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Induction characteristics and optimization of production conditions of Xylanase by selected fungal species on lignocellulosic substrates

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ABSTRACT

Five xylanolytic fungi were isolated from saw dust samples collected from a saw mill dump in Ibadan, Nigeria. The effect of lignocellulosic substrates on the production of xylanases was studied in shake flasks. *Trichoderma* sp. Fd4 produced high yield of the enzyme when grown in 2% wheat bran. Similarly, xylan, lactose and maltose, used as carbon sources induced the production of high levels of xylanase. *Trichoderma viride* Fd18 produced 10.4 U/ml of xylanase using NH_4Cl as nitrogen source, while NH_4NO_3 repressed enzyme production by *Aspergillus ustus* Fb2. The fungal isolates produced good amount of xylanase at 30-40 ^oC, pH 4.5-6.0, 7 days of incubation, initial spore size of 10^6 and in presence of NaCl and KCl in the basal medium.

Keywords: Xylanase, fungi, lignocellulosic waste, submerged fermentation, optimization.

INTRODUCTION

Hemicellulolytic enzymes have aroused great interest due to their biotechnological potential in many industrial processes. Plant hemicellulosic biomass, the second most abundant and renewable organic compound in nature, can be converted to reducing sugars by means of xylanolytic enzymes produced by microorganisms. These sugars are eventually converted to useful products by other microorganisms. The major component of hemicellulose is xylan, a non-crystalline complex polysaccharide consisting of a backbone of β–D–1,4–linked xylopyranoside units substituted with acetyl, glucuronosyl and arabinosyl side chains (La Grange et al., 2001). A complete and efficient enzymatic hydrolysis of xylan depends mainly on two types of enzymes: endoxylanases (EC 3.2.1.8) which hydrolyse the xylanopyranose of the central chain and β–xylosidases (EC 3.2.1.37) which hydrolyse xylobiose and other xylooligosaccharides. Lignocellulosic waste forms a large proportion of solid waste in our cities, thus, constituting an environmental problem. Studies have shown that conventional waste treatment strategies have failed to ameliorate this problem. The use of microbial enzymes in lignocellulosic waste treatment has been shown to be an alternative that is efficient and cost-effective. Therefore, considering the industrial potentials of xylanases and their potential use in

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lignocellulolytic waste treatment, it becomes imperative to obtain new enzymes and enzyme-producing microbial strains that produce highly active xylanases at low cost. Microbial enzymes are the preferred catalyst for hydrolysis due to their high specificity, mild reaction conditions, negligible substrate loss and side product generation (Kulkarni et al., 1999).

Filamentous fungi are particularly useful producers of xylanases from the industrial point of view, due to the high production level and extra cellular secretion of enzymes, as well as elative ease of cultivation. In general, xylanase activity levels from fungal cultures are typically much higher than those from yeasts or bacteria (Tallapragada and Venkatesh, 2011; Irfan and Syed, 2012). The purpose of this research was to evaluate xylanase production by 5 fungal isolates grown in submerged fermentation using some natural lignocellulosics and to standardize some enzyme production parameters.

MATERIALS AND METHODS

Isolation and identification of fungi

The fungi were isolated from saw dust samples collected from the Bodija saw mill dump in Ibadan, Nigeria. The isolated fungi were identified after growth on PDA medium by observing its macroscopic (colour, texture, appearance and diameter of colonies) and microscopic (microstructures) characteristics according to Barnett (1960), and Domsch et al. (1980). The pure cultures were then transferred to PDA slants and maintained by sub-culturing every four weeks.

Xylanase production by fungal isolates in submerged fermentation

One millilitre of fungal spore suspension (~ 10^7 spores/ml) from a 6-day-old PDA slants were inoculated into the modified medium of Mandels and Weber (1969) containing 1% (w/v) oatspelt xylan (20 ml in 50 ml flasks) and incubated at 30 ^oC with continous agitation (180 rev/min) for 6 days. The content of each flask was filtered through Whatman filter paper No.1 and the supernatant solution was used as crude enzyme preparation.

Xylanase production on various lignocellulosics

Fungal isolates were screened for their ability to utilize various natural lignocellulosics (wheat bran, sugarcane bagasse and saw dust) to produce xylanase in submerged cultivation at 30 ^oC for 6 days as described by Franco et al. (2004).

Xylanase production by solid state fermentation

Lignocellulosic substrates (wheat bran, sugarcane bagasse and saw dust) served as carbon source as well as matrix for SSF. Five gram (5 g) of the solid substrate was moistened with 5 ml of modified medium of Mandels and Weber (1969) in 250 ml conical flasks. The flasks were autoclaved at 121 ^oC for 20 min, after which they were each inoculated with 1 ml of spore suspension (~10^7 spores ml^{-1}) and incubated for 6 days at 30 ^oC. Enzyme was harvested by shaking thoroughly with 100 ml 0.05 M sodium citrate buffer (pH 5.0). The contents of the flasks were filtered using Whatman No.1 filter paper and the clear supernatant was assayed for enzyme activities (Jeya et al., 2005).

Optimization of production conditions

The optimization of fermentation parameters was done by evaluating the effect of an individual parameter and to incorporate it at standardized level before standardizing the next parameter. Process parameters optimized included pH (3.0 – 8.0) adjusted with 0.1 M sodium citrate and 0.1 M sodium phosphate buffers, incubation temperature (30 ^oC – 55 ^oC), and additional nutrients: NH_4Cl,

$(NH_4)_2SO_4$, $NaNO_3$, $NH_4H_2PO_4$, Urea, KNO_3, $(NH_2)_2HPO_4$, NH_4NO_3, and Peptone each at concentration of 0.1% w/v as nitrogen sources, and maltose, lactose, mannitol, sorbitol, and glycerol each at 0.5 - 2.5% concentration as additional carbon sources. Effect of mineral salts ($CaCl_2$, $CoS0_4$, $FeSO_4$, $MgS0_4$, and $ZnCl_2$) and incubation period were also optimized.

Enzyme assay

Xylanase activity was assayed according to the method of Bailey et al. (1992) using 1% (w/v) oat spelt xylan (Sigma Chemical Co., St. Louis, Mo.) as substrate and incubation of the entire mixture carried out at 50 ^oC. Xylose (Sigma Chemical Co., St. Louis, Mo.) was used as standard. One unit of xylanase was defined as the amount of enzyme required to release 1 µmol of xylose/minute.

