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Unveiling Xylanolytic Enzymes Production of *Talaromyces wortmannii* DR49 on Industrial Agro Wastes

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HIGHLIGHTS

- Process development to produce xylanolytic enzymes by *Talaromyces wortmannii*.
- First report *of T. wortmannii* cultivation in bioreactors using industrial agro wastes.
- The best condition for xylanolytic enzymes production were 29 °C and pH 6.0.

Abstract: Xylan degradation is an important step in different industries, such as in biorefinery for biomass hydrolysis. *Talaromyces wortmannii* is a known fungus due to second metabolite production but only few works showed the xylanolytic potential of this fungus. In this way, the aim of this study was to evaluate the production of xylanolytic enzymes from *T. wortmannii* DR49 on industrial agro wastes. Cultivation in shake flask showed highest xylanase titration (10.3 U/mL; 9.5 U/mL) for wheat bran (WB) and hydrothermal pretreated sugar cane bagasse (HB); in β-xylosidase production WB and xylose were the best carbon sources (0.57 U/mL; 0.34 U/mL) respectively. STR cultivation revealed that 29°C and pH 6.0 were the best conditions for xylanase (14.5 U/mL) and β-xylosidase (1.7 U/mL) production. *T. wortmannii* DR49 showed to be a potential candidate for xylanolytic enzymes production using agro wastes in bioreactors, which has never been previously reported in this fungus.

Keywords: *Talaromyces wortmannii*; xylanase; β-xylosidase; agro wastes; sugar cane bagasse.

INTRODUCTION

Xylan degradation can be an important step in different industry products, such as breadmaking and in biorefinery [1]. Several studies showed that enzymatic plant biomass deconstruction can be improved by the enzymatic hemicellulose removal that lead for higher cellulose hydrolysis yields [2-4]. Xylan degradation is mainly performed by endo 1,4-β-xylanases (EC 3.2.1.8) that cleaves glycosidic bonds in the xylan backbone and β-xylosidase (EC 3.2.1.37) that acts in the hydrolysis of xylobiose and larger xylooligosaccharides release [1]. It is important to highlight that xylanolytic enzymes can also be applied to xylan recovery from lignocellulosic biomass and contribute to biomass valorization. One high value-added that can be obtained is xylooligosaccharides (XOS), these oligomers are considered non-digestible carbohydrates that present prebiotic effect [5].

Lignocellulosic biomass substrates have been extensively applied in glycohydrolases production by filamentous fungi with the objective of media cost reduction. Pretreated sugar cane bagasse, wheat straw, cotton seed hulls, soybean hulls had been used in media formulation to xylanolytic enzymes production by several ascomycetes species [2, 6, 7].

Talaromyces genera contains species with medical, industrial and agriculture importance. It was firstly describe as a sexual state of *Penicillium*, and further was redefined by polyphasic taxonomy approach [8, 9]. Some species present biotechnological potential due to enzyme production, such as *Talaromyces cellulolyticus* for cellulase [10] and *Talaromyces emersonii* for xylanase [11]. Furthermore, *Talaromyces* spp can produce xylanolytic enzymes with desires biochemical characteristics. Wang and coauthors [12] identified in *Talaromyces leycettanus* JCM 12802 highly thermostable xylanase able to hydrolyze wheat straw. Nieto-Domínguez and coauthors [13] produced and purified a pH-stable β-xylosidase from *Talaromyces amestolkiae* with regioselective transxylosylation activity.

Talaromyces wortmannii belongs to section Islandici which is a group easily recognized by its slow or restricted growth and conspicuous yellow aerial mycelium [14]. In one hand *T. wortmannii* is a known fungal species due to the production of second metabolites with biological activities [15]. Some examples are wortmannilactones, that presented cytotoxic activity against some human cancer cell lines [16]; skyrin and rugulosin A, that presented antibiotic activity against some Gram positive pathogenic bacteria [17]. In the other hand, few works showed glycohydrolase production by this fungus. The first report of xylanolytic enzymes production by *T. wortmannii* was made by Lee and coauthors [18], which screened xylanolytic *Penicillium* spp isolated from woods materials. Further Robl and coauthors [19] showed that a strain isolated from spoiled books, *T. wortmannii* DR49, produces a wide profile of several hemicellulases capable to hydrolases plant cell wall and that its enzymatic production is mainly related to media carbon source.

