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# Laccase production by *Aspergillus niveus* on SSF using wheat bran as alternative carbon source and its synergistic effect on pulp biobleaching using a mix of laccase/xylanase from the same microorganism

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## Abstract

Aiming to investigate the production oflaccases by *Aspergillus niveus* RP05, the performance of this enzyme on cellulose pulp biobleaching wasanalyzed and tests of mixing laccase/xylanases from the same microorganism were also included. *A.niveus* was cultivated at 30ºC, for 2 weeks on solid state fermentation (SSF) composed by wheat bran and 0.05 M $(NH_4)_2HPO_4$ (1:2 – w/v) for laccase production. Optima of temperature and pH reaction were 60ºC and 4.5 – 7.0, respectively. The correlation between pH and temperature was investigated using the experimental design and the highest enzyme activity levels were obtained at the central points (55°C, pH 5.0). It was verified that only the temperature had significance in the linear level with the confidence interval of 95%.The enzyme was stable up to 70ºC (more than 80% of residual activity); after 1 hour of incubation laccase lost at least 50% of its initial activity from 75 – 80ºC. Crude extract containing laccase activity itself was used (STRATEGY I) or mixed with xylanase (STRATEGY II) to clarify cellulose pulp. The most interesting result was obtained with a mix of xylanase/laccase, resulting in Kappa efficiency around 56% and an increase in whiteness on the order of 17.2 points.

**Keywords:***Aspergillus*, laccase, xylanase, biobleaching, cellulose pulp

## Introduction

The number of studies concerning the development of non-toxic methods for industrial processes environmentally clean, including paper manufacturing, has increased considerably (Betini et al. 2009; Michelin et al. 2010). In this context, the inclusion of an enzymatic step in cellulose pulp biobleaching might contribute to reduce the use of chlorine-containing reagents because using enzymes, particularly xylanases, results in an easier bleaching in subsequent stages and a better pulp brightness (Valcheva et al. 2001; Peixoto-Nogueira et al. 2009; Thakur et al. 2012). Endo-1,4-β-xylanase (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8) catalyzes the hydrolysis of the glycoside bonds in the xylan backbone bringing about a reduction in the degree of polymerization of the substrate (Polizeli et al. 2005), improving the access of bleaching reagents into cellulose fibers and facilitating lignin removal by subsequent alkaline extraction (Zhao et al. 2002; Woldesenbet et al. 2013).

Another enzyme that has attracted considerable interest in pulp biobleaching application is the laccase (p-diphenol oxidase; EC 1.10.3.2). This enzyme attacks the phenolic subunits of lignin, resulting in its degradation. Since the remaining lignin in pulp during the bleaching is a major cause of residual color in the pulp, laccase has been considered one of the most promising enzyme for such application (Madhavi and Lele 2009; Woldesenbet et al 2013; Quintana et al 2015; Sing et al. 2015; Yan et al. 2015).

There are many studies about industrial xylanase application in biobleaching of cellulose pulp in the literature (Beg et al. 2001; Zhao et al. 2002; Techapun et al. 2003; Rizzatti et al. 2004; Salles et al. 2005; Sandrim et al. 2005; Betini et al. 2009; Michelin et al. 2010; Quintana et al 2015), including pilot-scale production (Lu et al. 2003) and also studies about ligninases (Katagiri et al. 1995) applied in cellulose pulp.

Among the microorganisms employed to produce xylanase, filamentous fungi from *Aspergillus* genus are the most explored. For example, the production of xylanase and biobleaching assays of cellulose pulp were reported for *A. niveus* (Peixoto-Nogueira et al. 2009), *A. caespitosus* (Sandrim et al. 2005), *A. nidulans* and *A. awamori* (Techapun et al. 2003), *A. niger* An76 and *A. aculeatus* (Polizeli et al. 2005). Agricultural residues can be used as alternative carbon sources to produce these enzymes reducing the production costs and also lowering the final product price (Betini et al. 2009; Peixoto-Nogueira et al. 2008; 2009). Laccase can also be

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produced from agricultural residue (Gomes et al. 2009) and generally using a solid-state system (Katagiri et al. 1995; Gomes et al. 2009), but in the literature, data about ligninases are generally from basidiomycete fungi (De Boer et al. 1987; Faison and Kirk 1985; Levit and Shkrob 1992) as *Phanerochaetechrysosporium*(De Boer et al. 1987; Faison and Kirk 1985).However, filamentous fungi are also able to efficiently degrade lignin. In this context, the aim of the present study was to optimize laccase production by *A. niveus* using wheat bran as carbon source, a by-product formed during wheat processing; and also, analyze laccase efficiency on cellulose pulp bleaching when used alone (STRATEGY I) or in a mix of laccase and xylanase (STRATEGY II).

