

## Original Research Article

# Potential of the *Aspergillus labruscus* ITAL 22.223 as a producer of cellulolytic enzymes and xylanase under solid-state fermentation

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## ABSTRACT

**Background:** The enzymatic hydrolysis of the lignocellulosic biomass to obtain saccharides that can be used for the production of bioethanol is an important field in the renewable energy area. For this purpose, fungal cellulases and xylanases can be applied.

**Methods:** *Aspergillus labruscus* ITAL 22.223 was cultured under SSF with agroindustrial residues and by-products as substrates, humidified with different moistening agents, at different proportions (1:0.5, 1:1, 1:1.5 and 1:2; m/v), for different periods (24-216 h) at 25°C. The extract obtained was used for determination of the cellulase and xylanase activities. The influence of temperature, pH and different compounds on xylanase activity was analyzed.

**Results:** *A. labruscus* produced cellulases and xylanase under solid-state fermentation (SSF) using agroindustrial by products and residues as carbon source/substrates. The best production of  $\beta$ -glucosidase (6.3 U/g of substrate) was obtained in the presence of rye bran, whereas for the CMCase it was in the presence of crushed soybean (5.1 U/g of substrate) and xylanase using oat bran (74.8 U/g of substrate) as substrates, for 168 h of cultivation at 25°C. Considering the high xylanase production, the best moistening agent and its proportion (tap water, 1:2 m/v) were determined. Optimum of temperature and pH for xylanase activity was determined as 55°C and pH 5.5. The xylanase activity was inhibited by different salts, with exception of  $\text{MnSO}_4$ . It was also inhibited by organic solvents, detergents, EDTA, urea and  $\beta$ -mercaptoethanol.

**Conclusions:** The fungus *A. labruscus* presented potential to produce enzymes from the cellulolytic complex and xylanase using low cost substrates.

**Keywords:** *Aspergillus*, Cellulase, Solid-state fermentation, Xylanase

## INTRODUCTION

The agroindustrial activities generate tons of lignocellulosic residues all over the world. The lignocellulosic biomass is composed of three polymeric fractions: the cellulose, the hemicellulose and the lignin.<sup>1,2</sup> The use of the lignocellulosic biomass to obtain saccharides that can be used for the production of bioethanol, throughout the fermentation process, is a straight focus in the renewable energy area. For this purpose, the cellulose should be hydrolyzed by an enzymatic complex constituted by different enzymes as

endo- $\beta$ (1-4) glucanase (EC 3.2.1.4), exo-  $\beta$ (1-4) glucanase or celobiohydrolase (EC 3.2.1.91) and celobiase or  $\beta$ (1-4) glucosidase (EC 3.2.1.21).<sup>3</sup> However, the other polymeric constituents, i.e. hemicellulose and lignin, hinder the access of these enzymes to the cellulose molecule. This problem can be reduced with the treatment of lignocellulosic biomass featured as chemical or physical pre-treatment, and through enzymatic hydrolysis. Different enzymes can be involved in the hydrolysis of hemicellulose according to the origin and structure of this polymer, such as exo-hydrolases, endo-hydrolases and enzymes that act on the linkages in the

lateral chains. Xylan is an important type of hemicellulose that can be hydrolyzed by xylanase.<sup>4</sup> Lignin, a recalcitrant compound found in lignocellulosic biomass, is hydrolyzed by different enzymes, such as laccase (EC 1.10.3.2), lignin peroxidase (EC 1.11.1.14) and manganese peroxidase (EC 1.11.1.13).<sup>5</sup>

The necessary enzymes to completely hydrolyze the lignocellulosic biomass can be produced by microorganisms, with prominence of the filamentous fungi, which are able to produce and secrete enzymes able to act on cellulose, hemicellulose and lignin. According to Aguiar and Lucena, fungi from the genera *Aspergillus* and *Trichoderma* deserve some attention due to their being the main producers of cellulolytic and hemicellulolytic enzymes.<sup>6</sup> Solid-state fermentation (SSF) is a good option to produce enzymes for both cellulolytic and hemicellulolytic complexes, offering conditions very close to the natural environments, reduction of the microbial contamination, high metabolite recovery and use of lignocellulosic biomass from agro-industrial residues as substrates. According to that, the search for new fungal sources of these enzymes is attractive since new species have been described each year, such as *Aspergillus labruscus* isolated from *Vitis labruscas* L.<sup>7</sup> Until this moment, the enzymatic potential of this species was not described and considering this aspect, our aim was to investigate the *A. labruscus* potential to produce enzymes from the cellulolytic complex and xylanase using agro-industrial byproducts/residues as substrates under SSF.

