Characterization of Hemicellulolytic Enzymes Produced by *Aspergillus niger* NRRL 328 under Solid State Fermentation on Soybean Husks

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This manuscript describes the analysis of xylanase production by *Aspergillus niger* NRRL 328 in solid state fermentation (SSF) of soybean husks. A maximum value of extracellular xylanase activity of approximately 950 U g^{-1} was achieved after 96 h. Proteomic analyses performed on the enzymatic mixture responsible for the maximum value of xylanase activity in SSF revealed the presence of two xylanases. This xylanolytic mixture was partially purified and characterized. It followed Michaelis-Menten kinetics towards xylan, with a K_M of 7.92 ±0.97 mg xylan/mL and a V_{max} of 262.2 ± 27.8 g L^{-1} s^{-1}. The optimum pH for the enzyme is 5.3, and the optimal temperature is 50 °C. The enzyme retains 100% of its activity at 40 °C for at least 1 month. It shows very high stability in a broad pH range, with a half-life of 40 days at pH 5.3, pH 6.0, pH 6.5, pH 7.0, and pH 8.0.

Keywords: Aspergillus niger; Solid state fermentation; Xylanase

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INTRODUCTION

Hemicellulose represents 20 to 35% of lignocellulosic biomass and is a very complex polysaccharide composed of a wide variety of sugars, including xylose, arabinose, mannose, and galactose (Saha 2003). The hemicellulose enzymes degrading hemicellulose are divided into two major groups: depolymerizing enzymes, which cleave the backbone, and auxiliary enzymes, which remove substituents. These enzymes act synergistically to hydrolyze lignocellulosic polysaccharides (Van Dyk and Pletschke 2012). Depolymerizing enzymes include endo-β-1,4-xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37), while auxiliary enzymes comprise α-glucuronidases (EC 3.2.1), α-arabinofuranosidases (EC 3.2.1.55), acetyl esterases or acetyl xylan esterases (EC 3.1.1.6), and β-mannanases (EC 3.2.1.78).

Most studies on hemicellulases have focused on enzymes that hydrolyze the xylan backbone. These enzymes include, according to the CAZY (Carbohydrate-Active enZYmes) database, GH 3, 30, 39, 43, 52, 54, 116, and 120. Endo-1,4-β-xylanase (1,4-b-d-xylan xylanohydrolase, EC 3.2.1.8) cleaves the glycosidic bonds in the xylan backbone, releasing shorter xylo-oligosaccharides; β-xylosidase (1,4-b-d-xylan xylohydrolase, EC 3.2.1.37) cleaves the small xylo-oligosaccharides and cellobiose into xylose. These enzymes are produced by microorganisms such as fungi, bacteria, yeast, and marine algae.

Filamentous fungi are interesting producers because the enzymes are excreted at higher levels than those of yeasts and bacteria.

The use of xylanases is very well established in the food industry, for the digestion of animal feed, in the textile industry, as well as in the paper and pulp industry for bleaching processes to replace the chlorine utilization and to reduce the environmental impacts (Juturu and Wu 2012). Lately, the application of hemicellulases in lignocellulose conversion is increasing because of the progress in lignocellulose biorefinery installation (Kumar and Satyanarayana 2012). In this case, a finely planned cocktail of enzymes, consisting not only of cellulases but also of xylanases, is required to achieve the highest yields of fermentable sugars from the polysaccharidic components of lignocellulosic biomass. However, the high cost of enzymes is one of the major economic bottlenecks for the implementation of economically feasible lignocellulosic biorefineries (Hasunuma *et al.* 2013).

A major challenge therefore entails the development of a suitable efficient and economically viable hydrolysis process step, through discovery of new enzymes with higher catalytic efficiency, recycling enzymes during the hydrolysis and fermentation process, and on-site enzyme production in the biorefinery facility. In this way, the development of processes, with the employment of agro-industrial residues, is a good alternative for enzyme production. Among processes used for enzyme production, solid-state fermentation (SSF) is an attractive one. SSF systems resemble the natural habitats of microbes and, therefore, may prove to be efficient in producing certain enzymes and metabolites (Haltrich *et al*. 1996). This biotechnological process allows the use of inexpensive substrates that decrease the cost of enzyme production (Pandey *et al.* 2000; Soccol and Vandenberghe 2003). The cost of carbon source plays a major role in the economics of xylanase production. An approach to reduce the cost of xylanase production is the use of lignocellulosic materials as substrates rather than opting for the expensive pure xylans (Haltrich *et al*. 1996).

