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Research Article

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Production and biochemical characterization of alkaline, thermostable xylanase secreted by recombinant *Escherichia coli*

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ABSTRACT

Xylanase gene was expressed in Escherichia coli. The enzyme production was induced in E. coli and liquid broth was used for enzyme estimation and characterization. Total three positive clones were used in the study naming sample 1, 2 and 3. Sample no 2 was found the best in expression of xylanase. To compare all the various parameters of the recombinant enzyme all 3 sample were studied simultaneously. The enzyme showed maximum activity at alkaline pH 7.5 and high temperature 60 °C respectively. The enzyme showed good thermal stability with half life of 3 hrs at 60 °C. Molecular weight of recombinant xylanase (determined by SDS-PAGE) was found 22 kDa. K_m and V_{max} of the recombinant xylanase was found to be 8.17 mg/ml and 3.7 IU/ml, respectively.

Keywords: *Xylanase, E. coli, Birchwood, endo-1,4-β-xylanases*

INTRODUCTION

Enzymes are an important class of globular proteins of biological origin that act as biochemical catalysts. The most distinguishing property of an enzyme in its catalytic action is its specificity and selectivity. Another major characteristic of enzymes is their sensitivity to the working conditions – they are functional only within a specific range of pH, temperature and presence of inhibitors, cofactors etc. A very useful property of enzymes as catalysts is that they are generally required in very small quantities. Today, enzymes are commonly used in many industrial applications and demand for more enzymes is increasing rapidly as they can convert complex compounds in to valuable products.

Xylans are polysaccharide, a major component of hemicelluloses present in plant cell wall, usually accounting for 20%–30% of their total dry mass. Structurally, xylans are linear homopolymers that contain d-xylose monomers linked through β-1, 4-glycosyl bonds. The acetylated xylan of hardwoods and arabinoxylan of softwoods are the two major forms of xylan in woods. Depending on the origin, the backbone structure is substituted to various degrees with acetyl, L-arabinofuranosyl, glucuronyl and 4-*O*-methylglucuronyl groups[1]. Degradation of xylan which is a complex polysaccharide is very difficult. Thus, it requires an efficient xylanolytic enzymatic system that consist of several enzymes including endo-β-1,4-xylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase etc. Complete degradation of xylans requires the synergistic action of these enzymes of which EC3.2.1.8 endo-1,4-β-xylanases are the crucial enzymes for de-polymerization of xylan[2].

Pulp and paper industries use harsh chemicals to break down the complex structure of hemicelluloses during the paper making process. Looking to the environmental issues related to the pollution caused by these harsh chemicals, there is a big demand of enzyme based eco-friendly bio-bleaching process.

Xylanase is one the promising alternative used for the bio-bleaching of the kraft pulp but the supply of the enzyme is not meeting the current market demand as for its application in bio-bleaching process xylanase should be active in alkaline pH and should be stable at high temperature.

In the past few decades' researchers have used molecular techniques to improve xylan degrading enzymes and increased its expression rates. However the search of new microbial strains and over expression of xylanase should be intensified in order to develop viable enzyme production technologies[3]. In the same direction the present study was carried out to study the production and characterization of a thermo stable recombinant xylanase enzyme expressed by *E. coli.* So that, this system could be exploited for thermo stable and alkaline pH active xylanase enzyme required for pulp bleaching in paper and pulp industry.

MATERIALS AND METHODS

Microbial culture and induced expression:

The recombinant strain of *E.coli* was maintained in glycerol stock at -80 °C. A loopful of *E. coli* was streaked of LB agar plate under laminar flow. A loopful of strain was extracted from agar plate and was inoculated in 5 ml LB supplemented with 5 µl Kanamycin antibiotic and 50 µl of 2M sterile glucose solution. 100 µl of overnight grown culture was used for inoculation of 20 ml fresh LB broth. 20 µl IPTG (100 mM) was added after 4 hours growth at 37 °C with 200 rpm in incubator shaker. Culture was grown for three days and then supernatant after centrifugation of culture at 8000 rpm for 10 min were used for xylanase activity and protein analysis.

