Production of Cold-Active Xylanase by Novel *Trunctella angustata* (BPF5) Strain Under Solid State Fermentation with Wheat Bran, Rice Bran and Saw Dust as Substrates

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Abstract
Agro-industrial wastes are the most abundant renewable resources on earth, affordable and easily available in large quantities. Recently cold-xylanase which plays an important role in breakdown of xylan, has achieved a great attention due to its remarkable biotechnological applications. The aim of this study was to screen three filamentous fungi, *Penicillium canesence* (BPF4), *Truncatella angustata* (BPF5), and *Pseudogymnoascus roseus* (BPF6) available as laboratory stocks for their ability to produce extracellular xylanases at cold temperature. *T. angustata* was found predominant to produce the highest amount of xylanase followed by *Pseu. roseus and P. canesence* in that order and was selected for further studies. Under solid state fermentation, a comparative study of three substrates, wheat bran, rice bran and saw dust was investigated. Xylanase was maximally produced at 30ºC and pH 9. At 30°C and in presence of substrate wheat bran an enzyme activity 0.22/1/7.6=1.672 IU/ml was obtained which was far better that rice bran and saw dust. Effect of carbon source, nitrogen source, metal ions and surfactants were also observed and fungus showed remarkable production in presence of carbon source xylose followed by CMC both in presence of solid substrate wheat bran and saw dust. Most suitable nitrogen source was ammonium nitrate followed by yeast extract. All the optimized parameters (substrate- wheat bran, carbon source-xylose, nitrogen source-NH_4NO_3, temperature-30°C, pH-9) were added and comparative studies for xylanase production were made against basal SSF medium. With this integration of selected cultural and nutritional parameters an enzyme activity of 0.22/1/8.2= 19.4 IU/ml was obtained. All the metal ions and surfactants tested were found to inhibit enzyme yield by the fungus. The enzyme was also tested for some of the applications including biomass conversion and immobilization which showed wonderful results. Activity of xylanase production was confirmed by measuring the amount of reducing sugars liberated from the medium by the DNS method using crude extract. Xylanases production from *T. angustata* has thus been studied under various conditions in SSF and as a result an optimized production strategy has been achieved. Also, this is the first report of the fungus *T. angustata* having cold-active xylanases producing ability.

Keywords*:* Wheat bran, Rice bran, Saw dust, *Truncatella angustata*, Xylanase, Biomass hydrolysis, Enzyme Immobilization

Introduction

Hemicellulose is a linear and branched heterogeneous polymer characteristically comprising of five non-identical sugars, L-arabinose, D-galactose, D-glucose, D-mannose, and D-xylose (Fengel and Wegener, 1989). Plant cell wall comprises of three layers, which include primary cell wall, middle lamella and secondary cell wall. Xylan is present in secondary cell wall, at the interface between lignin and cellulose via covalent and non-covalent bonds to provide cell wall integrity and fibre cohesion (Motta *et al*., 2013; Corral and Ortega, 2006; Butt *et al*., 2008). Xylanases are responsible for the hydrolysis of xylan, hence they subsequently release xylo- oligosaccharides (Gupta *et al*., 2013). The hydrolysed products in the

form of soluble sugars have widespread range of biotechnological significance in a number of fermentation processes and practical value in food industry (Li *et al*., 2012). Illustrations of literature review shows that, a wide variety of microorganisms, including bacteria, yeast and filamentous fungi have been reported, which produce xylanase (Wong *et al*., 1988). From an industrial viewpoint, fungi are remarkable due to higher amount of extracellular production of xylanases, in addition to release of numerous auxiliary enzymes that are significant for debranching of the substituted xylans (Haltrich *et al*., 1996). Lignin signifies about 15% - 25% of lignocellulosic biomass and is a therefore a large cross-linked macromolecule composed of three monomers: *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Davin and Lewis, 2005). White rot fungi have received a great attention due to their commanding extracellular lignin degrading enzymatic systems (Chandra, *et al*., 2007). Other groups of fungi have also been testified and reported as effective producers of lignolytic enzymes (James, and Natalie, 2001).

In addition to this, psychrophilic or psychrotrophic microorganism are well-known to produce cold active enzymes (Huston *et al.,* 2000), which include, xylanase (Collins *et al.,* 2002), cellulases and xylanase (Akila and Chandra, 2003). α-amylase (Feller *et al.,* 1998; Aghajari *et al.,* 1996), lipase (Jeon *et al.,* 2009; Suzuki *et al.,* 2001). In recent times, an efficient investigation has been carried out in order to understand the rules governing their molecular adaptation to low temperatures. These enzymes comprise an essential group having high catalytic properties at relatively low temperatures. Psychrotrophic and psychrophilicmicrobes can play a vital role in the degradation of organic matter during cold seasons. From time to time, qualities of cold active enzymes and the microbes producing them, have been assessed (Georlette *et al.,* 2004; Margesin *et al.,* 2002; Feller and Gerdey, 2003). Low activation energies and high catalytic activities at low temperature are conferred by cold active enzymes which are constructive properties for the production of various compounds.

Xylanases have been a great attention of research owing to their industrially important applications in many fields. They are, for instance, advantageous in pulp and paper industries predominantly for the facilitative role they play in the bio-bleaching of craft pulp, by reducing the amount of chlorine required for target pulp brightness (Gerber *et al*., 1999). Xylanases are also very efficient in animal foodstuff industry for improving the quality and nutritive standard of animal food and juices. They are helpful in lignin extraction, thus releasing chromophores from pulp. Xylanases have also been found very effective in the baking industry for improving dough quality and volume of bread, making it easy to digest. Furthermore, lignocellulosic conversion of biomass (materials to fuels and chemicals), by xylanases is the most recent and advanced investigation.

For the production of any industrially important enzyme, an economical substrate and an effectual process of fermentation are needed for commercial viability. It has been recognized that solid-state fermentation (SSF) has several advantages over submerged fermentation (SmF), due to resulting in higher productivity per unit volume, smaller volume of solvent needed for product recovery, very low contamination and foaming problems and an efficient exploitation of numerous agro-residues as substrates (Nigam and Singh, 1994; Grajek, 1987; Archana and Satyanarayan, 1997; Kewalrami *et al*., 1988). Hence, an attempt of xylanase production in bulk, using xylan rich agro-residues including wheat bran, wheat straw, rice bran, rice straw, saw dust, sugarcane bagasse and corn cob has been done by several workers using fungi (Qinnghe *et al*., 2004; Bakir *et al*., 2001; Anthony *et al*., 2003; Singh *et al*., 2000), bacteria (Archana and Satyanarayan, 1997; Bataillon *et al*., 1998; Virupakshi *et al*., 2005), actinomycetes (Kohli *et al*., 2001; Beg *et al*., 2002; Nascimento *et al*., 2002) and yeasts (Liu *et al*., 1999).

