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Screening of yeasts capable of producing cellulase-free xylanase

Otero, D. M.[1]*, Cadaval, C. L.[1], Teixeira, L. M. [1], Rosa, C. A[2], Sanzo, A. V. L.[1] and Kalil, S. J[1]

[1]Universidade Federal do Rio Grande, Escola de Química e Alimentos, Laboratório de Microbiologia e Biosseparações, Rio Grande, Brazil.
[2]Universidade Federal de Minas Gerais, Laboratório de Taxonomia, Biodiversidade e Biotecnologia de Fungos, Belo Horizonte, Brazil.

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Xylanases have largely been obtained from filamentous fungi and bacteria; few studies have investigated the production of this enzyme by yeasts. The aim of this study was to isolate yeasts from different sources, such as vegetables, cereal grains, fruits, and agro-industrial waste and to obtain yeasts capable of producing celulase-free xylanase. Samples were enriched using yeast malt broth, and yeasts were isolated on Wallerstein nutrient agar. In all, 119 yeast strains were isolated and evaluated in terms of their ability to degrade xylan, which was found in the medium by using agar degradation halos, the basis of this polysaccharide, and Congo red dye. Selected microorganisms were grown in complex medium and the enzymatic activities of endo-xylanase, β-xylosidase, carboxymetilcellulase, and filter paper cellulose were determined over 96 h of cultivation; the pH and biomass concentration were also evaluated. The yeast strain 18Y, which was isolated from chicory and later identified as *Cryptococcus laurentii*, showed the highest endo-xylanase activity (2.7 U.mL^{-1}). This strain had the ability to produce xylanase with low levels of cellulase production (both CMCase [0.11 U.mL^{-1}] and FPase [0.10 U.mL^{-1}]). This result gives this strain great biotechnological potential since this enzyme can be used for industrial pulp and paper bleaching.

Key words: *Cryptococcus laurentii*, endo-xylanase, xylan.

INTRODUCTION

Xylan, the major component of the hemicellulose complex, is a heterogeneous polysaccharide found in the plant cell wall, formed by xylopyranosyl residues linked by β-1,4-glycosidic bonds (Flores, 1997). An enzymatic complex is responsible for the total hydrolysis of xylan, but its main enzymes are endo-1,4-β-xylanase and β- xylosidase (Polizeli, 2005). These enzymes are responsible for the hydrolysis of the main chain: the former attacks the main internal chain of xylosidic linkages and the latter yields xylosyl residues by endwise attack of xylooligosaccharides (Subramaniyan and Prema, 2002).

Endo-1,4-β-xylanase and β-xylosidase are divided into two categories: one is associated with polysaccharidases (for example, cellulases) and the other is cellulase-free (Biely, 1985). The former is used for the production of hydrolysates in food and beverage processing (Sreenath,

*Corresponding author. E-mail: deborah.m.otero@gmail.com. Tel: +(55)(53)32935381.

1996), in animal feed, to reduce viscosity and improve the digestibility of nutrients (Ahmad et al., 2013; Viana, 2011; Mathlouthi, 2003) and production of xylitol used as a natural sweetener (Parajó et al., 1998; Usama et al., 2013). Cellulase-free xylanase is used for paper and vegetable fiber bleaching (Damiano, 2003; Bocchini et al., 2003; Techapun et. al., 2003; Shangi, 2009).

Cellulase-free xylanase is used mainly in the pulp and paper industries for pretreating pulp prior to bleaching to minimize the use of chlorine, the conventional bleaching agent. This application has great potential as an environmentally safe method (Subramaniyan, Prema, 2000).

These enzymes are produced by molds, bacteria, yeasts, marine algae, among other organisms, but their main commercial sources are filamentous fungi (Polizeli et al., 2005). Xylanases from filamentous fungi are generally associated with the production of cellulases (Steiner, 1987), which may not be desirable, depending on the application of the enzyme.

Xilanolitic systems have largely been obtained from filamentous fungi (Adesina, 2013; Rao, 2002, Cacais, 2001) and bacteria (Rajagopalan et al. 2013; Chavez et al., 2006); there are few studies on the production of this enzyme by yeasts (Lara et al., 2014; Lopes, 2011; Pe9trescu, 2000). To the best of our knowledge, there have been no studies on the production of hydrolytic cellulases (CMCase and FPase) and β-xylosidase associated with the production of xylanases by yeasts; the only related studies have been conducted on filamentous fungi (Malabadi, 2007; Petrescu, 2000; Gottschalk, 2013).

