SCREENING AND PRODUCTION OF COLD-ACTIVE XYLANASE FROM *TRUNCATELLA ANGUSTATA* (BPF5) UNDER SUBMERGED FERMENTATION

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Abstract

In this study, three cold-active fungi, *Penicillium canesence* (BPF4), *Truncatella angustata* (BPF5), and *Pseudogymnoascus roseus* (BPF6) available as laboratory stocks have been screened for their ability to produce extracellular xylanases at cold temperature. The selection of hyper producing strains of xylanase was carried out on Potato Dextrose Agar (PDA) medium fortified with 1% (w/v) of xylan incubated at 20ºC for seven days. Selection for best producer of xylanase was done on the basis of breadth of clear zones observed after flooding the plates with Gram's iodine indicating the hydrolysis of xylan by xylanase around the colonies. The fungus *T. angustata* was found to produce the highest amount of xylanase followed by *P. roseus and P. canesence* in that order. The cold-active xylanase-secreting ability of the fungal species was verified by incubating them in xylanase producing medium at 20ºC. Consequently, *T. angustata*, *P. roseus* and *P. canesence* were found to produce xylanase activity equal to 11.0 IU/ml, 7.0 IU/ml and 5.9 IU/ml respectively. Under submerged fermentation xylanase was maximally produced at 20ºC and pH 4.5 and 9. Effect of carbon source, nitrogen source, metal ions and surfactants was also observed and fungus showed remarkable production of 0.166 IU/ml in presence of fructose. The nitrogenous compound $NaNO_3$ was found the best for xylanase production. Among the metal salts tested, $MnSO_4$ was the unchallenged source of metal that induced xylanase production, other metals seemed rather to inhibit xylanase production by the fungus. Both the surfactants tested were also inhibitory to the xylanase production. Activity of xylanase production was confirmed by measuring the amount of reducing sugars liberated from the medium by the DNS method using crude extract. This is the first report of the fungus *T. angustata* having cold-active xylanases producing ability.

Keywords: Cold active xylanase, Xylan, *Penicillium canesence*, *Truncatella angustata*, *Pseudogymnoascus roseus*, Screening, Production, Submerged Fermentation

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INTRODUCTION

Xylanases (O-glycoside hydrolases, EC 3.2.1.8) catalyze the hydrolysis of xylan, the major hemicellulose component in plant cell walls. Depending on its origin, the structure of xylan can differ to a great extent [1]. Xylan is a branched heteropolysaccharide constituting a backbone of 1,4 linked xylopyranosyl units substituted with arabinosyl, glucuronyl and acetyl residues [2]. The hydrolysis of the xylan backbone is basically carried out by endoxylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37) along with a diversity of debranching enzymes including, α-L-arabino furanosidases, α-D-glucuronidases and acetyl esterases [3].

Xylanases are a group of microbial enzymes that have attained great interest in recent times due to their biotechnological potential in a number of industrial processes, for example, in the production of xylitol and ethanol [4], in food [5], cellulose and paper industries [6], in the manufacturing of oligosaccharides [7], liquid fuels, cellular proteins and other chemical substances [8], and in poultry, pork and caprine feeding [9]. Cold-active enzymes on the other hand are also gaining importance as they have potential to save energy since they are produced and applied at low temperature. These enzymes have reduced cost of production as there is least care for controlling contamination at lower temperature [10].

Potentially, filamentous fungi are best known for the production of xylanases from industrial point of view as they are efficient secretor, capable of producing high levels of extra cellular enzymes and cultivated easily [11]. Therefore, screening of naturally occurring fungal species is an excellent way to obtain novel and better source of enzymes in question for commercial applications [12]. The present study was undertaken to find novel fungal species as source of cold-active xylanases.

MATERIALS AND METHODS

Chemicals

All the chemicals used were of analytical grade. Potato Dextrose Agar was obtained from Hi-media, Mumbai, India. Birch wood xylan was purchased from Sigma chemicals Co., USA.