The aim of this work was therefore to screen an efficient producer of xylanase enzyme using wheat bran, rice bran and saw dust as sole carbon sources, supplemented with mineral salt solution (MSS), under solid state fermentation (SSF). These studies were performed with a psychrotrophic xylanolytic fungal species producing cold-active xylanase enzyme (high catalytic activity at low temperature), as these cold-adapted xylanases are more significant for industrial application, and also as xylanase production by psychrotrophic fungi (*Trunctella angustata*, BPF5), under SSF using wheat bran, rice bran and saw dust has not been reported.

Materials and Methods

Microorganism and Maintenance

Psychrotrophic fungal isolates (*Penicillium canescens* BPF4, *Truncatella angustata* BPF5, *Pseudogymnoascus roseus* BPF6) were obtained from laboratory stocks. These isolates were maintained as glycerol stocks and routinely subcultured on PDA medium at 20°C.

Chemicals

All the Chemicals used during the research period were of analytical grade, obtained from different manufactures like Sigma-Aldrich USA, Hi-media Laboratories Pvt. Ltd. Mumbai, Fisher scientific Mumbai, SD fine chemicals Mumbai etc.

Media

The following media and buffers were used for the study.

PDA (Potato, Dextrose and Agar)

Potato (200g) was peeled and cut into pieces and boiled in 500 ml distilled water. The extract was filtered through muslin cloth and to which Dextrose-(15g) and Agar-(20g) was added and final volume was made up to 1000 ml. The pH was adjusted to 6.0 and autoclaved. The media was supplemented with an antibiotic Chloramphenicol at 200mg/100ml in cold molten form after autoclaving it.

MM (Minimal Media)
Minimal medium was Czapek-Dox medium prepared by adding $NaNO_3$ -3g, K_2HPO_4-1g, $MgSO_4$ -500mg, KCl-500mg, $FeSO_4$ $7H_2O$-10mg, Carbon source (Glucose or else)-10g, pH - 6.5 and Agar -20g in 1000ml of distilled water, then autoclaved. The medium was used for screening xylanase producing fungus after replacing glucose with xylan as sole carbon source.

Enzyme Production Medium
The enzyme production medium contained (g/L) K_2HPO_4 2.0; KH_2PO_4 2.0; NH_4NO_3 2.0; $MgSO_4$ 0.6; $CaCl_2$ 0.5; birch wood xylan 10.0, pH 8.5.

Screening for Xylanases Producing Fungus Primary Screening
The fungal isolates were inoculated onto xylan agar plates containing (g/L) K_2HPO_4 2.0; KH_2PO_4 2.0; NH_4NO_3 2.0; $MgSO_4$ 0.6; $CaCl_2$ 0.5; birch wood xylan 10.0, pH 8.5, where xylan was as the sole carbon source. The plates were inoculated at 20°C. The isolates showing positive growth and clear zones around the colonies on this plate were considered xylan utilizer ones. The clear zones observed after flooding the plates with Gram's Iodine will indicate the secretion of xylanase. The area where there is no hydrolysis (clear zone) will be stained blue black.

Secondary Screening
After six days of incubation, colonies that showed areas of clear zones with a minimum radius of 1 cm were selected for further screening in liquid medium where birch wood xylan was the main carbon source. Those producing enzyme, xylanases were then considered as xylanase producing fungi.

Solid-State Fermentation
Cultivation was performed in 500ml Erlenmeyer flask containing 100g of solid substrate (wheat straw/bran). Mineral solution (50 ml) containing (g/L) K_2HPO_4 2.0; KH_2PO_4 2.0; NH_4NO_3 2.0; $MgSO_4$ 0.6; $CaCl_2$ 0.5 at pH 8.5 was added to the solid substrate. The production medium and the mineral solution were autoclaved separately. The flask was cooled down at room temperature and then was inoculated with pieces of 1 mm^2 size of freshly grown colonies (five days old) and incubated at 20ºC for 5 days on a static platform.

Enzyme Extraction
A hundred ml of cold sterile distilled water (4°C) was added to SSF medium (100g substrate) after cultivation. The mixture was shaken for 30 minutes on shaker at 200 rpm. The suspension was decanted in beaker and was then spun down at 10000rpm at 4°C using refrigerated centrifuge (REMI, BL 20). The cell free supernatant was used as the extracellular crude enzyme preparation.

Protein Determination
Protein contents of the enzyme extracts was determined by following the method of Lowry *et al.,* (1951) with Bovine Serum Albumin as standard.

Determination of Xylanase
Xylanase (Endo-xylanase, l,4-β-D-xylan xylanohydrolase EC 3.2.1.8.) activity was determined by following the method of Rickard and Laughlin (1980), using the standard curve of known concentration for xylose. A suitably diluted solution of the enzyme (1ml) was mixed with 1.0 ml of 0.05M citrate buffer (pH 4.8) and (1ml) of xylan. Enzyme blanks were prepared by adding all the reagents except sugars. The reagent blank was prepared in the same manner but here instead of sugars, enzyme was excluded. The test sample was placed in a water bath for 60 min. The reaction was terminated by adding 3:0 ml of dinitrosalicylic acid reagent, which was then kept in boiling water bath for 5 minutes. The concentration of reducing sugars released was estimated against xylose standard by noting the absorbance at 540nm. One unit of enzyme activity was defined as the amount of enzyme that releases 1µmol of xylose per ml per min under the above assay conditions. Specific enzyme activity was expressed as units/mg of protein.

Effect of Various Physico-Chemical Conditions and Nutritional Parameters
The effect of various physico-chemical (temperature, pH, surfactants and metal ions) and nutritional parameters like substrates/carbon sources and nitrogen sources was investigated and optimization of these factors under SMF and SSF conditions was carried out in order to achieve maximal production of xylanases.

Solid State Fermentation (SSF)
Cultivation was performed in 500ml Erlenmeyer flask containing 10g of solid substrate (Wheat straw/bran, Rice bran, Saw dust) with the addition of mineral solution (5ml) containing (% w/v): with (g/L) K_2HPO_4 2.0; KH_2PO_4 2.0; NH_4NO_3 2.0; $MgSO_4$ 0.6; $CaCl_2$ 0.5 at pH 8.5. The production medium and the mineral solution were autoclaved separately, then inoculated with fungal spores, incubated at 20°C for 7 days in a rotary shaker (200 rpm).

Effect of Different Growth and Nutrition Parameters
Effect of different growth and nutrition parameters was investigated and enzyme activity was read as per the above-mentioned procedure of enzyme extraction and enzyme assay for all the parameters.

Effect of Temperature
The selected fungus was grown in xylanase production medium (SSF) at varying temperatures ranging from 4-50°C (4, 20, 30, 40 and 50°C). The fermentation was carried out for a period of 7 days. Samples were used for analysis as described earlier.

