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Screening of potential xylanase producing fungal strains under solid state fermentation condition

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Abstract
Xylanases are industrially important group of hydrolytic enzymes associated with the cleavage of β-1,4 backbone of the complex plant cell wall polysaccharide xylan. Several microbial sources namely bacteria, actinomycetes and fungi have been reported to be important sources of xylanases. In the present study, a total of 40 fungal strains were screened to assess the potential for the production of xylanases under solid state fermentation (SSF) conditions. Xylanase from three of the promising strains namely *Aspergillus tubingensis* MTCC1781, *Oidiodendron echinulatum* MTCC1356 and *Aspergillus flavus* MTCC10938 were further characterized for pH optima, pH stability, temperature optima, temperature stability and effect of metal ions. The pH optima observed for xylanase from *Aspergillus tubingensis*, *Oidiodendron echinulatum* and *Aspergillus flavus* was found to be 9.0, 7.0 and 9.0 respectively while pH stability ranged from pH 3.0-9.0. The temperature optima was 40°C**,** 60°Cand 50°C for xylanase from *Aspergillus tubingensis*, *Oidiodendron echinulatum* and *Aspergillus flavus* respectively while temperature stability was observed in the range of 20ºC- 60ºC, 10ºC-60ºC and 10ºC-50ºC respectively. Among the different metal ions tested, $Cu^{+2}$and Mn^{+2}showed slight enhancement in enzyme activity while Hg^{+2} inhibited the xylanase activity.

Keywords: xylan, xylanase, solid state fermentation, *Aspergillus tubingensis*, *Oidiodendron echinulatum*, *Aspergillus flavus*

1. Introduction

Plant cell wall is composed of polysaccharides comprising of three major constituents namely cellulose, hemicelluloses and lignin. Xylan is one of the major hemicellulosic constituent, having a linear backbone of β-1, 4 linked xyloses (Ju *et al*., 2013; Collins *et al*., 2005; Shallom and Shoham, 2003) [21, 10, 33]. The degradation of xylan is mainly facilitated by xylanases group of enzymes, including- Endo-1, 4-β-xylanase (1,4-β-D-xylanxylanohydrolase; EC 3.2.1.8) that cleaves the glycosidic bonds in the xylan backbone, bringing about a reduction in the degree of polymerization of the substrate, β-D- xylosidases (1, 4-β-D-xylanxylohydrolase; EC 3.2.1.37) that act on these xylooligomers releasing xylose (Juturu and Wu 2014; Knob *et al*., 2010) [20, 22]. Other related enzymes like arabinofuranosidases (EC 3.2.1.55) removes L-arabinose residues substituted at positions 2 and 3 of the β-D-xylopyranosyl; acetylxylan esterase (EC 3.1.1.6) removes the O-acetyl substituents at the 2 and 3 positions of xylose residues in acetylated xylans; α- glucuronidases (EC 3.2.1.131) hydrolyzes the α-1, 2 bonds between the glucuronic acid residues and β-D-xylopyranosyl backbone units found in glucuronoxylan (Amaretti *et al*.,2013;Alvira *et al*.,2011; Zhang *et al*., 2011) [6, 5, 44].

Among all xylanases, Endo-1, 4-β-xylanase are the promising one based on their direct involvement in cleaving the glycosidic bonds and in liberating short xylooligosaccharides (Verma and Satyanarayana 2012) [36]. Several microorganisms including bacteria, fungi and actinomycetes have been reported to be important sources for xylanases (Walia *et al*., 2017) [39]. Most of the commercial xylanases have been reported to be produced by *Trichoderma, Bacillus, Aspergillus, Penicillium* and *Aureobasidium* (Li *et al*., 2000) [26]. In general, both submerged state fermentation (SmF) and solid-state fermentation (SSF) have been successfully used in xylanase production using different microbial strains.

Xylanases have several industrial applications like lignocellulosic bioconversion into fermentative sugars, juice clarification, bioethanol production, animal feed, paper and pulp industries and also for fuels and chemicals (Ali *et al*., 2017; Shi *et al*., 2013; Singh *et al*., 2013; Golugiri *et al*., 2012; Li *et al*., 2000; Hatanaka., 2012) [3, 34, 35, 15, 26, 18]. Though xylanases have been reported to have several applications but still there exists several constraints for its subsequent commercialization due to unreachability of substrate, limited hydrolysis due to their diverged branched nature, narrow pH range, thermal instability and economical feasibility (Walia *et al*., 2017) [39]. Screening of microbial sources, production optimization using solid state fermentation, cloning and expression of relevant xylanase genes are some of the approaches being utilized to overcome the constraints.