RESULTS

A high level of xylanase was produced when xylan, lactose and maltose were used as carbon sources (Table 1). The maximum level of xylanase activity (10.8 Uml^{-1}) was produced by *Aspergillus ustus* Fb2 using lactose (2%). *Trichoderma* sp Fd7 produced 9.62 Uml^{-1} xylanase using 2% maltose; whereas *Trichoderma* sp Fd4, *A. ustus* Fd12 and *T. viride* Fd18 produced 9.7, 9.32 and 9.56 Uml^{-1} xylanase, respectively, using 2% oat-spelt xylan as carbon source.

The agricultural substrates, wheat bran and sugarcane bagasse, were better in synthesizing xylanase than saw dust. *Trichoderma* sp. Fd4 and *Trichoderma viride* Fd18 generated 17.2 Uml^{-1} and 17.0 Uml^{-1} of xylanase in 2% wheat bran respectively, showing no difference ($p > 0.05$) in the enzyme activity. *A. ustus* Fb2 (15.2 Uml^{-1}, 1%), *Trichoderma* sp Fd7 (14.1 Uml^{-1}, 2%) and *A. ustus* Fb12 (14.1U ml^{-1}, 3%) produced their highest values of xylanase by utilizing sugarcane bagasse as carbon source (Table 2).

Result of experiments to compare the production of xylanase using solid state fermentation (SSF) and submerged fermentation (SmF) is shown in Table 3. Compared to SmF, the yield of xylanase in SSF was significantly lower at $p \leq 0.05$. *Trichoderma viride* Fd18 and *A. ustus* Fb2 generated 17.8 Uml^{-1} and 13.2 Uml^{-1} xylanase activities in SmF, and 16.3 Uml^{-1} and 11.8 Uml^{-1} in SSF respectively using wheat bran as substrate. When saw dust was used as substrate, *A. ustus* Fb2 produced 7.9 Uml^{-1} of xylanase in SSF compared to 5.0 Uml^{-1} produced in SmF. Wheat bran was the most suitable substrate for the generation of xylanase activities in both SSF and SmF.

Trichoderma viride Fd18 produced significantly ($p \leq 0.05$) high xylanase yield of 10.4 Uml^{-1} and 10.0 Uml^{-1} using NH_4Cl and $(NH_4)_2S0_4$ as nitrogen source respectively. *Trichoderma* sp Fd7 produced 9.7 Uml^{-1} of xylanase enzyme by utilizing $(NH_4)_2S0_4$ and urea as source of nitrogen. The enzyme activity was completely repressed by NH_4N0_3 when used as nitrogen source for xylanase production by *Aspergillus ustus* Fb2 (Figure 1).

The optimal temperature for xylanase production by all the test organisms was 40 ^oC, except for *T. viride* Fd18 whose optimum temperature for xylanase synthesis was 30 ^oC. The least xylanase activity was 0.3 Uml^{-1} at 55 ^oC produced by *A. ustus* Fb2. *Trichoderma* sp Fd7 produced significantly higher ($p \leq 0.05$) (8.8 Uml^{-1}) xylanase at 40 ^oC (Figure 2).

The least xylanase activity of 0.9 U ml^{-1} was obtained with *Trichoderma* sp Fd4 at an initial medium pH of 8.0 and 9.0. The pH 9 was the initial pH at which the lowest activity for xylanase was obtained by all the organisms. The best initial pH for xylanase production was pH 5.0 (10.4 Uml^{-1}) for *T. viride* Fd18. This figure was significantly different from the xylanase activities at other pH values. High xylanase activities were also produced by

Trichoderma sp Fd4 at pH 4.5 (10 Uml^{-1}), pH 5 and 6 (9.5 Uml^{-1}); and by *T. viride* Fd18 at pH 6.0 (10 Uml^{-1}) (Figure 3).

Xylanase was best produced on day 7 of incubation by all the fungal isolates. The amount of enzyme produced increased up to the maximum level and then decreased. *T. viride* Fd18, with an activity of 9.2 Uml^{-1}, had the highest xylanase activity that was significantly different ($p \leq 0.05$) from the others. The xylanase activity of *T. viride* Fd18 is about 2.5 fold that produced by *A. ustus* Fb2 (3.95 Uml^{-1}) (Figure 4).

All the fungal isolates showed a general trend of increased production of xylanase in the presence of NaCl and KCl in the basal medium. The presence of $CuCl_2$, $ZnCl_2$ and $AlCl_3$ in the basal medium decreased the production of xylanase by the five fungal isolates (Table 4).

The effect of spore inoculum size on xylanase production is shown in Figure 5. Higher inoculum concentration of 1×10^6 spore's ml^{-1} enabled the maximum enzyme production to be reached, giving a maximum xylanase activity (16.7 Uml^{-1}, *T. viride* Fd18) in seven days. Higher inoculum size require shorter time to attain maximum enzyme yields, e.g., *T. viride* Fd18 spore concentration of 1×10^6 spores ml^{-1} gave a significantly higher xylanase yield of 15.2 Uml^{-1} in 5 days compared to 6.5 Uml^{-1} obtained in seven days using 1×10^4 spores ml^{-1}. The same pattern was recorded by the other fungal isolates in the production of the enzyme.

