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Fibrolytic enzyme production of *Myceliophthora thermophila* M.7.7. using inexpensive carbon sources and mineral nutrients

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This study investigated the effect of inexpensive carbon and nitrogen sources on enzyme production by *Myceliophthora thermophila* M.7.7 in solid-state fermentation. Three kinds of lignocellulosic waste (corn straw, sugarcane bagasse and sugarcane straw) and six nitrogen sources (urea, calcium nitrate, analytical ammonium sulphate, yeast extract, agricultural fertilizer NPK 20-05-20 and fertilizing grade ammonium sulphate) were tested. Some physical-chemical parameters of the fermentation, such as temperature, initial pH and moisture content of the substrate on enzyme production, were evaluated. The maximum activities of xylanase (446.9 U/ml), endoglucanase (94.7 U/ml) and β-glucosidase (2.8 U/ml) were observed in a mixture of corn straw and wheat bran (1:1 w/w) as the carbon source using fertilizer grade ammonium sulphate as the nitrogen source. This production occurred for an incubation period of 96 h, at 40°C, with initial moisture content of 70% and pH 5.0. These results have significant interest since they could be used for the future production of enzymes in a low-cost industrial process.

Key words: *Myceliophthora thermophila*, solid-state fermentation, xylanase, endoglucanase, β-glucosidase.

INTRODUCTION

The hydrolysis of cellulose and hemicellulose present in plant cell walls into glucose and xylose requires the cooperative action of complex enzymes of cellulase and xylanase groups, respectively (Panagiotous et al., 2003; Soni et al., 2010; Danmek et al., 2014). These enzymes have great application potential in several biotechnological processes such as the bioconversion of biomass wastes to fermentable sugars (Fang et al., 2010; Huang et al., 2013).

Enzyme production by filamentous fungi is attractive technologically and has advantages over bacteria and yeasts due to their ability to grow on solid substrates and secrete a higher quantity of extracellular enzyme. These properties make possible the use of agro-industrial residues as substrates in solid state fermentation (SSF) allowing the production of low-cost enzymes (Jecu, 2000;

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Gao et al., 2008; Singh et al., 2009; Longwei et al., 2014). In the fermentation process, it is also important to consider the cost of nitrogen and other macronutrient sources (Su et al., 2011) because, it is very important to find alternatives of using inexpensive mineral nutrients to the culture media. The fermentation parameters such as pH, temperature, moisture, aeration and incubation time influence the expression and secretion of the enzymes significantly affecting the targeted product (Lakshmia et al., 2009).

The heat released by the microbial activity during the fermentation process causes increase of the temperature of the system requiring cooling of the bioreactor. The use of thermophilic fungus in SSF has been very promising since they can adapt to variations in temperature during the process and do not require cooling (Gomes et al., 2009).

This study aimed to evaluate the conditions of xylanase and cellulase production searching an inexpensive fermentative process to make them viable for future industrial application. The strategy used was to cultivate the thermophilic fungus *Myceliophthora thermophila* M.7.7 in SSF using inexpensive agro-industrial waste as carbon sources and commercial agricultural fertilizer as nutrients sources. In addition, the effect of various physico-chemical conditions was evaluated on enzyme production.

MATERIALS AND METHODS

Microorganism and effect of temperature on fungal growth

The strain *M. thermophila* M.7.7 used in this study was isolated from decaying sugarcane bagasse piles and identified by the data derived from BLASTn results using the ITS-rDNA region as a molecular marker which had 99% sequence identity with *M. thermophila* strain ATCC 42464 (Moretti et al., 2012). The culture was maintained on slanted Sabouraud agar (g/L: 40.0 dextrose, 10.0 peptone, 20.0 agar and pH 5.6) under water and mineral oil, at room-temperature (25 ± 2°C) and by spores immersed in 20% glycerol at -80°C.

In order to investigate the performance of the strain at various temperatures, mycelia from pure cultures were spotted on agar plates and incubated at 37, 40, 45, 50 and 55°C. The diameters of the colonies were measured at 12 h intervals. All experiments were performed in replicates of three.