In the present study, a total of 40 fungal strains predominately representing *Aspergillus* genera were screened for xylanase production under solid state fermentation conditions and further xylanase produced from three promising fungal strains namely *Aspergillus tubingensis* MTCC1781, *Oidiodendron echinulatum* MTCC1356 and *Aspergillus flavus* MTCC10938 were partially characterized.

2. Materials and Methods

Chemicals

Xylan was purchased from Himedia Laboratories Pvt. Ltd. Rests of the chemicals were procured either from Merck (Navi Mumbai, India) or S.D. Fine (Mumbai, India).

Organism and culture condition

The fungal strains were procured from the culture collection centers namely Microbial Type Culture Collection & Gene Bank (MTCC), Institute of Microbial Technology (IMTECH, CSIR) Chandigarh, India and National Institute of Interdisciplinary Culture Collection (NIICC) Science and Technology, Thiruvananthapuram, Kerala, India. The list of fungal strains used in the present study is shown in Table-1. The culture was maintained by cultivation on Czapek-Dox agar slants at 26°C. Screening of fungal strains for xylanases under solid-state fermentation (SSF) conditions was achieved by media comprising of wheat bran 4.5 g, tea extract 0.5 g and 5 ml salt solution. The salt solution composition was 4 g/L each of K_2HPO_4, KH_2PO_4, and $(NH_4)_2SO_4$ and the final moisture content was kept upto50%.The Erlenmeyer flask (2 x 250ml) used for production media was inoculated by inoculum (10%w/v) and incubated at 26°C for 5 days and were allowed to grow under stationary condition.

Table 1: List of 40 different fungal strains used for xylanase production under solid-state fermentation (SSF).

S. No.	Strain	Accession Number
1.	*Aspergillus oryzae*	MTCC 1122
2.	*Aspergillus tubingensis*	MTCC 2587
3.	*Aspergillus tubingensis*	MTCC 1781
4.	*Aspergillus niger*	MTCC 478
5.	*Aspergillus niger*	MTCC 872
6.	*Aspergillus var. terreus*	MTCC 3006
7.	*Aspergillus fumigatus*	MTCC 2584
8.	*Aspergillus fumigatus*	MTCC 3070
9.	*Aspergillus fumigatus*	MTCC 2508
10.	*Aspergillus fumigates*	MTCC 870
11.	*Aspergillus flavus*	MTCC 7589
12.	*Aspergillus flavus*	MTCC 10938
13.	*Aspergillus flavus*	MTCC 8835
14.	*Aspergillus versicolor*	MTCC 3071
15.	*Aspergillus flavus*	NIICC 08142
16.	*Aspergillus terricola*	MTCC 7588
17.	*Aspergillus ficcum*	MTCC 7591
18.	*Penicillium citrinum*	MTCC 8897
19.	*Byssochlamys fulva*	MTCC 505
20.	*Oideodendron echinulatum*	MTCC 1356
21.	*Aspergillus niger*	MTCC 404
22.	*Aspergillus oryzae*	MTCC 634
23.	*Aspergillus oryzae*	MTCC 3782
24.	*Aspergillus awamori*	MTCC 2456
25.	*Aspergillus terreus*	MTCC 2580
26.	*Aspergillus oryzae*	MTCC 6993
27.	*Aspergillus flavus*	MTCC 8837
28.	*Aspergillus flavus*	NIICC08143
29.	*Aspergillus flavus*	NIICC08145
30.	*Aspergillus flavus*	NIICC08147
31.	*Fusarium moniliforme*	MTCC 2088
32.	*Fusarium decemcellulare*	MTCC 2079
33.	*Fusarium oxysporum*	MTCC 1755
34.	*Fusarium udum*	MTCC 3829
35.	*Fusarium lateritium*	MTCC 8794
36.	*Fusarium avenaceum*	MTCC 10572
37.	*Fusarium subglutinans*	MTCC 9916
38.	*Fusarium culmorum*	MTCC 349
39.	*Fusarium solani*	MTCC 3004
40.	*Fusarium graminearum*	MTCC 2089

Production of Xylanase

For production of xylanases from different fungal strains, approximately 1 ml of spore suspension was inoculated in (2 x 250ml) Erlenmeyer flask containing SSF media and incubated at 26°C in incubator shaker (LabTech, Korea). After-day, the culture was mixed with 15 ml of cold distilled water in each flask and was properly mixed with glass rod. Finally the culture was filtered with the help of cheese cloth and filtrate was centrifuged at 10,000 rpm for 20 min at 4°C. The pellet was discarded and supernatant was used as crude enzyme.

