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Characterization of Crude Xylanase Produced by Edible Mushroom *Pleurotus eryngii*

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Abstract

Xylanase has been increasingly forthcoming in recent years because of their possible involvement in numerous industrial processes including bioconversion of lignocellulose derived sugars in to fuels, processing food and the paper and fiber industries. Edible mushrooms are emerging as important source of xylanolytic enzymes and this study has concentrated to produce and characterize xylanases by *Pleurotus eryngii*. The crude enzyme was characterized on the basis of various parameters such as incubation time, substrate specificity, substrate concentration, enzyme volume, buffer, pH, pH stability, temperature, temperature stability, and effect of various metal ions or compounds. The xylanase activity was noted maximum at 15 minutes of incubation time, 2.0% xylan and 0.5 ml enzyme volume. The highest enzyme activity was found at pH 4.5, whereas xylanase exhibited maximum stability in the range of pH 4.0 to 10.0. The maximum xylanase activity was noted at 60°C, while enzyme was most active and retains more than 40% activity at 90°C within 10 minutes of incubation. $ZnCl_2$ (10mM) stimulated the xylanase activity as compare to other metal ions or compounds. It is concluded that *Pleurotus eryngii* is capable to produce pH stable and thermostable xylanase for industrial purposes.

Keywords: Characterization; Xylanase; Edible mushroom; *Pleurotus eryngii*

Introduction

The plant cell wall is the most abundant reservoir of lignocellulosic material in the biosphere and degradation takes place by microbial enzymes is a key biological process that is central to the carbon cycle, herbivore nutrition and host invasion by phytopathogenic fungi and bacteria. Xylan, the main component of hemicellulose consists of α-1, 4-linked d-xylosyl residues backbone branched with other pentoses, hexoses and uronic acids. Xylanases and associated debranching enzymes produced by a variety of microorganisms, which bring about the hydrolysis of Xylobiose of hemicelluloses [1,2].

While xylanases have been reported from fungi [3], bacteria [4], actinomycetes [5,6], and yeast [7-9], there have been few studies using edible fungi as a xylanase source [10]. There are three reasons for using edible fungi as xylanase sources. First, mushrooms produce a wide range of extra-cellular enzymes that enable them to degrade complex lignocelluloses substrates into soluble substances [11]. Second, xylanase from edible fungi is an extra-cellular enzyme, with more than 90% of the xylanase from the mushroom secreted out of cells by *T. Clypeatues* [12]. Third edible mushroom protein is safe and highly nutritional [13]. Many of these fungi are also the source of high-value metabolites of interest to the pharmaceutical, food and cosmetic industries [14,15]. The objective of this study was to characterize xylanase enzyme, produced by *Pleurotus eryngii* grown on 0.6% starch as a main substrate in liquid-state culture and examined the effects of pH, temperature and metal ions on crude xylanase stability. To our knowledge, this is the first report describing the characterization of xylanase by edible mushroom *Pleurotus eryngii*.

Materials and Methods

Fungal strain

In this study, mushroom strain *Pleurotus eryngii* was purchased from Edible fungi Institute, Shanghai Academy of Agricultural Sciences Shanghai, China. Stock cultures of these fungi are maintained on Potato dextrose agar slants at 4°C.

Inoculum preparation

The inoculum was prepared by growing mushrooms on a rotary shaker at 120 rpm and 27 ± 2°C in 250 ml Erlenmeyer flasks containing 50 ml of following synthetic medium (per liter) 6.0 g glucose, 0.2 g yeast extract, 0.5 g peptone, 1.0 g KH_2PO_4 and 0.5 g $MgSO_4{\cdot}7H_2O$. The medium was adjusted to pH 5.5 with 0.1 N HCl or NaOH. After 4 days of cultivation mycelial pellets of *Pleurotus eryngii* were harvested and homogenized with a laboratory blender.