Effect of pH
In order to study the effect of initial pH (4, 7, 8, 9 and 11) of the culture medium on the formation of extracellular endo-xylanases, the mineral medium was prepared in respective buffer (of a particular pH) and 5ml of such medium was mixed per 10g of solid substrate. The medium was inoculated as above and incubated for 7 days.

Effect of Carbon Source
Xylanase production medium as mentioned above was used to carry out optimization of carbon source. Different carbon sources (Xylose, Glucose, Fructose, Sucrose and Carboxymethyl cellulose) were added to the production medium replacing xylan in such a way that the medium contained only one carbon source. The aim was to identify the carbon source effecting the highest xylanase production. All the experiments were conducted for a period of 7 days.

Effect of Nitrogen Source
The production of xylanase was analysed in different nitrogen sources (Sodium nitrate, Yeast extract, Potassium nitrate, Ammonium nitrate and Urea). The nitrogen source giving highest enzyme yield in comparison with the control was treated as the potent one and used for further studies. All the experiments were conducted for a period of 7 days.

Effect of Metal ions
Studies on the influence of different metal ions using $ZnCl_2$, $CaCl_2$, $PbCl_2$, $MnSO_4$ and $FeSO_4$ (10mM) was carried out by adding respective metal salts in the production medium. All other parameters were kept constant.

Effect of Surfactants
Studies on the influence of selected surfactants such as Tween-80 and Triton-x 100 (0.2%) were carried out at 0.2% concentration adding respective surfactant in the production medium. All other parameters were kept constant.

Optimization of Nutritional Parameters
The optimization studies were conducted to evaluate the higher production environment using three different strategies i.e. studies on the optimization of carbon sources, nitrogen sources and mineral ions for the enhanced production of xylanases from the selected psychrotrophic fungus.

Application of Xylanase Biomass source
Three types of plant biomasses were taken for hydrolysis. They were wheat bran, saw dust and mahaneem (*Ailanthus excelsa*) wood. Wheat bran was collected from a local shop. It was milled with particle size about 1mm, dried in pre dried container in convection oven maintaining the temperature at 45 ± 3°C for 48 h and stored at -20°C in a polyethylene bag. Small branches of mahaneem were plucked from the tree present in the college campus and broken into pieces of 1cm long. A large branch was also used, after getting it sheer-milled to small chips of size about 2x20x25mm. Saw dust was obtained from local timber shop. All these forms of woods were dried at room temperature (30-35°C) for a week before subjecting them to hydrolysis.

Pre-treatment of biomass
10 gm of above prepared biomass was taken and 10 ml of 0.7% (v/v with water) sulphuric acid was added in separate 250ml Erlenmeyer flasks and then impregnated for 4 h (Sahay and Rana, 2017). The surplus acid was decanted after impregnation. Flasks were autoclaved at 121°C at 15psi for 20 minutes. Cooked wood taken and 100 ml distilled water was added, mixed. The hydrolysate (Hemicellulosic hydrolysate) was filtered by whatman's No. 1 paper and discarded. The fibrous of substrate was again dried and taken for hydrolytic experiments.

Saccharification of substrates by the crude enzyme
Saccharification of substrates by the crude enzyme extract was determined by the method of (Tanguchi *et al.*, 2005; Kalogeris *et al.*, 2003). To 1g dilute acid pre-treated substrates *viz.* wheat bran, saw dust and *A. excelsa* wood pieces were added 5ml of 0.1M citrate buffer (pH 4.8), 10 ml distilled water and 500µL of crude xylanase enzyme. The mixture was incubated at 20°C on a rotatory shaker at 150rpm. Samples were withdrawn after 72 h (3 days) and 120h (5 days) time interval. The samples were heated to 100°C, cooled and centrifuged for 10min at 800rpm. The supernatant was used for the reducing sugar analysis. The liberated reducing sugar was assayed by DNS method as described by (Ghose, 1987).
% Sacharification = Reducing sugar (mg/mL) × 0.9 × 100/Initial substrate (mg/mL)

Enzyme Immobilization

Immobilization of xylanase enzyme was carried out by addition of water to polyvinyl alcohol (PVA) to obtain 12.5 % w/v solution. The solution was then heated to a temperature of about 60°C to dissolve PVA. Sodium alginate solution (0.5% w/v in water) was prepared by smoothly stirring for 45 min and then added to the PVA solution. The PVA-alginate solution was then cooled to around 35°C and 10 ml concentrated xylanase solution was added to this solution and mixed well.

Beads containing xylanase were formed by cross-linking PVA with the boric acid. The mixture of beads was extruded in a solution of saturated boric-acid as drop wise which containing 2% (w/v) $CaCl_2$. The beads were stirred gently in this solution for 24h at 4°C to complete solidification and then washed with DW to separate any excess boric-acid.

Statistical Analysis

Enzyme activity was measured in triplicates and three times (N). Standard error was calculated in three-step method. Mean value (ΣX) was calculated first, followed by calculation of standard deviation.

Results

Primary Screening for Cold-Active Xylanase Production

In the present work screening and identification of xylanase producing fungi from laboratory stock was studied. A total of three psychrotrophic isolates *Penicillium canescens* (BPF4), *Truncatella angustata* (BPF5) and *Pseudogymnoascus roseus* (BPF6) were selected to ensure the best producer of xylanase on Czapek-Dox medium supplemented with 1% (w/v) of xylan as sole carbon source and kept at 20°C for three days. All the three fungal species showed positive growth, though rate of growth was different. *T. angustata* showed best growth performance (**Fig.4.1**).

Second Round of Screening for Cold-Active Xylanase Production

After a time period of seven days it was found that *T. angustata* (BPF5) demonstrated more vigorous growth on xylan-medium. It formed broader zone due to hydrolysis of xylan in comparison with those formed by *P. roseus* and *P. canescens* and thus was selected as the potential producer of xylanase (**Fig.4.2**). *T. angustata* shows potential xylanase activity and resulting zone of clearance in xylan plate assay as it produced large amount of xylanase. The size of the zone was taken as the measure of the amount of xylanase production.

