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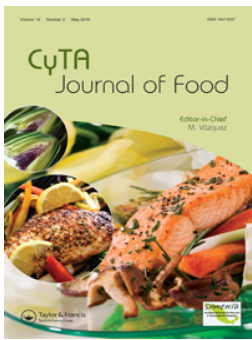
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## Production of thermostable xylanase by thermophilic fungal strains isolated from maize silage Producción de xilanasa termoestable por cepas de hongos termófilos aisladas de ensilaje de maíz

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The search for microorganisms able to produce thermostable xylanases with high yield and characteristics desired for industrial applications has been strongly encouraged since such enzymes are widely used in large-scale processes. In the present study, thermophilic fungal strains able to grow at high temperatures ( $\geq 55$  °C) were isolated from maize silage. The strains were molecularly identified and used for the production of extracellular xylanase by solid-state fermentation using corn cobs as support-substrate material. Species from the genera *Rhizomucor* and *Aspergillus* were identified among the isolated strains and these species demonstrated good ability to produce xylanase under solid-state fermentation conditions. Maximal values of enzymatic activity (824 U/g) and productivity (8.59 U/g.h) were obtained with *Rh. pusillus* SOC-4A (values per g dry weight of fermented medium). The xylanase produced by this fungus presented thermal stability at 75 °C, with maximum activity at 70 °C and pH 6.0, revealing, therefore, great potential for application in different areas.

**Keywords:** maize silage; thermophilic fungi; thermostable enzymes; molecular identification; solid-state fermentation; xylanase

La búsqueda de microorganismos capaces de producir xilanasas termoestables con alto rendimiento y características deseadas para aplicaciones industriales ha sido fuertemente alentada ya que tales enzimas son ampliamente utilizadas en procesos de gran escala. En el presente estudio, cepas de hongos termófilos capaces de crecer en elevadas temperaturas ( $\geq 55$  °C), fueron aisladas a partir de ensilaje de maíz. Las cepas fueron identificadas molecularmente y utilizadas para la producción de xilanasa extracelular por fermentación en estado sólido usando mazorcas de maíz como material de soporte-sustrato. Especies de los géneros *Aspergillus* y *Rhizomucor* fueron identificadas entre las cepas aisladas y demostraron una buena capacidad de producir xilanasa bajo condiciones de fermentación de estado sólido. Los valores máximos de actividad enzimática (824 U/g) y productividad (8,59 U/g.h) fueron obtenidos con *Rh. pusillus* SOC-4A (valores son por g en peso seco de medio fermentado). La xilanasa producida por este hongo presentó estabilidad térmica a 75 °C, con actividad máxima a 70 °C y pH 6,0; revelando, por lo tanto, un gran potencial para aplicación en diferentes áreas, incluyendo en las industrias de alimentos, papel y celulosa, biocombustibles y textiles.

**Palabras clave:** ensilaje de maíz; hongos termófilos; enzimas termoestables; identificación molecular; fermentación en estado sólido; xilanasa

### Introduction

Xylanases (EC 3.2.1.8) are enzymes with the ability to hydrolyze xylan, a polysaccharide constituted by xylose units linked through  $\beta$ -(1 $\rightarrow$ 4) bonds usually found as the most abundant component in hemicellulose structures (Carvalho, Mussatto, Candido, & Almeida e Silva, 2006; Saha, 2003). Xylanases have extensive applications in different industrial sectors. In the food industry, for example, they are used for the clarification of fruit juices (Dhiman, Garg, Sharma, & Mahajan, 2011), to improve the texture, loaf volume, and shelf-life of baked products (Bajaj & Manhas, 2012), and to reduce the viscosity and filtration rate of the brewery mash (Qiu et al., 2010). In the pulp and paper industry, xylanases are used to increase the brightness of the pulp in order to produce papers of superior quality. This application is also advantageous from an environmental point of view because it avoids or reduces the use of chemicals such as chlorine (Li et al., 2010; Saleem, Tabassum, Yasmin, & Imran, 2009). Xylanases are also employed to convert xylan structures present in lignocellulosic biomass wastes to xylose sugar, which can be further used as carbon source for the production of a variety of valuable compounds such as

xylitol (Mussatto & Roberto, 2008) and ethanol (Silva, Mussatto, Roberto, & Teixeira, 2012), among others.

In some cases, the use of xylanase produced by mesophilic organisms is limited as these enzymes generally undergo denaturation at temperatures higher than 55 °C. As a consequence, the efficiency of hydrolysis is decreased and higher enzyme loadings are required to overcome this problem, increasing the costs of the process. The use of thermostable enzymes able to carry out hydrolysis at high temperatures is needed for these kinds of applications. One example of industrial application for thermostable xylanase enzymes is in the pulp and paper industry. For the pulp production, wood is treated at high temperature and basic pH. As a consequence, the enzymatic procedure requires enzymes with high thermostability and active in a broad pH range. Treatment with xylanase at elevated temperatures disrupts the cell wall structure, facilitating lignin removal during the bleaching stages (Georis et al., 2000).