It is important to highlight that xylanolytic enzymes of section Islandici species is unexplored and that *T. wortmannii* glicohydrolases may present biochemical and catalytic characteristics potential to industrial application. Antonopoulou and coauthors [20] produced by heterologous expression and tested three feruloyl esterases from *T. wortmannii* with ability for the transesterification and syntheses of feruloyl derivatives. In this way, this study aimed to evaluated xylanolytic enzymes production by *T. wortmannii* DR49 on plant biomass materials and study the influence of temperature and pH parameters in bioreactor fungus cultivation.

MATERIAL AND METHODS

Fungal strain

T. wortmannii DR49 is an ascomycete isolated from spoiled books and screened as a potential xylanolytic producer [19]. The strain was kindly given by the fungal collection of the Laboratório de Microbiologia e Biologia Molecular of Universidade Federal do Paraná (LabMicro/UFPR). The microorganism was stored in cryotubes with glycerol 20% at -80 °C.

Lignocellulose materials

It was tested five industrial agro waste rich in cellulose and hemicellulose: Hydrothermal pre-treatment of sugarcane bagasse (HB), steam-exploded sugar cane bagasse (EB), steam-exploded delignified sugar cane bagasse (DEB), liquor, soybean bran (SB) and wheat bran (WB). EB and DEB were prepared and characterized by Rocha and coauthors [21] and dos Santos Costa and coauthors [7] and sugar cane bagasse was provided from Rosário Mill (Orlândia, Brazil). Hydrothermal pre-treatment of sugarcane bagasse was

performed previously by Robl and coauthors [22] to produce the solid (HB) and liquid fractions (Liquor) used in this study. Both materials had chemical composition determined also by Robl and coauthors [22]. Other lignocellulose materials such as SB and WB were provided from Agricola (São Carlos, Brazil) [23]. The material composition is presented at tables S1 and S2 (Supplementary material).

Culture media

The culture medium [24], originally applied to cellulase production, was adapted according to Robl and coauthors [19] and used in this study: 1 mL Tween 80; 0.3 g/ L urea; 2.0 g/L KH_2PO_4; 1.4 g/L $(NH_4)_2SO_4$; 0.4 g/L $CaCl_2.2H_2O$; 0.3 g/L $MgSO_4.7H_2O$; 1.0 g/L proteose peptone; 5.0 mg/L $FeSO_4.7H2O$; 1.6 mg/L $MnSO_4.4H_2O$; 1.4 mg/L $ZnSO_4.7H_2O$; 2.0 mg/L $CoCl_2.6H_2O$; 10 g/L glucose (carbon source). The medium pH was settled to 5.0 and then sterilized (121 °C for 20 min). All the culture medium used present identical composition, with exception of carbon sources, which varied among assays.

Shake flask cultures

Fungal conidia were harvested by the addition of sterilized Tween 80 (0.01% v/v) on *T. wortmannii* colonies (7^{th} day growth on potato dextrose agar, PDA). Erlenmeyer flasks (500mL) with 200 mL of culture medium with glucose (10g/L) was inoculated with *T. wortmannii* conidia (3 x 10^6 conidia/mL of medium) and incubated for 48 h at 29 °C at 200 rpm. This pre-culture (20 mL) was transferred to 500 mL Erlenmeyer flasks containing 180 mL of the production medium and cultivated at 29 °C at 200 rpm for 144 h. This assay was performed in triplicates.