Culture conditions

Lignocellulosic substrates submerged fermentation has been carried out on a rotary shaker at 120 rpm and 27 ± 2°C in 250 ml Erlenmeyer flasks containing 50 ml of above-mentioned medium with different carbon sources (glucose, xylose, sucrose, starch, xylan, wood straw and rice husk powder) with 0.2, 0.4 and 0.6% concentrations g/L. The initial pH of the medium was adjusted to 5.5 prior to sterilization by using 0.1 N HCl or NaOH. 1.0 ml of mycelial homogenate was used to inoculate the Erlenmeyer flasks containing media. After 96 hours of mushroom cultivation, when cultures reached end of logarithmic-stationary phase of growth, the extracellular enzymes were extracted in

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order to measure the pH of culture medium and enzyme activity. The mycelial biomass was separated by filtration through Whatman filter paper No.1 and followed by centrifugation (5000 g; 15 min) at 4°C and the final pH was measured by (WAPA) pH meter.

Enzyme assay

Xylanase activity was determined by mixing 0.5 ml sample with 0.5 ml of oat to xylan (Fluka, Germany) (1% w/v) in 50mM citrate buffer (pH 5.3) at 60°C for 15 min reported by Bailey et al. [16]. Xylose standard curve was used to calculate the xylanase activity. In assay the release of reducing sugars were measured by using dinitrosalicylic acid reagent method [17]. One International unit of enzyme activity was defined as the amount of enzyme, releasing 1 µmol of reducing sugars per minute ml^{-1}.

Results and Discussion

The effect of time period and carbon sources on the production of xylanase by *Pleurotus eryngii* was checked and it is concluded from the outcome of the present study as shown in Figures 1-3 and Table 1 that the addition of different carbon sources were enhanced the xylanase production and result reveals that incubation period varies from carbon source to carbon source. However, after 96 hours a decrease in enzyme production was observed when starch was used as a carbon source for the growth and xylanase production that may be due to the exhaustion of nutrients from the medium that affected the organism's growth. Other factor, glucose is the end product of starch so in my study glucose has shown repressed expression, which is also supported by early investigators [18-20]. After optimizing carbon source, nitrogen sources were optimized with two different concentrations (0.5 and 1.0%) and incorporated with 0.6% starch in the fermentation medium for the growth and xylanase production of *Pleurotus eryngii*. Among the nitrogen sources used 1.0% corn steep liquor was found best for xylanase production as shown in Table 2.

The crude xylanase produced by *Pleurotus eryngii* when grown on 0.6% starch, 0.5% corn steep liquor and 0.05% valine incubated at 30°C for 96 hours and pH was adjusted to 7.0 was characterized on the basis of time of incubation, substrate specificity, substrate concentration, enzyme volume, buffer, pH, pH stability, temperature, thermostability, activators and inhibitors.

The xylanase activity of *Pleurotus eryngii* was observed highest up to 15 minutes at 60°C and then gradually declined as shown in Figure 4. The reason behind the decrease in xylanase activity might be inactivation of enzyme on prolong incubation or self digestion [21,22]. Similar results are reported from earlier studies [23,24]. Figure 5 shows the substrate specificity xylan was replaced with various substrates such as CM-cellulose, avicel, cellulose and starch. The 100% relative higher activity was observed by 2.0% xylan. Figure 6 shows the effect of substrate concentration (xylan 0.5-4.0%) on xylanase activity produced by *Pleurotus eryngii*. Xylanase activity was increased with increase of xylan concentration up to 2.0% and later on activity was gradually decreased. The declination of xylanase activity may be due to alteration in enzyme and substrate ratio. The effect of crude enzyme volume (0.1-1.0 ml) was also checked on *Pleurotus eryngii* xylanase activity. The higher xylanase activity was found using 0.5 ml crude enzyme while low xylanase activity was observed when less amount of enzyme volume was used so it could be due to insufficient amount of enzyme to hydrolyze available substrate as shown in Figure 7. Figure 8 shows the effect of different buffers (sodium phosphate, sodium citrate and universal buffer (0.05 M. pH 5.5) on xylanase activity. The 100%

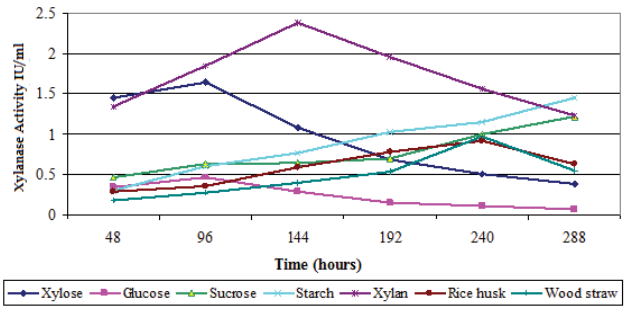


Figure 1: Effect of 0.2% carbon sources on growth and xylanase production by *Pleurotus eryngii* after 288 hours of incubation period.