The optimum temperature for activity of most xylanases is around 50–60 °C only, with a half-life of about 1 h at 55 °C (Georis et al., 2000). In the past decade, several microorganisms

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including fungi and bacteria have been reported to produce xylanases with thermostable properties, active at temperatures between 50 and 80 °C (Haki & Rakshit, 2003). However, taking into account the amount of enzymes required for large-scale applications, the search for microorganisms able to produce thermostable xylanases with high yield and characteristics desired for industrial applications is still being pursued. Some thermophilic fungi able to produce xylan-degrading enzymes have been isolated from soil and plant materials. Maize silage is the main source of forage for lactating dairy cows in Europe and North America (Cavallarin, Tabacco, Antoniazzi, & Borreani, 2011). To the best of our knowledge, this material has been understudied for the isolation of xylanase producers' fungal strains.

Agricultural, agro-industrial, and forest residues are usually rich in xylan, which can act as a natural inducer of xylanases by microorganisms. The reuse of such residues has been strongly encouraged in the past years for economic and environmental reasons, in order to minimize pollution and generate compounds of industrial interest from available and low-cost resources. Corn cobs, a broad by-product generated from the corn harvest, are composed of approximately 30% (w/w) xylan (Garrote, Domínguez, & Parajó, 2002) and can be then considered as a material of interest for use on the production of xylanases. The enzyme production by fungi can be done by submerged or solid-state fermentation (SSF) systems. However, in recent years, SSF has received more interest because this process may lead to higher yields and productivities or better product characteristics than submerged fermentations. In addition, capital and operating costs are also lower than in submerged fermentation (Mussatto, Aguiar, Marinha, Jorge, & Ferreira, 2015), and the downstream step for separation of the produced compound is facilitated due to the low water volume used for fermentation (Martins et al., 2011; Mussatto, Ballesteros, Martins, & Teixeira, 2012).

In the present study, efforts were made in order to isolate new thermophilic fungal strains with the ability to produce thermostable xylanase. The fungal strains were isolated from maize silage taking into account that this material has been little explored for this purpose. The selected strains were molecularly identified and used in a subsequent stage for the production of extracellular xylanase by SSF using corn cobs as support-substrate material. Finally, the thermostability of the produced enzymes as well as the pH and temperature where the activity is maximized were determined.

## Material and methods

### Isolation of thermophilic fungal strains

Maize silage was collected from a local farm in the province of Chihuahua, México. Soon after collection, the samples were cooled in ice and transported to the laboratory within 6 h. For the experiments, 1 g of maize silage was dissolved in 100 mL of sterilized distilled water, and the obtained solution was diluted up to  $10^4$  times. Afterwards, 0.1 mL of the diluted solution was added to potato dextrose agar (PDA) plates, which were incubated at 55 °C for 5 days. Fungi were isolated from each plate and continuously subcultured on new fresh PDA medium until pure isolates were obtained. Stock cultures were maintained in PDA medium at 4 °C.

### Selection of the xylanase producers' fungal strains (plate-screening method)

In order to select the xylanase producers' fungal strains, cultures of the previously isolated fungi were transferred to a solid media

containing birch wood xylan 0.5% as carbon source, yeast extract 0.1% as nitrogen source, Congo red dye 0.5% as chromogenic reagent, and agar 1.5%. The inoculated media were incubated at 55 °C for 5 days. Then, the xylanase enzyme activity was determined by analyzing the clear zone formed around the fungal colony as a result of the reaction between the enzymes secreted by the fungi and the chromogenic substances present in the solid medium (Yoon et al., 2007).

### Identification of the isolates

The strains were identified by rDNA 18S amplification, which was carried out by obtaining the genomic DNA and amplifying an 18S rDNA fragment corresponding to 500 bp approximately. High-molecular-weight DNA was extracted using the protocol described by Barth and Gaillardin (1996), and the primer pairs PN3 (5'-CCGTTGGTGAACACGCGAGGGATC-3') and PN10 (5'-TCCGCTTATTGATATGCTTAAG-3') were used to amplify a fragment of 18S rDNA. The PCR reaction system consisted of 0.5 µL of 1 U/µL Taq DNA polymerase, 2.5 µL of 10X buffer stock solution, 0.5 µL of 10 mM deoxynucleotides (dNTP mixture), 2.0 µL of 10 µM oligonucleotide, 2 µL template DNA, and sterile distilled water to obtain 25.0 µL as the final volume. Amplification was done in a PCR Thermal Cycler Px2 (Thermo Electron) with the following program: initial denaturation at 95 °C for 10 min; 35 cycles (each one of them at 94 °C for 1 min), annealing temperature of 54 °C, 72 °C for 1 min, and final extension at 72 °C for 20 min. PCR products were visualized on 1.5% (w/v) agarose gel stained with ethidium bromide. PCR amplicons were sequenced using Big Dye® terminator cycle sequencing kit, in the 3730XL DNA Automatic Sequencer. The sequences obtained were compared with data available in GenBank database using the Mega BLAST network service of the National Centre for Biotechnology Information (NCBI) (Figure 1).

### Corn cobs characterization and xylanase production by solid-state fermentation (SSF)

Corn cobs samples were supplied by Instituto del Maíz, Universidad Autónoma Agraria Antonio Narro (Saltillo, Coahuila, México). As soon as obtained, the material was milled to particle sizes between 0.7 and 1.4 mm, and was characterized to determine the water absorption index (WAI) (Anderson, 1982) and critical humidity point (CHP) (Oriol, Schettino, Vinięra-González, & Raimbault, 1988). To be used as a solid substrate during fermentation, milled corn cob was boiled for 10 min, washed three times with distilled water, dried at 60 °C to constant weight, and autoclaved at 121 °C for 20 min, as reported by Mussatto, Aguiar, Rodrigues, and Teixeira (2009a).

