Original Research Article

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

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ABSTRACT

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).

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. Ca2+ Cu2+ and Fe2+ 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.
Xylanase, a hydrolytic enzyme involved in depolymerization of xylan, finds application in many industrial processes such as enzymatic bleaching of paper pulp, juice and 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).

On an 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 titers 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 xylanase

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$_2$O, 0.005; NH$_4$Cl, 6.4; FeSO$_4$.7H$_2$O, 0.0016; MnSO$_4$, 0.0014; K$_2$HPO$_4$ (10%), 7.00 mL; KH$_2$PO$_4$ (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 substrate was 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 tap 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. 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. 200 μl 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, 50 and 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 Triton 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 by three 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 50°C 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. Srivedi 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 *Neurospora* sp. 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 BSSF
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>Basic BSSFM*</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td>A + Dextrose (1%)</td>
</tr>
<tr>
<td>3</td>
<td>C</td>
<td>A + CMC (1%)</td>
</tr>
<tr>
<td>4</td>
<td>D</td>
<td>A + CAH (1%)</td>
</tr>
<tr>
<td>5</td>
<td>E</td>
<td>A + YE (1%)</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>A + CSS (1%)</td>
</tr>
<tr>
<td>7</td>
<td>G</td>
<td>C + YE (1%)</td>
</tr>
<tr>
<td>8</td>
<td>H</td>
<td>C + CSS (1%)</td>
</tr>
</tbody>
</table>

*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 BSSF
<table>
<thead>
<tr>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>G1</td>
<td>G + PE: CSL (1:1)</td>
</tr>
<tr>
<td>2</td>
<td>G2</td>
<td>G + PE: CSL (2:8)</td>
</tr>
<tr>
<td>3</td>
<td>H1</td>
<td>H + PE: CSL (1:1)</td>
</tr>
<tr>
<td>4</td>
<td>H2</td>
<td>H + PE: CSL (2:8)</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>I-1</th>
<th>I-2</th>
<th>I-3</th>
<th>I-4</th>
<th>I-5</th>
<th>I-6</th>
<th>I-7</th>
<th>I-8</th>
<th>I-9</th>
<th>I-10</th>
<th>I-11</th>
<th>I-12</th>
<th>I-13</th>
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</thead>
<tbody>
<tr>
<td>Xylanase Activity (U/ml)</td>
<td>1.63</td>
<td>2.37</td>
<td>1.92</td>
<td>2.51</td>
<td>2.65</td>
<td>1.60</td>
<td>1.62</td>
<td>1.76</td>
<td>1.62</td>
<td>1.96</td>
<td>1.81</td>
<td>1.79</td>
<td>1.65</td>
</tr>
</tbody>
</table>

I= Isolates

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

<table>
<thead>
<tr>
<th>Samples</th>
<th>T</th>
<th>T</th>
<th>T</th>
<th>T</th>
<th>T</th>
<th>N</th>
<th>N</th>
<th>N</th>
<th>N</th>
<th>N</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Activity (U/ml)</td>
<td>2.10</td>
<td>2.31</td>
<td>2.45</td>
<td>2.62</td>
<td>2.37</td>
<td>1.94</td>
<td>2.12</td>
<td>2.24</td>
<td>2.44</td>
<td>1.97</td>
<td></td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Samples</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity(U/gds)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>22.57</td>
<td>24.77</td>
<td>107.22</td>
<td>38.35</td>
<td>43.85</td>
<td>41.20</td>
<td>565.40</td>
<td>569.90</td>
</tr>
<tr>
<td>Day 5</td>
<td>24.75</td>
<td>22.80</td>
<td>103.20</td>
<td>34.20</td>
<td>30.19</td>
<td>54.40</td>
<td>568.70</td>
<td>573.45</td>
</tr>
</tbody>
</table>
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 uninoculated 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 moderate cellulase 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) & Beget 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. Xylanase 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 Wardet 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

<table>
<thead>
<tr>
<th>Samples</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
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<tbody>
<tr>
<td>Activity (U/gds)</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Day 3</td>
<td>0.39</td>
<td>0.07</td>
<td>0.05</td>
<td>0.04</td>
<td>0.04</td>
<td>0.06</td>
<td>0.04</td>
<td>0.05</td>
</tr>
<tr>
<td>Day 5</td>
<td>0.05</td>
<td>0.05</td>
<td>0.06</td>
<td>0.06</td>
<td>0.19</td>
<td>0.04</td>
<td>0.04</td>
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</tr>
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</table>

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

<table>
<thead>
<tr>
<th>Samples (day 3)</th>
<th>PE: CSL (1:1)</th>
<th>PE: CSL (2:8)</th>
<th>PE: CSL (1:1)</th>
<th>PE: CSL (2:8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variation of BSSFM</td>
<td>G1</td>
<td>G2</td>
<td>H1</td>
<td>H2</td>
</tr>
<tr>
<td>Activity (U/gds)</td>
<td>643</td>
<td>661</td>
<td>695</td>
<td>722</td>
</tr>
</tbody>
</table>

Table 9 Purification of xylanase by TPP method

<table>
<thead>
<tr>
<th>Steps</th>
<th>Enzyme activity (U/ml)</th>
<th>Protein concentration (mg/100ml)</th>
<th>Specific activity (U/mg)</th>
<th>Partition coefficient</th>
<th>Activity recovery (%)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSF</td>
<td>661</td>
<td>78.79</td>
<td>839</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>TPP</td>
<td>374</td>
<td>6.25</td>
<td>5985</td>
<td>0.56</td>
<td>56.58</td>
<td>7.13</td>
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</tbody>
</table>

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; Deesukonet *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.

![Graph showing xylanase activity vs temperature](image1)

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

![Graph showing xylanase activity vs pH](image2)

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

![Bar chart showing relative xylanase activity](image3)
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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