Microorganism

Psychrotrophic fungal isolates, *Penicillium canesence*, BPF4 [13], *Truncatella angustata* BPF5 [14] *Pseudogymnoascus roseus*, BPF6 [13] were obtained from laboratory stocks. The fungal strains were maintained on Potato Dextrose Agar (PDA) plates at 4°C. The culture was observed daily and fungal growth was sub cultured onto fresh plates of PDA until pure isolates were obtained.

First round of screening for xylanases producing fungal isolates

First round of screening of fungal isolates was done on the basis of their abilities to grow and utilize xylan as sole carbon source. This was carried out by using Czapek-Dox medium containing (g/l): sucrose – 30, $NaNO_3$ – 2.0, K_2HPO_4 – 1.0, $MgSO_4$ – 0.05, KCl – 0.5, $FeSO_4$ – 0.01, xylan – 1.0, Agar Agar – 20 with pH adjusted to 5. After autoclaving at 121°C and 15 lbs pressure, the medium was poured into petri-plates and allowed to solidify. Cavities of 6 mm size were made in the solidified medium and inoculated with 0.1 mm size of fungal colony from 7 day old culture. The plate was incubated at 20 ± 2°C for three days to allow fungal growth [15].

Second round of screening

To confirm the xylanase producing ability, the fungi inoculated on the xylan-medium were allowed to continue growing till ten days at 20 ± 2°C. Afterward, the culture plate was flooded with Gram's iodine. The plate was then washed with sterile distilled water to see the clear zone around colony.

Confirmation test for cold-active xylanase production

Enzyme production was carried out in 50 mL of basal medium, containing Czapek-Dox medium with 1% xylan substrate as carbon source in Erlenmeyer flasks (250 mL). The medium was inoculated with pieces (1 mm^2) of 7 days old colony, followed by incubation at 20°C for 5 days in an orbital incubator shaker with constant shaking (120 rpm).

Extraction of enzymes

The broth was directly filtered through a four layered cheese cloth and the filtrate obtained was centrifuged at 5000 rpm for 10 min at 4°C. The clear supernatant was used as crude enzyme and stored at -20°C until used.

Submerged Fermentation

Enzyme production by the selected fungus was carried out in 250 mL Erlenmeyer flasks containing 50 mL of medium. The composition of isolating media was (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. Pieces of 1 mm^2 size

were cut out of the carpet like freshly grown colonies (five days old) and added to the sterilized medium and cultivated at 20ºC for 72h in a rotary shaker (200 rpm). The clear supernatant of the culture was assayed for enzyme activity, which was obtained by centrifugation (10000rpm for 10 min at 4ºC) followed by filtration (Whatman's no. 1 paper). The growth of the fungus was estimated on the basis of biomass dry weight (mg/mL).

Effect of Temperature

Optimum temperature needed for enzyme production was estimated by incubating the reaction mixture (25ml medium) for 7 days at different temperatures (5, 10, 15, 20, 25 and 30°C). After 7 days of incubation the clear supernatant of the culture was assayed for enzyme activity, which was obtained by centrifugation (10000rpm for 10 min at 4 ºC) followed by filtration (Whatman's no. 1 paper). Enzyme was assayed above and absorbance was read at 540nm.

Effect of pH

The effect of pH on the production of enzyme was studied by maintaining the enzyme production medium at different pHs (4, 5, 7, 8, 9 and 11) using citrate buffer (pH 3.0- 5.0), phosphate buffer (pH 6.0-7.0), Tris-HCL (pH 8.0-9.0) and carbonate /bicarbonate buffer (pH10, 11.0). The mixture was incubated at 20°C for 7 days in a rotary shaker (200 rpm). After 7 days of incubation the clear supernatant of the culture was assayed for enzyme activity, which was obtained by centrifugation (10000rpm for 10 min 4ºC) followed by filtration (Whatman's no. 1 paper). Absorbance was read at 540nm by above mentioned enzyme assay method.