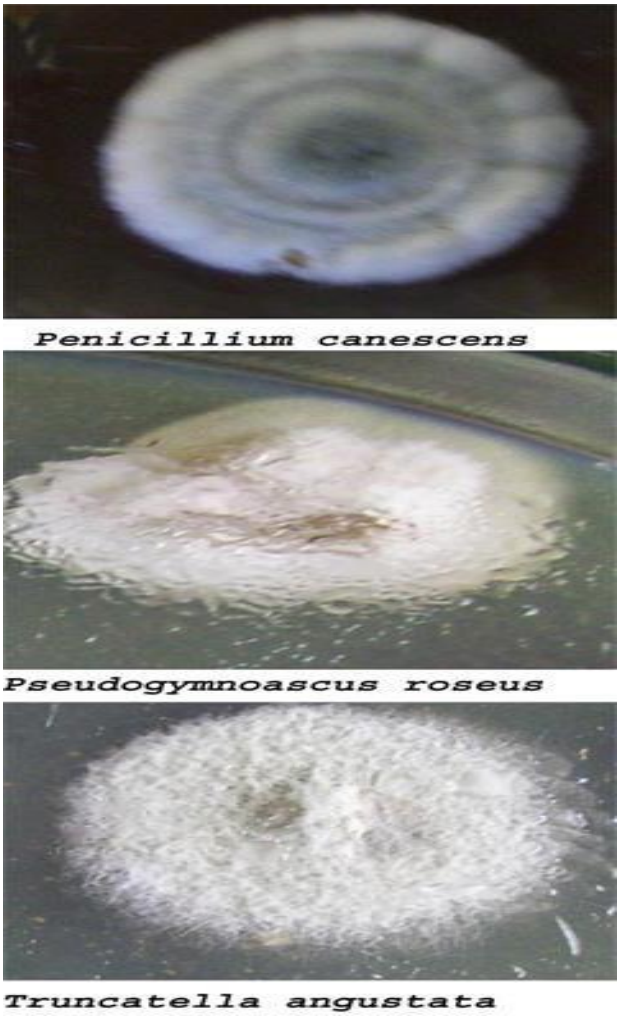

Fig. 1. Fungal colonies on PDA medium.

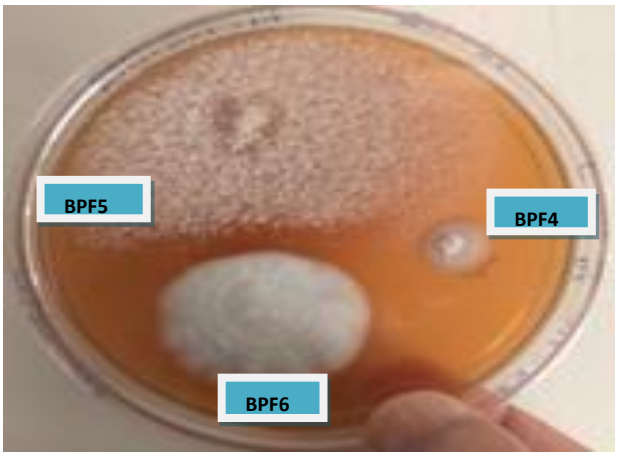

Fig. 2. Growth of selected psychrotrophic fungi on screening (xylan –medium)

Production Optimization Under SSF Effect of Temperature

The SSF media with different substrates were prepared as given in materials and methods (**Fig. 3**). The best substrate among the three tested was the wheat bran and best production temperature was 30°C for the production of the xylanase by the selected fungus. Other substrates such as saw dust and rice bran legged far behind. At 30°C and in presence of substrate wheat bran an enzyme activity 0.22/1/7.6=1.672 IU/ml was obtained (**Fig. 4**).

Effect of pH

Initial alkaline condition of the medium was found more suitable for the production of xylanase as in case of wheat and rice bran as substrate were used. But this was not true to saw dust substrate which gave variable results **(Fig. 5).**

Effect of Carbon Source

The experiment with different carbon sources in presence of various substrates was found to yield surprising results. Wheat bran in combination with xylose showed best performance, rice bran performed best in presence of CMC. Saw dust was found to yield maximum enzyme in presence of carbon source xylose and CMC (**Fig. 6**).

Effect of Nitrogen Source

The experiment with different nitrogen sources in presence of various substrates was found to yield reliable results. NH_4NO_3 was found to be the best nitrogen source followed by yeast extract in presence of wheat bran and saw dust as the substrates. Rice bran did not give any response to change in nitrogen source (**Fig. 7**).

Effect of Metal Ions and Surfactants

Although MnSO4 was found to be the unchallenged source of metal that induced xylanase production, in SSF no metal ion was found to induce enzyme production. Iron was the most inhibitory causing up to 80% inhibition, while Manganese caused at least 50% inhibition of enzyme production. Surfactants used were also found to inhibit the xylanase production by the fungus as in case of SSF (**Fig. 8**).

Enzyme Yield Under Optimized SSF

The optimized conditions (substrate- wheat bran, carbon source-xylose, nitrogen source- NH_4NO_3, temperature-30°C, pH-9) was used to find out yield of xylanases. Under optimized SSF conditions an enzyme activity of 0.22/1/8.2= 19.4 IU/ml was obtained (**Fig. 10**). This activity was 19.4/0.132 = 146.9 times more than what obtained under basic SMF condition (0.132 IU/ml).

Fig. 3. Preparation of SSF media with different solid substrates

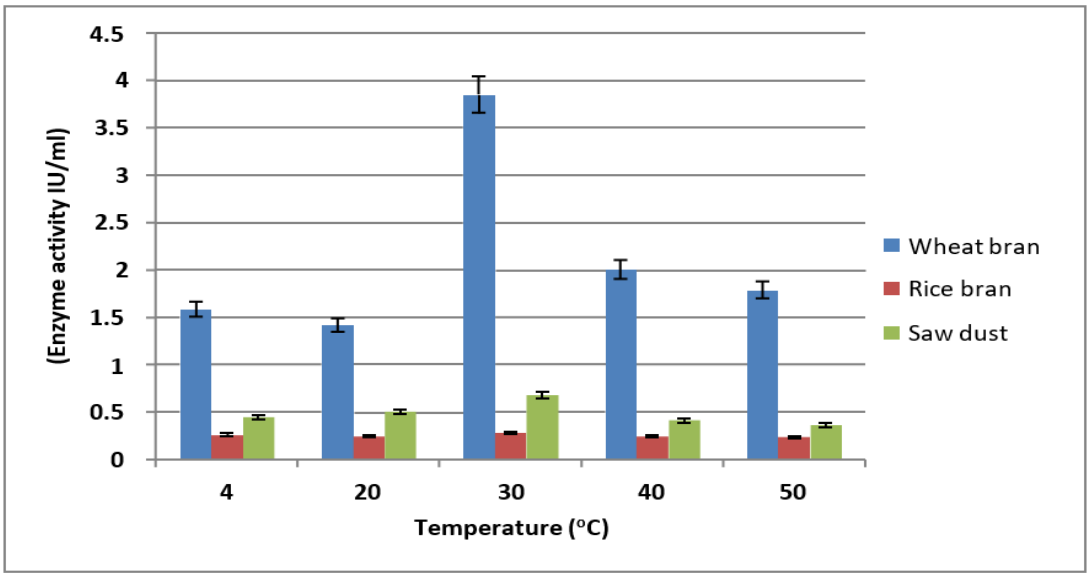

Fig. 4. Effect of substrate (wheat bran, saw dust and rice bran) and temperature on the production of xylanase by *T. angustata* under SSF

