Journal of Biochemical & Microbial Technology

Andrade CCP et al., J Biochem Microb Technol 2015, 3(2):8-17
http://dx.doi.org/10.14312/2053-2482.2015-2

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Optimization of xylanase production by *Cryptococcus flavescens* LEB-AY$_{10}$ from steam exploded sugarcane bagasse

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Abstract

The production of a thermostable xylanase was evaluated using steam exploded sugarcane bagasse as substrate. Initially, the selected yeast isolate was identified as *Cryptococcus flavescens* LEB-AY$_{10}$ (CCT 7725) by sequencing the ITS1-5.8S-ITS2 region and D1/D2 domains of the large subunit (26S) ribosomal DNA. Then, sugarcane bagasse submitted to three different conditions of steam explosion (188.5°C, 198°C or 210°C) and their corresponding soluble C5 fractions were assayed as enzyme inductors, while raw bagasse and xylan from beech wood were used as controls. Molasses or compounds from synthetic media (peptone, yeast extract, magnesium and ammonium sulphate) were used to supplement the cultivation broth. Additionally, two-phase fermentations or activated charcoal treatments were also assayed with the purpose of removing inhibitors. After selection of C5 fractions from the mild pretreatment and their supplementation with synthetic compounds, an experimental design was used to study and optimize the fermentation conditions, as well as to enhance the production of xylanase. At the optimized conditions, according to the results of the experimental design, the enzyme activity after 96 h of fermentation was 4.67±0.23 U/mL (at 50°C) or 8.33±0.36 U/mL (at 80°C), which is 5.6 times higher compared to the activity in the preliminary tests using bagasse and sugarcane molasses, and 2.4 times higher than the activity in the initial assays using C5 fractions. Therefore, mild pretreatment of sugarcane bagasse showed to be a suitable C5 source for the production of xylanase by *C. flavescens* LEB-AY$_{10}$, instead of expensive purified xylan.

Keywords: xylanase; steam exploded bagasse; *Cryptococcus flavescens*; experimental design

Introduction

Bio-bleaching of paper pulp, bioconversion of lignocellulose materials to biofuels, waste treatment, digestibility of animal feed and various segments of the food industry [1–3] are some of the potential applications of xylanases. These enzymes, also known as endo-1,4-β-xylanases (E.C. 3.2.1.8), are the main xylan degrading enzymes which randomly cleave the β-1,4 glycosidic bonds between xylose residues, to release xylooligosaccharides and xylobiose.

Although xylan is, after cellulose, the most abundant polysaccharide in nature [4], xylanase production utilizing purified xylan as a substrate is uneconomical. Therefore, enzyme production costs may be substantially reduced when using agricultural by-products [5]. In addition to low cost, great interest in these lignocellulosic materials due to the fact that they do not compete with food products [6]. Hence, wastes such as corn cob, corn husk, oat husk, sugarcane bagasse [7], rice straw [8] wheat straw and bran [9] have been used by researchers to replace pure xylan in xylanase induction.

Sugarcane bagasse is an efficient alternative for xylanase induction since it is inexpensive and is often available in tropical countries such as Brazil [10]. Bagasse corresponds to the fibrous residue of cane stalks leftover after extraction of the juice [11], and is composed mainly by 32-44% cellulose, 19-24% lignin, 27-32% hemicellulose and 4.5-9.0% ash [12].

Due to the recalcitrant nature of lignocellulosic biomass, pretreatment of the raw material is important prior to enzymatic hydrolysis [13]. Its main purpose is to separate lignin from cellulose and hemicellulose, reduce cellulose crystallinity and increase porosity of the material [14]. From the same point of view, pretreatment favours increased enzyme production since it makes the fibre components more accessible to microorganism.

Several pretreatment techniques for lignocelullosic material have been described [15–17]. Among them,

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Received 12 January 2015 *Revised* 10 March 2015 *Accepted* 18 March 2015 *Published* 25 March 2015

Citation: Andrade CCP, Santos TP, Franco SF, Rodrigues MI, Pereira GAG, Maugeri Filho F. Optimization of xylanase production by *Cryptococcus flavescens* LEB-AY$_{10}$ from steam exploded sugarcane bagasse. J Biochem Microb Technol. 2015; 3(2):8-17. doi:10.14312/2053-2482.2015-2

steam explosion (SE) is widely used as a physicochemical method for fractionation of biomass components [16,18]. In SE, biomass is heated using high pressure steam for a few min, then the steam condenses under the high pressure and is "exploded" by rapid depressurization of the reactor. SE causes both hemicellulose solubilization (by partial hydrolysis) and disaggregation of the lignocellulosic matrix, breaking down inter- and intra-molecular linkages [19–21].

The use of SE technique to treat sugarcane bagasse is not limited to laboratory-scale, since it has also been demonstrated at both pilot- [22] and industrial-scales [23]. However, some conditions applied in this thermal process may degrade hemicellulose-derived sugars and solubilize and transform the lignin-related compounds to chemicals that can inhibit downstream processes, or even affect enzymes in the hydrolysis step, reduce sugar conversion during fermentation, and depress the rate of fermentation after biomass hydrolysis [24, 25]. Therefore, one of the main challenges in the SE process is to find optimal operational conditions to avoid problems in downstream steps.

Experimental designs are common practices in biotechnology and have been applied by various researchers, especially for optimization of culture conditions [26]. The main advantages to the use of these tools include: the reduced number of assays, analysis of the interaction among factors and exploration of the entire experimental space [27].

Lopes et al.[28] selected 9 strains for the production of xylanases out of 349 wild yeasts isolated from different Brazilian biomes. Among these isolates, the *Cryptococcus* sp. LEB-AY$_{10}$ strain secreted a xylanase which showed an optimal activity at 80°C. The authors increased the activity of this enzyme by up to 600% by means of an experimental design, using Birch wood xylan as substrate. Therefore, the main goals of the present work were to identify the species of *Cryptococcus* sp. strain LEB-AY$_{10}$, select steam explosion conditions for sugarcane bagasse and optimize the fermentation medium in order to enhance xylanase production by this strain.