Effect of Carbon and Nitrogen Source

The effect of different carbon sources (Xylan, Glucose, Fructose, Sucrose, Carboxymethyl cellulose and Mannitol) and nitrogen sources (Sodium nitrate, Yeast extract, Potassium nitrate, Peptone, Ammonium nitrate and Urea) on the production of enzyme was studied. Xylan was replaced by different carbon sources and ammonium nitrate was replaced by different nitrogen sources in the medium such that the medium had one carbon/nitrogen source different from the standard medium. The inoculated medium was incubated at 20°C for 7 days. After 7 days of incubation the clear supernatant of the culture was assayed for enzyme activity, which was obtained by centrifugation (1000rpm for 10 min 4ºC) followed by filtration (Whatman's no. 1 paper). Absorbance was read at 540nm by above mentioned enzyme assay method.

Effect of Metal ions and Surfactants

The effect of different metal ions such as $ZnCl_2$, CaCl2, $PbCl_2$, $MnSO_4$ and $FeSO_4$ (10mM) and surfactants such as Tween-80 and Triton-x 100 (0.2%) on xylanase enzyme production was determined. After adding heavy metals or surfactants to 25 mL of medium, it was incubated for 7 days and enzyme activity was carried out following the above mentioned enzyme assay.

Enzyme assays and protein determination

Xylanase activity was determined as described by [16]. The assay mixture, in a total volume of 2 mL, contains 0.5 mL of 1 mM of xylan in 50 mM citrate buffer (pH 4.8) and 0.5 mL of diluted crude enzyme. The mixture was incubated at 20°C for 30 min. After completion of incubation period, DNS mixture was added, boiled for 5 min and transferred immediately to a cold water bath. Then 20 mL of distilled water was added to the tubes, mixed and the developed colour was measured at 540 nm to estimate the amount of reducing sugars released [17]. The enzymatic activity of xylanase was defined in international units (IU). One unit of enzymatic activity was defined as the amount of enzyme that released 1µmol reducing sugars (xylose) per mL per min.

Protein assay

In addition, the protein assay by Lowry method (1951) was carried out in order to calculate the specific enzymatic activity.

Reproducibility of results

All results are the means of at least three (n=3) independent experiments.

RESULTS AND DISCUSSION

First round of screening for 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 canesence* (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 20ºC for three days. All the three fungal species showed positive growth, though rate of growth was different. *T. angustata* showed best growth performance. Most fungal species are known for

the secretion of xylanase but species belonging to the genera *Aspergillus* and *Trichoderma* have been reported to produce the enzyme on an industrial scale [18].

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 xylanase-medium. It formed broader zone due to hydrolysis of xylan in comparison with those formed by *P.. roseus* and *P. canesence* and thus was selected as the potential producer of xylanase **(Fig. 1).**

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. Earlier, xylanase producing fungi were screened from mangrove forest soil on solid medium and eight isolates *Aspergillus sp., Aureobasidium sp., Colletotrichum sp., Fusarium sp., Paecilomyces sp., Guignardia sp., Penicillium sp.,* and *Phomopsis sp.* were selected as positive for xylanase production [19]. Another prospective xylanase producing fungus *Aspergillus candidus* was also isolated from soil sample [20]. Screening of xylanase has been reported from many microbial strains including cold active microbes [21, 22, 23, 24, 25], but no report claiming screening and production of cold active xylanase from *T. angustata* has been reported.

Cold-active xylanase production under SMF

All the three fungi were grown under submerged condition in the xylanase production medium for five days of incubation. The enzyme activity was assayed at 20ºC after five days of incubation. The enzyme activity for *T. angustata*, *P. roseus* and *P. canesense* was found to be 11.0 IU/mL, 7.0 IU/mL and 5.9 IU/mL (µmol/min/mL) **(Fig. 2).** The activity is far better than reported earlier, for example the specific activity of xylanase from *Glaciecola mesophila* [22], *Flavobacterium sp.* [21], *Bacillus sp.*HJ2 [24], *Sorangium cellulosum* [26] and *Zunongwangia profunda* [25] has been reported to be 98.31, 142.00, 16.10, 8.91 and 2.98 µmol/min/mg respectively.

Psychrotrophic fungi are thought to have potential to improve the climatic conditions not only by bringing down the increasing temperatures but as well by producing these enzymes themselves at relatively lower temperatures [10].

