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Xylanases from *Cryptococcus flavus* isolate I-11: Enzymatic profile, isolation and heterologous expression of *CfXYN1* in *Saccharomyces cerevisiae*

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ABSTRACT

The aim of this study was to characterize the xylanolytic activity of *Cryptococcus flavus* isolate I-11. This microorganism was isolated from the Brazilian Cerrado, and enzyme plate assays showed that it also produces amylase and CMCase activity. The xylanolytic production of *C. flavus* isolate I-11 was improved by using a suitable combination of the carbon and nitrogen sources, reaching 130 U/mL. A zymogram assay was performed showing three xylanase activity bands. The cDNA of one xylanase gene, *CfXYN1*, was obtained and preliminary expression analysis was performed on RNA samples collected after yeast growth on different carbon sources. This indicated that the *CfXYN1* gene is transcribed in the presence of xylose, sugar cane bagasse and carboxymethyl cellulose, but not in the presence of glucose, as carbon source. The cDNA of *CfXYN1* was cloned and expressed in *Saccharomyces cerevisiae*. The recombinant enzyme was partially characterized and showed an optimum at a pH of 3.0 and temperature of 50 °C. The recombinant enzyme retained 70% of its initial activity after pre-incubation for 30 min at the optimum pH and temperature. Computational analysis predicted a molecular weight of 21.2 kDa, and an isoelectric point of 7.02. The *Cfxyn1p* has 209 amino acids, including a signal peptide consisting of 16 amino acids.

1. Introduction

Xylanases are enzymes responsible for the hydrolysis of xylan, the main constituent of hemicellulose, and are the second most abundant polysaccharide in nature [1]. Endoxylanases catalyse the hydrolysis of the xylan backbone, producing xylooligosaccharides. These can further be converted into xylose by β-D-xylosidases, which can be used as an energy source by different microorganisms [2].

The importance of xylanases in industrial processes is evidenced by the number of patent applications regarding this group of enzymes. The North American and European patent offices registered 1153 and 1003 patents concerning xylanases, respectively, in 2007. Xylanases have been applied in various industrial processes, such as the production of bread [3], the extraction and clarification of juice pulp [4] and textile refinery [5]. However, the main application of xylanases is in the pulp and paper industry, where they are used in pre-treatment prior to bleaching [6]. Enzymatic treatment with xylanases reduces the amount of chlorine required in this process. Most xylanases utilized in the pulp and paper industry have an optimum at a pH above 6.0, and various commercial xylanases with optima at basic pH are available [7].

Interest in xylanases with other biochemical properties has increased recently, due to their applicability in the production of bioethanol from lignocellulosic materials [8]. In this process, lignocellulosic materials are usually pre-treated in an acid environment, and further enzymatic hydrolysis is performed to hydrolyse the remaining cellulose and hemicellulose into monomeric sugars [9]. Despite the number of hydrolytic enzymes, including xylanases, described in the literature, the hydrolysis of lignocellulosic materials is still hampered by enzyme inhibition [10], by the substrate and/or pH (non-optimal conditions). The characterization of new xylanases is therefore necessary in order to supply the demand for such enzymes in industrial applications.

Due to their variable biotechnological applications, xylanolytic enzymes have been extensively isolated from different microorganisms, including filamentous fungi [11], bacteria, protozoa, insects, seeds [12] and even wheat flour [13]. Surprisingly, there are few reports on the isolation of xylanases from yeast [14–16]. The yeast *Cryptococcus flavus* isolate I-11, which exhibits amylase, carboxymethyl cellulase (CMCase) and xylanase activities, was recently isolated from leaves and flowers of the Brazilian Cerrado [17]. The Brazilian Cerrado is the second largest Brazilian biome, after Amazonia [18]. It covers 21% of the country, and is

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the most extensive savannah region in South America. To the best of our knowledge, this is the first yeast isolated from the Brazilian Cerrado whose enzymatic profile has been described and characterized.

The amylase activity of this microorganism has been reported previously [17,19]. In the current study, the xylanolytic profile of the yeast *C. flavus* isolate I-11 and the best growing conditions for the production of such hydrolases were studied. The *CfXYN1* gene encoding for xylanase activity (accession number EU330207) was identified and characterized, and its expression profile on different carbon sources was analysed. Furthermore, *CfXYN1* was expressed in *Saccharomyces cerevisiae* and the recombinant protein was partially characterized. Finally, the biochemical properties and possible applications of such an enzyme are discussed.

