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SELECTION OF THERMOPHILIC AND THERMOTOLERANT FUNGI FOR THE PRODUCTION OF CELLULASES AND XYLANASES UNDER SOLID-STATE FERMENTATION

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ABSTRACT

Twenty-seven thermophilic and thermotolerant fungal strains were isolated from soil, decaying organic matter and sugarcane piles based on their ability to grow at 45°C on medium containing corn straw and cardboard as carbon sources. These fungi were identified in the genera *Aspergillus, Thermomyces, Myceliophthora, Thermomucor* and *Candida*. The majority of the isolated strains produced xylanase and cellulases under solid state fermentation (SSF). The highest cellulase and xylanase productions were obtained by the cultivation of the strains identified as *Aspergillus fumigatus* M.7.1 and *Myceliophthora thermophila* M.7.7. The enzymes from these strains exhibited maximum activity at pH 5.0 and at 60 and 70ºC. The endo-glucanase from *A. fumigatus* was stable from 40°C to 65°C and both endo-glucanase and xylanase from *M. thermophila* were stable in this temperature range when in absence of substrate. The enzymes were stable from pH 4.0 to 9.0.

Key words: Thermophilic fungi, solid-state fermentation, xylanase, cellulase

INTRODUCTION

Lignocellulosic biomass from crops, whose main components are polysaccharides polymers as cellulose and hemicellulose (75-80%) is an important renewable energy source. Cellulose is a linear homopolysaccharide of D-glucose residues linked by β-1,4 glycosidic bonds. Hemicellulose is heteropolysaccharide composed by pentoses (xylose and arabinose), hexoses (glucose, mannose and galactose) and sugar acids. The dominant component of hemicellulose is the xylan that consists of a backbone chain of β-1,4-linked xylosyl residues. The lignin, the main non-carbohydrate portion of lignocellulosic material, is an amorphous phenolic polymer composed of monolignols such as *p*-hydroxyphenyl, guaiacyl and syringyl which are able to form a variety of ether or carbon-carbon bonds, resulting in a complex and stable branched network (7, 22, 33).

Many microorganisms are able to produce enzymatic complexes that degrade cellulose and hemicellulose releasing sugars that can be used for obtainment of products with high

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economical value such as bioethanol (1). However, the costs of enzymes production is the major limitation to the application of this technology on industrial scale (12, 23, 28). Therefore, the main challenges to be overcome are the search for microorganisms with high potential to produce fibrolytic enzymes and the development of a low-cost fermentative process.

The majority of commercial enzymes are obtained from mesophilic microorganisms. Since industrial processes using high temperatures are becoming more and more common, a supply of thermostable enzymes for industrial use is needed. Thermophilic fungi produce enzymes with activity at high temperatures and they usually have a higher thermostability, broad tolerance to pH variation and greater resistance to denaturing agents. One valuable advantage of conducting of hydrolysis lignocellulosic material at elevated temperatures is reducing the risk of contamination by mesophiles microorganisms. Allowing a higher operation temperature decrease in viscosity and an increase in higher mass-transfer rates leading to better substrate solubility (17, 31).

That way we intended to select novel thermophilic fungi strains that produce cellulases and xylanases with potential for biotechnological applications including the saccharification of lignocellulosic material. In this work, we report the isolation and selection of thermophilic fungal strains able to grow under lignocellulosic-suplemented medium as carbon sources, and the use of selected isolates *A. fumigatus* M.7.1 and *M. thermophila* M.7.7 for cellulase and xylanase production by SSF using crops and agro-industrial wastes as carbon sources and some properties of enzymes were reported.

MATERIALS AND METHODS

Isolation, identification and maintenance of fungi

The thermophilic and thermotolerant fungal strains were isolated from soil, decaying organic matter and sugarcane piles in the north-west of São Paulo State (São José do Rio Preto (49º 22' 45" W – 20º 49' 11 S), Fernandópolis (50º 14' 46" W – 20º 17' 03" S), Santa Rita D' Oeste (50º 49' 54" W – 20º 08' 42" S), Olímpia (48º 54' 53" W – 20º 44' 15" S) and Jales (50º 32' 45" W – 20º 16' 08" S), Brazil. Samples (0.5 g) were collected and homogenized in a sterile medium containing ($g.L^{-1}$): corn straw, 5.0; cardboard, 5.0; $(NH_4)_2SO_4$, 1.4; K_2HPO_4, 2.0; $CaCl_2$, 0.3; peptone, 2.0; yeast extract, 2.0; glucose, 1.0; urea, 0.3; $MgSO_4$ $7H_2O$, 0.2 and 10.0 mL of nutrient solution. The nutrient solution was composed of (mg/L): $FeSO_4$ $7H_2O$, 5.0; $MnSO_4.H_2O$, 1.6; $ZnSO_4$ $7H_2O$, 1.4; $CoCl_2$, 2.0, at pH 5.0. The inoculated medium was incubated at 45 °C for 48 h and then an aliquot of liquid medium was streaked on agar plates containing the same medium plus chloramphenicol and ampicillin (0.2 g/L each one) and agar (25.0 g/L) and incubated at 45 ºC for 24 to 72 h. All morphologically-contrasting colonies were isolated by repeated streaking. Pure cultures were sub-cultured on slants of the same medium for subsequent identification and enzymatic studies.