Enzyme production under solid state fermentation (SSF) on different agricultural wastes and mineral nutrients

The effect of lignocellulosic wastes (mixtures of corn straw, sugarcane bagasse or straw with wheat bran, w/w 1:1) on enzyme production was studied according Moretti et al. (2012). The dried substrates were ground to particles of 3 mm and 5 g of the substrate mixture was placed in polypropylene bags (size 12 x 20 cm). The chemical composition of substrates were (% w/w): corn straw was cellulose 28%, hemicellulose 15% and lignin 19%; sugarcane bagasse was cellulose 47%, hemicellulose 16% and lignin 27%; sugarcane straw was cellulose 43%, hemicellulose 15% and lignin 23%.

The nitrogen sources urea, calcium nitrate, ammonium sulphate, yeast extract, agricultural fertilizer NPK (20:05:20 -Heringer) and ammonium sulphate fertilizer grade were tested. Additionally, nutrient solutions were composed of (g/L) 3.0 KH_2PO_4, 0.5 $MgSO_4.7H_2O$, 0.5 $CaCl_2$ and Tween 80 (0.1%), pH 5.0 (modified Mandels and Sternberg, 1976 method) and of a solution formulated with agricultural fertilizer containing (g/L) 10.0 ammonium sulphate, 3.0 mono-ammonium phosphate (MAP) (9% nitrogen and 48% phosphorus) and 2.0 potassium chloride (Heringer), 0.5 $MgSO_4.7H_2O$, 0.5 $CaCl_2$ and Tween 80.

The substrates containing nutrient solution were autoclaved at 121°C for 30 min. The inoculum consisted of 2 x 10^7 spores/g substrate and the final moisture content of the medium was 80%. After inoculation, the material was incubated at 45°C for 10 days and samples were collected at 48 h intervals. Crude enzyme solutions were obtained using suspensions of the fermented material in 100 mL distilled water. The filtrate was centrifuged at 10000 ×*g* for 15 min at 10°C and the supernatant liquid was used as crude enzyme.

Enzyme assays

Xylanase and endoglucanase activities were assayed in 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 added xylan (birchwood) (10.0 g/L) or carboxymethylcellulose (CMC) (40.0 g/L), which were then incubated at 60°C for 10 min. The free xylose and glucose units produced as a result of xylanase and endoglucanse activity, respectively; react with 1.0 mL of 3-5 dinitrosalicylic acid (DNS) reagent (Miller, 1959). This final reaction forms a colored complex that was measured by spectrophotometer at 540 nm. The enzyme activity was defined in International Units (U), as the amount of enzyme required to release 1 µmol of product per 1 min in the assay conditions.

The β-glucosidase activity was determined according to Leite et al. (2008) in a reaction mixture composed of 0.050 mL of crude enzyme solution, 0.250 mL of sodium acetate buffer (0.1 M; pH 5.0) and 0.250 mL of 4-nitrophenyl-β-D-glucopyranoside (4 mM), (PNPG, Sigma) incubated at 60°C for 10 min. The reaction was stopped by the addition of 2.0 mL of Na_2CO_3 (2 M) and was measured at 410 nm. One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenol per 1 min in the assay conditions.

Effect of incubation temperature, initial pH and moisture on enzyme production

To evaluate the effect of incubation temperature, the two substrate and nutrient supplements which had high enzyme activity in the previous assay were used. The inoculated substrates were incubated at 40, 45, 50°C for 10 days. In all the experiments, the pH and moisture content were maintained as previously described (Gautam et al., 2011).

The effect of initial pH of the media on enzyme production was evaluated at pH 5.0, 5.5, 6.0, using a substrate at 80% of moisture under a temperature that allowed high enzyme activity, for 10 days. The effect of substrate moisture was studied for 60, 70 and 80% using the best substrate and nutrient conditions. For the enzyme extraction, distilled water was added to fermented material at proportions of 1:10 (w/v).

Evaluation of the amount of eluent and use of buffer and surfactant in the enzyme extraction

The fermented material was mixed and divided into five equal parts. Three were used to evaluate the proportion of eluent (1:10, 1:20

