one laccase to another (Dittmer and Kanost, 2010). These peculiarities make it a very interesting enzyme for
industrial applications such as in the food industry (Osma et al., 2010), biopulping (Zhou et al., 2013), sewage treatment (Majeau et al., 2010) and degradation or discoloration of dyes (Zeng et al., 2011).

Agaricus blazei Murrill sensu Heineman (Heinemann, 1993), considered as Agaricus brasiliensis Wasser et al. by Wasser et al. (2002) and Agaricus subrufescens Peck by Kerrigan (2005), is an edible native Brazilian mushroom. Many biological actions are attributed to this fungus such as immunomodulation (Zhong et al., 2005; Hetland et al., 2011), inhibition of tumor cell growth (Ohno et al., 2001), inhibition of cell migration or tumor-induced neovascularization (Jumes et al., 2010), and protection against allergies (Ellersten and Hetland, 2009).

A. blazei is a litter-decomposing fungus (LDF) and grows naturally on the surface layers of soil that are rich in organic matter and humus in forests and fields (Wasser et al., 2002). Due to its ecological characteristics, this fungus is able to degrade lignin and compounds that are structurally similar to lignin. However, the degradation rate is smaller as compared to white rot fungus, essentially ligninolytic ones (Durrant et al., 1991). The production of ligninolytic enzymes by LDF should not be discarded, since maximal levels of production can be improved by employing optimization of culture conditions (Elisashvili and Kachlishvili, 2009) as well as by using heterologous expression (Piscitelli et al., 2010) or structural modification (Robert et al., 2011). A. blazei has been described before as a laccase producer (Soden and Dobson, 2001; D’Agostini et al., 2011) and a producer of other enzymes such as amylase, cellulase, xylanase, mannanase and pectinase (Siqueira et al., 2010; Jonathan and Adeoyo, 2011).

The growing demand for lower cost in industrial processes that are also highly specific and environmentally safe has stimulated the search for new enzymes. The use of agro-industrial residues in bioprocesses has enabled the production of enzymes employing alternative substrates at low cost as well as reducing environmental degradation caused by the disposal of these residues (Elisashvili et al., 2008; Karp et al., 2013).

The sugar and ethanol industry produces different residues and many of them have great potential for bioprocesses application (Pandey et al., 2000). Sugarcane molasses is dark syrup obtained during sugar production from sugarcane or beets, resulting from the final stage of crystallization from which further recovery of sugar is no longer economically viable (Arakaki et al., 2011). Sugarcane molasses has an average of 50% total sugars in which sucrose predominates (Villavicencio et al., 1999; Feltrin et al., 2000; Arakaki et al., 2011). The production of sugarcane in Brazil in 2012/2013 harvest reached 589 million tons, yielding from 23 to 35 tons of molasses (CONAB, 2013). Its composition and abundance makes this residue a potential substrate for the development of biotechnological processes, including the production of enzymes and other products (Miranda et al., 1999).

The objective of this study was to develop a bioprocess for A. blazei laccase production in submerged cultivation using agro-industrial byproducts.

MATERIALS AND METHODS

Microorganisms and inoculum preparation

A. blazei U2-4 strain, available at the culture collection of the Molecular Biology Laboratory of Paranaense University, was selected to optimize the culture conditions for laccase production. The mycelium was kept on 1% malt extract agar medium (MEA; m/v) at 25°C and subcultured to 2% MEA (m/v) at 28°C for 7 days in order to prepare the inoculum.

For the experimental phase, the submerged cultivation (SmC) was done in a 250 mL Erlenmeyer. Each flask had 100 mL of autoclaved (121°C for 20 min) minimum medium. This medium consisted of 1.5 g/L KH₂PO₄, 0.5 g/L MgSO₄, 0.5 g/L KCl, 0.036 g/L FeSO₄. H₂O and 0.035 g/L ZnSO₄. H₂O. All flasks were inoculated with three agar discs, 6 mm diameter each, containing mycelium. The mycelial growth was carried out at 28°C for 10 days in the dark. For all experiments, at the third day of cultivation, CuSO₄ (300 g/L) was aseptically added until obtaining 150 µM in the culture medium. This elemental SmC scheme was used to evaluate the effect of the addition of substrates, nitrogen sources and salt concentrations on laccase production.

