International Journal of Agricultural Research

ISSN 1816-4897

Academic Journals Inc.

www.academicjournals.com

International Journal of Agricultural Research 7 (1): 46-57, 2012
ISSN 1816-4897 / DOI: 10.3923/ijar.2012.46.57
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Production of Xylanase by *Aspergillus niger* and *Trichoderma viride* using Some Agriculture Residues

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ABSTRACT

Aspergillus niger and *Trichoderma viride* are used for xylanase (EC 3.2.1.8) production using some lignocellulosic substrates under Solid State Fermentation (SSF). The results indicate that Barley Bran (BB) is the most potential substrate for higher xylanase production compared with other used substrates. The influence of various culture conditions including; fermentation period, incubating temperature, initial pH, initial moisture level, nitrogen sources, mineral sources, inoculum size and different levels of barley bran were studied. Generally, *A. niger* is more active in xylanase production compared with *T. viride* when grown on 1.0 g barley bran with moisture levels of 1:3 (75%), at pH 5.5 and 35°C after 2.0 days incubation in absence of additive nitrogen source. Parametric optimization of xylanase yielded 11.1 and 6.9 fold increase on barely bran compared with rice straw as initial solid substrate by *A. niger* and *T. viride*, respectively.

Key words: Xylanase (EC 3.2.1.8), SSF, *Aspergillus niger*, *Trichoderma viride*, barley bran

INTRODUCTION

Microbial enzyme is a fast growing field in biotechnology. The global market of industrial enzymes was closed to a billion dollars in 1990 and crossed the $2.0 billion in 2005 Krishna (2005). The market is estimated at $3.3 billion in 2010 and expected to reach $4.4 billion by 2015 (http://www.bccresearch.com/report/enzymes-industrial-applications-bio030f.html). The production cost and the yields of enzymes are considered the major problems. Therefore, investigations on the ability of fungi to utilize inexpensive substrate have been done (Kang *et al*., 2004). Most literatures have been directed to develop the hyper-production of industrial enzymes from fungi, focusing an improvement of fermentation processes (Haltrich *et al*., 1996). Solid State Fermentation (SSF) offers a lot of advantages over submerged fermentation for enzyme production (Pandey *et al*., 1999). For this reasons, lignocelluloses have been looked as a promising feedstock due to their abundance, cheapness and huge potential availability (Yang *et al*., 2006).

Hemicellulose is the second abundant renewable biomass in nature. Of them, xylan is the major hemicellulose component and approximately accounts 20-35% of plant cell wall dry weights (Jiang *et al*., 2010). The basic structure of xylan is a β-D-1,4-linked xylopyranosyl residue with a few relatively short side chains. The heterogeneity of xylan led to a diversity of xylan-degrading enzymes (Dwivedi *et al*., 2009). Xylanases have considerable interest because of their applications in food processing, bleaching of pulp during paper industry, bio-conversion of biomass wastes to fermentable sugars and clarification of fruit juices (Romanowska *et al*., 2006). They also have application in improvement nutrient digestibility in animal diets and in the production of

3xylooligosaccharides and xylose (Shah and Madamwar, 2005). From industrial point of view, xylanase in pre-bleaching process can replace up to 20-30% of chlorine and can reduce up to 50% organic halogens that are known to form toxic dioxins (Sonia *et al*., 2005). There are many reports on the production of xylanase by SSF using different fungi and several lignocelluloses residues (Panagiotou *et al*., 2003; Yang *et al*., 2006; Botella *et al*., 2007).

The present study was to produce xylanase from two local isolates of *Aspergillus niger* and *Trichoderma viride* through SSF using some agriculture wastes. Fermentation factors including; incubation time, temperature, initial pH and moisture, nitrogen sources, mineral sources, inoculum size and different levels of most suitable substrate are studied.

MATERIALS AND METHODS

Microorganisms: Two local fungal strains namely *Aspergillus niger* and *Trichoderma viride* were isolated from fermented rice straw and sub-cultured on xylan containing medium. The strains on PDA media at 30°C for 7.0 days were maintained at 4°C. PDA slants were mixed with 10 mL of the basal media to prepare spore suspensions. The spore counted in the suspension was about 2.0×10^{7} spore mL^{-1}.