2. Materials and methods

2.1. Isolation of C. flavus isolate I-11 from the Brazilian Cerrado

C. flavus isolate I-11 was isolated from samples of leaves and flowers collected in the Brazilian Cerrado using YNB agar plates (6.7% yeast nitrogen base without amino acids, 2% glucose, 1.5% agar) supplemented with 1% xylan (from oat spelts, Sigma–Aldrich, Brazil). Xylanolytic activity was visualized by the formation of clear halos around individual colonies when stained with Congo red (1%) [20]. The colony with the largest hydrolytic halo was isolated by plating several times on agar plates with xylan as the sole carbon source. The ability of the same colony to grow on different carbon sources, such as starch and carboxymethyl cellulose, was investigated.

2.2. Optimization of growth conditions for xylanase production

C. flavus isolate I-11 was pre-grown in YPD medium (2% bactopeptone, 1% yeast extract and 2% glucose) overnight. It was subsequently inoculated to an initial $OD_{620\,nm}$ of 1.0 in minimal medium containing 1% (w/v) sugar cane bagasse (SCB) or xylan (from oat spelts, Sigma–Aldrich, Brazil) as the carbon source. In combination with each carbon source, six nitrogen sources were tested: YNB (1.34% w/v), yeast extract (1.34% w/v), urea (1.34% w/v), ammonium sulphate (1.34% w/v), yeast extract with ammonium sulphate (0.67%:0.67%) and yeast extract plus urea (0.67%:0.67%). The yeast cells were cultivated at 28 °C on a rotational shaker at 180 rpm. Five-mL samples were withdrawn at different times, centrifuged at 5000 rpm for 5 min, and the supernatants were assayed for xylanase activity.

2.3. Xylanase activity assay

The xylanase activity was assayed as previously described [21]. Briefly, the assay mixture consisted of 100 μL of culture supernatant and 900 μL of a 1% (w/v) xylan suspension in 50 mM citrate buffer (pH 5.0). The mixture was incubated at 50 °C for 5 min followed by immediate chilling on ice for 5 min. The amount of reducing sugars released was determined using the dinitrosalicylic acid (DNS) method [22]. One unit of xylanase was defined as the amount of enzyme necessary to release 1 μmol of xylose equivalent per minute.

2.4. Profile of secreted xylanases

The profile of the secreted xylanases was analysed using the supernatant from a 72-h culture of yeast under the optimal conditions. Total proteins were precipitated by the addition of 10% trichloroacetic acid (TCA) and analysed using SDS-PAGE, as previously described [23]. The proteins were visualized by silver staining [24]. A zymogram was obtained using SDS-PAGE. Xylan was added in the polyacrilamide gel for the final concentration of 1%. After electrophoresis, the gel was incubated for 1 h in Triton X-100 (2.5%) under agitation, followed by incubation at 40 °C in MES buffer. The presence of xylanolytic activity was detected by staining with 1% (w/v) Congo red solution [20].

2.5. Isolation and cloning of CfXYN1 from C. flavus isolate I-11

The *CfXYN1* was isolated from genomic DNA and RNA by PCR and reverse transcriptase PCR (RT-PCR), respectively. *C. flavus* isolate I-11 genomic DNA isolation was performed as previously described [25]. The *CfXYN1* gene from genomic DNA was amplified with the primers 5xynA (5′ CGGATCCGCGATTGGTAGCATCCGCT 3′) and 3xynA (5′ CGGATCCTTATCCGGAGATGAGCTTCATGT 3′), which were designed based on a xylanase sequence previously described for *Cryptococcus* sp. [14].

For RT-PCR experiments, total RNA from *C. flavus* isolate I-11 grown in YNB medium (6.7% yeast nitrogen base w/o amino acids) supplemented with 1% xylose, 1% sugar cane bagasse, 1% carboxymethyl cellulose or 2% glucose, was extracted using Trizol® (Invitrogen, Brazil) following the manufacturer's instructions. First-strand cDNA was obtained using the *Superscript First-Strand Synthesis System* (Invitrogen, Brazil).

