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CLONING AND EXPRESSION OF *CHAETOMIUM THERMOPHILUM* XYLANASE 11-A GENE IN PROKARYOTE

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The xylanase gene was cloned into pET32a(+) and expressed in *E. coli* BL21 under T7 promotor alongwith fusion protein. The SDS-PAGE and western blot analysis showed a protein of ~42 kDa. The best expression of xylanase enzyme was found by using xylose as carbon source and lactose as an inducer. The maximum activity of xylanase expressed in *E. coli* was 6.02 U/mL in the presence of 2 % xylose in DS medium. The activity of recombinant xylanase was observed on 1 % xylan LB agar plates, showed halos of xylan clearance when lactose was used as an inducer.

Keywords: Thermophilic fungi, Xylanase, Cloning and gene expression, *Cheatomium thermophilum*

1. Introduction

Xylanase has many industrial applications, including the conversion of lignocellulosic material to fuels and chemicals [1] and the processing of hemi-cellulose to paper [2], nutritional improvement of foods (e.g. clarification of juices; making bread fluffier; separation of wheat or other cereal gluten from starch), increasing animal feed digestibility [3], biobleaching of pulp in the pulp and paper industry, biopulping [4], nutritional improvement of lingo-cellulosic food stock, production of ethanol, methane and other products and in processing of food [5]. During the process of pulp bleaching, xylanases are used instead of chlorine to increase the extractability of lignin for the production of high quality paper [6]. The use of xylanase to either replace or reduce the amount of chlorine used in pulp bleaching would have a strong positive effect on the environmental impact of the process.

Asperigillus niger and *Trichoderma ressei* produce xylanase [7] enzyme that have applications in poultry feed and also in paper and pulp industry. The yeast *Saccharomyces cerevisiae* can neither utilize nor degrade xylan [8], but it possesses a number of attributes that render it an attractive host for the expression and production of β-xylanases [9]. *Aspergillus kawachii* is a common fungus used in the fermentation of *Shochu* and this strain makes a large quantity of citric acid and simultaneously produces many interesting acid-stable enzymes including several xylanases and cellulases. Xylanase A encoded by Xyn A gene which is a major component of the xylanase family of *A.kawachii* was also studied [10]. Fungi are the most common industrial sources for hemi-celluloses such as glucanases, xylanases, galactanases, mannases, galactomanases and pentosanases.

Xylanases are commonly induced with cellulases and secreted into the medium. Geographical isolates of the same thermophilic fungus may differ in enzyme productivity and in structural and biochemical properties of xylanases [11, 12]. Some thermophilic fungi produce multiple forms of xylanases that differ in molecular size, stability, adsorption or activity on insoluble substrates [13]. They are generally single-chain glycoproteins, ranging from 6 to 80 kDa, active between pH 4.5 to 6.5, and at temperatures between 55° to 65° C.

Thermophilic fungi are a small assemblage in mycota that have a minimum temperature of growth at or above 20ºC and a maximum temperature growth extending upto 60-62ºC. Some extra cellular enzymes from thermophilic fungi are being produced commercially and a few others have commercial prospects. Genes of thermophilic

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fungi encoding lipase, protease, xylanase and cellulose have been cloned and over expressed in heterologous fungi [14]. *Chaetomium thermophilum* is a thermophilic fungus that produces thermostable xylanase [15]. At NIBGE there is a large collection of thermophilic fungi [16], which have potential application in the poultry feed industry, paper and pulp industry. *Chaetomium thermophile* shows large amounts of extra cellular cellulase and xylanase activity when grown on cellulosic or lignocellulosic substrates as carbon sources. *Chaetomium* belongs to the kingdom fungi and phylum Ascomycota. It is a filamentous fungus frequently found in soil, air and plant debris. The major inducible endoxylanase secreted by these fungi is Xyn 11-A.

2. Materials and Methods

2.1. Strains and culture conditions

C. thermophilum was isolated from root rhizosphere of wheat at Faisalabad, Pakistan. The primers were designed to amplify the gene, *E. coli* 10b, DH5α and BL21 strains, restriction enzymes; taq polymerase and pTZ57R used in this study were purchased from MBI Fermentas. pET32a(+) expression system from Novogen, Germany was used. *E. coli* was grown in Luria-Bertini (LB) medium on rotary shaker at 100 rpm and 37 °C. Ampicillin (100 µg/mL) was added for the selection of pTZ57R and pET32a(+). Oat spelt xylan from Sigma was used as substrate for the enzyme assay.