Nutrient basal solution: The composition of basal mineral medium was: 3.0 (g) $NaNO_3$; 0.1 (g) KH_2PO_4; 0.5 (g) $MgSO_4.7H_2O$ and 0.5 (g) KCl $2H_2O$. All contents were dissolved in 1000 mL 0.05 M acetate buffer pH 5.5.

Natural substrates: Rice Straw (RS), Wheat Straw (WS), Corn Straw (CS), Bagasse (B), Corn Cob (CC), Wheat Bran (WB), Rice Bran (RB), Corn Bran (CB), Barley Bran (BB) and Soybean (SB) were collected locally from farms near El-Mansoura district. Each substrate was air dried, then milled into suitable sizes (about 0.1-0.2 cm) and utilized as carbon source for SSF.

Solid-state fermentation and xylanase production: The design of SSF medium was prepared according to Purkarthofer *et al*. (1993) with some modification. After incubation period, 50 mL of 0.1 M acetate buffer (pH 5.5) was added to each flask and kept at room temperature (25±2°C) for 1.0 h under mild shaking. The resultant slurry was filtered through a cloth and centrifuged at 5000×g for 10 min and finally, the extracts were used for protein and enzyme assay.

Protein determination: Soluble protein in enzyme solution was determined according to Bradford (1976) by measuring the optical density of the colour developed at 595 nm using a Spectro UV-VIS RS spectrophotometer. The μg of protein was estimated using Bovine Serum Albumin (BSA) as standard curve.

Xylanase assay: Xylanase was assayed by measuring the reducing sugars liberated from Larchwood xylan (Sigma chemicals) dissolved in 0.1 M acetate buffer (pH 5.5). The reaction mixture contains 0.1 mL of 1.0% (w/v) xylan and 0.2 mL of enzyme was incubated at 40°C for 30 min. The reducing sugars were measured by Smogyi (1952) method against a boiled enzyme as control, with D-xylose as standard. One unit of xylanase was defined as the amount of enzyme that released one μmol xylose per minute per μg protein under assay conditions. Xylanase activity was expressed as U g $substrate^{-1}$.

Optimization of process parameters: The SSF medium containing solid substrate and moistening media described in the previous section were taken as a basal medium and the process parameters under study were varied. Different agriculture wastes Rice straw, wheat straw, corn straw, bagasse, corn cob, wheat bran, rice bran, corn bran, barley bran and soybean, incubation time (1-8 days), initial pH levels (3.5-8.0), initial moisture content (0.5-7.0 mL flask), incubation temperature (20-55°C), supplementation with different organic nitrogen sources of 1.0% (Tryptone, L-Glutamine, L-Asparagine, Urea), inorganic nitrogen sources (ammonium nitrate, ammonium chloride, sodium nitrate, potassium nitrate, ammonium sulphate), different mineral salts (tap water, KCl, NaCl, $CaCL_2$, $FeSO_4$, $CUSO_4$, $MnSO_4$), inoculum size (ranged from 0.03-4.0×10^7 spores/flask) and finally different levels of barley bran as suitable solid substrate (0.25-5.0 g flask) were conducted. Incorporating all the optimized parameters, the specific enzyme activity was determined. The procedure adopted for optimization of various process parameters influencing xylanase production was to evaluate the effect of individual parameters (keeping all other parameters as constant) and to incorporate it at the optimized level in the experiment before optimizing the next parameter.

Reproducibility: All the experiments were repeated at least three times and the results were reproducible. The data points represented the Mean value within ±5.0% of the individual values.