The inoculum was prepared by activating the selected strains in slants of potato dextrose agar medium for 4 days at 30 °C. Subsequently, spores were recovered by washing the cultures with sterile aqueous solution of Tween 80 (0.01% v/v). Spore concentration was determined using a Neubauer chamber.

For the SSF experiments, 3.0 g of sterilized corn cobs were moistened with 11.0 mL of minimum Czapek-Dox medium (composed of 7.65 g/L NaNO<sub>3</sub>, 3.04 g/L KH<sub>2</sub>PO<sub>4</sub>, 1.52 g/L MgSO<sub>4</sub>·7H<sub>2</sub>O and 1.52 g/L KCl, with the final pH adjusted to 6.0 before sterilization) and the moistened material was transferred to a Petri dish. Then, each dish was inoculated with  $1 \times 10^7$  spores per gram dry corn cob, and incubated at 55 °C for 5 days.

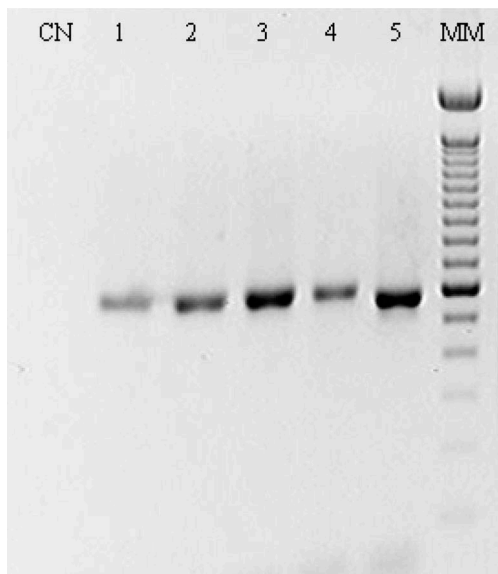


Figure 1. 18S rDNA strains fragment. CN, negative control; 1, 2, 3, 4, and 5 correspond to the strains SOC-4A, SOC-4B, SOC-4C, SOC-4D and SOC-5A, respectively; MM, molecular marker 1000 bp. PCR products were visualized on 1.5% (w/v) agarose gel stained with ethidium bromide.

Figura 1. 18S rDNA fragmento de cepas. CN, control negativo; 1, 2, 3, 4, and 5 corresponden a las cepas SOC-4A, SOC-4B, SOC-4C, 4D y SOC-SOC-5A, respectivamente; MM, marcador molecular de 1.000 bp. Los productos de PCR fueron visualizados en 1,5% (w/v) gel de agarosa teñido con bromuro de etidio.

For the enzyme recovery, 50 mL of sodium phosphate buffer (0.1 M, pH 7.0) was added to the fermented material and the mixture was homogenized during 30 min at 166 g and 30 °C. The sample was then centrifuged (3327 g, 15 min, and 10 °C; Sorball, Primo R Biofuge Centrifugation, Thermo, USA) to separate the enzyme extract, which was further used for the xylanase activity determination.

#### ***Xylanase activity determination in the extracts produced by SSF***

Xylanase activity in the extracts produced by SSF was determined by mixing 0.05 mL of extract with 0.05 mL of birch wood xylan 1.0% (w/v) prepared in 50 mM acetate buffer pH 6.0. The enzyme-substrate mixture was incubated at 50 °C for 30 min. The released reducing sugars were quantified by the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959) using xylose as standard. One unit of xylanase was defined as the amount of enzyme that liberates 1  $\mu$ mol of xylose equivalent per minute under the assay conditions.

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

To evaluate the effect of the pH on xylanase activity, enzyme solutions at different pH values (4–10) were prepared using 0.05 M acetate buffer, 0.05 M Tris-HCl buffer or 0.05 M glycine-NaOH buffer. For the experiments, 0.3 mL of xylanase solution was added to 0.7 mL of 1% birch wood xylan and the mixture was incubated at 55 °C for 5 min, following which the reaction was stopped in ice-water bath and the enzyme activity was determined under standard assay conditions. To evaluate the

effect of the temperature on enzyme activity, the crude enzyme extract obtained by SSF was incubated at different temperatures (55–80 °C) for 5 min. The reaction was then stopped in ice-water bath and the enzyme activity was determined under standard assay conditions. To determine the thermal stability of the enzyme, the crude extract produced by SSF was incubated at different time intervals (0–60 min) at fixed temperatures (65–85 °C).

#### ***Data analyses***

All the experiments and analyses were performed in triplicate and average values are reported. Differences among mean values were identified by the Tukey's range test ( $p \leq 0.05$ ) using the software Minitab®.

## **Results and discussion**

### ***Isolation and identification of xylanase producers' thermophilic fungal strains***

Twenty-one fungal strains were initially isolated from maize silage. Xylanase producer strains were selected using the plate-screening method, which consists in the detection of clear zones formed as a consequence of the enzymatic activity that hydrolyses the Congo red dye substrate (Yoon et al., 2007). Among the 21 isolated strains, seven were able to growth on xylan-Congo red plates; however, two of them were unable to promote discoloration of the agar medium, probably because they did not secrete the enzyme out of the cell. The other five strains, referred to as SOC-4A, SOC-4B, SOC-4 C, SOC-4D, and SOC-5A, were then selected at this stage. When compared with data available in GenBank database, the sequence identified as SOC-4A (Access number KC711060), SOC-4B (Access number KC711061), and SOC-4 C (Access number KC711062) showed 100% homology with the sequence available for *Rhizomucor pusillus* 1341; while the sequence SOC-4D (Access number KC711063) showed 98% homology with that for this same fungal strain. On the other hand, the sequence identified as SOC-5A (Access number KC711064) presented 99% homology with the sequence available for *Aspergillus fumigatus* BF18.