Stirred tank reactor cultivation

Based on the results obtained in shake flask assays and based on ascomycetes bioreactor cultivations [2,22], stirred tank reactor (STR) batches were conducted using 3.0-L BioFlo® 115 bioreactors (New Brunswick Scientific Co.) with 1.0 L working volume to evaluate the kinetics of enzyme production. The pH was controlled with the addition of 0.4 M H_2SO_4 or 1:3 (v/v) NH_4OH:H_2O and the temperature was kept at 29°C. Stirring and aeration varied respectively (200 – 500 rpm; 0.3 – 1.0 L/min) to maintain dissolved oxygen level above 30% of air saturation. The medium was inoculated with fungi cells 10 % (v/v) from the shaken flask pre-culture as described in the item above. Polyglycol antifoaming (FluentCane 114, DOW Chemical, Brazil) was added manually if required. Samples were periodically collected, cells were removed by centrifugation (10,000 x *g*, 10°C for 15 min) and supernatants were used for enzymatic activities assays.

Carbon source, temperature and pH influence in enzyme production

Evaluation of xylanolytic enzymes production under industrial agro-industrial were accessed in shake flask cultures at 29°C at 200 rpm for 144 h. Six lignocellulose materials and one sugar were evaluated at 10 g/L: HB, EB, DEB, SB, WB, liquor and xylose. Then two carbon sources with promising results were used with higher dry matter (20g/L for each carbon source) for shake flask cultures (200 rpm for 144 h) at 29° 32° and 35°C. The pH influence was performed in STR cultivations at pH 4.0, 5.0 and 6.0 to access enzymatic production under a pH controlled process (Duplicates only for pH 5.0). In bioreactor assays, temperature and carbon source were selected based on the highest results obtained previously in shaken flask cultivation.

Analytical methods

Xylanolytic activity was determined as xylanase and β-xylosidase titration. Xylanase activity was determined employing Beechwood xylan (0.5% w/v) and DNS method for reducing sugars quantification (xylose as standard). The reactions were carried out at pH 5.0 with 90 µL of 50 mM citrate buffer and 10 µL diluted centrifugation supernatant [19]. β-xylosidase activity was measured using 10 µL of sample and 90 µL of the 4-nitrophenyl β-D-xylopyranoside (Sigma-Aldrich, USA) at 0.5 mM, diluted in 50 mM citrate buffer at pH 5.0. The reactions were incubated for 10 min at 50°C and enzymatic hydrolysis were interrupted by 100 µL of DNS or 1 M Na_2CO_3 for xylanase and β-xylosidase respectively [19]. The absorbances were measured by the micro plate reader Tecan Infinite® 200 (Switzerland) at 400 nm (β-xylosidase) and 540 nm (xylanase). One unit of glycohydrolases activity (in International Units, U) corresponds to 1 µmol of xylose or pNP released per minute.

Statistical analysis

Enzymatic activities produced at 29° 32° and 35°C were evaluated by analysis of variance (ANOVA), means were compared by Tukey test at 1% with Statistica 10.0 software (Statsoft, Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

To evaluate the influence of different carbon sources on xylanolytic enzymes by *T. wortmannii* DR49 shake flask cultivation were performed. It was tested six carbon sources rich in cellulose and hemicellulose (HB, EB and DEB), hemicellulose (SB and WB), xylooligomers (Liquor) and pure xylose at 1 % (w/v), material composition is presented at tables S1 and S2 (Supplementary material). Figure 1 shows the xylanase and β-xylosidase activities as a time course over 144 h of cultivation.