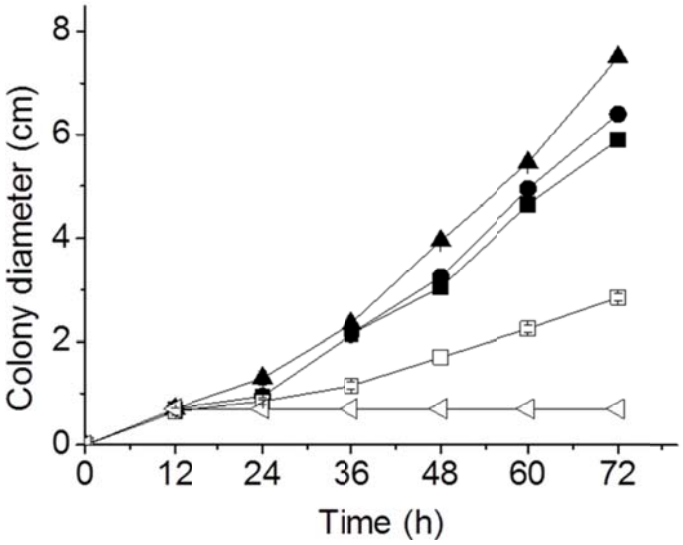

Figure 1. Growth curves of the fungus *Myceliophthora thermophila* M.7.7. in Petri dishes containing solid medium agar Saboroud, at different temperatures, for 72 h. ■ 37°C; ● 40°C; ▲ 45°C; □ 50°C; ◁ 55°C.

and 1:30 w/v) using distilled water and two of them were used to evaluate the type of eluent (Tween 80 at 0.2 mL/L and sodium acetate buffer 0.1M, pH 5.0) at 1:10 (w/v) proportions.

SDS-PAGE analysis and xylanase, endoglucanase and β-glucosidase activity detection by zymogram analysis

Polyacrylamide gel (SDS-PAGE) 10% (w/v) was used for detection of protein bands from crude enzyme solution as described by Laemmli (1970). The molar mass of proteins, under denaturizing conditions, was determined with reference standard proteins (SDS-PAGE Molecular Weight Standards, Broad Range, Bio-Rad from 6.5 to 200 kDa). Protein bands were stained with silver.

For zymogram activities, samples of crude enzymes from *M. thermophila* M.7.7. were mixed in the loading buffer (2% SDS (w/v), 87% glycerol, 0.1 M Tris-HCl buffer pH 8.8 and bromophenol blue). After electrophoretic running, the gels were incubated for 30 min at 60°C in solutions containing xylan or carboxymethylcellulose for xylanase and endoglucanase activity, respectively. After incubation, the gels were stained with 0.1% Congo red solution under gentle shaking for 15 min at 25°C. Subsequently, the gels were immersed in 1 M NaCl solution until the appearance of clear bands on the red background. For better resolution of the bands, 0.1 M HCl was added.

For the β-glucosidase zymogram, after running, the gel was incubated for 10 min at 25°C in 0.2 M acetate buffer, pH 5.0. Subsequently, the gel was incubated for 1 h at 60°C in 0.2 M acetate buffer, pH 5.0 containing 0.1% esculin and 0.03% ferric chloride until the appearance of dark bands when the gel was dipped in 10% glucose solution to stop the reaction.

RESULTS AND DISCUSSION

Effect of temperature on fungal growth

M. thermophila M.7.7 showed maximum growth rate (7.5 cm colony diameter) at 45°C on a solid medium after 72 h of incubation. Colony diameters of 6.4 and 5.9 cm were obtained at 40 and 37°C, respectively. These results confirm the thermophilic profile of the fungus (Figure 1). This evaluation is necessary because the growth response of fungus on a solid medium may differ from that in a liquid medium and provides substantial guidance for the solid state fermentation process. This methodology has been used by other authors showing results consistent (Martin et al., 2010; Silva et al., 2005).

Enzyme production using different agricultural wastes and mineral nutrients in solid state fermentation (SSF)

Corn straw with wheat bran was the best carbon source for xylanase and endoglucanase production by *M. thermophila* M.7.7 (120 and 40 U/mL respectively) while the mixture of sugar cane bagasse and wheat bran allowed the highest β- glucosidase production (Figure 2). Badhan et al. (2007) obtained 62.0 U/ml of xylanase when using cultivated fungus *Myceliophthora* sp. IMI 387099 in sugar cane bagasse but lower amounts of endoglucanase (0.7 U/ml) and β-glucosidase (0.2 U/ml).
In a medium with fertilizer grade ammonium sulphate and analytical ammonium sulphate, maximum xylanase and endoglucanase activity were observed (Figure 2). These values were higher as compared to other studies with fungus of the same genus. According to data from Badhan et al. (2007), the measured activities of xylanase, endoglucanase and β-glucosidase were 90.0, 3.2 and 0.7 U/mL, respectively, when *Myceliophthora* sp. IMI 387099 was cultivated on rice straw with addition of ammonium sulfate (0.3%).