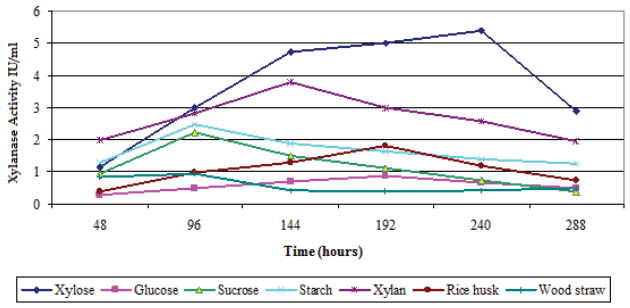


Figure 2: Effect of 0.4% carbon sources on growth and xylanase production by *Pleurotus eryngii* after 288 hours of incubation period.

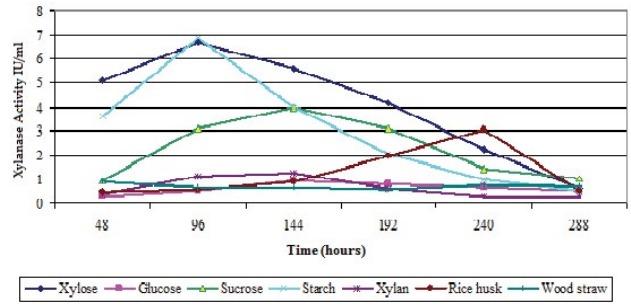
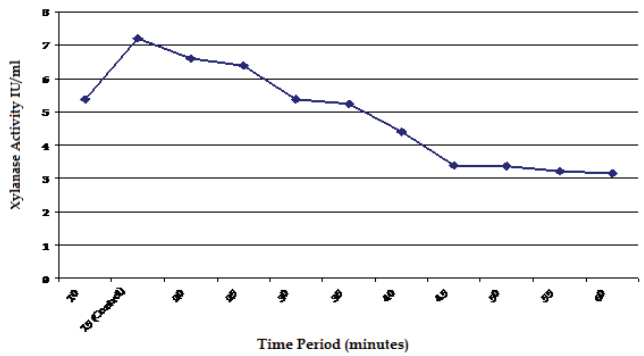
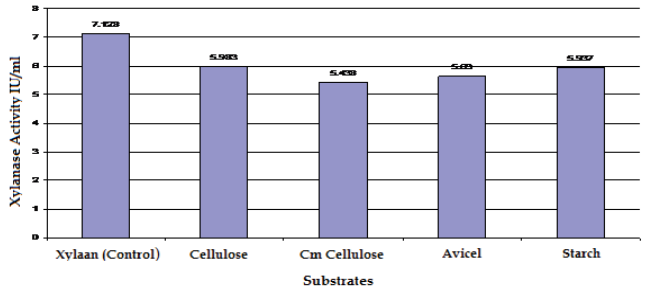


Figure 3: Effect of 0.6% carbon sources on growth and xylanase production by *Pleurotus eryngii* after 288 hours of incubation period.



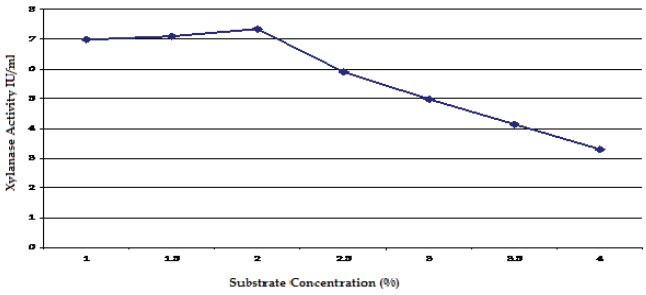
SD: Standard Deviation; SE: Standard Error; Minimum: 2.1150; Maximum: 7.4800; Sum: 42.296; Mean: 5.2870; Variance: 3.5130; SD: 1.8743; SE Mean: 0.6627

Figure 4: Effect of time of incubation on crude xylanase.