Xylanase assay

Enzymatic activity of xylanase was determined by the liberating of reducing-end products, the reaction solution (2 ml) consisting of 0.5 ml of 1% xylan, 1.4 ml of 100 mM citrate -phosphate buffer (pH 5.0) and 0.1 ml of enzyme solution. A control was simultaneously prepared taking thermal denatured enzyme. It was incubated for 30 min at 40°C in a water bath. Added 3ml of Dinitrosalicylic acid (DNSA) reagent and 1ml of distilled water, total volume was made to 6 ml. The solution was boiled for 10 min, cooled and absorbance was read at 580 nm by Colorimeter (Aimil Limited, New Delhi).The xylanase activity was determined by measuring the amount of xylose released from 1% (w/v) xylan by the standard DNSA method (Miller, 1959) [28]. One unit of xylanase activity was defined as the amount of enzyme which liberated1µmol of reducing sugars per min. under assay condition.

Biochemical characterization of crude xylanase

The pH optimum was determined by using 0.5 ml of 1% xylan in the buffered reaction solution, using different buffers at 100 mM in the pH range 1.0–12.0 and incubating the reaction mixtures at 40°C for 30 min. The different buffers used were: citrate–phosphate (pH 3.0–8.0), glycine–sodium hydroxide (pH 9.0–10.0) and sodium phosphate–sodium hydroxide (pH 11.0). The pH stability of the enzyme was studied by exposing the enzyme to buffers of different pH for 24 h at 4° C. After 24 h, the activities of the enzyme exposed to different pH were assayed and plotted in the form of IU versus pH at which enzyme was exposed for 24 h. The optimum temperature for the enzyme activity was determined by assaying activity of the enzyme at different temperatures in the range 10–70° C and plotting a graph of the IU versus temperature of the reaction solutions. The temperature stability was tested by 0.1ml enzyme incubated at temperatures 10°C to 70°C for 30min. After that the crude enzyme was inoculated at 30min with the substrate xylan 0.5 ml and buffer 1.4 ml. The xylanase activity was measured by DNSA method.

Effect of metal ions

The effect of metal ions on enzyme activity was achieved by using 0.1ml of different salts ($CaCl_2$, $CoCl_2$, $MgCl_2$, $MnCl_2$, $CuSO_4$, EDTA, $K_3Fe(CN)_6$, $AgNO_3$, KCl, $KMnO_4$, NaCl, $ZnSO_4$ and $HgCl_2$) at a final concentration of 5 mM along with 0.1 ml of crude xylanase, 1.3 ml buffer and 0.5 ml of the substrate xylan for 30 minutes and xylanase activity was measured by DNSA method.

3. Results and Discussion

Screening of Xylanase from fungal strains

A total of 40 fungal strains as listed in Table-1 were screened for xylanase production under solid state fermentation (SSF) condition, incubated at 24±2°C for a maximum of 5 days. The production status of the xylanase is shown in Figure-1. Three of the fungal strains namely *Aspergillus tubingensis* MTCC1781, *Oidiodendron echinulatum* MTCC1356 and *Aspergillus flavus* MTCC10938 seems to be promising based on production status of xylanases. The enzyme productivity is comparatively cheaper in solid-state fermentation (SSF) and normally much higher than that of submerged fermentation (Agnihotri *et al*., 2010; Walia *et al*. 2013a, b) [2, 37, 38]. Isolation of fungal sources showing xylanase production under solid state (SSF) and submerged state fermentation (SmF) from different soil sample, saw dust, degraded wood has been reported (Kulkarni *et al*.,2013; Adesina *et al*., 2013; Ramanjaneyulu *et al*., 2015) [24, 1, 30]. Attempts have been made for screening of xylanase from different strains of *Fusarium* under SSF conditions (Arabi *et al*., 2011) [7]. Screening of xylanase from fungal sources isolated from environment samples revealed predominance of *Aspergillus* and *Trichoderma* (Ja'afaru, 2013) [19].