Materials and methods

Yeast identification

C. flavescens LEB-AY$_{10}$ was isolated from the Atlantic Forest [29]. Species identification was carried out by sequencing the gene encoding the 5.8S region of ribosomal RNA, the spacer regions ITS1 and ITS2, and D1/D2 domains of the large subunit 26S. DNA extraction of the culture grown overnight was performed according to Ausubel et al. [30]. The universal primers ITS1F (5'TCCGTAGGTGAACCTGCGG3') and ITS4R (5'TCCTCCGCTTATTGATATGC3') were used for ITS1-5.8S-ITS2 amplification, and primers NL1F (5'GCATATCAATAAGCGGAGGAAAAG3') and NL4R (5'GGTCCGTGTTTCAAGACGG3') were used for D1/D2 amplification, according to the methodology described previously [31–33]. PCR products were purified using Exo 1 (Exonuclease 1) and SAP (Shrimp Alkaline Phosphatase), and quantified using a ND-1000 Spectrophotometer (Thermo Scientific, USA). Both strands were sequenced using the BigDye® Terminator Sequencing Kit (Applied Biosystems) and the DNA ABI PRISM 377 Genetic Analyzer (Applied Biosystems), at the Brazilian Biosciences National Laboratory (LNBio, Brazil). Identification of the yeast species was based on comparison of nucleotide sequences with those deposited in GenBank (NCBI, http://www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool (Blastn).

Substrates

Xylanase production was performed by induction using steam exploded sugarcane bagasse (Centro de Tecnologia Canavieira - CTC, Piracicaba, Brazil) submitted to three different pretreatment conditions: mild (12 kgf/cm²; 188.5°C), moderate (15 kgf/cm²; 198°C) and high (19 kgf/cm²; 210°C). For each pretreated sample, the soluble fraction (C5 fraction, containing the hemicellulosic compounds of bagasse) was extracted as described below. Beech wood xylan (Sigma) and raw sugarcane bagasse (Usina Ester, Cosmópolis, Brazil) were utilized as controls.

Soluble fraction preparation

C5 fractions of pretreated bagasse (PTB) were extracted by consecutive washings. For each steam explosion condition, PTB was added with distilled water up to a final concentration of 10% (dry weight/volume), and incubated in a rotatory shaker for 1 h, at 150 rpm, and at room temperature. Subsequently, the suspensions were vacuum filtered and the filtrates were stored, while the retentates were washed twice and treated as described above, except for incubation was only for 15 min. The filtrates from all washes were pooled and concentrated in a rotary evaporator Marconi AM-120 (São Paulo, Brazil) at 70°C until reaching the desired concentration. Therefore, unless otherwise stated, concentration of the C5 fraction described hereafter indicates the amount of PTB (dry weight) used to obtain the final volume after vacuum evaporation.

Inoculum and enzyme production

The microorganism was activated in 50 mL GYMP (2.0% glucose, 0.5% yeast extract, 1.0% malt extract and 0.2% monobasic sodium phosphate, pH 5.5) broth and pre-inoculum was performed in 200 mL of the C5 fraction extracted from 5% (wet weight/volume) of moderately pretreated bagasse and containing 0.2% (total reducing sugars) of molasses. The fermentation broth was incubated at 30°C for 48 h.

Cells of the pre-inoculum were centrifuged and inoculated in a 500 mL baffled Erlenmeyer containing 150 mL of broth with different compositions (as described below). The flasks were incubated under rotational shaking at 150 rpm and 28°C. Samples were collected every 12 h for pH measurement and determination of enzymatic activity. The samples were centrifuged at 10,000 x g in a centrifuge model RC26Plus Sorvall (DuPont) for 10 min at 4°C.

Assay #1: Preliminary test using bagasse and molasses

The fermentation broth first consisted of 2.5% (dry weight/volume) of PTB (high, moderate or mild) supplemented with 0.2% (on the base of total reducing sugars) molasses.

Synthetic medium containing xylan (1% beech wood xylan; 0.5% peptone; 0.3% yeast extract; 0.01% $MgSO_4.7H_2O$; 0.1% $(NH_4)_2SO_4$) was also used as a control.

Assay #2: Removal of possible inhibitors

This assay sought to compare the PTB supplemented with molasses or with synthetic compounds. In addition to this comparison, two treatment methods for the removal of potential inhibitors in the PTB soluble fraction were also assayed. In the first, the C5 fraction was treated with activated charcoal before preparation of the medium. For this purpose, 4% (w/w) of activated charcoal ANFC were added to the C5 fraction, agitated at 150 rpm and 60°C for 15 min, centrifuged at 6,000 x g and filtered [34, 35] to recover the filtrate for media preparation. The second treatment was performed during fermentation, using biodiesel (from castor oil) as solvent, generating a two-phase fermentation system, as reported by Maugeri Filho et al. [36]. According to the authors, this method is based on different partition coefficients of the media compounds, in which some undesirable compounds may "migrate" to the organic phase.

The two treatments described above and the control (C5 fraction with no treatment) were applied to the two types of fermentation media, totalling six assays:

- *C5-MOL Broth:* C5 fraction corresponding to 3% (dry weight/volume) of PTB (moderate) and 0.2% molasses (corresponding to the amount of total reducing sugars present);

- *C5-SYNT Broth:* C5 fraction corresponding to 3% (dry weight/volume) of PTB (moderate) and 0.5% peptone, 0.3% yeast extract, 0.01% $MgSO_4.7H_2O$, 0.1% $(NH_4)_2SO_4$.

Assay #3: Enzyme production using bagasse and synthetic medium

The third test sought to verify the induction of enzyme production for all pretreatments, as determined by the above assays. For this purpose, the three types of pretreated bagasse (PTB, 2.5% dry weight/volume) and their respective soluble fractions (C5, corresponding to 5% dry weight/volume) were used instead of xylan in synthetic medium (0.5% peptone, 0.3% yeast extract, 0.01% $MgSO_4.7H_2O$, 0.1% $(NH_4)_2SO_4$). Untreated bagasse and xylan (1% dry weight) were used for comparison.

Optimization strategy

As a preliminary study, a Plackett-Burman (PB) design [27, 37] was performed to evaluate the effects of eight variables (temperature, agitation, substrate, pH, yeast extract, peptone, $MgSO_4.7H_2O$, $(NH_4)_2SO_4$) on xylanase production (response), using a matrix of 12 trials and 4 replicates of the central point.

The analysis of effects was performed by the Student's t-test, using the Statistica 8.0 software (Statsoft, Brazil). After selection of the variables, a Central Composite Rotational Design (CCRD) was used to evaluate the experimental space and verify the interactions among variables [27].