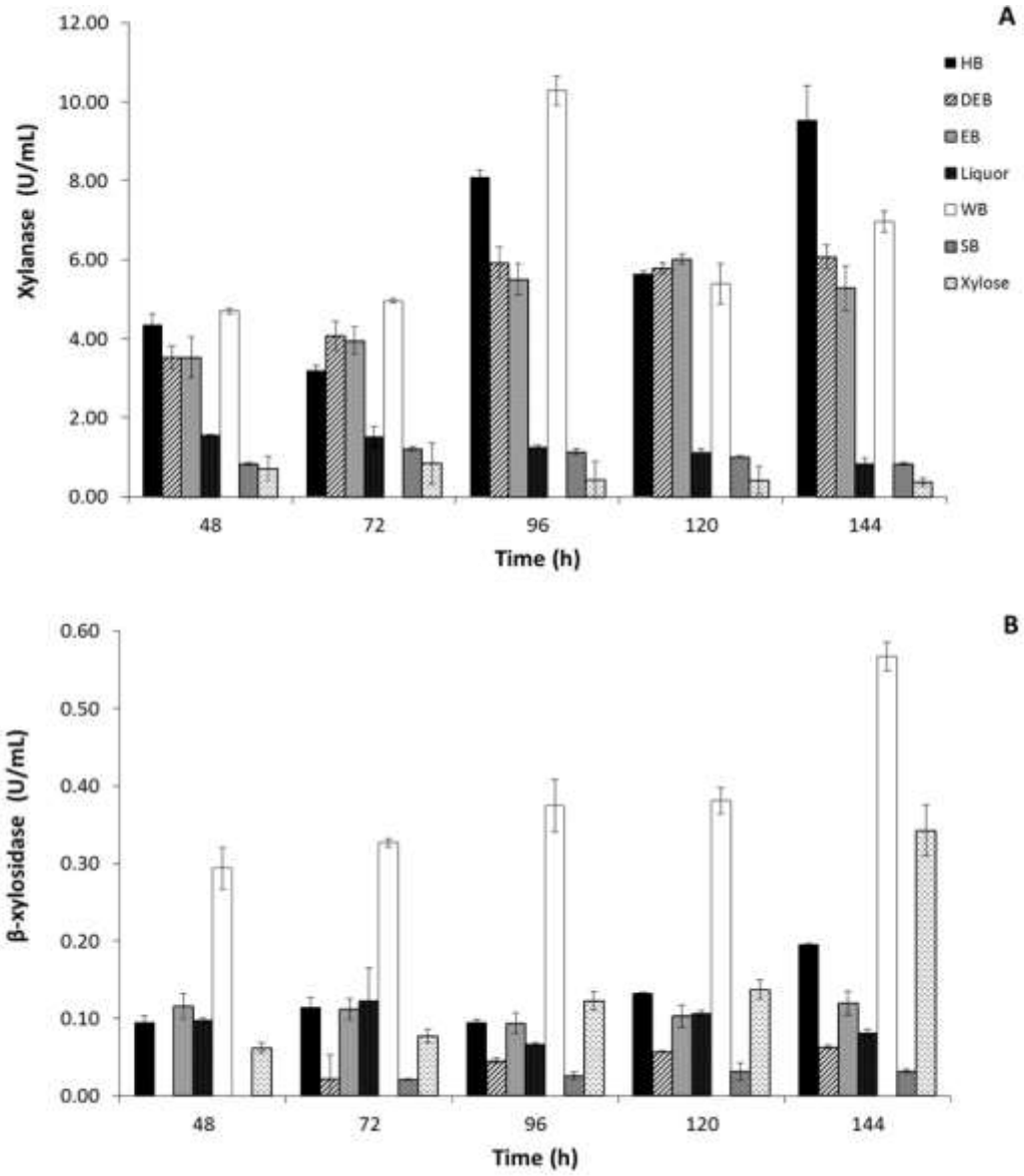

Figure 1. Influence of different carbon sources on xylanase (A) and β-xylosidase (B) production by *Talaromyces wortmanii* DR49 in shake flask cultivation.

Evaluation of xylanase production under industrial agro-industrial wastes showed that the highest enzyme concentration was obtained on materials rich in hemicellulose. The best results obtained were for WB (10.3 U/mL) at 96h and HB (9.5 U/mL) at 144 h. WB has been used in others works to induce xylanolytic enzymes due to high proportions of arabinoxylan [25]. Romdhane and coauthors [26] produced xylanase and β-xylosidase by *Talaromyces thermophiles* in WB shaken flask cultivation and obtained enzymatic activities approximately of 4.5 U/mL and 1.5 U/mL respectively. According to Robl and coauthors [22] HB presented the following composition: cellulose (56.03 %), hemicellulose (4.59%), lignin (36.36%) ashes (4.24%). Despite of the fact that HB presented high percentage of lignin which may hamper fungal growth and

enzymatic production, the presence of hemicellulose induced xylanase production. The same results were observed by Robl and coauthors [2], in which xylanase production by *Aspergillus niger* was higher in HB instead of EB and DEB.