PCR reactions consisted of either 2 μL of the reverse transcriptase reaction or 10 ng of genomic DNA, 10 pmoles of each primer, 2.5 mM $MgCl_2$, and 1 U of Platinum® Taq DNA Polymerase High Fidelity (Invitrogen). Amplification was carried out under the following conditions: denaturation at 94 °C for 2 min; 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The resulting genomic and cDNA amplicons were cloned into the pGEM-T vector and sequenced.

2.6. Sequence analysis

The nucleotide sequence obtained from sequencing of three independent clones was analysed using the programs PHRED [26], PHRAP and CONSED [27]. The web interface of the National Center for Biotechnology Information [28] was used to conduct the search for databank similarity with the aid of BLAST search tools. Multiple alignments were performed using the program ClustalW [29]. Signal sequence prediction was made using the program SignalP [30]. Putative N- and O-glycosylation sites were analysed using the programs NetNGlyc [31] and NetOGlyc [32]. The tertiary structure was predicted by homology modelling through alignment of the primary protein sequence with the SWISS-MODEL protein structure homology-modelling server [33].

2.7. Expression of CfXYN1

Plasmid pGEM-T containing the *CfXYN1* cDNA was restricted with BamHI and inserted into the *S. cerevisiae* YEp351PGK vector [34] previously restricted with BglII. The resulting plasmid was used to transform the *S. cerevisiae* MFL strain. *S. cerevisiae* MFL is an auxotrophic mutant for leucine, obtained by *LEU2* gene disruption of the industrial strain FTPT472. The recombinant clones were selected by plating the transformants on minimal medium plates containing 0.5% (w/v) xylan from oat spelts. Expression of xylanase was observed after 72 h incubation at 30 °C by staining the agar plates with Congo red (1% w/v). *S. cerevisiae* transformed with the expression vector without insert was used as a negative control. The transformant that showed the largest hydrolytic halo was used for further analysis.

2.8. Partial characterization of the recombinant Cfxyn1p enzyme

The effect of pH on enzyme activity was studied by replacing the citrate buffer (50 mM, pH 5.0, described in Section 2.3) with 100 mM sodium citrate buffer at pH 2.0 and 3.0, 100 mM sodium acetate buffer at pH values of 4.0–6.0, 100 mM MOPS buffer at pH 7.0, 100 mM Tris–HCl buffer at pH 8.0 and 100 mM Tris–glycine at pH 9.0. The effect of temperature on the enzymatic activity was determined at pH 3.0, in the range 0–90 °C. The enzyme stability was tested over time at the optimal pH and temperature. Enzymatic activity was calculated from the amount of reducing sugars released, using the DNS method, as described previously [22].

3. Results and discussion

3.1. Xylanase production

The xylanolytic profile of a given microorganism can vary depending on the carbon source on which it is grown. Therefore, xylanase production in a native microorganism can often be improved by optimization of the culture medium, with regard to carbon source, nitrogen source and pH [35]. The xylanolytic activity exhibited by *C. flavus* isolate I-11 was evaluated in two carbon sources, sugar cane bagasse and xylan, and in six different nitrogen sources (see Fig. 1). Xylan as the carbon source led to the highest xylanolytic activity *in vitro* (120 U/mL), which was 10 times higher than the activity obtained when the yeast was grown in SCB (Fig. 1A). Higher xylanolytic activities have also been observed in *A. phoenicis* [36] and *C. adeliae* [37] when xylan was the sole carbon source.

The nitrogen source has also been found to influence the xylanolytic activity of *C. flavus* isolate I-11 and other microorganisms [38,39]. The highest *in vitro* activity (120 U/mL) obtained in this study was with ammonium sulphate (Fig. 1B). Cells grown in YNB medium also showed high xylanolytic activity, whereas cultivation with the yeast extract and urea led to very low or no activity (Fig. 1B). The highest xylanolytic activity in *A. nidulans* [38] has also been found when this microorganism was grown on an inorganic nitrogen source (ammonium sulphate). However, the opposite effect was observed in *Aspergillus awamori* [39], *Cryptococcus* sp. S-2 [14] and *C. adeliae* [37], where the xylanolytic production increased when yeast extract was used as nitrogen source. During all carbon and nitrogen growth tests, the maximum xylanolytic activity was detected in the supernatant after 72 h of cultivation, which corresponds to the beginning of the stationary phase for *C. flavus* isolate I-11 (data not shown). The optimized medium containing xylan and ammonium sulphate (1.34%) was used throughout this study.