The fungal strains selected as the best producers of xylanase and cellulase were identified by conventional and molecular approaches. Morphological identification was carried out by examining the colony characteristics on a stereoscope (Leica MZ6, Wetzlar, Germany). Wet mounts stained with lactophenol and cotton blue were prepared to examine the morphology of microscopic reproductive structures under a light microscope (Leica DM LS, Wetzlar, Germany) (3). Molecular identification was performed by sequencing the ITS1-5.8S-ITS2 rDNA region and results were queried at the NCBI-GenBank database. Particularly for strain M7.7, phylogenetic analyses were carried out as described in Sette *et al*. (24).

The stock cultures were maintained in Sabouraud slant agar, under water and mineral oil, at room-temperature (25 ± 2°C).

Enzyme production by solid-state fermentation (SSF)

All the isolated species were cultivated under SSF using

as carbon source a mixture (5.0 g) (1:1 w/w) of wheat bran and sugar cane bagasse. The wheat bran used was bought from local retailer and was washed, ground, oven dried (60 ^{o}C for 24h) and sieved (Tyler mesh 10 – 12) to particles of diameter 1.4 mm. The sugar cane bagasse was obtained from a Brazilian ethanol industrial plants (Usina Cerradinho, Catanduva-SP) and was also washed, oven dried (60 °C for 24h) and sieved. The particles with 2.0 mm were used. The solid substrate was placed in polypropylene bags (12 x 20 cm) and sterilized at 120 °C for 40 min. Conidia or mycelial biomass of cultures with 3 days growth were suspended in a sterile nutrient solution composed of (g L^{-1}): $(NH_4)_2SO_4$, 3.5; KH_2PO_4, 3.0; $MgSO_4$ $7H_2O$, 0.5; $CaCl_2$, 0.5 and Tween 80 (1.0 % v/v) and a volume equivalent to 10^7 spores or 5g equivalent of dry mycelial biomass per gram of substrate was used as inoculum and more 20 mL of the same nutrient solution were added to the substrate.

The fermentation was carried out at 45 °C for 14 days. After 24 h, one bag was taken and the fermented material was mixed with distilled water (20 mL per gram of fermented material), stirred for 30 min, filtered and centrifuged at 10000 x *g*, at 10 °C. The other samples were taken each 48h. The supernatant was used as a crude enzyme solution.

The fungi that presented the higher enzymes productions were then cultivated by SSF using different mixtures of lignocellulosic material (w/w): sugarcane bagasse and wheat bran (9:1), banana tree leaves and wheat bran (9:1), rice husk and wheat bran (9:1), rice husk and barley bran (9:1), corn straw and wheat bran (9:1), corn straw and barley bran (9:1), wheat bran and sugarcane bagasse (8:2), and wheat bran. The material was washed in tap water to remove impurity and until absence of detectable reducing sugars, dried at 65 ºC, ground and sieved to select particles of 1.0 to 3.0 mm. The fermentation procedures were identical to those described above but the sample were taken each 48h.

Enzyme activity measurements

Endo-glucanase and xylanase activities were assayed in a reaction mixtures containing 0.1 mL of crude enzyme and 0.9 mL of sodium acetate buffer solution at 0.1 M, pH 5.0 containing carboxy methyl cellulose (CMC) or xylan (birchwood) (5.0 g/L) which were incubated at 60 ^{o}C for 10 min. The quantification of released reducing sugar (expressed as glucose or xylose) was carried out by the DNS (3,5-dinitrosalicylic acid) method (19), using a glucose and xylose standard curves. Cellulase activity on filter paper (FPase) was determined using a piece of paper Whatman nº 1 (1.0 cm x 6.0 cm) dipped in 1.0 mL acetate buffer 0.05 M, pH 5.0 and 0.5 mL of crude enzyme solution The reaction was carried out at 60 °C for 60 min and stopped with 3.0 mL of DNS according to Ghose, T.K. (1987). One unit of enzyme activity (U) was defined as the amount of enzyme required to release one μmol of reducing sugar per minute under assay conditions.

The β-glucosidase activity was determined according to Leite *et al*. (13) in reaction mixture composed of 50 μL of crude enzyme by solution, 250 μL of sodium acetate buffer (0.1 M; pH 5.0) and 250 μL of 4-nitrophenyl-β-D-glucopyranoside (4 mM), (PNPG, Sigma) incubated at 60 ^{o}C, for 10 min. The reaction was stopped by the addition of 2.0 mL of Na_2CO_3 (2 M). One unit of enzyme activity (U) was defined as the amount of enzyme required to release one μmol of *p*-nitrophenol per minute in the assay conditions.