In β-xylosidase production the highest titration observed were for WB (0.57 U/mL) and xylose (0.34 U/mL) at 144 h. Xylose was not able to induce xylanase production, although this saccharide seems to play a role in β-xylosidase production. Llanos and coauthors [27] studied the influence of carbon source in the transcriptional levels of glicohydrolase genes in *Talaromyces versatilis* and verified that xylose did not induce dose-dependent inhibitory effect for xylanases and β-xylosidase synthesis.

Xylose cost can makes unfeasible its use for industrial enzyme production. In this way, liquor from hydrothermal sugar cane pretreatment which is rich in xylose and xylooligomers was tested. Although liquor presented xylose (4.7 ± 0.41 g/L) and xylooligomers (9.98 ± 1.13 g/L), low xylanases and β-xylosidase activities were obtained (Figure 1). This result corroborates with Robl and coauthors [2], according to the authors xylanolytic batch enzyme production by *A. niger* DR02 was negatively impacted by liquor xylose concentration. In this way, a fedbatch cultivation was developed to keep xylose concentration at low levels. Consequently it reduced CCR effect and increased xylanolytic enzymes production. Besides liquor contain inhibitors (Table S1) that can affect negatively enzyme production such as lignin (3.15 g/L), furfural (1.05 g/L) and hydroxymethylfurfural (0.18 g/L). In ascomycetes fungi SB has been extensively used in glycohydrolase production such as β-glucosidase, pectinase and xylanase [7, 22]. However in our work this agro waste was not able to induce none of the measured enzymes activities. Even though SB is composed by 18.13% of hemicellulose, the high amount of protein is presented (43.22%) [23]. This fact could induce proteases production and also increase media pH due to protein metabolism.

As presented in figure 1 WB and HB were best the carbon source to produce both enzymes. In this way, the fungus was grown in both carbon source in higher concentration, HB (20g/L) and WB (20 g/L) to evaluate the influence of temperature and pH in xylanolytic enzymes production. Previous works with ascomycetes showed that higher polysaccharides concentration could lead to higher glycohydrolase production [7, 22]. Robl and coauthors [22] showed that pectinase and β-glucosidase production by *Annulohypoxylon stygium* were higher when citrus bagasse and SB concentration were increased and use simultaneously (20g/L for each carbon source).

It was investigated the xylanolytic production under three different temperatures (29, 32 and 35°C). Samples were collected at 96, 120 and 144 h, once that the production peak occurs during this period (Figure 1). *T. wortmannii* DR49 was able to growth and produced xylanase and β-xylosidase activities under the three conditions tested (Table 1). The highest enzymatic activity obtained for both enzymes were at 29°C at 144h and differed from higher temperatures by Tukey test ($p<0.01$). A drastically decrease in enzyme production was observed at 35°C for both enzymes. According to Yilmaz and coauthors [14] *T. wortmannii* is able to growth poorly at 37 °C, but is not able to growth at 40°C in Czapek Yeast Autolysate Agar (CYA).

The pH is also a parameter that impacts directly glycohydrolases production. Due to fungal metabolism, during cultivation, pH fluctuation occurs on non pH controlling medium. In order to minimize that, bioreactor cultivations at pH 4.0, 5.0 and 6.0 were performed to evaluate the kinetics of enzyme production in a controlled batch environment (Figure 2).

Xylanase and β-xylosidase presented similar production profiles in batch bioreactor for each different pH. The highest xylanase titration was obtained for pH 6.0 with production peaked at 120 h (14.5 U/mL) (Figure 2A). Regarding to β-xylosidase, pH 6.0 and 5.0 showed similar results in enzyme production with the highest enzymatic activity (1.7 U/mL and 1.5 U/mL) at 144 respectively (Figure 2B). These data indicates that acid pH, values above 5.0, can influence negatively in xylanolytic enzymes production. In ascomycetes pH plays a role in xylanolytic enzymes production. The pH can affect the expression of some *Aspergillus* genes encoding glycohydrolases [28]. According to MacCabe and coauthors [29] *Aspergillus nidulans* xylanase genes (*xlnA* and *xlnB*) are pH regulated via PacC factor and depending on media pH one is expressed instated another.