Selection of alternative substrates for laccase production

Five agro-industrial byproducts were tested as substrates: sugarcane molasses, soybean molasses, coffee husks, soybean husks and pellet citrus pulp. The molasses were added until total sugars reached 10 g/L in the minimum medium and the other particulated residues were used at 50 g/L. As nitrogen source, urea (300 g/L) was filtered (0.22 µm Millipore membrane) and added to the autoclaved medium in order to reach 100 mM.

Effect of nitrogen source on laccase production

Different nitrogen sources were added to the minimum medium in order to achieve 2.8 g/L of nitrogen. Sugarcane molasses was used as substrate until total sugars reached 10 g/L. The evaluated nitrogen sources were 13.2 g/L ammonium sulfate; 7.0 g/L yeast extract; 6.0 g/L urea; a mixture of 3.0 g/L urea and 6.6 g/L ammonium sulfate; and a mixture of 6.6 g/L ammonium sulfate and 3.5 g/L yeast extract. Water solutions of urea and ammonium sulfate were filtered (0.22 µm Millipore membrane) and aseptically added to the culture medium. The yeast extract was autoclaved with SmC media at 121°C for 20 min.

Screening of media components for laccase production

Plackett-Burman statistical experimental design is used for the screening of major constituents of the media with significant effects on laccase response (Bari et al., 2009). To achieve the best conditions for laccase production by submerged cultivation with sugarcane molasses, the concentration of salts of the minimum medium as well as the addition of urea and ethanol as inducers of laccase production were analyzed. First, a seven-factor Plackett-Burman design, resulting in eight runs, was used to determine the
effect of salt concentrations of the minimum medium such as KH₂PO₄, MgSO₄, KCl, FeSO₄·H₂O and ZnSO₄·H₂O, and the ethanol (Lomasco et al., 2003; Meza et al., 2007) on laccase production by A. blazei. Subsequently, a 2⁴⁻¹ incomplete factorial design was used and four components were evaluated in 11 experiments with three replications of the central point to test the concentration effect of sugarcane molasses (6; 10 or 14 g/L), urea (2; 7 or 12 g/L), MgSO₄·7H₂O (2; 7 and 12 g/L) and ethanol (0.2; 0.7 and 1.2 g/L) on laccase production.

The significant urea variable was again studied at concentrations below 2 g/L, the lowest concentration evaluated in the 2⁴⁻¹ incomplete factorial design. For this experiment, the composition of the culture medium was sugarcane molasses (6 g/L), magnesium sulfate (12 mM), ethanol (1.2 mM), the best conditions determined in the 2⁴⁻¹ incomplete factorial design, and urea added at 0; 0.5; 1.0; 1.5 and 2.0 g/L.

Kinetics of laccase production by SmC with sugarcane molasses

After determining the best cultivating conditions (6 g/L sugarcane molasses, 1.5 g/L urea, 12 mM MgSO₄ and 1.2 mM ethanol), 10 day-kinetics was carried out by SmC. The pH of the cultivated medium, the amount of reducing sugars, the production of biomass, laccase and manganese peroxidase activities were also determined.

Enzymatic assay

Laccase (EC 1.10.3.2) activity was determined by monitoring the oxidation of ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid]. The reaction mixture contained: 200 μL culture media, 700 μL water, 450 μL sodium acetate buffer (0.1 M; pH 5.0) and 150 μL ABTS (1 mM). The mixture was kept for 10 min at 30 °C in a bath water and the reaction was interrupted by the addition of 100 μL of 5% trichloroacetic acid (m/v). The increase of the absorbance was measured at 420 nm using the ε = 36000 M⁻¹ cm⁻¹. The controls were: a) 200 μL culture media, 850 μL water and 450 μL sodium acetate buffer; and b) 900 μL water, 450 μL sodium acetate buffer and 150 μL ABTS. One unit (U) of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per minute.

