Comparative study for production and charcterization of D-Xylanase produced from locally isolate *Aspergillus niger* by submerged and solid state fermentation

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Abstract: Xylanase was pooled from *Aspergillus niger* that was cultivated on czepak dox with addition rice husk. Condition affecting xylanase production were assessed these included p H, temperature of cultivation, carbon source, nitrogen source, incubation period, and inoculums size for both types of culture. Results obtained were as follow ion exchange DEAD-Cellulose specific activity from solid state fermentation (I, II) were (902.43,3666.6 U/mg), with yield (0.602,0.212%) and No. of fold (1.24,5.034). The specific activity from submerged fermentation (P 1, P2) were (1861.76,910 U/mg), with yield (1.033, 0.531%) and No. of fold (5.570, 2.790). the optimum and stability p H for enzyme activity type (II) produced by solid state fermentation (7.0 and 7.0) respectively. the optimum and stability temperature for enzyme type (II) produced by solid state fermentation **50°C and 70 °C** respectively. and the optimum and stability p H for enzyme activity type (P1) produced by submerged (7.0 and 7.0) respectively. the optimum and stability temperature for enzyme activity type (P1) produced by submerged fermentation 30 and 60 °C respectively.
Keywords: xylanase, solid state fermentation, purification, submerged fermentation

Introduction

Xylan is the major hemicellulose constituent of hard wood and soft wood, and is the next most abundant renewable polysaccharide after cellulose. Xylan is a heterogeneous carbohydrate, Process of removing lignin from wood in pulp and paper industry is facing serious problem due to the use of chlorine as a bleaching agent since this compound is very toxic to the environment. An alternative method using xylanase for delignifying lignin is a promising method since, in addition to its environmentally friendly technology; this method is easy to apply which does not require major modification of the existing pulping / bleaching processes. Production of xylanase has been performed by many researchers as reported in many reviews (Subramaniyan *et al.*, 2002; Collins *et al.*, 2005). Xylanases have mainly been produced from fungal and bacterial strains of microorganism. Although bacterial xylanase were reported to have higher value of optimum pH which is beneficial from the view point of applying in biobleaching process, xylanase produced from fungi usually has higher activity (Subramaniyan *et al*, 2002). Type of strains is close related to type of media used for cultivation. Fungal strains usually prefer solid state fermentation media which has low water content. On the contrary, bacterial strains prefer submerged fermentation which has high water content. These media types are one of the most responsible factors for the higher value of crude enzyme activity obtained from fungal strains.

Although many reports are available concerning the purification of xylanase, application of xylanase to biobleaching process as well as molecular studies of xylanase, all factors responsible for increasing enzyme activity and productivity, especially in its crude form, need be investigated since these are of vital importance from industrial point of view. xylanohydrolase, (E.C. 3.2.1.8) is the key enzyme for xylan depolymerization. A large number of bacteria and fungi are known to produce xylanases (Subramaniyan *et al.*, 2002; Kulkarni *et al.,* 1999). Filamentous fungi are industrially important producers of this enzyme due to extracellular release of xylanases, higher yield compared to yeast and bacteria and production of several auxiliary enzymes that are necessary for debranching of the substituted xylans (Haltrich *et al.*, 1996).

However, fungal xylanases are generally associated with concurrent production of cellulases. Xylanases are produced by either solid state or submerged fermentation. Enzyme productivity in solid

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state fermentation (SSF) is usually much higher than that of submerged fermentation (Haltrich *et al*., 1996). Therefore, solid state fermentation has gained interest from researchers in recent years and has often been employed for the production of xylanases because of economic and engineering advantages (Pandey *et al*., 1999). Microbial xylanases are used in the animal feed, textile and food processing industries, and in the production of several valuable products like xylitol and ethanol (Salles *et al*., 2005). Biobleaching of pulps using xylanase is one of the most suitable applications in the pulp and paper industry to reduce and/or eliminate the use of chlorine and chlorine dioxide. Main objectives of the study included xylanase-producing fungi from soil and the maximum amount of xylanase during fermentation. The work also aimed to produce xylanase under solid state and submerged fermentation conditions.

Materials and Methods

Xylanase production on Czapek's agar medium

To produce extracellular xylanase, fungal isolate was grown on Czapek's agar medium containing xylan as the substrate (quality test). The composition of the medium was (g.L-1): rice husk xylan, 5.0; peptone, 5.0; yeast extract, 5.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.2 and agar, 20.0 (Nakamura *et al.,* 1993). The inoculated plates were incubated for 7 days at 25ºC.