The highest productions of β-glucosidase (4.1 and 3.5 U/mL) were obtained in the media containing yeast extract and urea, respectively. On the other hand, it is clear that the endoglucanase and xylanase production were affected by supplementation with urea (Figure 2). Similar behavior was observed by Kalogeris et al. (2003) using thermophilic *Thermoascus aurantiacus*.

In this study, we can conclude that the corn straw with wheat bran was the best carbon source while ammonium sulfate was the most suitable nitrogen source for endoglucanase and xylanase production. Since there was no difference in enzyme production between the analytical grade and the fertilizer grade of the ammonium sulphate, we opted for the latter as the nitrogen source in the continuity of experiments considering the lower cost of input.

Evaluation of the amount of eluent and use of buffer and surfactant in the enzyme extraction

For enzyme extraction from the fermented solid substrate, three volume and different eluents were used.

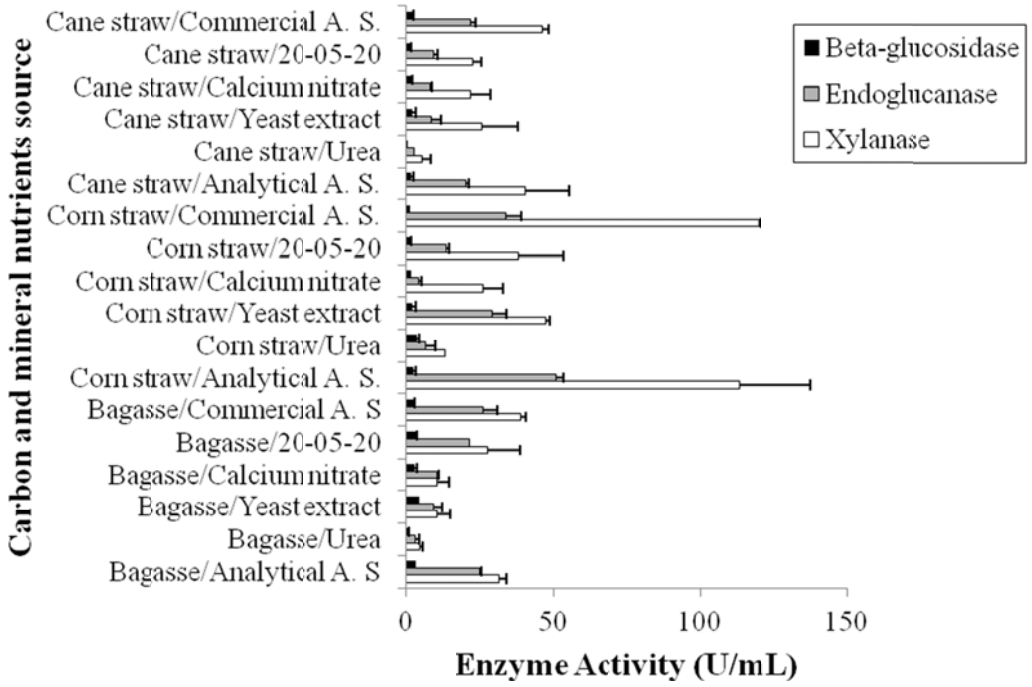

Figure 2. Maximum production of xylanase, endoglucanase and β-glucosidase by *M. thermophila* on different agricultural wastes and mineral nutrient sources. The fermentations were carried out at 45 ºC, pH 5.0, 80% moisture for 240 h. Data are the averages of two assays. Where: analytical A.S. = analytical grade ammonium sulphate, Comercial A.S. = fertilizer grade ammonium sulphate and 20:05:20 = NPK.