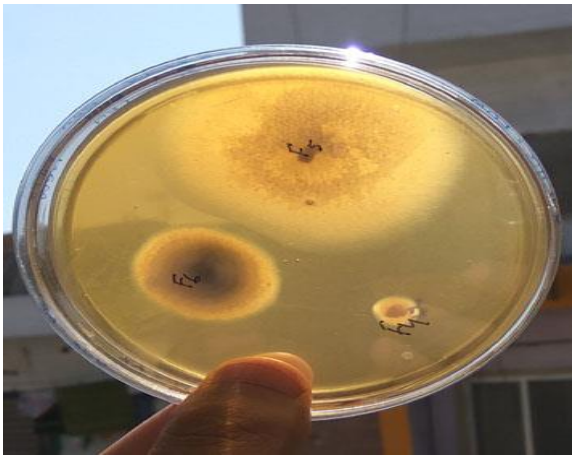

Fig.1. Xylanase screening

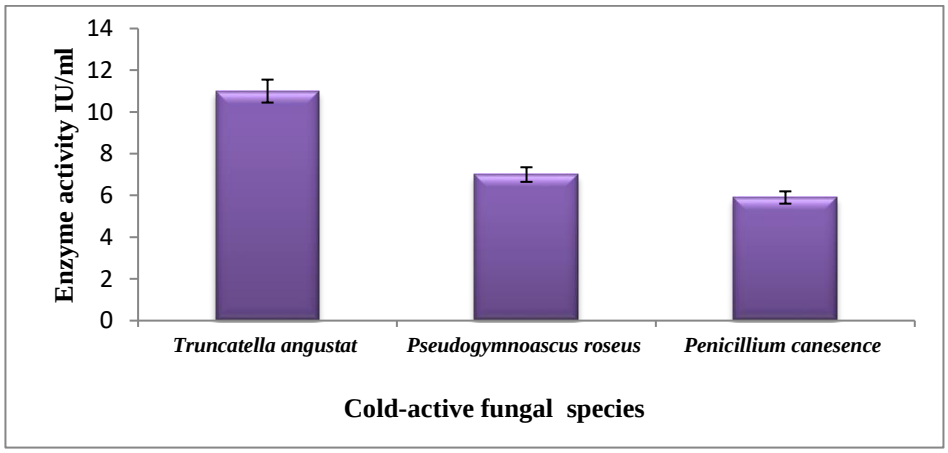

Fig. 2. Xylanase assay after 5 days of incubation at 20ºC under SMF

Production Optimization Under SMF

Effect of Temperature

The xylanase was produced maximally at 20°C, though a residual production of more than 50% was also observed at all the temperatures examined (**Fig. 3**). An enzyme activity of 0.132 U/ml was obtained at 20°C. The temperature is justified as this is also the optimum temperature

for the growth of the psychrotrophic fungus. Earlier, optimal temperature for xylanase production has been reported to be 35°C from mesophilic bacterium **(27)** and 50°C from thermo-tolerant bacteria **(28).**