2.2. Isolation and amplification of xylanase gene

Xylanase (Xyn 11-A) gene was isolated from *Chaetomium thermophile* strain NIBGE-1. A set of primers was designed based on the reported sequence from Genebank nucleotide sequence database accession no. AJ508931. Specific primers Xyn 11-A (F) and Xyn 11-A (R) as shown in Table 1 were designed to amplify an 860 bp DNA fragment. This fragment was cloned into T/A cloning vector pTZ57R (MBI Fermentas). The cloned gene was sequenced from Microsynth GmbH, Switzerland. The sequence was submitted to Genebank and assigned accession no.

Table 1. A set of primers for cloning Xylanase gene.

Xyn 11-A (F)	5´-CGATAGCTAGCATGGTCAACTTCTCA AC TCTC-3´ (34 MERS)
Xyn 11-A (R)	5´-GGAAGGGCCCGCACTGCATGCTTGT TA GC-3´(29 MERS)
Xyn 1 (P_1)	5´-GCATGGTCAACTTCTCAACTC-3´ (21 MERS)
Xyn 3 (P_3)	5´-AGACTCGAGTCGAACCCCGGTATCG AC-3´ (27 MERS)

AY366479. Sequencing and characterization of gene information revealed a 35 bp intron with two exons in the isolated gene fragment. The gene sequence AY366479 was compared with other xylanase genes from other organisms using online software. Intron was removed by amplifying the insert alongwith vector backbone except intron by using primers P_3 and P_4 as indicated in Table 1. This gene was named as Xyn 11-A, cloned in T/A vector pTZ57R. The construct was named as pSSZ810(a) as given in Fig. 1.

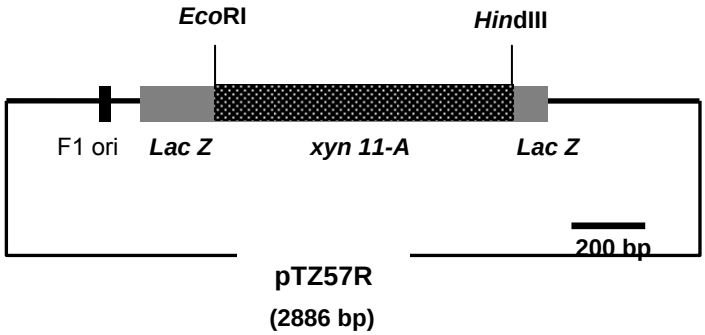

Figure 1. Recombinant clone pSSZ810(a) with *Eco*RI and *Hin*dIII.

2.3. Cloning of recombinant xylanase into pET32a(+)

Cloned Xyn 11-A gene from pTZ57R with *Eco*RI and *Hin*dIII, fragment was eluted from 0.5 % agarose gel and ligated into restricted pET32a(+) with similar enzymes. Transformed into *E. coli* 10b through heat shock at 42 °C. The transformants were selected on LB medium plates supplemented