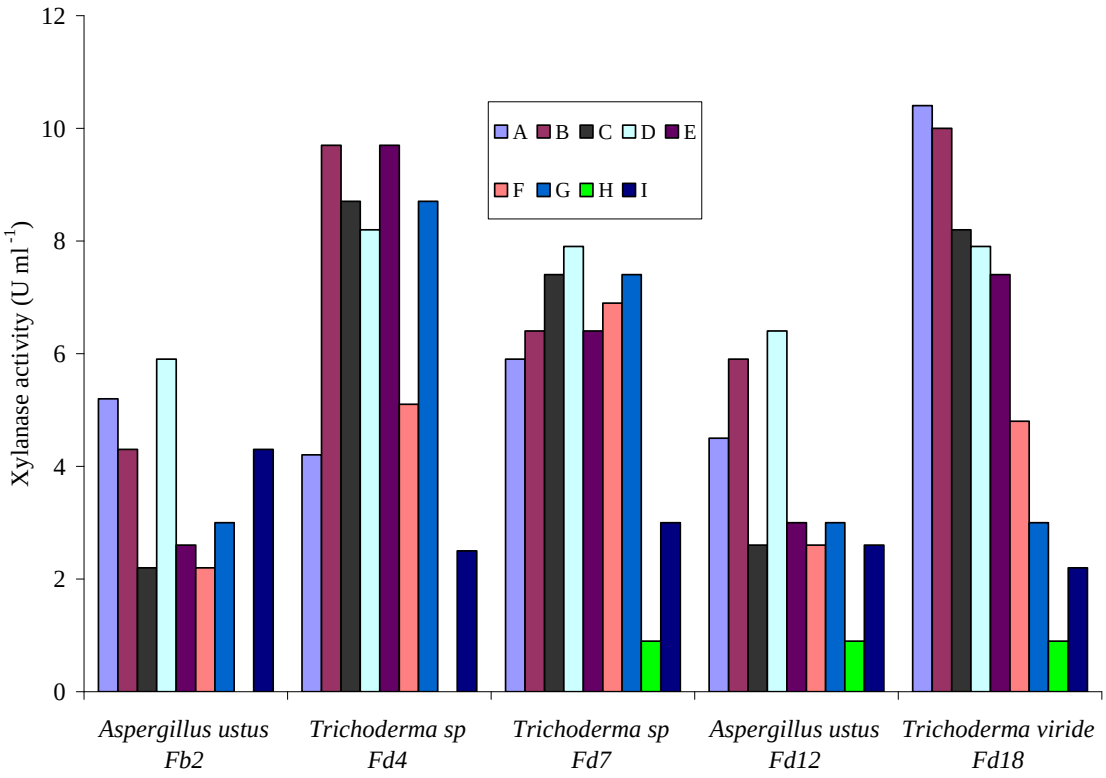

Figure 1: Effect of nitrogen sources on xylanase synthesis by selected fungal isolates. Data are presented as means of 2 replicates, having standard deviation within the range 0 – 8%. A = NH_4Cl, B = $(NH_4)_2SO_4$, C = $NaNO_3$, D = $NH_4H_2PO_4$, E = Urea, F = KNO_3, G = $(NH_2)_2HPO_4$, H = NH_4NO_3, I = Peptone.

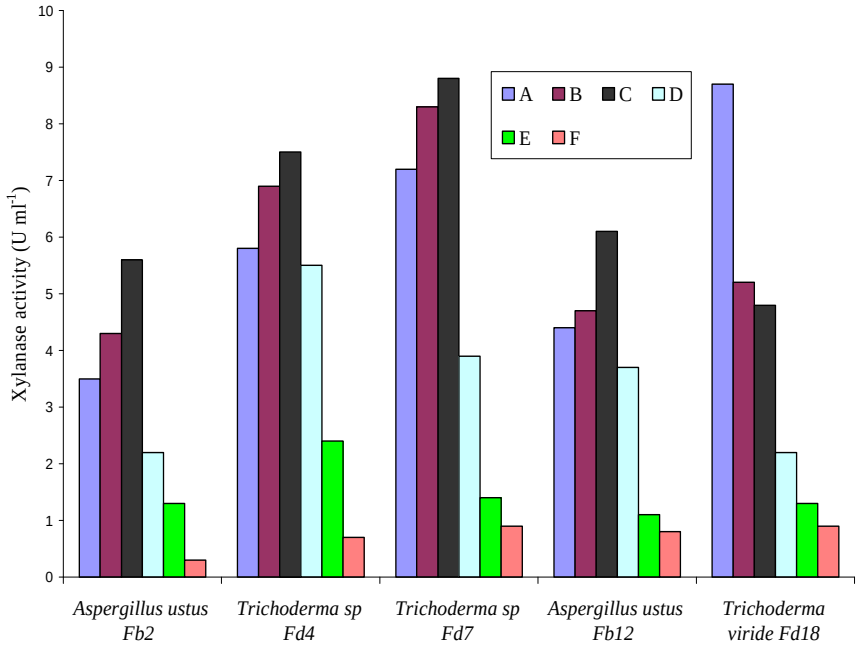

Figure 2: Effect of incubation temperatures on xylanase synthesis by fungal isolates. Data are presented as means of 2 replicates, having standard deviation within the range 0 – 8%. A = 30 ºC, B = 35 ºC, C = 40 ºC, D = 45 ºC, E = 50 ºC, F = 55 ºC.

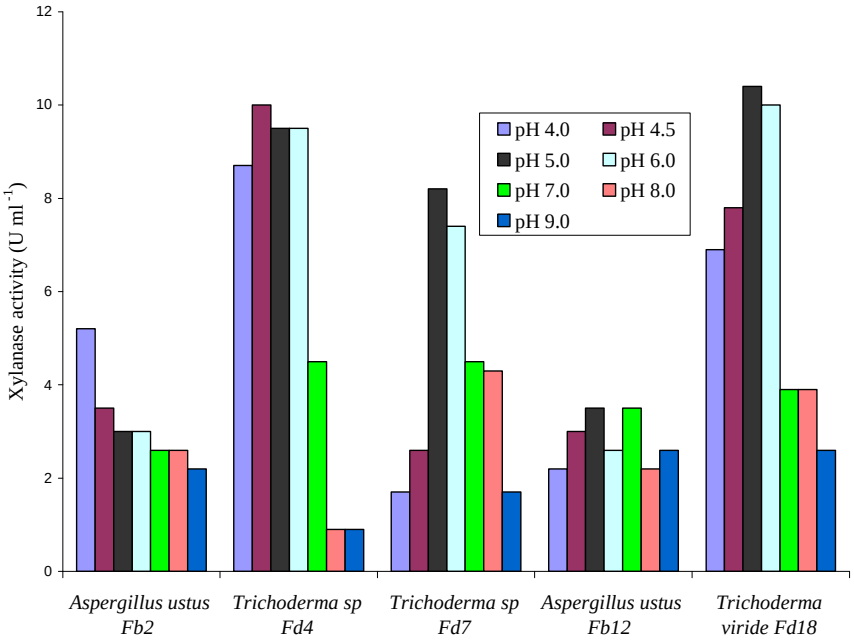

Figure 3: Effect of pH on xylanase synthesis by fungal isolates. Data are presented as means of 2 replicates, having standard deviation within the range 0 – 8%.