Enzymes properties

The effect of pH on the enzyme activities was investigated by incubating the reaction mixture at 60 ^{o}C for 10 min, using solutions containing 5.0 g L^{-1} of CMC or xylan in several buffers at 0.1 M: sodium acetate (pH 3.5-5.0), MES (pH 5.5-6.5), HEPES (pH 7.0-8.0), glycine-NaOH (pH 8.5-10.5). Optimum temperature was determined by incubating the reaction mixture at temperatures ranging from 30 ^{o}C to 80 ^{o}C, at the optimum pH.

The thermal stability was investigated by measuring the enzyme activity after keeping the aqueous enzyme solution for 1 h, at temperatures between 35 ºC and 80 °C in absence of substrate. Remaining enzyme activity was determined at

optimum pH and temperature.

The pH stability of the crude enzyme was evaluated by mixing the enzyme solution and the buffers quoted above to give final proportion of 1:1 (v/v). These solutions were incubated at 25 ºC for 24 h. An aliquot was taken to determine the remaining enzymatic activity at the optimum pH and temperature.

RESULTS AND DISCUSSION

Isolation of thermophilic and thermotolerant fungi

From 73 collected samples, a total of 26 filamentous fungi and only one yeast were isolated. This number of thermophilic fungi isolated is in accordance with those cited by Martin *et al.* (16), who isolated thermophilic pectinolytic fungi from a pile of compost. Both results corroborate those obtained by Maheshwari *et al.* (14) that reported that few thermophilic fungi species have been described being around 30 of the 75,000 fungi already described. The increasing in temperature during the decomposition of the lignocellulosic material by microorganisms provide the succession from mesophilic, to thermotolerant and thermophilic microorganisms and a number of thermophilic cellulolytic and hemicellulolytic fungi specimens could be found . In relation to genus detected, the prevalence of *Aspergillus* in the samples (Table 1) is consistent with the reports of Wareing (32), Ghatora *et al*. (5) and Martin *et al*. (16) and it is possible that these results are due to the technical approach used, once representatives of this genus are fast growing and easily obtained from many substrates. Culture methods alone have not showed to be sufficient to measure the quantitative and qualitative microbial community in function of its complexity and of the medium and culture conditions standardization (29). Maybe the association between culture and culture-independent methods could improve the detection of the microbial cellulolytic and hemicellulolytic community associated in degradation of plant biomass.

Table 1. Maximum production of xylanase and cellulase (FPase) by thermophilic fungal strains in SSF using sugarcane bagasse and wheat bran as substrate (1:1 w/w) throughout the fermentation process.

Sample	Strain	Maximum enzyme activity obtained (U g^{-1})			
		Xylanase	t* (h)	FPase	t* (h)
Manure	*Aspergillus* sp. M.1.3	588.0	96	0.2	24
Sugar cane bagasse pile	*Aspergillus* sp. M.2.8	708.0	96	0.4	24
	Aspergillus sp. M.2.11	374.0	96	0.6	288
	Aspergillus fumigatus M.7.1	1040.0	144	0.8	48
	Aspergillus fumigatus M.7.3	1239.0	192	1.0	48
	Thermomucor sp. M.7.6	15.0	336	ND	-
	Myceliophthora thermophila M.7.7	1292.0	96	2.0	48
	Aspergillus sp. M.7.8	354.0	96	1.8	96
	Aspergillus sp. M.7.14	602.0	96	0.4	24
Decaying grass	M.3.4	338.0	144	0.4	24
Silage	M.4.7	ND	-	0.4	288
	M.4.10	674.0	48	0.8	288
Crops wastes	*Aspergillus* sp. M.6.1	392.0	144	3.2	288
	M.6.2	386.0	96	1.4	144
	M.6.3	55.0	96	0.4	24
	Aspergillus sp. M.6.3.E	290.0	144	0.8	48
	Candida sp. M.6.3.L	ND	-	ND	-
	M.6.4	ND	-	ND	-
	Aspergillus sp. M.6.5	234.0	48	0.6	48
	M.6.9	669.0	144	1.0	96
	Thermomyces sp. M.6.10	ND	-	0.6	192
	M.6.11	213.0	336	0.6	48
	Thermomyces sp. M.6.12	147.0	96	0.6	24
	M.6.14	355.0	240	0.2	24
	M.8.1	300.0	192	0.4	24
	M.8.5	326.0	96	0.4	24
	M.8.6	922.0	144	0.8	144