RESULTS AND DISCUSSION

Effect of different substrates: The effect of different lignocellulosic substrates on xylanase production by *A. niger* and *T. viride* were investigated. The results (Table 1) showed significant variation between *A. niger* and *T. viride* in their abilities for xylanase production, the variation value was reached to 18.0%. Also, *A. niger* was found to be the most active xylanase producer on all used substrates except on wheat bran compared to *T. viride*. The variation (63.4%) between different substrates and its use as xylanase inducers were significant. This result may be attributed to the value of xylan or cellulose: Xylan ratios in each substrate (Haltrich *et al*., 1994; Ghanem *et al*., 2000). The results also indicated that Barley Bran (BB) has the most potential xylanase inducer compared with the other used substrates. Barley bran was increased the enzyme to about 12.5 and 11.0 Ug substrate^{-1} by *A. niger* and *T. viride,* respectively. With that result,

Table 1: Effect of different substrates on xylanase production

	Xylanase activity (Ug substrate^{-1})	
Different substrates	*A. niger*	*T. viride*
Rice straw	03.8±0.05	03.2±0.11
Wheat straw	08.7±0.14	08.1±0.21
Bagasse	02.0±0.11	00.7±0.05
Wheat bran	02.0±0.17	05.8±0.24
Rice straw+wheat bran (3:1)	10.2±0.27	05.6±0.14
Rice straw+wheat bran (1:1)	08.1±0.15	02.5±0.17
Rice bran	12.1±0.13	04.0±0.03
Barley bran	12.5±0.13	11.0±0.13
Corn bran	08.0±0.20	01.7±0.22
Corn straw	06.5±0.21	03.7±0.30
Corn cob	11.7±0.25	06.5±0.06
Soy bean	05.0±0.12	00.9±0.09

barley bran was selected as the most suitable substrate for further experiments. More or less similar results were obtained by different substrates for production of xylanase from *A. tamarii* (De Souza *et al.*, 2001). In this connection, most literatures proved that fungal xylanase could produce on different natural substrates during SSF (Fadel, 2001a; Lu *et al.*, 2003; Shah and Madamwar, 2005).

Effect of different incubation periods: In this experiment, the production of xylanase by *A. niger* and *T. viride* were studied along 8.0 days incubation when cultivated on BB. Three flasks from each organism were harvested daily, then, the enzyme was extracted and estimated. The results in Fig. 1 indicated that xylanase activities were started after the first day of incubation. A significant decrease of xylanase activities were seen from the fourth day onwards. For both fungi, 2.0 days incubation was the optimum for higher xylanase production. The highest levels of xylanase were 26.25 and 24.22 Ug substrate^{-1} for *A. niger* and *T. viride,* respectively. Our optimum incubation period for xylanase production on barely bran is very short compared with the most other studies on different substrates. The short incubation time offers significant advantages such as; reducing the risk of contamination and decrease costs of enzyme production. This result is in a good agreement with that obtained by Fadel (2001a). Generally, the optimum fermentation period for maximum xylanase production during SSF was depending upon the nature of substrate, organism, additive nutrients and many other fermentable conditions (Dekker, 1983; Mishra *et al.*, 1985).

Effect of different pH levels: The effects of initial pH on xylanase production has synergistic and an antagonistic effect. The microorganism holds a range of pH for its growth and activity with an optimum values, between these ranges the initial pH influences enzymatic system and the transport of enzyme across cell membrane (Poorna and Prema, 2007; Mohana *et al.*, 2008). The results (Fig. 2) showed that the maximum xylanases were showed at pH range from 4.5-6.5 for both fungi. However, xylanase was decreased at low pH (under pH 0.4) and high pH (above pH 6.5). pH 5.5 was the optimum for xylanase production by *A. niger* (22.53 Ug substrate^{-1}) and