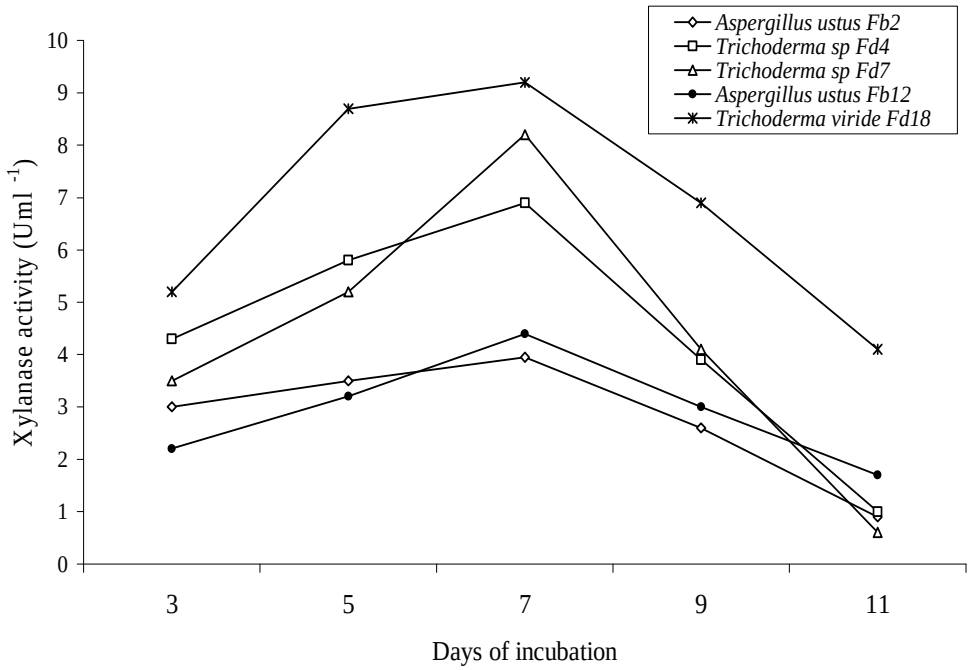

Figure 4: Effect of number of days of incubation at 30°C on xylanase synthesis by fungal isolates. Data are presented as means of 2 replicates, having standard deviation within the range 0 – 8%.

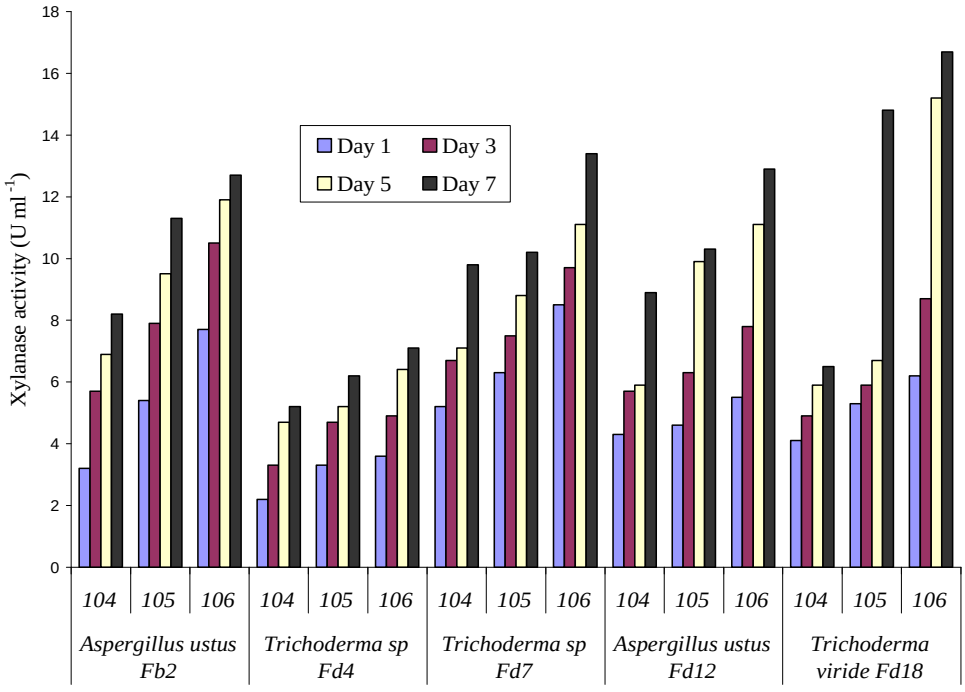

Figure 5: Effect of spore inoculum size on xylanase synthesis by fungal isolates. Data are presented as means of 2 replicates, having standard deviation within the range 0 – 8%. 104 = 1 × 10^4 spores ml^{-1}; 105 = 1 × 10^5 spores ml^{-1}; 106 = 1 × 10^6 spores ml^{-1}.

Table 1: Effect of different concentrations of carbohydrates on the production of xylanase enzymes by fungal isolates.

Carbon source	Concentration (%) of carbon source	Isolate / Xylanase production (U/ml)				
		***A. ustus* Fb2**	***Trichoderma* sp Fd4**	***Trichoderma* sp Fd7**	***A. ustus* Fd12**	***T. viride* Fd18**
Maltose	0.5	0.4±0.00^{e}	2.0±0.00^{c}	5.9±0.14^{a}	1.0±0.00^{d}	4.0±0.00^{b}
	1.0	1.7±0.14^{c}	8.5±0.28^{b}	8.2±0.28^{b}	1.0±0.00^{d}	9.1±0.14^{a}
	1.5	2.0±0.00^{d}	8.9±0.14^{c}	9.62±0.00^{a}	2.3±0.21^{d}	9.2±0.00 ^{b}
	2.0	2.2±0.28^{d}	9.45±0.00^{a}	2.64±0.00^{c}	1.2±0.14^{e}	7.63±0.00^{b}
	2.5	1.2±0.28^{b}	2.63±0.00^{a}	2.60±0.00^{a}	1.2±0.00^{b}	2.65±0.00^{a}
Lactose	0.5	7.0±0.07^{a}	0.4±0.00^{e}	3.3±0.42^{b}	0.9±0.00^{d}	2.3±0.00^{c}
`	1.0	8.0±0.00^{a}	0.8±0.00^{d}	3.8±0.28^{b}	1.8±0.28^{c}	3.7±0.00^{b}
	1.5	10.5±0.71^{a}	5.7±0.00^{b}	5.1±0.07^{bc}	2.5±0.42^{d}	4.1±0.41^{c}
	2.0	10.8±0.42^{a}	1.7±0.00^{d}	5.9±0.00^{b}	4.3±0.28^{c}	4.8±0.00^{c}
	2.5	10.1±0.14^{a}	1.5±0.00^{e}	4.7±0.14^{b}	4.0±0.00^{d}	4.3±0.00^{c}
Glycerol	0.5	0±0.00^{c}	0±0.00^{c}	0.2±0.00^{b}	0.2±0.00^{b}	0.4±0.00^{a}
	1.0	0±0.00^{c}	0.2±0.00^{b}	0.2±0.00^{b}	0.4±0.07^{a}	0.4±0.00^{a}
	1.5	0.2±0.28^{ab}	0±0.00^{b}	0.2±0.00^{ab}	0.4±0.00^{a}	0.2±0.00^{ab}
	2.0	0.2±0.00^{b}	0.2±0.00^{b}	0.2±0.00^{b}	0.4±0.00^{a}	0.2±0.00^{b}
	2.5	0.4±0.00^{a}	0±0.00^{c}	0.4±0.00^{a}	0.20±0.00^{b}	0.2±0.00^{b}
Xylan	0.5	7.55±0.00^{b}	5.5±0.00^{d}	4.5±0.00^{e}	6.55±0.00^{c}	7.56±0.00^{a}
`	1.0	8.0±0.07^{a}	6.1±0.14^{c}	6.78±0.00^{b}	6.8±0.00^{b}	8.1±0.14^{a}
	1.5	8.25±0.00^{b}	7.5±0.00^{d}	7.08±0.00^{e}	7.56±0.00^{c}	8.85±0.00^{a}
	2.0	8.8±0.28^{b}	9.7±0.42^{a}	8.05±0.07^{c}	9.32±0.00^{ab}	9.56±0.00^{a}
	2.5	7.8±0.00^{d}	8.4±0.28^{c}	8.10±0.00^{cd}	8.77±0.00^{b}	9.50±0.00^{a}
Carboxymethyl	0.5	1.0±0.00^{d}	2.2±0.28^{b}	1.7±0.28^{c}	1.0±0.00^{d}	3.3±0.00^{a}
cellulose	1.0	1.2±0.28^{c}	3.5±0.00^{a}	2.2±0.28^{b}	1.8±0.28^{b}	4.0±0.00^{a}
	1.5	1.7±0.28^{d}	3.5±0.00^{b}	2.55±0.00^{c}	2.2±0.28^{c}	5.5±0.00^{a}
	2.0	2.61±0.00^{d}	4.0±0.00^{b}	3.32±0.00^{c}	3.3±0.28^{c}	4.9±0.14^{a}
	2.5	2.41±0.00^{d}	4.5±0.00^{a}	3.56±0.00^{b}	2.8±0.00^{c}	--

