

## Optimization and biochemical characterization of extracellular xylanase from *Trichoderma harzianum* MH-20 under solid state fermentation

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**Abstract:** The production of extracellular xylanase was investigated employing ten fungal isolates grown on different agroindustrial wastes such as wheat bran, rice bran and sugarcane baggase. *Trichoderma harzianum* showed maximum enzyme production (85.3 U/mg) in a combination of wheat bran and rice bran in a ratio of 3: 1, respectively after 7 days of incubation. Different physical and chemical factors were optimized for the highest xylanase production. The maximum xylanase production was achieved at 75% moisture content, pH 5 and incubation temperature 30°C with peptone as a suitable nitrogen source. Enzyme characterization was also investigated. The optimum pH value and temperature for xylanase activity were 6 and 55°C, respectively. Xylanase exhibited remarkable stability over a broad pH and temperature range so; it has the potential to be used in biofuels, animal feed and food industry applications. SDS-PAGE analysis showed that the relative molecular mass of xylanase was about 23.0 kDa. Thus, the present study proved that *Trichoderma harzianum* is promising in xylanase production.

[Abo-Elmagd H. I. **Optimization and biochemical characterization of extracellular xylanase from *Trichoderma harzianum* MH-20 under solid state fermentation** . *Life Sci J* 2014;11(3s):188-195] (ISSN:1097-8135).

<http://www.lifesciencesite.com>. 28

**Keywords:** *Trichoderma harzianum*, Solid state fermentation, Xylanase.

### 1. Introduction

Xylanases (endo-1, 4-β-D-xylanohydrolase; EC 3.2.1.8) are glycosidases involved in depolymerization of xylan, the major renewable hemicellulosic polysaccharide of plant cell wall. Recently, interest in xylanase has markedly increased due to its wide variety of biotechnological applications such as pre-bleaching of pulp, improving the digestibility of the animal feed stocks, modification of cereal based stuffs, bioconversion of lignocellulosic material and agro waste to fermentable products, clarification of fruit juices and degumming of plant fibers (Kuhad and Singh, 1993; Ratanakhanokchai, et al., 1999; Kapoor et al., 2001) etc. Although xylanase from eubacteria and archaeobacteria have considerable higher temperature optima and stability than those of fungi, the amount of enzyme produced by these bacteria is comparatively lower than that produced by fungi. In general, the level of xylanase in fungal culture is typically much higher than those from yeasts and bacteria (Singh et al., 2003), moreover, (Zhang and Lynd, 2004) stated that most commercial cellulases are produced by filamentous fungi of the genera *Trichoderma* and *Aspergillus*.

The use of agriculture residues as low cost substrates for the production of industrial enzymes is a significant way to reduce production cost. The use of solid-state fermentation (SSF) is particularly advantageous for enzyme production by filamentous

fungi, since it simulates the natural habitat of the microorganisms (Hölker and Lenz, 2005) From the environmental point of view, the main benefit of SSF is the ability to use agroindustrial waste (sugarcane bagasse, wheat bran, soybean meal, etc.) as a solid substrate that acts as a source of both carbon and energy (Raimbault, 1998).

The main objective of this study is the production, purification and characterization of xylanase enzyme by solid state fermentation using different agroindustrial wastes.

### 2. Materials and Methods

#### Materials

##### Chemicals

Birch wood xylan and 3, 5-dinitrosalicylic acid (DNS) were purchased from Sigma Pharmaceutical Industries, Nasr City, Cairo, Egypt.

##### Microorganisms and culture conditions

Ten fungal species were isolated from soil in Cairo governorate and identified in Taxonomy Department, Ain-Shams University, Cairo city, Egypt. These fungal isolates were *Aspergillus flavus*, *A. niger*, *A. terreus*, *A. ustus*, *Penicillium chrysogenum*, *P. citrinum*, *P. olsonii*, *Chaetomium sp.* *Trichoderma harzianum* and *T. viride*. These isolates were maintained as single spore isolated on potato-dextrose-agar (PDA) medium, subcultured on PDA slopes and incubated at 30°C for 7 days. The

tested isolates were screened for the production of xylanase enzyme.