Xylanase production under solid state fermentation

Fungal isolate was cultured in Erlenmeyer flasks (250 ml) containing 10 g of wheat bran (particle size 300 - 500 mesh) moistened with 50 ml of mineral salts solution. The composition of the mineral salts solution was (g.L-1): KCl, 0.5; $MgSO_4.7H_2O$, 0.5; NaH_2PO_4, 0.5; $CaC_{12}.2H_2O$, 0.01; $FeSO_4.7H_2O$, 0.01; $ZnSO_4.7H_2O$, 0.002 and rice husk xylan, 1.0. The pH was adjusted to 5.0 The medium was then autoclaved for 20 min at 121ºC (15 lbs). After cooling, the flasks were inoculated with 1 ml of spore suspension containing ($1x10^6$) spores/ ml. The spore suspension was obtained from 7 day-old pure cultures. After mixing, flasks were incubated at 40ºC under static conditions for 7 days. After incubation, the enzyme was extracted with 70 ml cold sodium citrate buffer (50 mM, pH 5.3) with stirring for 1h. The fermented slurry was filtered through cheese cloth and centrifuged at 6000 rpm for 20 min at 4ºC. The clear supernatant was used for enzyme activities assays.

Xylanase production under submerged fermentation: The composition of mineral salts medium was the same as that of the solid state fermentation with (xylan) rice husk as the substrate. The pH of the medium was adjusted to 5.0. Fifty ml of the medium was transferred into a 250 ml Erlenmeyer flask, and after autoclaving was inoculated with $1 x 10^6$ spore/ ml of spore suspension. The flasks were incubated at 40ºC on a rotary shaker (100 rpm) for 7 days. After incubation, the medium was filtered through Whatman No.1 filter paper and the filtrate was centrifuged at 6 000 rpm for 15 min at 4ºC. The clear supernatant was used as source of xylanase assay.

Enzyme assays:

Xylanase activity was determined by mixing 0.9 ml of 1% (w/v) rice husk xylan (prepared in 50 mM Na-citrate buffer, pH 5.3) with 0.1 ml of crude enzyme and the mixture was incubated at 50ºC for 5 min (Bailey, 1992). The reaction was stopped by addition of 1.5 ml of 3.5-dinitrosalicylic acid (DNS) and the content was boiled for 5 min (Miller, 1959). After cooling, the colour developed was read at 540 nm. The amount of reducing sugar liberated was quantified using xylose as standard. One unit of xylanase is defined as the amount of enzyme that liberates 1 mmol of xylose equivalents per minute under the assay conditions.

Determination of protein:

The concentration of soluble protein was estimated by lowery method using bovine serum albumin as the standard (Lowry, 1951).

Precipitation by Ammonium Sulfate

Solid ammonium sulfate was added to the culture supernatant to 40% saturation under cooled conditions with continuous stirring for 30 min. The resulting precipitate was collected by centrifugation at 6,000 rpm for 30 min at 4ºC. The precipitate was redissolved in a 50 mM sodium acetate buffer (pH 5.3).

Dialysis: Dialysis was performed using dialysis tube. The buffer 50 mM sodium acetate buffer (pH 5.3) was altered three times (after 2, 4 and 19 h) to ensure removal of ammonium sulfate which may interfere separation during ion exchange chromatography.

Enzyme purification by Ion exchange
The dialyzed solution was loaded onto a column (2 x 17 cm) of DEAE (Sigma, U.S.A.) previously equilibrated with the same buffer, The column was eluted with the same buffer. The bound proteins were then eluted with a linear gradient of NaCl (0.05 to 1M) in the same buffer; the flow rate was 40 ml/ 1h at 280 nm. Active fractions were determined for xylanase activity.
Determine the optimum pH The optimum pH of the xylanase activity was determined by using Buffer solutions were pre-incubated in the absence of a substrate at different pH (4.0,5.0,6.0,7.0,8.0,9.0,10 and 11) at 50ºC for 10 min.
Determine the optimum temperature for enzyme activity Buffer solutions were pre-incubated in the absence of a substrate at different temperatures from (30, 40, 50, 60, 70, 80,90,100)º C for 10 min.
Determine the stability temperature Enzyme solution was pre-incubated in the absence of a substrate at different temperatures from (30, 40, 50, 60, 70, 80, 90, 100)º C for 30 min; there after the residual activity was determined under optimal conditions.
Determine the stability pH Enzyme solution was pre-incubated in the absence of a substrate at different pHs from (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10 and 11) at 50ºC for 30 min; there after the residual activity was determined under optimal conditions.