*Each value is a mean of two replicates; ± stands for standard deviation among replicates; means with different superscripts within each column differ significantly ($p \leq 0.05$).

Table 2: Effect of different lignocellulosic substrates on xylanase production by the fungal isolates.

Isolate	Concentration (%) of lignocellulose	Substrate / Enzyme activity (U/ml)		
		Xylanase		
		Saw Dust	Sugarcane Bagasse	Wheat Bran
A. ustus Fb2	1	4.8±0.28^{b}	15.2±0.28^{a}	13.7±2.83^{a}
	2	13.1±2.97^{a}	12.7±0.00^{d}	9.4±0.00^{b}
	3	4.0±1.41^{b}	12.2±2.55^{a}	13.4±0.57^{ab}
Trichoderma sp Fd4	1	7.4±0.57^{a}	14.5±0.00^{a}	16.9±0.14^{a}
	2	6.9±1.41^{b}	14.1±0.00^{b}	17.2±0.14^{a}
	3	5.0±0.00^{a}	12.3±0.42^{a}	15.6±1.98^{a}
Trichoderma sp Fd7	1	3.8±0.00^{d}	9.8±0.28^{b}	9.8±0.57^{b}
	2	5.2±0.28^{b}	14.1±0.14^{b}	9.4±0.28^{b}
	3	3.8±0.00^{a}	14.1±0.28^{a}	9.4±0.14^{b}
A. ustus Fd12	1	5.7±0.14^{b}	14.3±2.40^{a}	10.1±0.00^{b}
	2	3.8±0.00^{b}	13.4±0.00^{c}	12.7±0.00^{b}
	3	5.9±0.00^{a}	14.1±0.14^{a}	13.8±0.14^{ab}
T. viride Fd18	1	4.3±0.00^{cd}	16.4±0.57^{a}	16.4±0.00^{a}
	2	4.8±0.00^{b}	16.4±0.00^{a}	17.0±2.83^{a}
	3	7.4±2.83^{a}	14.5±2.12^{a}	12.3±1.41^{b}

SD – Saw dust ; SB – Sugarcane bagasse; WB – Wheat bran ; *Each value is a mean of two replicates,
± stands for standard deviation among replicates; means with different superscripts within each column differ significantly ($p \leq 0.05$).

Table 3: Comparison of production of xylanase, filter paper activity (FPA) and cellulase on various lignocelluloses in solid state and submerged fermentation.

Isolate / Substrate		Xylanase		FPA		Cellulase	
		SSF	SmF	SSF	SmF	SSF	SmF
Aspergillus ustus Fb2	Wheat bran	*11.8±1.13^{c}	13.2±0.28^{b}	4.8±0.57^{b}	5.4±0.00^{b}	14.8±0.42^{a}	16.7±0.99^{a}
	Sugarcane bagasse	14.5±0.71^{a}	14.8±0.42^{ab}	6.2±0.28^{a}	3.6±0.14^{a}	10.5±0.71^{b}	12.2±0.28^{a}
	Saw dust	7.9±0.57^{a}	5.0±0.00^{ab}	3.0±0.00^{b}	3.2±0.28^{a}	10.1±2.83^{a}	8.9±2.83^{a}
Trichoderma sp Fd4	Wheat bran	15.2±0.00^{a}	16.5±0.71^{a}	7.7±0.00^{a}	8.0±1.41^{a}	12.8±0.42^{b}	14.3±0.00^{b}
	Sugarcane bagasse	10.1±0.14^{b}	13.0±0.71^{bc}	3.5±0.71^{bc}	4.1±0.00^{b}	8.7±0.28^{cd}	11.0±1.41^{b}
	Saw dust	6.3±0.42^{ab}	6.0±1.41^{a}	1.8±0.57^{c}	2.1±0.00^{b}	5.3±0.28	6.8±0.28^{ab}
Trichoderma sp Fd7	Wheat bran	8.5±1.41^{d}	8.0±0.00^{d}	4.3±0.42^{b}	9.5±0.42^{b}	9.5±0.71^{c}	12.5±0.28^{c}
	Sugarcane bagasse	7.1±0.14^{c}	9.2±0.28^{d}	5.0±1.41^{ab}	6.2±0.28^{b}	9.7±0.28^{cb}	11.2±0.28^{b}
	Saw dust	4.9±0.00^{bc}	4.1±0.14^{b}	5.7±0.28^{a}	2.0±0.00^{b}	2.1±0.00^{e}	4.5±1.41^{bc}
Aspergillus ustus Fd12	Wheat bran	10.5±0.71^{b}	13.3±0.42^{c}	4.5±0.71^{b}	3.5±0.71^{bc}	11.7±0.28^{a}	15.2±1.41^{c}
	Sugarcane bagasse	9.4±0.57^{b}	11.5±0.71^{c}	4.2 ±0.28^{abc}	2.8±0.28^{b}	12.7±1.41^{a}	10.6±0.14^{b}
	Saw dust	3.9±0.57^{c}	5.1±0.14^{ab}	2.2±0.28^{c}	1.8±0.14^{b}	3.5±1.41^{d}	2.7±0.00^{c}
Trichoderma viride Fd18	Wheat bran	16.3±0.42^{a}	17.8±0.35^{a}	1.5±0.14^{c}	2.9±0.14^{c}	2.7±0.28^{d}	9.1±0.07^{d}
	Sugarcane bagasse	14.3 ±0.42^{a}	15.9±0.42^{a}	2.6±0.14^{c}	3.8±0.14^{b}	8.3±0.00^{d}	12.7±0.99^{a}
	Saw dust	6.4±0.57^{c}	4.6±0.42^{b}	0.85±0.07^{d}	1.8±0.00^{b}	4.2±0.28^{b}	3.8±1.13^{c}

SSF – Solid state fermentation; SmF – Submerged fermentation; *Each value is a mean of two replicates; ± stands for standard deviation among replicates; means with different superscripts within each column differ significantly ($p \leq 0.05$).