*ND- No Detected

t*=period of fermentation when was obtained the maximum activity in the

Production of xylanase and cellulase (FPase) by the isolated fungal strains

The first evaluation of the isolated fungi ability to produce xylanolytic and cellulolytic enzymes was carried out by SSF using a mixture of sugarcane bagasse and wheat bran (1:1 w/w). Xylanase and FPase productions ranging from 15 to 1292 and from 0.2 to 3.2 U. g^{-1}, respectively, were observed. The strains M.7.1, M.7.3 and M.7.7 were the best producers of xylanase (1040, 1239 and 1292 U. g^{-1}, respectively) and the higher FPase activities were observed from the culture of M.6.1, M.6.2, M.7.7 and M.7.8 (3.2, 1.4, 2.0, 1.8) (Table 1). Lower value for FPase activity (0.7 U. g^{-1}) was obtained by Badhan *et al*. [4] when cultivating *Myceliophthora* sp. IMI 387099 on mixture of sugarcane bagasse and wheat bran, although higher values (243 U. g^{-1} and 19.5 U. g^{-1}) were obtained by Gao *et al.* (4) and Kang *et al*. (9) in cultures of *A. terreus* and *A. niger* KK2 on wheat bran.

The xylanase activity was comparable to that obtained from *Myceliophthora* sp IMI387099 (900 U. g-1) and *Fusarium oxysporum* (1840 U. g^{-1}) (1, 20).

The sugarcane bagasse is composed of, approximately, 15% of lignin, 30-35 % of celullose and 30-35% of hemicellulose, and it is a good substrate to induce the microbial production of cellulolytic and hemicellulolytic complex. Wheat bran has also been an excellent medium for fungal growth and for the production of these enzymes in function of its high nutrient content (13, 18, 23, 25).

The data from Table 1 shows that, although the value of FPase activity was not high, the isolated fungi showed to be good xylanase producer. According to Percival Zhang *et al.* (21) the use of the filter paper method to quantify cellulolytic activity can be subject to several variations that could influence the results. In this way, the endo-glucanase (CMCase) and β-glucosidase activities were determined in subsequent assays.

The strains M.7.1 and M.7.7 were chosen to give continuity to the research since they showed a good potential for FPase and xylanase production. These strains were subjected to taxonomic characterization by morphological and molecular approaches. Analyses of macro and micro-morphological characteristics classified the isolate M.7.7 and M.7.1 in the genus *Myceliophthora* and *Aspergillus*, respectively. Data derived from BLASTn results using the ITS-rDNA region as a molecular marker showed that isolate M.7.7 had 99% sequence identity with *Myceliophthora thermophila* strain ATCC 42464. Phylogenetic analyses using the neighbor-joining algorithm indicated that isolate M.7.7 clustered with strain ATCC 42464 and this grouping was confirmed by 99% of bootstrap support (Figure 5). In addition, BLASTn results indicated that strain M.7.1 showed 100% sequence identity with ITS-rDNA sequences of several different strains of *Aspergillus fumigatus* deposited at NCBI-GenBank database.

Considering the combined methods used for fungi identification, the isolate M.7.1 was identified as *Aspergillus fumigatus* and isolate M.7.7 as *Myceliophthora thermophila*.

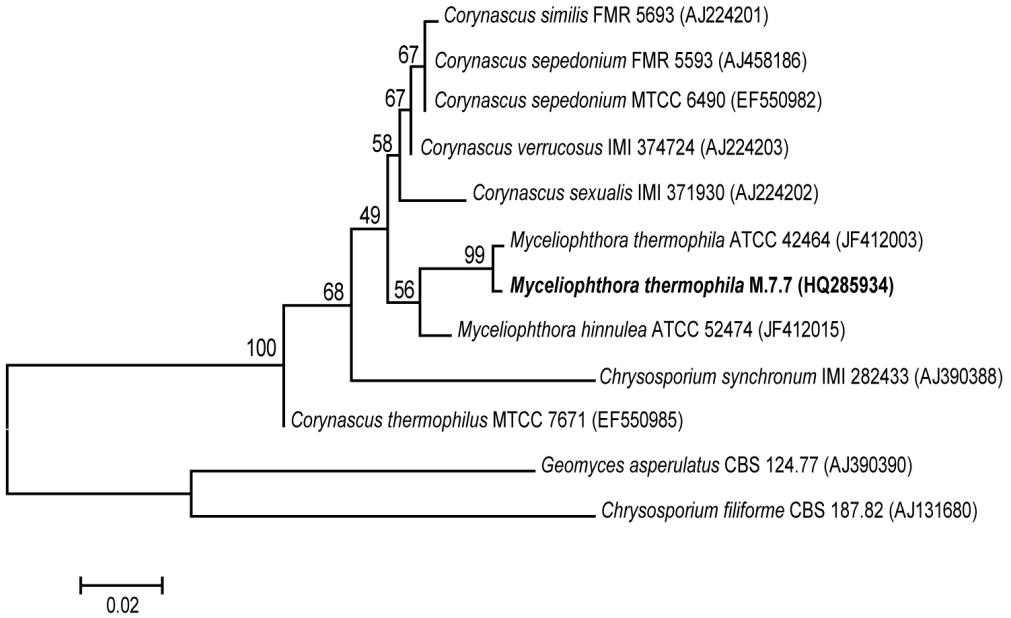


Figure 5. Neighbor-joining phylogenetic tree inferred from a 464-base pair fragment of ITS-rDNA region of strain M7.7. and close fungal relatives. The Kimura 2-parameters model was used as the nucleotide substitution model. Numbers on branches are bootstrap support values obtained from 1,000 pseudoreplicates. Names of fungi are followed by the voucher accession number (in culture collections) and the NCBI-GenBank accession number in parentheses.