Enzyme study

Preparation of substrate solution:

The substrate used for xylanase assay was birchwood xylan obtained from Sigma Aldrich. Substrate solution was prepared by adding the finely powdered xylan (1% w/v) to phosphate buffer (0.2M, pH 7.0), (preheated at 60 °C for 10 mins) and stirring the mixture at 25-35 °C for 10 mins. The xylan formed a suspension which was stirred uniformly to distribute the xylan in the buffer.

Measurement of Xylanase activity

Xylanase activity was measured by adding 0.2 ml of appropriately diluted enzyme solution in 0.2M, pH 7 phosphate buffer to 1.8 ml of substrate solution. The reaction mix was incubated at 70 °C for 5 min. The reaction was terminated by adding 3 ml DNS reagent and boiling reaction mix for 10 min in a boiling water bath and immediately cooling it in melting ice. The un-degraded xylan was removed by centrifugation at 2000 rpm at room temperature for 5 mins. The reducing sugar generated by xylanase action was estimated in the supernatant by deducting the absorbance value at 540 nm using UV-Visible spectrophotometer.

Time optimization:

The xylanase activity was optimized by ranging the first incubation time from 0 to 35 minutes at the intervals of 5 mins and reading absorbance at 540 nm.

Temperature optimization

The xylanase activity was optimized by ranging the temperature of first incubation of enzyme and substrate mix from 30 °C to 90 °C at an interval of 10 °C.

Substrate optimization

The xylanase activity was optimized by changing the substrate concentration from 1 to 20mg, and the optimum activity was measured by reading absorbance at 540 nm.

pH optimization

The optimum activity of xylanase was measured by changing the pH of buffers ranging from 3 to 10. Citrate buffer (0.1 M) was used for pH 3 to 5.5, phosphate buffer for pH 6 to 8 and glycine- NaOH buffer was used for pH 8.5 and 10.

SDS-PAGE:

The molecular mass of xylanase was determined by SDS-PAGE following the procedure of Lammeli[4]. The gel was stained with Cossmic brilliant blue. BIO-RAD mini-protean kit was used for performing this technique

RESULTS

Three samples were chosen for expression and further characterization of xylanase. The crude enzyme was obtained through expression using IPTG and the plasmid possessed Kanamycin resistance gene as selectable marker. The crude enzyme was characterized for various parameters like pH, time, temperature, substrate concentration and thermal stability.

Characterization of Xylanase:

Incubation time optimization:

The reaction mixtures of xylanase were incubated for varied time intervals from 0 to 30 minutes. Figure 1 shows the incubation time profile for all three clones selected for the present study. Optimum time of incubation for xylanase activity was found 5 minutes as the enzyme showed maximum activity at the incubation time of 5 min and after that no significant increases was noticed even increasing the incubation time up to30 minutes.

Temperature and pH optimization

Optimum temperature of xylanase was determined by varying the temperature from 30 $^{\circ}$C to 90 $^{\circ}$C. Enzyme obtained from clone no 1 and 2 showed maximum activity at 60 $^{\circ}$C while, enzyme of clone no 3 did not show any significant at 60 $^{\circ}$C (Fig. 2). The pH of reaction mixtures were varied from 3 to 9 as shown in figure 3. Maximum enzyme activity was observed at pH 7.5 for all three samples.

Substrate concentration optima:

The concentration of xylan was varied from 1 to 20 mg and it was found that enzyme activity is increasing with increase of substrate concentration as shown in Figure 4 and reaches near to platue at the concentration of 10mg/ml. Further increase in the substrate did not make any significant increase in the enzyme activity. Decrease in the enzyme activity was observed near 15 mg/ml afterwards. Lineweaver-Burk plot was prepared by plotting (data not shown) 1/V versus 1/[S] and Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) were found to be 8.17 mg/ml and 3.7 IU/ml, respectively.

Thermal Stability:

Thermal stability of the expressed xylanase was estimated at 60 $^{\circ}$C. Nearly 100% activity was observed till 80 min (Figure 5). Nearly 80% enzyme activity was retained up to 2 hours and after that rapid drop in the enzyme activity was observed.

Molecular mass determination (SDS-PAGE)

The SDS-PAGE gel contains band in two lanes which corresponding to the first lane of protein marker gives molecular mass of approximately 22 kDa (Figure 6).