## Materials and Methods

*Organism and culture conditions*

*A. niveus* RP05 was isolated from soil and decomposing leaves in a reforestation area in the Campus of São Paulo University in RibeirãoPreto, SP, Brazil, as previously reported (Peixoto-Nogueira et al. 2009). Stock cultures were propagated on slants of solid oatmeal medium (Emerson 1941), at 35°C using 002CD FANEM (Brazil) incubator. Culture medium was composed by wheat bran added by nitrogen salt solution (1:2 - w/v). Different nitrogen solutions were tested such as 0.05 M $(NH_4)_2SO_4$ – N1; 0.05 M $(NH_4)_2HPO_4$ – N2; a combination of N1 and N2 at a concentration of 0.025 M each one – N3, Khanna (Khanna et al. 1995), Vogel (Vogel et al. 1964), SR (Rizzatti et al. 2001) and Czapek (Wiseman 1975). The incubation time varied according to each experiment and the cultivation temperature corresponded to 30ºC, it was inoculated with 1 mL of $10^7$ spore solution and the equipment used for incubation was the same described above. Growth was estimated as dry weight of mycelial pads (collected by filtration through filter paper on a Buchner funnel), dried at 60ºC for 4 days.

*Enzyme extraction, enzymatic assays and protein determination*

After cultivation, the enzyme was extracted from the growth medium by the addition of 25 mL of distilled water and agitation for 15 minutes, at 4ºC, followed by vacuum filtration (Vacuum pump Prismatec, Brazil). The filtrate was centrifuged at 15,000xg (Centrifuge 5810 R, Eppendorf, Germany) to remove solid residues and the supernatant was used as crude enzyme source.

Laccase activity was assayed using classic method described by Buswell et al. (1995), using 0.1 M ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) as substrate in 0.05 M sodium acetate buffer, pH 5.0 as substrate. One unit was defined as the amount of enzyme that releases 1 μmol of ABTS per minute. Xylanase activity was determined by biobleaching assays as described by Peixoto-Nogueira et al. (2009) and, to ensure that cellulases were absent, their activities were determined by the sugar reducing formation (Miller, 1959) using CM-cellulose or avicel in 0.1 M citrate-phosphate buffer (McIlvaine, 1921), pH 5.0, as substrate. One unit was defined as the amount of enzyme that releases 1 μmol of glucose per minute, in the assay conditions. Total protein was estimated according to Lowry et al. (1951), using bovine serum albumin as standard.

*Laccase production simulation*

Considering that the rate of laccase production is little at initial growth time, and then there is a sharply increase in the rate of enzyme production, reaching a climax and finally drops with the culture ageing, the equation of Boltzmann could be applied to simulate laccase production including the ascending and descending branches:

$$Y = a1 - a2 / 1 + e^{(t-to)/dt} + a2$$

where y is the laccase production (U $g^{-1}$) at time t (day), t is time (day) for a period of enzyme production, a1 (U $g^{-1}$ $d^{-1}$) and a2 (U $g^{-1}$ $d^{-1}$) are constants and mean the enzyme production at initial and final period, respectively, to is the time (day) where the production of laccase is 50% of the maximal, and dt is the time constant.

Assuming that laccase production follows the normal distribution over the time of culturing, the Gaussian equation could be applied to simulate the production including ascending and descending branches:

$$y = yo + \frac{a}{w\sqrt{\frac{\pi}{2}}} e^{-2}(t - to)/w^2$$

where y is the laccase production (U g-1) at time t (day), yo is basal line, a (U g-1) and w (day) are constants, to is the time (day) where maximal laccase production rates occur. All regression models were performed by Origin plot, version 6.1.

*Effects of temperature and pH*

Optima of temperature and pH for laccase activities were analyzed using crude filtrate from *A. niveus* cultures obtained in SSF. Optimum temperature was determined in assays carried out in a temperature range of 25 – 80ºC, with intervals of 5ºC. Optimum pH was assayed using citrate-phosphate buffer (McIlvaine, 1921) in the pH range 2.5 – 8.0. The reaction was performed at the optimum temperature previously determined. Thermal stability was analyzed after enzyme incubation for 1 hour at different temperatures (25 – 80°C). The assays were performed at the standardized optima temperature and pH established in the previous test. Stability to pH was analyzed incubating the enzymes at 25ºC with citrate-phosphate buffer (McIlvaine, 1921) in different pH (2.5 – 8.0) for 1 hour, after that, the assay was carried out in the optima conditions of temperature and pH previously established. The molarity of reaction buffer was doubled in order to maintain the reaction pH in optimal conditions.The enzymatic assays were performed using a water bath (TE-054, Tecnal, Brazil).

*Experimental design*

Enzymatic activity was determined using a $2^2$ experimental design, using two independent variables in their axial levels (± 1.44), factor (± 1) and central (0). Results were calculated in relative activity using the test number 12 of set 3 (328.3) as a benchmark 100%. Furthermore, analysis of variance were performed (ANOVA) with 95% confidence interval, significance of $p<0.05$. Analyzes and response surfaces were generated using Statistica 7.0 (StatSoft).

*Biobleaching*

The amount of enzyme used for this treatment corresponded to 10 U/g of dried cellulose pulp extracted from *Eucaliptusgrandis*. All these calculations and proceedings were done according to TAPPI methodology (TAPPI, 1996).