Brazil is the second largest producer of soybean, with 30.2% of the world production or 86.5 million tons in 2014. According to the Ministry of Agriculture, Livestock and Supply of Brazil (IBGE 2014), the exportation of soybean grains generates more than $5.5 billion dollar per year and its local processing to produce products with high value is of paramount importance for the economic development and job creation in the country (MAPA 2014). Soybean husks (SH) are one of the byproducts/wastes obtained in the process of extraction of seed oil. For each ton of processed soybeans, up to 3% shells, are generated (Karp *et al.* 2011).

Several innovative processes and bioproducts linked to the productive chains of soybean, which were transferred to the productive sector, have already been developed. Among these, are bioproducts obtainable from soybean hulls and molasses that include bioethanol and acids (Siqueira *et al*. 2008, Sanada *et al*. 2009; Karp *et al*. 2011; Letti *et al*. 2012), and extracts of soybean meal as bacterial growth inhibitors (Noseda *et al*. 2012). This substrate could also be used for xylanases and other enzymes production.

This manuscript describes the production of xylanases from the strain *Aspergillus niger* NRRL 328 through solid state fermentation (SSF) on soybean husks, as well as partial purification, proteomic analyses for enzymatic identification, and functional characterization of extracellular xylanolytic enzymes.

EXPERIMENTAL

Materials

Microorganisms

The fungus *Aspergillus niger* NRRL 328, from the Agricultural Research Service – ARS Culture Collection, was cultivated on potato dextrose agar (Sigma-Aldrich) slants at 28 °C for 168 hand stored at 4 °C. The culture was maintained at the Culture Collection of the Biotechnological Process Laboratory, Bioprocess Engineering and Biotechnology Division, Federal University of Paraná, Curitiba, Brazil. The strain was previously selected as good xylanase producer.

Pre-inoculum

The pre-inoculum of *A. niger* NRRL 328 was cultivated in Erlenmeyer flasks (125 mL) containing 20 mL of potato dextrose agar (Sigma-Aldrich, Milan, Italy) and incubated at 28 °C for 144 h. Spore suspensions were obtained by mixing pre-cultured fungus in 20 mL of sterile solution Tween 80® 0.01% containing glass pearls in constant agitation at 120 rpm for 10 min. The concentration of spores/mL was determined by counting using a Bürker camera.

Methods

SSF for xylanase production analysis

Approximately 10^7 spores/g of *A. niger* NRRL 328 were inoculated on 2.5 g of soybean husks, between 0.8 and 2.0 mm, in 100-mL Erlenmeyer's flasks, in triplicate. Previous studies with other lignocellulosic wastes had shown that this is the best dimension for SSF (Soccol and Vandenberghe 2003; Maciel *et al*. 2008; Iandolo *et al*. 2011). The initial moisture was adjusted to 70% utilizing a nutritive solution containing (g/L) manganese sulfate (1.0), and urea (0.7). The flasks were incubated at 28 °C for 168 h. Enzymes produced by SSF were extracted by solid-liquid extraction using a 50 mM sodium citrate buffer, pH 5.0, at the proportion of 1:10 (w/v) with vigorous agitation for 10 min. The mix was filtered in TNT paper and centrifuged at 8000 rpm, 4 °C for 15 min. The supernatant was filtered using Whatman nº 1 filter paper and subjected to analyses.

Xylanase activity assay

The xylanase activity assay was performed using a modified version of the methodology reported by Bailey *et al*. (1992). An aliquot of 20 µL of enzymatic filtrate (appropriately diluted in 50 mM sodium citrate buffer, pH 5.0) was mixed with 180 µL of 1% beech wood xylan and incubated for 5 min at 50 °C. Released reducing sugars were quantified using the dinitrosalicylic acid reagent (DNS) method (Miller 1959), adding 300 µL of DNS solution and then incubating the mixture at 95 °C for 5 min. Absorbance was measured at 540 nm. One unit of enzyme is defined as the amount of enzyme catalyzing the release of 1 µmol of xylose equivalent *per* min.