DISCUSSION

In 1973 Horikoshi and Atsukawa reported first time xylanase from alkaliphilic bacteria[5]. The purified xylanase of *Bacillus* sp. C-59-2 showed a broad pH optimum ranging from 6 to 8. Xylanase multiplicity in *Bacillus* spp. reveals that bacteria produce two types of xylanases: one having basic (pH 8.3-10.0) with low molecular weight (16-22 kDa) and the other having acidic (pH 3.6-4.5) with high molecular weight (43-50 kDa). Our xylanase falls in the first type as it has low molecular weigh 22 kDa and was more active on pH higher than 6.0. Many of the xylanases produced by alkaliphilic organisms such as *Bacillus* sp. and *Aeromonas* sp. 212 showed stability at pH 9-10[6,7]. Xylanase from *Bacillus* sp. TAR-1, C-125, *Bacillus* sp. NTU-06, *Bacillus arseniciselenatis* DSM 15340 were reported optimally active at pH 9-10[8,9,10,11].

In our study, we found that xylanase expressed by recombinant *E. coli* has pH and temperature optima of 7.5 and 60 °C, respectively. The interesting part of this study is that the enzyme was stable in a broad range of temperatures (40-90 °C) and showed good thermal stability at 60 °C with half life of 2 hours and was able to retain 80% enzyme activity up to 3 hours. Generally most of the xylanases from bacteria and fungi or showed optimal activities at neutral and slightly acidic pH respectively[12,13]. Sa-Pereira[14] reported that the difference in pH and temperature tolerance for xylanase may be due to the excretion of a mixture of different enzymes mixtures, and/or due the post-translational modifications in xylanase excretion process that makes it stable at more extreme pH and temperature conditions.

Many researchers have tried to express xylanase genes in *E. coli*, isolated from different microbial organisms but, the xylanase expression level was generally lower than the parent organism[15,16,17,18,19,20].

Yang [21] have reported the hyperexpression of xylanase gene in *E. coli* with xylanase activity of 7 IU/ml found in intracellular fraction of *E. coli.* Expression of xylanase in the extracellular fluid had also been reported earlier in recombinant *E. coli* for the bacterial xylanases from alkaliphilic Bacillus[8] , and alkaliphilic, and thermophilic *Bacillus* species [22]. In the present study we have studied the production and characterization of bacterial xylanase in *E. coli*. The most significant part of this study is the expression of xylanase in extracellular medium by *E. coli* and maximum activity was found 80IU/ml, possibly due to induced expression. Further studies on the expressed xylanase for bio-bleaching may give promising results for its application in bio-bleaching of kraft pulp as in this study enzyme was found active in alkaline pH and at high temperature required in the bleaching process of Kraft pulp during the paper making process.