It is important to isolate yeast strains that produce the xylanase enzyme to develop new process for synthesizing products of biotechnological interest. Therefore, the aim was to isolate and select yeasts from food and residues capable of producing cellulase-free xylanase enzymes.

MATERIALS AND METHODS

Sample collection

Samples were obtained from different cities in southern Rio Grande do Sul: Pelotas (31º63´S/52º33´W), Rio Grande (31º21´S/52º38´W), São Lourenço (31º25´S/52º13´W), Quinta (32º08´S/52º26´W), and Povo Novo (31º93´S/52º30´W). The samples collected were vegetables (chayote, chicory, radicci, tomato), grains (soybeans, corn, rice), fruits (banana, jelly palm, grape), and agro-industrial wastes (rice husk, corn cobs, soybean hulls). Samples (10 g) were collected, stored in sterile plastic bags and transported under refrigeration to the laboratory within 24 h. For the isolation of yeasts, parts of the fruits and vegetables (stems, leaves, roots) were evaluated separately.

Yeast isolation

For enrichment, about 2.5 g of each sample was inoculated into 25 mL yeast malt broth composed of 3 $g.L^{-1}$ yeast extract; 3 $g.L^{-1}$ malt extract; 5 $g.L^{-1}$ peptone; and 10 $g.L^{-1}$ glucose (pH 6.0); the samples were then incubated at 25ºC for 72 h at 150 rpm (Maugeri, 2007).

Cultures were streaked onto Petri dishes with Wallerstein nutrient agar composed of 4 $g.L^{-1}$ yeast extract; 5 $g.L^{-1}$ bactocasitone; 50 $g.L^{-1}$ dextrose; 550 $mg.L^{-1}$ KH_2PO_4; 125 $mg.L^{-1}$ KCl; 250 $mg.L^{-1}$ $MgSO_4 \cdot 7H_2O$; 2.5 $mg.L^{-1}$ $FeCl_3$; 2.5 $mg.L^{-1}$ $MnSO_4$; 22 $mg.L^{-1}$ Bromocresol green, and 20 $g.L^{-1}$ agar at pH 5.5. Cultures were grown at 25°C for 72 h. Colonies were transferred and maintained in GYMP agar (20 $g.L^{-1}$ glucose, 5 $g.L^{-1}$ yeast extract, 10 $g.L^{-1}$ malt extract, 2 $g.L^{-1}$ KH_2PO_4, and 20 $g.L^{-1}$ agar at pH 5.5) at 4°C in a slant tube (Maugeri, 2007). HCl was used to adjust the pH.

Screening yeast for xylanase activity

For the selection of yeasts capable of producing xylan-degrading enzymes, all isolated yeasts were tested using two different methods. In Method 1 (Whitaker, 2002), only a medium containing xylan (2 $g.L^{-1}$) and agar (17 $g.L^{-1}$) was used. In Method 2 (Lopes, 2011), the following medium was used: 10 $g.L^{-1}$ Beechwood xylan (Sigma); 0.6 $g.L^{-1}$ yeast extract; 7.0 $g.L^{-1}$ KH_2PO_4; 2.0 $g.L^{-1}$ K_2HPO_4; 0.1 $g.L^{-1}$ $MgSO_4.7H_2O$; 1.0 $g.L^{-1}$ $(NH_4)_2SO_4$, and 15 $g.L^{-1}$ agar; HCl was used to adjust the pH to 5.0. From the yeasts in GYMP agar, the inoculum was transferred to xylan agar plates, by picking one by one using a toothpick and incubated at 30°C for 72 h. The clearance zones produced around the yeast colonies in the medium were visible when Congo red was used. They were discolored by sodium chloride at different concentrations.

The diameters of the zones of clearance and colonies were measured in millimeters and the enzymatic index (EI) was calculated by Equation 1:

$$EI = \frac{diameter\ hydrolysis\ zone}{diameter\ colony}. \quad (1)$$

Strains with an EI above 1.0 were considered to be potential producers of xylanase in Method 1 (Whitaker, 2002), whereas strains with an EI above 2.5 were considered to be potential producers in Method 2 (Lopes, 2011).