Results and Discussion

Xylanase production in SSF was recored specific activity for crud enzyme (728.442 U/mg) higher than that in submerged fermentation (SmF) was recored (326.151 U/mg). The results are in agreement with the results of (Malarvizhi *et al.*, 2003) on *Aspergillus nidulans*. Javier *et al.*, (1998) suggested that low molecular mass degradation products of xylan and cellulose hydrolysis penetrate into the cells and induce the production of hydrolytic enzymes. Haltrich *et al.*, (1996) reported that xylose, the ultimate breakdown product of xylan, serves as a good inducer of this enzyme. (Marta *et al.*, 2000) observed enhancement more of xylanase production in solid state fermentation than liquid culture when wheat bran with rice husk was used as the substrate for a culture of *A. niger*. Xylanase is an inducible enzyme and the xylan present in wheat bran as well as rice husk (xylan) acted as good inducers for enzyme production. Javier *et al.*, (1998) reported that addition of small amounts of purified xylan to complex lignocellulosic substrates like wheat bran resulted in considerable enhancement of xylanase production. Among the best xylanase producers, *Aspergillus* was the most common genus and represented 55% of the selected strains. *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus* and *Aspergillus ochraceous* were the most prevalent species. *A. niger* constituted 52.6% of the total *Aspergilli* isolated. Similar reports were obtained by Kulkarni *et al.,* (1999), Gawande (1999), Abdel-Sater (2001). *Aspergillus sydowii*, a rare species, was also isolated. All strains produced xylanase along with cellulase during SSF *Aspergillus sp*. was the best xylanase producers (Suprabha, 2008). Wheat bran proved to be a suitable substrate along with 0.1% rice husk xylan for the production of xylanase during SSF. Several workers reported the suitability of wheat bran for xylanase production by SSF Marta *et al.*, (2000) and Ghosh, (1993). Commercial wheat bran consists of 30% cellulose, 27% hemicellulose, 21% lignin and 8% ash (Ghosh, 1993).

Purification by ion exchange chromatography:

The extracellular xylanase was purified to homogeneity from the culture filtrates of *A.niger* grown on SSF and SMF medium. The ammonium sulphate ranging from 60% to 70% has been reported for the purpose of precipitation of xylanase. In the present study, xylanase was fractionated with 40% ammonium sulphate. A recovery rate of 0.691% with 0.937 fold purification was achieved with the present process. A similar study reports a recovery rate of 78.9% xylanase from a fungus *Paecilomyces themophila* upon partial purification using 20-50% ammonium sulphate saturation (Kulkarni *et al.,* 1999).

The summary of purification stepes of xylanase is presented in Table 1 and 2 for two methods SSF and SMF. Xylanase obtained from *B. pumilus* ASH by fermentation, without purification was used for commercial application (Marta *et al.*, 2000); (Bindu, 2007) however, purification would enhance the extent of its efficacy as a biobleaching agent and hence purity is warranted. xylanase was purified by a -step chromatographic procedure, purified by using ion exchange chromatography. Two peaks of proteins with xylanase activity could be separated using DEAE-cellulose column (Figure 1). For SSF the two peaks Xylanase (**I, II**) representing the major portion of xylanase specific activity

were eluted (902.43 and 3666.6 (U/mg)) respectively and no. of fold 1.24 and 5.034 respectivily. And for SmF the two peaks Xylanase (**P1, P2**) representing the two peaks of xylanase specific activity were eluted (1861.76 and 910 (U/mg)) respectively and no. of fold 5.570 and 2.790 respectively.

Table 1. Purification steps of xylanases production by *Aspergillus niger* using solid state fermentation.

purification Step	Volume (ml)	Enzyme Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)	Total Activity (U)	Yield%	Fold of purification
Crude	870	95.426	0.130	728.442	83020.62	100	1
Precipitate with 40% saturation ammonium sulfate and desalting by dialysis	15	38.233	0.056	682.732	573.490	0.691	0.937
DEAE (xylanase I)	9 ml	33.39	0.037	902.43	500.51	0.602	1.24
(xylanase II)	5 ml	35.20	0.0096	3666.6	176	0.212	5.034