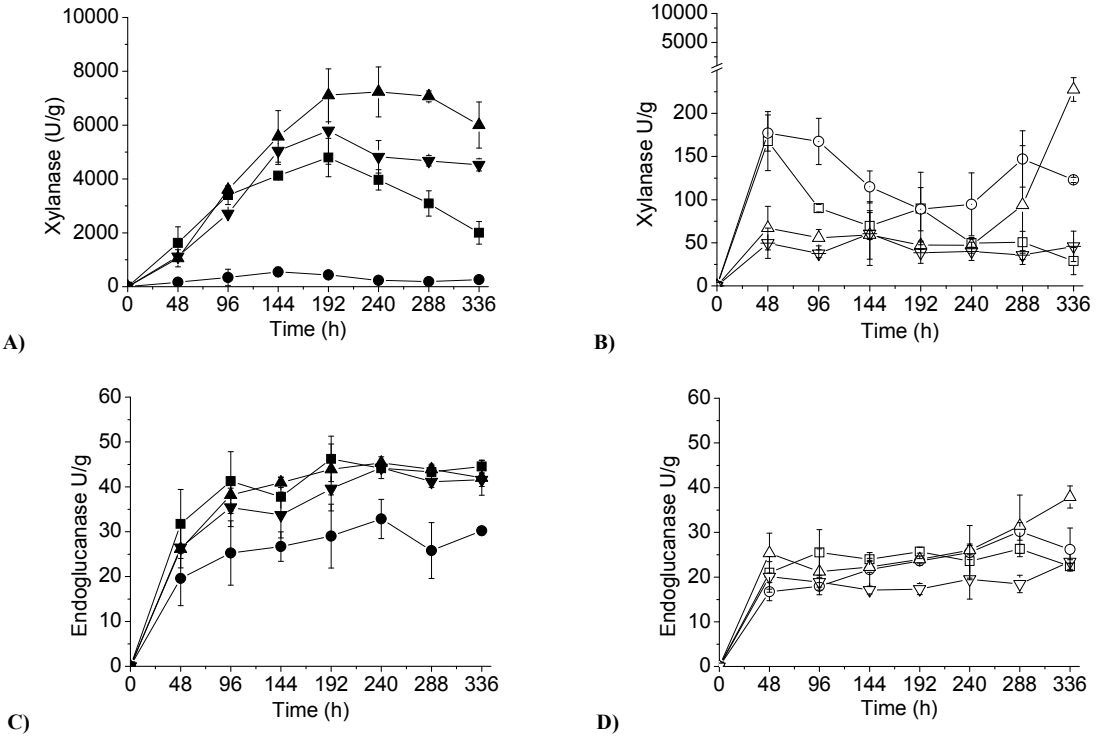
Production of xylanase, endoglucanase (CMCase) and β-glucosidase by *Myceliophthora thermophila* M.7.7 and *Aspergillus fumigatus* M7.1.

A. fumigatus M.7.1 and *M. thermophila* M.7.7 were cultivated under SSF using different lignocellulosic materials as substrates. The maximum xylanase (7238 U. g^{-1}) and β-glucosidase (40.4 U. g^{-1}) productions by *A. fumigatus* M.7.1 were obtained by culture on a mixture of corn straw and wheat bran and on wheat bran, respectively (Figure 1A, B, E, F). CMCase production by *A. fumigatus* was similar on mixtures of sugarcane bagasse and wheat bran, corn straw and wheat bran and corn straw and barley bran with values varying from 40 to 47 U. g^{-1} (Figure1C, D).

M. thermophila M.7.7 produced 1044.6 U. g^{-1} of xylanase and 54 U. g^{-1} of CMCase in medium composed of a sugarcane bagasse and wheat bran mixture (Figure 2A, B, C, D) after 192 h of fermentation. Similar to that observed for *A. fumigatus*, the maximum β-glucosidase activity was detected after 336h of fermentation on wheat bran (Figure 2E, F). These data indicate that the kind of enzyme produced depends on the substrate used for both microorganisms which could have different potential of enzyme induction, i.e. the medium containing only wheat bran improved the β-glucosidase production.

The presence of complex carbon sources such as rice straw decreased the production of all enzymes (Figure 1, 2B, D, F). Probably the high silicate content this residue difficult the degradation and access of microorganism to the polysaccharides.