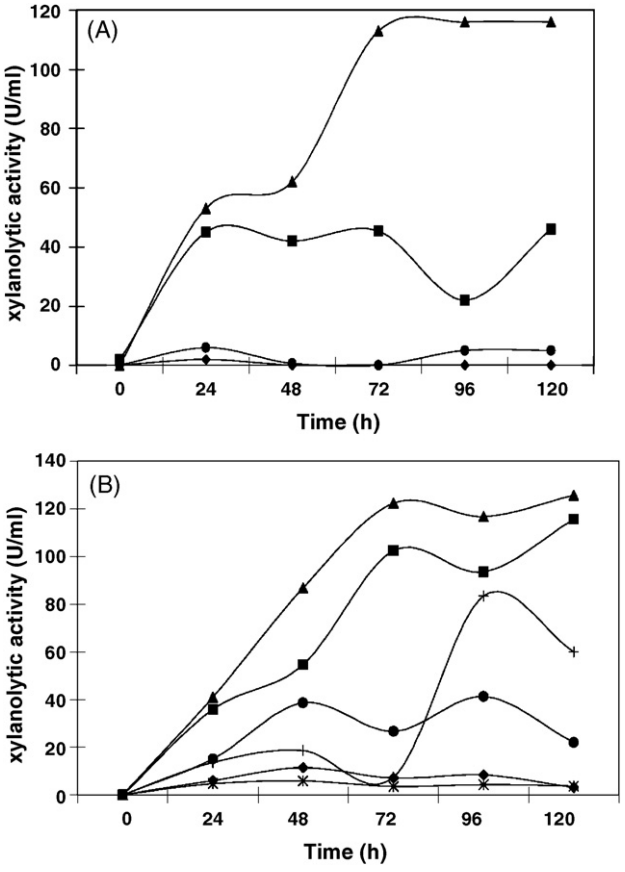

Fig. 1. Xylanase activity detected in the supernatant of *Cryptococcus flavus* isolate I-11 grown on different carbon (A) and nitrogen (B) sources. The experiments were performed in duplicate. Symbols in (A): (▲) xylan + YNB, (■) xylan + yeast extract, (●) SCB + YNB and (♦) SCB + yeast extract. Symbols in (B): (▲) 1.34% ammonium sulphate, (■) 1.34% YNB, (●) 0.67% yeast extract and 0.67% ammonium sulphate, (×) 1.34% urea, (♦) 1.34% yeast extract and (*) 0.67% yeast extract and 0.67% urea.

3.2. Profile of secreted xylanase

The profile of xylanase activities secreted by *C. flavus* isolate I-11 was evaluated using 72-h culture in xylan and ammonium sulphate. The culture supernatant was analysed with SDS-PAGE and simultaneously assayed for xylanase activity using a zymogram (Fig. 2). The zymogram showed three activity bands (Fig. 2, lane 4) with approximate sizes of: 20 kDa, 40 kDa and 50 kDa. In the control, no bands could be seen and no xylanase activity was detected (data not shown).

3.3. Cloning and sequence analysis of CfXYN1

The gene responsible for one of the xylanolytic activities in *C. flavus* isolate I-11 was cloned in PCR and RT-PCR experiments. Initially, the *Cryptococcus* sp. DNA sequence encoding for xylanase [14] was used to design primers for amplification of the xylanase-encoding gene for *C. flavus* isolate I-11. Genomic DNA from *C. flavus* isolate I-11 was extracted and used as a template for PCR reactions with the primers 3XynA and 5XynA. A fragment of 756 bp was obtained and cloned into a pGEM-T vector. This fragment was sequenced and after analysis with BLAST was confirmed as a gene encoding for a xylanase.