## METHODS

### Microorganism and culture conditions

The filamentous fungus *Aspergillus labruscus* ITAL 22.223 was kindly provided by the Institute of Food Technology, Campinas, São Paulo Brazil. The fungus was maintained on PDA slants at 4°C and new cultures were obtained periodically. The agro-industrial byproducts/residues (oat bran, oat meal, cassava peels, rice bran, crushed soybean) were kept on 125 mL Erlenmeyers flasks and humidified with different moistening agents (tap water, distilled water, Vogel salt solution, Khanna salt solution and SR salt solution at different proportions (1:0.5, 1:1, 1:1.5 and 1:2; m/v).<sup>8-10</sup> The culture media were autoclaved at 120° C, 1.5 atm for 30 min. Thereafter, the media were inoculated with 1 mL of spore suspension (10<sup>6</sup> spores/mL).

### Obtainment of the enzyme extracts

After cultivation, the cultures were added with 50 mL distilled water at 4°C and submitted to agitation at 200 rpm for 30 min at 4°C. Thereafter, the erlenmeyer content was harvested using filter paper Whatman no. 1 and gauze with a vacuum pump. The free cell filtrate was used for the determination of enzyme activities and for protein quantitation.

### Determination of enzyme activities and protein quantitation

The enzymatic activities were determined as follow: i) The FPase activity was determined as described by Ghose using Whatman no.1 filter paper as substrate.<sup>11</sup> ii) The enzyme activities of CMCase and xylanase were determined according to Miller using DNS.<sup>12</sup> For CMCase, carboxymethylcellulose (CMC) was used as substrate in sodium acetate buffer 50 mM, pH 4. For xylanase activity, 1% (m/v) "birchwood" xylan (Sigma®) was used as substrate in sodium acetate buffer 50 mM pH 5.5. One unit of enzyme activity was defined as the amount of enzyme necessary to produce 1 µmol of reducing sugars per min under the assay conditions. iii) The β-glucosidase activity was determined according to Coston and Loomis using pNPβG as substrate in sodium acetate buffer 50 mM, pH 5.5.<sup>13</sup> One unit of enzyme activity was defined as the amount of enzyme necessary to produce 1 µmol of *p*-nitrophenolate per min under the assay conditions. The reactions were performed at 50° C.

Protein quantitation was carried out by means of the Bradford methodology using bovine serum albumin (BSA) as standard and expressed as mg of protein per mL of sample.<sup>14</sup>

### Influence of temperature and pH on xylanase activity

The xylanase activity was determined under different temperatures (40-70°C) and pH values (3-8). For the determination of influence of pH, it was used 50 mmol L<sup>-1</sup> citric acid buffer (pH 3-4.5), 50 mmol L<sup>-1</sup> sodium acetate buffer (pH 5-6) and 50 mmol L<sup>-1</sup> Tris-HCl buffer (pH 6.5-8.0).

### Influence of different compounds on xylanase activity

The influence of different salts (1 mmol L<sup>-1</sup>: AgNO<sub>3</sub>, BaCl<sub>2</sub>, CaCl<sub>2</sub>, CoCl<sub>2</sub>, CuCl<sub>2</sub>, Fe<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>, FeCl<sub>3</sub>, MgCl<sub>2</sub>, MgSO<sub>4</sub>, MnCl<sub>2</sub>, MnSO<sub>4</sub>, NaCl, NH<sub>4</sub>Cl and ZnSO<sub>4</sub>), detergents (1 mmol L<sup>-1</sup> SDS, 0.01% Tween-20), organic solvents (1% v/v: acetone, acetonitrile, butanol, ethanol, isopropanol and methanol), 1% (v/v) EDTA, 1 mmol L<sup>-1</sup> urea and 1 mmol L<sup>-1</sup> β-mercaptoethanol, on xylanase activity was analyzed.

### Reproducibility

All experiments were done in triplicate and the results were expressed as the media values±standard error.