Xylanase, CMCase and β-glucosidase production of both fungi were lower than those reported for some *Thermoascus aurantiacus* strains (6193, 1572 and 101.6 U. g^{-1}, respectively), since this is a species known as an excellent xylanase and cellulase producer (7, 11). On the other hand, the enzyme production by both *A. fumigatus* M.7.1 and *Myceliophthora* sp M.7.7 are similar to those observed for many fungal species, including other *Thermoascus* strains. For example, the cultivation of *Thermoascus aurantiacus* CBMAI 756 on wheat bran (13) provided a β-glucosidase production of 58 U. g^{-1}, a value close to those reported in the present work. Similar values were also cited by Heidorne *et al*. (8), when cultivating *Ceriporiopsis suvermispora* by SSF on wood chips. The β-glucosidase and CMCase productions cited by these authors were around 53- 43 and 15-31 U. g^{-1}, respectively.



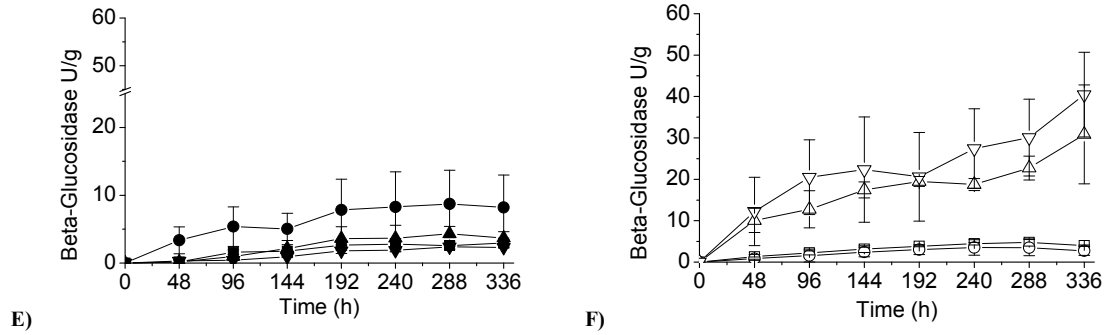


Figure 1. Xylanase (A, B), endoglucanase (C,D) and β-glucosidase (E, F) production by *Aspergillus fumigatus* M.7.1 in SSF using different crops and agro-industrial wastes mixtures (9:1 w/w). (■) sugar cane bagasse and wheat bran; (●) banana wastes and wheat bran; (▲) corn straw and wheat bran; (▼) corn straw and barley bran; (□) rice husk and wheat bran; (○) rice husk and barley bran; (△) wheat bran and sugar cane bagasse (8: 2 w/w); (▽) wheat bran.