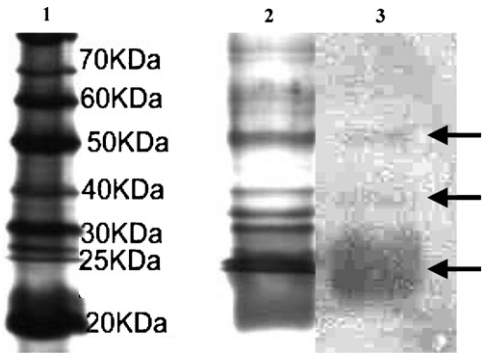

Fig. 2. Xylanolytic profile of *C. flavus* isolate I-11 obtained using SDS-PAGE (lane 2) and a zymogram (lane 3). Cells were grown in xylan and 1.34% ammonium sulphate, and samples were taken immediately (data not shown) and after 72 h (lane 2). The gel resulting from SDS-PAGE was silver stained. The zymogram was obtained in parallel with the sample preparation (lane 3) by adding xylan to the gel. The black arrows indicate the presence of the xylanolytic bands.

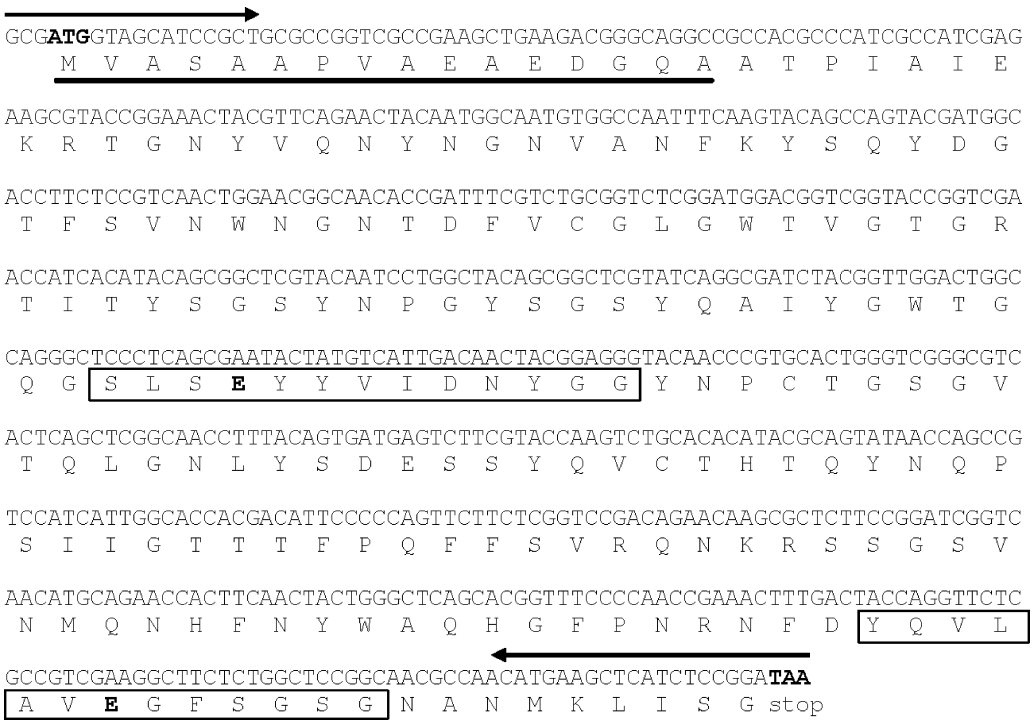

Fig. 3. Nucleotide and deduced amino acid sequence of the *CFXYN1* gene. The deduced signal peptide is underlined. Arrows indicate the orientation and position of the primers 5xynA and 3xynA. The deduced signature sequence is in the box, and the catalytic glutamate residues are shown in boldface.

Possible presence of introns in *CfXYN1* was evaluated by cloning and sequencing the cDNA amplified with the same primers as those used in the genomic PCR reaction. For these experiments, total RNA from *C. flavus* isolate I-11, grown in 1% (w/v) xylan, was extracted and used in RT-PCR as a template. A fragment of 633 bp was obtained, cloned into the pGEM-T vector and then sequenced. The alignments of genomic and cDNA sequences showed that the isolated fragment corresponded to the *CfXYN1* gene and contained 3 introns with consensus sequence for its removal (data not shown). The complete sequence of the *CfXYN1* gene is available at the NCBI portal, accession number EU330207.