Protein concentration determination

Protein concentration was determined by the method of Lowry *et al*. (1951), using the BioRad Protein Assay (BioRad Laboratories S.r.l.; Segrate, MI, Italy), with bovine serum albumin as a standard.

Enzyme enrichment

Supernatant obtained by extraction of the enzymes produced by SSF was precipitated by the addition of $(NH_4)_2SO_4$ up to 80% saturation at 4 °C and centrifugation at 10,000×g for 30 min. The precipitate was resuspended in 0.02 M Tris-HCl at pH 7.5 and loaded on HiTrap Phenyl FF high sub (GE Healthcare; Uppsala, Sweden), equilibrated in buffer A (0.02 M Tris-HCl, 1.2 M $(NH_4)_2SO_4$, pH 7.5), and the proteins were eluted isocratically with buffer B (0.02 M Tris-HCl pH 7.5). Fractions containing activity were combined and concentrated on an Amicon PM-10 membrane (Millipore, Vimodrone, Italy) and analyzed by SDS-PAGE (Sodium Dodecyl Sulphate–Poly-Acrylamide Gel Electrophoresis) according to Laemmli (1970).

Optimal temperature and thermo-resistance

To determine the optimum temperature of the partially purified enzyme, the substrate of the activity assay (1% beech wood xylan) was dissolved in 50 mM sodium citrate buffer at pH 5.3 and the incubation (5 min) was performed at 40, 50, 60, 70, and 80 °C. The thermo-resistance was studied by incubating the partially purified enzyme preparation in sodium citrate buffer at pH 5.3 at 40, 50, and 60 °C. The samples withdrawn were assayed for residual xylanase activity, performing incubation (5 min) at 50 °C. The reported values are representative of three experiments, and each experiment was performed in duplicate.

Optimal pH and pH stability

To determine the optimum pH of the partially purified enzyme, the substrate of the activity assay (1% beech wood xylan) was dissolved in citrate phosphate buffers (McIlvaine 1921) with pH values between 3.0 and 7.0, and in 50 mM Tris-HCl with pH values of 7.0, 8.0, and 9.0, and then incubated (5 min) at 50 °C. The pH stability of the purified enzyme preparation was studied by diluting it in citrate phosphate buffers, pH 5.3 – 6.5 – 7.0 – 8.0, and incubating at 25 °C. From time to time, samples were withdrawn and immediately assayed for residual xylanase activity, performing incubation (5 min) at 50 °C. The reported values are representative of three experiments, and each experiment was performed in duplicate.

Determination of k_{cat} and K_M

For determination of the Michaelis-Menten constants K_M and k_{cat}, the activity assay was performed at beech wood xylan concentrations ranging from 0.2 mg/mL to 25 mg/mL at pH 5.3, performing incubation for 5 min at 50 °C. The reported values are representative of three experiments, and each experiment was performed in duplicate.

Zymogram analyses

Semi-denaturing gel electrophoresis was carried out by loading non-denatured and not-reduced samples on a SDS polyacrylamide gel, performed as described by Laemmli (1970).

Proteins showing xylanolytic activity purified by HIC were visualized as follows. After electrophoresis, the gel was soaked in the same buffer used for dissolving proteins and gently shaken to remove SDS and to renature the proteins in the gel.

Protein identification by mass spectrometry

Active fractions were diluted in denaturant buffer (final concentrations: Tris 300 mM pH 8.8, urea 6 M, EDTA 10 mM), and disulfide bridges were reduced with 10 mM dithiothreitol at 37 °C for 2 h and then alkylated by adding 50 mM iodoacetamide at room temperature for 30 min in the dark. Protein sample was desalted by size exclusion chromatography on a Shephadex G-25M column (GE Healtcare, Milan, Italy) equilibrated in 50 mM NH_4HCO_3 buffer, pH 8.0. Protein-containing fractions were frozen at -80 °C, and lyophilized by Vacuum Freeze Dryer Lyophilizer system (Lio 5Pascal) at -50 °C and with a forced vacuum of 0.30 mbar. Lyophilized fractions were dissolved in 50 µL of 10 mM NH_4HCO_3 buffer, pH 8.0, and enzymatic digestion was performed with 5 µg of trypsin at 37 °C for 16 h.