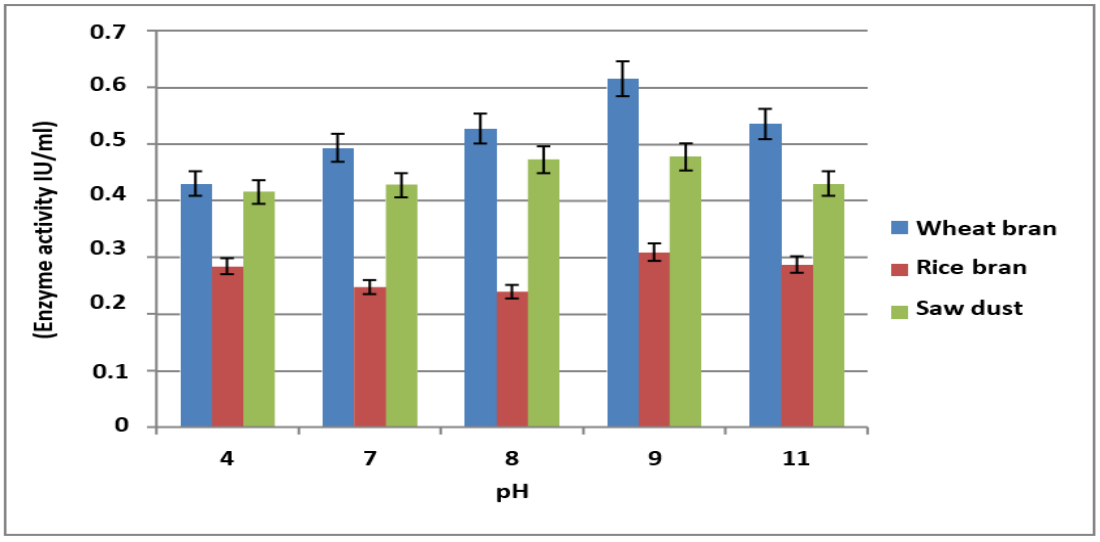

Fig. 5. Effect of pH on the production of xylanase by *T. angustata* under SSF

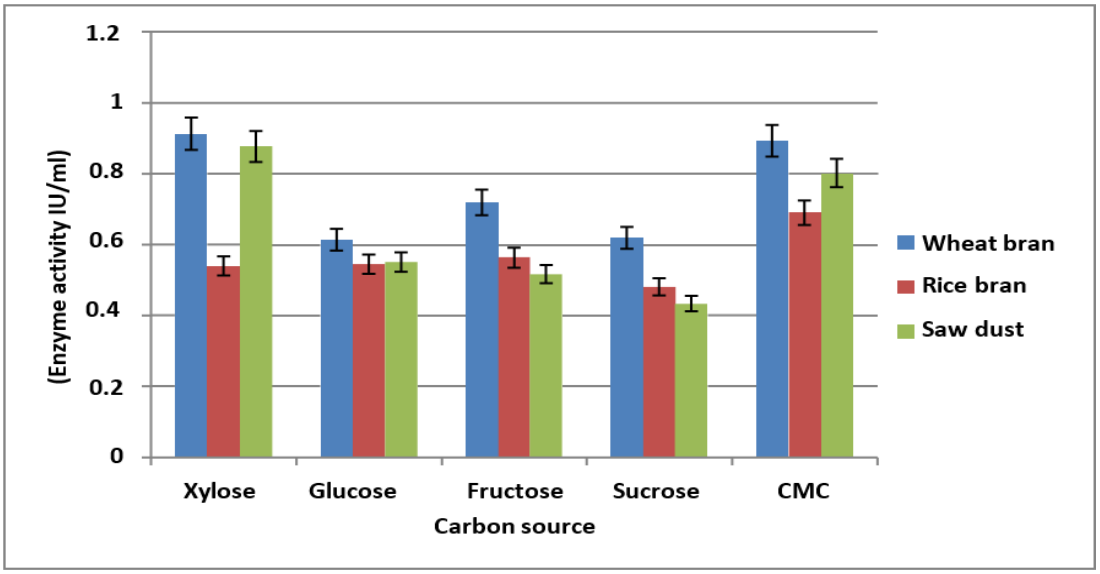

Fig. 6. Effect of carbon source (chemical form of carbon) on the production of xylanase by *T. angustata* under SSF and in presence of different substrates

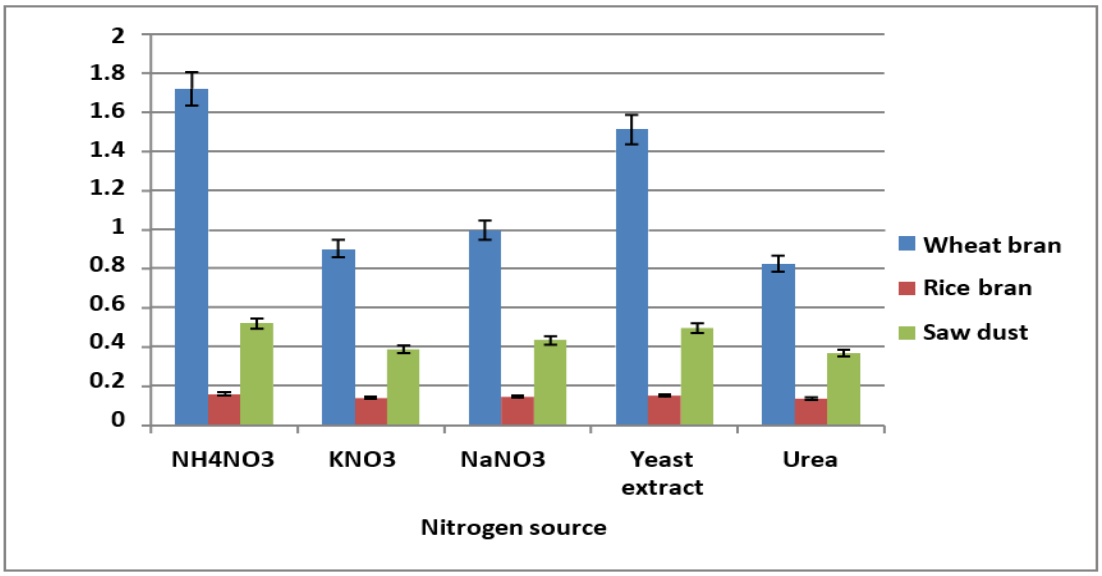

Fig. 7. Effect of nitrogen source on the production of xylanase by *T. angustata* under SSF