Production of xylanase in liquid medium

Only the yeast strains considered to be potential producers of xylanase (which met the criteria described above) were pre-grown in liquid medium. The inoculum was grown in 150 mL medium, as follows: 10.0 $g.L^{-1}$ Beechwood xylan; 3.0 $g.L^{-1}$ yeast extract; 7.0 $g.L^{-1}$ KH_2PO_4; 2.0 $g.L^{-1}$ K_2HPO_4; 0.1 $g.L^{-1}MgSO_4.7H_2O$; 1.0 $g.L^{-1}$ $(NH_4)SO_4$ and 5.0 $g.L^{-1}$ peptone; the samples were grown at pH 6.0 (adjusted using HCl), 30ºC, and 150 rpm for 24 h (Lopes, 2011). The cultivation was carried out in 500 mL Erlenmeyer flasks containing 150 mL of the same medium and was inoculated with cellular suspension (10^8 cells.mL^{-1}) obtained from a 24 h inoculum, incubated in a rotary shaker at 30°C and 150 rpm. The samples, which had been collected over 96 h of fermentation (samples were taken every 12 h until peak activity was observed, after which they were immediately withdrawn every 24 h), were centrifuged at 6,000 × *g* at 4°C for 10 min. The cell-free supernatant was used to determine pH and the activities of enzymes (endo-xylanase, cellulase, and β-xylosidase). The precipitated cells were then used for biomass determination; the tests and analytical measurements

were performed in triplicate.

Analytical methods

Enzyme assay

Carboxymethylcellulase activity was assayed using a reaction medium containing 0.5 mL substrate (2% [w/v] solution of carboxymethylcellulose) and 0.5 mL crude enzymatic extract and incubated for 30 min at 50ºC. Reducing sugars were assayed as glucose using the DNS method (Miller, 1959). Filter paper activity was assayed using a reaction medium containing 0.5 mL crude enzymatic extract, 1.0 mL citrate sodium buffer (pH 4.8), and 50 mg filter paper incubated for 60 min at 50ºC (Ghose, 1989). One international unit of filter paper activity was considered as the amount of enzyme that forms 1 µmol glucose (reducing sugar as glucose) per min during the hydrolysis reaction. One enzyme activity unit (U) of Carboxymethylcellulase was defined as the amount of enzyme required to produce 1 µmol of glucose per min under the assay conditions..

For all enzymatic activities, blanks were performed with enzymes (buffer + enzyme) and substrates (buffer + substrate). All tests of enzymatic activities were performed in triplicate.

Biomass determination

The biomass concentration was estimated by measuring absorbancy in a spectrophotometer at 620 nm and relating the readings to biomass dry weight with a calibration curve (Rech et al., 1999).

Kinetic parameter determination

Kinetic parameters were determined for each growth curve: maximum biomass concentration (X_{max}, $g.L^{-1}$), maximum specific growth rate (μ_{max}, $1.h^{-1}$), and enzymatic productivity (P, $U.mL^{-1}.h^{-1}$) of each selected yeast grown in liquid medium, as described in "production of xylanase in liquid medium" section of this work.

The productivity (P) was calculated when the time of maximum activity was reached, and the maximum specific growth rate (μ_{max}) was calculated using Equation 2 by exponential regression of the logarithmic growth curve (Bailey and Ollis, 1986). The enzymatic productivity was calculated using Equation 3, according to Schmidell et al. (2001).

$$\mu = \frac{1}{x}\frac{dx}{dt} \quad (2)$$

$$P = \frac{A - A_0}{t - t_0} \quad (3)$$

Where, µ is the specific growth rate (h^{-1}), X is the biomass concentration ($g.L^{-1}$), A_o is initial enzymatic production, t is cultivation time at which maximum enzymatic activity occurs and t_o is the start point of cultivation.

Yeast identification

Only the yeast strain with the highest xylanase activity was identified. Identification was based on the sequencing of the D1/D2 domains of the large subunit of the rRNA gene. The D1/D2 domains were amplified by PCR, directly from whole cells as previously described (Lachance et al., 1999). Sequencing was performed on an ABI 3130 automated DNA gene analyzer according to the manufacturer's instructions. The sequences were assembled, edited, and aligned using the program MEGA6 (Tamura et al., 2013). The sequences obtained were compared to those included in the GenBank database using the Basic Local Alignment Search Tool (BLAST at http:// www.ncbi.nlm.nih.gov).

Statistical analysis

The results were evaluated statistically through Variance Analysis and Tukey test at 95% confidence level ($p < 0.05$), using Statistica 5.0 software.

RESULTS AND DISCUSSION

In all, 119 yeasts strains were isolated from different types of samples to obtain microorganisms capable of producing xylanolytic enzymes. Strains that were capable of degrading xylan present in the medium and that met the criteria of both methods were chosen. Results were expressed in terms of rings of hydrolysis, revealed by the addition of Congo red dye. Among the strains tested, 23 were able to hydrolyze xylan, but only 7 of those (6% of all microorganisms) met the criteria of both methods and thus were selected (Table 1). These results were considered promising since they are similar to those of previous studies. For example, Rao et al. (2008) obtained 374 yeasts from tree bark and decaying fruits that were evaluated for the ability to ferment xylose, and found that 27 isolates (7.2%) converted it into ethanol. Similarly, Lopes et al. (2011) evaluated 349 yeasts in Petri plates, and found that 9 (2.6 %) showed an enzymatic index above 2.5.