In order to determine the enzyme volume that should be used in this treatment to reach 10 U, it was necessary to calculate the pulp consistency to estimate the liquid volume that was present in the cellulose, according to the equation:

$$Pulp\ consistency\ (\%) = \left(\frac{pulp\ dry\ weight}{pulp\ humid\ weight}\right) \times 100$$

Enzyme extract volumes or distilled water (controls were prepared adding distilled water instead of enzyme extract) were added up to 10% pulp consistency, and samples were incubated inside polyethylene bags at 60ºC for one hour. The treatments were performed using the same water bath described above. After that, cellulose pulp was filtrated on Büchner funnel, rinsed with 200 mL of distilled water and used to determine Kappa, viscosity and brightness parameters. Pulp pH was determined and adjusted to 5.0 when necessary. Two strategies were used in these tests: STRATEGY I – cellulose pulp treated with crude extract containing mainly laccase from *A. niveus*; STRATEGY II – cellulose pulp treated with crude extract containing laccase plus xylanases from the same fungus.

*Scanning electron microscopy*

Samples of pulp fibers were processed for scanning electron microscopy (SEM). Fibers were put on circular coverslips and dried overnight at 40ºC using the same incubator described above. After that, the samples were washed thrice in PBS buffer (8.0 g NaCl; 0.2 g KCl; 0.2 g $KH_2PO_4$; 2.16 g $Na_2PO_4$; and $H_2O$ Milli-Q – final volume of 1000 mL), added by 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$, pH 7.0, for2 hours. The materials were gradually dehydrated with 30 – 100% (v/v) ethanol gradient and submitted to drying by the critical point method in camera of carbon dioxide. Critical-point-dried pulp material was cemented to aluminum stubs and sputter-coated with gold using fine coat, BALTEC® SCD 050. Samples were examined at 25KV under SEM (Model JSM 6100, JEOL) at various magnifications.

All results described corresponded to at least three independent experiments.

## Results and Discussion

*Optimization of laccase production*

*A. niveus* was used to produce xylanases in a previous study, aiming the application in paper pulp biobleaching (Peixoto-Nogueira et al. 2009). According to Gutiérrez et al. (2006) other important enzymes that can be used in this kind of process are the ligninolytic enzymes. Therefore, Mn-peroxidase, lignin-peroxidase and laccase produced by *A. niveus* were investigated and only good levels of laccase activity were verified. The use of an enzymatic mix (xylanases plus laccase – current work) could be a strategy to improve the results obtained only with xylanase.

Even though the literature frequently describes basidiomycetes as good laccase (and other ligninases) producers (Levit and Shkrob 1992; Raghukumar et al. 2008), *A. niveus*, a filamentous fungus, could synthesize at least one of these three ligninases, laccase enzyme. In order to accomplish laccase synthesis wheat bran was used in this work. It was also described by other authors as Doniniet al. (2006).

Seeking to find the best conditions to produce laccase, *A. niveus* was grown on solid state fermentation (SSF) using wheat bran as carbon source, humidified with Czapek salt solution (1:2 – w/v) (Wiseman 1975), at 30ºC, with relative humidity around 70%. SSF fermentation was used in this work because in the literature many reports describe SSF as the best condition to produce ligninases (Stajic et al. 2004). Furthermore, SSF is less onerous and agro industrial residues can be used (Stajic et al. 2004; Sandrim et al. 2005; Peixoto-Nogueira et al. 2008).

Aiming to determine the time course for laccase production *A. niveus* was cultivated during different periods, 72 – 144 hours, with intervals of 24 hours each. After 144 hours, laccase activity was evaluated weekly, up to sixth weeks (42 days), during this time a growth curve based on dry mycelium was made (Figure 1). The equation of Boltzmann ($R^2$ = 0.968) was best for fitting the data growth in function of the time of culturing for the synthesis and secretion of ligninases by the filamentous fungus *A. niveus*. According to some authors (Tien et al. 1987) ligninases (such as laccase) production occurs after weeks of growth. When the microorganism starts its secondary metabolism it also initiates the expression of the ligninolytic enzymes. However, this data refers to basidiomycetes fungi and not to filamentous ones. The results presented on Figure 2.A showed that the best enzymatic levels were obtained after 2 weeks. Therefore, long cultivation periods were also necessary for the synthesis and secretion of ligninase by the filamentous fungus *A. niveus*. The analysis of the model of laccase production by *A. niveus* showed that $R^2$ of Boltzmann plots were better than Gaussian plot (Figure 2.B-D). $R^2$ of