## RESULTS

### Production of cellulolytic enzymes and xylanase by *A. labruscus*

The fungus *A. labruscus* was able to grow on all agroindustrial byproducts/residues used as substrate in SSF producing enzymes from the cellulolytic complex

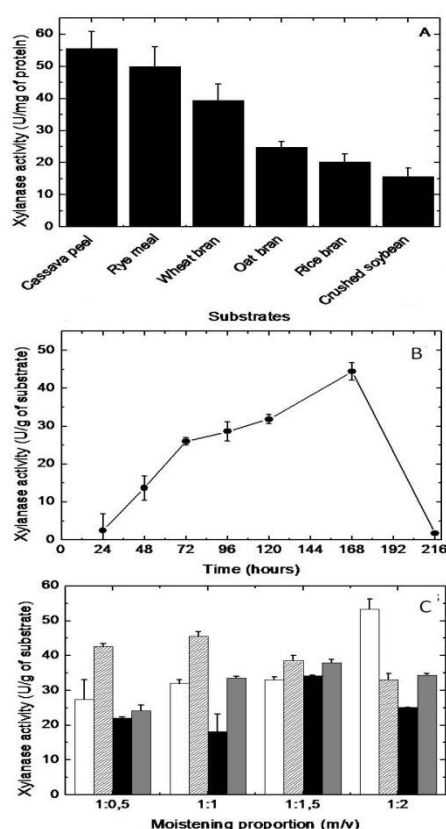
and xylanase (Table 1). The FPase production was extremely reduced for the SSF conditions analyzed. On the other hand, the best production of CMCase was obtained in the presence of crushed soybean (5.1 U/g of substrate), 16-fold higher than that obtained in the presence of cassava peel. The best production of  $\beta$ -glucosidase was observed when oat bran was used as substrate (6.35 U/g of substrate) and the minor

production (0.21 U/g of substrate) in the presence of rice bran. The highest production of xylanase was achieved using wheat bran as substrate (74.83 U/g of substrate), followed by rye meal (44.23 U/g of substrate). The xylanase productions using crushed soybean and oat bran were similar. Among all substrates used, rice bran was that of the least importance for the production of cellulolytic enzymes and xylanase.

**Table 1: Production of cellulolytic and hemicellulolytic enzymes by *Aspergillus labruscus* ITAL 22.223 under solid-state fermentation using agroindustrial byproducts/residues.**

Substrates	Enzyme activities (U/ g of substrate)			
	FPase	CMCase	$\beta$ -glucosidase	Xylanase
Oat bran	0.01*	3.51 $\pm$ 0.24	6.35 $\pm$ 0.32	35.44 $\pm$ 1.41
Wheat bran	0.03 $\pm$ 0.01	2.95 $\pm$ 0.08	4.41 $\pm$ 0.01	74.83 $\pm$ 5.37
Crushed soybean	0.01*	5.1 $\pm$ 0.29	4.33 $\pm$ 0.21	37.44 $\pm$ 6.12
Cassava peels	0.02 $\pm$ 0.01	0.33 $\pm$ 0.2	0.58 $\pm$ 0.02	12.38 $\pm$ 1.96
Rye meal	0.02*	2.42 $\pm$ 0.44	2.11 $\pm$ 0.08	44.23 $\pm$ 5.16
Rice bran	0*	0.44 $\pm$ 0.2	0.21 $\pm$ 0.08	6.35 $\pm$ 1.06

The cultures were maintained for 168 h at 25° C. Thereafter, the cultures were added with 50 mL of cold distilled water, shaken at 200 rpm for 30 min at 4°C and filtered throughout Whatman no.1 filter paper to obtain the enzyme filtrate.



**Figure 1: (A) Influence of the agroindustrial byproducts/residues used as substrate; (B) cultivation period; (C) moistening proportion for tap water (□), distilled water (▨), Khanna salt solution (■), and SR salt solution (▩) on the production of the xylanase by *A. labruscus* ITAL 22.223 cultured under SSF at 25° C.**

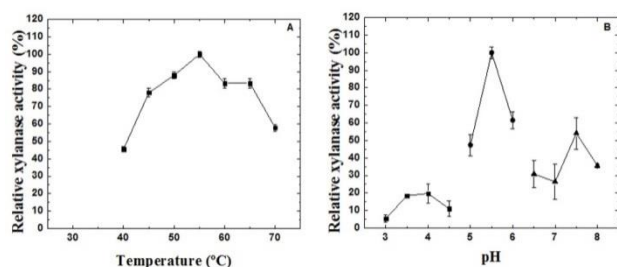
According to the Figure 1A, the highest specific activity for xylanase was observed when cassava peels was used as substrate, followed by the value obtained using rye meal as substrate. However, the enzyme production was better in the presence of wheat bran, as mentioned above, and this substrate was maintained for the xylanase production by *A. labruscus*.