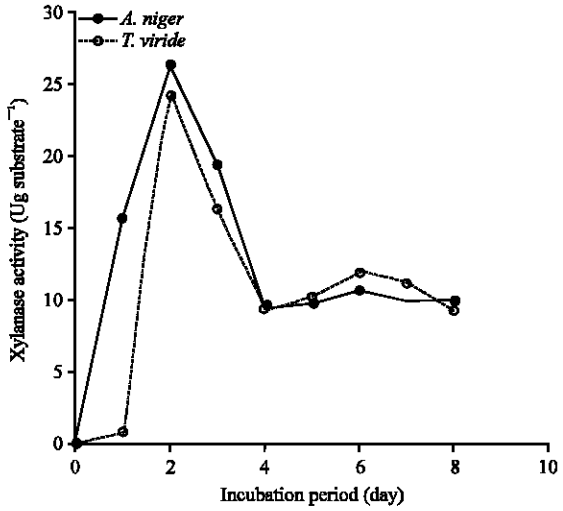

Fig. 1: Effect of different incubation periods on xylanase production

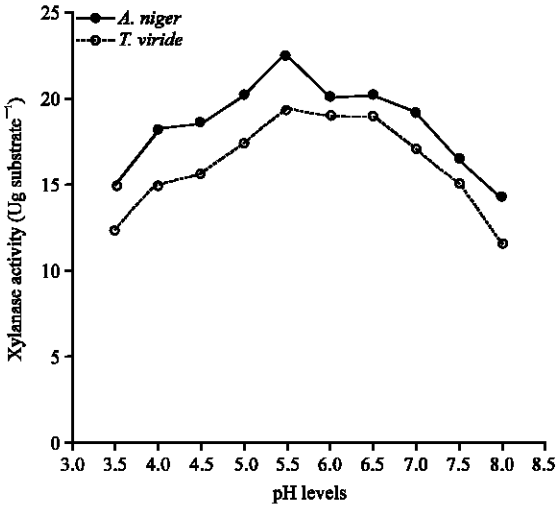

Fig. 2: Effect of different pH levels on xylanase production

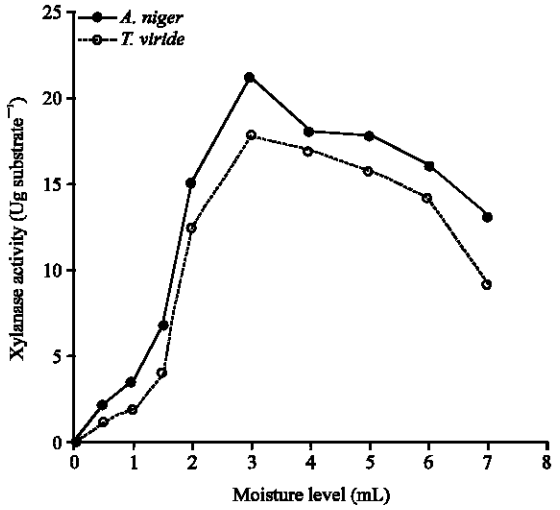

Fig. 3: Effect of different moisture levels on xylanase production

T. viride (19.42 Ug substrate^{-1}). pH 4.5 was the optimum for higher xylanase production by *T. harzianum* on sorghum flour (Fadel, 2001b). In contrary, pH 7.0 was the optimum for xylanase production from *P. themophila* when grown on wheat straw (Yang *et al*., 2006).

Effect of different moisture levels: The results (Fig. 3) indicate that xylanase activities were increased at moderate moisture levels (75-80%); While, decreased under lower (33-66%) and higher (83-88%) moisture contents. 75% was the optimum initial moisture for xylanase production by *A. niger* (21.32 Ug substrate^{-1}) and *T. viride* (17.9 Ug substrate^{-1}). These results are in agreement with those reported by Smits *et al.* (1996).

The importance of water during SSF is attributed to the fact that the majority of microbial viable cells require about 70-80% moisture content for new cell synthesis. Furthermore, moisture level is very limiting factor affecting stability, biosynthesis and secretion of fungal enzymes

(Pandey *et al.*, 1994). The optimum moisture was closely depended on some other parameters such as; nature of substrate (i.e., physical, chemical and their water-binding characteristics, in addition with particle size), organism and studied enzyme. Low moisture may reduce the solubility and swelling capacity of substrate causing high-water tension, decreasing growth and enzyme production. A reduction in enzyme biosynthesis at higher moisture than the optimum is due to steric hindrance of microorganisms growth through reduction in inter particle space, decreased porosity, gummy texture, alteration in particles of substrate structure and impaired oxygen transfer (Poorna and Prema, 2007).