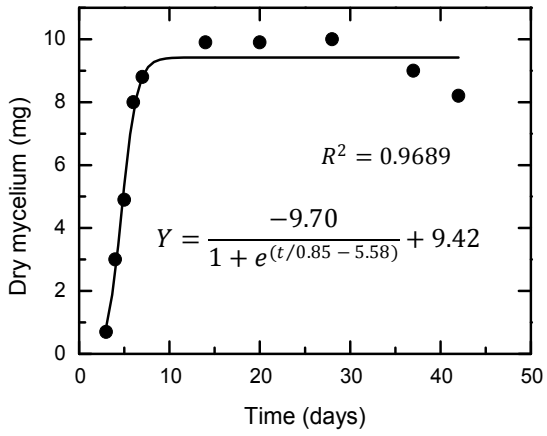


*Figure 1: Boltzmann plot of A. niveus growth on SSF.*

Boltzmann plots in the ascending and descending branches range from 0.9940 to 0.9967, while the $R^2$ of Gaussian plot exhibited a value of 0.8626.Still aiming at improving laccase production, *A. niveus* was grown in different salt solutions to determine the best one. The tested solutions were those present in the media Khanna (Khanna et al. 1995), Vogel (Vogel 1964), SR (Rizzatti et al. 2001) and Czapek (Wiseman 1975). Three other solutions were also tested: 0.05 M $(NH_4)_2SO_4$ – N1; 0.05 M $(NH_4)_2HPO_4$ – N2; and $(NH_4)_2SO_4$ plus $(NH_4)_2HPO_4$, 0.025M each one – N3. The cultivation period was 2 weeks, as the results obtained in the previous experiment (Figure 2.A).

According to the results summarized on Figure 3, cultures humidified with Khanna salt solution presented higher growth rates. That is important to emphasize that wheat bran is an agro industrial residue that presents high protein quantity, which could interfere in protein determination. However, as the protein results from all cultures should be very close because

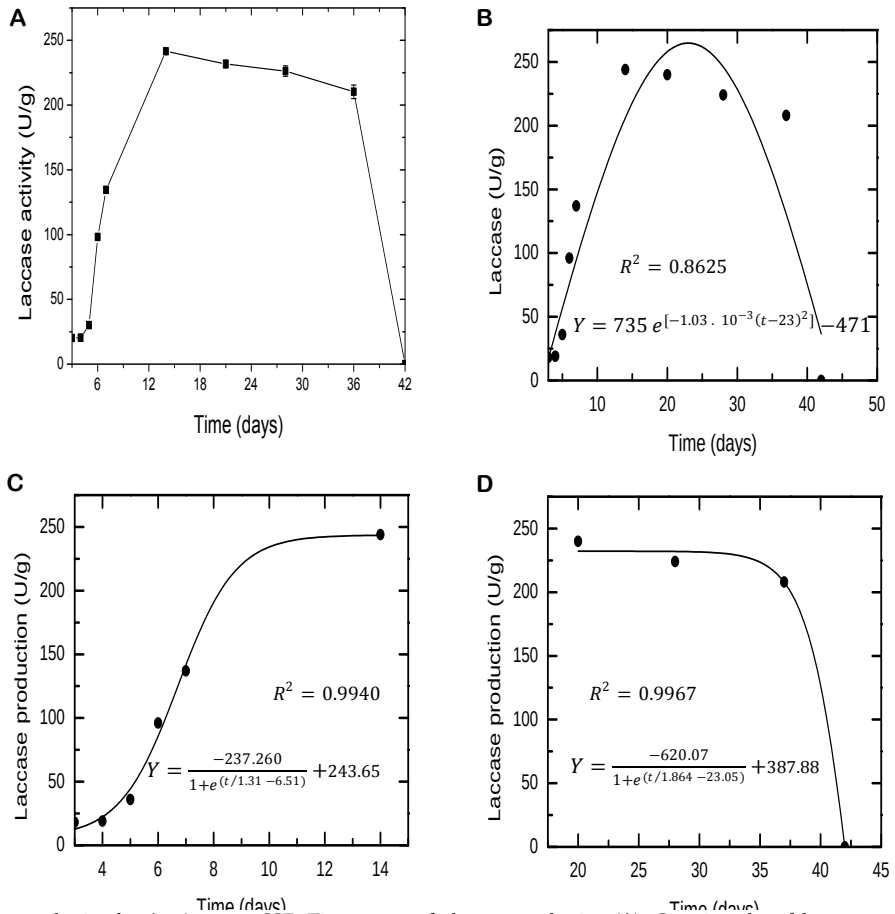


Figure 2: Laccase production by *A. niveus* on SSF. Time-course of laccase production (A); Gaussian plot of laccase production rate (B); Boltzmann plots of laccase production rates of ascending (C) and descending limbs (D). *A. niveus* was cultivated on SSF composed by wheat bran and Czapek salt solution (1:2 – w/v), at 30ºC and relative humidity around 70%. 42 days: activity not detected.

in all media the same quantity of wheat bran and salt solutionswere used, the differences obtained in protein values were attributed to microorganism development.

Laccase production was higher in cultures humidified with N3 or N2 salt solutions, respectively. Probably this higher result occurred because of the presence of inorganic nitrogen, which was promptly assimilated and also because of the presence of ion phosphate in N2 solution, an important cofactor of numerous enzymes.

Therefore, besides time-course, nutritional factors can influence the synthesis and secretion of any enzymes (Ooijkaas et al. 2000). According to the literature, the ligninolytic enzymes synthesis could vary specially according to the quantity and the sort of nitrogen present (inorganic or organic) (Gomes et al. 2009).