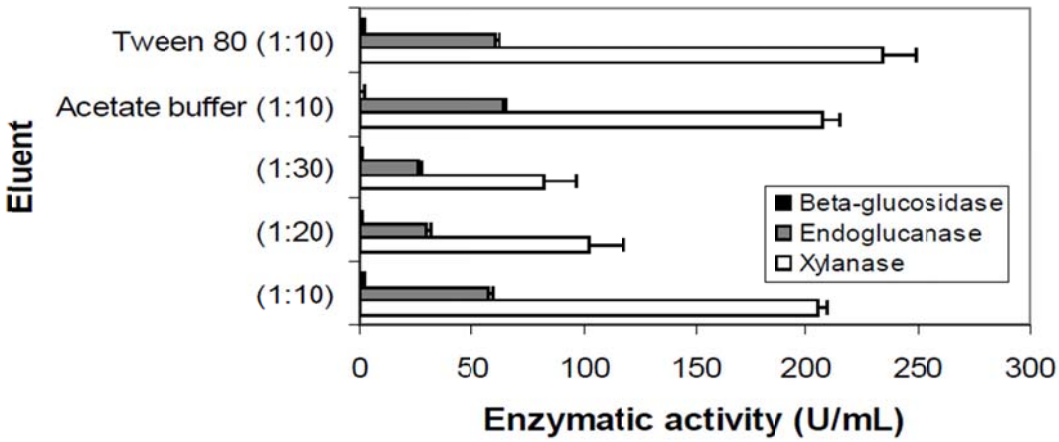

Figure 3. Effect of different eluents and their volume on the enzyme extraction: 1:10, 1:20, 1:30 (1 g of fermented material per distilled water w/v), 1:10 sodium acetate buffer (0.1 M, pH 5.0) and 1:10 Tween 80 (0.2 mL/L). The fermentation was carried out on corn straw and wheat bran (w/w 1:1) and a nutrient solution composed of analytical ammonium sulphate at 45°C, pH 5.0, 80% moisture and incubation for 96 h. Data are the averages of two assays.

According to Figure 3, the ratio of fermented material to water of 1:10 resulted in the highest extraction of enzymes and there were no significant differences among the three eluents, with only slightly higher extraction occurring when tween was used. The specific activities (data not shown) confirm these results. Therefore, it was decided by using water as eluent extraction because the buffer and tween could interfere with the subsequent steps of the study and application of the crude enzymes.

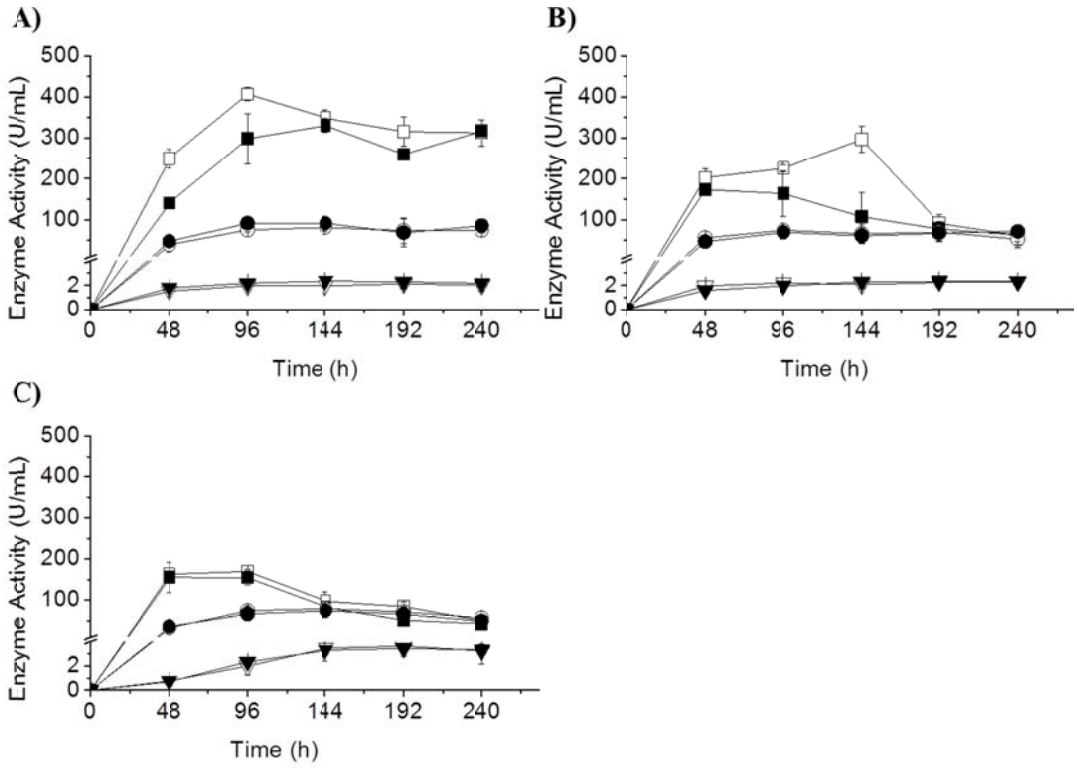

Figure 4. Effect of incubation temperature on SSF. At 40°C (A) 45°C (B) and 50°C (C) on production of xylanase (square), endoglucanase (circle), and β-glucosidase (triangle) by *Myceliophthora thermophila* using a medium consisting of corn straw and wheat bran. The open symbol: analytical grade ammonium sulphate and full symbol: fertilizer grade ammonium sulphate