Table 4: Effect of different mineral salts on xylanase synthesis by fungal isolates.

Mineral salts	**Fungal isolates / Xylanase activity (U ml^{-1})**				
	***A. ustus* Fb4**	***Trichoderma* sp Fd4**	***Trichoderma* sp Fd7**	***A. ustus* Fd12**	***T. viride* Fd18**
Control	*9.3±2.83 [a]	9.5±0.71 [a]	8.1±0.00 [a]	9.3±0.42 [a]	9.5±1.41 [a]
$CaCl_2$	10.6±0.85 [a]	8.2±0.28 [b]	6.1±0.14 [b]	9.7±0.00 [a]	9.9±0.00 [a]
$FeSO_4$	10.3±0.00 [a]	9.4±0.57 [ab]	8.0±1.41 [a]	9.3±4.24 [a]	9.4±2.83 [a]
$MgSO_4$	10.4±0.00 [a]	9.6±0.42 [a]	8.1±0.14 [a]	9.5±0.00 [a]	9.9±0.57 [a]
NaCl	10.9±0.57 [a]	10.1±0.00 [a]	9.2±0.28 [a]	9.8±1.70 [a]	10.7±0.00 [a]
KCl	11.7±0.99 [a]	9.9±0.57 [a]	9.3±0.42 [a]	10.2±0.28 [a]	10.5±0.71 [a]
$CuCl_2$	3.9±0.00 [b]	4.7±0.99 [d]	2.9±0.00 [c]	1.8±0.00 [b]	2.7±042 [b]
$ZnCl_2$	3.3±1.41 [b]	4.1±0.00 [d]	2.7±0.57 [c]	1.2±0.00 [b]	2.0±0.00 [b]
$AlCl_3$	5.7±0.00 [b]	6.3±0.42 [c]	3.3±0.00 [c]	2.8±0.57 [b]	3.5±0.71 [b]

*Each value is a mean of two replicates; ± stands for standard deviation among replicates; means with different superscripts within each column differ significantly ($p \leq 0.05$).

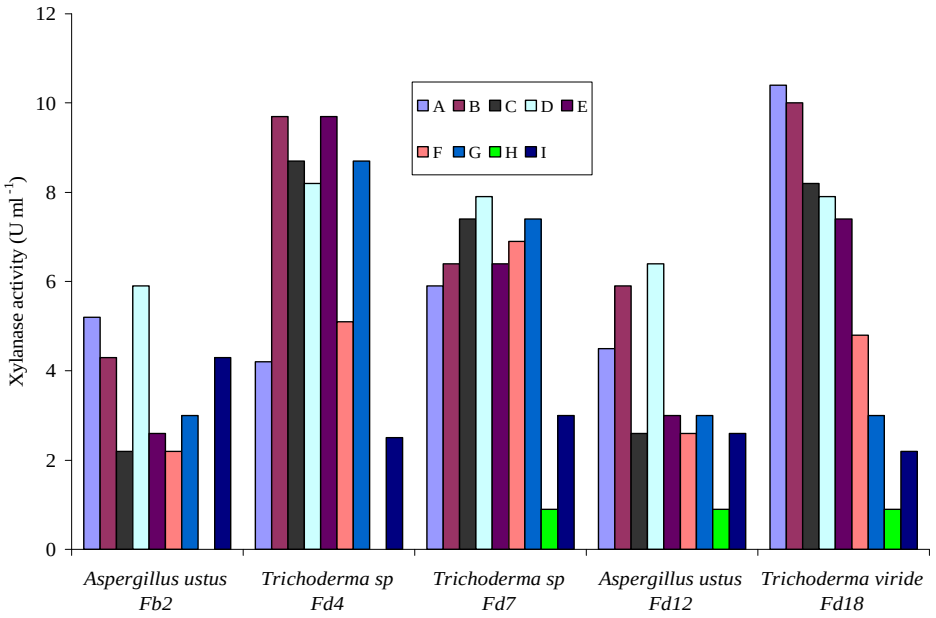

Figure 1: Effect of nitrogen sources on xylanase synthesis by selected fungal isolates. Data are presented as means of 2 replicates, having standard deviation within the range 0 – 8%. A = NH_4Cl, B = $(NH_4)_2SO_4$, C = $NaNO_3$, D = $NH_4H_2PO_4$, E = Urea, F = KNO_3, G = $(NH_2)_2HPO_4$, H = NH_4NO_3, I = Peptone.

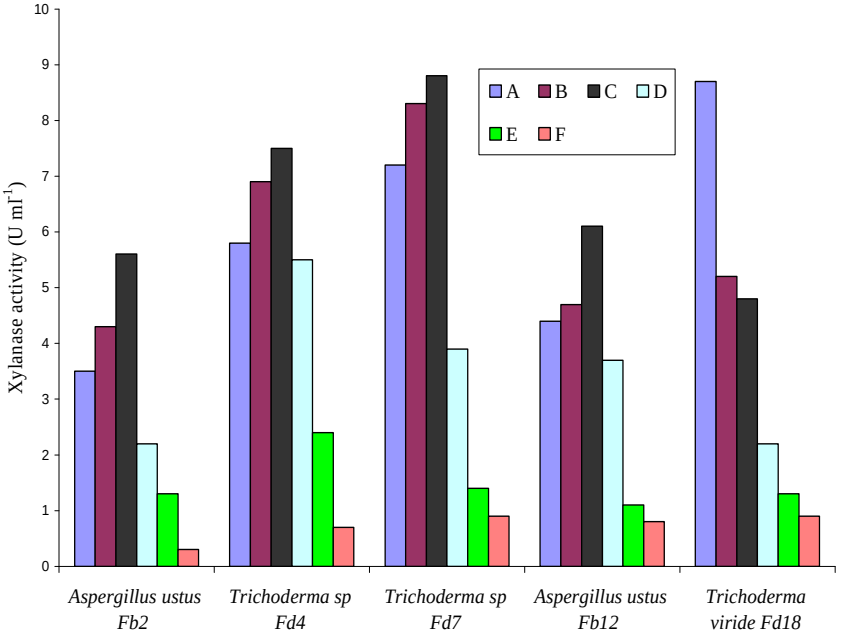

Figure 2: Effect of incubation temperatures on xylanase synthesis by fungal isolates. Data are presented as means of 2 replicates, having standard deviation within the range 0 – 8%. A = 30 ^oC, B = 35 ^oC, C = 40 ^oC, D = 45 ^oC, E = 50 ^oC, F = 55 ^oC.