Analysis

Enzymatic activity

The enzymatic solution (0.3 mL) was incubated with 2.7 mL of 1% beech wood xylan (Sigma) in 50 mM sodium citrate buffer (pH 5.3) at 50°C [38] or at 80°C, in a shaker bath. Samples were collected at constant time intervals and xylanase activity was determined by the amount of reducing sugar (xylose) released, measured by the DNS method [39]. In this work, one unit of xylanase activity was defined as the amount of enzyme necessary to produce 1 μmol of xylose from xylan per min at 50°C, and unless informed differently.

Moisture content

Moisture was measured by the gravimetric technique at 105°C, in an oven with air circulation, until the samples reached constant weight.

HPAEC-PAD analysis

HMF, sucrose and monomeric sugars (arabinose, mannose, glucose, xylose, galactose and fructose) were analysed using a HPAEC-PAD DX-500 system (Dionex, USA) equipped with a CarboPac PA-1 column (0.4×25 cm; Dionex, USA) and pre-column (0.4×5 cm; Dionex, USA). Elution was performed at 1 mL/min and involved an isocratic step of 1 mM NaOH for 8 min followed by a linear gradient of 1-5 mM NaOH for 8 min and an isocratic step of 5 mM NaOH for 14 min. The column was cleaned between runs for 5 min with 150 mM NaOH and equilibrated for 5 min with 1 mM NaOH.

Results and discussion

Yeast identification

The yeast LEB-AY_{10}, previously classified as *Cryptococcus* sp. by Lopes et al. [28], did not grow in temperatures exceeding 30°C. On GYMP agar at 28°C, 48-hours-old colonies were creamy, smooth and glossy, with soft texture, circular shape and slightly raised. According to the microscopic analysis at the same conditions, cells were single, ovoid, encapsulated and showed some buddings.

A fragment of 493 bp sequenced for the ITS1-5.8S-ITS2 region was compared with others deposited in the GenBank databases using the NCBI's BLAST tool (http://www.ncbi.n1m.nih.gov/blast/Blast.cgi). For this region, the sequence showed 100% of identity (493/493, e-value 0.0) with *C. flavescens* IMUFRJ 52043 (emb|FN428902.1|) and 99% (487/493, e-value 0.0) with *C. flavescens* AUMC 7794 (JQ425400.1).

The 640 bp fragment in the D1/D2 domains of large subunit (26S) ribosomal DNA also showed high similarity (99%, e-value 0.0) with *C. flavescens* isolates, as *C. flavescens* strain ATT120 (FJ743610.1, 638/640) and *C. flavescens* HB 1178 (AM160631.1, 638/640). Thus, according to morphological and molecular characteristics, the strain LEB-AY_{10} was classified as *Cryptococcus flavescens* and deposited in the "Coleção de Culturas Tropical" (Fundação André Tosello, Campinas, Brazil) bank as CCT 7725.

Although some species of *Cryptococcus* have been described as xylanase producers [40–42], Lopes et al. [28]

were the first to report the production of this enzyme by the species *C. flavescens* (strain LEB-AY$_{10}$), followed by the screenings performed by Morais et al. [43] and Carvalho et al. [44].

Enzyme production

In order to evaluate enzyme production using PTB as an inductor, three series of assays were performed. Moisture of PTB samples varied from 50.3 to 70.5%, and they were used to adjust the dry weight of each PTB before the assays.

Assay #1: Preliminary tests using bagasse and molasses

Preliminary tests were performed to evaluate the production of xylanase on PTB supplied with 0.2% molasses as a nutrient source. The maximum activity achieved was 0.84 U/mL at 72 h for the mild PTB. This amount was approximately three times lower than those obtained with the standard medium (1% xylan in synthetic medium). Due to low enzyme production, the soluble C5 fraction of PTB was extracted and tested with molasses or synthetic compounds (mainly nitrogen sources), as described in assay #2.

Assay #2: Removal of possible inhibitors

Fermentation of the microorganism LEB-AY$_{10}$ was performed with the C5 fraction extracted from 3% (dry weight/volume) of moderate PTB supplemented with molasses or synthetic compounds. These nutrients, instead of molasses, were added in order to induce and enhance enzyme production.

Two treatments were also carried out to remove potential inhibitors while the C5 fraction with no treatment was used as control. In the first treatment, the C5 fraction was treated with activated charcoal, as described, and filtered for the recovering of the liquid phase, which was then used in preparation of the media. The other treatment consisted of a two-phase fermentation, adding biodiesel as solvent to the media and collecting the aqueous phase for enzymatic analysis. Results of these six different combinations are shown in Figure 1.

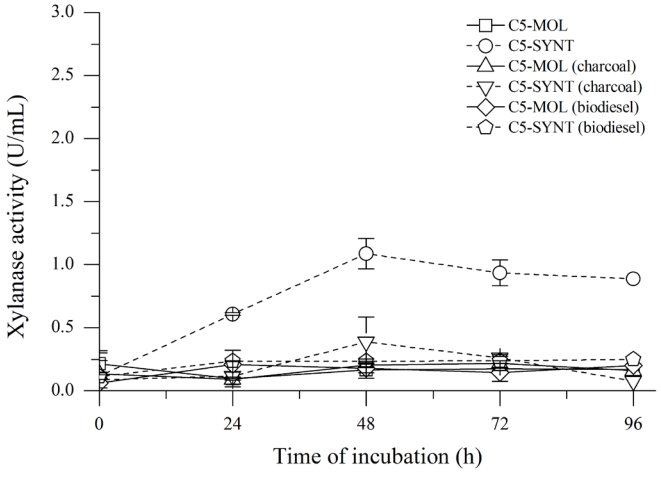

Figure 1 Xylanase activity for *Cryptococcus flavescens* LEB-AY$_{10}$ cultivated in the soluble fraction (C5) treated with activated charcoal, submitted to biphasic fermentation (biodiesel) or without treatment. Synthetic compounds (SYNT) or molasses (MOL) were added.

According to this figure, fermentation of the C5 fraction in synthetic medium showed the highest enzyme production, reaching 1.1 U/mL at 48 hours of fermentation. These results demonstrate that the presence of molasses in the fermentation broth was a negative factor for enzyme production, which may have resulted from the reducing sugars or due to an incidental lack of nitrogen and other nutrients.