Peptide mixtures were filtered on a 0.22-µm PVDF membrane (Millipore) and analysed by LC-MS/MS (liquid chromatography coupled with tandem mass spectrometry) on a 6520 Accurate-Mass Q-TOF LC/MS (liquid chromatography mass spectrometry) System (Agilent Technologies, Palo Alto, CA), equipped with a 1200 HPLC (High-Performance Liquid Chromatography) system and a chip cube (Agilent Technologies, Palo Alto, CA). After loading, the peptide mixture was concentrated and washed in a 40-nL enrichment column (Agilent Technologies chip, Palo Alto, CA), with 0.1% formic acid in 2% acetonitrile as the eluent.

The sample was then fractionated on a C18 reverse-phase capillary column (Agilent Technologies chip, Palo Alto, CA) at a flow rate of 400 nL/min, with a linear gradient of eluent B (0.1% formic acid in 95% acetonitrile) in A (0.1% formic acid in 2% acetonitrile) from 7 to 80% in 50 min. Peptide analysis was performed using data-dependent acquisition of one MS scan (mass range from 300 to 1800 m/z) followed by MS/MS scans of the five most abundant ions in each MS scan. The MS/MS spectra were measured automatically when the MS signal was over the threshold of 50,000 counts. Double- and triple-charged ions were preferably isolated and fragmented over single-charged ions. The acquired MS/MS spectra were transformed in *mzData* (.XML) format and used for protein identification with a licensed version of MASCOT software (www.matrixscience.com), version 2.4.0.

Raw data from nano LC-MS/MS analysis were used to query the NCBInr database (the nr database compiled by the National Center for Biotechnology Information), NCBInr 20121120 (21,582,400 sequences; 7,401,135,489 residues), with taxonomy restriction to *Fungi* (1,569,912 sequences). The MASCOT search parameters were as follows: trypsin as enzyme; 3, as allowed number of missed cleavages; carboamidomethyl (C) as fixed modification; oxidation of methionine and pyro-Gluformation at N-term Q as variable modifications; 10 ppm as MS tolerance and 0.6 Da as MS/MS tolerance; and peptide charge from +2 to +3.

The peptide score threshold provided from MASCOT software to evaluate the quality of matches for MS/MS data was 40. Spectra with a MASCOT score of < 20 were rejected as having low quality.

Trypsin, dithiothreitol, iodoacetamide, and NH_4HCO_3 were purchased from Sigma-Aldrich (Milan, Italy). Trifluoroacetic acid (TFA)-HPLC grade was from Carlo Erba (Milan, Italy). All other reagents and solvents were of the highest purity available from Baker (Milan, Italy).

RESULTS AND DISCUSSION

Xylanase Production by Solid State Fermentation

Different substrates were already tested in previous studies by the group for xylanase production (Maciel *et al*. 2008). Soybean husks were chosen for xylanase production due to their main lignocellulolytic composition (34.5% fibers, 13.2% proteins), which could promote not only the production of xylanases, but the production of other enzymes such cellulases, *etc*. The choice of the use of only soybean husks was done due to the interest of concomitantly producing mannanases. Seeds present an interesting composition of galactomannans. So, soybean husks certainly would present the favorable composition for that.

Analysis of xylanase activity production by the strain *A. niger* NRRL 328 showed that a maximum production of xylanase activity of around 950 U g^{-1} was achieved by SSF on soybean husks at 96 h (Fig. 1), with a productivity of 9.89 U $g^{-1}h^{-1}$.

