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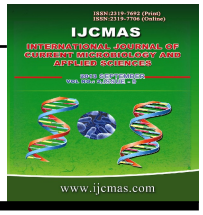

Original Research Article

Utilization of fortified rice husk for the fermentative production of xylanase by *Trichoderma* sp.

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A B S T R A C T

Mineral salt agar plates containing rice husk powder were used for the isolation of xylanase producing microorganisms. Two of the fungal isolates namely *Trichoderma* sp. and *Neurospora* sp. showed better xylanase activities. *Trichoderma* sp. produced maximum xylanase activity (22.5 U/ gds 72 h) when grown in solid substrate than that in submerged cultivation (2.62 U/mL in 96 h). It was further revealed that carboxy methyl cellulose (CMC) as additional carbon source produced maximum rise in xylanase activity (107 U/gds, 72 h) in solid culturing. Similarly the nitrogen sources (casein acid hydrolysate, yeast extract & corn steep solid) supplemented also gave enhanced enzyme activities. Synergistic effect of CMC & corn steep solid resulted in enhanced xylanase activity as high as 570 U/gds in 72 h. The same further enhanced to 722 U/gds when this combination was altered by replacing pressmud extract (PE) with a combination of PE: Corn steep liquor (2:8). The crude xylanase activity was more pronounced at 50°C and at pH 5. Enzyme was stable at 40 to 70°C. Ca^{2+} Cu^{2+} and Fe^{2+} acted as strong inhibitors for xylanase activity. Three phase partition resulted in 7 fold rise in enzyme activity i.e. 5985 U/mg.

Keywords

Rice husk; *Trichoderma* sp.; SSF; Pressmud *Neurospora* sp.; Xylanase.

Introduction

Xylan, the main hemicellulose component of lignocellulose, is a polymer with a linear backbone of β-1,4-d-xylopyranoside residues that are commonly substituted by acetyl, arabinosyl, and glucuronosyl groups. Microorganisms growing on xylan-containing substrates generally produce a variety of enzymes, including endoxylanases, exoxylanases, xylosidases, β-glucuronidases, β-arabinofuranosidases, and esterases for the degradation of xylan. Among the various xylanolytic enzymes, endo- β-1,4- xylanase (EC 3.2.1.8), frequently called xylanase, can randomly cleave the β-1,4 glycosidic bonds in the xylan backbone, thus bringing about a major reduction in the degree of polymerization of the substrate (Collins *et al*., 2005).

Xylanase, ahydrolytic enzyme involved in depolymerization of xylan, finds application in many industrial processes such as enzymatic bleaching of paper pulp, juiceand wines clarification, extraction of oils from plants, texture improvement in bakery, bioconversion of agricultural wastes, bioscouring in textiles and improving digestibility of animal feed. Xylanases can be used for the tailor designing of drugs and modifying the properties of food (Polizeli *et al*., 2005; Butt *et al*., 2008; Dhiman *et al*., 2008; Liu *et al*., 2007; Nortey *et al*., 2007;Uhlig *et al*., 1998). Xylanases are used to convert the polymeric xylan into fermentable sugars for the production of ethanol and xylitol from plant biomass (Galbe and Zacchi, 2002; Beg *et al*., 2001).

Onan industrial scale, xylanases are produced mainly by *Aspergillus* sp. and *Trichoderma* sp. (Polizeli *et al*., 2005; Pal and Khanum 2010). The xylanase can be produced in submerged fermentation as well as in solid-state fermentation either using commercially available substrates (xylan) or using agro waste materials (wheat bran, wheat straw, corn cob, sugarcane bagasse, etc.) as production substrates (Manimaran 2009; Nagar *et al*., 2011).

In the present work, a set of local isolates of microbes were graded as per their xylanase production ability in rice husk incorporated mineral salt agar (MSAM). *Trichoderma* sp. exhibiting better enzyme activity was studied both under submerged and solid state fermentation conditions for optimized production of the enzyme. Influence of supplementation of the basic culture medium for solid state fermentation (SSF) was studied to find the enhanced enzyme yields. Enzyme titters followed under the combined influence of more than one fortifying substances. The crude enzyme was characterized in terms pH, temperature and presence of metal ions. The specific enzyme activity after three phase partition purification was also dealt in the various experiments described hereunder.

Materials and Methods

Materials

All the chemicals used were of analytical grade and procured from Renkem, New Delhi, Himedia, Mumbai and Merck, Mumbai, India. Soil samples were collected from around a local oil mill in Sathyamangalam.

Isolation of microbes producing xlanase

Samples of infected vegetables, infected fruits, soil, and water collected and transported to the laboratory under sterile conditions. 1 g or 1mL of these samples were serially diluted up to 10^4 dilutions and plated in mineral salt agar medium (MSAM) (modified medium of Mandels and Sternburg, 1976) with the substrate rice husk (1%)as carbon source and salts such as (in g/L) NaCl, 0.3; $CaCl_2$, 0.3; $MgSO_4$, $7H_2O$ 0.005; NH_4Cl, 6.4; $FeSO_4.7H_2O$, 0.0016; $MnSO_4$, 0.0014; K_2HPO_4 (10%), 7.00 mL; KH_2PO_4 (10%), 3.0 mL: and agar 20g (pH 7).The plates were incubated at 30°C for 48 hours in order to isolate individual colonies and test the same for their ability to produce xylanase enzyme.