The enzymatic index, a semi-quantitative parameter applied to evaluate the ability of yeast strains to produce enzymes in solid medium, has been used by several authors (Ribeiro et al., 2014; Adesina, 2013; Florencio, 2012; Nagar, 2012; Samanta et al., 2011; Tallapragada, 2011), who have considered it an efficient method for screening for microorganisms. The next selection step was performed according to the capacity of yeast strains to produce the xylanolytic enzyme in liquid medium with xylan. Based on the activity of endo-xylanases in the seven yeasts under study (Figure 1a), yeast 18Y showed the highest xylanolytic activity (2.7 $U.mL^{-1}$), followed by yeast 34Y (2.1 $U.mL^{-1}$), after 36 h of cultivation. Yeast 19Y (0.5 $U.mL^{-1}$) had the lowest ability to produce the enzyme endo-xylanase. As shown in Figure 1, yeast 19Y had the highest peak activity in the fastest culture time (12 h), followed by yeast 13Y, which needed 24 h to reach its maximum enzymatic activity. Yeast 40Y needed most time (48 h) to reach its maximum compared to the other yeasts, which had high production up to 36 h of cultivation followed by a significant decrease after 48 h of cultivation. The only exception was yeast 60Y since it did not show any meaningful decrease in its activity. β-Xylosidases are glycoside hydrolases, which catalyze the

Table 1. Enzymatic index of yeasts isolated from different samples

Strain	Samples	Enzymatic index (>1.0) (Method 1)	Enzymatic index (>2.5) (Method 2)
13Y	Tomato	6.0	4.0
18Y	Chicory	3.0	3.0
19Y	Chicory	2.3	2.5
34Y	Tomato	3.0	3.5
40Y	Corn Seed	2.0	2.7
53Y	Tomato	2.5	3.0
60Y	Chicory	4.0	2.5

release of xylose units from xylo-oligosaccharides derived from the degradation of xylan; thus, their presence may prove to be unsatisfactory when the aim is to obtain xylo-oligosaccharides (Subramaniyan and Prema, 2000). Therefore, the low levels of β-xylosidases produced by yeasts 18Y (0.003 $U.mL^{-1}$) and 34Y (0.0006 $U.mL^{-1}$) can be considered a positive factor. Low activities similar to the ones found in this study (Figure 1b) were also found by Biely (1980), who reported β-xylosidase levels below 0.1 $U.mL^{-1}$ produced by *Cryptococcus albidus* CCY 1741 in medium containing Beechwood xylan, and Bastawde (1994), who reported low levels of β-xylosidase by a yeast isolated from decaying wood (NCIM 3574). However, when cultivation is conducted with filamentous fungi, the levels of β-xylosidase tend to be higher, as reported by Lemos (2000). This author described β-xylosidase activities of 1.3 $U.mL^{-1}$ when cultivating *Aspergillus awamori* in medium with sugar cane bagasse.

In the present study, the production of cellulases (CMCase and FPase) was extremely low since all yeast activities were lower than 0.11 $U.mL^{-1}$ (Figure 1c and d). Therefore, xylanases produced by the yeasts under study could be applied to the textile (degumming of natural fibers), pulp, and paper industries. The values of cellulase found in this study agreed with the results of free-cellulase xylanase (0.02 $U.mL^{-1}$ for CMCase and FPase) from *Thermomyces lanuginosus* reported by Mendoza (2006). Boddireddy (2011) obtained free-cellulase xylanase from different isolated fungi with CMCase activities of 0.1 to 0.6 $U.mL^{-1}$ in submerged cultures. Alves-Prado (2010) cultivated *Lysinibacillus sp*. strain P5B1 in submerged cultures with xylan to produce endo-xylanase and reported a CMCase activity of 0.58 $U.mL^{-1}$. Therefore, considering these previous results, the findings obtained by the present study are promising.

The pH of the medium ranged from 6.0 to 7.2 (Figure 2a) during the cultivation of yeasts aimed at the production of xylanase. The only exception was yeast Yeasts 18Y and 34Y reached maximum enzyme 60Y: its initial pH was 6.2 but it reached 7.6 after 96 h. production within the first 36 h of culture at pH 7.1 and 6.7, respectively. Lopes (2011) isolated environmental yeasts and produced xylanase in medium with xylan. They observed that yeasts LEB-AY 10 and LEB-AAD5 reached their maximum enzymatic activity when the pH was around 6.5.