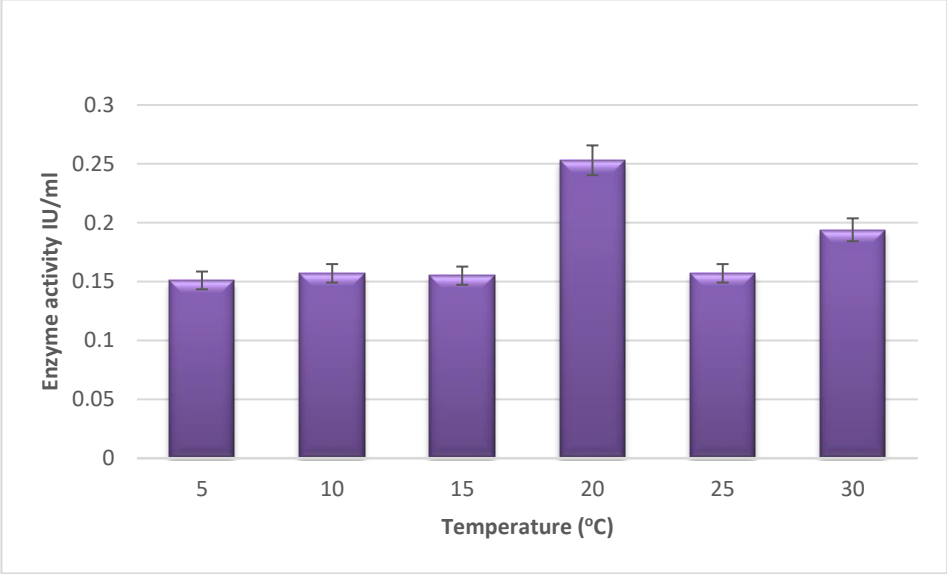

Fig. 3. Effect of temperature on the production of xylanase by *T. angustata*

Effect of pH

The xylanase was produced maximally at pH 4, 5 and 9, though a residual production of more than 50% was also observed at pH 7 and 8 (**Fig. 4**). It seems that acidic pH is preferred condition for the production of xylanase, though a peak of production was also observed at mild alkaline condition. 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 **(29).** Earlier, optimum pH range 7-9 has also been reported for the production of xylanases **(30).** Moreover, most of the xylanases have been reported to show optimum pH condition for production in the range of pH 5-8 **(31).**

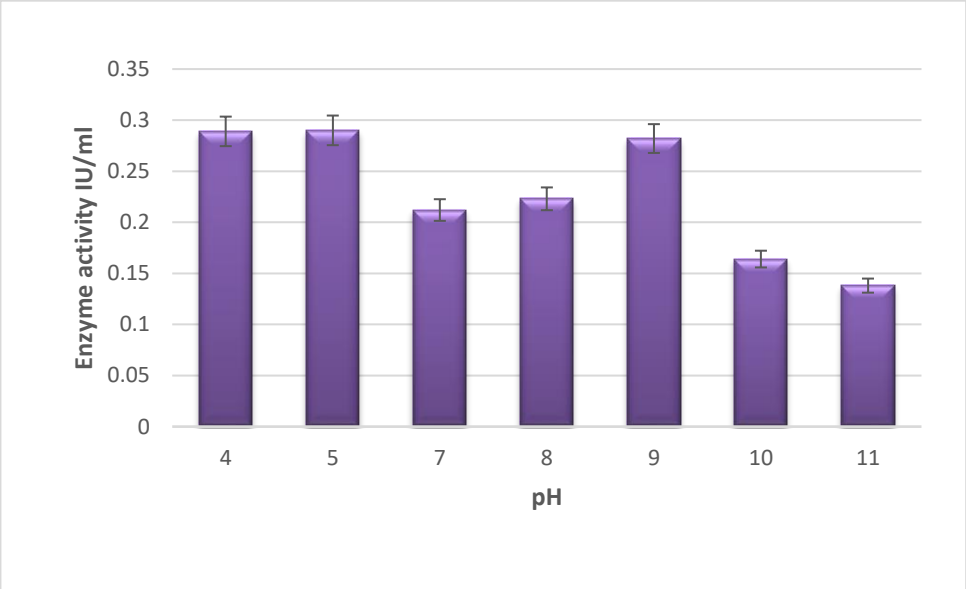

Fig. 4. Effect of pH on the production of xylanase by *T. angustata*

Effect of Carbon Source

Medium supplemented with various compounds as sole carbon source were prepared and incubated as given in materials and methods. Fructose was found the best form of carbon that gave maximum yield of the enzyme xylanase by the fungus. The carbon source fructose was also clear choice as other carbon sources legged far

behind (**Fig. 5**). In presence of fructose an enzyme activity of 0.22/1/3= 0.166 IU/ml was obtained indicating that carbon source required by the fungus was related both as energy source and metabolism of enzyme of interest. Often the most readily usable sugars cause rapid growth but lower down the productivity of metabolites **(32).** Xylanase stimulation by xylose has been reported in bacteria and fungi **(33; 34)** and is generally considered as an inducer of the enzyme.