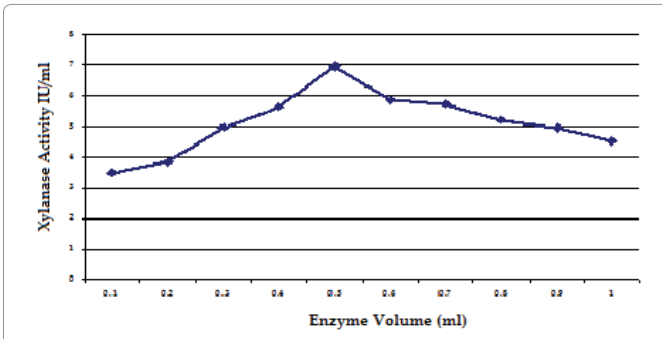
SD: Standard Deviation; SE: Standard Error; Minimum: 5.4380; Maximum: 7.1280; Sum: 30.116; Mean: 6.0232; Variance: 0.4317; SD: 0.6570; SE Mean: 0.2938

Figure 5: Effect of substrate specificity on crude xylanase.



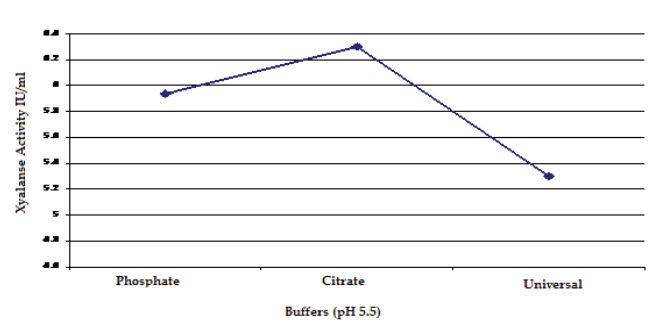
SD: Standard Deviation; SE: Standard Error; Minimum: 3.3020; Maximum: 7.3450; Sum: 39.759; Mean: 5.6799; Variance: 2.5078; SD: 1.5836; SE: 0.5985

Figure 6: Effect of substrate concentration on crude xylanase.



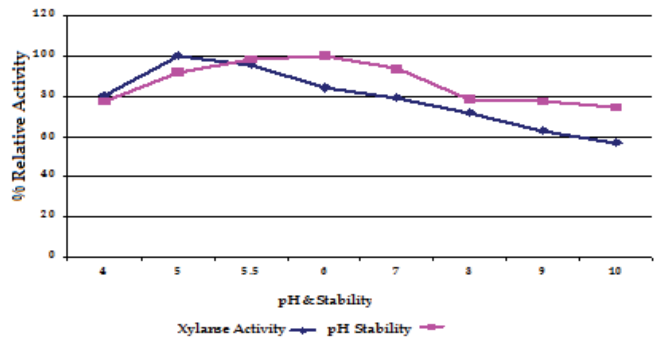
SD: Standard Deviation; SE: Standard Error; Minimum: 3.4910; Maximum: 6.9670; Sum: 30.739; Mean: 5.1232; Variance: 1.7009; SD: 1.3042; SE: 0.532

Figure 7: Effect of enzyme volume on crude xylanase.



SD: Standard Deviation; SE: Standard Error; Minimum: 5.2990; Maximum: 6.3010; Sum: 17.538; Mean: 5.8460; Variance: 0.2573; SD: 0.5073; SE: 0.2929

Figure 8: Effect of different buffers on crude xylanase.



(a) Effect of pH (b) pH stability; SD: Standard Deviation; SE: Standard Error; Minimum a: 74.420; Maximum a: 100.00; Sum a: 664.62; Mean a: 83.077; Variance a: 82.316; SD a: 9.0728; SE a: 3.2077; Minimum b: 74.420; Maximum b: 100.00; Sum b: 664.62; Mean b: 83.077; Variance b: 82.316; SD b: 9.0728; SE b: 3.2077

Figure 9: Effect of pH and stability on crude xylanase.

relative activity was determined by using sodium citrate buffer which was used throughout the study. The use of sodium citrate buffer in xylanase activity is reported by Salama et al. [25].