Manganese peroxidase (EC 1.11.1.13) was determined by the oxidation of 10 mM MnSO₄ at 30°C in 50 mM sodium malonate buffer (pH 4.5) and in the presence of 0.5 mM hydrogen peroxide (Warishii et al., 1992). The oxidation was monitored by absorbance increase at 270 nm (ε = 11590 M⁻¹ cm⁻¹) caused by the complex formed by the Mn³⁺ ion with malonate. The mixing of the enzyme extract and sodium malonate buffer and the mixture of MnSO₄ and sodium malonate buffer were used as analytical controls. One unit (U) of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of MnSO₄ per minute.

Statistical experiments and analysis were carried out using the software package STATISTICA 10 (StatSoft, Tulsa, OK, USA). All experiments and procedures were done in triplicate and the results evaluated using ANOVA or Tukey’s test (p <0.05).

RESULTS AND DISCUSSION

Selection of alternative substrates

The selection of alternative substrates for laccase production was carried out in SmC containing various agro-industrial residues. The sugarcane molasses, soybean molasses and coffee husks increased laccase activity when compared with the control with glucose (Table 1). The highest (p≤0.05) laccase activity was with sugarcane molasses and it was 1.7, 1.5 and 3.8 times higher than soybean molasses, coffee husk and glucose (control), respectively. Also, sugarcane molasses was the best for productivity, reaching 58.5 U/Lh. Thus, in our study, sugarcane molasse was chosen as an alternative substrate for the next experiments.

Easily assimilated substrates seem to favor the laccase production for some fungal species, but several reports showed otherwise (Galhaup et al., 2002; Elisashvili and Kachlishvili, 2009; Majeau et al., 2010). Our results showed that the highest value for laccase activity (14052 U/L) was obtained with sugarcane molasses. This residue has 540 g/L total sugars (Rodrigues et al., 2009), 70 to 91% of which is sucrose, and 2 to 4% is glucose (Arakaki et al., 2011). For A. blazei, the easily assimilated substrates present in sugarcane molasses seem to increase the laccase production.

Our results show that when coffee husks was used as substrate, the laccase production was 3566 and 5140 U/L when citric pulp and soybean hulls were used respectively. The production of laccases in SmC using agro-industrial residues can be stimulated by the presence of aromatic and/or phenolic compounds (Elisashvili and Kachlishvili, 2009). Coffee husks, for example, are rich in caffeine, tannins and polyphenols (Pandey et al., 2000). Thus, it can be suggested that aromatic and/or phenolic compounds can improve laccase production by A. blazei. Previous data from our laboratory suggested that these compounds stimulated A. blazei laccase production (data not shown) and also stimulate laccase production of other basidiomycetes (Silva et al., 2012). Additionally, the fungal ability of producing high levels of laccase in different agro-industrial residues demonstrates the versatility of this strain as a laccase producer. Previous reports of A. blazei laccase production in SmC with agro-industrial byproducts showed lower levels of laccase activity of 500 U/L and productivity of 12.2 U/Lh (Ullrich et al., 2005) against 14052 U/Lh for laccase activity, and 58.5 U/Lh for productivity obtained in our work with sugarcane molasses.

Selection of alternative nitrogen source

Following the selection of substrates for laccase production, aproteic (urea and ammonium sulfate), proteic (yeast extract), and combinations of aproteic and proteic nitrogen sources were evaluated. Different nitrogen sources, as well as combinations thereof, did not affect (p≤0.05) the laccase activity (Figure 1). The highest laccase activities were obtained with urea (11427 U/L) and a urea and ammonium sulfate combination.
Table 1. Laccase produced by *A. blazei* with different agro-industrial wastes after ten days of submerged cultivation.

| Laccase activity (U/L) | Sugarcane molasses | Soybean molasses | Citric pulp | Coffee husk | Soybean hull | Glucose |
|------------------------|--------------------|------------------|-------------|-------------|--------------|---------|
| *14052*                | 14052*             | 8340*            | 3566*       | 9253*       | 4104         | 3666    |
| Productivity (U/Lh)    | 58.5               | 34.7             | 14.8        | 38.5        | 34.7         | 15.3    |

*Significant increase of laccase activity related to glucose by Tukey's test (p<0.05).