Regarding the production of biomass (Figure 2b), yeast 34Y stood out in terms of cell growth since it reached 12.27 $g.L^{-1}$. Yeast 18Y showed a maximum peak of endo-xylanase activity of 2.7 $U.mL^{-1}$, coinciding with the maximum increase in biomass production (5.75 $g.L^{-1}$), which occurred after 36 h.

The X_{max} found in the yeasts under study (Table 2) ranged from 2.54 $g.L^{-1}$ (yeast 53Y) to 12.27 $g.L^{-1}$ (yeast 34Y), whereas the productivity ranged from 0.015 $g.L^{-1}.h^{-1}$ (yeast 53Y) to 0.07 $g.L^{-1}.h^{-1}$ (yeast 18Y). The highest maximum specific growth rate (0.23 h^{-1}) was obtained by yeast strain 53Y while yeast 34Y had the lowest one (0.05 h^{-1}).

Yeast 18Y, which showed the highest endo-xylanase activity, was submitted to sequencing of the D1/D2 domains of the large subunit of the rRNA gene, and identified as *Cryptococcus laurentti*. Studies using micro-organisms for xylanase production are mainly performed using filamentous fungi (Guimarães, 2006) and bacteria (Vieira, 2007). The yeasts described in the literature as producing endo-xylanase include *Trichosporon* (Stevens, 1977), *Pichia stipitis* (Lee et al., 1986), *Aureobasidium pullulans* (Leathers et al., 1986), *Cryptococcus albidus* (Biely, 1980), and *Cryptococcus flavus* (Parachin et al., 2009; Yasui, 1984). Lopes (2011) isolated two strains of *Cryptococcus* that produced endo-xylanase with activities of 0.67 and 0.73 $U.mL^{-1}$. Morais et al. (2013) isolated yeasts from decaying wood and tested their ability to ferment xylose and produce endo-xylanase; the main yeasts were *Cryptococcus laurentti*, *Cr. humicola*, *Cr. flavences*, and *Cr. podzolicus*. Lara et al. (2014) isolated *C. laurentti* UFMG-HB-48, which
produced extracellular xylanase with an activity of nearly 1.3 $U.mL^{-1}$.