Effect of different temperatures: Incubating temperature is one of the most significant factors strongly affected the production of enzyme. The type of fermentable wastes may also affect the value of incubating temperature. The results (Fig. 4) showed maximal xylanases at temperature ranged from 30 to 40°C. However, 35°C was the optimum for xylanase from *A. niger* (25.32 Ug substrate^{-1}) and *T. viride* (19.55 Ug substrate^{-1}). More or less similar result for xylanases was previously reported (Fadel, 2001b; Lu *et al.*, 2003). On the other hand, Suh *et al.* (1988) reported doubled reduction in *T. reesei* mutant RL-P37 xylanase when the temperature increased from 25°C to 37°C.

Effect of different nitrogen sources: The effects of supplementation of different inorganic and organic nitrogen sources on xylanase production are evaluated. The results in Table 2 indicated that barely bran alone is more suitable for xylanase production. *A. niger* reaches the maximum xylanase activity (42.1 Ug substrate^{-1}) in absence of additive nitrogen to BB medium; while, the highest level of *T. viride* xylanase was detected in the presence of NH_4NO_3 (31.2 Ug substrate^{-1}) and $(NH_4)_2SO_4$ (29.7 Ug substrate^{-1}), respectively. The richness of natural barley bran with some nitrogen sources such as; hordeins, glutelins, amino acids and vitamins (Kulp and Ponte, 2000) may be enough to stimulate growth and enhancing xylanase production. Generally, this result is in a good agreement with that obtained by Bailey and Poutanen (1989) and Purkarthofer *et al.* (1993), they reported that wheat bran and corncob alone are efficient to induce high levels of xylanase during SSF. On the other hand, some fungi need additive nitrogen compounds to

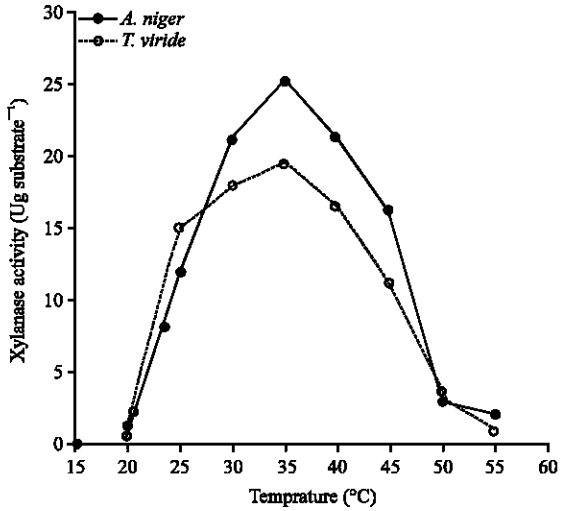

Fig. 4: Effect of different temperatures on xylanase production

Table 2: Effect of different nitrogen sources on xylanase production

	Xylanase activity (Ug substrate^{-1})	
Different nitrogen sources	*A. niger*	*T. viride*
Control	42.1±0.60	23.9±0.25
NH_4NO_3	20.2±0.17	31.2±1.28
NH4Cl	26.5±0.21	21.9±3.01
$NaNO_3$	23.8±0.13	19.9±0.27
KNO_3	11.9±0.22	21.9±0.16
$(NH_4)_2SO4$	17.1±0.17	29.7±0.30
Tryptone	19.9±0.26	27.6±0.14
L-Glutamine	17.3±0.13	23.6±0.15
L-Asparagine	20.2±0.17	17.2±0.12
Urea	15.9±0.11	14.6±0.18

Table 3: Effect of different mineral sources on xylanase production

	Xylanase activity (Ug substrate^{-1})	
Different mineral sources	*A. niger*	*T. viride*
Control	29.6±0.38	12.5±0.17
Tap water	39.5±0.16	22.4±0.14
Kcl	43.0±0.12	25.1±0.12
NaCl	22.1±0.13	14.2±0.23
$CaCL_2$	33.2±0.12	18.9±0.19
$FeSO_4$	12.5±0.15	11.1±0.12
$CUSO_4$	13.7±0.21	10.5±0.15
$MnSO_4$	24.6±0.20	20.8±0.70

stimulate xylanase production (Haltrich *et al*., 1994, 1996) as in the case of *T. viride*. In this connection, NH_4 residue was required for 1.7 fold increase of xylanase production by *A. terreus* grown on wheat straw (Ghanem *et al*., 2000).