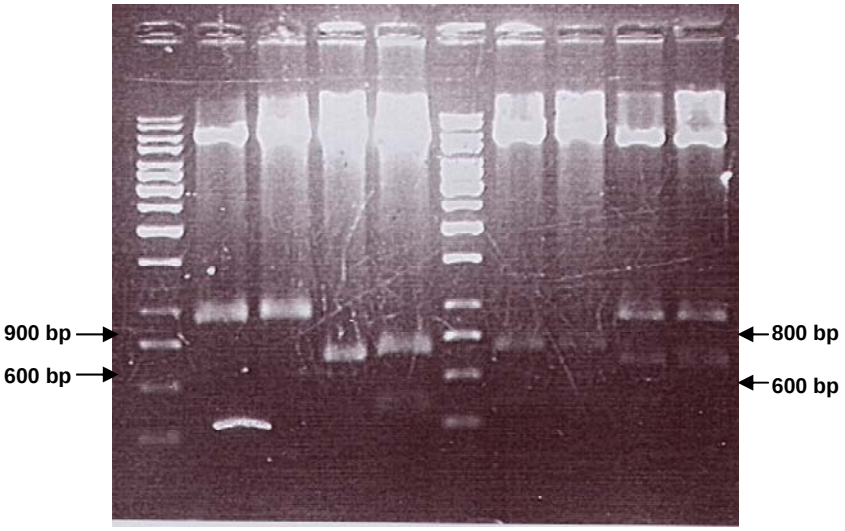

Figure 2. Confirmation of cloning of xylanase gene in pET 32a(+) through restriction/digestion with combination of enzymes Lane 1 and 2: pET 32a(+) digestion with *Hin*dIII and *Eco*R1, Lane 3: pET 32a(+) digestion with *Hin*dIII and *Xho*1, Lane 7 and 8: pET 32a(+) digestion with *Hin*dIII and *Xba*1, M: 1 Kb DNA ladder.

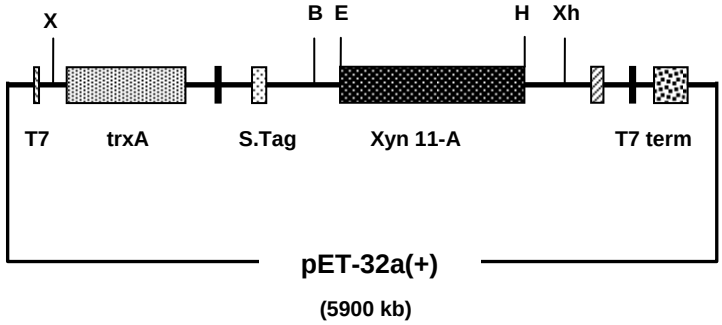

Figure 3. Recombinant clone pSSZ810(b) with restriction sites. E: *Eco*RI; H: *Hin*dIII; B: *Bam*HI; X: *Xba*I; Xh:*Xho*I; T7: T7 promoter T7 term: T7 terminater.

with 100 mg/mL ampicillin. The construct was confirmed through restriction/digestion analysis with *Eco*RI, *Hin*dIII, *Xho* I and *Xba* I as indicated in Fig. 2.

2.4. Expression of recombinant xylanase 11-A in E. coli

The resultant recombinant clone pSSZ810(b) as represented in Fig. 3 was transformed into BL21 through heat shock at 42 °C. The transformants were selected on LB medium plates supplemented with 100 mg/mL ampicillin and choloroamphenicol 34 mg/mL. The integration of transforming DNA was verified through PCR analysis. The colonies were cultured in LB liquid media supplemented with ampicillin (100 mg/ml) and 34 mg/ml chloroamphenicol. Two ml of overnight culture was transferred to 50 ml LB broth medium with ampicillin and chloroamphenicol and grown for further 3 hours at 37 °C. After three hours growth, 70 μl of (1 mM) IPTG was added which act as inducer. The culture was equally divided into 9 test tubes. The cells were incubated at 37 °C in a shaker and allowed to grow for 2.5 hours. Samples were taken after each 0.5 hours. Cells were pelleted down and given freeze thaw treatment. Cell lysate was dissolved in 1 ml citrate buffer and sonicated for complete lysis. The lysate was centrifuged at 13,500 rpm and supernatant was used for further analysis.

2.5. Xylanase activity assay

The xylanase activity from *E. coli* was assayed against oat spelt xylan by the method described [17]. Two mL of reaction mixture containing one mL of cell lysate, 0.5 mL of 1 % oat spelt xylan and

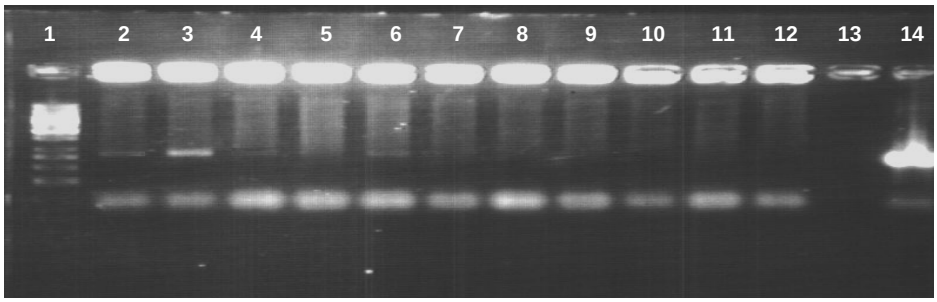

Figure 4. Agarose gel electrophoresis of pSSZ810(b) in *E. coli* BL21 strain through PCR analysis using xylanase specific primers.Lane 1: 1 kb DNA ladder, Lanes 2- 9: selected transformed BL21 colonies containing pSSZ810(b), Lanes 10 – 12: transformed BL21 colonies containing pET 32a(+), Lane 13: water control, Lane 14: positive control.