Cultures supplemented with wheat bran still have about 2.4% of nitrogen present in its composition since wheat bran is a complex agro industrial by-product rich in many kinds of nutrients, including proteins. Though, laccase production by *A. niveus* improved in the presence of inorganic nitrogen and a combination of $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$ which increased its secretion (Figure 3). The effect of these mixture components was also observed by Gomes et al. (2009).

*Characterization of laccase crude extract produced by A. niveus and the experimental design for their enzymatic activities*

Biotechnological applications of enzymes are successful if the characteristics of theprotein usedwere compatible and resistant to temperature or pH of the process where it will be used. Thus, it was very important to determine the temperature and pH of reaction of the laccase studied, besides its respective stability to temperature and pH.

The optimum temperature for laccase assaycorresponded to 60ºC (Figure 4A), such as the xylanase from the same fungus previously reported (Peixoto-Nogueira et al. 2009). Laccase pH reaction corresponded to 4.5 – 7.0 (Figure 4B). These results are in accordance with literature data that describes laccase from *Coriolopsisbyrsina* and *Lentinus* sp. with a similar reaction temperature (60 – 65°C) (Gomes et al. 2009). The assay temperature of laccase from *A. niveus* was also similar to a commercial product from NOVO NORDISK produced by an *Aspergillus* genetically modified and cultivated on submerged fermentation (Soares et al. 2001).

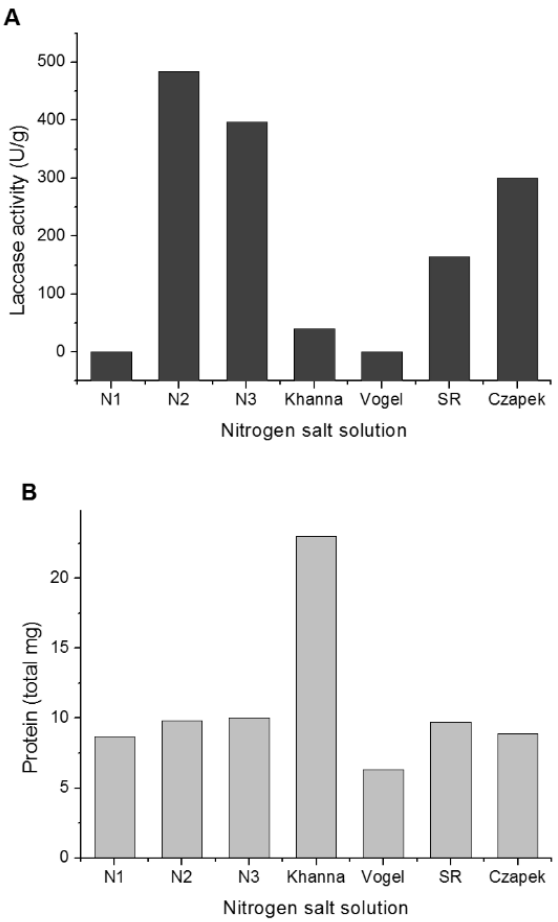


Figure 3: Determination of the best nitrogen salt solution to induce laccase production by *A. niveus*. The fungus was cultivated on SSF composed by wheat bran humidified with different salt solutions (1:2 – w/v), at 30ºC and with relative humidity around 70%. (A) Laccase activity, (B) Protein.

In order to determine laccase thermostability, different aliquots of enzyme were incubated in temperatures from 25 to 80ºC, and the residual activity was measured after 1 hour of incubation. The enzyme presented a good thermostability up to 70ºC, losing 50% of the initial activity at 75 and 70% at 80ºC (Figure 4C) only after 1 hour of exposure. Comparing to a laccase from *Trametestrogii* LK13, 50% of initial activity was lost after only 5 minutes of exposure to 80ºC (Yan et al. 2015).

Laccase stability at different pH values was also analyzed (Figure 4D) and it maintained about 100% of its initial activity at pH from 4.5 to 7.0. These results showed that laccase from *A. niveus* was more stable to chemical or thermal inactivation comparing to others described in literature (Soares et al. 2001; Sorensen et al. 2007). Only manganese peroxidase (another kind of ligninase) from *Phanerochaetechrysosporium* presented this considerable stability to different pH values (Alam et al. 2009).

The influence of the independent variables, temperature and pH, on enzyme activity was also analyzed using an experimental design. The highest enzyme activity levels were obtained at the central points (55°C, pH 5.0) (Table 1). Both independent variables were significant in their quadratic levels and only the temperature had significance in the linear level with the confidence interval of 95% ($p > 0.05$) with $R^2$ of 93.5% (Table 2). According to the F test, F-value was 33.9 times higher than the F-tabulated (Table 3), allowing obtaining an equation model and predictive significant second order polynomial equation as the follow:

$$Relative\ activity = 95 + 10{,}8\ (°C) - 49{,}9\ (°C)^2 + 1{,}2\ (pH) - 16{,}3\ (pH)^2 + 0{,}4\ (°C)(pH)$$

A response surface (Figure 5A) analysis showed high levels of enzyme activity at intervals near the central point for both variables, but the variations of pH conditions in the middle-points of temperature had activities exceeding 50%. Desirability graph (Figure 5B) demonstrates that temperature and pH desirable levels that maximize response (laccase relative activity) are level 0 (central points), for both independent variables. However, pH has lower influence than temperature.