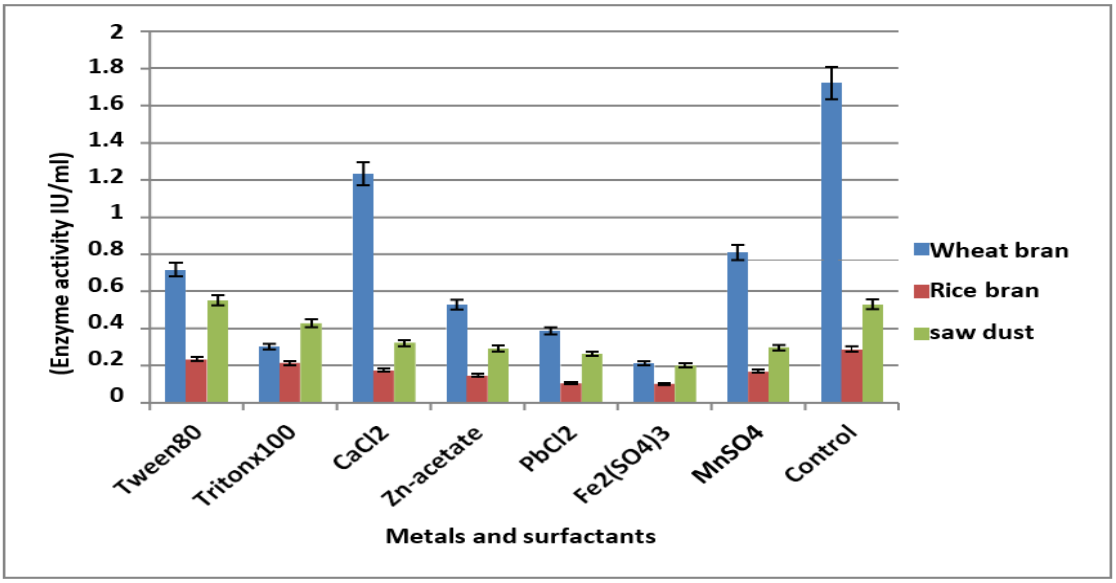

Fig. 8. Effect of selected metal ions and surfactants on the production of xylanase by *T. angustata* under SSF

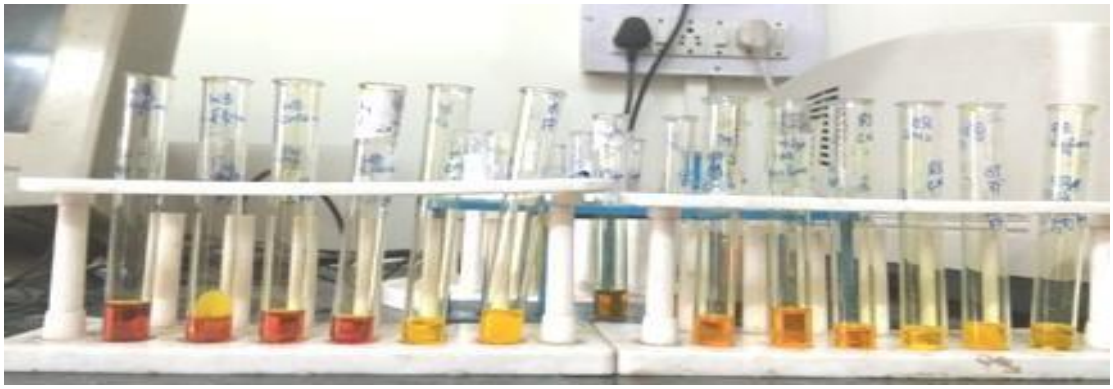

Fig. 9. Assay of xylanases applying DNS method

Application of Xylanases Plant Biomass Hydrolysis

Three types of plant biomasses (wheat bran, saw dust and wood of *Ailanthus excelsa*) individually and in mixed sample were taken to examine the activity of xylanase from *T. angustata* on the hydrolysis of their cellulosic materials. The enzyme could hydrolyze mostly wheat bran followed by saw dust and *A. excelsa* wood chips. The hydrolysis rate increased till fifth days, highest yield was found on the fifth day (**Fig. 11**).

Immobilization of Enzyme

The enzymes were tested for its immobilization by entrapping them in polyvinyl beads and subsequent assaying the activity of the enzyme in immobilized state. The activity of the enzyme tested for five days, once every day. Only, 20% of erosion in activity was found in five days (**Fig. 12**). The immobilized xylanases in polyvinyl beads are shown in (**Fig. 13)**.

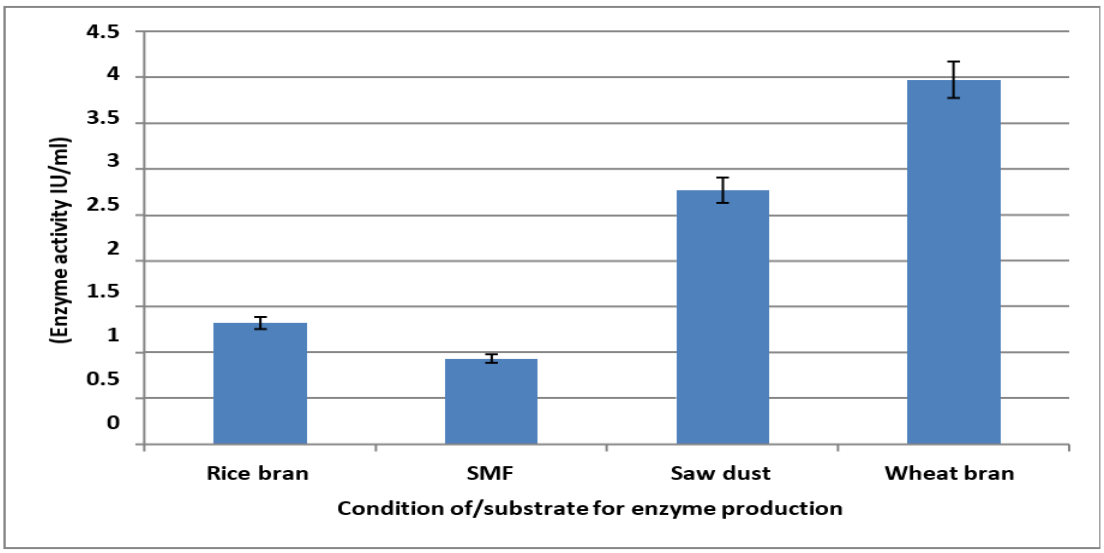

Fig. 10. Xylanase production level under optimized SMF and SSF (with various substrates) conditions