The *CfXYN1* open reading frame has 633 nucleotides encoding a 209-amino-acid peptide (Fig. 3). Sequence analysis verified that the *CfXYN1* gene is 91% similar to the xylanase gene from *Cryptococcus* sp. S-2 [14], and the translated primary protein sequence revealed high similarity with other xylanases: 96% with xylanase from *Cryptococcus* sp. S-2, 51% with xylanase from *Aspergillus niger*, 51% with xylanase from *Aspergillus awamori* and 50% with xylanase from *Aspergillus usamii*. The primary sequence alignment showed that these xylanases have some conserved residues, such as the glutamic acid residues, which are important for the catalytic activity. It was also possible to identify the signature signal of xylanase (Fig. 3). The residues responsible for the maintenance of the xylanolytic activity at acid pH [40] were also present in *C. flavus* isolate I-11. The primary protein sequence submitted to the SWISS-MODEL 32 server generated a 3D structure with high similarity to xylanases that belong to the G/11 family of hydrolases.

The deduced amino acid sequence was analysed using the ExPASy server [41]. The predicted molecular weight and isoelectric point were 21.2 kDa and 7.02, respectively. Although it was not possible to predict the signal peptide of Cfxyn1p using the ExPASy platform, the signal peptide was deduced using SignalP [42], and is underlined in Fig. 3. No sites for N-acetylation or O-glycosylation were identified using the ExPASy platform [41], indicating that *Cfxyn1p* might not undergo any post-translation modification. This hypothesis is supported by the experimental observation that there was no difference in size between the native and recombinant *Cfxyn1p* and the predicted size of the protein.

3.4. Expression analysis of CfXYN1

Gene expression for xylanolytic enzymes is regulated by the carbon source in the medium. Preliminary expression analysis of *CfXYN1* was performed with RT-PCR, utilizing RNA extracted from *C. flavus* isolate I-11 grown in different carbon sources: glucose, xylose, SCB and CMC. Gene expression was confirmed when *C. flavus* isolate I-11 was grown in xylose, SCB and CMC (Fig. 4). No transcripts were detected when the yeast was grown on glucose as the sole carbon source (Fig. 4). Transcription of *CfXYN1* in xylose, SCB and CMC can be explained by the absence of catabolic repression previously observed for other microorganisms such *C. adeliae* [43] and *A. niger* [44]. Furthermore, it has been reported that the induction of xylanase takes place in the presence of an inducer molecule [1] (xylose, xylobiose and small xylooligosaccharides), which corroborates the expression of *CfXYN1* in SCB medium. *CfXYN1* expression

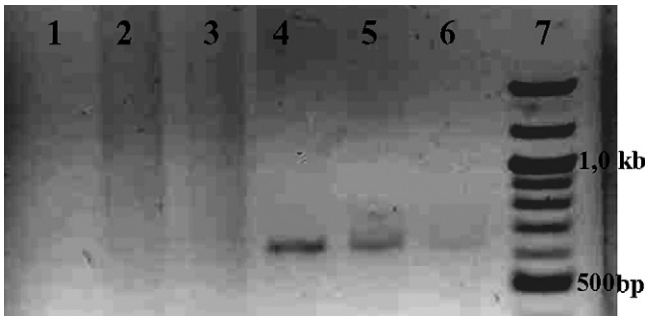

Fig. 4. Results of a RT-PCR experiment showing preliminary expression analysis of *CfXYN1*. Total RNA was extracted from *C. flavus* isolate I-11 grown on different carbon sources: glucose (lane 3), xylose (lane 4), SCB (lane 5) and CMC (lane 6). The negative controls are without DNA (lane 1) and without primers (lane 2).