#### *Media*

*Fungal isolates maintenance and subculturing (PDA) medium* ( $g L^{-1}$ ).

Potato slices, 200; dextrose, 10; agar, 20 and distilled  $H_2O$ .

*Fungal inoculum (growth enhancement) medium* ( $g L^{-1}$ ).

Peptone, 5; Yeast extract, 1; glucose, 10;  $MgSO_4 \cdot 7H_2O$ , 0.5;  $KH_2PO_4$ , 0.5 and distilled  $H_2O$ .

*Xylanase production medium (SSF)*

The fungal cultivation in the SSF system was performed in a 500 ml Erlenmeyer flask containing 10 g of each substrate lonely (wheat bran, rice bran and sugarcane baggase) moistened with known volume of moistening agent. The moistening agent solution consists of ( $g L^{-1}$ )  $NH_4NO_3$ ; 5.00, corn steep liquor; 2.00, NaCl; 1.00, and  $MgSO_4 \cdot 7H_2O$ ; 1.00 and was supplemented with trace elements consisting of ( $mg L^{-1}$ ):  $FeSO_4 \cdot 7H_2O$ ; 5.00,  $MnSO_4 \cdot 4H_2O$ ; 1.60,  $ZnSO_4 \cdot 7H_2O$ ; 3.45 and pH 7.0.

#### **Methods**

##### *Fungal inoculum preparation*

The fungal inoculum was prepared by cultivation of the fungal strain in growth enhancement medium and the culture was incubated in bench-top shaker at  $30^\circ C$  for 48 h. 5 ml of inoculum pellets were used for fungal culture inoculation. 5 ml fungal pellets of each fungal culture were transferred to 500 ml Erlenmeyer flask containing xylanase medium production. The fungal culture was incubated in bench-top shaker at  $30^\circ C$ , and the culture was lasted for 7 days. The content of each flask was gathered up and thoroughly mixed with 10 ml cooled sterilized distilled water, then the mixture was filtered through a double-layered cotton wool and was centrifuged at 10,000 rpm for 10 min. The enzyme activity was determined in the supernatant.

##### *Enzyme assay*

Xylanase activity was determined by mixing 0.9 ml of 1% (w/v) birch wood xylan (in 50 mM Na-citrate buffer, pH 5.3) with 0.1 ml of filtrate and the mixture was incubated at  $50^\circ C$  for 5 min (Bailey et al., 1992). The reaction was stopped by addition of 1.5 ml of 3, 5-dinitrosalicylic acid (DNS) and the contents were boiled for 5 min (Miller, 1959). After cooling, the resulting color was read at 540 nm. The amount of reducing sugar liberated was quantified using glucose as standard. One unit of xylanase is defined as the amount of enzyme that liberates 1 mmol of glucose or xylose equivalents per minute under the assay conditions (Maria and Samia, 2006)

##### *Protein estimation*

The concentration of soluble protein was estimated using bovine serum albumin as the standard (Lowery et al., 1951).

##### *Optimization of xylanase production on SSF*

The optimization of SSF medium for xylanase production was performed by modification of the physical parameters such as moisture content 60 -85% (v/w), pH 4 -10, cultivation temperature in the range of 20 -  $50^\circ C$ . The effect of supplementation of additional nitrogen source on xylanase production was examined. The nitrogen sources consist of peptone, urea, tryptone, yeast extract and sodium nitrate with a concentration of 5.0% (w/w). All experiments were carried out in triplicates.