### ***Corn cobs characterization for use as support-substrate in SSF***

In SSF processes, the microorganisms are grown on solid particles in the absence (or near absence) of free water. However, the support-substrate material must present enough moisture to allow the growth and metabolism of the microorganism (Martins et al., 2011; Mussatto et al., 2012). Therefore, determining the WAI and CHP of the material that is desired for use as support-substrate is very important because these properties are directly related to the capacity of the material to absorb water and, as a consequence, to be invaded and colonized by the microorganisms. In brief, the WAI and CHP values allow estimating if the material presents characteristics suitable for use as support-substrate in SSF systems (Mussatto, Aguilar, Rodrigues, & Teixeira, 2009b; Mussatto et al., 2009a).

WAI reflects the ability of the material to absorb water, and depends on the availability of hydrophilic groups to be bonded with water molecules as well as on the gel-forming capacity of the macromolecules (Mussatto et al., 2009a). Materials with high WAI are preferred for use in SSF systems because they facilitate

the species growth and development (Mussatto et al., 2009b). In the present study, the corn cobs sample presented WAI value of 2.55 g gel per g dry material, which is similar to that reported by other authors for this same substrate (Buenrostro-Figueroa et al., 2014; Mussatto et al., 2009a) as well as for other substrates already used in SSF processes, including cork oak (Mussatto et al., 2009a), wheat bran, and pecan nutshell (Orzua et al., 2009).

CHP represents the quantity of water linked to the support material, which cannot be used by the microorganism. Therefore, materials with low CHP values are desired (Mussatto et al., 2009b; Robledo et al., 2008). The CHP value obtained for corn cobs in the present study was 32.6%. This value is within the range of values acceptable for use in SSF systems. A maximum limit of 40% CHP was recommended for *Aspergillus niger* strains grow in solid-state cultures (Moo-Young, Moreira, & Tengerdy, 1983). The value of CHP found for corn cobs was similar to those found for other materials such as lemon peel and apple pomace (Orzua et al., 2009).

Finally, the results of WAI and CHP obtained for corn cobs reveal that this material presents characteristics suitable for use as support-substrate in SSF processes.

#### Xylanase production by the isolated fungal strains under SSF conditions

In this step of the study, the ability of the five fungal strains previously isolated from the plate-screening method (SOC-4A, SOC-4B, SOC-4 C, SOC-4D, and SOC-5A) to produce xylanase under SSF conditions was evaluated. All the strains were able to grow and synthesize xylanase when cultivated in corn cobs under SSF conditions. The fermentation runs were carried out during 120 h, but the highest production of xylanase was observed at 96 h of fermentation. Figure 2 shows the relative xylanase activity recorded for all the strains at this time. *Rhizomucor pusillus* SOC-4A provided the highest value of xylanase activity, which was not different at  $p < 0.05$  when compared with the value achieved with *Rh. pusillus* SOC-4B. *Aspergillus fumigatus* SOC-5A presented also a good ability to produce xylanase, providing an enzyme activity value similar to that observed for *Rh. pusillus* SOC-4B.

Since fungi from different species presented good ability to produce xylanase, a kinetic study was then carried out with the strains *Rh. pusillus* SOC-4A and *A. fumigatus* SOC-5A in order to better understand and compare the enzyme production by these fungi. As can be seen in Figure 3, the highest xylanase production (824 U/g) by *Rh. pusillus* SOC-4A occurred at 96 h of fermentation. *Aspergillus fumigatus* SOC-5A produced maximum enzyme activity (488 U/g) at 72 h, but this maximum value was 40% lower than that obtained with *Rh. pusillus* SOC-4A. In addition, the xylanase production by *A. fumigatus* SOC-5A occurred with lower productivity (6.78 U/g.h) when compared with the production by *Rh. pusillus* (8.59 U/g.h). These results reveal that *Rh. pusillus* SOC-4A was a more efficient xylanase producer strain than *A. fumigatus* SOC-5A, since it was able to produce more enzymes in a shorter fermentation time. In terms of stability, the xylanase produced by both fungi presented high thermostability, since the enzyme activity decreased only 2.0% and 6.0% for *Rh. pusillus* SOC-4A and *A. fumigatus* SOC-5A, respectively, after 15 min at 75 °C. Taking into account the high thermal stability of the xylanase produced, and mainly the high enzyme production and productivity, *Rh.*

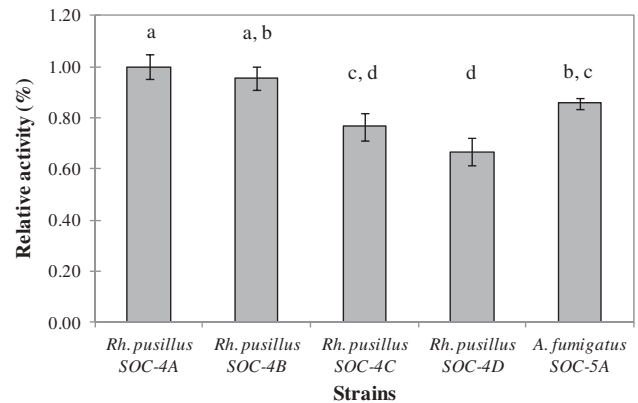


Figure 2. Relative xylanase activity (%) for the five isolated fungal strains cultivated for 96 h in corn cobs under solid-state fermentation conditions. Each value corresponds to a mean of 3 experiments. Different letters in each column means that differences between mean values are significant at  $p < 0.05$ . Relative activity was calculated using the xylanase activity of the strain *Rhizomucor pusillus* SOC-4A as reference.