Culture maintenance

The selected isolates were grown and maintained in nutrient agar medium (NAM) or potato dextrose agar (PDA) slants under refrigeration conditions.

Based on cell and colony morphology characteristics, 13 microbial types were isolated using standard reference manuals (Ellis, 1976).

Screening of Xylanase producing microbial isolates through Submerged Fermentation (SmF)

Rice husk (1g) as substratewas incorporated to 10ml of sugar cane pressmud extract (source of minerals and salts; this was prepared by mixing 100g of pressmud with 400 mL of tape water followed by stirring for 30 min and filtration) and 40ml tap water containing 0.5% ammonium sulphate. This was used as the screening medium (SM) for the aforesaid selected cultures isolated from the MSAM. 0.5 mL each of spore suspension in tween water (0.5: 99.5) from PDA slants or two loops each of bacterial cells from NAM slants were used as the inoculum of isolates and were separately added to the 50 mL each of the sterile SM held in 250 mL EM flasks. The cultures were incubated at 30°C& under shaking (120 rpm) for 48-72 hrs. Samples were collected periodically and the extracts obtained (for fungal cultures) by filtration using Whatmann No.1 filter paper (or supernatants of centrifuged (4000 rpm for 10 min at room temperature) bacterial cultures) used for the enzyme assay. Only those cultures showing consistently good enzyme activity alone were taken up for further studies.

Xylanase production by the selected fungal isolates under submerged batch process

About 0.5ml each of spore suspension as inoculum from the PDA slant of the selected fungal cultures was transferred into 50 mL of Xylanase production medium (Mandels and Sternburg's basal medium (MSBM, for composition see above as given in ASAM) (modified), pH 6.0)held in 250 mL EM flask and supplemented with 1 % rice husk as carbon source. Inoculated flasks were incubated at 30 ± 2°C in an orbital shaker at 130rpm for 5 days. Periodic samples collected during the 5 days of fermentation, were filtered using Whatmann no.1 filter paper. 10 ml each of the samples were collected and centrifuged at 6000 rpm for 10 min and the supernatants of the samples were assayed for the enzyme activity.

Xylanase production by the selected fungal isolates under SSF

25 g of rice husk as solid substrate was mixed with 46.25 ml of diluted pressmud extract (pressmud extract: water = 1:4) to homogeneity. The sterilized solid substrate matrix (BSSFM) thus prepared was inoculated with 5 % (v/wt) of spore suspension (as mentioned above) of the select fungal isolates separately. This was carried in wide sized glass petri plates. The plates were incubated for 120 h (static, 30°C) with provision for deriving periodic samples.

Optimization of medium for SSF

SSF was carried out for *Trichoderma* sp. (best xylanase producing isolate obtained in this study) with the addition of different nutrients to pressmud extract-moistened rice husk medium (BSSFM). The effect of supplementation of additional carbon source to rice husk was examined using various carbon sources (incorporated separately) such as carboxy methylcellulose (CMC) & dextrose (1% each). The role of additional nitrogen sources other than the nitrogen containing

salts present in the typical solid substrate namely BSSFM was tested by incorporating separately casein acid hydrolysate (CAH); yeast extract (YE) and corn steep solids (CSS) (1% each).Table 1 depicts briefly the various supplementations done to BSSFM. Xylanase and residual cellulase activities were found from the samples collected on day 3 & day 5.

The synergistic effect of various carbon & nitrogen sources which showed high xylanase activity was also examined to determine the best combinations of carbon & nitrogen sources for the xylanase activity. CMC along with YE (1% each) and CMC along with CSS (1% each) were the combinations tested for supplementation to BSSFM (see G&H as variations of BSSFM as given in Table.1). Impact of replacement of pressmud extract (PE) with PE: corn steep liquor (CSL) (1:1) and PE: CSL (2:8) in the preparation of G & H variants of BSSFM (See table .2 for the variants used for the study) were also checked for xylanase activity.

Characterization of crude xylanase of *Trichoderma* sp. grown in fortified BSSFM

The activity of xylanase was estimated at varying temperature, pH, and in the presence of metal ions and detergents as described below

Optimum pH determination

In order to determine the optimum pH of enzyme activity, acetate buffers (pH 5.0 and 5.5), phosphate buffers (pH 6.0 and 6.5) and Tris–HCl buffers(pH 7.0, 7.5, 8.0, 8.5, 9.0 and 9.5) were used for the xylanase assay. 200µl each of 0.5% xylan in respective buffers was incubated with diluted enzyme samples (20 µl of enzyme samples) at 37°C for 40 min and subsequently assayed as mentioned below (see Analytical methods).

Optimum temperature determination

For determination of the optimum temperature, enzyme activity was assayed at different temperatures (in °C)such as 25, 30, 40, 50and 60 for 40 min. The desired temperatures were provided by using a water bath. pH was kept constant at 7.0.The reaction was arrested by adding 1 ml DNS reagent and assayed as mentioned below (see Analytical methods).