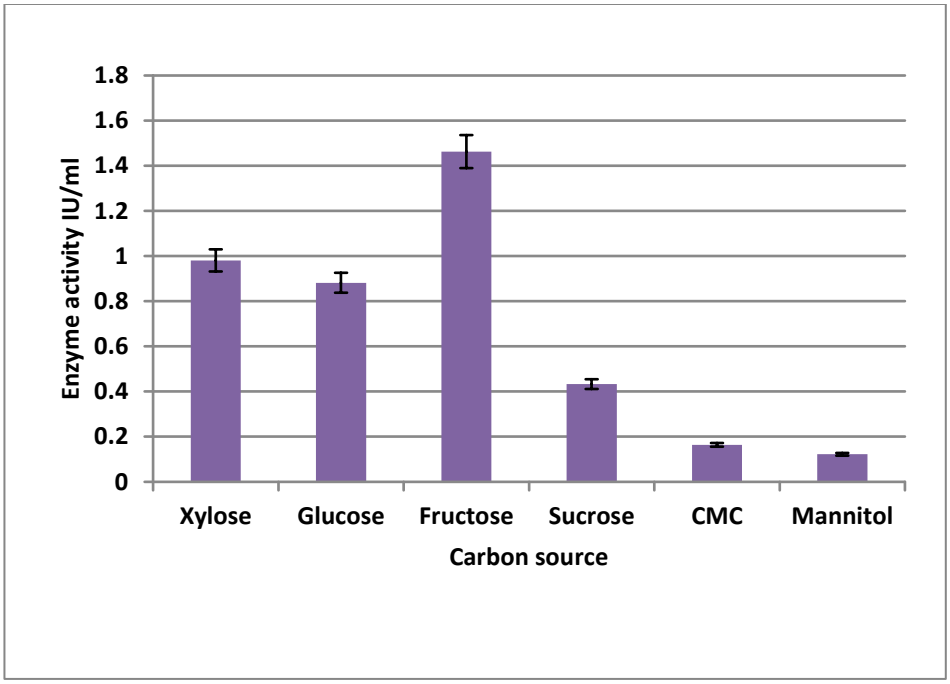

Fig. 5. Effect of carbon source (chemical form of carbon) on the production of xylanase by *T. angustata*

Effect of Nitrogen Source

The nitrogenous compound $NaNO_3$ was found the best as nitrogen source for xylanase production, NH_4NO_3 legged only slightly behind it (Fig. 6). Screening for suitable nitrogen source as an essential step to enhance xylanase expressions from microbes including bacteria has been reported (35; 36; 37; 38).

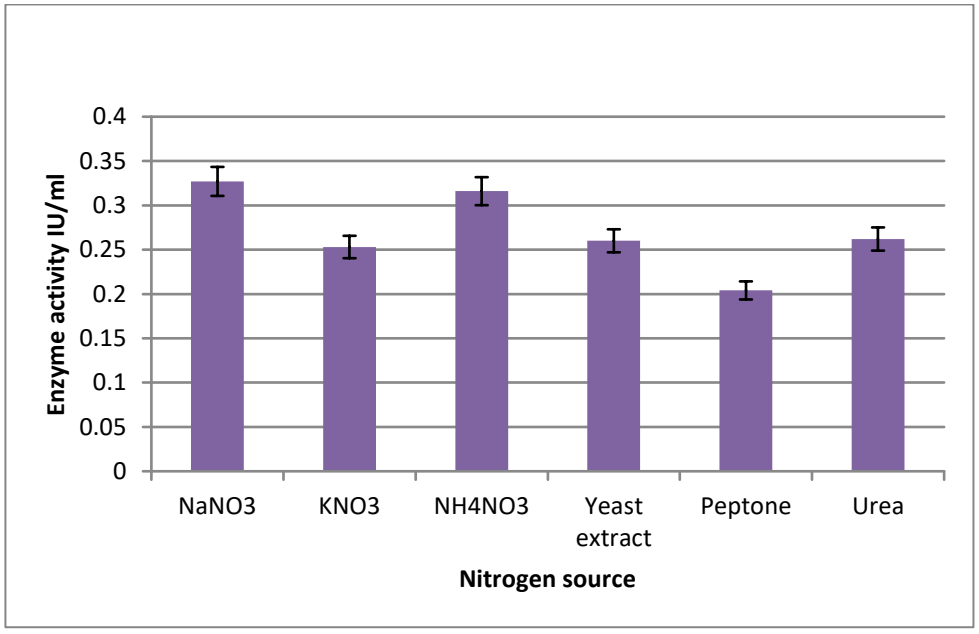

Fig.6. Effect of nitrogen source (chemical form of nitrogen) on the production of xylanase by *T. angustata*