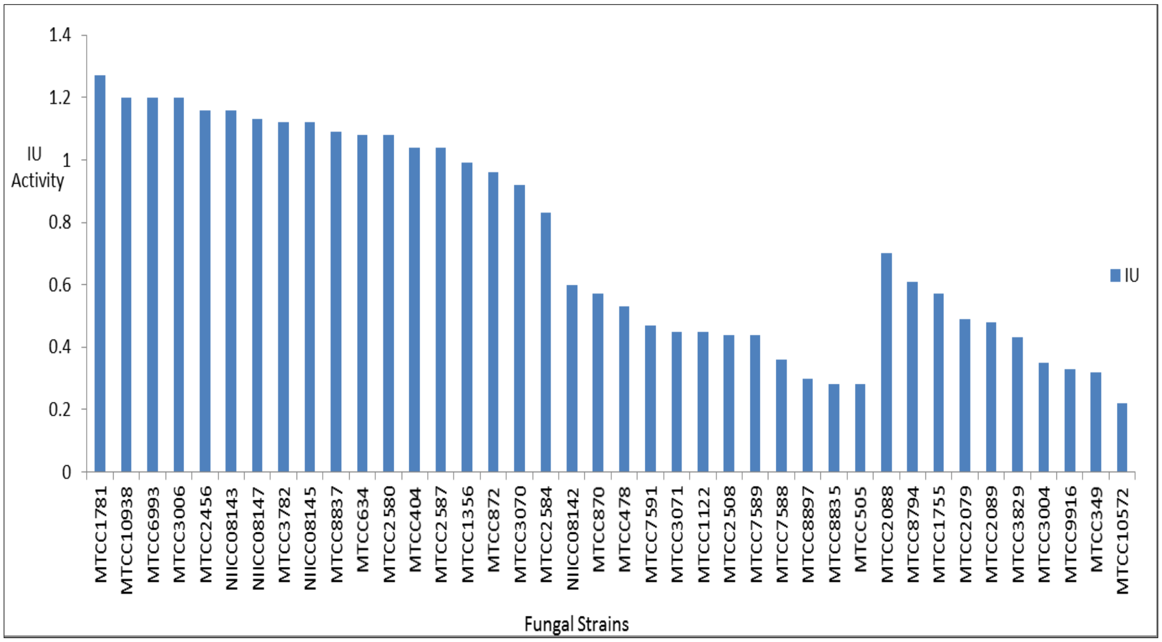

Fig 1: Screening of xylanase from different fungal strains *(IU represent International Unit; µmol/ml/min.)*

Biochemical characterization of xylanase

Xylanases produced by three promising strain *viz. Aspergillus tubingensis* MTCC1781, *Oidiodendron echinulatum* MTCC1356 and *Aspergillus flavus* MTCC10938 was characterized for pH optima, pH stability, temperature optima, temperature stability and effect of metal ions from the crude preparation to elucidate its potential application and further purification to homogeneity. Comparative analysis of xylanase from these fungal strains for basic enzymatic attributes is discussed.

pH optima and stability

The optimum pH of xylanase from *Aspergillus tubingensis* MTCC 1781,*Oidiodendron echinulatum* MTCC 1356 and *Aspergillus flavus* MTCC 10938 was found to be 9.0, 7.0 and 9.0 respectively (Figure-2A). The neutral pH of xylanase reported from *Oidiodendron echinulatum* could be interesting as only few reports of neutral xylanase is available in literature like xylanase from *Penicillium citrinum* (Ghosal *et al.*,2012) [14] and *Streptomyces lividans* (Nadia *et al.*, 2010) [29]. Xylanase showing alkaline pH has been reported from *Streptomyces olivaceus* (Sanjivkumar *et al.*, 2017) [32] and *Cladosporium oxysporum* (Guan *et al.*, 2016) [16]. In general most of the fungal xylanases have their pH optima in acidic range (Korkmaz *et al.*, 2017; Yegin., 2017; Deshmukh *et al.*, 2016; Youzhi *et al.*, 2015) [23, 42, 11, 43].

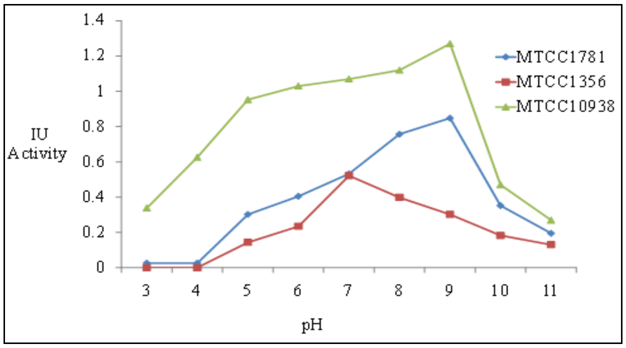

Fig 2A: pH optima of xylanase produced by fungal strains *Aspergillus tubingensis* MTCC 1781, *Oidiodendron echinulatum* MTCC 1356 and *Aspergillus flavus* MTCC 10938