Impacts of metal ions& detergents

To determine the effects of metal ions & detergents on xylanase activity, 5 mM concentrations of the following metal ions (as salts)- sodium selenite pentahydrate, magnesium sulphate, cupric sulphate, zinc sulphate, calcium chloride, manganese sulphate, ferrous sulphate, SDS and Trition X 100 were added separately into the reaction mixture (buffer incorporated) and the assays were performed at 37°C for 40 min. The reaction was arrested by adding 1 ml each of DNS reagent and the colour developed read as mentioned earlier. The enzyme activities were measured, and control tubes made with no metal ion incorporation taken as 100% activity. All experiments were conducted in triplicates and their mean values represented.

Partial purification bythree phase partition (TPP)

Three-phase partitioning (TPP) method as reported earlier by Akardere *et al.*, (2010) was used for the partial purification of the

crude enzyme sample (filtered extract of solid matrix used for SSF). Ammonium sulphate was added to the leachate to reach 50% saturation. To the precipitated supernatant half the volume of t- butanol was added and the mixture was kept undisturbed for 1hr at room temperature and then subjected to centrifugation (4000rpm for 10min at 4°C) to facilitate the separation of phases. The upper t-butanol layer was removed carefully and the lower aqueous layer was collected. The lower aqueous layer obtained was dialysed against the sodium acetate buffer with pH 5 for 24 hours. Dialysis was carried out using cellulose tubing (molecular weight cut off 13,000 KDa). The enzyme activity was determined in the dialysed sample.

Analytical methods

Quantitative assay for Xylanase activity

Xylanase activity was determined by measuring the increase in concentration of reducing sugars formed by enzymatic hydrolysis of Birchwood Xylan. The assay mixture consisted of 20 µl of crude enzyme sourced from cell free supernatant containing 100 µg total protein and 200 µl of 0.5% Xylan in 100 mM Tris-HCl buffer (pH 7.0). The mixture was incubated at 37°C for 40 min and the reaction was arrested by addition of 1 ml DNS reagent.

The reducing sugar generated was quantified at A540, by addition of 7 ml of water and using D-xylose as a standard by spectro-colorimetric estimation (Marta *et al.*, 2000). One unit of enzyme activity was defined as 1 µM of xylose equivalent produced per minute under the assay conditions. Xylan without enzyme was the control (reagent blank) to eliminate the possibility of substrate having any reducing sugar residues.

Quantitative assay for cellulase activity

Cellulase (CMCase/endocellulase) activity was assayed by adding 0.5 ml of appropriately diluted enzyme to 0.5 ml of 1% (w/v) carboxymethyl cellulose (CMC) (Sigma) in 50 mM sodium acetate buffer (pH 6) and incubating at 50ºC for 30 min (Saha*et al*., 2002). The amount of reducing sugars released during the reaction was measured using the DNS method and D-glucose solution was used as the standard. One unit of cellulase activity was defined as the amount of enzyme that liberated 1 mol of glucose equivalents under the assay conditions.

Protein assay

Protein concentration was determined by Bradford's method using Coomassie Blue G- 250 dye as a reagent and bovine serum albumin (BSA) as a standard. Specific activity was expressed as enzyme units per milligram of protein.

Results and Discussion

Isolation and selection of microbes producing Xylanase

About 13 isolates exhibited the ability to grow on the mineral medium supplemented with rice husk as carbon source. They were isolated from samples of infected vegetables, soil and air using mineral salt agar plates (MSAM) with rice husk as the sole carbon source. Sridevi and Charya (2011) had reported about the screening of 30 fungal strains from various sources for the production of xylanase. *Aspergillus* sp. and *Penicillium* sp were mostly found to be producing higher amount of xylanase in submerged fermentation. 70 fungal strains from soils were collected from different parts of southern Kerala, India & most of the

strains produced xylanase in SSF & SmF but in SmF cellulose free xylanase production was reported as more (Nair *et al.,* 2008).Rice husk is a rich source of xylan (28%) and xylose (23%). Therefore it is an attractive substrate for production of xylanase enzyme (Wong *et al*., 1988).

Screening of isolates and selection of the potent Xylanase producers

Screening of these isolates carried out using specially designed typical xylanase production medium (SM) indicated two better isolate during the assay (2.51 & 2.65U/ml of Xylanase activity) (Table 03) and these isolates were found to be *Trichoderma & Neurospora sp.,* by colony morphological examination and through microscopic observation. As similar to our result Sridevi *et al*., (2011) reported earlier that *Trichoderma* sp. produced maximum xylanase & *Neurospora* sp. produced less xylanase titres among the 30 fungal isolates.

Xylanase Production

SmF and SSF cultivations were carried out for the two selected cultures of *Trichoderma sp.* and *Neurosporasp.* It was found that SmF using *Trichoderma sp.* showed better enzyme activity(2.62 U/ml) when compared to that of *Neurospora sp.* in liquid culturing. Samples collected on the fourth day showed the maximum activity in SmF (Table 4) for both these fungal cultures. In SSF when the rice husk moistened with PE was used, the increase in xylanase titer was very pronounced (22.5 U/gds on day 3 for *Trichoderma* & 15.9 U/gds on day 3 for *Neurospora*) (Table 05). Mohan *et.al* (2011) has checked the tolerance of *Trichoderma sp.* for the production of xylanase enzyme. Maximum growth of the organism was found at 48h under submerged condition in xylan containing enriched medium. Seyis *et.al* (2005) reported production of xylanase by *Trichoderma harzianum 1073D3* with maximum activity of 26.5 U/mg in SSF using melon peel as substrate. Pang *et al.,* (2006) studied the production of xylanase by a local isolate of *Trichoderma*sp. FETL c3-2 via solid state fermentation system using sugar cane bagasse: palm kernel cake as substrates. 75.0 U per mg of xylanase productivity was obtained after 4 days of fermentation at 30°C.