##### *Xylanase Purification*

All purification steps were done at  $4^\circ C$ . The crude lyophilized culture supernatant (200 ml) was precipitated by isopropanol (1:1, v/v) and dissolved in 50 mM citrate phosphate buffer, pH 6.0, (5 ml) then dialyzed against the same buffer for 24 h at  $4^\circ C$ . The enzyme sample was gel filtered through a Sephadex G-100 column (18×2 cm), preequilibrated with the same buffer. The elution was performed by the same buffer at a flow rate of 20 ml/h. Fractions of 5 ml were collected and assayed for their protein, xylanase activity. The active fractions with the highest specific activity of enzyme were pooled, mixed and dialyzed. The pooled fractions were further fractionated separately through a DEAE-cellulose column, and eluted with a 0-0.8 M-NaCl gradient in citrate-phosphate buffer (240 ml) at a flow rate of 10 ml/h. 5 ml fractions were collected and assayed for their protein and enzyme activity. The most active fractions were pooled, mixed and dialyzed once again (Peterson and Sober, 1962; Palmer, 1991). The purified enzyme was lyophilized and stored at  $5^\circ C$  for further investigations (Plummer, 1978).

##### *Characterization of purified xylanase*

The effect of pH on enzyme activity was assessed by adding 1 ml of purified xylanase enzyme to 1 ml 1% (w/v) xylan at different pH values (from 3 to 10) then the reaction mixture was incubated for 30 min at  $50^\circ C$ . Liberated reducing sugar was then estimated by 3,5 Dinitrosalicylic acid (DNS) reagent. For determining pH stability, the purified enzyme was incubated at pH values ranging from 3 to 10 for two time intervals of 20 and 60 min. The original pH value was then restored and the residual activity for each enzyme was estimated under standard assay conditions. To determine the optimum temperature of purified xylanase, the enzyme was incubated with 1% of the substrate at different temperatures for 30 min in 50 mM potassium phosphate buffer, pH 5.5. The enzyme activity was estimated at each temperature as mentioned before. Thermostability of xylanase was

determined by incubating the purified enzyme in 50 mM potassium phosphate buffer, pH 5.5 at different temperatures (from 20 to 90°C) for 1 and half hour. After incubation, the mixture was subjected to centrifugation to precipitate the denatured protein which may generate due to thermal denaturation, and then the residual activity of the enzyme was measured.

#### SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis (SDS-PAGE) analyses was performed using 10% (w/v) polyacrylamide gel with 25 mM Tris / 192 mM glycerin buffer (pH 8.3) that contained 0.1% (w/v) SDS as the running buffer as described by (Laemmli, 1970). After electrophoresis, the gel was stained for 1 h with Coomassie Blue R250 dye in a methanol-

acetic acid-water solution (4:1:5, by volume) and then destained in the same solution without the dye.

#### Statistical analysis

All the results are expressed as mean  $\pm$  SD from triplicate. Values of  $P \leq 0.05$  were statistically significant. The mean values and standard deviation were calculated using the Excel program from Microsoft Office 2010 package.

### 3. Results

Different fungal species were tested for their capability to grow on different agroindustrial wastes (wheat bran, rice bran and sugarcane baggase) under solid state fermentation (SSF). The superiority of *Trichoderma harzianum* in xylanase activity (85.3 U/g) grown on wheat bran followed by rice bran after 7 days of incubation is clear from Table. 1.

**Table 1. Changes in xylanase activity in some fungal cultures during different incubation periods using various agroindustrial wastes.**