Since the treatments for inhibitor removal reduced the enzyme production, composition of the synthetic medium (without pure xylan) was selected for fermentation of PTB and its C5 fractions.

Assay #3: Enzyme production using bagasse and synthetic medium

The fermentation broths were formulated like the synthetic media composition (carbon source, 0.5% peptone, 0.3% yeast extract, 0.01% $MgSO_4.7H_2O$ and 0.1% $(NH_4)_2SO_4$) in which the carbon sources were: raw bagasse, PTB (2.5% dry weight/volume) or C5 fraction (extracted from 5% of dry weight of PTB).

Figure 2 shows the results of xylanase production by LEB-AY$_{10}$ at the defined conditions for each pretreatment (mild, moderate or high). Based on this figure, it can be observed that the microorganism showed the best activity when induced with bagasse pretreated under mild conditions. Using this substrate source, the xylanase activity was 1.97 U/mL for the PTB and 1.92 U/mL for the C5 fraction, both at 36 h of fermentation.

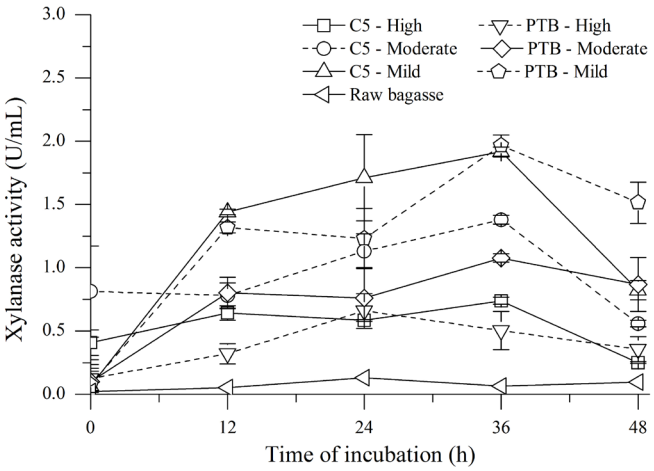

Figure 2 Xylanase activity for *C. flavescens* LEB-AY$_{10}$ using pretreated bagasse (PTB), the soluble fraction (C5) or raw bagasse, in synthetic medium.

In an attempt to understand the different activities, different substrate sources were considered. The composition of HMF and sugars released in the pretreatments are showed in Table 1. However, as can be seen in this table, sugars released and HMF produced are meaningless, especially in the fermentation medium composition, and may not significantly affect microorganism behaviour.

According to Table 1, xylose is the main sugar in pretreated bagasse while sucrose is predominant in raw bagasse. For galactose and xylose, the more intense the treatment, greater is the quantity of sugars released. However, high pretreatment showed lower levels of sugars than

Table 1 HMF, sucrose and monomeric sugars analyzed by HPAEC-PAD (averages of duplicate analysis).

Media	*HMF*	*Arabinose*	*Galactose*	*Glucose*	*Sucrose*	*Xylose*	*Mannose*	*Fructose*
				Composition after extraction of the C5 fraction (mg/L)				
C5-High	86.6	167.2	116.1	446.5	nd	2515.5	68.8	118.2
C5-Moderate	198.8	281.6	114.1	803.5	nd	2322.8	90.0	306.2
C5-Mild	< 20	412.0	< 20	nd	nd	401.7	nd	nd
				Composition of media at the beginning of fermentation (mg/L)				
C5-High	< 20	78.8	41.4	205.1	< 20	953.4	21.7	40.6
C5-Moderate	< 20	87.9	36.3	250.4	nd	720.7	28.3	90.9
C5-Mild	< 20	158.2	< 20	40.3	nd	143.8	60.1	nd
PTB-High	< 20	79.4	69.9	177.6	nd	1097.9	119.8	66.2
PTB-Moderate	< 20	137.9	63.8	373.8	nd	1016.4	44.2	152.8
PTB-Mild	< 20	275.8	38.0	nd	nd	311.4	nd	nd
Raw bagasse	Nd	Nd	45.2	574.6	1203.8	24.5	< 20	620.7

Abbreviations: nd: not detected.

moderate treatment in cases of arabinose, glucose, mannose and fructose. The trend of sugars degradation at higher temperatures was previously reported by Ruiz et al. [18]. These authors also reported that the increase in pretreatment temperature led to a higher quantity of sugars up to a certain temperature, above which greater sugar degradation took place. The authors studied the composition of sunflower stalks hydrolyzates after steam explosion, between 180°C and 230°C, and verified that the higher sugar yields corresponded to intermediate temperatures (around 210°C). Solubilisation and stability were also dependent on the sugar type.

Comparing the composition of the C5 fraction after its extraction with the composition of media after sterilization at 121°C for 20 min in Table 1, it can be observed that the amount of free sugar and HMF were reduced substantially. Hemicellulosic sugars may degrade to weak acids, furan and phenolic derivatives as a function of thermal processes and pretreatment conditions [45]. Sterilization with heat may have also contributed to the formation of these compounds, and they may be responsible for decreased pH after autoclaving (data not shown). They may inhibit subsequent fermentative processes, leading to low yields and productivities [46]. In the case of xylanase production, greater amounts of free sugars may also repress enzyme induction. Based on the studies of Mandal et al. [47], despite the presence of xylan and low amounts of xylose and arabinose in the media, supplementation with xylan increased the production of xylanase; higher concentrations (>0.1%) of these sugars repressed xylanase biosynthesis by *Bacillus cereus* BSA1, and addition of 0.1% glucose also repressed xylanase production.

According to the results of xylanase activities, the microorganism LEB-AY_{10} can grow well in a broth containing the C5 fraction of bagasse subjected to mild pretreatment, supplemented with components of the synthetic medium. Although the amount of substrate needed to generate the C5 fraction is twice as high as in the PTB, the C5 fraction was selected to allow for monitoring of both biomass and enzyme activities during fermentation. Furthermore, because the C5 fraction is obtained by water washing of the PTB, the solid retentate may be reused in cellulase induction or hydrolysed and used as a glucose-rich substrate for biotechnological applications. As stated by Aguiar et al. [48], washed substrates release higher amounts of glucose upon hydrolysis by cellulolytic enzymes due to the presence of water-soluble inhibitory compounds in unwashed material. Therefore, the C5 fraction is often a "waste" from these processes and is very suitable for xylanase induction or pentose extraction.