Table 2. Purification steps of xylanases production by *Aspergillus niger* using submerged fermentation

| purification Step	Volume (ml)	Enzyme Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)	Total Activity (U)	Yield%	Fold of purification
Crude	520	100.101	0.307	326.151	52052	100	1
Precipitate with 40% saturation ammonium sulfate and desalting by dialysis	15	44.787	0.221	202.656	671.805	1.290	0.621
DEAE (xylanase P1)	17	31.65	0.017	1861.76	538.05	1.033	5.570
(xylanase P2)	16	17.29	0.019	910	276.64	0.531	2.790
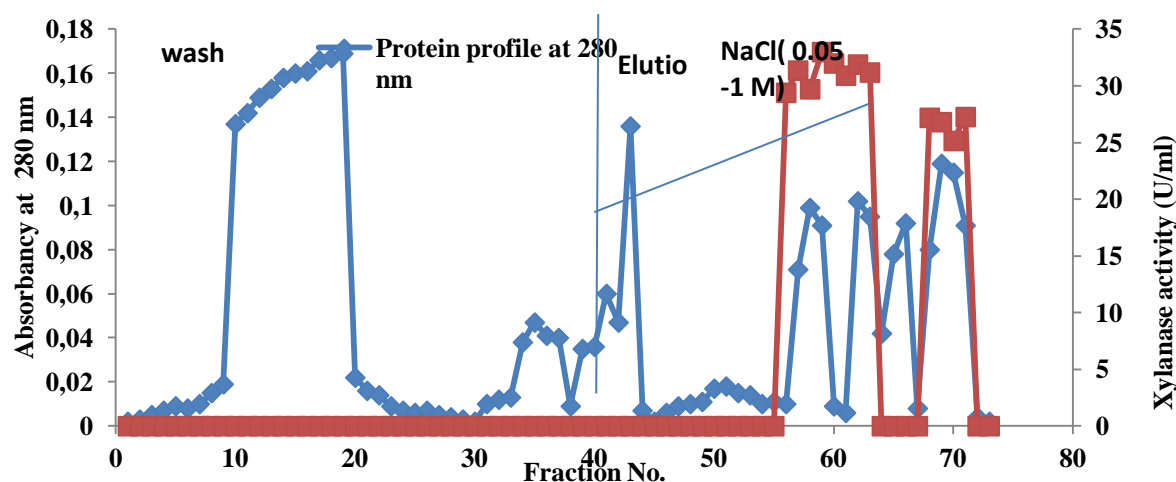
Figure 1. Ion exchange chromotography of D-xylanase produced by Solid state fermentation on DEAE-cellulose column (2 x 17 cm) elution was carried out with alinear grediant from of NaCl (0.05 to 1M) 50 mM Na-citrate buffer, pH 5.3. fraction 5 ml /7 min. were collected and the flow rate was 40 ml/ 1h.

Effect of Optimum pH on xylanase activity and stability by solid state fermentation
The relative xylanase activity using 0.1% (w/v) ricehusk xylan was determined at various pHs. The pH range used varied from 4 to 11. Three different buffers (0.05 M) were used. Citrate buffer was used for pH 4 to 6. Phosphate buffer was used for pH from 6 to 8 and glycine-NaOH buffer was used for pH from 8 to 11. To test the optimum pH and pH Stability, the purified enzyme recored (21.9 U/ml at pH=7.0 and 27.11, 28.16 at pH 7 and 9) were using respective buffers having pH ranging from 4 to 11 as described above and were incubated for 30 min at 30º C. The residual enzyme activity was