#### **Influence of the cultivation period and moistening agents on xylanase production**

The fungal development and the enzyme production are affected directly by the period of cultivation. According to this, it was observed that the highest xylanase production (46 U/g of substrate) was obtained when the fungus was cultured for 168 h, period used for the analysis of the influence of different moistening agents on enzyme production. After 168 h, the presence of xylanase in the extracellular filtrate was drastically reduced (Figure 1B). High xylanase production (53.3 U/g of substrate) was achieved using tap water as moistening at 1:2 (m/v) (Figure 1C). When the Vogel salt solution was used, the xylanase production by *A. labruscus* was not detected for all moistening proportion used. On the other hand, the enzyme production was also observed using distilled water, Kanna salt solution and SR salt solution.

#### **Influence of temperature and pH on xylanase activity**

Optimal of temperature and pH for xylanase activity were achieved at 55°C and 5.5, respectively (Figure 2A and B). At 70 °C, around 55% of the xylanolytic activity was maintained.



**Figure 2: (A) Influence of the temperature (40-70°C) and (B) pH on the xylanolytic activity. Influence of pH was determined at pH values from 3 to 8, using citric acid buffer 50 mM (pH 3-4.5), sodium acetate buffer 50 mM (pH 5-6) and Tris-HCl buffer (pH 6.5-8).**

**Table 2: Influence of different salts on xylanase activity.**

Salts (1 mmol L <sup>-1</sup> )	Relative xylanase activity (%)
AgNO <sub>3</sub>	69.96±4.93
BaCl <sub>2</sub>	36.15±5.22
CaCl <sub>2</sub>	47.60±2.32
CoCl <sub>2</sub>	72.70±5.62
CuCl <sub>2</sub>	49.79±3.10
CuSO <sub>4</sub>	60.77±116
Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub>	53.64±2.32
FeCl <sub>3</sub>	6.04±2.52
MgCl <sub>2</sub>	36.63±0.58
MgSO <sub>4</sub>	35.12±1.35
MnCl <sub>2</sub>	82.72±1.87
MnSO <sub>4</sub>	105.83±7.55
NaCl	58.98±3.47
NH <sub>4</sub> Cl	68.72±5.71
ZnSO <sub>4</sub>	55.69±6.20
Without	100

100% relative activity corresponds to 72.9 U/g of substrate.

**Table 3: Influence of different compounds on xylanase activity.**

Compounds	Relative xylanase activity (%)
<b>Detergents</b>	
SDS (1 mmol L <sup>-1</sup> )	24.01±2.32
Tween-20 (0.01%)	49.04±9.40
<b>Organic solvents</b>	
Acetone	37.11±8.43
Acetonitrile	33.13±4.94
Buthanol	24.42±5.39
Ethanol	44.10±9.79
Isopropanol	42.94±3.38
Methanol	51.71±3.19
<b>Others</b>	
EDTA (1%)	34.09±1.64
Urea (1 mmol L <sup>-1</sup> )	35.60±3.28
β-mercaptoethanol	41.36±4.17
Without	100

100% relative activity corresponds to 72.9 U/g of substrate.

## Influence of different compounds on xylanase activity

The xylanase activity was inhibited by all salts used, except MnSO<sub>4</sub> (Table 2). The highest inhibition was promoted by the use of FeCl<sub>3</sub> (- 94%), followed by the use of MgCl<sub>2</sub> and MgSO<sub>4</sub> (-65%). The enzyme activity was also inhibited by detergents (SDS and Tween 20), organic solvents (-29-76%), EDTA (-66%), urea (-65%) and β-mercaptoethanol (-59%) (Table 3).

## DISCUSSION

### Production of cellulytic enzymes and xylanase by *A. labruscus*

Studies on cellulosic biomass degradation is an increasing subject around the world and many works have been done considering the production of cellulases and hemicellulases by microorganisms as filamentous fungi. According to this, the use of SSF is an interesting alternative to produce these enzymes. The SSF allows the use of lignocellulosic biomass from different origins, including agro industrial residues. Some advantages of SSF are the similarity with the natural conditions found by the microorganisms, reduced water activity, minimizing the bacterial contamination, reduction of the reagent and energy consume and increased productivity.<sup>15,16</sup> In this context, both cellulases and xylanase were produced by *A. labruscus* using different agro industrial residues and by-products as substrates for SSF. However, the most pronounced production was obtained for xylanase, with eminence of wheat bran, what can be explained by its composition. In general, wheat bran is a heterogeneous mixture of compounds such as cellulose, hemicellulose, lignin and complex xylans.<sup>17</sup> The presence of hemicellulose and complex xylan in the medium can stimulate the xylanase production by the fungus. The production of xylanases using wheat bran as carbon source has also been reported for other fungi such as *Aspergillus niger*, *Aspergillus oryzae* and *Aspergillus fumigatus*, but they were minor if compared to the xylanase level obtained throughout *A. labruscus* cultivation.<sup>18-20</sup>