Effect of incubation temperature on enzyme production

The maximum activity of xylanase (407.0 U/mL) was obtained when *M. thermophila* was grown at 40°C (Figure 4A). When incubated at 45 and 50°C there was a large reduction in the production of xylanase (Figure 4B and C) while the endoglucanase production was little affected by temperature of incubation. The production of β-glucosidase peaked (3.6 U/mL) at 50°C (Figure 4C). These data suggest that temperatures above 40°C could affect the xylanase stability although the growth of fungus was higher at 45°C and also indicated that cellulases were more thermostable than xylanase. Similar results were obtained by Roy et al. (1990) where there was a higher production of β-glucosidase (0.12 U / ml) when *M. thermophila* D14 was cultured at 50°C.

Effect of initial pH and moisture on enzyme production

Figure 5A shows the effect of the pH of the culture medium on the production of cellulases and xylanases. There were no significant differences in the production of enzymes in the extensive pH range tested, with less than 10% variation between the maximum and the minimum activity obtained in the different pH tested, throughout the cultivation period of 240 h. These data corroborate other reported in the literature such as those of Xiong et al. (2004) with xylanase production by *Trichoderma reesei* Rut C-30, those of Shingh et al. (2009) with xylanase from *Coprinellus disseminatus* and those of Sohail et al. (2009) with endoglucanase from *Aspergillus niger* MS82.

The effect of substrate moisture on the production of enzymes by *M. thermophila* is shown in Figure 5B. The maximum xylanase activity (446.9 U/mL) was obtained on the substrate containing 70% moisture after 96 h of cultivation. When the moisture was 60 and 80%, there was a reduction in the xylanase activity (19 and 15%, respectively). The highest endoglucanase (94.7 U/ml) and β-glucosidase (2.8 U/ml) activities were observed after 144 and 240 h of fermentation respectively, with the same profile of xylanase, with a higher production at 70% moisture.

The production of enzymes from *M. thermophila* M.7.7 was quite stable under the effects of a wide range of pH and moisture contents. This characteristic is very interesting for use in industry, since the control of pH and moisture are the most critical parameters to be controlled