There are reports indicating SSF as the method for hyper-production of endo-xylanase (Pandey *et al.,* (2000). Agro industrial residues are considered as the best substrate for SSF process for xylanase production by microorganisms (Poorna & Prema 2006; Pandey *et al.,* (2000). Low cost agro residues have been excessively used for hyper production of xylanase using fungal SSF system.

Thermomyces lanuginosus (D2W3) has been reported as one of the best thermophilic fungi, which produced 48,000 U/g of xylanase using sorghum straw after 6 days of incubation (Sonia *et al*., 2005). *T. lanuginosus* SSBP was able to produce 19,320 U/g of xylanase using bagasse pulp after 5 days of incubation (Manimaran, 2009).

Paecilomyces thermophila J18 produced 18,580 U/g of extracellular xylanase using wheat straw under SSF after duration of 7 days (Yang, 2006). These findings indicated the need for further fine tuning our SSF process for xylanase production by *Trichoderma* sp. in order to achieve the optimized yields of xylanase and the findings given hereunder confirms the same.

Table.1 Supplementation of nutrients to the basic BSSFM for SSF

S. No.	Flask label	Variations of BSSFM
1	A	Basic BSSFM*
2	B	A + Dextrose (1%)
3	C	A + CMC (1%)
4	D	A + CAH (1%)
5	E	A + YE (1%)
6	F	A + CSS (1%)
7	G	C + YE (1%)
8	H	C + CSS (1%)

*25g rice husk and 46.25ml of diluted pressmud extract

Table.2 Modified variants of G & H compositions of BSSF used for SSF

S. No.	Flask label	Modifications of G & H variants of BSSFM
1	G1	G + PE: CSL (1:1)
2	G2	G + PE: CSL (2:8)
3	H1	H + PE: CSL (1:1)
4	H2	H + PE: CSL (2:8)

Table.3 Xylanase activity measured from the culture filtrates of various isolates obtained from MSAM plates incorporated with rice husk as the sole carbon source.

Microorganism	I-1	I- 2	I-3	I-4	I-5	I-6	I-7	I-8	I-9	I-10	I-11	I-12	I-13
Xylanase Activity (U/ml)	1.63	2.37	1.92	**2.51**	**2.65**	1.60	1.62	1.76	1.62	1.96	1.81	1.79	1.65

I= Isolates

Table. 4 Xylanase activity exhibited by *Trichoderma sp.* and *Neurospora sp.* in submerged fermentation

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Samples	T	T	T	T	T	N	N	N	N	N
Days	1	2	3	4	5	1	2	3	4	5
Activity (U/ml)	2.10	2.31	2.45	**2.62**	2.37	1.94	2.12	2.24	**2.44**	1.97

T- *Trichoderma* ***sp.*** **N-** *Neurospora* ***sp.***

Table.6 Xylanase activity exhibited by *Trichoderma* sp. in SSF when BSSFM was incorporated with different carbon and nitrogen sources

Samples	A	B	C	D	E	F	G	H
	Activity(U/gds)							
Day 3	22.57	24.77	107.22	38.35	43.85	41.20	565.40	569.90
Day 5	24.75	22.80	103.20	34.20	30.19	54.40	568.70	573.45

Medium Optimization

Supplementation by additional carbon and nitrogen sources influenced the xylanase production in SSF using the pressmud extract moistened rice bran mix (BSSFM) as substrate. As depicted in Table 6 & 7, maximum xylanase activity of 107.22 U/mL (72 h) was noticed for *Trichoderma* sp. in CMC incorporated medium. This was a remarkable jump to the tune of 4.75 times the value reported for the un-inoculated SSF medium (22.57 U/mL). YE and CSS when incorporated separately to the basic SSF medium used, they also produced enhanced xylanase titres of 43.85 U/ mL for day 3 (yeast extract) and 54.40 U/mL for day 5 (corn steep solids).The cellulase activities were found to be negligible for this strain in all the media combinations. Similar results were reported by Pang *et al.*,(2006)indicating a 180% increase in xylanase productivity after modification in the basic SSF system. Synergetic impacts of YE and CSS (separately) along with 1% each of CMC when incorporated to BSSFM (i.e. G & H variants of BSSFM) was well pronounced. Increased levels of enzyme activities (565.4 U/gds for G variant and 569.9 U/gds for the H variant (for 3rd day)) were reported in comparison with incorporation by CMC or YE or CSS alone to BSSFM. When PE in BSSFM mix was replaced with a mixture of PE and CSL (1:1) (G1), it resulted in still better production of xylanase in SSF (13.7 % increase). For G2 (PE: CSL as 2: 8) variant, the same was 16.9% increase. H1 & H2 variants also permitted a jump in enzyme titres to the tune of 22% and 26.7% respectively in comparison with the values obtained for the H variant of BSSFM.

An attractive feature of the strain of *Trichoderma* was that it did not yield higher levels of cellulases (Table 07). Similar results were shown for *Trichoderma* sp. earlier exhibiting moderatecellulase activity (Sridevi *et al*, 2011).