The pH stability of xylanases from these three fungal strains revealed wider pH range i.e., pH 3.0- 9.0, 4.0 to 9.0 and 5.0 to 9.0 respectively (Figure-2B) when exposed to buffer of variable pH for 24 hr. Similar pH stability (2.0 to 8.0) range has been reported from *Penicillium oxalicum* (Liao *et al.*, 2014) [25] while xylanases with stability in alkaline pH has been reported from *Aspergillus fumigatus* (Deshmukh *et al.*, 2016) [11].

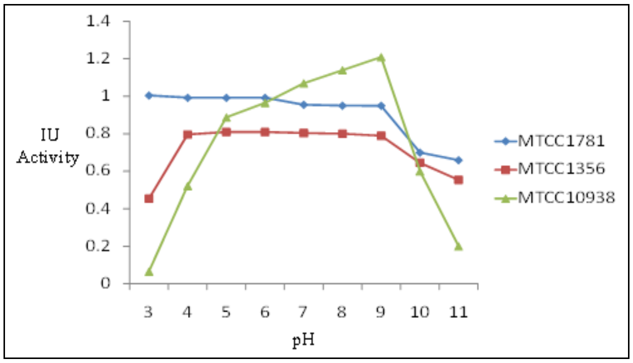

Fig 2B: pH stability of xylanase produced by fungal strains *Aspergillus tubingensis* MTCC 1781, *Oidiodendron echinulatum* MTCC 1356 and *Aspergillus flavus* MTCC 10938

Temperature optima and stability

The temperature optima of xylanase from *Aspergillus tubingensis* MTCC 1781,*Oidiodendron echinulatum* MTCC 1356 and *Aspergillus flavus* MTCC 10938 was found to be 40°C, 60°C and 50°C respectively (Figure-3A). Xylanases with temperature optima in the range of 40°C to 60°C has been reported from several sources like *Trichoderma pleuroticola* (Korkmaz *et al.*, 2017) [23], *Penicillium occitanis* (Driss *et al.*, 2014) [13], *Rhizopus oryzae* (Xioa *et al.*, 2014) [41], *A.niger* and *A. flavus* (De Alencar Guimaraes *et al.*, 2013) [12]. There are several reports of thermophilic xylanases showing temperature optima of 65°C or above from diverse sources like *Lichtheimia ramose* (Alvarez-Zúñiga *et al.*, 2017) [4], *Aspergillus fumigatus* (Lin *et al.*, 2017) [27], *Caldicoprobacter algeriensis* (Bouacem *et al.*, 2014) [9], *Aureobasidim pullulans* (Bankeeree *et al.*, 2014) [8].

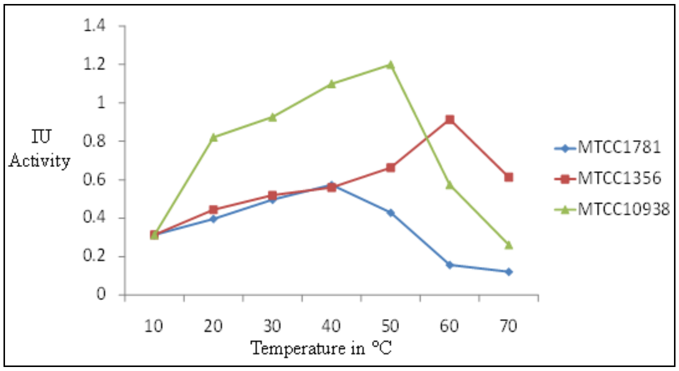

Fig 3A: Temperature optima of xylanase produced by fungal strains *Aspergillus tubingensis* MTCC 1781, *Oidiodendron echinulatum* MTCC 1356 and *Aspergillus flavus* MTCC 10938

Temperature stability of the xylanase from these three fungal strains were carried out by incubating the enzyme solution at various temperature ranges (10ºC to 70ºC) for 30 min. The temperature stability for fungal strains *Aspergillus tubingensis* MTCC 1781,*Oidiodendron echinulatum* MTCC 1356 and *Aspergillus flavus* MTCC 10938 was found to be in the range 20ºC- 60ºC, 10ºC-60ºC and 10ºC to 50ºC respectively as shown in Figure-3B.