0.5 mL of citrate phosphate buffer (pH 6) was incubated at 40 °C for 2 hrs at 60 rpm. The reducing sugar was determined by dinitrosalicylic acid (DNS) procedure.

2.6. Selection of transformants on 1 % LB xylan plates

One hundred µL of cell lysate were poured on LB 1 % xylan plates incubated at 45 °C for 2 days to observe the size of clearing zones.

2.7. HPLC (High Performance Liquid Chromatography)

The concentration of free xylose liberated from xylan after enzyme activity was also determined by HPLC with refractive index detection using Aminex HPX-87H column (Biorad) and 0.001 NH_2SO_4 as mobile phase. Samples were filtered through a 0.2 µm filter and 20 µL were injected into auto sampler vial of HPLC. The flow rate was 0.6 mL/min and column temperature was 45-65 °C. The peaks produced were compared to standard xylose peaks the concentration of free xylose was calculated with the help of linear regression equation.

2.8. SDS-PAGE analysis

The supernatant fluid from cell lysate of selected cultures of *E. coli* were separated by SDS-PAGE on 10 % and visualized by coomassie blue staining.

2.9. Western blot analysis

15 µg of protein from both transformed and non-transformed *E. coli* cells were run on 15 % SDS-PAGE gels and transferred to nitrocellulose paper. The polyclonal antibodies, raised against fusion part of the protein, conjugated to alkaline phosphatase detected bands that bound antibodies.

3. Results and Discussion

3.1. Recombinant xylanase gene expression in BL21 E. coli

The intron-less xylanase gene from pTZ57R was cloned into pET32a(+) with *Hin*dIII and *Eco*RI. The resultant recombinant clone was confirmed through combination of different enzymes and transformed into *E. coli* BL21 to get expression. The PCR confirmed transformant as shown in Fig. 4 was cultured in Dubose salt (DS) medium substituted with 2 % xylose as carbon source and the expression of enzyme was induced with IPTG and lactose. The maximum and minimum amount of total protein contents in the intracellular fluid in case of IPTG induction was 0.663 and 0.378 mg/mL as compared to lactose induction, the maximum and minimum amount of protein was 0.598 and 0.355 mg/mL respectively.

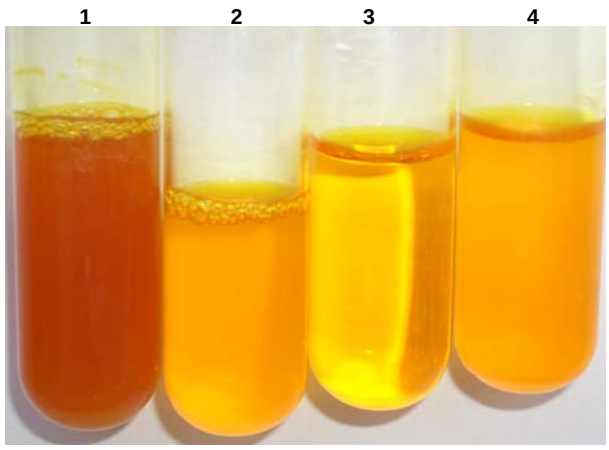

Figure 5. Xylanase enzyme assay to measure the xylanase activity of the recombinant clone. Lane 1: *E. coli*