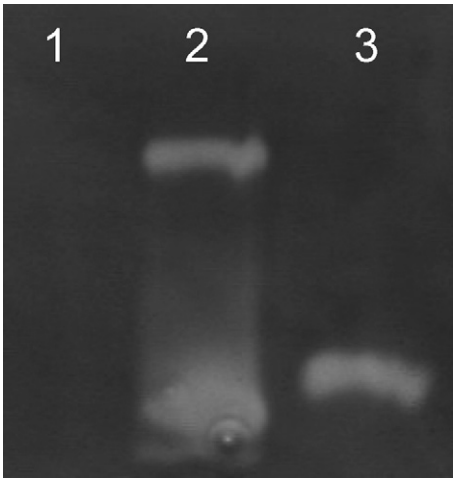

Fig. 5. Results of a zymogram assay using supernatants from *C. flavus* isolate I-11 grown on xylan (lane 2) and *Saccharomyces cerevisiae* expressing Cfxyn1p (lane3). The negative control (lane 1) is *S. cerevisiae* transformed with an empty plasmid.

was also induced by CMC. This can be explained if the hydrolytic genes in *C. flavus* isolate I-11 are under the regulation of only one transcriptional activator, as occurs in *Clostridium cellulovorans* [45]. Expression of *CfXYN1* in xylose could indicate basal expression levels in *C. flavus* isolate I-11. A study related to the regulation of gene expression of hydrolytic enzymes in *Cellulomonas flavigena* [46] showed a basal xylanase activity in the presence of glycerol, cellobiose and xylose, thus indicating that in this microorganism the de-repression caused by the absence of glucose is enough to trigger gene expression of hydrolytic at basal levels.

3.5. Heterologous expression of CfXYN1

The *CfXYN1* gene was expressed in the *S. cerevisiae* MFL strain under the control of the PGK promoter. The functional expression of *CfXYN1* in *S. cerevisiae* was confirmed in the zymogram (Fig. 5). The zymogram obtained from the supernatants of *S. cerevisiae* expressing Cfxyn1p confirmed that *CfXYN1* encodes for the smallest of the three xylanolytic activities detected in *C. flavus* isolate I-11 culture supernatants (Fig. 5). No xylanase activity was detected in the negative control.

The highest recombinant xylanolytic activity detected in the supernatant was 2.5 U/mL. The difference in xylanolytic activity observed between recombinant and native (120 U/mL) Cfxyn1p production could be due to the synergistic action of the three xylanolytic activities detected in the zymogram assay (Fig. 2), increasing the degradation of xylan molecules. Furthermore, the medium used for the production of the recombinant enzyme Cfxyn1p was not optimized. As previously noted, medium optimization could increase the amount of recombinant protein secreted by *S. cerevisiae* [47,48]. The choice of host for heterologous expression is also very important in achieving high levels of expression of the heterologous protein. For instance, the recombinant xylanase from *Aspergillus pullulans* [49] when expressed in *S. cerevisiae* had an activity of 1.6 U/mL, while the same gene expressed in *Pichia pastoris* showed a 25 times higher activity [50].

3.6. Partial characterization of the recombinant xylanase

The *S. cerevisiae* strain expressing the recombinant xylanase was grown in minimal medium and the culture supernatant was used in the partial biochemical characterization of the recombinant protein since no background activity was detected. The optimum pH for Cfxyn1p activity was assessed in the interval from 2 to 9. Enzymatic activity was seen between pH 2.0 and 6.0, and the optimal activity was found at pH 3.0 (Fig. 6A). At pH 2.0, the xylanase activity was 80% of the maximal, while at pH 6.0 only 44% of the optimal activity could be detected. No activity was observed at a pH above 7.0 (Fig. 6A).