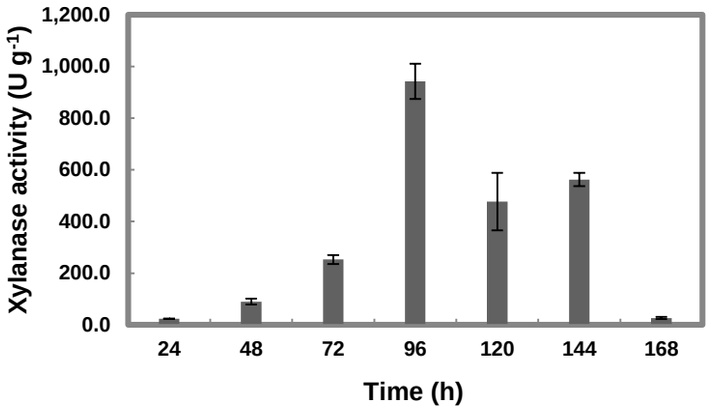

Fig. 1. Time course of xylanase activity (U g^{-1}) produced by *A. niger* NRRL 328 in SSFon soybean husks

When compared with xylanase activity produced by other *Aspergillus* spp., the maximum xylanase activity value obtained for *A. niger* NRRL 328 was similar or, in some cases, lower than those obtained by SSF with most *Aspergillus* strains (Table 1). Even so, this process presents good perspective for xylanase production. It must be pointed out that the enzyme production was not optimized and/or concentrated, which could lead to a 5- to 10-fold augmentation of enzyme activity. However, this fact may not restrain the direct application of the fermented material with the enzymes in feed. Rations are generally composed of the most commonly available feed ingredients in alfalfa, corn (grain and silage), grass hay, soybean meal, and pasture (Peters *et al*. 2014). The mixture of the fermented substrate, soybean husks, containing xylanases and other produced hemicellulolytic enzymes, with ration components can be a good alternative for their employment for animal feed. The complete analysis of other enzymes concomitantly produced will certainly define the real potential for the use of the fermented material. Besides, the direct use of the fermented material with enzymes would eliminate the need and costs of separation and purification steps.

Table 1. Comparison of Xylanolytic Activity Values Reported for Other *Aspergillus* spp.

Microrganism	Substrate	Maximum xylanase activity	Reference
Aspergillus niger NRRL 328	Soybean husks	950 U/g (SSF)	This study
Aspergillus fumigatus SK1	Untreated oil palm trunk	418.7 U/g (SSF)	Ang *et al*. 2013
Aspergillus fumigatus P40M2 *Aspergillus niger* P47C3	Sugarcane bagasse and wheat bran	1055.6 U/g (SSF) 1285.0 U/g (SSF)	Delabona *et al*. 2013
Aspergillus niger DFR-5	Wheat bran and soybean cake	2596.0 IU/g (SSF)	Pal and Khanum 2010
Aspergillus niger FGSCA733	*Jatropha curcas* seed cake	6087 IU/g (SSF)	Ncube *et al.* 2012
Aspergillus niger F3	*Citruc peels*	250 U/g (SSF)	Rodríguez-Fernández *et al*. 2011
Aspergillus terreus MTCC 8661	Palm fiber	115000 U/g (SSF)	Lakshmi *et al*. 2009

Enzyme Identification and Characterization

The xylanolytic enzymes produced by *A. niger*NRRL 328SSF were partially purified, as shown by SDS-PAGE (Fig. 2), and subjected to proteomic analyses and functional characterization as described below.

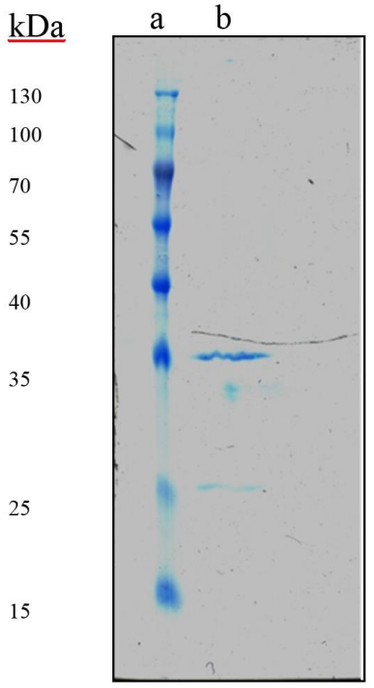

Fig. 2. SDS-PAGE profiling of the partially purified xylanase from *A. niger* NRRL 328: Lane 1: molecular marker; lane 2: partially purified xylanase

The sample positive for xylanolytic activity, as assessed by zymogram analyses, was enzymatically digested, and the peptide mixture was analyzed by LC-MS/MS. Database searches confidently assessed the presence of an endo-1,4-β-xylanase C and a xylanase that can account for the observed activity. Interestingly, anα-L-arabinofuranosidase axhA, an endoglucanase A, and an endo-β-1,4-glucanase B were also identified (Table 2) in the same sample.