estimated at 1 h intervals during the 2 h period of incubation. Xylanase from a fungal source *A. terreus* UL 4209 showed maximum pH activity at 6.0 (Winterhalter *et al*,1995).Thermostable alkaline xylanase from a *Bacillus sp.* showed three optimum peaks for pH 6.5, 8.5 and 10.5 (Zverlov *et al*,1996). A wide range of pH activity from 5.0 - 8.0 was observed in *B. circulans* BL53 upon solid state cultivation (Lowry *et al.*, 1951). The persistence of activity in a large range of pH is a desirable quality of an industrial enzyme.
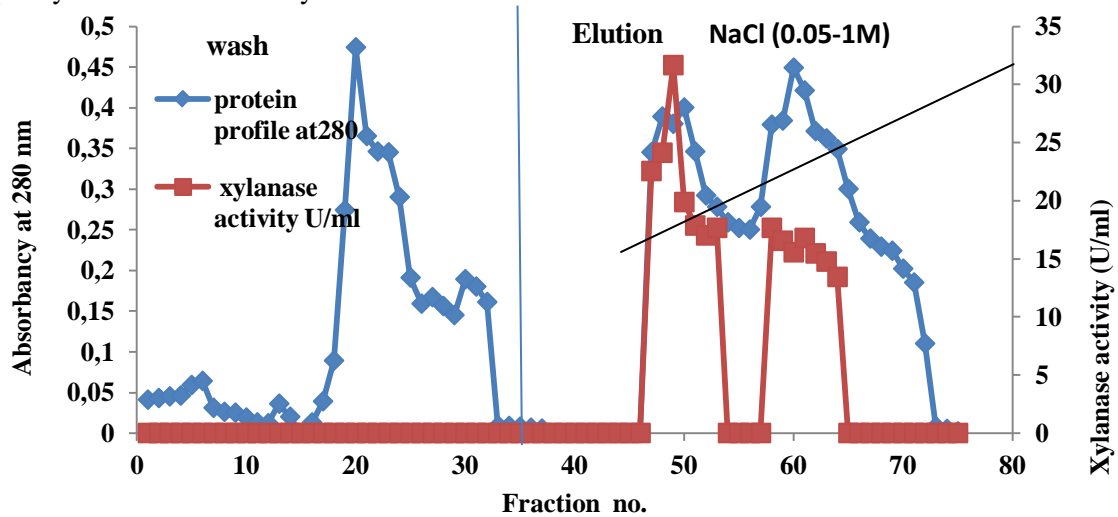
Figure 2. Ion exchange chromotography of D-xylanase produced by **Submerged fermentation** on DEAE-cellulose column (2 X 17 cm) equlibrate with elution was carried out with alinear grediant from of NaCl (0.05 to 1M) 50 mM Na-citrate buffer, pH 5.3. fraction 5 ml /7 min. were collected and the flow rate was 40 ml/ 1h.
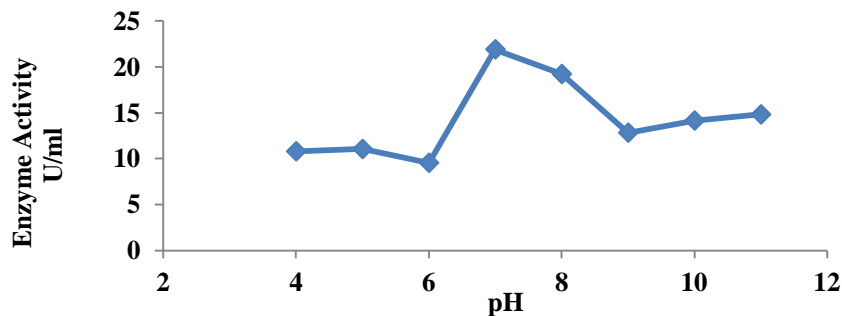

Figure 3. The effect of optimum pH values on the activity of purified xylanase from *Aspergillus niger* by solid state fermentation.
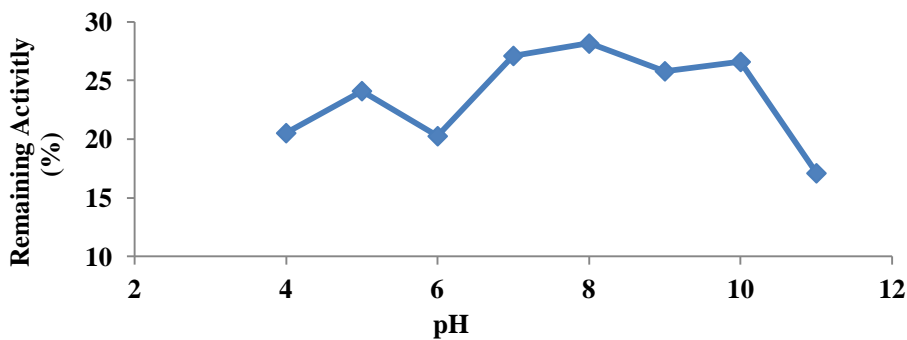

Figure 4. The effect of pH on the stability on the activity of purified xylanase from *Aspergillus niger* by solid state fermentation.

Effect of temperature on xylanase activity and stability by solid state fermentation

The activity of partially purified xylanase was found to be maximal at 30°C as indicated in (Figure 5) a finding similar to *A. terreus* UL 4209 that showed optimum activity at 35°C (Silas *et al.*,2008). Xylanase from a thermoalkaliphilic bacterium showed optimum activity at 50 °C (Sapre *et al.*,2005). Optimum activity of xylanase obtained from both *B. circulans* and *B. amyloliquefaciens* was at 50 °C (Júlio *et al*, 2006); Javier *et al,* 1998). The industrial importance of an enzyme will be more if the temperature input for its optimal activity is less.