Parameter	Specific xylanase activity (U/mg ml <sup>-1</sup> protein)								
	4 days			7 days			9 days		
Incubation period	Sugarcane baggase	Wheat bran	Rice bran	Sugarcane baggase	Wheat bran	Rice bran	Sugarcane baggase	Wheat bran	Rice bran
<i>Aspergillus flavus</i>	16.9 $\pm 0.22^b$	19.8 $\pm 0.31^a$	18.5 $\pm 0.34^a$	19.5 $\pm 0.33^a$	22.6 $\pm 0.11$	20.7 $\pm 0.15^a$	20.7 $\pm 0.18^a$	29.8 $\pm 0.33^a$	26.8 $\pm 0.33$
<i>A. niger</i>	20.6 $\pm 0.41^c$	32.7 $\pm 0.54^b$	29.8 $\pm 0.21^b$	21.7 $\pm 0.41^b$	33.0 $\pm 0.31^a$	30.5 $\pm 0.23^b$	23.3 $\pm 0.24$	28.3 $\pm 0.32^a$	22.6 $\pm 0.22^a$
<i>A. terreus</i>	24.5 $\pm 0.71$	40.4 $\pm 0.32$	39.4 $\pm 0.42$	23.2 $\pm 0.43$	45.6 $\pm 0.35$	42.3 $\pm 0.22$	25.1 $\pm 0.34$	43.4 $\pm 0.21$	41.5 $\pm 0.12$
<i>A. ustus</i>	12.6 $\pm 0.74^a$	22.8 $\pm 0.82$	19.8 $\pm 0.52^a$	18.9 $\pm 0.23^a$	33.6 $\pm 0.22^a$	29.7 $\pm 0.14^b$	22.7 $\pm 0.33$	35.7 $\pm 0.19^b$	31.4 $\pm 0.52$
<i>Penicillium chrysogenum</i>	17.4 $\pm 0.90^b$	33.8 $\pm 0.60^b$	23.5 $\pm 0.34$	25.8 $\pm 0.21$	43.7 $\pm 0.11^b$	34.5 $\pm 0.17$	26.7 $\pm 0.21$	44.7 $\pm 0.31$	45.6 $\pm 0.52$
<i>P. citrinum</i>	13.8 $\pm 0.47^a$	18.3 $\pm 0.12^a$	17.1 $\pm 0.62^a$	17.5 $\pm 0.33^a$	24.4 $\pm 0.11$	20.6 $\pm 0.18^a$	20.0 $\pm 0.42^a$	23.9 $\pm 0.23$	22.7 $\pm 0.33^a$
<i>P. olsonii</i>	20.7 $\pm 0.64^c$	33.9 $\pm 0.11^b$	30.0 $\pm 0.12$	19.5 $\pm 0.22^a$	37.9 $\pm 0.33$	35.1 $\pm 0.15$	19.7 $\pm 0.56^a$	34.7 $\pm 0.62^b$	31.4 $\pm 0.22$
<i>Chaetomium sp</i>	22.8 $\pm 0.23$	30.4 $\pm 0.22$	28.9 $\pm 0.11^b$	20.6 $\pm 0.13^b$	32.4 $\pm 0.32^a$	30.7 $\pm 0.12^b$	21.5 $\pm 0.31^b$	33.7 $\pm 0.71^b$	29.6 $\pm 0.12$
<i>Trichoderma harzianum</i>	27.8 $\pm 0.31$	47.3 $\pm 0.33$	43.2 $\pm 0.45$	30.5 $\pm 0.32$	66.7 $\pm 0.41$	59.2 $\pm 0.62$	22.8 $\pm 0.23^b$	55.1 $\pm 0.41$	51.4 $\pm 0.32$
<i>T. viride.</i>	19.7 $\pm 0.60^c$	37.0 $\pm 0.25$	33.5 $\pm 0.22$	16.9 $\pm 0.34$	44.5 $\pm 0.33^b$	41.2 $\pm 0.34$	14.3 $\pm 0.24$	40.8 $\pm 0.21$	36.4 $\pm 0.14$

The same letters are non significant ( $P < 0.05$ ). Values are expressed as mean  $\pm$  SD.

The effect of the substrate ratio of wheat bran and rice bran was examined as a comparison to the use of wheat bran or rice bran only. As indicated in Table 2, it was observed that the combination

between wheat bran and rice bran with a ratio of 3: 1, respectively improved the xylanase production significantly.

**Table 2. Potentiality of *Trichoderma harzianum* MH-20 to produce exo-xylanase on a mixture of wheat bran and rice bran at different ratios.**