Figure 9 shows the effect of pH and pH stability on xylanase activity synthesized by *Pleurotus eryngii* using sodium citrate buffer ranging from pH 3.0-10.0. The maximum activity was determined at pH 5.0. The similar results have been reported by Franco et al., Ximenes et al. and Chipeta et al. [26-28]. The pH stability of xylanase was also checked using sodium citrate buffer ranging from pH 3.0-10.0 when enzyme was incubated for 10 minutes without substrate. After 10 minutes substrate was added and assay was carried out at 60°C. Xylanase showed maximum pH stability in-between pH 5.0 to 7.0. The similar results are reported by Ref. [29-32].

Figure 10 shows the effect of temperature and temperature stability ranging from 10-100°C on crude xylanase activity. The xylanase activity increased due to the enzyme catalyzed reaction up to 60°C and also due to the increase in the number of collisions between the reacting molecules. But, the enzyme probably got denatured on exposure to higher temperatures than its optimum and therefore, steadily lost its activity when incubated for 15 minutes with sodium citrate buffer pH 5.0. These findings are in accordance with several earlier reports showing optimal xylanase activity at 60°C by Balakrishnan H et al. [33-35]. 100% relative xylanase activity was measured after incubation at 60°C for 15 minutes and high optimum temperature may have striking effect on industrial purpose. The thermostability was checked by heating enzyme for 10 minutes at different temperatures ranging from 10-100°C. More than 40% activity was retained within 10 minutes at 100°C that proves xylanase activity is thermostable.

Figure 11 shows the effect of various metal ions or compounds (5 mM) on xylanase activity produced by *Pleurotus eryngii*. The xylanase activity was activated by metal ions such as Zn^{2+} and Ca^{2+} that suggests xylanase is a metalloenzyme**.** Xylanase is strongly inhibited by EDTA. The maximum activity was noted with 10 mM $ZnCl_2$ 114% and $CaCl_2$ 105% respectively. It has been reported that the divalent metals enhance xylanase activity [36-39]. Figure 12 shows effect of different concentrations (2.5-15.0 mM) of the best activator and was observed that xylanase activity increased with the increase of $ZnCl_2$ concentration and 100% relative activity was noted with 10 mM $ZnCl_2$. This was also observed that with the increase of $ZnCl_2$ concentration decreased the enzyme activity and it may be due to the higher amount

	Carbon sources %	Biomass g/50 ml	Xylanase Activity IU/ml
Xylose	0.2	0.05	1.635
	0.4	0.02	5.35
	0.6	0.02	6.068
Glucose	0.2	0.043	0.46
	0.4	0.11	0.88
	0.6	0.11	0.92
Sucrose	0.2	0.19	1.21
	0.4	0.27	2.205
	0.6	0.25	6.53
Starch	0.2	0.02	1.443
	0.4	0.027	2.46
	0.6	0.026	6.83
Xylem	0.2	0.048	2.383
	0.4	0.048	3.77
	0.6	0.051	1.258
Wood straw	0.2	0.02	0.961
	0.4	0.02	0.832
	0.6	0.012	0.85
Rice husk	0.2	0.007	0.912
	0.4	0.007	1.818
	0.6	0.011	3.069

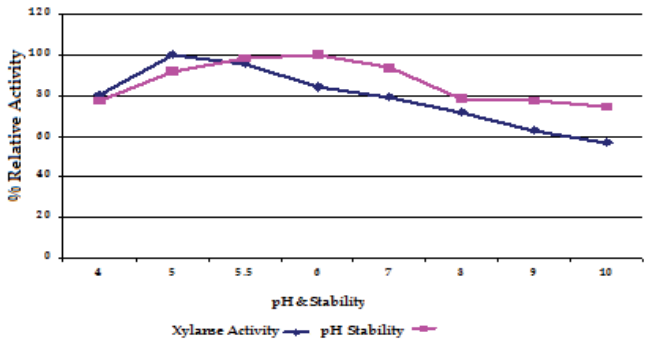
a: Biomass g/50 ml; b: Xylanase Activity IU/ml; SD: Standard Deviation; SE: Standard Error; Minimum a: 0.007; Maximum a: 0.0.270; Sum: 1.360; Mean: 0.065; Variance a: 0.006; SD a: 0.078; SE a: 0.017; Minimum b: 0.460; Maximum b: 6.830; Sum: 50.844; Mean: 2.421; Variance b: 4.071; SD b: 2.018; SE b: 0.440; ± indicated standard deviation from mean value

Table 1: Effect of different carbon sources on growth and xylanase production by *Pleurotus eryngii*.