Table 2. Proteins Identified in the Sample with Xylanolytic Activity

Protein name [Species] (Accession Number)	Score	Nominal mass (Mr)	N° peptides	Sequence coverage (%)	Sequence
1,4-β-D-xylan xylanohydrolase C [*Aspergillus niger*] (gi|292495278)	342	35578	9	27	DSVFYK; QASVSIDTK - Gln->pyro-Glu (N-term Q); VIGEDYVR; WDATEPSR; NTLIEVMK – Oxidation (M); TGMVSHVK;ADFGALTPENSMK; YLGNIGDQYTLTK; LYINDYNLDSASYPK
endoglucanase A [*Aspergillus niger*] (gi|3757552)	162	25957	4	18	QIATATVGGK; YGNIQPIGK; SYSNSGVTFNK; LVSDVSSIPTSVEWK
xylanase [*Aspergillus niger*] (gi|13242071)	121	11059	2	33	GTVTSDGSVYDIYTATR; YIVESYGDYNPGSGGTYK
endo-β-1,4-glucanase B [*Aspergillusniger* CBS 513.88] (gi|145238644)	69	36649	2	7	ITDATQWLK; VGFIGEYAGGSNDVCR
α-L-arabinofuranosidase axh A [Aspergillus niger CBS 513.88] (gi|145234699)	67	35928	2	9	YLMIVEAIGSTGHR - Oxidation (M); ANSGATWTDDISHGDLVR

The NCBInr database was searched with MS/MS ion search MASCOT software (www.matrixscience.com), with carboamidomethyl (C) as fixed modification and oxidation on Metand cyclization Pyro-Glu of Gln at N-terminus of the peptides as variable modifications. Only proteins identified with at least two peptides were considered significant. Peptides with individual ion score < 20 were rejected

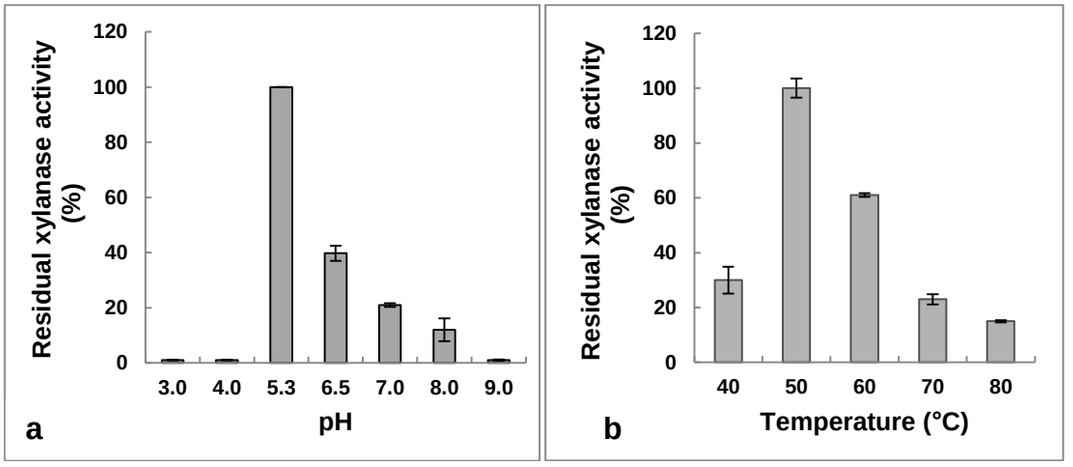

Fig. 3. Effect of pH (A) and temperature (B) on xylanase activity produced by *A. niger* NRRL 328

Functional characterization of the enriched xylanolytic mixture showed that xylanase activity follows Michaelis-Menten kinetics towards xylan: the K_M for this substrate was 7.92 ±0.97 mg xylan/mL and the V_{max} was 262.2 ± 27.8 g L^{-1} s^{-1}. The optimum pH for the xylanase activity (assayed in a range from 3.0 to 8.0) was 5.3 (Fig. 3a), and the optimal temperature was 50 °C (Fig. 3b).