Optimization of culture media

Selection of factors

A PB experimental design was utilized, considering the results of previous assays, in order to improve the fermentation process and consequently increase enzyme production. Table 2 shows the PB matrix and the main responses for xylanase activity. Effects of the variables were analysed at 24, 36 and 48 h according to the PB design, and are shown in Figure 3. The effects for maximum activity of each assay divided by the respective fermentation time (MAX/t) were also estimated, as a parameter of productivity, so as to aid in interpretation of the results.

Considering the effect of the estimates in Figure 3 the curvature showed positive effects, and according to Cockshott and Sullivan [49] this parameter indicates the degree to which the centre points differ from the non-centre points, and a positive effect indicates that responses occur at or beyond the extremes.

Only the variable magnesium sulphate had no significant effect, at 90% confidence, for xylanase production for up to 48 h of incubation in the studied concentration ranges. It was therefore fixed at the low level (0.01 g/L). The increase

Table 2 PB matrix showing the coded levels and real values (in brackets) followed by responses of xylanase activity at 24 h, 36 h, 48 h and maximum activity/time (MAX/t) of incubation.

Assay	*Temperature (°C)*	*Agitation (rpm)*	*Substrate (g/L)*	*pH*	*Yeast extract (g/L)*	*Peptone (g/L)*	*$MgSO_4.7H_2O$ (g/L)*	*$(NH_4)_2SO_4$ (g/L)*	*24 h*	*36 h*	*48 h*	*MAX/t*
1	1 (35)	-1 (100)	1 (150)	-1 (5)	-1 (1)	-1 (0)	1 (0.5)	1 (2)	0.53	0.69	0.58	0.0191
2	1 (35)	1 (200)	-1 (50)	1 (7)	-1 (1)	-1 (0)	-1 (0.1)	1 (2)	0.47	0.88	0.69	0.0245
3	-1 (25)	1 (200)	1 (150)	-1 (5)	1 (3)	-1 (0)	-1 (0.1)	-1 (0)	0.79	0.47	0.81	0.0168
4	1 (35)	-1 (100)	1 (150)	1 (7)	-1 (1)	1 (4)	-1 (0.1)	-1 (0)	0.81	0.63	0.31	0.0336
5	1 (35)	1 (200)	-1 (50)	1 (7)	1 (3)	-1 (0)	1 (0.5)	-1 (0)	0.28	0.00	0.22	0.0117
6	1 (35)	1 (200)	1 (150)	-1 (5)	1 (3)	1 (4)	-1 (0.1)	1 (2)	0.44	0.28	0.53	0.0111
7	-1 (25)	1 (200)	1 (150)	1 (7)	-1 (1)	1 (4)	1 (0.5)	-1 (0)	0.77	0.68	0.39	0.0321
8	-1 (25)	-1 (100)	1 (150)	1 (7)	1 (3)	-1 (0)	1 (0.5)	1 (2)	1.38	1.00	1.49	0.0310
9	-1 (25)	-1 (100)	-1 (50)	1 (7)	1 (3)	1 (4)	-1 (0.1)	1 (2)	1.93	2.16	1.36	0.0601
10	1 (35)	-1 (100)	-1 (50)	-1 (5)	1 (3)	1 (4)	1 (0.5)	-1 (0)	0.51	0.33	0.21	0.0214
11	-1 (25)	1 (200)	-1 (50)	-1 (5)	-1 (1)	1 (4)	1 (0.5)	1 (2)	1.04	0.61	0.44	0.0433
12	-1 (25)	-1 (100)	-1 (50)	-1 (5)	-1 (1)	-1 (0)	-1 (0.1)	-1 (0)	0.70	0.58	0.43	0.0293
13	0 (30)	0 (150)	0 (100)	0 (6)	0 (2)	0 (2)	0 (0.3)	0 (1)	1.48	1.51	1.83	0.0382
14	0 (30)	0 (150)	0 (100)	0 (6)	0 (2)	0 (2)	0 (0.3)	0 (1)	1.19	1.53	1.58	0.0329
15	0 (30)	0 (150)	0 (100)	0 (6)	0 (2)	0 (2)	0 (0.3)	0 (1)	1.14	1.32	1.57	0.0327
16	0 (30)	0 (150)	0 (100)	0 (6)	0 (2)	0 (2)	0 (0.3)	0 (1)	1.12	1.19	1.06	0.0330

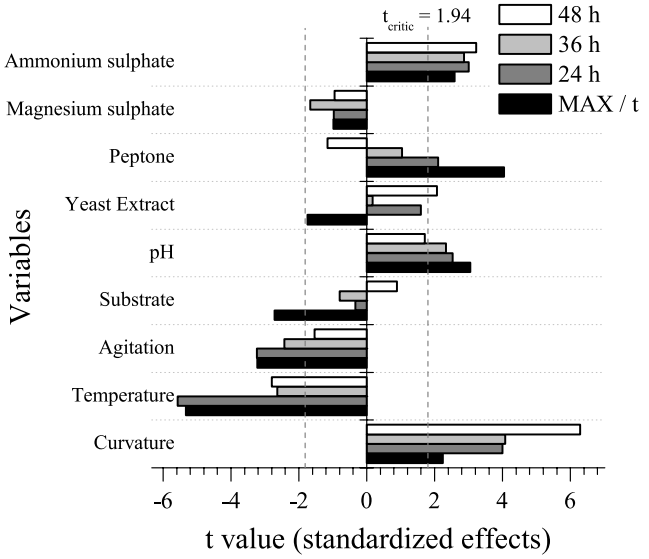

Figure 3 Bar chart showing the estimate of effects in the PB design at 90% confidence determined by the Student's t-test. Vertical dashed lines represent t_{critic}=1.94 (two-tailed test, p<0.1; 6 degrees of freedom). MAX/t: maximum activity for each fermentation divided by its time of occurrence.

in pH apparently had a positive effect on enzyme induction, however, because the culture media is not buffered, the initial value decreased after sterilization and all trials presented an initial pH in the range from 4.9 to 5.7 (except the trial 9 that showed a pH of 6.4). Because the use of phosphate buffered media was prejudicial for xylanase production by this microorganism [28], this variable was fixed at 8.0 (before sterilization).