Effect of different mineral sources: In this experiment, the results (Table 3) indicated that xylanase production is highly affected by different additive mineral salts. KCl is the most suitable mineral source for maximum xylanase production by both *A. niger* (43.0 Ug substrate^{-1}) and *T. viride* (25.1 Ug substrate^{-1}). This result is in agreement with that obtained by Kunamneni *et al.* (2005), who reported the impotence of minerals during SSF process on several agriculture residues. in addition, Krishna (2005) reported that supplementation of fermentation medium with a mixture of salt solution such as Na^+, Ca^{++}, Ni^+, Cu^+, Fe^{++}, Mn^{++}, K^+, Zn^{++}, Mg^{++} and Mo^{++}, etc. stimulate the growth and enzyme production yield.

Effect of different inoculum size: The size of inoculum plays a significant role in the production of enzymes under SSF (Pandey *et al*., 1994). The effect of inoculums size may depend on other culture conditions such as; incubation period, moisture, type of microbe and the used substrate (Soccol *et al*., 1994). Lower levels of inoculum may not be sufficient for initiating growth and enzyme synthesis on different substrates. An increase in the number of spores however, ensures a rapid proliferation of biomass and enzyme synthesis (Kashyap *et al*., 2002). After a certain limit,

the enzyme production could decrease or established because of depletion of nutrients. A balance between the proliferating biomass and available material would yield maximum enzyme (Sabu *et al.*, 2005). In the present study, different inoculum sizes were studied to improve xylanase production. The results in Fig. 5 showed that the higher inoculum sizes (1.0-4.0×10^7 spore $flask^{-1}$) of both *A. niger* and *T. viride* are increased the yield of xylanases; while, small inoculum sizea (0.03-0.25×10^7 spore $flask^{-1}$) are decreased the enzyme production. These results are in agreement with that obtained by Ghanem *et al.* (2000). Further increase in inoculum size resulted in decreasing xylanase yield due to limitation of nutrients as reported by Kunamneni *et al.* (2005).

Effect of different substrate levels of barley bran: Substrate level is a vital factor at SSF. It has a lot of influences on other culture fermentation factors; such as substrate depth, aeration, heat transfer, moisture content, inoculum size, rate of microbial growth etc.. The suitable level is highly depends on substrate type, substrate size, organism and studied enzyme (Fadel, 2000). The results (Fig. 6) indicated that 1.0 g barley bran is being optimum in depth, moisture level, inoculum size. It was optimum to make substrate suitable for the optimum xylanase production from *A. niger* (42.02 Ug $substrate^{-1}$) and *T. viride* (22.02 Ug $substrate^{-1}$). The decrease of xylanase activity at lower substrate levels ranged from 0.25-0.75 g flask may be attributed to shallow depth, high moisture content, high inoculum size and increasing the amount of soluble protein (Fig. 7). These results are in agreement with those obtained by Fadel (2000) and Kumar and Satyanarayana (2004). On the other hand, the reduction of xylanase activity at higher barley bran levels (2.0-5.0 g $flask^{-1}$) may attributed to greater substrate depth, lowering in moisture content, lowering inoculum size, reduction of substrate swelling and nutrient diffusion and lowering in amount of soluble protein. Similar results were reported by Lonsane *et al.* (1985) and Fadel (2000).

Optimization for xylanase production revealed that it become around 11.1 and 6.9 folds increase for *A. niger* and *T. viride* respectively when the fungi grown on barley bran instead of rice straw which used as first substrate in survey. It was also, 3.36 and 2.00 fold increase for *A. niger* and *T. viride*, respectively (Table 4) after the optimization of different culture conditions on barely bran