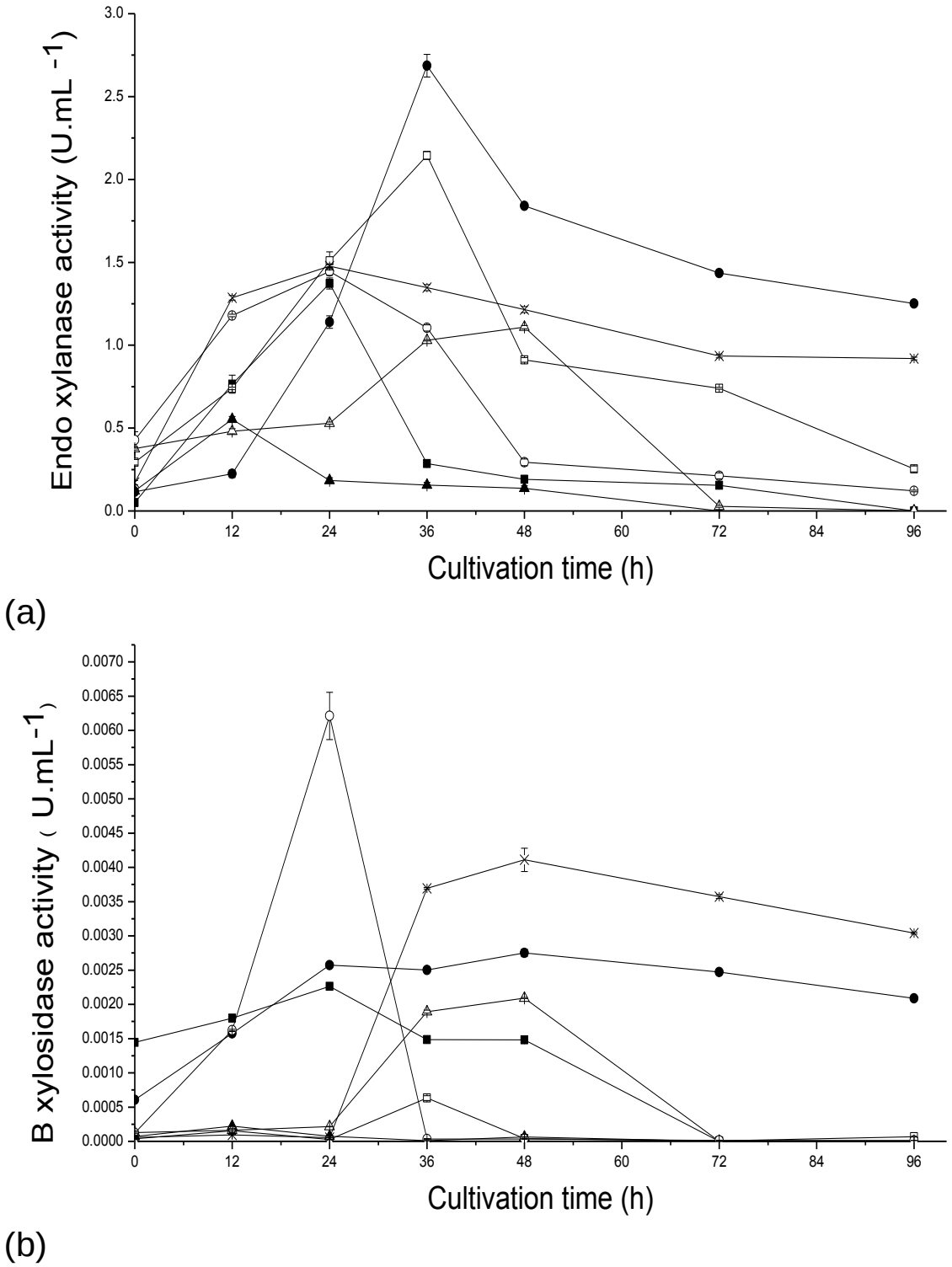
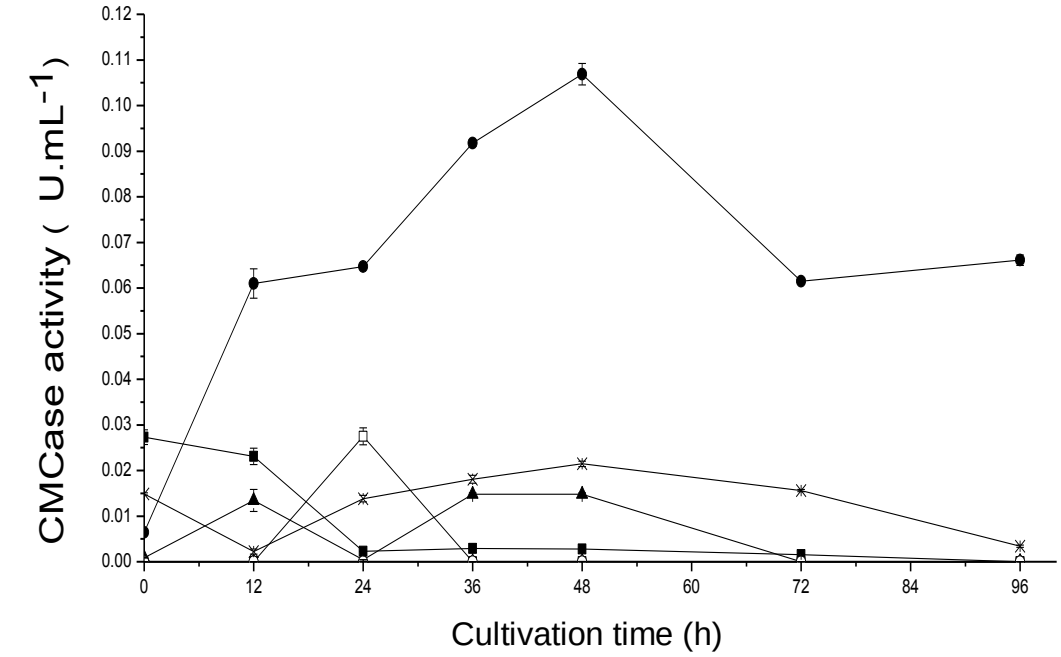
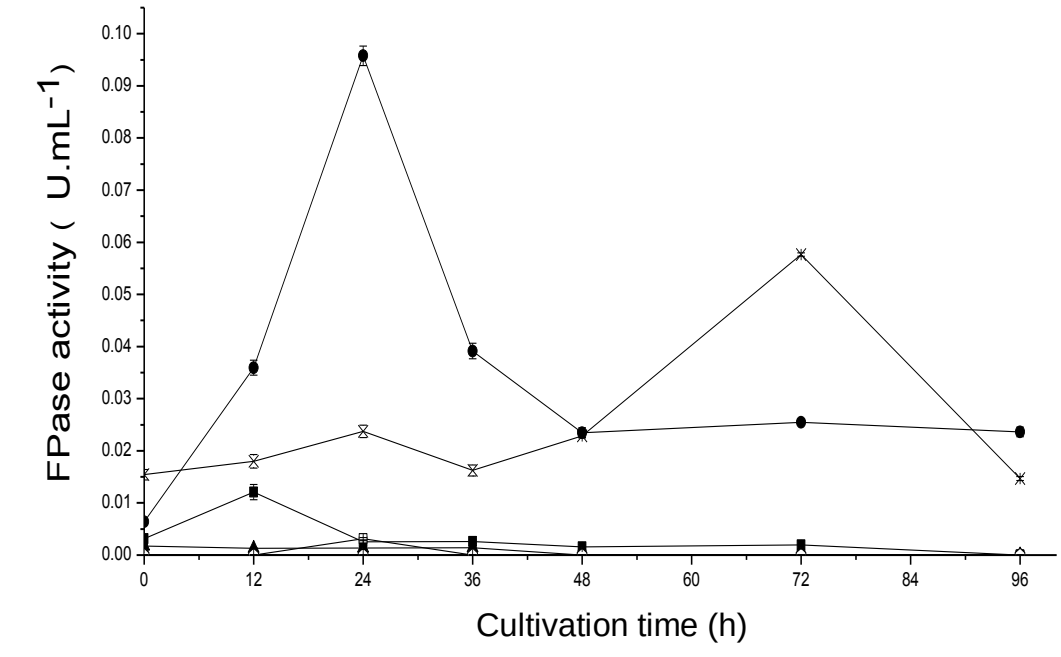