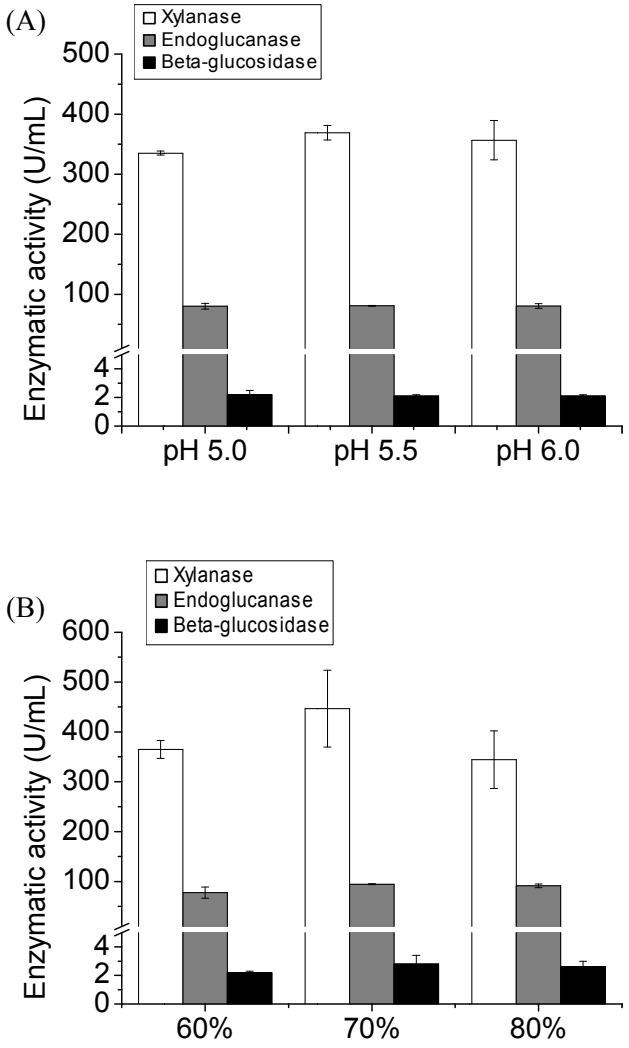

Figure 5. Xylanase, endoglucanase and β-glucosidase production by *M. thermophila* with different initial pH (A) and moisture content (B). The fermentation conditions were carried out on corn straw and wheat bran mixture (w/w 1:1) and a nutrient solution composed of fertilizer grade minerals and ammonium sulphate at 40°C, for 240 h. Data are the averages of two studies.

due to the heterogeneity and the consistency of the solid material normally used as substrate (Lonsane et al., 1985).

SDS-PAGE analysis and xylanase endoglucanase and β-glucosidase activity detection by zymography

The objective of this assay was to verify if the enzyme extract produced by *M. thermophila* M.7.7 on two culture media, using fertilizer grade (1) and analytical grade (2) ammonium sulphate exhibited similar profiles, since ions and metals contained in the first one could inhibit the expression or the activities of the enzymes.

In Figure 6, line B revealed four isoform bands for

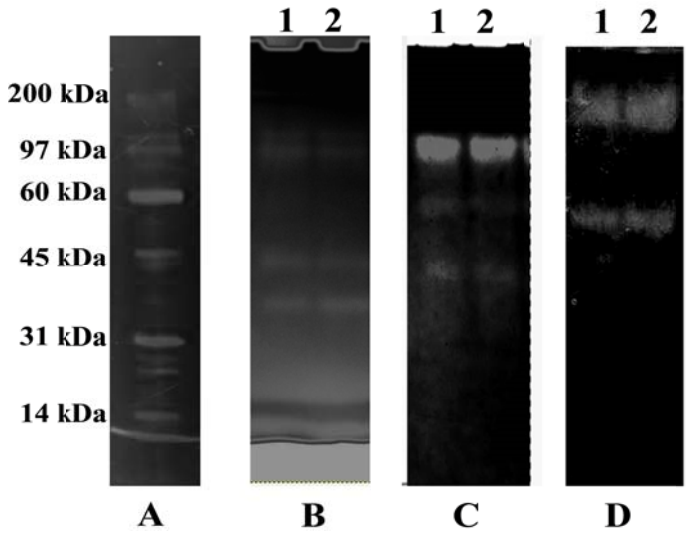

Figure 6. SDS-PAGE and zymogram analysis: (A) molecular weight marker, (B) crude endoglucanase, (C) crude xylanase, (D) crude β-glucosidase. The sample of crude enzymes from *M. thermophila* M.7.7. were obtained under SSF in: corn straw and wheat bran using two different nutrient solutions, fertilizer grade (1) and analytical grade (2) ammonium sulphate.

endoglucanase, corresponding to about 38, 45, 97 and 166 kDa. For xylanase activity (line C), three active isoforms were observed with approximately 43, 60 and 100 kDa. β-Glucosidase appeared in two bands corresponding to 50 and 200 kDa (line D). This similarity in the expression of enzymes in different culture media (1 and 2) shows that nutrient solutions formulated with fertilizer grade ammonium sulphate did not affect the enzymes expression or their activities.

The ability of *M. thermophila* to produce xylanase and endoglucanse in media composed of inexpensive carbon sources and mineral nutrients has commercial interest considering that the substitution of analytical chemical reagents for agricultural fertilizer in the cultivation of fungus reduces the cost of enzyme production. In addition, the fungus used in this study showed great stability in a certain range of temperatures, pH and moisture contents providing a much easier approach to the fermentation process.

Conclusions

In this study, it was established that the best incubation time for enzyme production by the thermophilic fungus *M. thermophila* M.7.7 was 96 h, at which time the maximum activity of xylanase (446.9 U/ml), endoglucanase (77.6 U/ml) and β-glucosidase (2.4 U/ml) was achieved. Corn straw and wheat bran (w/w 1:1) and fertilizer grade minerals can be successfully used as carbon and nitrogen sources. Temperature of 40°C, initial pH 5.0 and moisture content 70% afforded the highest enzyme production.

Conflict of Interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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