	Nitrogen sources %	Biomass g/50 ml	Xylanase Activity IU/ml
KNO_3	0.5	0.043	2.82
	1.0	0.037	3.671
$NaNO_3$	0.5	0.047	3.541
	1.0	0.064	3.863
$(NH_4)_2 HPO_4$	0.5	0.061	2.64
	1.0	0.054	3.01
Urea	0.5	0.062	2.95
	1.0	0.067	3.16
Corn steep liquor	0.5	0.072	6.530
	1.0	0.083	7.185

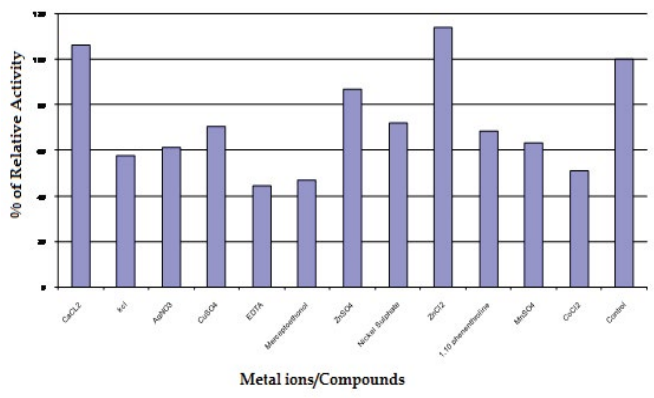
a: Biomass g/50 ml; b: Xylanase Activity IU/ml; SD: Standard Deviation; SE: Standard Error; Minimum a: 0.037; Maximum a: 0.083; Sum: 0.590; Mean: 0.059; Variance a: 0.000; SD a: 0.014; SE a: 0.004; Minimum b: 2.640; Maximum b: 7.185; Sum: 39.370; Mean: 3.937; Variance b: 2.541; SD b: 1.594; SE b: 0.504; ± indicated standard deviation from mean value.

Table 2: Effect of nitrogen sources on growth and xylanase production by *Pleurotus eryngii*.



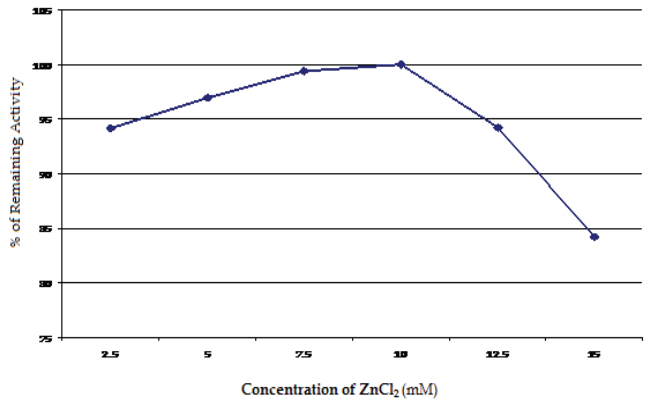
(a) Temperature (b) Temperature stability; SD: Standard Deviation; SE: Standard Error; Minimum a: 45.550; Maximum a: 100.00; Sum a: 652.14; Mean a: 65.214; Variance a: 333.27; SD a: 18.256; SE a: 5.7729; Minimum b: 41.580; Maximum b: 100.00; Sum b: 796.36; Mean b: 79.636; Variance b: 662.75 SD; b: 25.744; SE b: 8.1409

Figure 10: Effect of temperature and stability on crude xylanase.