Fig.1: Incubation time optimization

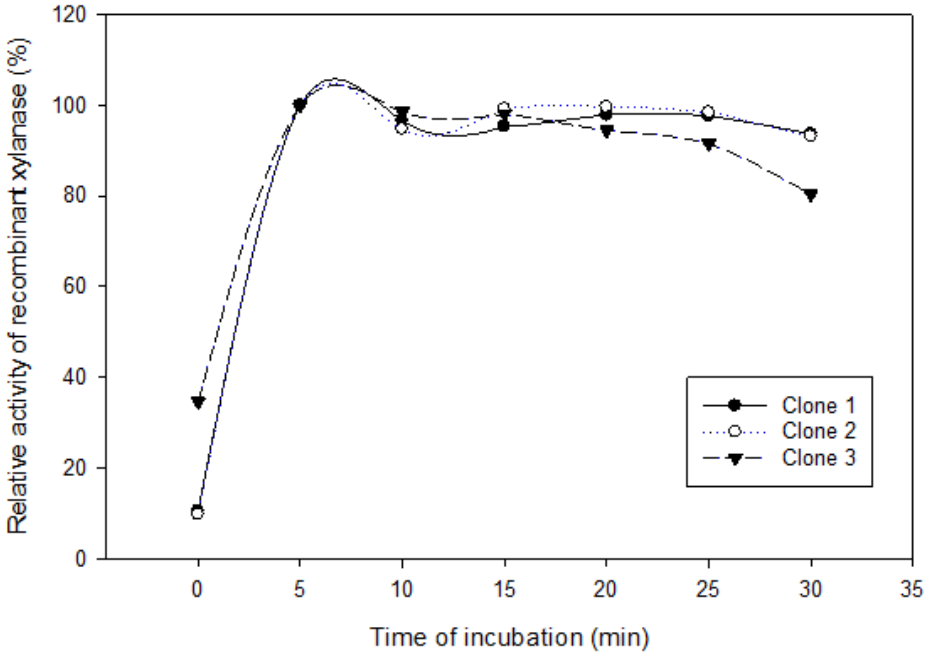


Fig. 2: IncubationTemperature optimization of Xylanase activity

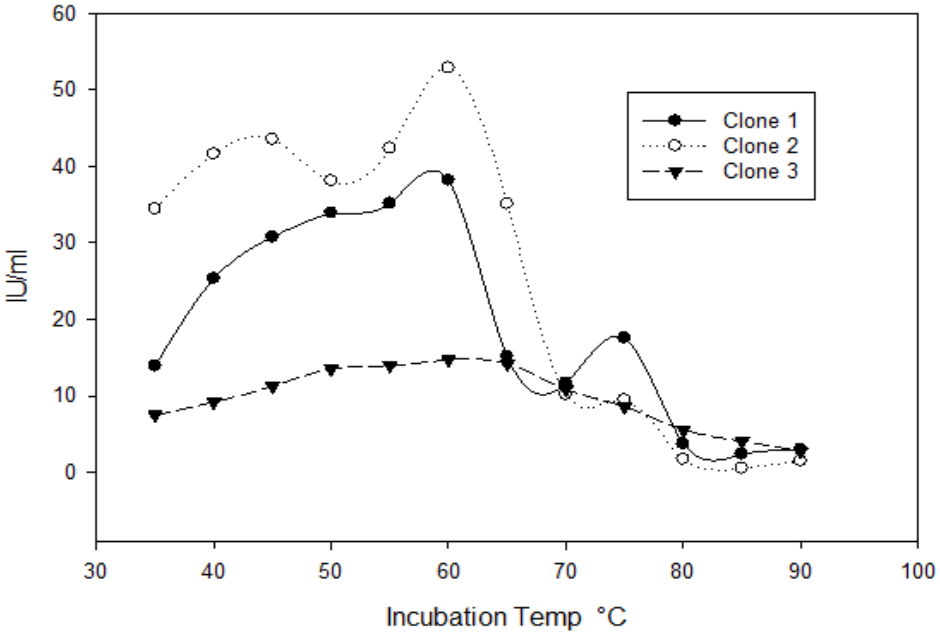


Fig. 3: Optimization of pH for Xylanase activity

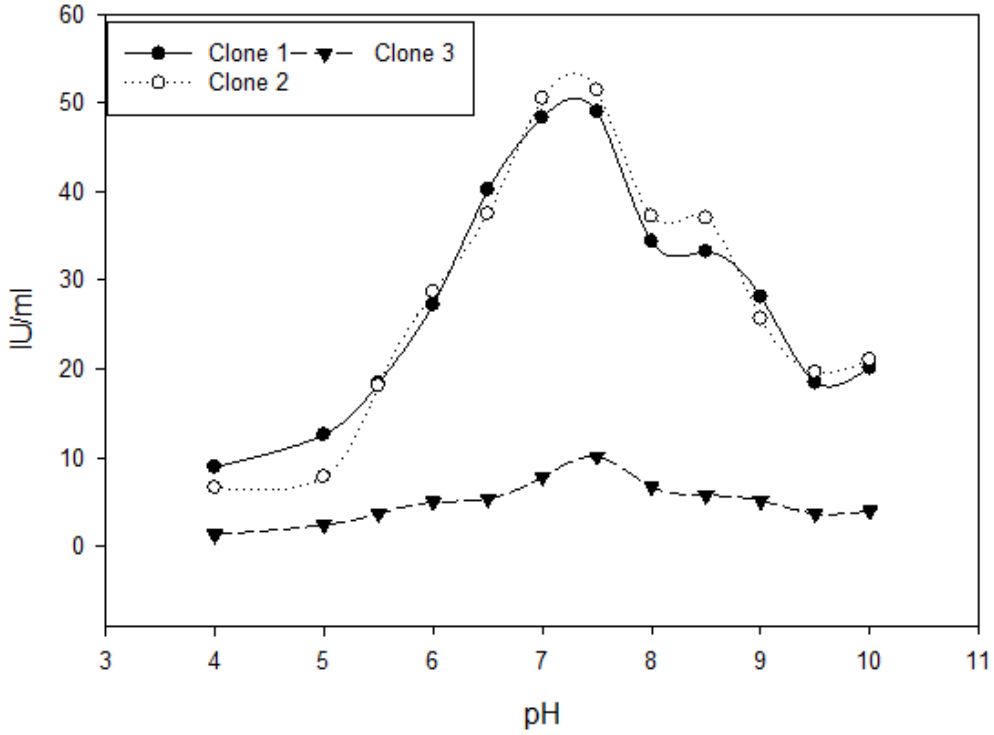


Fig. 4: Effect of Substrate concentration on Xylanase activity

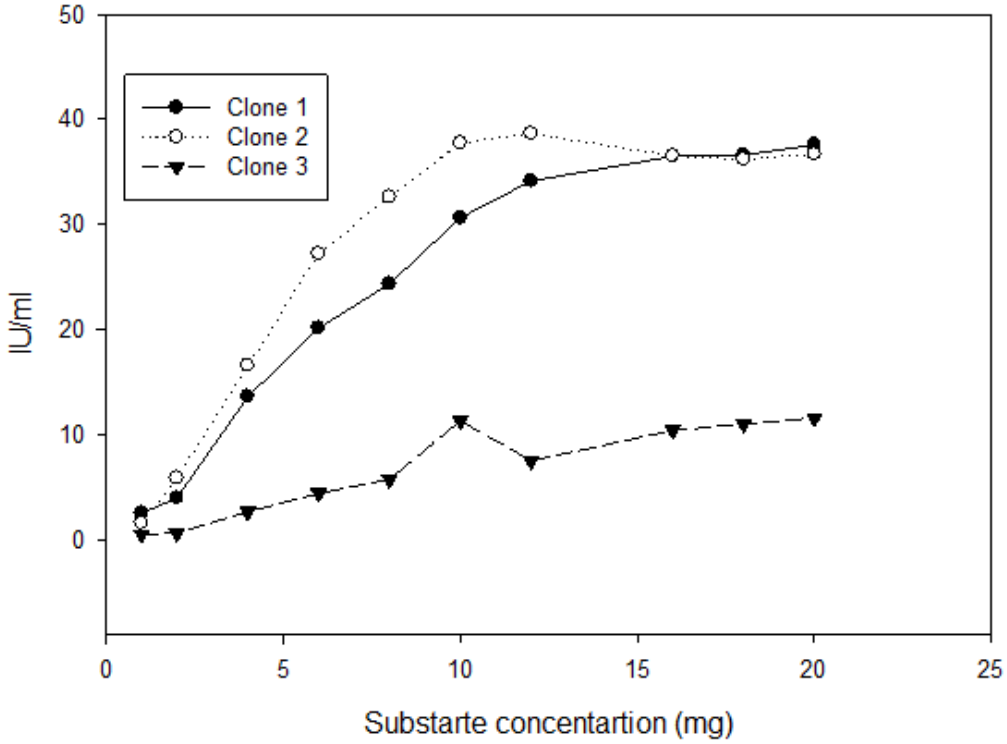