Ratio of wheat bran: Rice bran (w/w)	Xylanase activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg ml <sup>-1</sup> protein)
1:1	203.98±0.21	3.1±0.10 <sup>b</sup>	65.8±0.33
1:2	189.84±0.23	2.8±0.30 <sup>c</sup>	67.8±0.31 <sup>a</sup>
1:3	169.2±0.34	2.4±0.31 <sup>a</sup>	70.5±0.45
1:4	143.85±0.25	2.1±0.23 <sup>a</sup>	68.5±0.12 <sup>a</sup>
1:5	197.4±0.27 <sup>a</sup>	2.8±0.22 <sup>c</sup>	70.5±0.56
1:6	224.4±0.41	3.0±0.21 <sup>b</sup>	74.8±0.22
2:1	210.08±0.27	2.6±0.11	80.8±0.45 <sup>c</sup>
3:1	129.66±0.17	1.5±0.17	85.3±0.24
4:1	196.08±0.20 <sup>a</sup>	2.4±0.34 <sup>a</sup>	81.7±0.32 <sup>c</sup>
5:1	213.84±0.51	2.7±0.42 <sup>c</sup>	79.2±0.37 <sup>b</sup>
6:1	164.64±0.26	2.1±0.41 <sup>a</sup>	78.4±0.65 <sup>b</sup>

The same letters are non significant ( $P < 0.05$ ). Values are expressed as mean  $\pm$  SD.

#### Optimization of xylanase production on solid state fermentation

Optimization conditions were monitored in Table 3. Moisture content is an important parameter in solid state fermentation processes. Water affects the physical properties of the substrate mainly by causing swelling of the substrates and facilitates effective absorption of the nutrients from the substrates for growth and metabolic activities (Pandey, 1992). As shown in Table 3 the moisture content of 75% was the most appropriate ratio for higher enzymatic activity. SSF processes were influenced by the environmental pH of the process (Jecu, 2000; Panagiotou et al., 2003) In This respect, the results clearly indicated that the optimum pH for the maximal xylanase production was 5 and significantly decrease above this limit. In regard to temperature effect, temperature plays a prominent role in solid state fermentation. The maximum enzyme productivity was achieved at 30°C. The effect of different nitrogen sources in SSF system on enzyme production is also investigated. The maximum xylanase productivity was achieved with the addition of peptone followed by yeast extract.

#### Xylanase purification

The *T. harzianum* xylanase was purified by protein precipitation with ammonium sulfate and

molecular exclusion chromatography. Enzyme yield (about 48.38%) was observed in the 60% ammonium sulfate saturation supernatant. The molecular exclusion chromatography elution onto Sephadex G-100 resulted in 22.37% of the original activity with a purification fold of 28.34. These resulted fractions were pooled, lyophilized and applied onto DEAE-cellulose column which resulted in 58.76 purification fold with yields of 19.33% of the initial xylanase activity (Table 4). The electrophoretic analysis revealed that xylanase corresponded to a single molecular mass band of 23 kDa (Fig. 1).

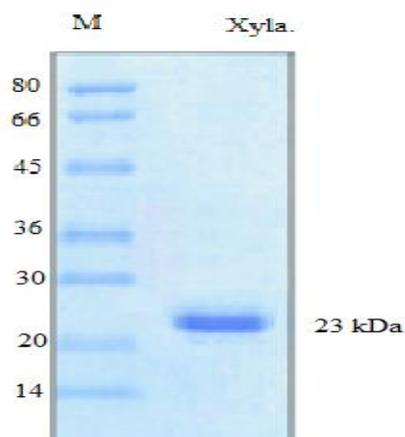
Table 3. Optimization conditions for xylanase production by *Trichoderma harzianum* MH-20

Parameter	Specific activity (U/mg ml <sup>-1</sup> protein)
Optimum moisture content (%)	75
Optimum pH value	5
Optimum temperature (°C)	30
Optimum nitrogen source	Peptone

Table 4. Purification steps of xylanase from *Trichoderma harzianum* MH-20 grown on a mixture of wheat bran and rice bran

Purification step	Total activity Uml <sup>-1</sup>	Total protein (mg ml <sup>-1</sup> )	Specific activity U/mg ml <sup>-1</sup> protein	Yield %	Purification fold
Crude enzyme	129.66	1.52	85.3	100.00	1.00
Dialysis	98.96	0.420	235.62	76.32	2.76
Cell free precipitate	62.73	0.050	1254.6	48.38	14.71