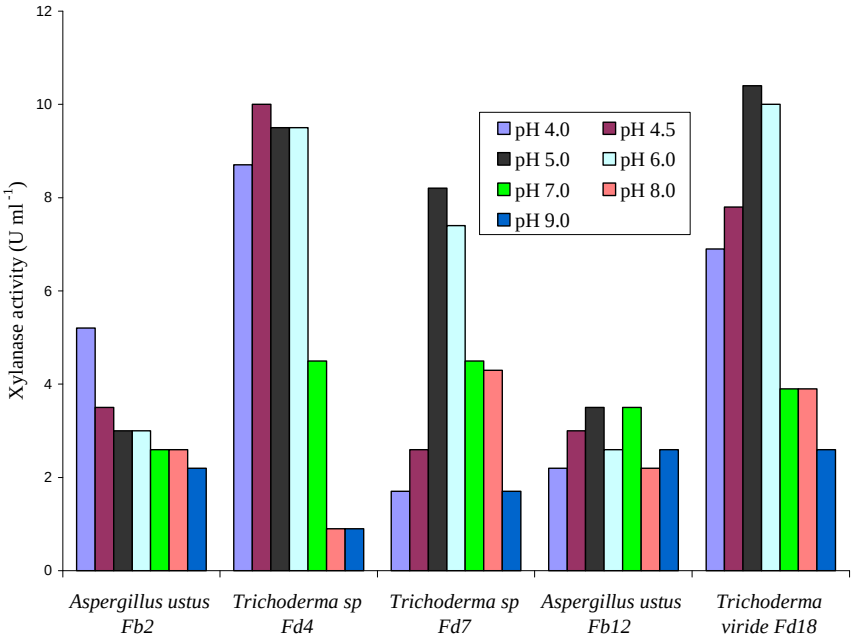

Figure 3: Effect of pH on xylanase synthesis by fungal isolates. Data are presented as means of 2 replicates, having standard deviation within the range 0 – 8%.

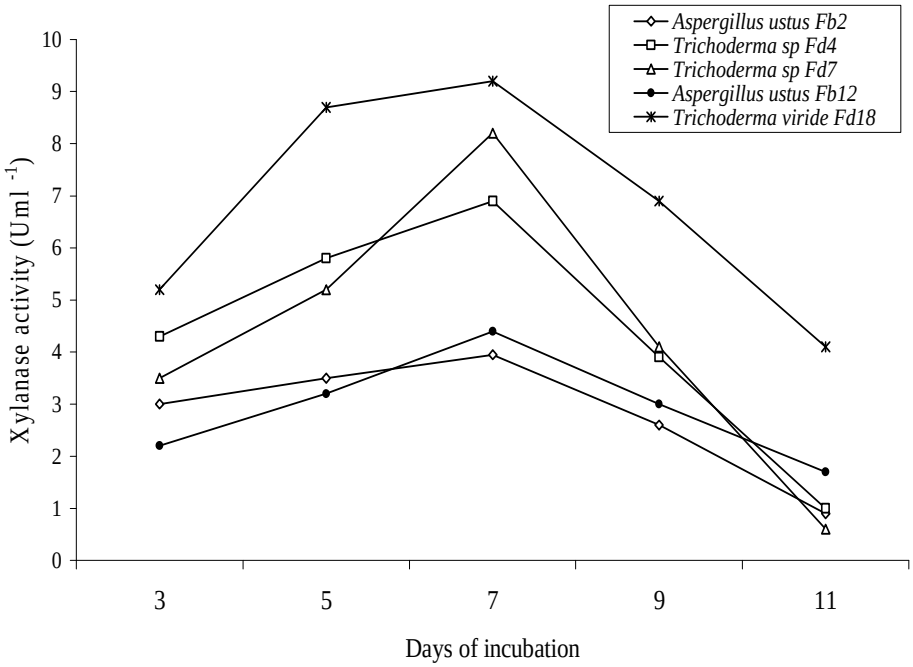

Figure 4: Effect of number of days of incubation at 30°C on xylanase synthesis by fungal isolates. Data are presented as means of 2 replicates, having standard deviation within the range 0 – 8%.

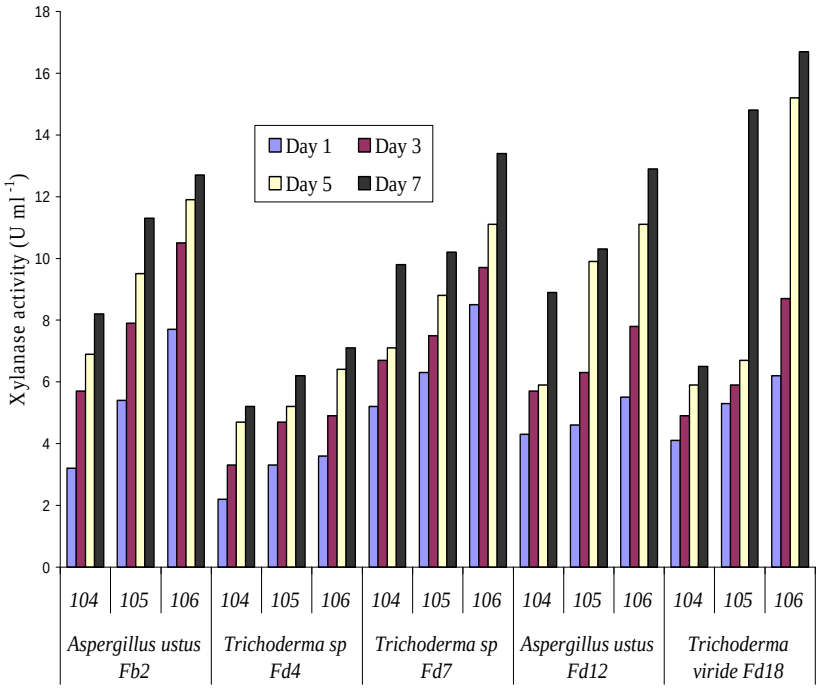

Figure 5: Effect of spore inoculum size on xylanase synthesis by fungal isolates. Data are presented as means of 2 replicates, having standard deviation within the range 0 – 8%. 104 = 1 ×10^4 spores ml^{-1}; 105 = 1 ×10^5 spores ml^{-1}; 106 = 1 ×10^6 spores ml^{-1}.