Fig. 5: Thermal stability of xylanase at 60 °C

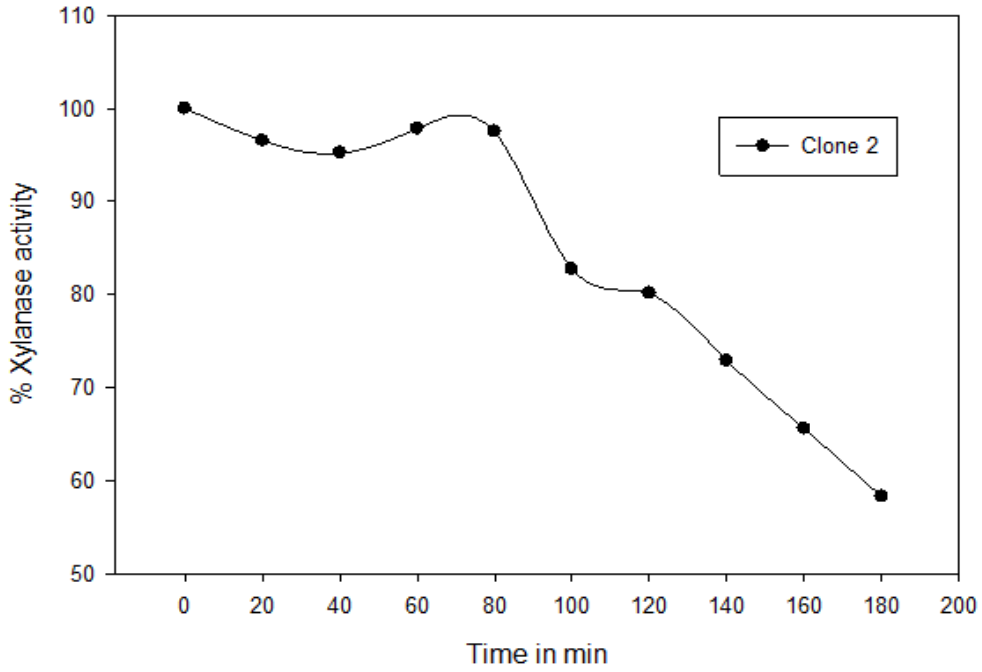
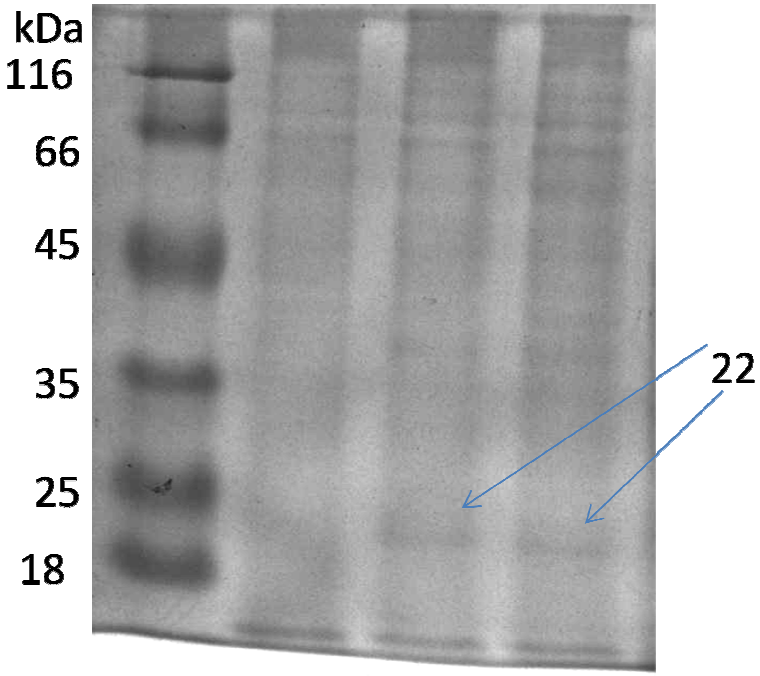


Fig. 6: SDS-PAGE



Lane1- Protein marker, Lane 2- Xylanase sample of clone no. 1,
Lane 3- Xylanase sample of clone no. 2, Lane 4- Xylanase sample of clone no. 3.

CONCLUSION

The present study was centered on expressing and optimizing the xylanase for various parameters necessary for the enzyme activity. Recombinant xylanase enzyme showed very good activity in alkaline pH and high temperature (60 °C) required of application of xylanase to be used as bio-bleaching agent in the pulp and paper industry. In future the enzyme could be exploited to study its effect on the kraft pulp as a bio-bleaching agent.

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