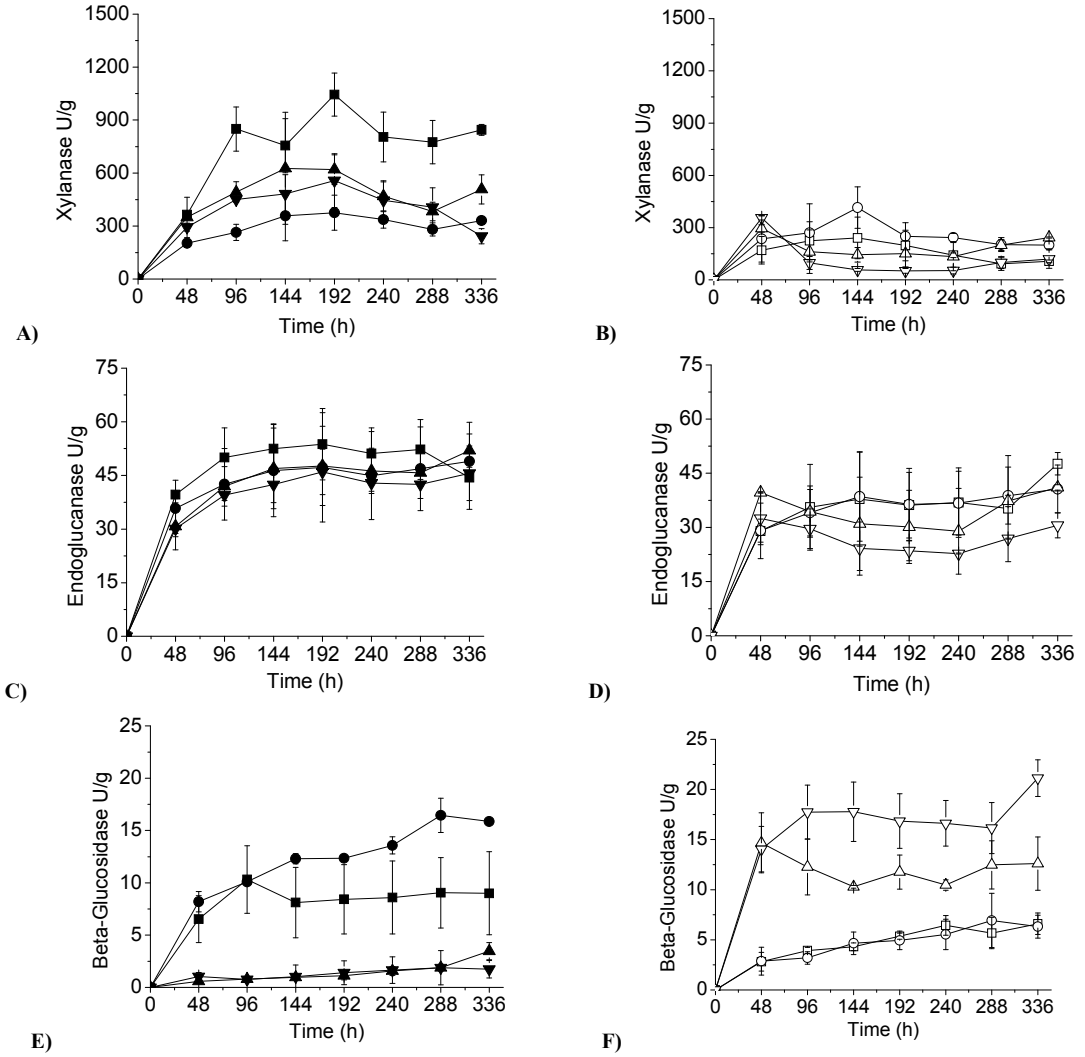


Figure 2. Xylanase (A, B), endoglucanase (C, D) and β-glucosidase (E, F) production by *Myceliophthora* sp. M.7.7 in SSF using different crops and agro-industrial wastes mixtures (9:1 w/w). (■) sugar cane bagasse and wheat bran; (●) banana wastes and wheat bran; (▲) corn straw and wheat bran; (▼) corn straw and barley bran; (□) rice husk and wheat bran; (○) rice husk and barley bran; (△) wheat bran and sugar cane bagasse (8: 2 w/w); (▽) wheat bran.

Enzymes properties

The optimum pH for endo-glucanase and xylanase from *A. fumigatus* M.7.1 were 4.5 and 4.5-5.5, respectively (Figure 3A) and 5.0 for both enzymes from *M. thermophila* M.7.7 (Figure 3B). These results were near to related by Silva *et al*. (26). The maximum activities were observed at 60 ºC for xylanase and 70 ºC for CMCase from *A. fumigatus* M.7.1. Xylanase and CMCase from *M. thermophila* M.7.7 exhibited maximum activities at 70 ºC (Figure 3C, D). All enzymes from both fungi were stable in the large pH range (5.0-10.0) (Figure 4A, B). The enzymes showed themselves to be thermostable, maintaining 90% of the initial activity when kept for 1 h at 65 ºC in absence of substrate, except the xylanase from *A. fumigatus* M.7.1 that lost 50% of the activity after 1 h at 55 ºC (Figure 4C, D). The thermostability of both enzymes is higher than that reported for xylanase and endo-glucanase from mesophilic fungi such as *Aspergillus* sp 2M1, *Trichoderma reesei* (2), *Penicillium canescens* CP10, *P. janthinellum* and *Penicilium* sp (30) and was similar to described for thermophilic as *T. aurantiacus* and *Thermomyces lanuginosus*, which were stable up to 60 ºC (11, 27).

The stability of an enzyme is an important parameter for its application in an industrial process. Enhanced stability and the maintenance of a desired level of activity over a long period are two important points to be considered for the selection and design of enzymes (10).

With this study, we have been able to isolate thermophilic and thermotolerant fungi. Furthermore, the strains studied presented potential for enzymes production such as xylanase and cellulase, which can be used in hydrolyses of plant biomass.