The optimal temperature for the purified xylanase (39.03 U/ml) was obtained by assaying the enzyme activity at different temperatures. The enzyme activity at temperatures from 30°C to 100 °C was determined by incubating the enzyme with the substrate for 10 min at respective temperature using water bath. The temperature effect on the activity of the enzyme was further reconfirmed using a water bath (50°C to 100 °C). Optimum activity of xylanase obtained from both *B. circulans* and *B. amyloliquefaciens* was at 50 ^{0}C (Júlio *et al.*, 2006; Javier *et al.,* 1998). The optimal temperature of xylanase from SmF under standard assay condition was 50°C (Figure.9). At 60 °C the enzyme was stable for 10 min and there after the stability decreased with the increase in the incubation period and at 60°C 81 % of the activity was retained after 2 h of the incubation period. At 70°C (39 %), at 80 °C (40%), 90°C (41 %)and(33%) at 100°C of the enzyme activity was retained after 1 h, respectively. To the 30 best of our knowledge there are no published reports on xylanase exhibiting optimal activity at 100 °C when the reaction buffer of pH 9.0 was used. However, Xyn A and Xyn B from *Thermotoga maritima* showed optimal temperature of 92 °C and 105 ^{o}C,respectively at pH 6 (Winterhalter *et al.*,1995). Similarly, xylanase isolated from *Thermotoga neapolitana* had optimal temperature of 102 °C at pH 5.5 (Zverlov *et al.*, 1996)
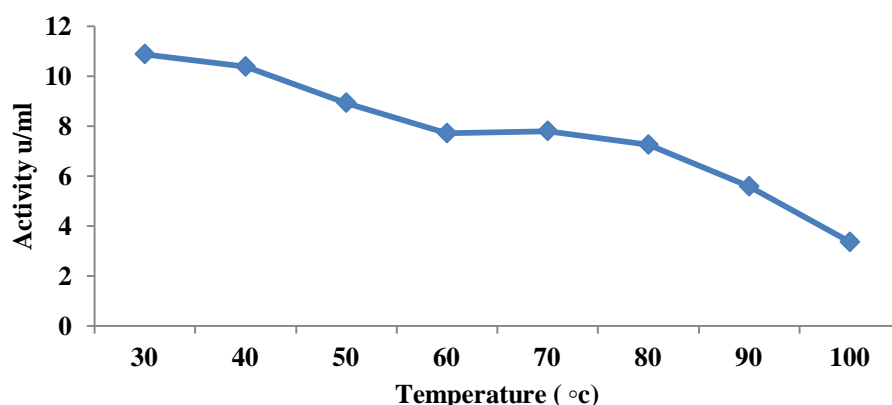

Figure 5. The effect of Temperature on the activity of purified xylanase from *Aspergillus niger* by solid state fermentation.