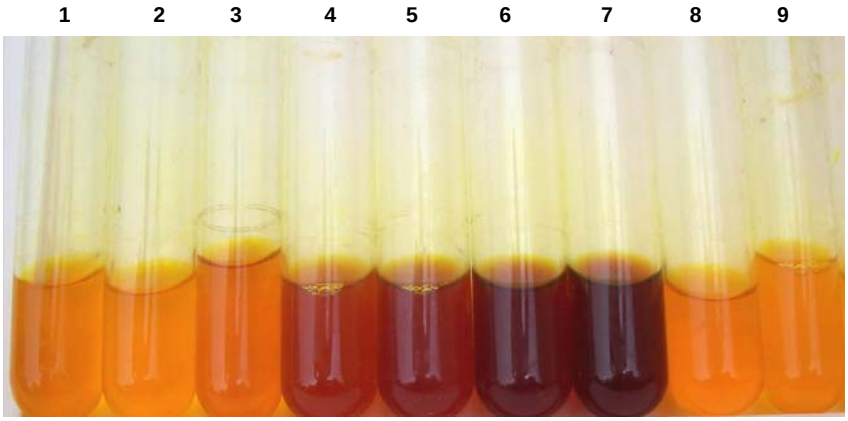

Figure 6. Xylanase enzyme assay to estimate the xylanase activity of the recombinant clone pSSZ810(b) after digestion with enterokinase enzyme. Lane 1: undigested pET 32a(+) control, Lane 2: digested pET 32a(+) control, Lane 3: Enzyme control, Lane 4 and 5: Digested pSSZ810(b), Lane 6 and 7: Undigested pSSZ810(b), Lane 8: Water control (blank), Lane 9: Substrate control.

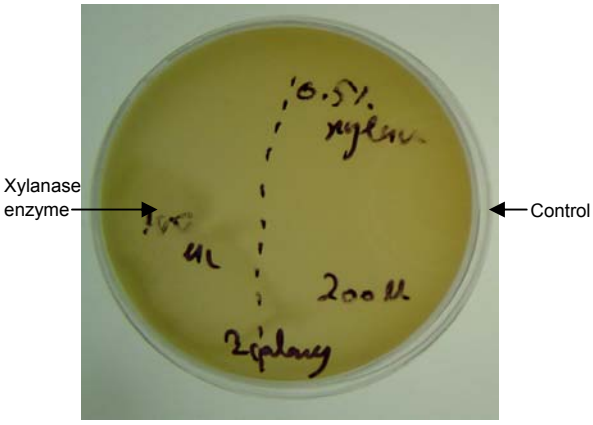

Figure 7. Xylanase activity of *E. coli* strain BL21 having pSSZ810(b) on 1 % xylan LB agar plates.

having pSSZ810(b), Lane 2: Substrate control, Lane 3: Water control (blank), Lane 4: Enzyme control

3.2. Optimization of expression of recombinant Xyn 11-A in E. coli

The maximum and minimum activity of xylanase 4.62 and 3.99 U/mL was obtained after incubation with xylan for 2 hrs at 40 °C as compared to control as shown in Fig. 5. The maximum and minimum activity of digested xylanase protein was 0.387 and 0.345 U/mL respectively whereas the maximum and minimum activity of undigested xylanase protein was 3.99 and 3.48 U/mL as indicated in Fig. 6 as compared to the cloning of the gene was done in *Bacillus subtilis* using a shuttle vector pHB 201, which resulted in increasing the basal level xylanase activity from 14.02 to 22.01 U/mL [18]. The fusion protein did not increase enzyme activity rather it decreased the activity (data not shown), which may be due to the presence of enterokinase site within the expressed recombinant xylanase as represented in Fig. 6.

3.3. Selection of transformants on 1 % xylan LB agar plates

The activity of recombinant xylanase was observed on 1 % xylan LB agar plates which show halos of xylan clearance similarly as the positive clones were screened on the selected LB agar plates supplemented with xylan by Congo-red staining method [19]. Moreover, higher xylanase activity for clearance of xylan was obtained when lactose was used as an inducer as indicated in Fig. 7.

3.4. HPLC analysis of free xylose

The amount of xylose liberated from xylan was determined by HPLC analysis alongwith standard concentration of 1 % xylose. The maximum