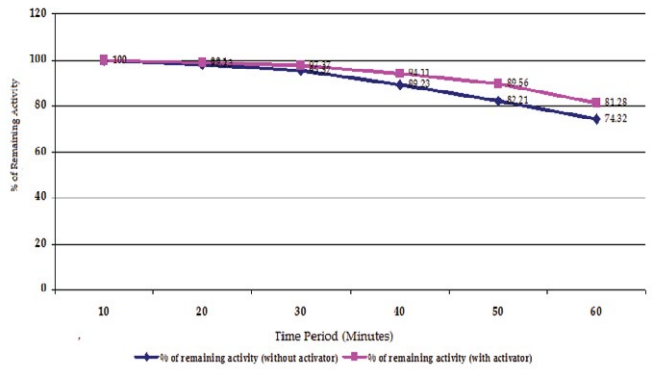
SD: Standard Deviation; SE: Standard Error; Minimum: 44.650; Maximum: 114.05; Sum: 980.45; Mean: 75.419; Variance: 519.21; SD: 22.786; SE: 6.3198

Figure 11: Effect of (5mM) metal ions or compounds on crude xylanase.



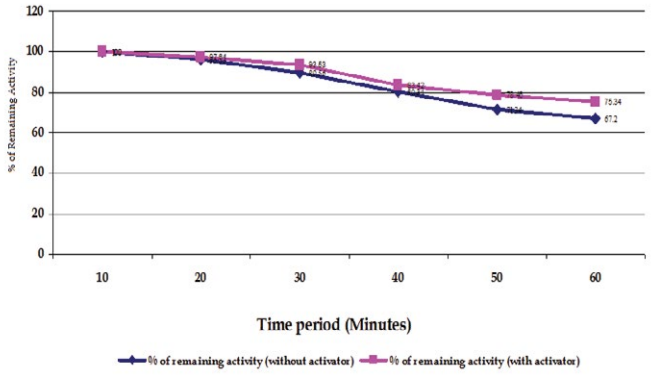
SD: Standard Deviation; SE: Standard Error; Minimum: 84.230; Maximum: 100.00; Sum: 568.91; Mean: 94.818; Variance: 33.082; SD: 5.7517; SE: 2.3481

Figure 12: Effect of different concentrations (2.5-15.0mM) of $ZnCl_2$ as activator.



(a) Without activator (b) With activator; SD: Standard Deviation; SE: Standard Error; Minimum a: 74.320; Maximum a: 100.00; Sum a: 539.31; Mean a: 89.885; Variance a: 100.62; SD a: 10.031; SE a: 4.0951; Minimum b: 81.280; Maximum b: 100.00; Sum b: 561.42; Mean b: 93.570; Variance b 50.756; SD b: 7.1243; SE b: 2.9085

Figure 13: Effect of thermostability at 60°C with and without activator (10mM $ZnCl_2$) on different time periods.



(a) Without activator (b) With activator; SD: Standard Deviation; SE: Standard Error; Minimum a: 67.200; Maximum a: 100.00; Sum a: 504.97; Mean a: 84.162; Variance a: 179.93; SD a: 13.414; SE a: 5.4762; Minimum b: 75.340; Maximum b: 100.00; Sum b: 528.39; Mean b: 88.065; Variance b: 107.95; SD b: 10.390; SE b: 4.2417

Figure 14: Effect of thermostability at 70°C with and without activator (10 mM $ZnCl_2$) on different time periods.

of activator, which acts as inhibitor.

Figures 12 and 13 show thermostability of crude xylanase at fixed temperatures 60°C and 70°C respectively with varying time of incubation (10-60 minutes) with and without $ZnCl_2$. It was observed that xylanase activity was decreased with increasing time of incubation in all cases but in the case of without activator, activity was lower in comparison to 10 mM $ZnCl_2$ as an activator at 60°C and 70°C. More than 80% and 75% xylanase activity was retained at 60°C and 70°C respectively after 60 minutes of incubation with activator ($ZnCl_2$) (Figure 14).

Conclusion

According to this study, crude enzyme characterization proved that enzyme is thermostable and pH stable, which is suitable for industrial use and xylanase produced by *Pleurotus eryngii* can be cost effective and helpful for pulp and paper industries by saving large amount of foreign exchange.

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