Figura 2. Actividad relativa de xilanasas (%) para las cinco cepas fúngicas aisladas cultivadas durante 96 h en mazorcas de maíz en condiciones de fermentación en estado sólido. Cada valor corresponde a una media de 3 experimentos. Letras diferentes en cada columna significa que las diferencias entre los valores medios son significativas para  $p < 0.05$ . La actividad enzimática relativa fue calculada teniendo como referencia a la actividad de xilanasas de la cepa *Rhizomucor pusillus* SOC-4A.

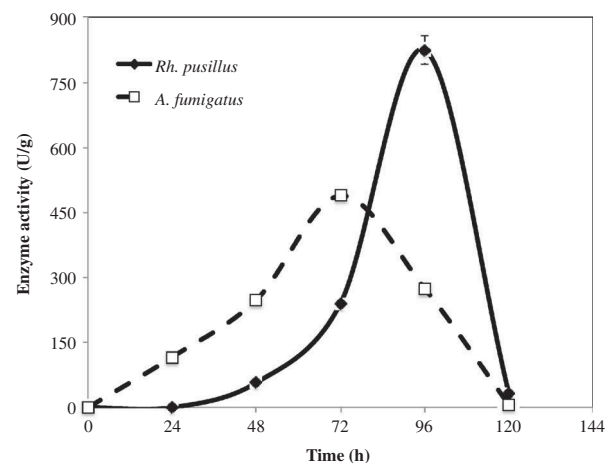


Figure 3. Kinetic profile of the xylanase activity produced by *Rhizomucor pusillus* SOC-4A and *Aspergillus fumigatus* SOC-5A cultivated under solid-state fermentation conditions using corn cobs as support-substrate.

Figura 3. Perfil cinético de la actividad de xilanasas producida por *Rhizomucor pusillus* SOC-4A y *Aspergillus fumigatus* SOC-5A cultivados bajo condiciones de fermentación en estado sólido utilizando mazorcas de maíz como soporte-sustrato.

*pusillus* SOC-4A was selected as the best xylanase producer thermophilic fungal strain isolated in the present study.

The xylanase activity values (824 U/g, 8.59 U/g.h) obtained with *Rh. pusillus* SOC-4A can be well compared to values reported in other SSF studies on the production of this enzyme by different microorganisms (Kapilan & Arasaratnam, 2011; Sadaf & Khare, 2014). It is important to emphasize that the results of the presented study were obtained without optimizing the fermentation conditions and, therefore, it is expected that they can be further improved. Selecting the best operational

conditions for use during the fermentation process is fundamental to achieving maximum product formation. These conditions can be selected by using statistical tools such as experimental designs and surface response methodology, for example (Mussatto & Roberto, 2008; Mussatto et al., 2013).

Higher values of xylanase activity have been reported when using submerged fermentation systems (Bakri, Masson, & Thonart, 2010; Bokhari, Rajoka, Javaid, & Latif, 2010). Nevertheless, the use of SSF systems presents important advantages from the downstream point of view. Since the volume of medium used in SSF is low, the enzyme can be more easily recovered from this medium than from the large volumes of liquid medium used in submerged fermentation systems (Martins et al., 2011; Mussatto et al., 2012). This fact may have important influence in the practical and economic aspects of the global process (Mussatto et al., 2015).

Another interesting aspect presented in Figure 3 is that the xylanase production by both fungi, *Rh. pusillus* SOC-4A and *A. fumigatus* SOC-5A, decreased after attained the maximum value. Such behavior can be related to a possible hydrolysis of the enzyme caused by proteases secreted by the microorganisms. Some studies report that *Rh. pusillus* (Macchione, Merheb, Gomes, & Da Silva, 2008; Yegin, Fernández-Lahore, Guvenc, & Goksungur, 2010) and *A. fumigatus* (Neustadt et al., 2009; Oguma et al., 2011) are protease producer strains.

#### Effect of pH and temperature on activity of the xylanase produced by *Rhizomucor pusillus*

It is well known that pH and temperature are two important process variables affecting the enzyme activity. Therefore, this part of the study consisted in establishing the conditions of these variables in which the activity of the xylanase produced by *Rh. pusillus* SOC-4A can be maximized. A pH range varying from 4 to 10 and temperature range varying between 55 and 80 °C were studied. The results obtained in these experiments indicated that the xylanase was highly active in a range of pH between 5.0 and 7.0, with maximum value at pH 6.0 (Figure 4). A pronounced drop in the xylanase activity was observed above pH 7.0. Similar pH range was reported for the xylanase produced by *Rh. miehei* (Fawzi, 2010), while different results were reported for other fungi. For example, xylanase purified from *Arthrobacter* sp. MTCC 5214 presented maximum activity at higher and narrower pH range (between 7.0 and 8.0) (Khandeparkar & Bhosle, 2006), whereas the xylanase produced by *Thermomyces lanuginosus* presented maximum activity in a wider pH range, within 5.5–10.0 (Singh, Pillay, & Prior, 2000).