Characterization of crude xylanase of *Trichoderma* sp. grown in fortified BSSFM

Effect of Temperature & pH

Xylanase activity of *Trichoderma sp*., a moderate thermophile, was monitored at temperatures ranging from 25°C to 60°C.The enzyme activity was more pronounced at 50°C. At pH 5.0, the xylanase activity was maximum for the crude enzyme obtained from G variant of BSSFM. The enzyme was stable at the range of 40°C to 70°C.

Results by Michelin *et al*. (2008) &Beg*et al*.,(2000) corroborate our findings. Studies conducted using *A. Fumigates* & *A. Niveus* showed the optimum temperature for xylanase activity as 60°C to 70°C and pH optimum as 4.5 to 5.5. Xylanse produced from *Streptomyces* sp. by SmF was having optimum pH (6.0) & temperature (40°C) for xylanase activity (Grabskit and Jeffris, 1991).

However, most of the xylanases from fungi or bacteria showed optimal activities at neutral (in particular for bacterial xylanases) or slightly acidic (in particular for fungal xylanases) pH values and at mesophilic temperatures (approximately between 40°C and 55°C (Collins *et al*.,2005; Wang *et al*., 2012). Most of the fungal species favoured acidic pH (Subramaniyan and Prema, 2002). According to Ward*et al*., (1988) for every 10°C rise in temperature, reaction rates approximately double, assuming the

Table.7 Cellulase activity exhibited by *Trichoderma* sp. in SSF when BSSFM was incorporated with different carbon and nitrogen sources

Samples	A	B	C	D	E	F	G	H
Activity(U/gds)								
Day 3	0.39	0.07	0.05	0.04	0.04	0.06	0.04	0.05
Day 5	0.05	0.05	0.06	0.06	0.06	0.19	0.04	0.04

Table.8 Xylanase activity of *Trichoderma* sp. when PE was replaced with combinations of PE & CSL in the variants of BSSFM namely G & H in SSF

Samples (day 3)	PE: CSL (1:1)	PE: CSL (2:8)	PE: CSL (1:1)	PE: CSL (2:8)
Variation of BSSFM	G1	G2	H1	H2
Activity (U/gds)	643	661	695	**722**

Table.9 Purification of xylanase by TPP method

Steps	**Enzyme activity (U/ml)**	**Protein concentration (mg/100ml)**	**Specific activity (U/mg)**	**Partition coefficient**	**Activity recovery (%)**	**Purification fold**
SSF	661	78.79	839	-	-	1
TPP	374	6.25	5985	0.56	56.58	7.13

enzyme is stable at the higher temperature, the amount of enzyme needed can be reduced or the conversion time be shortened. Sa-Pereira *et al*., (2002) reported that the difference in pH and temperature tolerance for xylanase excreted maybe due to the effect of different enzymes mixtures excreted, and or the post-translational modifications in xylanase excretion process, such as glycosylation, that improve stability in more extreme pH and temperature conditions.

Effect of Metal Ions and Detergents

The effects of different metal ions and detergents on the activity of the crude enzyme were determined. None of the metal ions tested such as Cu, Mg, Mn, Zn, Ca,Fe,Se and detergents SDS and Triton X 100 gave an enhanced xylanase activity when compared with the xylanase activity from un-supplemented samples. Poorna & Prema (2006) reported that most of the metal ions tested had little influence on the activity and similar findings were reported earlier (Breccia *et al*., 1998; Liu *et al*., 1998;Saha, 2002;Chivero*et al*., 2001; Pradeep *et al*., 2013; Deesukon*et al*., 2011).Inactivation due to SDS has already been reported for xylanases of different origins (Fujimoto *et al*., 1995) and the activity reported was just 66.88%. Boucherba *et al*., (2011) reported similar results showing 87.57% relative activity.

Figure.1 Effect of Temperature on xylanase activity by *Trichoderma* sp.

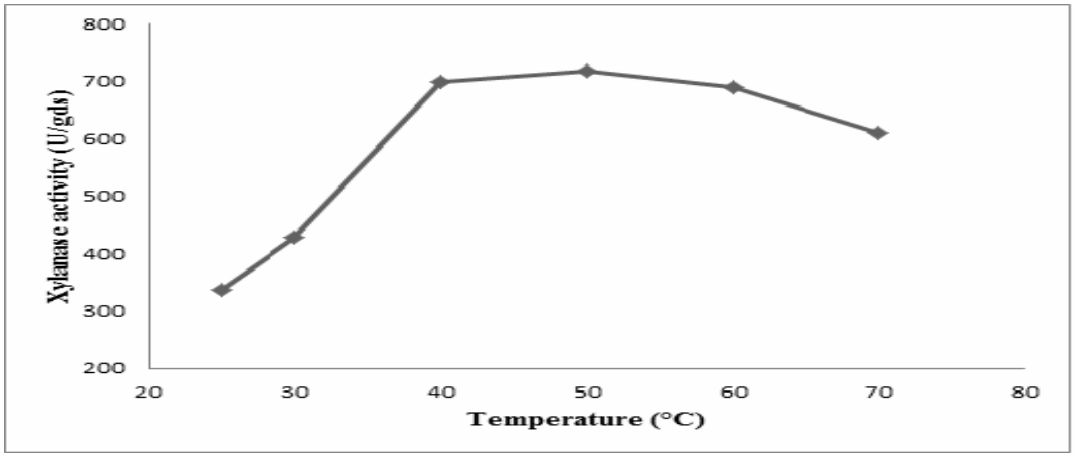


Figure.2 Effect of pH on xylanase activity by *Trichoderma* sp.