The results presented in this experiment are similar to those described above for pH activity, where the best results were from 4.5 - 7.0, confirming that pH changes do not have much effect on laccase activity. However, analyzing enzyme activity against reaction temperature, it the same was not observed, because when the assay was carried out using an experimental design combining both variables (temperature and pH), different from the previous experiment, performed in batch, which only temperature was varied.

The values of temperature and optimum pH were also interesting because are not so far from those described for the crude extract containing xylanase (65ºC; pH 4.5 – 5.5) produced by *A.niveus*(Peixoto-Nogueira et al. 2009), which favors the application of these enzymes in cellulose pulp biobleaching (STRATEGY I), or affords the utilization of an enzyme *mix* (xylanases plus laccase from *A. niveus* - STRATEGY II). In the second strategy, the xylanases would act in the degradation of the xylan fibers and would leave the lignin polymers exposed to laccase action.

*Assays of cellulose pulp biobleaching using two different strategies and scanning electron microscopy*

The efficiency of bleaching process can be determined by the Kappa number in cellulose pulp clarification for paper manufacturing. Kappa number is empirically determined so, the better the biobleaching process, the lower Kappa number.

In this assay, the quantity of laccase used corresponded to 10 U/g of dry cellulose pulp, for1 hour at the optimum reaction temperature (STRATEGY I). In another treatment a mixture of xylanase from *A. niveus* previously studied was used (Peixoto-Nogueira et al. 2009) – 5 U/g of dry cellulose pulp plus the laccase presented in this work – 5 U/g of dry cellulose pulp) - STRATEGY II.

Comparing to the literature data it was possible to observe that the treatment of cellulose pulp by the xylanase from*A. niveus*,resulted in good efficiency (Kappa efficiency - 39.6%; brightness - 58.1). Good results were alsoobtained using ligninases from *A. niveus* (STRATEGY I – Kappa efficiency 26% and brightness 56.3), but using theenzymemix (STRATEGY II – xylanases plus laccase from *A. niveus*) the results were more interesting: Kappa number diminished 6.5 points comparing to the control, Kappa efficiency was 56% and the brightness improvement corresponded to 17.2 points. The viscosity reduced 1% in STRATEGY I but, there was lower changes in STRATEGY II (Table 3).