Regarding the temperature, the xylanase produced by *Rh. pusillus* SOC-4A presented maximum activity between 65 and 75 °C, with optimum value at 70 °C (Figure 5). Fawzi (2010) reported an optimal temperature of 75 °C for xylanase produced by *Rh. miehei*. In the present study, when the temperature reached 75 °C, the relative xylanase activity was decreased to 77% of the maximum value achieved at 70 °C.

#### Conclusions

New thermophilic fungal strains with the ability to produce thermostable xylanase were isolated from maize silage. Species from the genera *Rhizomucor* and *Aspergillus* were identified among the selected strains and demonstrated good

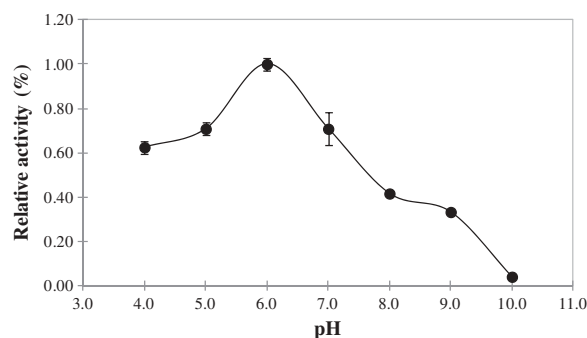


Figure 4. Effect of pH on the activity of xylanase produced by *Rhizomucor pusillus* SOC-4A grown on corn cobs under solid-state fermentation conditions. Relative activity was determined at 55 °C.

Figura 4. Efecto del pH sobre la actividad de xilanasa producida por *Rhizomucor pusillus* SOC-4A cultivado en mazorcas de maíz en condiciones de fermentación en estado sólido. La actividad relativa fue determinada a 55 °C.

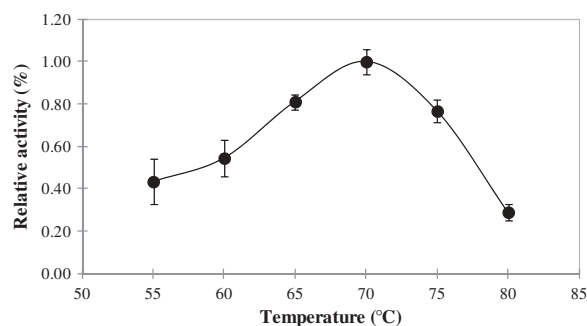


Figure 5. Effect of temperature on the activity of xylanase produced by *Rhizomucor pusillus* SOC-4A grown on corn cobs under solid-state fermentation conditions. Relative activity was determined at pH 6.0.

Figura 5. Efecto de la temperatura sobre la actividad de xilanasa producida por *Rhizomucor pusillus* SOC-4A cultivado en mazorcas de maíz en condiciones de fermentación en estado sólido. La actividad relativa fue determinada en pH 6,0.

ability to produce xylanase under solid-state fermentation conditions using corn cobs as support-substrate. The best results of activity and productivity were obtained for the thermostable xylanase produced by the isolated strain *Rhizomucor pusillus* SOC-4A. The enzyme produced by this fungus presented high activity within a pH range from 5.0 to 7.0, and temperature from 65 to 75 °C, with maximum value at pH 6.0 and 70 °C. This enzyme showed also good thermal stability since the activity decreased only 2.0% after 15 min at 75 °C. These characteristics suggest that the xylanase produced by the new isolated fungus *Rh. pusillus* SOC-4A has potential for applications in different areas, including in the pulp and paper, food, biofuel, and textile industries.

#### Disclosure statement

No potential conflict of interest was reported by the authors.