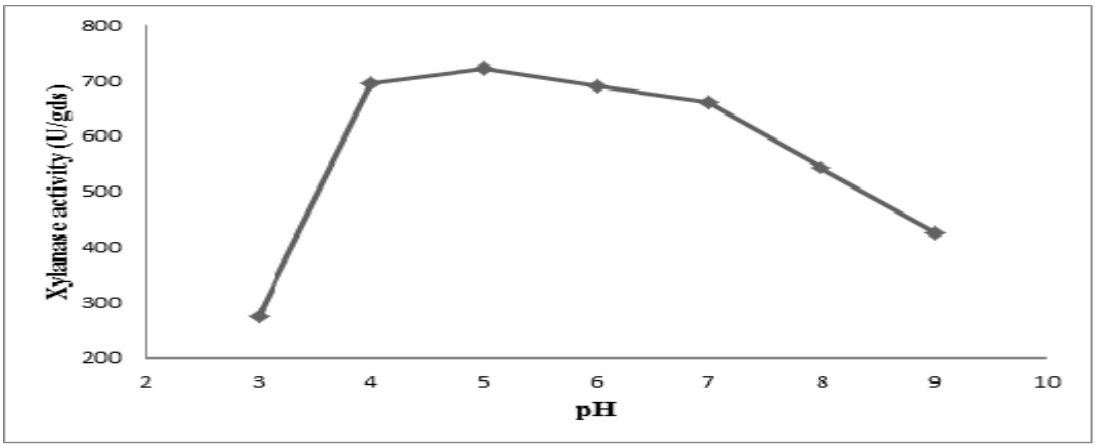
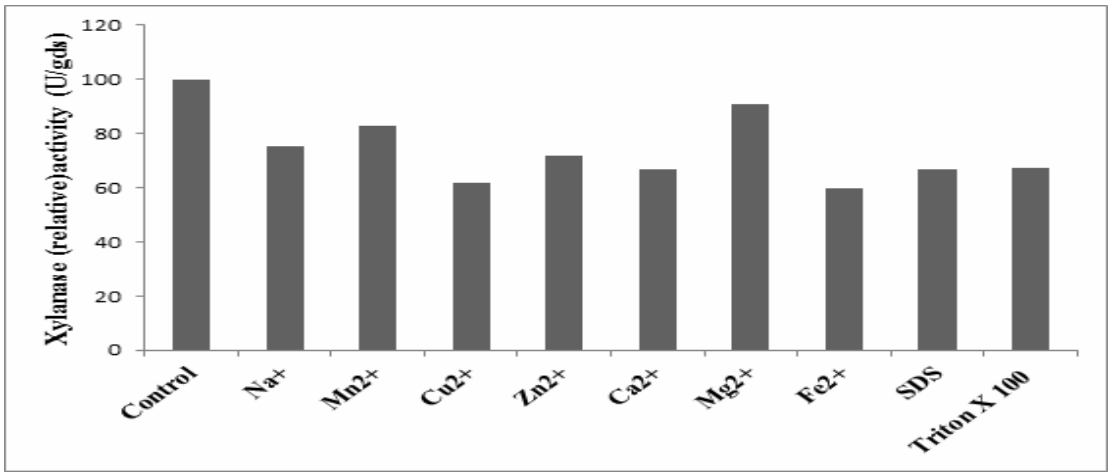


Figure.3 Effect of metal ions & detergents on xylanase activity by *Trichoderma* sp.



The xylanase activity was stimulated by Cu^{2+} up to 115.8%, (Lu *et al*., 2008). However, the results reported by us in this work were similar to other reports, where Cu^{2+} was inhibitory (Fialho and Carmona, 2004; Lin *et al*., 1999).Cu ions are known to catalyze the auto-oxidation of cysteines to form intra molecular disulfide bridges or the formation of sulphenic acid (Vieille *et al*., 2001). Pradeep *et al.*, (2013) reported enhanced activity by Ca^{2+}which is contradictory to our result. The result reveals what kinds of metals should be included or excluded for the industrial applications. In case of detergents xylanase activity was inhibited by Triton-X 100, this is contradictory to earlier reports (Pradeep *et al*., 2013; Mohana *et al*., 2008). The inhibitions of xylanase activity by SDS have been previously reported (Elegir *et al*., 1994 ; Wang *et al*., 2003).

Purification of Enzyme

The xylanase from *Trichoderma* sp. grown in BSSFM supplemented with 1% each of CMC & YE along with PE changed to PE:CSL (2:8) (i.e. G2variant of BSSFM) was purified using three phase partition (TPP). The specific activity of purified sample was 5985 U/mg and about 7 fold of purification for aqueous phase obtained (Table 8). Akardere *et al*., (2010) had found 15 fold of purification for aqueous phase from Baker's yeast by TPP method for invertase enzyme. Ozer *et al*., (2010) had found 8.6 fold of purification for interfacial phase from tomato by TPP method for invertase enzyme. A TPP process was reported as giving purification folds of 9 for the interphase of tomato pectinase (Sharma and Gupta 2001).