Both the optimum temperature and pH of *A. niger* NRRL 328 xylanase activity were similar to those of most xylanases from *Aspergillus* spp. (Table 3).

Table 3. Properties Reported for Xylanases from Other Filamentous Fungi

Microorganism		Kinetic Parameters		Optimal Parameters		Half-life		Stability			Reference
		K_M (mg/mL)	V_{max}	pH	T (°C)	Time (min)	T (°C)	Time (min)	T (°C)	pH	
Aspergillus niger NRRL 328		7.92 ± 0.97	262.2 ± 28.0 g $L^{-1}s^{-1}$	5.0	50	120 15	50 60	5760 0	40	5.3 6.0 6.5 7.0 8.0	This study
Aspergillus fumigatus SK1		-	-	4.0	60	70.0 %	< 50	30	60	4.0 – 6.0	Ang *et al*. 2013
Aspergillus fumigatus P40M2		-	-	5.5 – 6.0	50 – 65	-	-	30	60	5.0	Delabona *et al*. 2013
Aspergillus niger wild-type strain from Brazil		-	-	4.5	55	1458 0 5400	37 50				Farinas *et al*. 2010
Chaetomium sp. CQ31		0.86	-	7.5	65	437 49 15	55 60 65	30	55	4.5 – 11. 0	Jiang *et al*. 2010
Aspergillus terreus		10.4	188.7 µM/ml/min	5.0	50	-	-	240	50	5.0	Kamat *et al*. 2013
Aspergillus niger DFR-5		-	4126 IU/ml	5.0	40	462 267 193 144 110 92.4	45 50 55 60 65 70	180	40	4.0 – 6.5	Pal and Khanum 2011
Penicillium oxalicum GZ-2		-	-	4.0	50	-	-	30	50	4.0 – 8.0	Liao *et al*. 2012
Aspergillus awamori IOC-3914		-	-	4.8	50.8	5400	50.8	-	-	-	López *et al*. 2013
Aspergillus terreus	Xyl T1	-	-	6.0	50	-	-	120	50	4.0 – 7.5	Moreira *et al.* 2013
	Xyl T2	-	-	5.0	45	-	-	210		4.0 – 7.0	
Aspergillus niger FGSCA733		-	-	5.0	50	-	-	-	-	-	Ncube *et al.* 2012
Sporotrichum thermophile ATCC 34628	StXyn1	-	-	5.0	60	60	60	60	50	5.0	Vafiadi *et al.* 2010
	StXyn2	-	-			115	60				

The *A. niger* NRRL 328 xylanase activity showed a half-life of 2 h at 50 °C and 15 min at 60 °C, with a similar behavior to that of most xylanases from *Aspergillus* spp. (Table 3). It is worth noting that the enzymatic mixture retained 100% of its activity for at least 1 month at 40 °C. It showed very high stability in a broad pH range, with a half-life of 40 days at pH 5.3, pH 6.0, pH 6.5, pH 7.0, and pH 8.0.

CONCLUSIONS

1. The highest level of *Aspergillus niger* NRRL 328 xylanase activity achieved by SSF on soybean husks was around 950 U g^{-1} at 96 h.
2. Proteomic analyses on partially purified enzymatic mixtures produced by *A. niger* NRRL 328SSF on soybean husks at 96 h revealed the presence of endo-1,4-β-xylanase C and xylanase that can account for the observed xylanase activity.
3. Functional characterization of the enriched xylanolytic mixture showed that xylanase activity follows Michaelis-Menten kinetics towards xylan: the K_M for this substrate is 7.92 ±0.97 mg xylan/mL, and the V_{max} is 262.2 ± 27.8 g L^{-1} s^{-1}.
4. The optimum pH for the xylanase activity is 5.3, and the optimal temperature is 50 °C.
5. The xylanase activity shows a half-life of 2 h at 50 °C and 15 min at 60 °C.
6. The enzymatic mixture retains 100% of its xylanase activity for at least 1 month at 40 °C. It shows very high stability in a broad pH range, with a half-life of 40 days at pH 5.3, pH 6.0, pH 6.5, pH 7.0, and pH 8.0.

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