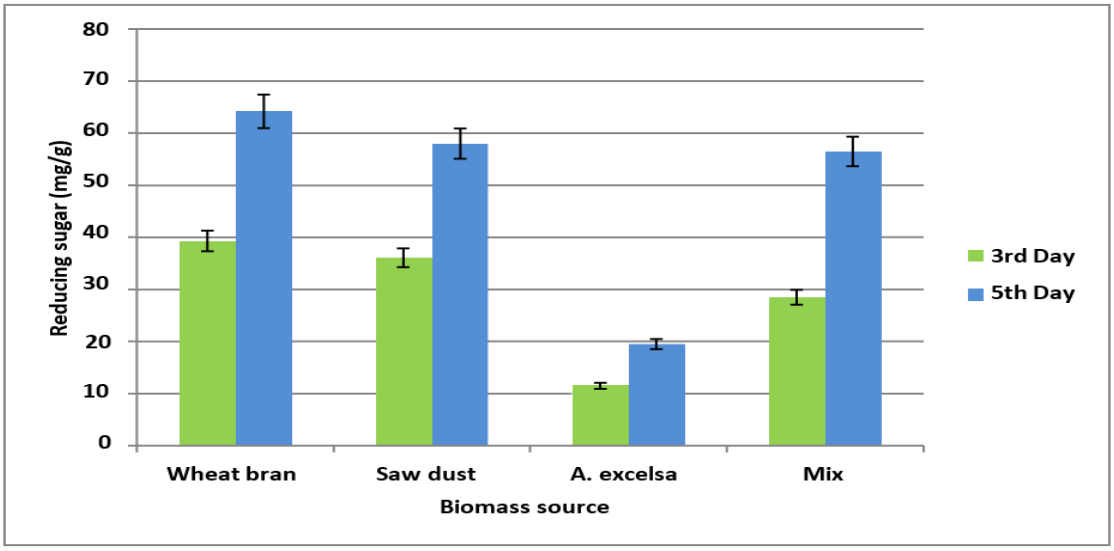

Fig. 11. Yield of reducing sugars by the treatment of selected plant biomass types with the enzyme after third and fifth days of incubation

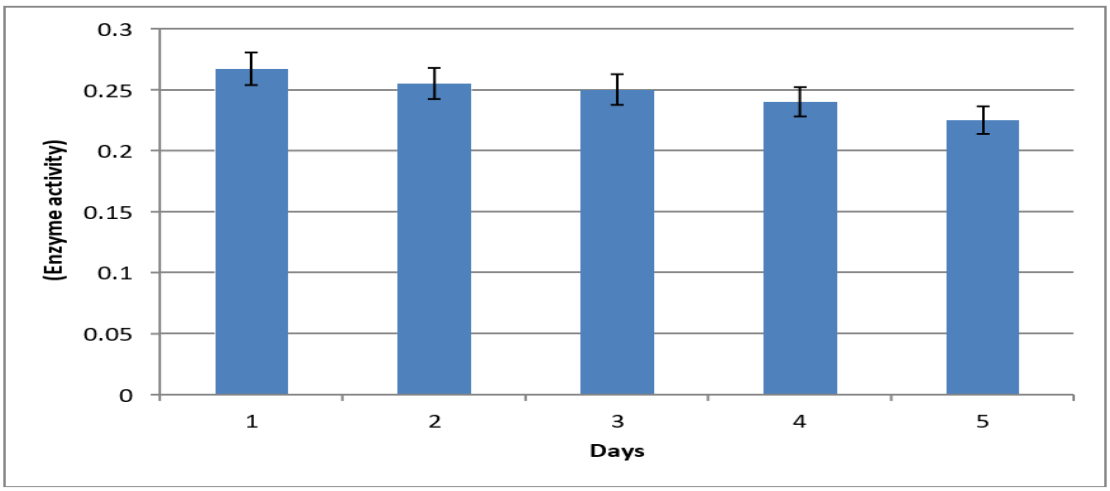

Fig. 12. The residual activity of immobilized xylanases in polyvinyl beads as a function of time (days)

Fig. 13. Polyvinyl beads enclosing xylanases

Discussion

A two-stage screening procedure was followed to identify the best xylanase producing one among available psychrotrophic fungi. The screening was based on the hypothesis that the best enzyme producer would form broader good clear zone on xylan supplemented agar medium (Aunstrup, 1974). Also, because xylan is a large molecule, too large to transport across plasma membrane, the extracellular endo-xylanase is essential to hydrolyse it into smaller molecule and therefore usually saprophytic microorganisms utilizing wood/xylan produce extracellular xylanases in nature (Biely, 1985). The fungus *T. angustata* BPF5 was found to show well- formed clear zone on the xylan agar medium. The selected fungus *T. angustata* showed clear zones of minimum 1cm diameter and thus was selected for further investigation.

The optimum temperature of *T. angustata* xylanases production was found to be 30°C. Earlier, optimal temperature for xylanase production has been reported to be 35°C from mesophilic bacterium (Lopez *et al.,* 1998) and 50°C from thermo-tolerant bacteria (Dubeau *et al.,* 1987). Kheng and Omar, (2005) have obtained an optimal temperature for xylanase activity at 28˚C for *A. niger*, while Chidi *et al.,* (2008), reported 35˚C for *A. terreus* UL4206 and Lu *et al.,* (2003) has reported optimum temperature for xylanase production by *A. sulphurous* cultivated from agro industrial wastes to be between 30˚C and 45˚C. The optimum temperature for growth of fungus may not necessarily be the optimum temperature for production of enzymes, although incubation temperature is a critical factor in enzyme production according to (Kheng and Omar, 2005).

Initial pH of the media is very important parameter that need to be standardized as it affects not only conformation of most of the enzymes but also their transport across the cell membrane (Moon and Parulekar, 1991). The enzyme was found to be produced at mild acidic and alkaline initial pH (Fig. 4.4). At other pHs also, 50% residual activities were observed.
Earlier, optimum pH range 7-9 has also been reported for the production of xylanases (Subramaniyan and Prema, 1998). Moreover, most of the xylanases have been reported to show optimum pH condition for production in the range of pH 5-8 (Krulwich and Guffanti, 1989).

A suitable growth condition including nutritional requirements is very important for all the microorganisms (Stanier *et al.,* 1987). Among various nutritional requirements, a carbon source is very important as it generally affects production of biomass and also primary or secondary metabolites. Often the most readily usable sugars cause rapid growth but lower down the productivity of metabolites (Inamine *et al.,* 1969). In solid state fermentation process xylose was the most favoured carbon source followed by CMC both in presence of solid substrate wheat bran and saw dust. This is to note that under SSF, solid substrate is already present as the main carbon source and the supplementary carbon sources tested seem to be used as inducer. Therefore, xylose was used as the favoured chemical for the production of xylanase. Earlier, negative effect of glucose on the xylanase synthesis has been reported (Nakanishi and Yasui, 1980; Saraswat and Bisaria, 1997; Siedenberg *et al.,* 1997). Glucose executes this negative effect through both catabolite repression at the transcriptional level (Saier and Fagan, 1992) and by catabolite inhibition (inducer exclusion) at the level of inducer transport across the cell membrane (McGinnes and Paigen, 1973). Xylanase stimulation by xylose has been reported in bacteria and fungi (Bataillon *et al.,* 1998; Leathers *et al.,* 1986) and is generally considered as an inducer of the enzyme. Also, Garapati *et al.,* (2009) supports the use of xylose as carbon source to improve xylanase production in filamentous fungi.