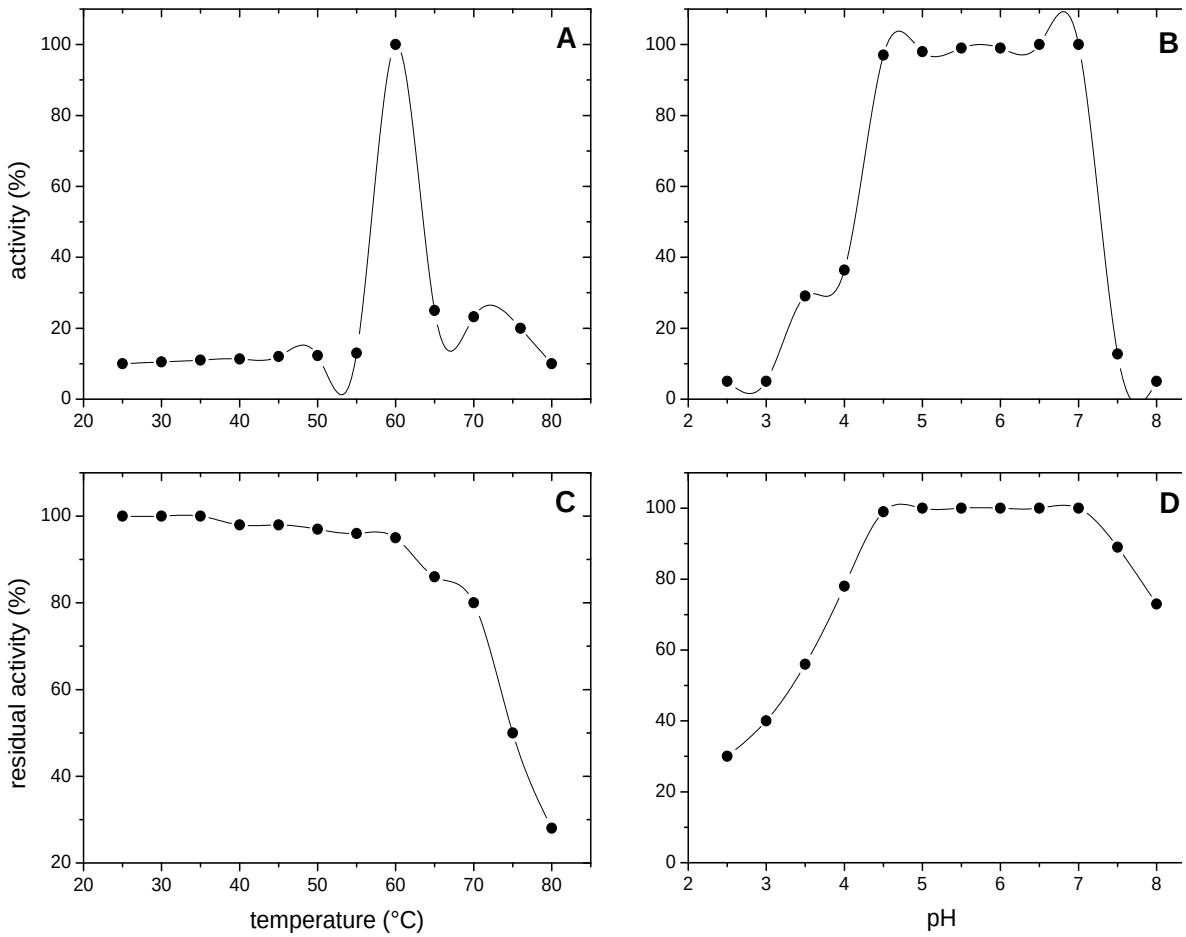


Figure 4: Effect of temperature and pH in the enzymatic activity. Optima of temperature (A) and pH (B); thermal stability (C) and pH stability (D) of extracellular laccase produced by A. niveus. The microorganism was cultivated on its respective standardized conditions and the enzymatic assays were done as described in Material and Methods.

Table 1. Experimental values obtained for the independent variables, temperature (°C) and pH.

| Test | Coded values | | Real values | | Experimental sets[a] |
|---|---|---|---|---|---|
| | Temperature | pH[b] | Temperature (ºC) | pH[b] | Mean of three experiments |
| 1 | 1 | 1 | 66 | 7.1 | 163.3 ± 5.0 |
| 2 | 1 | -1 | 66 | 2.9 | 138.9 ± 8.4 |
| 3 | -1 | 1 | 44 | 7.1 | 18.8 ± 2.2 |
| 4 | -1 | -1 | 44 | 2.9 | 0.2 ± 0.1 |
| 5 | 1.41 | 0 | 70 | 5 | 0.1 ± 0.1 |
| 6 | -1.41 | 0 | 40 | 5 | 0.4 ± 0.1 |
| 7 | 0 | 1.41 | 55 | 8 | 215.0 ± 3.3 |
| 8 | 0 | -1.41 | 55 | 2 | 223.9 ± 3.8 |
| 9 | 0 | 0 | 55 | 5 | 336.7 ± 20.1 |
| 10 | 0 | 0 | 55 | 5 | 290.0 ± 11.7 |
| 11 | 0 | 0 | 55 | 5 | 310.0 ± 7.5 |
| 12 | 0 | 0 | 55 | 5 | 326.7 ± 14.9 |

[a]Activity values presented on this table are in total U, but the analysis were done using relative activity (%). [b]pH (McIlvaine buffer 2.0 – 8.0).

The viscosity maintenance confirmed that the crude extract was free of harmful levels of cellulases. Besides, no activity was observed in the determination of cellulase by Miller (1959) (data not shown), although laccase production has been performed in wheat bran, which is a complex agro industrial by-product that presents in its composition xylan, lignin and cellulose.

Table 2.ANOVA for relative activity (%).

| Source of variation | Sum of squares | Degrees of freedom | Mean square | $F_{Calculated}$ | $F_{Tabulated}$ |
|---|---|---|---|---|---|
| Regression | 51090 | 5 | 10218 | 85.8 | 2.53 |
| Leavings | 3573 | 30 | 119.1 | | |
| Total | 54663 | | | | |

$R^2$ = 0.935 (93,5%)

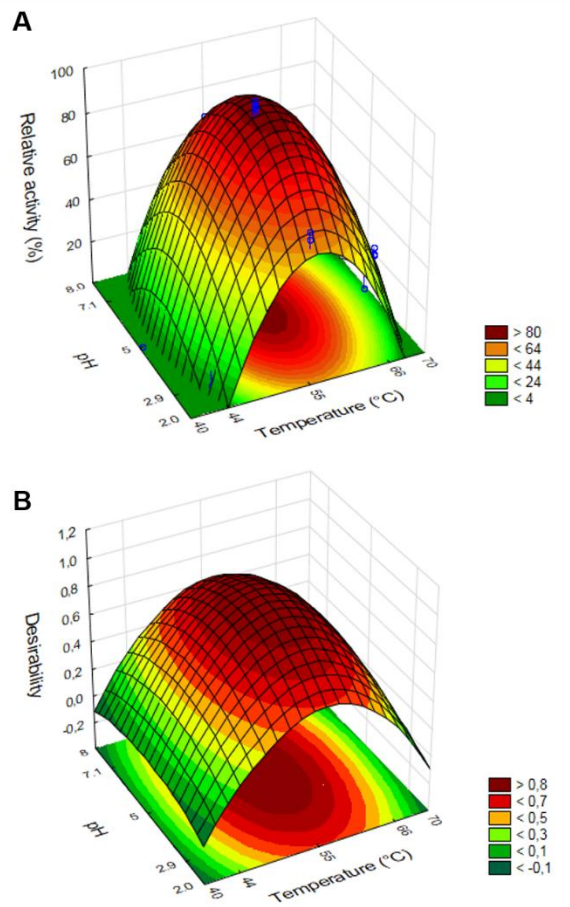


Figure 5: Laccase surface response. (A) Model of response surface showing interactive effects of temperature and pH on enzymatic activity of laccase from A. niveus; (B) Desirability surface.