Figure 1. Laccase activity of *Agaricus blazei* in media containing different nitrogen sources, urea (UR), ammonium sulfate (AS), yeast extract (YE), urea and ammonium sulfate (UR + AS), and (YE + AS) yeast extract and ammonium sulfate (YE + AS), after 10 days of cultivation. Averages indicated with the same letter do not differ statistically by Tukey's test (p<0.05).

Table 2. Plackett & Burman planning and the effect of the independent variables on laccase activity produced by *Agaricus blazei* in SmC with sugarcane molasses.

| Trial | Urea (g/L) | KCl (g/L) | KH₂SO₄ (g/L) | MgSO₄ (g/L) | FeSO₄ (g/L) | ZnSO₄ (g/L) | Ethanol (mM) | Laccase activity (U/L) |
|-------|------------|-----------|--------------|-------------|-------------|-------------|--------------|------------------------|
| 1     | 0          | 0         | 0            | 1.023       | 0.018       | 0.0175      | 0            | 10660 ± 972            |
| 2     | 6          | 0         | 0            | 0           | 0.0175      | 0.5         | 0            | 2097 ± 943             |
| 3     | 0          | 1.5       | 0            | 0           | 0.0175      | 0.5         | 0            | 10440 ± 368            |
| 4     | 6          | 0         | 0.5          | 1.023       | 0           | 0           | 0            | 3566 ± 44              |
| 5     | 0          | 0         | 0.5          | 1.023       | 0           | 0.5         | 0            | 11160 ± 403            |
| 6     | 6          | 0         | 0.5          | 0           | 0.018       | 0           | 0            | 2646 ± 236             |
| 7     | 0          | 1.5       | 0.5          | 0           | 0           | 0.0175      | 0            | 10788 ± 211            |
| 8     | 6          | 1.5       | 0.5          | 1.023       | 0.018       | 0.0175      | 0.5          | 3465 ± 894             |

Effect: -24.7**

p-value: <0.001

Selection of minimum medium components for the production of laccase by SmC

The results of the Plackett & Burman experiments for selection of significant variables in the laccase production are shown in Table 2. The obtained value for $R^2$ was 0.98, indicating that 98% of the data adjusted to the

(11736 U/L). Because the nitrogen sources did not affect the laccase production, it is supposed that the nitrogen concentrations could affect it. Thus, urea was chosen for the next experiments because it is an inexpensive source of nitrogen, produced high laccase activity in our work, and is used for laccase production by *A. blazei* in solid state cultivation (D’Agostini et al., 2011).
These results show that, the better the concentration of salt in the cultivation could be advantageous. The laccase production (77958 U/L) was the only variable that affected enzyme activity (13680 U/Lh) so the higher carbon/nitrogen (C/N) ratio, the better the laccase yield.

The variable and levels of sugarcane molasses, MgSO₄ and ethanol (ET) did not affect the laccase activity of A. blazei in SmC. Thus, these variables were kept at lower levels of 6 g/L sugarcane molasses, 12 mM MgSO₄, 1.2 mM ethanol, and urea was kept as a variable.

The results of the variable selection have pointed out that the laccase production can be facilitated by reducing the urea concentration, which means an increase in C/N ratio. The effect of nitrogen concentration on laccase production appears to vary according to the species and strain of the assessed fungus (Giardina et al., 2010). Pleurotus ostreatus cultivated in a medium containing urea (0.5 g/L) produced more laccase with lower concentration of nitrogen (Hou et al., 2004). Conversely, Stajić et al. (2006) showed that higher concentrations of nitrogen (20 g/L, peptone) slightly stimulate the laccase production of this species, which is an indication that different strains of the same species may respond differently to a specific nutrient. It suggests as well that laccase production depends on C/N ratio which differs for each employed strain and should be optimized for each case.