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## References

- Anderson, R. A. (1982). Water absorption and solubility and amylograph characteristics of roll-cooked small grain products. *Cereal Chemistry*, 59, 265–269. Retrieved from [http://www.aaccnet.org/publications/cc/backissues/1982/Documents/chem59\\_265.pdf](http://www.aaccnet.org/publications/cc/backissues/1982/Documents/chem59_265.pdf)
- Bajaj, B. K., & Manhas, K. (2012). Production and characterization of xylanase from *Bacillus licheniformis* P11(C) with potential for fruit juice and bakery industry. *Biocatalysis and Agricultural Biotechnology*, 1, 330–337. doi:10.1016/j.bcab.2012.07.003
- Bakri, Y., Masson, M., & Thonart, P. (2010). Isolation and identification of two new fungal strains for xylanase production. *Applied Biochemistry and Biotechnology*, 162, 1626–1634. doi:10.1007/s12010-010-8944-x
- Barth, G., & Gaillardin, C. (1996). *Yarrowia lipolytica*. In K. Wolf Ed., *Non-conventional yeasts in biotechnology: A handbook* (pp. 313–388). Berlin: Springer-Verlag. Retrieved from <http://www.springer.com/gp/book/9783642798580>
- Bokhari, S. A. I., Rajoka, M. I., Javaid, A., & Latif, F., & . (2010). Novel thermodynamics of xylanase formation by a 2-deoxy-D-glucose resistant mutant of *Thermomyces lanuginosus* and its xylanase potential for biobleachability. *Bioresource Technology*, 101, 2800–2808. doi:10.1016/j.biortech.2009.10.072
- Buenrostro-Figueroa, J., Ascacio-Valdés, A., Sepúlveda, L., De La Cruz, R., Prado-Barragán, A., Aguilar-González, M. A. . . . Aguilar, C. N. (2014). Potential use of different agroindustrial by-products as supports for fungal ellagitannase production under solid-state fermentation. *Food and Bioprocess Processing*, 92, 376–382. doi:10.1016/j.fbp.2013.08.010
- Carvalho, G. B. M., Mussatto, S. I., Cândido, E. J., & Almeida E Silva, J. B. (2006). Comparison of different procedures for the detoxification of eucalyptus hemicellulosic hydrolysate for use in fermentative processes. *Journal of Chemical Technology & Biotechnology*, 81, 152–157. doi:10.1002/jctb.1372
- Cavallarin, L., Tabacco, E., Antoniazzi, S., & Borreani, G. (2011). Aflatoxin accumulation in whole crop maize silage as a result of aerobic exposure. *Journal of the Science of Food and Agriculture*, 91, 2419–2425. doi:10.1002/jsfa.4481
- Dhiman, S. S., Garg, G., Sharma, J., & Mahajan, R. (2011). Characterization of statistically produced xylanase for enrichment of fruit juice clarification process. *New Biotechnology*, 28, 746–755. doi:10.1016/j.nbt.2010.11.004
- Fawzi, E. M. (2010). Highly thermostable purified xylanase from *Rhizomucor miehei* NRRL 3169. *Annals of Microbiology*, 60, 363–368. doi:10.1007/s13213-010-0052-1
- Garrote, G., Domínguez, H., & Parajó, J. C. (2002). Autohydrolysis of comcob: Study of non-isothermal operation for xylooligosaccharide production. *Journal of Food Engineering*, 52, 211–218. doi:10.1016/S0260-8774(01)00108-X
- Georis, J., Esteves, F. L., Lamotte-Brasseur, J., Bougnat, V., Giannotta, F., Frère, J.-M. . . . Granier, B. (2000). An additional aromatic interaction improves the thermostability and thermophilicity of a mesophilic family 11 xylanase: Structural basis and molecular study. *Protein Science*, 9, 466–475. doi:10.1110/ps.9.3.466
- Haki, G. D., & Rakshit, S. K. (2003). Developments in industrially important thermostable enzymes: A review. *Bioresource Technology*, 89, 17–34. doi:10.1016/S0960-8524(03)00033-6
- Kapilan, R., & Arasaratnam, V. (2011). Paddy husk as support for solid state fermentation to produce xylanase from *Bacillus pumilus*. *Rice Science*, 18, 36–45. doi:10.1016/S1672-6308(11)60006-1
- Khandeparkar, R. D. S., & Bhosle, N. B. (2006). Isolation, purification and characterization of the xylanase produced by *Arthrobacter* sp. MTCC 5214 when grown in solid-state fermentation. *Enzyme and Microbial Technology*, 39, 732–742. doi:10.1016/j.enzmictec.2005.12.008
- Li, X., She, Y., Sun, B., Song, H., Zhu, Y., Lv, Y., & Song, H. (2010). Purification and characterization of a cellulase-free, thermostable xylanase from *Streptomyces rameus* L2001 and its biobleaching effect on wheat straw pulp. *Biochemical Engineering Journal*, 52, 71–78. doi:10.1016/j.bej.2010.07.006
- Macchione, M. M., Merheb, C. W., Gomes, E., & Da Silva, R. (2008). Protease production by different thermophilic fungi. *Applied Biochemistry and Biotechnology*, 146, 223–230. doi:10.1007/s12010-007-8034-x
- Martins, S., Mussatto, S. I., Martínez-Avila, G., Montañez-Saenz, J., Aguilar, C. N., & Teixeira, J. A. (2011). Bioactive phenolic compounds: Production and extraction by solid-state fermentation. A review. *Biotechnology Advances*, 29, 365–373. doi:10.1016/j.biotechadv.2011.01.008
- Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, 31, 426–428. doi:10.1021/ac60147a030
- Moo-Young, M., Moreira, A. R., & Tengerdy, R. P. (1983). Principles of solid substrate fermentation. The filamentous fungi. In J. E. Smith, D. R. Berry, & B. Kristiansen (Eds.), *Fungal Technology* (Vol. IV, pp. 117–143). London: Edward-Arnold. doi:10.1016/0167-7799(84)90009-X
- Mussatto, S. I., Aguiar, L. M., Marinha, M. I., Jorge, R. C., & Ferreira, E. C. (2015). Economic analysis and environmental impact assessment of three different fermentation processes for fructooligosaccharides production. *Bioresource Technology*, 198, 673–681. doi:10.1016/j.biortech.2015.09.060
- Mussatto, S. I., Aguiar, C. N., Rodrigues, L. R., & Teixeira, J. A. (2009a). Fructooligosaccharides and  $\beta$ -fructofuranosidase production by *Aspergillus japonicus* immobilized on lignocellulosic materials. *Journal of Molecular Catalysis B: Enzymatic*, 59, 76–81. doi:10.1016/j.molcatb.2009.01.005
- Mussatto, S. I., Aguiar, C. N., Rodrigues, L. R., & Teixeira, J. A. (2009b). Colonization of *Aspergillus japonicus* on synthetic materials and application to the production of fructooligosaccharides. *Carbohydrate Research*, 344, 795–800. doi:10.1016/j.carres.2009.01.025
- Mussatto, S. I., Ballesteros, L. F., Martins, S., Maltos, D. A. F., Aguiar, C. N., & Teixeira, J. A. (2013). Maximization of fructooligosaccharides and  $\beta$ -fructofuranosidase production by *Aspergillus japonicus* under solid-state fermentation conditions. *Food and Bioprocess Technology*, 6, 2128–2134. doi:10.1007/s11947-012-0873-y
- Mussatto, S. I., Ballesteros, L. F., Martins, S., & Teixeira, J. A. (2012). Use of agro-industrial wastes in solid-state fermentation processes. In K. -Y. Show, & X. Guo, (Eds.), *Industrial Waste* (pp. 121–140). Rijeka, Croatia: InTech - Open Access Publisher. doi:10.5772/36310
- Mussatto, S. I., & Roberto, I. C. (2008). Establishment of the optimum initial xylose concentration and nutritional supplementation of brewer's spent grain hydrolysate for xylitol production by *Candida guilliermondii*. *Process Biochemistry*, 43, 540–546. doi:10.1016/j.procbio.2008.01.013
- Neustadt, M., Costina, V., Kupfahl, C., Buchheidt, D., Eckerskorn, C., Neumaier, M., & Findeisen, P. (2009). Characterization and identification of proteases secreted by *Aspergillus fumigatus* using free flow electrophoresis and MS. *Electrophoresis*, 30, 2142–2150. doi:10.1002/elps.200800700
- Oguma, T., Asano, K., Tomomatsu, K., Kodama, M., Fukunaga, K., Shiomi, T. . . . Ishizaka, A. (2011). Induction of mucin and MUC5AC expression by the protease activity of *Aspergillus fumigatus* in airway epithelial cells. *The Journal of Immunology*, 187, 999–1005. doi:10.4049/jimmunol.1002257
- Oriol, E., Schettino, B., Viniegra-González, G., & Raimbault, M. (1988). Solid-state culture of *Aspergillus niger* on support. *Journal of Fermentation Technology*, 66, 57–62. doi:10.1016/0385-6380(88)90130-6
- Orzua, M. C., Mussatto, S. I., Contreras-Esquivel, J. C., Rodriguez, R., De La Garza, H., Teixeira, J. A., & Aguilar, C. N. (2009). Exploitation of agro industrial wastes as immobilization carrier for solid-state fermentation. *Industrial Crops and Products*, 30, 24–27. doi:10.1016/j.indcrop.2009.02.001
- Qiu, Z., Shi, P., Luo, H., Bai, Y., Yuan, T., Yang, P. . . . Yao, B. (2010). A xylanase with broad pH and temperature adaptability from *Streptomyces megasporus* DSM 41476, and its potential application in brewing industry. *Enzyme and Microbial Technology*, 46, 506–512. doi:10.1016/j.enzmictec.2010.02.003
- Robledo, A., Aguilera-Carbó, A., Rodríguez, R., Martínez, J., Garza, Y., & Aguilar, C. (2008). Ellagic acid production by *Aspergillus niger* in solid state fermentation of pomegranate residues. *Journal of*