Addition of suitable nitrogen source is also very important for microbial growth and metabolism. Most suitable nitrogen

source was ammonium nitrate followed by yeast extract, sodium nitrate was pushed to third place. Screening for suitable nitrogen source as an essential step to enhance xylanase expressions from microbes including bacteria has been reported (Gomes *et al.,* 1993; Purkarthofer *et al.,* 1993; Christakopoulos *et al.,* 1996; Kuhad *et al.,* 1998). The explanation for ammonium nitrate being the best nitrogen source in SSF is the general observations that NH^{4+} is more efficient in influencing higher growth or cell material formation than NO^{3-} that can be formed per mol of ATP (Southamer, 1979). The second-best nitrogen source i.e., yeast extract has tendency to raise pH due to the release of excess nitrogen as ammonia that in turn stimulates growth rate and enzyme formation (Forage, 1985).

In case of SSF, all the metal ions and surfactants were found to inhibit enzyme yield. Metal ions have also been reported to be inhibitory to xylanases production by *Thermoascus aurantiacus* and *Bacillus* SSP-34 (Gomes *et al.,* 1994). Metal ions have been classified into Class A, Class B and border line ions (Nieboer and Richardson, 1980). Metal ions (Hg^{2+}, Pb^{2+}) belonging to class B however are toxic and not needed for biological function (Collins and Stotzky, 1996). Also, the cell surface of microbes is found negatively charged due to presence of various ionizable chemicals on their cell surfaces (Collins and Stotzky, 1992).

Of the three substrates tested in SSF for xylanase production, wheat bran was found to be best followed by saw dust and rice bran in that order, rice bran was found to be the poorest substrate (Fig. 4.15). Earlier, microbes utilizing wheat bran as preferred substrate for the production of xylanases has been reported such as *Bacillus thermoalkalophilus* and *Streptomyces chattanoogensis* UAH 23 (Fernandez *et al.,* 1995), *Streptomyces sp.* (Vyas *et al.,* 1990), *Trichoderma reesei* (Bailey *et al.,* 1993) and *Melanocarpus albomyces* IIS-68 (Saraswat and Bisaria, 1997). Unlike previous reports of *Bacillus thermoalkalophilus* (Rajaram and Varma, 1990). Wheat bran is a rich source of carbohydrate, however, it also contains certain nitrogenous components (Andersson *et al.,* 1994).

All the optimized parameters (substrate- wheat bran, carbon source-xylose, nitrogen source-NH_4NO_3, temperature-30°C, pH-9) were added and comparative studies for xylanase production were made against basal SSF medium. With this integration of selected cultural and nutritional parameters an enzyme activity of 0.22/1/8.2= 19.4 IU/ml was obtained. This was 146.9 times more than what obtained under basic SMF condition. This is phenomenal and justifies the optimization efforts. It was also found that wheat bran could be hydrolysed the most readily followed by saw dust and *A. excelsa* wood chips. The enzyme thus has potential to be applied for the production of 2G bioethanol. Since wheat with rice are the major food crops and thus can provide enormous biomass residues over the world (Bruun *et al.,* 2010; Talebnia *et al.,* 2010; Chou *et al.,* 2011). Saw dust also has been a byproduct of timber industry.

The enzyme was tested for its immobilization by entrapping in polyvinyl beads and subsequent assaying the activity of the enzyme in immobilized state. Immobilization enables reuse of enzymes many times thus much saving is possible on the purchase of costly enzymes, an essential consideration of full-scale process commercialization (Lynd *et al.,* 2005).Earlier, immobilization of the enzyme applying alginate–gelatin– calcium hybrid carriers has been successfully achieved that showed increased mechanical stability as well (Shen *et al.,* 2011). Supplementing the alginate with divalent ions (like Ca^{+2}) and glutaraldehyde as cross linker has also been reported to improve the stability of enzymes (Flores-Maltos *et al.,* 2011). Xylanases for example isolated from *Aspergillus niger* DFR-5, has been immobilized on the surface of alginate beads pre-activated with glutaraldehyde (Pal and Khanum, 2011). In another attempt, β-glucosidase isolated from *A. niger* has been immobilized in hen egg white (Karimpil *et al.,* 2011) and further study on the immobilized enzyme showed the retention of about 55% of the activity. Xylanolytic activity immobilized on Eudragit L-100 has also been reported (Roy *et al.,* 2003). The loss of enzyme activity during repeated use has been reported earlier (Bhushan *et al.,* 2015; Roy *et al.,* 2003) that may be due to enzyme inactivation (Nagar *et al.,* 2012). Immobilization of an enzyme often protects it against heat inactivation (Gouda and Abdel-Naby, 2002). Xylanase immobilized on various supports have been found toshow temperature stability (Kapoor and Kuhad, 2007; Gawande and Kamat, 1998). In view of the forgoing reports the performance of *T. angustata* xylanase within polyvinyl beads seems to be a better option.

Xylanase producing microorganisms have been reported from varied environments, such as antarctic environments (Bradner *et al.,* 1999a), marines (Annamalai *et al.,* 2009), thermal springs (Bouacem, 2014) and soda lakes (Huang *et al.,* 2015). Very few cold-adapted xylanase-producing microorganisms have been identified including three bacteria, *Clostridium* strain PXYL1 (Akila and Chandra, 2003), *Pseudoalteromonas haloplanktis* TAH3a (Collns *et al.,* 2002, 2003; Van Petegem *et al.,* 2002, 2003) and *Flavobacterium frigidarium sp.* nov. (Humphry *et al.,* 2001), some fungi, *Penicillium sp. Alternaria alternate* and *Phoma sp.* (Bradner *et al.,* 1999b), a yeast isolate, *Cryptococcus adeliae* (Petrescu *et al.,* 2000), krill *Euphasia superb* (Turkiewiz *et al.,* 2000) and a number of basidiomycetes, e.g., *Coprinus psychromorbidus* (Inglis *et al.,* 2000).

But a psychrotrophic fungus *T. angustata* from Bramullah (J & K, India) soil as producer of xylanases has been reported for the first time. Xylanases are one of the most important enzymes applied industrially, in particular biotechnological industries in the area of feed, paper and pulp industries (Polizeli *et al.,* 2005), food (Harris and Ramalingam, 2010). Xylanases application for biofuel production in order to increase the sugar recovery from agricultural residues has also been reported (Gonçalves, 2015). Xylanases production from *T. angustata* has thus been studied under various conditions in SSF and as a result an optimized production strategy has been achieved.

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