Considering that the best enzymatic production was obtained for xylanase in the presence of all substrates used, the focus was fixed on this enzyme. The use of wheat bran for the xylanase production by different fungal species has also been reported, such as the enzymes produced by *Aspergillus niveus*, *Aspergillus versicolor* and *Aspergillus niger*.<sup>21-23</sup>

Different factors can influence the fungal development and, consequently, the enzyme production under SSF. Among these factors, the humidity can be highlighted. Elevated humidity level can difficult the oxygen diffusion while a reduced humidity level can difficult the solubility of nutrients.<sup>16</sup> Xylanase production was possible using all moistening agents, with exception of Vogel salt solution, because the microorganism was not able to develop on



the substrate under this condition. Differing from the other salt solutions used, Vogel salt solution presents chloroform (0.05%),  $\text{CaCl}_2$  (0.05%) and sodium citrate (1.5%) in its composition. It is likely that one or more of these compounds had affected the fungal development negatively. On the other hand, tap water presents some salts at a lower concentration that can influence the fungal development positively, facilitating the xylanase production and secretion to the medium. For Khanna and SR salt solutions, in all moistening proportions, the xylanase production was lower than that observed for the maximal production using tap water.

### ***Influence of temperature and pH on xylanase activity***

The xylanases produced by the fungi *Aspergillus versicolor* and *Aspergillus niger* also presented optimal activity at 55°C.<sup>22-23</sup> Similarly, Betini et al reported xylanase produced by *Aspergillus niveus* with optimal activity at 55-65°C.<sup>21</sup> Considering the influence of pH on xylanase, a second peak of activity was noted at pH 7.5, suggesting the presence of xylanase isoform. The ability to produce xylanase isoforms is not an exclusive characteristic for *Aspergillus labruscus*, but also for others, such as *Aspergillus niger*.<sup>24</sup> The xylanases produced by *Aspergillus caespitosus* and *Aspergillus niveus* also presented optimum of activity at pH 5.5.

### ***Influence of different compounds on xylanase activity***

The xylanase activity from *A. labruscus* was drastically affected by  $\text{FeCl}_3$  as also reported for the enzyme produced by *Aureobasidium pullulans* that had its activity reduced to 30%.<sup>25</sup> Others ions also inhibited the xylanase activity, such as  $\text{MgCl}_2$ . On the other hand this same salt was responsible to increase the xylanase activity from *A. pullulans* and *Aspergillus nidulans*.<sup>25,26</sup> The use of  $\text{CuSO}_4$  also reduced the xylanase activity from *A. labruscus* as also reported for *A. nidulans* xylanase.<sup>26</sup> Ions can interact with amino acids in the molecule surface or with amino acids in the catalytic site, modulating positively or negatively the enzyme activity. This modulation depends on the type of ion and also the enzyme source.

The presence of SDS in the reaction promoted inhibition of 75% of the xylanase activity from *A. labruscus*. Similar results were also reported for the enzymes produced by *A. pullulans* and *A. nidulans*.<sup>25,26</sup> The SDS interacts with the hydrophobic regions of the enzyme molecule changing its tridimensional structure and promoting denaturation. The Tween-20 also can cause enzyme denaturation reducing the enzymatic activity as observed for *A. labruscus* xylanase.

The inhibition of the *A. labruscus* xylanase activity by  $\beta$ -mercaptoethanol, as also reported for the enzyme from *Aspergillus niger*, can be explained by its action on disulfide bonds in the molecule, compromising the enzyme structure.<sup>27</sup> On the other hand, xylanase

stimulation by  $\beta$ -mercaptoethanol was observed for *Aspergillus caespitosus* enzyme.<sup>28</sup>

Differing from our data, the presence of organic solvents in the reaction medium, stimulated the *A. niger* xylanase.<sup>29</sup> According to Do et al, organic solvents can facilitate the substrate solubilization, facilitating the enzymatic hydrolysis.<sup>27</sup> In addition, these solvents can act on the hydration coat of the enzyme, reducing its activity.

In conclusion, this is the first description on the enzymatic potential of the new filamentous fungus *Aspergillus labruscus* ITAL 22.223 aiming the production of cellulolytic enzymes and xylanase. The production of xylanase was influenced by the substrate, moistening agent and period of fermentation. This enzyme has biotechnological potential to be used in the saccharification of hemicellulose from biomass to obtain monosaccharides, which can be used for bioethanol production.

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