Xylanases with temperature stability in the range of 30 ºC to 70ºC for 60 min. has been reported from *Aspergillus* sources (Haiyan *et al.*, 2015) [17] with potential applications in biofuel and paper industries (Saleem *et al.*, 2012) [31].

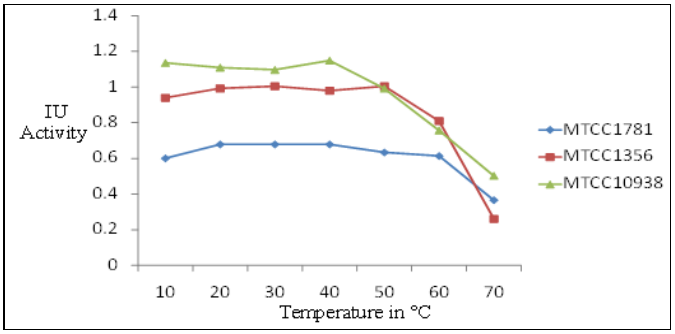

Fig 3B: Temperature stability of xylanase produced by fungal strains *Aspergillus tubingensis* MTCC 1781,*Oidiodendron echinulatum* MTCC 1356 and *Aspergillus flavus* MTCC 10938

Effect of metal ions

The effect of various metal ions has been assessed in terms of their relative percentage activity using 5mM concentration of each metal ion in reaction solution as shown in Figure-4. Among different metal ions, Cu^{+2} and Mn^{+2} slightly enhance the activity while Hg^{+2} completely inhibited the enzyme activity irrespective of the different sources. The increase in enzyme activity in the presence of Cu^{2+}, Co^{2+}, and Mn^{2+} has been reported for xylanase produced by *Pischia pastoris* (Wang *et al.*, 2017) [40]. The inhibitory enzyme activity in the presence of Hg^{2+} has been reported for xylanases (Bouacem *et al.*, 2014) [9]. In another report Mn^{+2} showed positive response for xylanase production while Cu^{+2} inhibited xylanase activity (Guan *et al.*, 2016) [16].

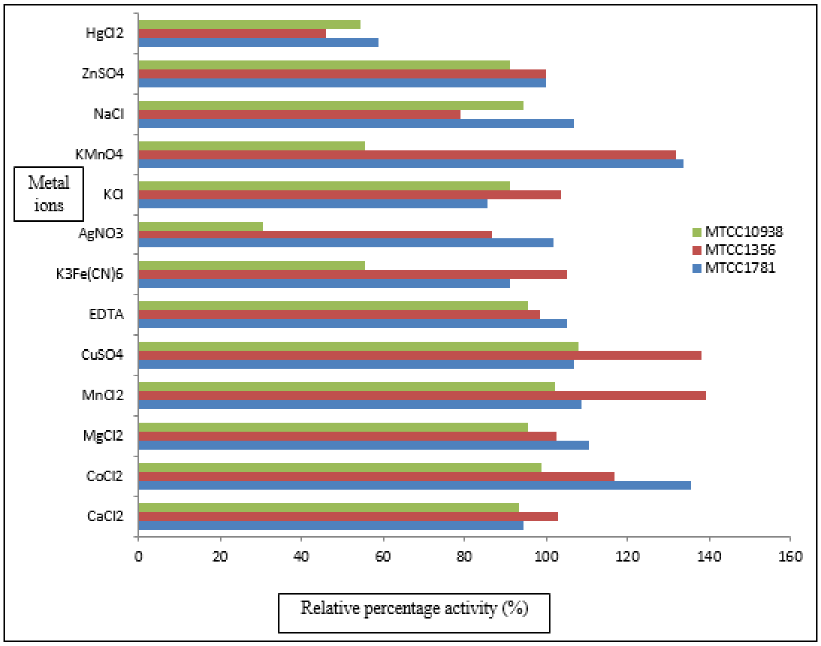

Fig 4: Effect of metal ions at (5mM) concentration on enzyme activity expressed in relative percentage activity.

4. Conclusion

In the present study assessment of 40 fungal strains for xylanase production under solid state fermentation condition revealed several potential strains, three of which namely *Aspergillus tubingensis* MTCC 1781, *Oidiodendron echinulatum* MTCC 1356 and *Aspergillus flavus* MTCC 10938 were partially characterized. Purification of xylanase produced by these fungal strains to homogeneity, followed by its extensive enzymatic characterization and elucidating its diverse application needs to be done. The xylanase showing neutral pH optima i.e. from *Oidiodendron echinulatum* MTCC 1356 could be interesting as only few reports are available.

5. Acknowledgment

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