- Industrial Microbiology & Biotechnology*, 35, 507–513. doi:10.1007/s10295-008-0309-x
- Sadaf, A., & Khare, S. K. (2014). Production of *Sporotrichum* thermophile xylanase by solid state fermentation utilizing deoiled *Jatropha curcas* seed cake and its application in xylooligosachharide synthesis. *Bioresource Technology*, 153, 126–130. doi:10.1016/j.biortech.2013.11.058
- Saha, B. C. (2003). Hemicellulose bioconversion. *Journal of Industrial Microbiology & Biotechnology*, 30, 279–291. doi:10.1007/s10295-003-0049-x
- Saleem, M., Tabassum, M. R., Yasmin, R., & Imran, M. (2009). Potential of xylanase from thermophilic *Bacillus* sp. XTR-10 in biobleaching of wood kraft pulp. *International Biodeterioration & Biodegradation*, 63, 1119–1124. doi:10.1016/j.ibiod.2009.09.009
- Silva, J. P. A., Mussatto, S. I., Roberto, I. C., & Teixeira, J. A. (2012). Fermentation medium and oxygen transfer conditions that maximize the xylose conversion to ethanol by *Pichia stipitis*. *Renewable Energy*, 37, 259–265. doi:10.1016/j.renene.2011.06.032
- Singh, S., Pillay, B., & Prior, B. A. (2000). Thermal stability of  $\beta$ -xylanases produced by different *Thermomyces lanuginosus* strains. *Enzyme and Microbial Technology*, 26, 502–508. doi:10.1016/S0141-0229(99)00193-3
- Yegin, S., Fernández-Lahore, M., Guvenc, U., & Goksungur, Y. (2010). Production of extracellular aspartic protease in submerged fermentation with *Mucor mucedo* DSM 809. *African Journal of Biotechnology*, 9, 6380–6386. Retrieved from <http://www.ajol.info/index.php/ajb/article/view/92273/81725>
- Yoon, J. H., Park, J. E., Suh, D. Y., Hong, S. B., Ko, S. J., & Kim, S. H. (2007). Comparison of dyes for easy detection of extracellular cellulases in fungi. *Mycobiology*, 35, 21–24. doi:10.4489/MYCO.2007.35.1.021