DISCUSSION

Production of microbial enzymes is dependent upon various nutritional and cultural parameters such as pH, temperature, carbon and nitrogen sources. These parameters were studied in order to optimize the production of lignocellulolytic enzymes by selected fungal isolates.

Oat-spelt xylan (2%) was the carbon source of choice for xylanase synthesis by *Trichoderma* sp. Fd4, *A. ustus* Fd12 and *T. viride* Fd18 (Table 1). Xylan or a xylan-containing carbon source is necessary for a satisfactory production of xylanase by many microorganisms, not only because it is the main carbon source, but probably also because its hydrolysis products act as inducers (Ding et al., 2004). Different carbohydrates were studied for xylanase production by *Trichoderma* with xylan being reported to induce high levels of xylanase (Gaanappriya et al., 2011). These findings are in agreement with the results in this work. The decrease in enzyme activity in the presence of high substrate concentration may be attributed to a partial adsorption of the enzymes to the substrate and to a strong decrease in pH that becomes inhibitory for the growth of the fungus and induction of the enzyme synthesis (Szczodrak, 1988).

The production of low levels of enzymes in the presence of glucose, glycerol or other carbon sources related to glycolytic mechanism may be due to catabolite repression, a regulatory mechanism known to control enzyme production in bacteria and fungi. In this case, the end product of lignocellulose hydrolysis may interact with a particular gene at the transcription level and represses enzyme synthesis (Lockington et al., 2002; Rajoka, 2004; Moussa and Tharwat, 2007).

When agro-residues - saw dust, sugarcane bagasse and wheat bran - were supplemented as sole carbon sources for xylanase production, lignocellulosic substrates

were observed to produce higher level of xylanase compared to the soluble sugars. Wheat bran was the best lignocellulosic substrate for the synthesis of xylanase by the fungal strains. Wheat bran was the substrate of choice by *T. viride* Fd18 in producing highest levels of xylanase (17.0 Uml^{-1}). Saw dust was a poor inducer of enzymes in this study. This result is in agreement with the findings of Xu et al. (2005) and Muthezilan et al. (2007), who reported the production enzymes using wheat bran as substrate. Wheat bran not only contains many nutrients needed for cell growth including various amino acids, but also serves as an inducer for xylanase production due to its high xylan content (30%). Wheat bran is also an abundant and cheap agricultural residue which remains loose and well aerated in mass, even in moist conditions (Xu et al., 2005).

The nitrogen source used in a production medium is one of the major factors affecting enzyme production and level (Moussa and Tharwat, 2007). In this study, NH_4NO_3 and urea were found to be poor sources of nitrogen for induction of xylanase activity. This is in agreement with the studies of Lieckfeldt and others (2000) who reported nitrate and urea as being not suitable for *Trichoderma reesei* cultivations. Ammonium compounds are reported to be the most favourable nitrogen compounds for protein and enzyme synthesis (Muthezhilan et al., 2007).

In this work, Xylanase production, except by *T. viride* Fd18, was optimal at 40 ^oC. The optimum temperature of 30 ^oC for xylanase production by *T. viride* fd18 is similar to other reported results that established the temperature range for xylanase production as being between 20 ^oC and 30 ^oC (Haq et al., 2004; Ikram-ul-Haq and Khan, 2006).

This study shows that any change, either increase or decrease in temperature resulted in the gradual decrease in enzyme production. According to Rahman et al. (2003) and Yuan et al. (2005), observed decreases in enzyme production at very low or high temperatures results from the inhibition of fungal growth at these temperatures, leading to a decrease in enzyme synthesis. High temperatures alter the cell membrane composition raising the maintenance energy requirement for cellular growth due to thermal denaturation of enzymes of the metabolic pathway (Ikram-ul-Haq and Khan, 2006). At lower temperature, the transport of substrates across the cells is suppressed and lower yield of products are attained (Rajoka et al., 1998).

High xylanase (Figure 2) values were obtained between pH 4.0 and 6.0. Ahmed et al. (2003) and Pang et al. (2006) reported pH 5.5 as the optimum for xylanase and cellulase production by *Aspergillus niger* MSK-7, *Trichoderma viride* MSK-10 and *Trichoderma harzianum*. According to Subramaniyan and Prema (2002) a pH of around 5.0, has in general, been the optimum for xylanase production by fungi and enzyme is normally stable at pH 2.0 to 9.0. The poor production of enzymes at high pH values might be due to the fact that alkaline pH has inhibitory effect on the growth of the fungi and enzyme production. The initial pH influences the transport of small amounts of enzymes across the cell membrane (Bakri et al., 2008).

All enzyme activities increased step wise and reached an optimum after 7 days of incubation (Figure 4). *T. viride* Fd18, with an activity of 9.2 Uml^{-1}, had the highest xylanase activity. The least activity of the enzymes was recorded on day 3 of incubation. Simoes and Tauk-Tornisielo (2006) reported that xylanase activity increased up to the fifth day and dropped considerably on the 6^{th} day of incubation. The depression of enzyme activity after the 7^{th} day may be due to cumulative effect of the reducing sugars, glucose and xylose, which are known to inhibit cellulases and xylanases respectively (Ojumu et al., 2003). Hatakka (1993) also suggested that delignification produces aromatic water-soluble products which can repress the cellulolytic action of the enzyme.

On varying inoculum size, it was observed that high inocula of 1 x 10^6 spores

ml^{-1} enabled maximum xylanase production in all fungal isolates. This result is similar to reports on other fungi (Pang et al., 2006). During enzyme production, the spore concentration in fungi cultivation must be high enough to colonize the substrates particles (Simoes and Tauk-Tornisielo, 2005). Studies however, have indicated that there can be a decline in this activity over a determined spore concentration. With higher inoculum size, the time taken for the inoculum to colonize the substrate will be shortened significantly with higher enzymes productivity (Pau and Omar, 2004).

In conclusion, the result obtained from the present work indicates xylanase production from new isolates of *Trichoderma* and *Aspergillus* using selective growth and nutrient conditions. This result will facilitate scale up processes for mass production. The xylanase so produced could be used in many industrial applications after detailed characterization.

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