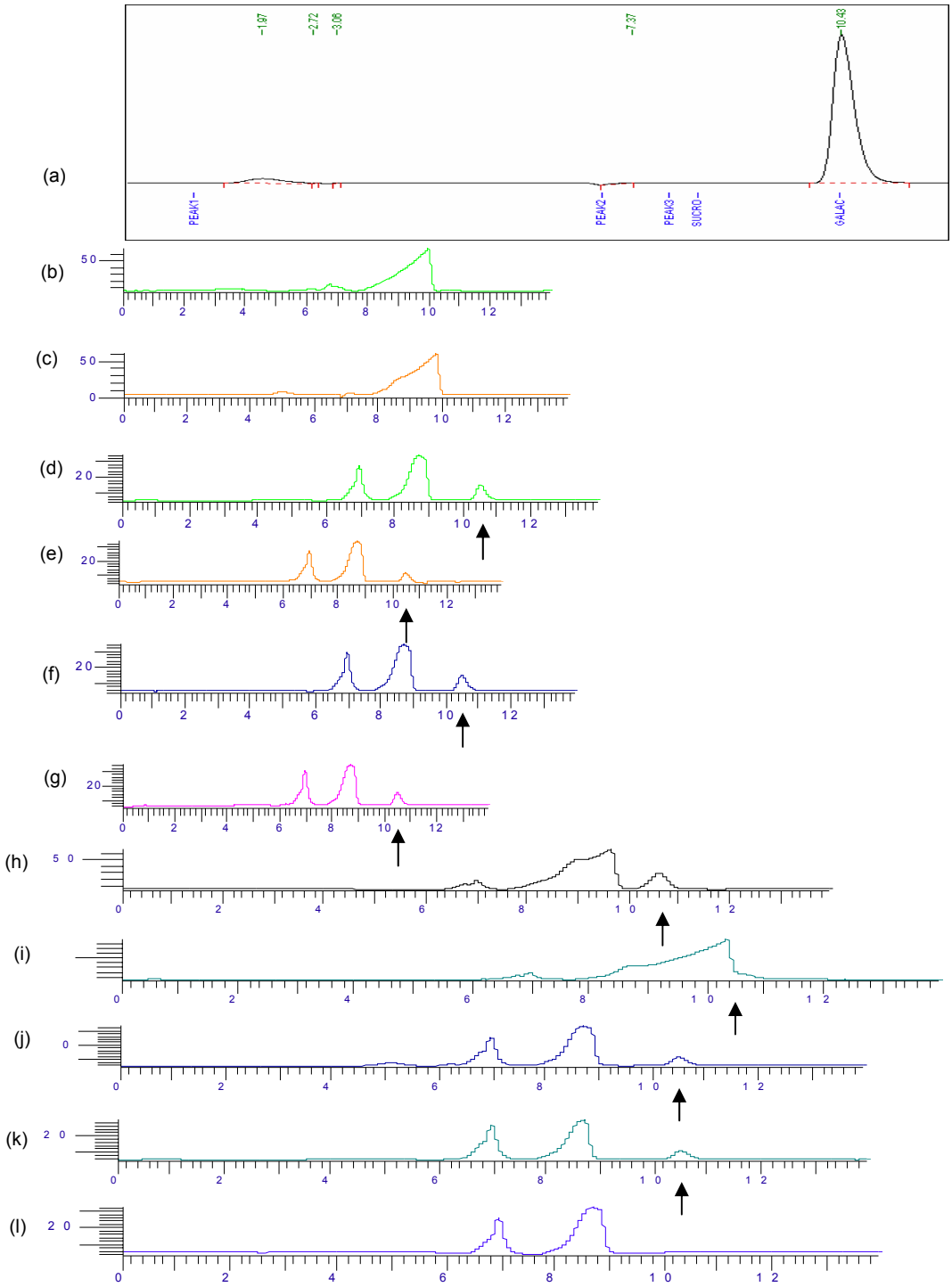

Figure 8 HPLC analysis for xylanase detection. (a) Standard 1% xylose, (b) Substrate control, (c) Enzyme control, (d), (e), (f), (g) Samples induced by 1 mM IPTG, (h),)i), (j), (k) Samples induced by 1 mM lactose, (l) pET 32a(+) control. Represent Xylose.

amount of xylose was produced when 1mM lactose was used as inducer as compared to 1mM IPTG as shown in Fig. 8.

3.5. SDS-PAGE analysis for xylanase protein induced by IPTG inducers

The production of recombinant xylanase by *E. coli* BL21 was indicated by the appearance of a 43