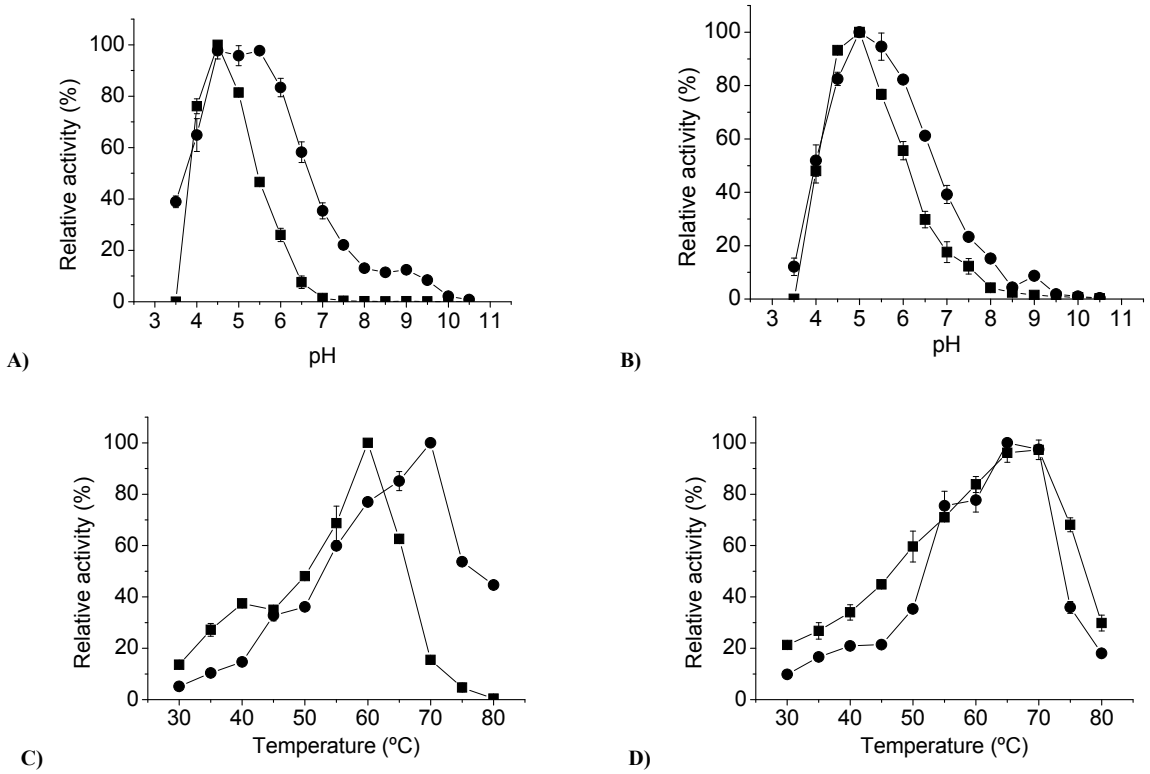


Figure 3. Effect of pH and temperature on activity of xylanase (■) and Endo-glucanase (●) produced by *Aspergillus fumigatus* M.7.1 (A, C) and *M. thermophila*. M.7.7.(B, D).

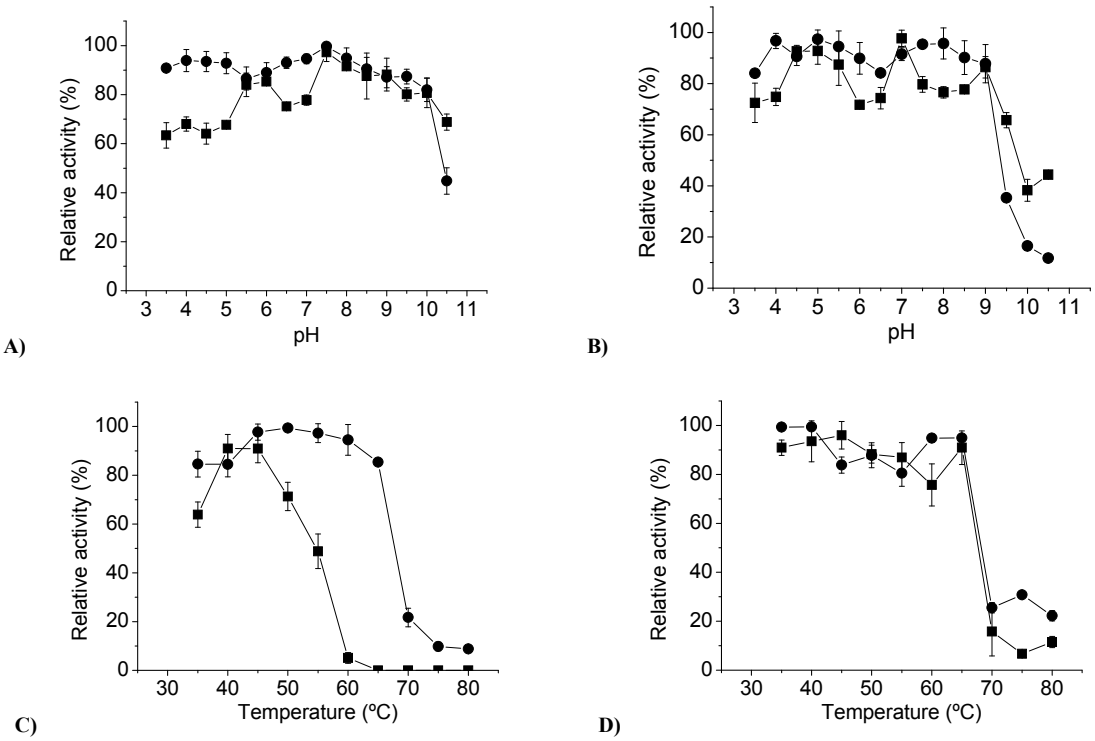


Figure 4. Effect of pH and temperature on stability of xylanase (■) and Endo-glucanase (●) produced by *Aspergillus fumigatus* M.7.1 (A, C) and *M. thermophila* M.7.7.(B, D) when maintained for 1h in absence of substrate.

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