Effect of pH on xylanase activity and stability by submerged fermentation

The relative xylanase activity using 0.1% (w/v) ricehusk xylan was determined at various pHs. The pH range used varied from 4 to 11. Three different buffers (0.05 M) were used. Citrate buffer was used for pH 4 to 6. Phosphate buffer was used for pH from 6 to 8 and glycine-NaOH buffer was used for pH from 8 to 11. To test the optimum pH and pH Stability, the purified enzyme recored (32.1 U/ml) at pH=7.0 and (29.83, 34.4,31.29,31.55 U/ml at pH 6 -9) were using respective buffers having pH ranging from 4 to 11 as described above and were incubated for 30 min at room temperature as indicated in (Figure 7, 8). The residual enzyme activity was estimated at 1 h intervals during the 2 h period of incubation. Xylanase from a fungal source *A. terreus* UL 4209 showed maximum pH activity at 6.0 (Nakamura *et al.*,1993) Thermostable alkaline xylanase from a *Bacillus sp.* showed three optimum peaks for pH 6.5, 8.5 and 10.5 (Sapre *et al.*,2005). A wide range of pH activity from 5.0 - 8.0 was observed in *B. circulans* BL53 upon solid state cultivation (Júlio *et al.*, 2006)The persistence of activity in a large range of pH is a desirable quality of an industrial enzyme.
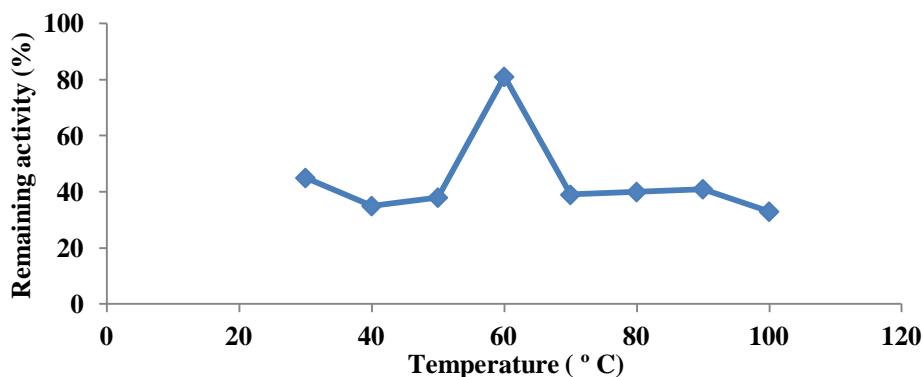
Figure 6. Thermal stability temperature of purified xylanase from *Aspergillus niger* by solid state fermentation.
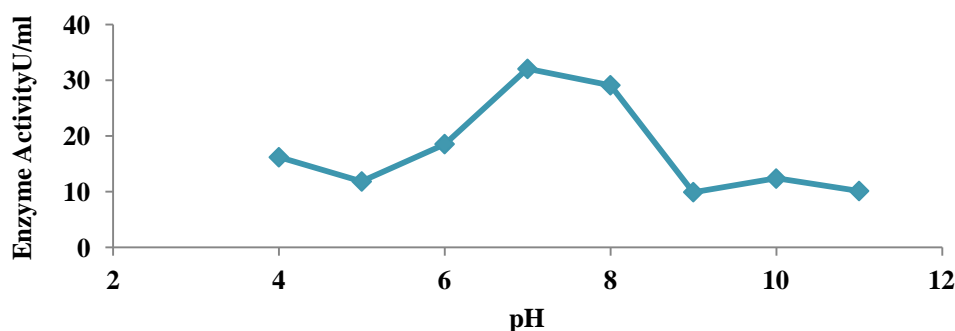

Figure 7. The effect of pH values on the activity of purified xylanase from *Aspergillus niger* by submerged fermentation.
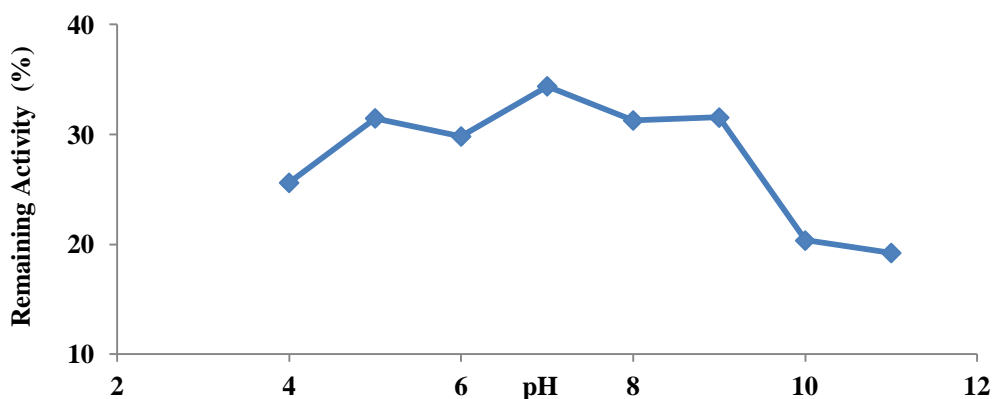

Figure 8. The effect of pH on the stability of purified xylanase from *Aspergillus niger* by submerged fermentation

Effect of temperature on xylanase activity and stability produced by submerged fermentation:

The optimal temperature for the purified xylanase producd by submerged fermentation (39.97 U/ml) was obtained by assaying the enzyme activity at different temperatures. The enzyme activity at temperatures from 30°C to 100 °C was determined by incubating the enzyme with the substrate for 10 min at respective temperature using a incubator. The temperature effect on the activity of the enzyme was further reconfirmed using a water bath (50°C to 100 °C). The activity of partially purified xylanase was found to be maximal at 50ºC as indicated in (Figure 9), a finding similar to *A. terreus* UL 4209 that showed optimum activity at 35ºC (Silas *et al*., 2008). Xylanase from a thermoalkaliphilic bacterium showed optimum activity at 50 ^{0}C (Sapre *et al*., 2005). Optimum activity of xylanase obtained from both *B. circulans* and *B. amyloliquefaciens* was at 50 ^{0}C (Júlio *et al*., 2006);(Javier *et al*., 1998). The industrial importance of an enzyme will be more if the temperature input for its optimal activity is less. In order to assess the stability, the enzyme solution was incubated

at 50 °C, 60 °C, and 70 °C for 2 h. and, the enzyme was incubated at 80 ℃, 90 ℃ and 100 ℃ for 2 h using incubators maintained at respective temperatures.

The optimal temperature of xylanase from SSF under standard assay condition was 50℃ (Figuare 9). At 60 °C the enzyme was stable for 10 min and there after the stability decreased with the increase in the incubation period and at 100°C 98.88% of the activity was retained after 2 h of the incubation period. At 90°C 11 % and at 60 °C 69.85%,70°C 85.53%, 80°C 89.55% and 80.81 % at 90 °C of the enzyme activity was retained after 1 h, respectively. To the 30 best of our knowledge there are no published reports on xylanase exhibiting optimal activity at 100 °C when the reaction buffer of pH 9.0 was used. However, Xyn A and Xyn B from *Thermotoga maritima* showed optimal temperature of 92 °C and 105 0C,respectively at pH 6 (Winterhalter *et al*.,1995). Similarly, xylanase isolated from *Thermotoga neapolitana* had optimal temperature of 102 °C at pH 5.5 (Zverlov *et al*.,1996)
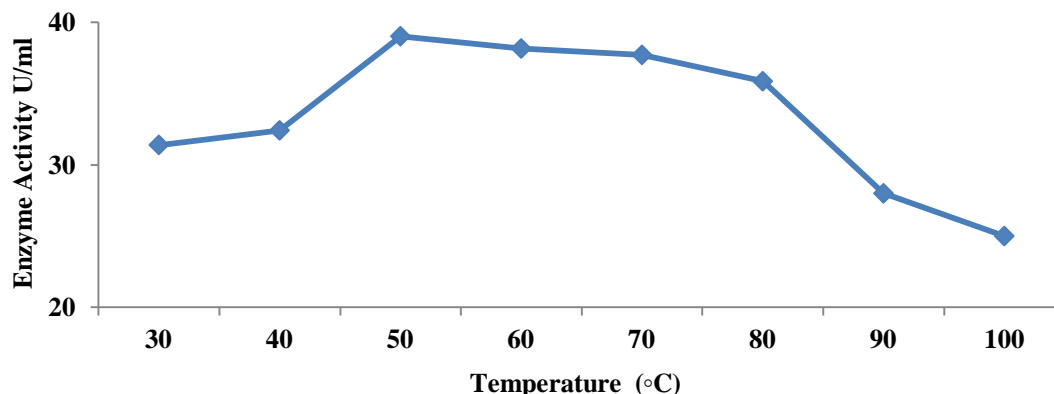

Figure 9. The effect of Temperature of purified xylanase from *Aspergillus niger* by submerged fermentation
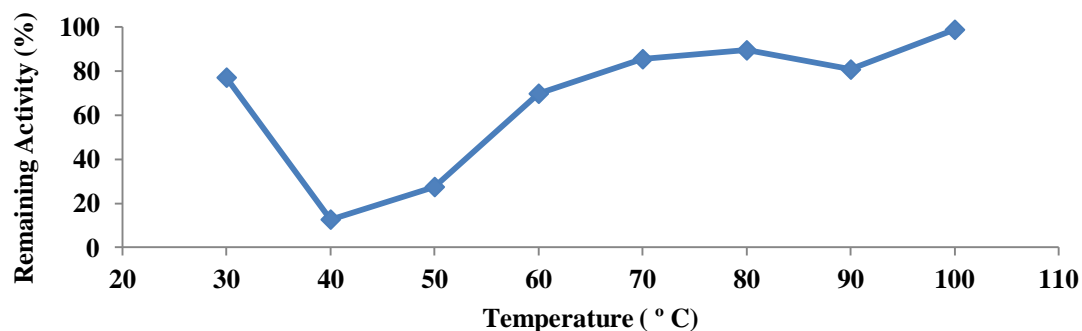

Figure 10. thermal stability of purified xylanase from *Aspergillus niger* by submerged fermentation.

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