Members of *Talaromyces* genera produce various glycohydrolases including cellulases and hemicellulases [30], although only few works reported the enzymatic production of *Talaromyces* section Islandici. The only studies of xylanlolytc enzymes production of *T. wortmannii* are from Lee and coauthors [18] and Robl and coauthors [19], although none of them verified the influence of pH, temperature and agro wastes on fungal enzyme production. Lee and coauthors [18] obtained high β-xylosidase titration by *T. wortmannii* cultivation at 25°C in pure substrates, such as beechwood xylan (3.82 U/mL) and birchwood xylan (2.17 U/mL). Similar result was obtained by Robl and coauthors [19], that cultivated *T. wortmannii* DR49 in beechwood xylan at pH 5.0 and 29°C and produced β-xylosidase activity (2.85 U/mL). Regarding to xylanase

activity our work, showed that it was possible to increase this enzymatic titration in 240% when compared to Robl and coauthors [19] that used pure xylan for xylanase production (6.00 U/mL).

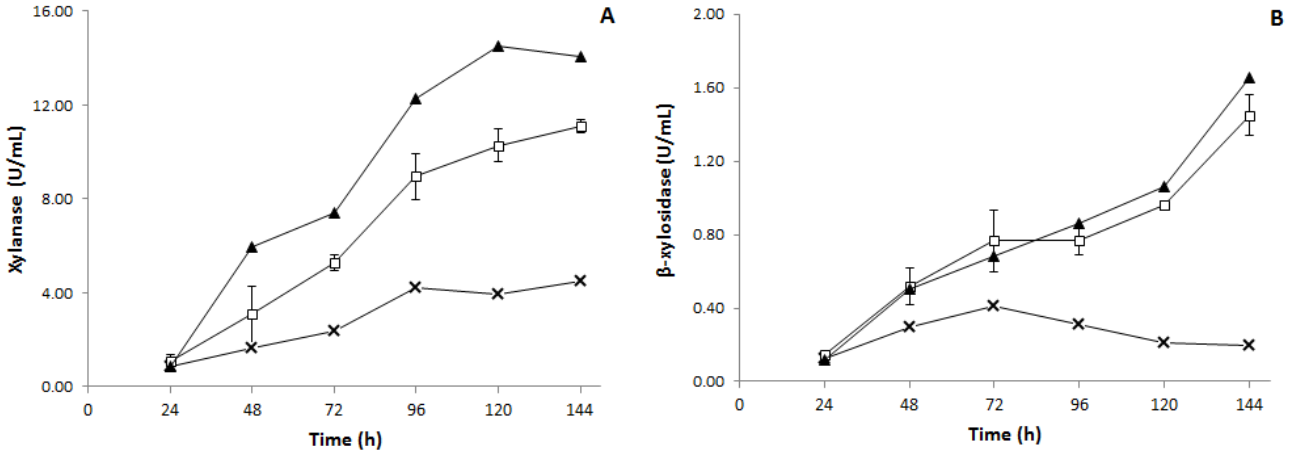

Figure 2. Xylanase (A) and β-xylosidase (B) activities of *Talaromyces wortmannii* DR49 cultivation on STR in pH 4.0 (X), pH 5.0 (□) and pH6.0 (▲) at 29°C.

CONCLUSION

T. wortmanii DR49 showed to be a potential candidate for xylanolytic enzymes production using plant biomass materials in bioreactors, which has never been previously reported in this fungus. Submerged cultivation revealed that the best temperature and pH for xylanase and β-xylosidase production are 29°C and 6.0. Biochemical characterization of *T. wortmanii* DR49 xylanase and β-xylosidase, as well as a culture media optimization must be performed to verify xylanolytic enzyme applicability.

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