Both agitation and temperature showed increasingly negative effects (p-value < 0.1). High agitation in baffled flasks may have been detrimental to the microorganism, but aeration is important for enzyme production. Thus, this parameter was fixed at 150 rpm and the volume of media was reduced to 10% of the total volume of the flask. Incubation of *C. flavescens* LEB-AY_{10} at 35°C hindered enzyme induction, different from the results of Castro et al. [50], who obtained 2.5 times greater xylanase production by *Aspergillus* FP-470 at 45°C than at 37°C, although growth was seriously affected. Because the highest activities were found in assays 9 (2.16±0.04 U/mL at 36h) and 13 (1.83±0.08 U/mL at 48h), and considering positive curvature effects, the temperature was fixed at 28°C.

Ammonium sulphate was statistically significant at all times and the increase in concentration proved to be beneficial for enzyme production. Yeast extract and peptone also appeared to be favourable for enzyme production, but the data were not statistically significant. With respect to the substrate concentration, it was statistically significant in the concentration range studied and showed a negative effect only when considering MAX/t. However, the substrate is usually an important parameter, and in this case it may be masked by the effects of other variables. Therefore, in order to define the best substrate concentration, other variables were fixed and the substrate was assayed at 37.5, 75 and 150 g/L, as shown in Figure 4.

The use of 37.5 g/L of substrate showed the lowest enzyme expression and biomass production (2.27 U/mL

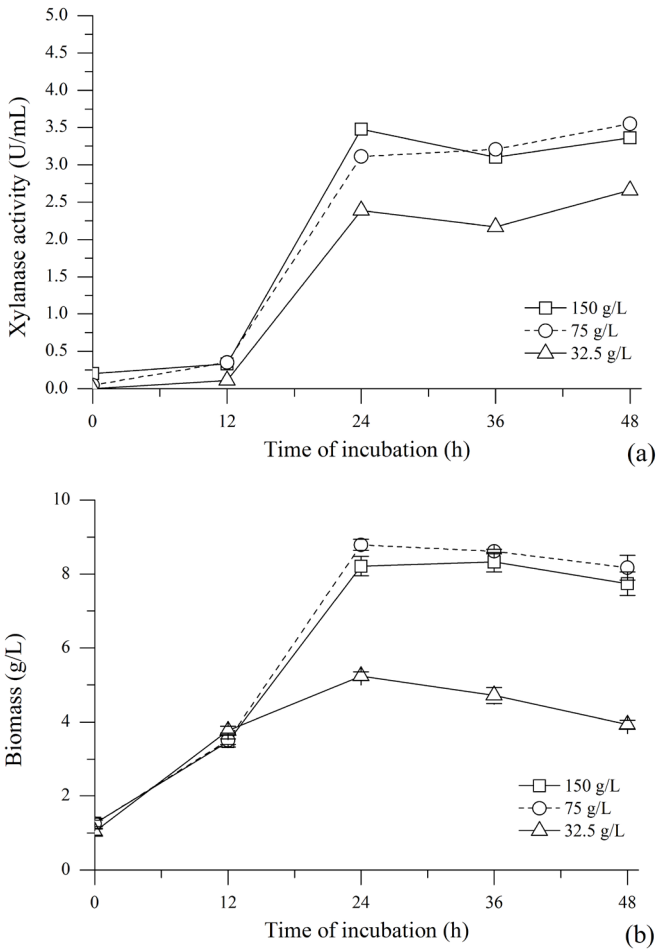

Figure 4 (a) Xylanase activity and (b) biomass for different concentrations of the C5 fraction.

and 3.93 g/L at 48 h, respectively), while the other two FC5 concentrations (75 and 150 g/L) showed the same tendency for both responses, as can be seen in Figure 4. Based on these trials, the C5 fraction concentration was fixed at 75 g/L (w/v) of PTB in mild conditions. Inoculum volume and the incubation time were also assayed (data not shown) to improve cell efficiency.

Optimization

After global analysis of the PB design effects, a CCRD experimental design was performed to evaluate the influence and interactions among higher levels of nitrogen sources on xylanase production (Table 3). Other variables were fixed as described in previous paragraphs: 75 g/L of substrate, 0.01 g/L magnesium sulphate, pH 8.0, 28°C and 150 rpm. Results for xylanase activity and biomass concentration are shown in Figure 5.

The pH values at the start of the process were 5.6-6.1 and at the end ranged from 8.2-8.6. According to Table 3 and Figure 5, at the end of the fermentation (60h) the activities were between 0.73 and 5.06 U/mL, while biomass oscillated between 3.68 and 5.69 g/L. The highest activities were obtained in trial 13, which corresponds to an axial point ($-\alpha$) of ammonium sulphate, reaching 5.06±0.17 U/mL, pH 8.53 and 5.69±0.10 g/L of biomass at 48h of induction.

Reproducibility of the assays at the central point condition (2.62 ± 0.22) allowed for analysis of the regression

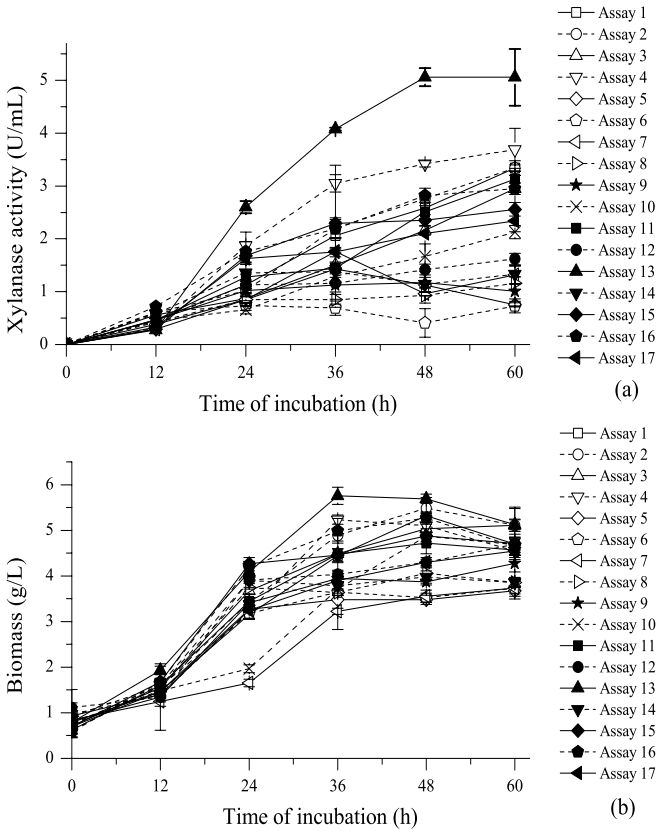

Figure 5 (a) Xylanase activity and (b) biomass concentration for the CCRD.