In the literature there are other studies showing that the use of wheat bran for *A. niveus* and other *Aspergilli* cultivation, not necessarily will induce cellulase production (Buswell et al. 1995).

The cellulose pulp used in biobleaching experiments was submitted to analysis of scanning electron microscopy (SEM). Cellulose pulp was analyzed before (control) and after the treatment with xylanase from *A. niveus*; only in the presence of laccase from *A. niveus* (STRATEGY I) and with an enzymatic mix of enzymes (xylanases plus laccase) from the same fungus (STRATEGY II). Figure 6A (cellulose pulp control) shows that the fiber remained intact, with integral structure, while after the enzymatic treatments a dissociation of the fibers occurred exposing the internal polysaccharides (Figures 6B, C and D). It was visible that the quantity of carbohydrates extracted from the cellulose fibers was higher and more efficient with the mix of enzymes. That can be confirmed by comparing the results of the SEM analysis (Figure 6 A - D) and biobleaching values presented in Table 3. Similar results were described by Salles et al. (2005), Kapoor et al. (2007) and Medeiros et al. (2007a, b), which also used SEM to observe and compare the differences in cellulose pulp biobleaching treatments. Then, the present study showed that improvement was still possible by using a mix of xylanases and laccase from *A. niveus*, such as Woldesenbet et al. (2013) described for an alkalophilic bacterial xylanase, mannanase and laccase-mediator system.

*Table 3. Bleaching of cellulose pulp by laccase or a mix of laccase and xylanases produced by A. niveus.*

| | *Strategy I* | | *Strategy II* | |
|---|---|---|---|---|
| | *Control* | *Treated* | *Control* | *Treated* |
| *Kappa number* | *11.5* | *8.5* | *11.5* | *5.0* |
| *Kappa efficiency (%)* | - | *26.0* | - | *56.0* |
| *Viscosity ($cm^3/g$)* | *896* | *886* | *890* | *889* |
| *Brightness (ISO %)* | *54.2* | *56.3* | *54.2* | *71.4* |

Microorganism cultivated in optimized conditions. Controls corresponded to the not treated samples. The treatments were done with extracellular enzymes (10 U/g dry cellulose pulp), for1h at 60ºC in both strategies. Xylanases were obtained from *A. niveus* crude extract cultivated in liquid medium Peixoto-Nogueira et al. (2009). Laccases were obtained on SSF as optimized in this study.
STRATEGY I – laccase – 10 U/g dry cellulose pulp. STRATEGY II – enzyme mix: xylanase 5 U/g dry cellulose pulp plus laccase 5 U/g dry cellulose pulp).

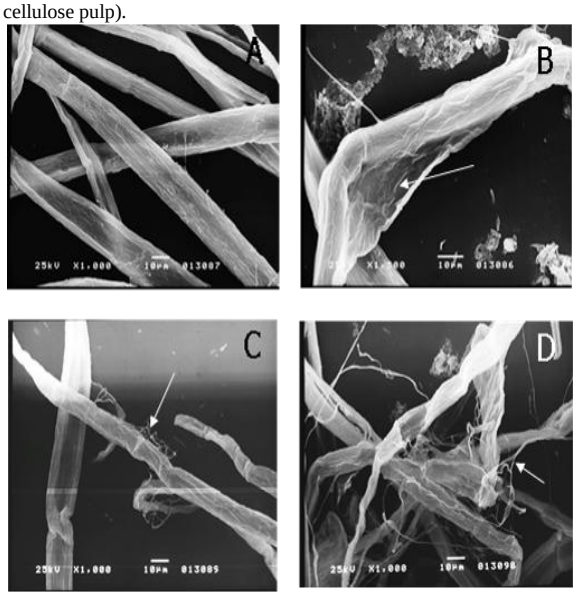


Figure 6: Scanning electron microscopy of Eucalyptus oxygen-bleached pulp: untreated pulp (control) (A); pulp treated with crude xylanase extract from *A. niveus* (B); pulp treated with crude laccase extract from *A. niveus* (C) and pulp treated with enzymatic mix of xylanase plus laccase from *A. niveus* crude extracts (D). Enzyme quantity was 10 U/g of dry cellulose pulp.

## Conclusions

The filamentous fungus*A. niveus* was described as a good laccase producer, which corresponded to an important data, because most of ligninases (including laccase) producers generally described in the literature are basidiomycetes. Laccase from *A. niveus* provided promising characteristics to be industrially applied in cellulose pulp biobleaching and

therefore should receive more attention in order to better understand its synthesis system and regulation and enlarge the number of enzymes available in the market to be used in paper industries.

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