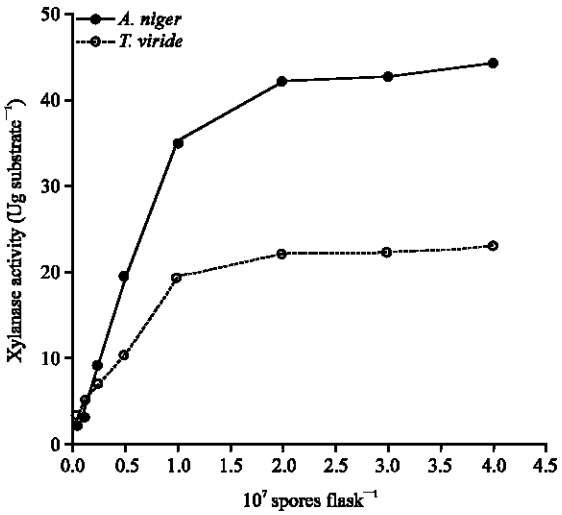

Fig. 5: Effect of different inoculum size on xylanase production

Table 4: Summary of xylanase production of *A. niger* and *T. viride* on rice straw and after optimization on barley bran

	Xylanase activity (Ug substrate^{-1})			Xylanase fold increasing	
Fungi	Xylanase activity on RS	Xylanase activity on BB before optimization	Xylanase activity after optimization on BB	Fold increase due to optimization on BB	Fold increase against RS
A. niger	03.8±0.05	12.5±0.13	42.0±0.22	3.36	11.1
T. viride	03.2±0.11	11.0±0.13	22.1±0.12	2.00	6.9

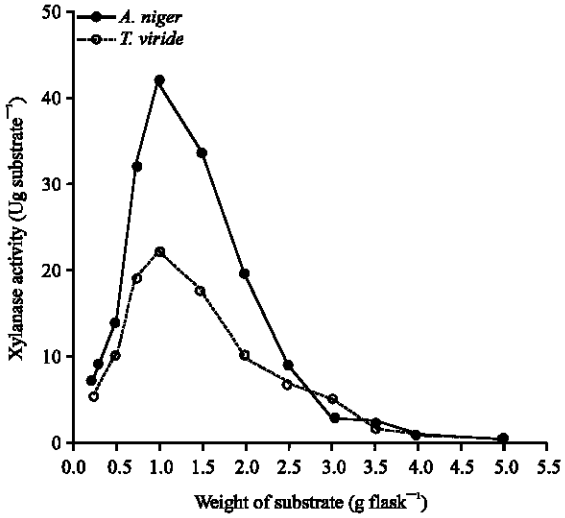

Fig. 6: Effect of different levels of substrates on xylanase production

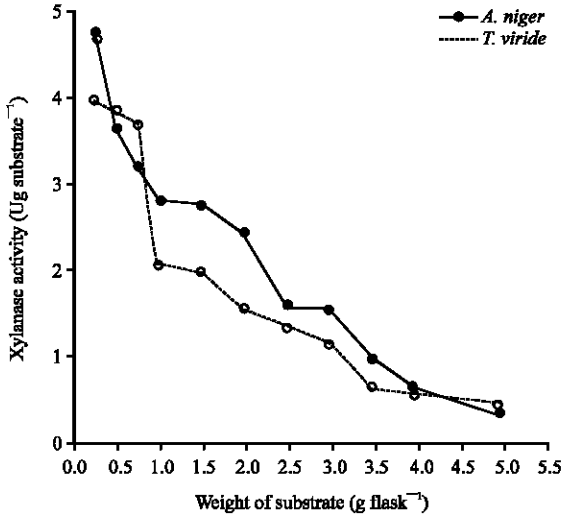

Fig. 7: Effect of different levels of substrates on protein production

medium. These may attribute to suitability and richens of barley bran as well as suitability of other SSF conditions which increase the yield of xylanase production by used fungi.

CONCLUSION

In conclusion, *A. niger* and *T. viride* as two local isolates could be use for several agriculture residues degradation and xylanase producers. Barley bran was the most suitable substrate for enzyme production compared with the other used substrates. *A. niger* is most potential fungal strain in xylanase production compared with *T. viride*. It produce about 42.5 Ug substrate^{-1} of enzyme on 1.0 g barley bran after 2.0 days incubation at temperature (35°C), pH level (5.5), moisture level (75%) and KCl as mineral salt without additive nitrogen sources. The optimization increased 11.1 and 6.9 fold of xylanase activity in case of *A. niger* and *T. viride* respectively on barley bran compared with their levels on rice straw.

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