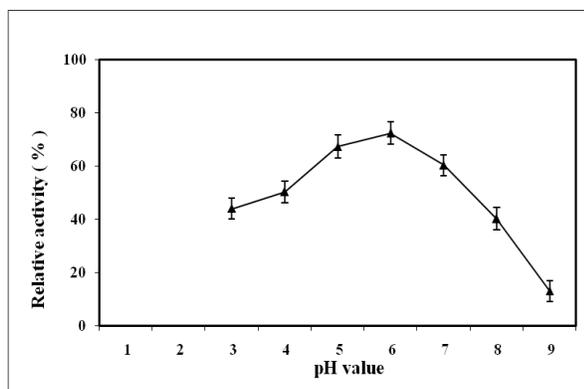
<b>Sephadex G-100</b>	29.01	0.012	2417.5	22.37	28.34
<b>DEAE- cellulose</b>	25.06	0.005	5012.00	19.33	58.76



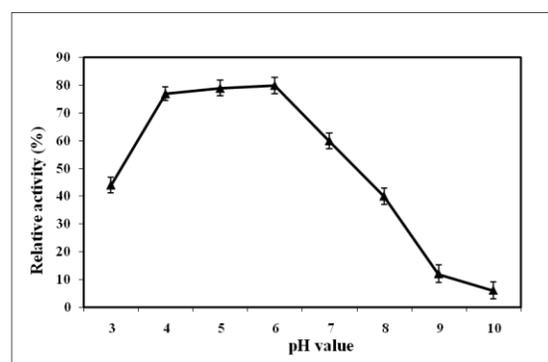
**Fig. 1 SDS-PAGE analysis of the purified xylanase from *Trichoderma harzianum* MH-20**

#### Purified xylanase properties

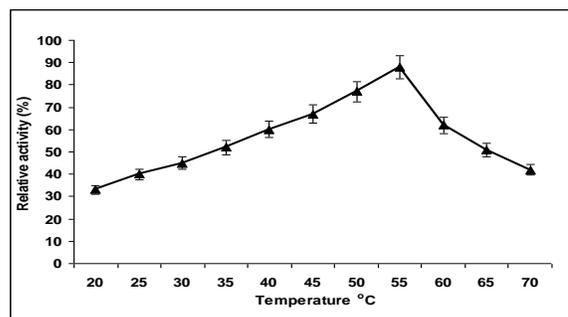
Testing the pH-dependence of xylanase activity, the results in Fig. 2 showed the significant effect of this factor on enzyme activity. It was clarified that the highest enzymatic activity was achieved at pH range between 5 and 6. Xylanase pH stability was also investigated. In this manner, xylanase produced by *T. harzianum* was stable over a broad pH range (from 4-6) (Fig. 3). Values of optimum temperature of xylanase hydrolysis vary according to the producing microorganism. Fig. 4 revealed that 55°C is the optimum temperature for xylanase activity. Thermal stability is an interesting enzyme character. the estimated half-lives of xylanase was determined and recorded at 60°C and 50°C after 65 and 70 min, respectively and retained 77 % of its activity over 20 min at 70°C (Fig. 5).



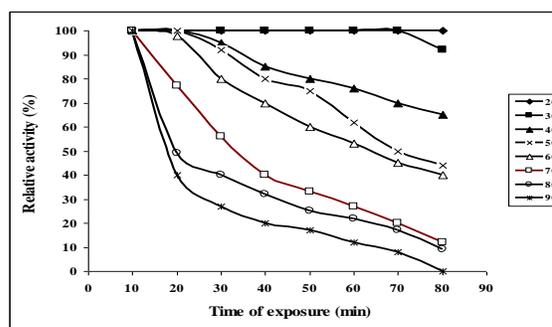
**Fig. 2 Optimum pH profile of purified xylanase from *Trichoderma harzianum* MH-20**



**Fig. 3 pH stability profile of purified xylanase from *Trichoderma harzianum* MH-20**



**Fig. 4 Optimum temperature profile of purified xylanase from *Trichoderma harzianum* MH-20**



**Fig. 5 Thermal stability profile of purified xylanase from *Trichoderma harzianum* MH-20**

#### 4. Discussions

The xylanase production has been reported from different fungal species such as *Thermoascus aurantiacus* (Kalegoris et al., 1998), *Fusarium oxysporum* F3 (Christakopoulos et al., 1996),