In this study, urea concentration ranged from 2 to 12 g/L. However, the results showed that concentrations of urea lower than 2 g/L could improve laccase production. Thus, an experiment was designed to refine the best urea concentration for enzyme production. The highest level of enzyme activity (13604 U/L) was obtained with 1.5 g/L urea (Figure 2). This result did not differ significantly from

Table 3. Variables and levels of the factorial planning 2⁴ on laccases activity and productivity: sugarcane molasses (SCM), urea (UR), MgSO₄ (Mg) and ethanol (ET) in the cultivation media.

| Level | SCM (g/L) | UR (g/L) | Mg (mM) | ET (mM) | Laccase activity (U/L) | Productivity (U/Lh) |
|-------|-----------|----------|---------|---------|-----------------------|---------------------|
| 6     | 2         | 2        | 0.2     | 0.2     | 12215                 | 50.9                |
| 14    | 2         | 2        | 1.2     | 1.2     | 13680                 | 57.0                |
| 6     | 2         | 2        | 1.2     | 1.2     | 7958                  | 33.1                |
| 14    | 2         | 2        | 0.2     | 0.2     | 4319                  | 18.0                |
| 6     | 2         | 12       | 1.2     | 0.2     | 14139                 | 60.0                |
| 14    | 2         | 12       | 0.2     | 0.2     | 13847                 | 57.7                |
| 6     | 12        | 12       | 1.2     | 1.2     | 9430                  | 39.3                |
| 14    | 12        | 12       | 0.2     | 0.2     | 6173                  | 25.7                |
| 10    | 7         | 7        | 0.7     | 0.7     | 11132                 | 46.4                |
| 10    | 7         | 7        | 0.7     | 0.7     | 11271                 | 47.0                |
| 10    | 7         | 7        | 0.7     | 0.7     | 13944                 | 58.1                |

Effect -1.1 -4.9 1.0 0.4 - -

p-value 0.3 0.003 0.4 0.7 - -

Optimization of sugar cane molasses, urea, MgSO₄ and ethanol for the production of laccase by SmC

The highest laccase activity (14139 U/L) was with 6 g/L of total sugars (sugarcane molasses), 2 g/L of urea, 12 mM MgSO₄ and 1.2 mM ethanol (Table 3). The average laccase production of 7 and 12 g/L of urea was of 12116 and 6970 U/L, respectively (Table 3). In addition, urea was the only variable that affected (p≤0.05) the laccase production (Table 3). However, the effect of MgSO₄ was very close to the limit of statistical significance, suggesting that an increased concentration of salt in the cultivation could be advantageous. The R² of this analysis was 0.81 indicating that 81% of the data fit in the statistical analysis model. These results show that laccase production by A. blazei in submerged cultivation is inversely proportional to the concentration of nitrogen, so the higher carbon/nitrogen (C/N) ratio, the better the laccase yield.
those obtained with 1 (13469 U/L) and 2 g/L urea (13590 U/L). Urea concentration of 0.5 or 0.0 g/L reduced (p≤0.05) the laccase activity to 12795 and 12996 U/L, respectively.

Thus, the best conditions for laccase production of *A. blazei* U2/4 in SmC is 6 g/L sugarcane molasses, 1.5 g/L urea, 12 mM MgSO$_4$, and 1.2 mM ethanol. Smaller amounts of nitrogen favor laccase production in SmC with sugarcane molasses that reached optimum values when C/N ratio was between 1.3 (2 g/L urea) and 3.0 (1 g/L urea).