coefficients of the variables at 90% of confidence. As can be observed in Table 4, no significant interactions ($p < 0.1$) were observed among factors. At 48 and 60 h of incubation, the times at which the highest activities were detected, only the quadratic term of yeast extract (x_1^2) and the linear term of ammonium sulphate (x_3) were statistically significant ($p < 0.10$). Therefore, the other terms were ignored and a new model (for 60 h) was generated, as represented by equation 1:

$$\text{Activity} = 2.66 - 0.42\, x_1^2 - 1.14\, x_3 \quad (1)$$

The analysis of variance (ANOVA) was used to verify the validity of the results. As the variance explained by the model was 85.9% and $F_{calculated}$ was 15.7 times higher than F_{listed} ($F_{14;2;0.1} = 2.73$), the model was considered acceptable and the response surface and contour plots were constructed as shown in Figures 6a and b, respectively.

By analysis of Figure 6, the best condition for enzyme production is from 2 to 4 g/L of yeast extract (from level -1 to 1) and 1.68 g/L of ammonium sulphate (level $-\alpha$), which are the same conditions of assay 13 for these factors. Thus, conditions of assay 13 were selected for model validation. Furthermore, since the peptone terms in equation 1 were not statistically significant, and by analysis of the response surface the use of yeast extract at the lower level is close to the optimal condition, it was decided to evaluate all variables at the lowest levels. All factors were also assayed at the condition $-\alpha$ (1.32 g/L yeast extract, 3.32 g/L peptone, 1.32 g/L ammonium sulphate), as this condition would decrease the costs of operation (by reduction of nutrient concentrations) without a significant loss in final enzyme activity. Results can be observed in Figure 7.

Table 3 Coded and real values (in brackets) for the CCRD matrix followed by responses of xylanase activity at 48 and 60h of incubation.

	Coded Levels (Real values, g/L)			*Xylanase activity (U/mL)*	
Assay	*Yeast extract*	*Peptone*	*Ammonium sulphate*	*48 h*	*60 h*
1	-1 (2)	-1 (4)	-1 (2)	2.58±0.21	3.34±0.14
2	1 (4)	-1 (4)	-1 (2)	2.76±0.06	3.34±0.06
3	-1 (2)	1 (6)	-1 (2)	2.16±0.09	2.95±0.38
4	1 (4)	1 (6)	-1 (2)	3.42±0.06	3.69±0.40
5	-1 (2)	-1 (4)	1 (4)	1.13±0.14	0.75±0.04
6	1 (4)	-1 (4)	1 (4)	1.17±1.33	0.73±0.13
7	-1 (2)	1 (6)	1 (4)	0.96±0.04	1.32±0.17
8	1 (4)	1 (6)	1 (4)	0.93±0.14	1.16±0.34
9	-1.68 (1.32)	0 (5)	0 (3)	1.17±0.03	1.01±0.28
10	1.68 (4.68)	0 (5)	0 (3)	1.67±0.23	2.13±0.12
11	0 (3)	-1.68 (3.32)	0 (3)	2.51±0.12	3.13±0.07
12	0 (3)	1.68 (3.32)	0 (3)	1.42±0.10	1.62±0.11
13	0 (3)	0 (5)	-1.68 (1.32)	5.06±0.17	5.06±0.54
14	0 (3)	0 (5)	1.68 (4.68)	1.14±0.08	1.33±0.05
15	0 (3)	0 (5)	0 (3)	2.35±0.11	2.56±0.13
16	0 (3)	0 (5)	0 (3)	2.82±0.14	2.95±0.11
17	0 (3)	0 (5)	0 (3)	2.10±0.72	2.34±0.20

Note: Means of analyses followed by ±standard error.

Table 4 Effects table of nitrogen sources for the CCRD of the strain *C. flavescens* LEB-AY10, where x_1 is the coded representation for yeast extract, x_2 for peptone and x_3 for ammonium sulphate; L and Q refer to the linear and quadratic terms, respectively.

Factors	*24 h*		*36 h*		*48 h*		*60 h*	
	Effect	*p-value*	*Effect*	*p-value*	*Effect*	*p-value*	*Effect*	*p-value*
Mean	1.71	0.0001	2.08	0.0001	2.44	<0.0001	2.63	<0.0001
x_1 (L)	0.06	0.7707	0.53	0.0493	0.33	0.1937	0.41	0.1608
x_1 (Q)	-0.73	0.0084	-0.63	0.0379	-0.82	0.0141	-0.83	0.0225
x_2 (L)	0.07	0.7235	0.04	0.8499	-0.29	0.2435	-0.23	0.4064
x_2 (Q)	-0.48	0.0508	-0.56	0.0552	-0.44	0.1267	-0.26	0.3861
x_3 (L)	-0.54	0.0216	-1.26	0.0008	-1.95	0.0001	-2.29	<0.0001
x_3 (Q)	0.07	0.7329	0.45	0.1120	0.36	0.1944	0.32	0.3011
$x_1.x_2$	0.25	0.3291	0.40	0.2160	0.29	0.3743	0.22	0.5422
$x_1.x_3$	-0.19	0.4446	-0.01	0.9643	-0.36	0.2656	-0.14	0.6902
$x_2.x_3$	-0.12	0.6322	0.05	0.8668	-0.17	0.5997	0.26	0.4706
	R^2=	0.807	R^2=	0.893	R^2=	0.933	R^2=	0.931