Effect of Metal Ions and Surfactants

Among the metal salts tested, $MnSO_4$ was the unchallenged source of metal that induced xylanase production. Other metals seemed rather to inhibit xylanase production by the fungus. Both the surfactants tested were also inhibitory to the

xylanase production, a 25-55% reduction was observed (Fig. 7). Metal ions have also been reported to be inhibitory to xylanases production by *Thermoascus aurantiacus* and *Bacillus* SSP-34 **(39).** Metal ions have been classified into Class A, Class B and border line ions **(40).** Metal ions (Hg^{2+}, Pb^{2+}) belonging to class B however are toxic and not needed for biological function **(41).**

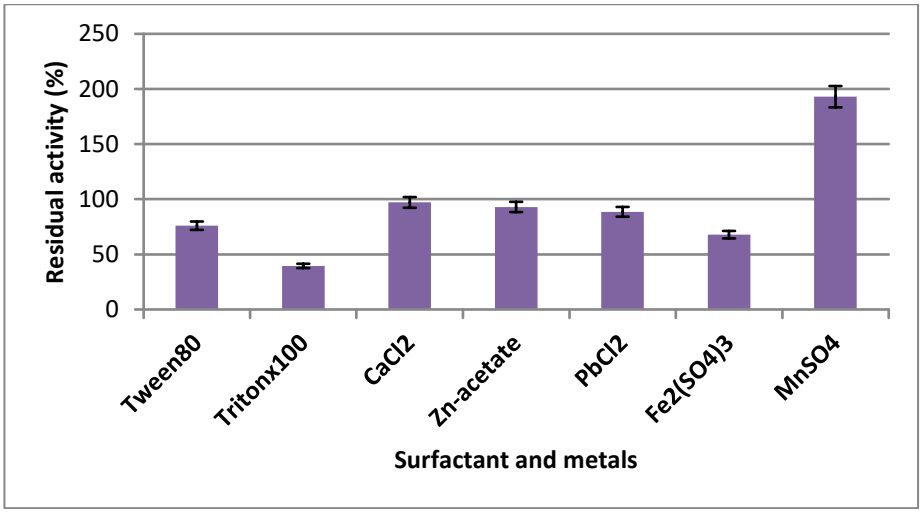

Fig. 7. Effect of selected metals and surfactants on the production of xylanase by *T. angustata*

Xylan is the major component of hemicellulose which is the second most abundant polysaccharide. Degradation of xylan into sugars is of much importance for many industries and so is the xylanase enzyme required for its degradation. Fungi are well known agents of decomposition of particularly xylan and cellulose containing organic matter. Xylanases are important industrial enzymes which depolymerizes xylan molecules into xylose units [42]. They have wide applications with potentials for use in the food processing, beverage, livestock feed, paper and pulp, detergent and textile industries as well as in the conversion of agricultural (lignocellulosic) biomass into products with commercial value [43, 44, 45]. With the increasing demand for alternative liquid fuels worldwide, the enzyme is used for enzymatic hydrolysis of lignocellulosic biomass in bioethanol production process [46].

CONCLUSION

The results obtained indicate that the selected fungal species yield variable xylanase activities at low temperature. One of the isolate *T. angustata* (BPF5) showed good xylanase activity in comparison to *P. roseus* (BPF6) and *P. canesence* (BPF4) as determined by the clear zone of hydrolysis around xylose-supplemented medium. Hence from present study, it is concluded that the characteristics of the produced xylanase by *T. angustata* (BPF5) revealed its enormous potential as a psychotolorent enzyme in xylan degradation process which would enhance and strengthen the competitive power of xylanase in global enzyme market. As a result, xylanase is anticipated to be a commercially high value bioproduct used in variety of industrial applications especially in the manufacturing of animal feeds, fruit juice and paper pulping. The cultivation systems can easily be modified to enhance the productivity of the enzyme formation by the fungus, which will facilitate the scale up processes for mass production.

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