**Kinetics of laccase production with the optimized conditions**

After determining the best cultivation conditions, our group studied the kinetics of laccase production in SmC by analyzing the enzyme activity every 24 h for a period of 10 days. The peak of laccase production and productivity, 9635 U/L and 44.6 U/Lh, respectively, were obtained at the 9$^{th}$ day of cultivation (Table 4). The pH gradually increased over time, from 5.6 to 8.0, at the peak of the enzyme activity, demonstrating that higher levels of activity occurred at higher pH and around neutrality. The microbial biomass production reached maximum value at day 8 when the enzyme production was near the maximum. This shows that laccase production in SmC is associated with *A. blazei* mycelium growth. The amount of reducing sugars showed no important variation up to half of the cultivation period, suffering a decrease from the increase of biomass and laccase production (Table 4). Thus, the laccase production was associated with the reduction of reducing sugars in the cultivation medium. The production of manganese peroxidase (Table 4) remained at low levels, reaching maximum activity at day 8 (1855 U/L).

There is scant information on the laccase production by *A. blazei*. Ullrich et al. (2005) achieved levels of enzymatic activity around 5000 U/L at the 17$^{th}$ cultivation day and productivity of 12 U/Lh with tomato juice as culture medium. Our results, after optimized for laccase production of *A. blazei*, surmount those described in the literature demonstrating an outstanding productivity of
44.6 U/L and laccase activity of 9635 U/L.

The production of manganese peroxidase (MnP), which remained at relatively low levels, was also determined (Table 4) reaching maximum activity at day 8 (1855 U/L). Fenice et al. (2003) reported activity peak of 292 U/L by *Panus tigrinus* in SmC with waste from olive oil production, whereas Hou et al. (2004) found peak of 1500 U/L for *Pleurotus ostreatus* in SmC.

The pH gradually increased over time, from 5.6 to 8.0, at the peak of enzyme activity, demonstrating that higher levels of activity occurred at higher pH and around neutrality. Similarly, Ulrich et al. (2005) reported that the laccase production of *A. blazei* in tomato juice also showed drastic change in pH which rose from 4.5 to 7.0 during 28 cultivation days. The stability of the majority of the laccases is higher under acid pH, although this characteristic varies greatly depending on the source of laccase (Majeau et al., 2010).

The microbial biomass production reached maximum value at day 8 when the enzyme production was near the maximum. This shows that laccase production is associated with mycelial growth. The amount of reducing sugars showed no important variations up to half of the cultivation period, suffering a decrease from the increase in biomass and laccase production.

### Conclusions

Sugarcane molasses is the best substrate for laccase production of *A. blazei* in submerged cultivation. Laccase production is negatively affected by the increase in nitrogen concentration. The highest level of laccase activity (9635 U/L) and productivity (44.6 U/hL) occurs in a cultivation medium comprising of 6 g/L sugarcane molasses, 1.5 g/L urea, 150 μM CuSO₄, 12 mM MgSO₄, 1.2 mM ethanol at pH of 8.0 after nine cultivation days. These results indicate the versatility of *A. blazei* in producing significant levels of laccase in SmC using abundant and inexpensive agro-industrial byproducts.

### ACKNOWLEDGEMENTS

The authors thank Paranaense University for part of experimental work fund and fellowship, and Federal University of Paraná. L. P. S. Vandenbergher thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

### REFERENCES

Arakaki AH, Vandenbergher LPS, Soccol VT, Masaki R, Rosa Filho EF, Gregório A, Soccol CR (2011). Optimization of biomass production with copper bioaccumulation by yeasts in submerged fermentation. Braz. Arch. Biol. Technol. 54:1027-1034.

Bari MN, Alam MZ, Muyibi SA, Jamal P, Mamun AA (2009). Improvement of production of citric acid from oil palm empty fruit bunches: Optimization of media by statistical experimental designs. Bioresource Technol. 100:3113-3120.

CONAB, Companhia Nacional de Abastecimento (2013). Acompanhamento da safra brasileira: cana-de-açúcar safra 2013/2014. Brasília. 2013. Available at http://www.conab.gov.br/OlalaCMS/uploads/arquivos/13_08_08_09_39_29_boletim_cana_portugues__abril_2013_10_lev.pdf. Accessed on 15 Nov 2013.