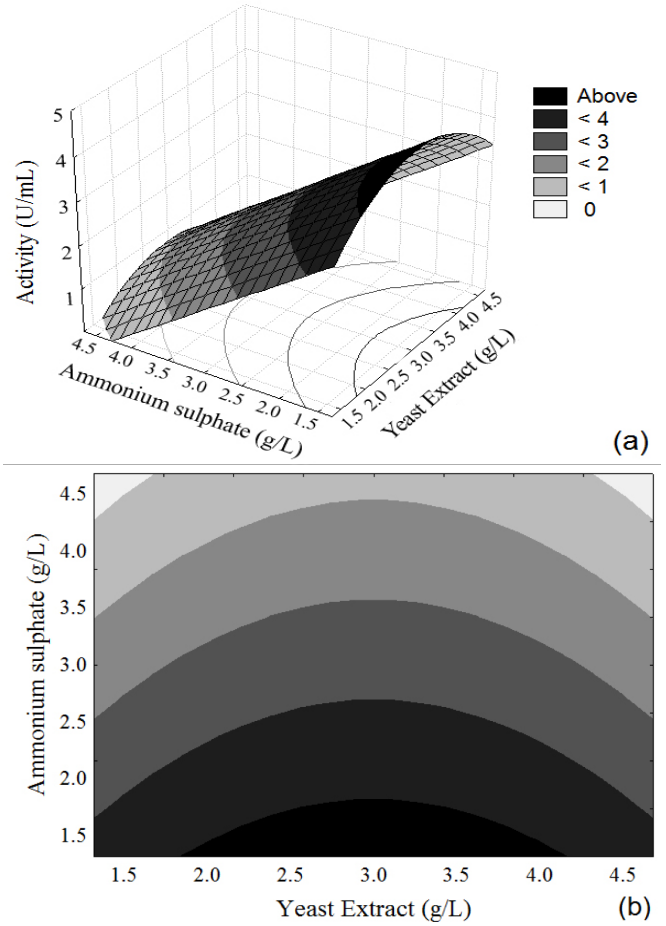

Figure 6 (a) Response surface and (b) contour plot for xylanase activity as a function of yeast extract and ammonium sulphate concentrations.

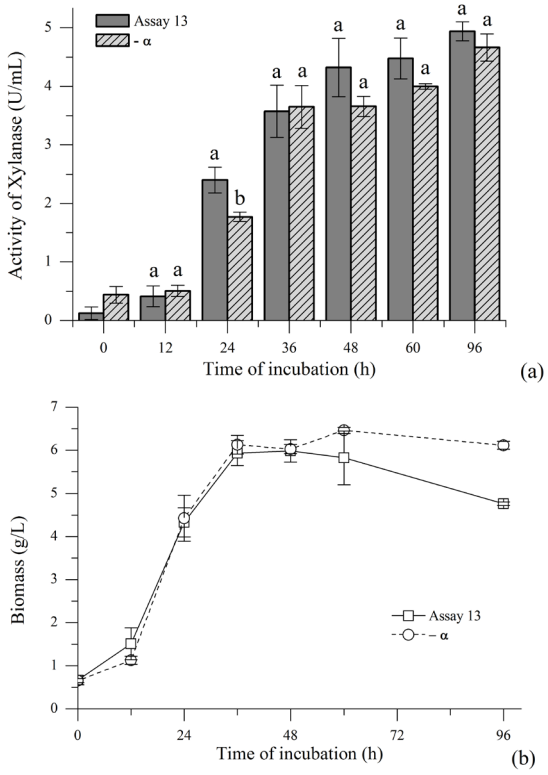

Figure 7 (a) Xylanase activity and (b) biomass for validation of results from assay 13 and study of all factors at the conditions of –α. Error bars represent the standard deviation among fermentation replicates and different letters [a,b] indicate that enzymatic activity differed significantly between means according to Tukey's test (α = 0.10) for different media compositions.

According to the analysis of variance and Tukey's test of the three replicates at a significance level of 10% ($p < 0.1$), there is no statistical difference between xylanase activity for assay 13 and condition -α. Therefore, because level -α is less expensive than assay 13, it was selected as the optimized media. Under these conditions, the maximum xylanase activity was 4.67±0.23 U/mL, reaching 6.12±0.09 g/L of biomass at 96 h. Similar activity values on sugarcane bagasse were reported for the thermolabile xylanase produced by *C. adeliae*, which showed 65 nkat/mL (equivalent to 3.9 U/mL) on optimized medium containing 24.2 g/L of alkali treated bagasse supplemented with 10 g/L of yeast extract [41].

Considering that the optimal temperature for xylan hydrolysis by xylanase enzymes from *C. flavescens* LEB-AY$_{10}$ is 80°C, according to Lopes et al. [28], activity may be up to 1.78 times higher at that temperature. Therefore, when measured at 80°C, the enzyme activity was 8.33±0.36 U/mL at 96 h of fermentation under conditions of level –α.

In the present study, activity in the optimized conditions was 5.6 times higher than the media containing molasses and 2.4 times higher than the initial tests using FC5 supplemented with synthetic media. Although no interactions among factors were significant, in the range of the studied factors, the effects of the factors were evaluated and the strategies utilized during this work permit for optimization of the process.

Conclusion

In this work, the strain LEB-AY$_{10}$, known as a thermostable xylanase producer, was identified as *Cryptococcus flavescens* by sequencing of the ITS1-5.8S-ITS2 region and D1/D2 domains of the large subunit (26S) ribosomal DNA. Sugarcane bagasse treated by SE under mild conditions (12 kgf/cm²) showed to be suitable for use as a xylanase inducer by this microorganism. The results suggest that increased temperatures during pretreatment may cause sugar or lignin degradation to inhibitory compounds which hinder enzyme expression. In this case, treatments with activated charcoal or two-phase fermentation were not effective. Likewise, supplementation of media with synthetic compounds was preferable compared to 0.2% of sugarcane molasses. Finally, experimental design tools helped to delineate strategies, and consequently increase xylanolytic activity by up to 5.6 times, reaching 4.67 U/mL (at 50°C) or 8.33 U/mL (at 80°C), and 6.12 g/L of biomass at 96 h of induced incubation. Because the enzyme produced by *C. flavescens* is thermostable, it can be recovered and reused many times either as soluble or immobilized enzymes in processes such as oligo-xylan production.

Abbreviations

FC5: fraction containing pentoses; MAX/t: maximum activity/time; PB: Plackett-Burman experimental design; PTB: pretreated bagasse; SE: steam explosion.

Acknowledgements

The authors thank the Brazilian National Council for Scientific and Technological Development (CNPq) and Foundation for Research Support of the State of São Paulo

(FAPESP) for the scholarships, the "Centro de Tecnologia Canavieira" (CTC, Piracicaba-SP, Brazil) for kindly providing the steam exploded bagasse and the "Usina Ester Ltda" (Cosmópolis - SP, Brazil) for supplying the raw bagasse.

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