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References

Akardere, E., B.Ozer, E.B. Celem and S. Onal. 2010. Three-phase partitioning of invertase from Baker's yeast. Sep. Purif. Technol. 72:335–339

Beg, Q.K., B.M. Bhushan, G.S. Kapoor and Hoondal. 2000. Production and characterization of thermostable xylanase and pectinase from *Streptomyces* sp.QC-11-3. J. Ind. Microbiol.Biotechnol. 24:396–402.

Beg, Q.K., M. Kapoor, M. Mahajanand G.S. Hoondal. 2001. Microbial xylanases and their industrial applications. Appl. Microbiol.Biotechnol. 56:326–338

Boucherba, N., S. Benallaoua, E.B..Copinet, H. Hebal and D. Francis. 2011. Production and partial characterization of xylanase produced by *Jonesiadenitrificans* isolated in Algerian soil. Process Biochemistry. 46:519–525.

Breccia, J.D., F. Sineriz, M.D. Baigori, G.R. Castro and R. Hatti-Kaul. 1998. Purification and characterization of a thermostable xylanase from *Bacillus amyloliquefaciens*. Enzyme Microb Technol. 22:42–9.

Butt, M.S., M. Tahir-Nadeem, Z. Ahmad and M.T. Sultan. 2008. Xylanases in baking industry.FoodTechnol. Biotechnol. 46:22–31

Chivero, E.T.,A.N. Mutukumira and R.

Zvauya. 2001. Partial purification and characterization of a xylanase enzyme produced by a micro-organism isolated from selected indigenous fruits of Zimbabwe. Food Chem. 72:179–85.

Collins, T., C. Gerday and G. Feller. 2005. Xylanases, xylanase families and extremophilic xylanases. FEMS Microbiol. 29:3-23.

Deesukon, W., Y. Nishimura, N. Harada, T .Sakamoto and W. Sukhumsirichart. 2011. Purification, characterization and gene cloning of two forms of a thermostableendo-xylanase from *Streptomyces sp*. SWU10. Process Biochem 46:2255–62.

Dhiman, S.S., J. Sharma and B. Battan. 2008. Industrial applications and future prospects ofmicrobialxylanases: a review. Bioresources. 3:1377–402.

Elegir, G., G. Szakacs, T.W. Jeffries. 1994. Purification and characterization and substrate specificities of multiple xylanases from Streptomyces sp. strain B-12-2. Appl Environ Microbiol. 60:2609–15.

Ellis, M.B. 1976. More dematiaceoushypomycetes', Knew, Survey, England: CABI; illustrated edition.

Fialho, M.B and E.C.Carmona. 2004. Purification and characterization of xylanases from *Aspergillus giganteus*. Folia Microbiol. 49:13–18.

Fujimoto, H., T. Ooi, S.L.. Wang, T..Takizawa, H. Hidaka and S. Murao. 1995. Purification and properties of three xylanase from *Aspergillus aculeatus*. Biosci Biotech Biochem. 59:538–40.

Galbe, M and G. Zacchi. 2002. A review of the production of ethanol from softwood, Applied Microbiology and Biotechnology. 59(6):618-628.

Grabskit, A.C and T. W. Jeffries. 1991. Production, purification characterisation of B- (1-4) endoxylanase of Streptomyces roseiscleroticus. Appl. Environ. Microbiol. 57:987-992.

Lin, J., L.M. Ndlovu, S. Singh and B. Pillay. 1999. Purification and biochemical characteristics of b-D-xylanase from a thermophilicfunfus, *thermomyceslanuginosus* -SSBP. Biotechnol. Appl. Biochem. 30:73–79.

Liu, W.H., W. Zhu, Y. Lu, J. Kong and G. MaG. 1998. Production, partial purification and characterization of xylanase from *Trichosporoncutaneum* SL409. Process Biochem. 33:331–6.

Liu, Z., Y. Li, F. Cui, Y. Xu and H. Zhao. 2007. Application of statisticalexperimental design to optimize culture requirements of *Aspergillus* sp. Zh-26 producing xylanase for degradation of arabinoxylans in mashing. Journal of Food Science.72:320-329.

Lu, F., M. Lu, Z. Lu, B. Xiaomei, Z. Haizhen and Y.Wang. 2008. Purification and characterization of xylanase from *Aspergillus ficuum* AF-9.8 Bioresource Technology 99:5938–5941

Mandels, M and D. Sternberg . 1976 Recent advances in cellulose technology. J .Ferment .Technol. 54: 267-286.

Manimaran, A., 2009. Hyper production of cellulase-free xylanase by *Thermomyceslanuginosus* SSBP on bagasse pulp and its application in biobleaching. Appl. Microbiol. Biotechnol. 81: 887–893

Marta, C. T. D., C. A. P. Ana, P.P. Edilberto, N.P. Alexandre, and T.F. Telma. 2000. Characterization of alkaline xylanases from Bacillus pumilus. Brazilian Journal of Microbiology. 31: 90-94.

Michelin, P and T.W. Jeffries. 2008.

Biochemistry and genetics of microbial xylanases. Curr.Opin. Biotechnol.7:337–342.

Mohan G., P. Guhankumar and V. Balakrishnan. 2011. Isolation of Xylan degrading enzyme from *Trichoderma* sp. Research in Plant Biology. 1(3): 15-20.