Couto SR, Toca-Herrera JL (2007). Laccase production at reactor scale by filamentous fungi. Biotechnol. Adv. 25:558-569.

D’Agostini EC, Mantovani TRD, Valle JS, Paccolla-Meireles LD, Colauto NB, Linde GA (2011). Low carbon/nitrogen ratio increases laccase production from basidiomycetes in solid substrate cultivation. Sci. Agric. 68: 295-300.

Dittmer NT, Kanost MR (2010). Insect multilipper oxidases: diversity, properties, and physiological roles. Insect. Biochem. Molec. 40:179-188.

Durrant AJ, Wood DA, Cain RB (1991). Lignocellulose biodegradation by *Agaricus bisporus* during solid substrate fermentation. J. Gen. Microbiol. 137:751-755.

Elisashvili V, Khalishvili E (2009). Physiological regulation of laccase and manganese peroxidase production by white-rot Basidiomycetes. J. Biotechnol. 144:37-42.

Elisashvili V, Khalishvili E, Penninckx M (2008). Effect of growth substrates, method of fermentation, and nitrogen source on lignocellulose-degrading enzymes production by white-rot Basidiomycetes. J. Ind. Microbiol. Biotechnol. 35:1531-1538.

Ellersten LK, Hetland G (2009). An extract of the medicinal mushroom *Agaricus blazei* Murill can protect against allergy. Clin. Mol. Allergy. 7; 6: doi: 10.1186/1476-7961-7-6

Feltrin VP, Sant’anna ES, Porto ACS, Torres RCO (2000). Produção de Lactobacillus plantarum em melão de cana-de-açúcar. Braz. Arch. Biol. Technol. 43. Available at http://www.scielo.br/pdf/babt/v43n1/v43n1a15.pdf Accessed on 15 November 2013. doi: 10.1590/S1516-89132000000100015.

Fenice M, Giovannozzi Spermman G, Federici F, D’Annibale A (2003). Submerged and solid-state production of laccase and Mn-peroxidase by *Panus tigrinus* on olive mill wastewater-based media. J. Biotechnol. 100:77-85.

Galhaup C, Wagner H, Hinterstoisser B, Haltrich D (2002). Increase of laccase production from basidiomycetes in solid substrate cultivation. Sci. Technol. 30:529-536.

Giardina P, Faraco V, Piscitelli A, Vanhulle S, Sannia G (2010). Laccases: a never-ending story. Cell. Mol. Life Sci. 67:369-385.

Hendermann P (1993). Agarici Austroamericani VIII, Agariceae from the intertropical region of South America. Bull. Jard. Bot. Nat. Bel. 62:355-384.

Hetland G, Johnson E, Lyberg T, Kvalheim G (2011). The mushroom *Agaricus blazei* Murill elicits medicinal effects on tumor, infection, allergy, and inflammation through its modulation of innate immunity and amelioration of Th1/Th2 imbalance and inflammation. Adv. Pharmacoloc. Sci. 2011:157015. doi: 10.1155/2011/157015.

Hou H, Zou J, Wang J, Du C, Yan B (2004). Enhancement of laccase production by *Pleurotus ostreatus* and its use for the decolorization of anthraquinone dye. Process Biochem. 39:1415-1419.

Jonathan SG, Adeoyo OR (2011). Evaluation of ten wild nigerian mushrooms for amylase and cellulase activities. Microbiol. 39:103-108.

Jumes FMD, Lugariini D, Pereira AL, Oliveira A, Christoff AO, Linde GA, Valle JS, Colauto NB, Acco A (2010). Effects of *Agaricus brasiliensis* mushroom inWalker-256 tumor-bearing rats. Can. J. Physiol. Pharm. 88:21-27.

Karp SG, Woiciechowski AL, Soccol VT, Soccol CR (2013). Pretreatment strategies for delignification of sugarcane bagasse: a review. Braz. Arch. Biol. Techn. 56:679-689.

Kerrigan RW (2005). *Agaricus subrufescens*, a cultivated edible and medicinal mushroom, and its synonyms. Mycologia 97:12-24.