Figure 1. Activities of endo-xylanase (a), β-xylosidase (b), CMCase (c), and FPase (d) in the isolated yeasts in medium with xylan as its carbon source. (■) Yeast 13Y; (●) Yeast 18Y; (▲) Yeast 19Y; (□) Yeast 34Y; (Δ) Yeast 40Y; (o) Yeast 53Y; and (×) Yeast 60Y.

The enzymatic activities obtained in the present study are relevant not only because xylanase was produce d from yeasts but also because the activities that were observed were found under conditions that had not been



(c)

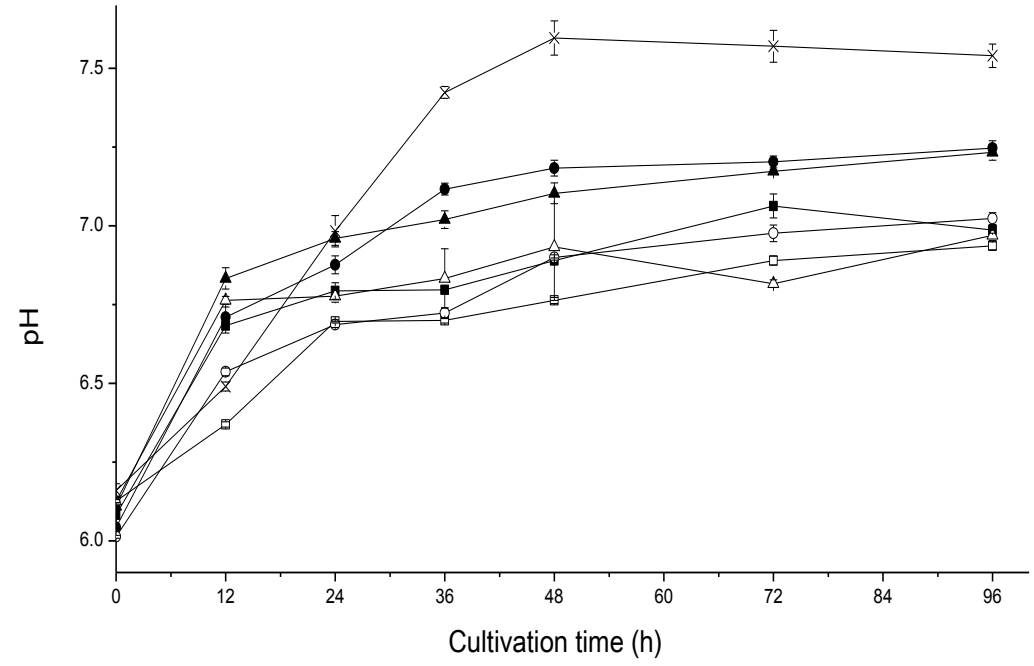


(d)