Mohana, S., A. Shah, J. Divecha and D. Madamwar.2008. Xylanase production by *Burkholderia sp.* DMAX strain under solid state fermentation using distillery spent wash. Bioresour.Technol .99:7553–64.

Nagar, S., A. Mittal, D. Kumar, L. Kumar, R. C. Kuhad and V. K. Gupta. 2011.Hyper production of alkali stable xylanase in lesser duration by *Bacillus pumilus* SV-85S using wheat bran under solid state fermentationuction. New Biotechnology. 28(6): 581-587

Nair, S.G., R. Sindhuand S. Shashidhar. 2008. Fungal xylanase production under solid state and submerged fermentation conditions. African Journal of Microbiology Research. 2:082-086.

Nortey, T.N., J.F.Patience and R.T. Zijlstra. 2007.Xylanase supplementation improves energy digestibility of wheat by-products in grower pigs Livestock science.109:96 - 99.

Ozer, B., E. Akardere, E.B. Celem and S. Onal. 2010. Three-phase partitioning as a rapid and efficient method for purification of invertase from tomato. Biochem.Eng.J. 50:110–115

Pal, A and F. Khanum. 2010. Production and extraction optimization of xylanase from*Aspergillusniger* DFR-5 through solid-state-fermentation. Biores Technol. 101:7563–9.

Pandey, A., C.R. Soccol, J.A. Rodriguez-Leon and P. Nigam.2000.Solid State Fermentation in Biotechnology.1st ed., Asiatech Publishers Inc. New Delhi.

Pang, P. K., I. Darah, L. Poppe, G.Szakacs and C.O. Ibrahim.2006. Xylanase Production by a Local Isolate, *Trichoderma* spp. FETL c3-2 via Solid State Fermentation Using Agricultural Wastes as Substrates. Malaysian Journal of Microbiology.2(1):7-14.

Polizeli, M.L.T.M., A.C.S. Rizzatti, R. Monti, H.F. Terenzi, J.A.Jorgeand D.S. Amori. 2005. Xylanasesfrom fungi: properties and industrial applications. Appl. Microbiol. Biotechnol. 67:577–91.

Poorna, C.A. and P. Prema. 2006. Production and partial characterization of endoxylanase by *Bacillus pumilus* using agro industrial residues. Biochem. Eng. J. 32: 106–112.

Pradeep, G.C., Y.H. Choia, Y. S. Choia, C.N. Seongb, S.S .Choc, H. J. Leed, and J. C. Yooab. 2013. A novel thermostablecellulase free xylanase stable in broad range of pH from Streptomyces sp. CS428. Process Biochemistry. 48:1188–1196

Saha, B.C. 2002. Production, purification and properties of xylanase from a newly isolated *Fusariumproliferatum*. Process Biochem 37:1279–84.

Sa-Pereira, A. P., J.C. Mesquita, M.R.A. Duarte, M. Barros and Costa-Ferreira. 2002. Rapid production of thermostablecellulase-free xylanase by a strain of *Bacillus subtilis* and its properties, Enzyme Microbiol. Technol. 30:924-933

Seyis, I and N. Aksoz. 2005. Xylanase production from *Trichodermaharzianum* 1073 D3 with alternative carbon and nitrogen sources. Food Technol. Biotechnol. 43 (1):37–40.

Sharma, A and M.N. Gupta. 2001. Purification of pectinases by three-phase partitioning. Biotechnol.Lett.

23:1625–1627

Sonia, KG., 2005. Sorghum straw for xylanase hyper-production by *Thermomyceslanuginosus* (D2W3) under solid state fermentation.Bioresour.Technol. 96:1561–1569.

Sridevi, B. and M.A. SingaraCharya. 2011. Isolation, identification and screening of potential cellulase-free xylanase producing fungi. African. J. Biotechnol. 10(22): 4624-4630.

Subramaniyan, S and P. Prema. 2002. Biotechnology of microbial xylanases: enzymology, molecular biology, and application. Crit. Rev. Biotechnol. 22:33– 64.

Uhlig, T and A. J. Jao. 1998. Purification and biochemical characterization of an endoxylanase from *Aspergillus versicolor*',*FEMS.* Microbiol. Lett.166:311–315.

Vieille, C and G.J. Zeikus. 2001. Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. Microbiol.Mol. Bio.65:1–43.

Wang, S.L., Y.H. Yen, I.L. Shih, A.C. Chang, W.T. Chang, W.C. Wu. 2003. Production of xylanases from rice bran by *Streptomyces actuosus* A-151. Enzyme Microb.Technol.33:917–25.

Wang, W., Z. Fu, H.Q.. Huang, H.S.. Zhang, B. Yao, H.R. Xiongand O. Turunen. 2012. Improved thermal performance of *Thermomyceslanuginosus* GH11 xylanase by engineering of an N-terminal disulfide bridge. Bioresour. Technol. 112: 275– 279

Ward, O.P and M. Moo-Young. 1988. Thermostable enzymes. Biotechnol Adv. 6:39–69.

Wong, K.K.Y., L.U. Tan and J.N. Saddler. 1988. Multiplicity of-1,4-xylanase in microrganisms: function and applications. Microbiol Rev. 52:305–17.

Yang, S.Q., 2006. High-level of xylanase production by the thermophilic*Paecilomycesthemophilis* J18 on wheat straw in solid state fermentation. Bioresour. Technol. 97:1794–1800.