Lomascolo A, Record E, Herpoël-Gimbert I, Dellatre M, Robert JM, Georis J, Dauvin T, Sigoillot JC, Asther M (2003). Overproduction of laccase by a monokaryotic strain of *Pycnoporus cinnabarinus* using
ethanol as inducer. J. Appl. Microbiol. 94:618-624.
Majeau JA, Brar SK, Tyagi RD (2010). Laccases for removal of recalitrant and emerging pollutants. Bioreavour. Technol. 101:2331-2350.
Meza JC, Auria R, Lomascolo A, Sigillotto JC, Casalot L (2007). Role of ethanol on growth, laccase production and protease activity in Pycnoporus cinnabarinus ss3. Enzyme Microb. Tech. 41:162-168.
Mikolasch A, Shauer F (2009). Fungal laccases as tools for the synthesis of new hybrid molecules and biomaterials. Appl. Microbiol. Biot. 82:605-624.
Miranda LAS, Sant’anna ES, Porto ACS (1999). The growth of Micrococcus varians by utilizing sugar cane blackstrap molasses as substrate. Braz. J. Microbiol. 30:125-129.
Morozova OV, Shumakovich GP, Gorbacheva MA, Shleev SV, Yaropolov AI (2007). “Blue” laccases. Biochemistry-Moscow+ 72:1136-1150.
Ohno N, Furukawa M, Miura NN, Adachi Y, Motoi M, Yadomae T (2001). Antitumor b-glucan from the cultured fruit body of Agaricus blazei. Biol. Pharm. Bull. 24:820-828.
Osma JF, Toca-Herrera JL, Rodriguez-Couto J (2010). Uses of laccases in the food industry. Enzyme Res. 2010:918761. doi: 10.4061/2010/918761.
Pandey A, Soccol CR, Nigam P, Brand D, Mohan R, Roussos S (2000). Biotechnological potential of coffee pulp and coffee husk for bioprocesses. Biochem. Eng. J. 6:153-162.
Piscitelii A, Pezzella C, Giardina P, Faraco V, Giovanni S (2010). Heterologous laccase production and its role in industrial applications. Bioeng. Bugs 1:252-262.
Robert V, Mekmouche Y, Pailléry PR, Tron T (2011). Engineering laccases: in search for novel catalysts. Curr. Genomics 12:123-129.
Rodrigues C, Vandenbergeh LPS, Teodoro J, Pandey A, Soccol CR (2009). Improvement on citric acid production in solid-state fermentation by Aspergillus niger LPB BC mutant using citric pulp. Appl. Biochem. Biotechnol. 158:72-87.
Silva JJ, Santana TT, Oliveira ACC, Almeida PH, Húise SG, Linde GA, Colauto NB, Valle JS (2012). Laccase production from basidiomycetes by submerged fermentation with coffee husks. Arq. Ciênc. Vet. Zool. Unipar 15:191-196. Available at http://revistas.unipar.br/veterinaria/article/viewFile/4234/2643
Accessed on 15 Nov 2013.
Siqueira FG, Siqueira AG, Siqueira EG, Carvalho MA, Peretti BMP, Jaramillo PMD, Teixeira RS, Dias ES, Félix CR, Filho EX (2010). Evaluation of holocellulase production by plant-degrading fungi grown on agro-industrial residues. Biodegradation 21:815-824.
Soden DM, Dobson ADW (2001). Differential regulation of laccase gene expression in Pleurotus sajor-caju. Microbiology 147:1755-1763.
Stajić M, Persky L, Friesem D, Hadar Y, Wasser SP, Nevo E, Vukojević-J (2006). Effect of different carbon and nitrogen sources on laccase and peroxidases production by selected Pleurotus species. Enzyme Microb. Tech. 38:65-73.
Ullrich R, Huang M, Dung NL, Hofrichter M (2005). Laccase from the medicinal mushroom Agaricus blazei: production, purification and characterization. Appl. Microbiol. Biotech. 67:357-363.