*Penicillium capsulatum* (Ryan et al., 2003) and *Trichoderma harzianum* (Tan et al., 1985). The substrates used mostly for xylanase production include wheat bran, corn cobs, sugarcane bagasse, bagasse pulp, spent sulphite liquor, rice straw, wheat straw, sorghum flour and eucalyptus pulp. However, lignocellulosic materials especially wheat bran has been more successful in production with higher titers being attributed to its hemicelluloses nature, favorable degradability and the presence of some nutrients in the carbon source (Singh et al., 2008).

Initial moisture content is one of the key factors affecting xylanase production especially in SSF. This may be attributed to the faster growth of microorganisms at higher moisture content and the subsequent early initiation of the enzyme production (Kalegoris et al., 1998). The optimum moisture content for xylanase production by *T. harzianum* (75%) is in agreement with many researchers (Ferrira et al., 1999).

The cultivation pH does not only affect the growth of the organism but also has a marked effect on xylanase production. In this manner, the favorable pH range obtained for xylanase production by the experimental fungus was similar to the results obtained by other microorganisms such as *Aspergillus* sp. (Khanna et al., 1995), *A. oryzae* (Kitamoto et al., 1999), *Fusarium verticillioides* (Saha, 2003), *Penicillium citrinum* (Tanaka et al., 2005) and *Penicillium* sp. AH-30 (Li et al., 2007).

The temperature in SSF system is influenced not only by the environmental temperature, but also by the increase in temperature generated from the metabolic activities of the fungi growing on the solid substrates (Pang et al., 2006). The optimal growth verified at 30°C is in accordance with the literature that describes this temperature as ideal for *Trichoderma* spp (Pang et al., 2006). While, (Azad et al., 2013) found that the optimum temperatures for xylanase production by *Rhizomucor pusillus* and *Thermomyces lanuginosus* were 45°C and 50°C respectively.

The effect of different nitrogen sources was also investigated. In this regard, (Seyis and Aksoz, 2005) found that the major organic nitrogen source for xylanase production from *T. harzianum* was peptone. The same finding was observed by (Bakri et al., 2003) and (Sakthiselvan et al., 2012) who found that organic nitrogen sources were more promising in xylanase production than inorganic ones.

Enzyme activity is markedly affected by pH because substrate binding and catalysis are often dependent on charge distribution on both substrate and, in particular, enzyme molecules (Kulkarni et al., 1999). In this manner, xylanase from *Trichoderma harzianum* 1073 D3 showing optimum activity at pH

5, and stable in pH range of 3-7 and also retained more than 50 % of its original activity after four months (Isil and Nilufer, 2005). Moreover, (Querido et al., 2006) found that xylanase from *Penicillium expansum* showed optimum activity at pH of 5.5 and stable from pH 5.5-6.5. Similar results were observed for other microorganisms such as *Penicillium* sp. AH-30 (Li et al., 2007) which presented xylanase with maximum activities at similar pH.

The optimum temperature for xylanase activity from different fungal species has been found to be similar or slightly higher than the results obtained. *Penicillium citrinum* (Tanaka et al., 2005), *Aspergillus niveus* RS2 (Sudan and Bajaj, 2007), *Aspergillus sydowii* SBS 45 (Nair et al., 2008) and *Trichoderma viride* (Irfan and Syed, 2012) presented xylanase with maximum activities at 50°C. The purified enzyme showed a single-protein band of ~23 kDa on SDS-PAGE. The present results were supported by (Zhang et al., 2007) who found that xylanase from a fungus *Plectosphaerella cucumerina* had a molecular weight of 19 kDa. While, the molecular mass of *Marasmius* sp xylanase was approximately 40 kDa (Ukrit et al., 2006).

#### Acknowledgments

The author wish to express her deepest gratitude to Prof. Dr. Ahmed Fouad Afifi, Professor of Microbiology and Formerly Head of Biological Sciences Department, Faculty of Education, Ain Shams University for his useful criticism and continuous encouragement.

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2/11/2014