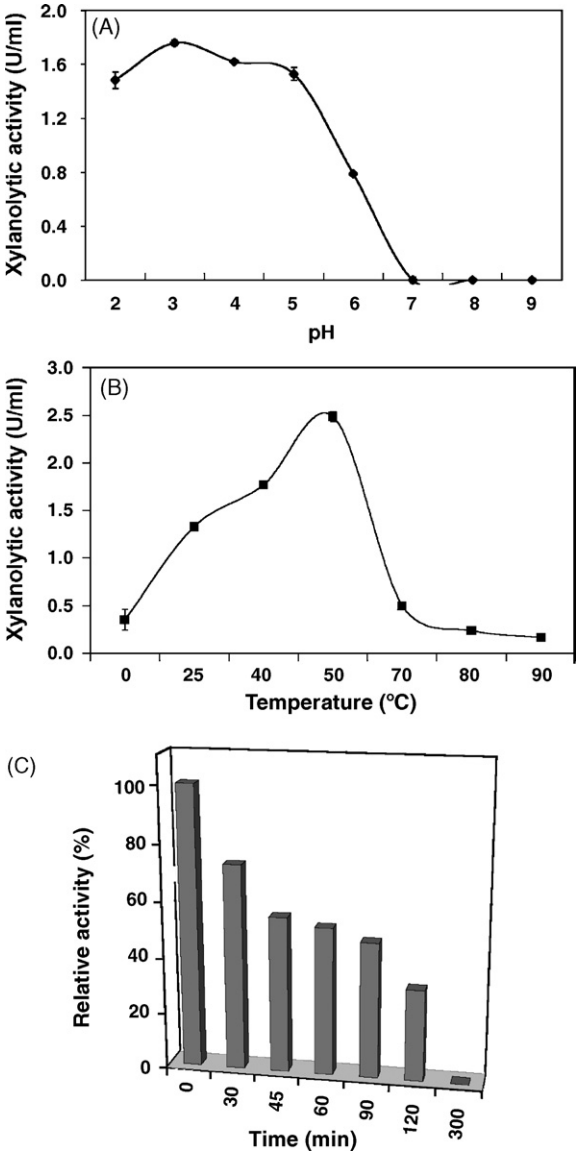

Fig. 6. Partial biochemical characterization of Cfxyn1p expressed in *S. cerevisiae*. Optimal pH (A) was established by determining the xylanase activity in the pH range from 2.0 to 9.0. The optimal temperature (B) was determined in the range 0–90 °C. Recombinant protein stability (C) was tested over time (min) with pre-incubation of the recombinant protein at the optimum pH and temperature. All experiments were performed in triplicate and the standard deviation is shown when relevant.

Xylanases can be grouped into alkaline or acid xylanases, according to their optimal pH. Among the several xylanases studied [4], the alkaline ones are more common and better characterized [6] due to their application in the pulp and paper industry. In contrast, acid xylanases are described only in a few microorganisms such as *Cryptococcus* sp. S-2 [14] (optimal pH 2.0), *Acidobacterium capsulatum* [51] (optimal pH 5.0), *A. pullulans* [52] (optimal pH 2.0), *A. kawachi* [53] (optimal pH 4.0) and *Penicillum* sp. 40 [54] (optimal pH 2.0). This group of xylanases could possibly be used to improve the bioconversion of lignocellulosic raw material where acid hydrolysis is required; for instance, during bioethanol production with simultaneous saccharification and fermentation [55].

The optimal temperature was determined in the range 0–90 °C at pH 3.0. The highest activity of the recombinant xylanase was found at 50 °C, and decreased to 57% when the temperature was lowered to 40 °C (Fig. 6B). At temperatures above 50 °C the enzyme activity was significantly reduced.

In order to determine Cfxyn1p stability at the optimal pH and temperature, the enzymatic activity of the recombinant enzyme incubated at 50 °C at pH 3.0 was followed over time. The supernatant assayed at 0 min was not incubated at 50 °C and its activity was assumed to be 100% (Fig. 6C). The stability assay indicated that the recombinant xylanase retained more than 50% of its initial activity at the optimal temperature and pH for at least 90 min (Fig. 6C). After 120 min, 40% of the initial activity remained. No activity was detected after 300 min.

4. Conclusions

The yeast *C. flavus* isolate I-11 produces at least 3 xylanases, and the gene encoding for a 20 kDa xylanase activity was isolated, successfully expressed in *S. cerevisiae* and partially characterized. Initial optimization of xylanase production in *C. flavus* isolate I-11 demonstrated that this microorganism is a potential xylanase producer. The highest activity obtained in this study (120 U/mL) is higher than that observed for *A. niger* (65 U/mL) [56] and for *Bacillus circulans* (19.28 U/mL) [57], both described as good xylanase producers. However, further studies are necessary for the cultivation of this microorganism on an industrial scale. Sequence analysis and partial biochemical characterization of Cfxyn1p showed that this protein can be classified as a mesophilic acid xylanase belonging to the G/11 family of glycosyl hydrolases. Overall *C. flavus* isolate I-11 is a potential xylanolytic producer and the biochemical characteristics of Cfxyn1p make it a suitable candidate for biotechnological processes.

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