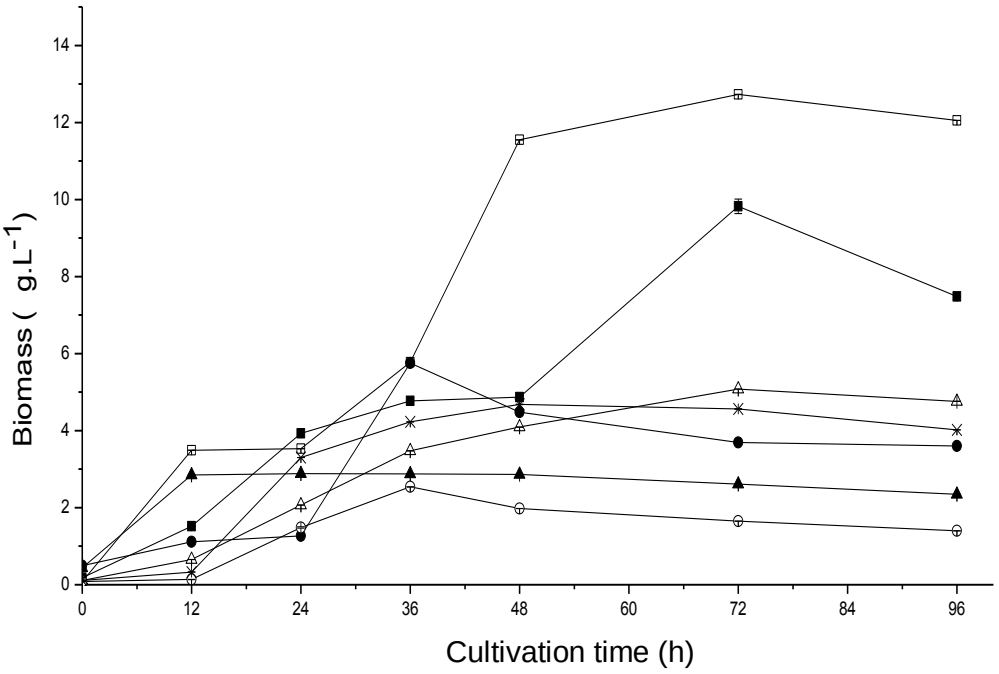
Figure 1. Contd.

optimized in terms of pH, temperature, stirring speed, the addition of ions, and so forth. Thus, their activities could be higher than observed.

The results also indicate that the samples used were suitable for the isolation of yeasts capable of producing xylan-degrading enzymes. Out of 119 isolated yeast strains, *C. laurentti* 18Y (isolated from chicory) showed the highest production of endo-xylanase (2.7 $U.mL^{-1}$); the averages of all the yeasts were evaluated statistically throughout the cultivation, as well as the maximum



(a)



(b)

Figure 2. The pH (a) and biomass (b) of the isolated yeasts in medium with xylan as its carbon source over 96 h, where: (■) Yeast 13Y; (●) Yeast 18Y; (▲) Yeast 19Y; (□) Yeast 34Y; (Δ) Yeast 40Y; (o) Yeast 53Y; and (×) Yeast 60Y.

temperatures were compared, where the yeast was statistically superior 18Y of other yeasts. This yeast species had the ability to produce endo-xylanase that was free of cellulase (CMCase and FPase) and showed low levels of β-xylosidade.

The potential industrial application of such cellulase-free xylanase, especially from yeast strains that show activity at a neutral pH, will be attractive to the paper and

Table 2. Kinetic parameters of isolated yeasts ± standard deviation.

Yeast	X_{max} ($g.L^{-1}$)	μ_{max} (h^{-1})	Productivity ($U.mL^{-1}.h^{-1}$)
13Y	9.82 ± 0.180	0.13 ± 0.002	0.05 ± 0.0021
18Y	5.75 ± 0.001	0.13 ± 0.001	0.07 ± 0.0016
34Y	12.27 ± 0.001	0.05 ± 0.001	0.05 ± 0.001
40Y	5.08 ± 0.001	0.11 ± 0.001	0.01 ± 0.001
53Y	2.54 ± 0.001	0.23 ± 0.007	0.05 ± 0.001
60Y	4.68 ± 0.005	0.13 ± 0.001	0.05 ± 0.001
19Y	2.88 ± 0.001	0.16 ± 0.001	0.03 ± 0.001

pulp industries to improve the quality of paper pulp as well as to minimize environmental pollution, which occurs due to the use of hazardous chemicals by these industries. In addition, these xylanases could also be used in agriculture; in the production of human food, cattle feed, and pet food; and in the production of xylo-oligosaccharides for pharmaceutical purposes.

Conflict of interests

The author(s) did not declare any conflict of interest.

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