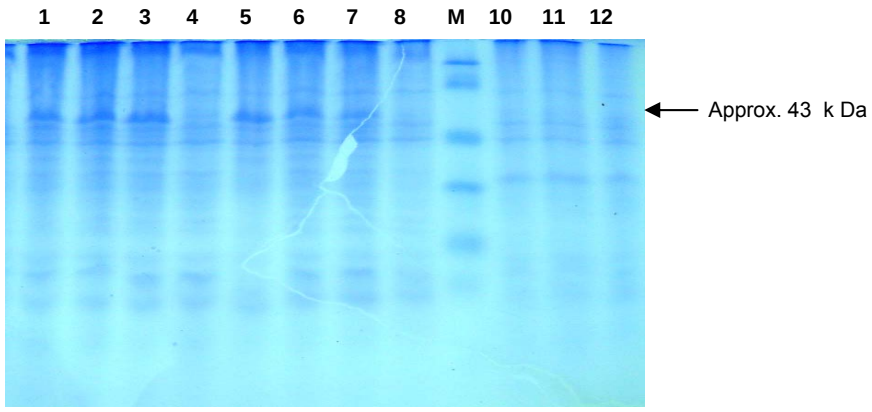

Figure 9 SDS-PAGE analysis of total protein isolated from bacterial strain BL21 transformed with pSSZ810(b) and pET 32a(+). Lanes 1-8: pSSZ810(b) transformed BL21. Lanes 10-12: pET 32a(+) transformed BL21. From right to left in lanes 12, 8 and 4: protein induced after zero minute, Lanes 11, 7 and 3: protein induced after 30 minutes, Lanes 10, 6 and 2: protein induced after 60 minutes, Lanes 5 and 1: protein induced after 90 minutes. M: represent prestained protein marker #SM0441 118 k Da (MBI Fermentas).

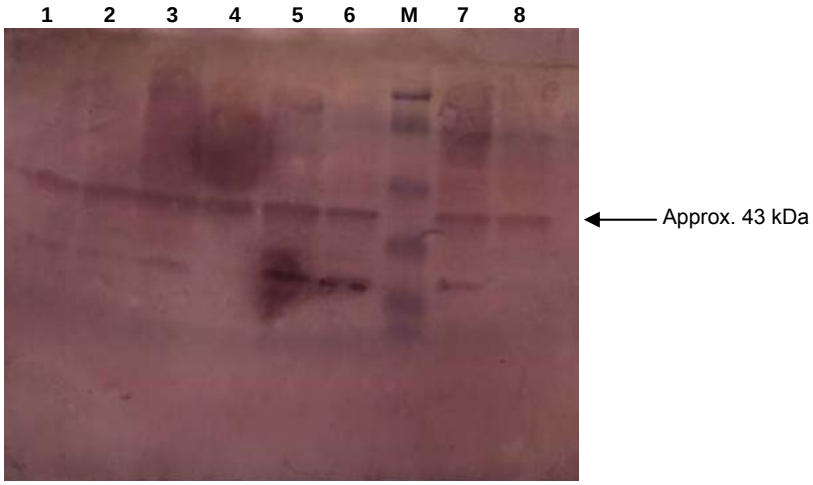

Figure 10. Western blotting of xylanase gene pSSZ810(b) vector transformed in *E. coli* BL21 strain. From left to right Lane 1, 2 and 3: Sample 1-4 (induced by 1 mM IPTG + DS + pSSZ810(b) + 2 % xylose), Lane 4: Sample 5 (induced by 1 mM IPTG + DS + pET 32a(+) + 2 % xylose), Lane 5 and 6: Sample 7 & 8 (induced by 1 mM lactose + DS media + pSSZ810(b) + 2 % xylose), Lane 7 and 8: Sample 9 & 10 (induced by 1 mM lactose + DS media + pSSZ810(b) + 2 % xylose). M: represents prestained protein marker #SM0441 118 k Da (MBI Fermentas).

kDa protein on SDS-PAGE. Maximum activity of xylanase was observed in case induction with lactose as compared to IPTG (data not shown). The amount of recombinant protein was increased with different induction times as indicated in Fig. 9.

3.6. Western blotting of recombinant xyalnase

Western blot analysis was carried out by using polyclonal antibodies raised against fusion protein. The western blot of fused Xyn 11-A protein of approx. 43 kDa is shown in Fig. 10.

4. Conclusion

The polypeptide consisted of 270 amino acid residues with a calculated mass of 29 KDa, which is very close to the value of 25 KDa determined for APX-II [20]. The C-terminal region is the glycine rich region, which is absent in other fungi. The hyper activity of this protein in comparison to xylanases from other sources may be because of this region and we also suggest that this region protect the active site from the protease activity and as a result enhance the activity of this enzyme

in comparison to other enzymes. Hence exact function is yet to be known.

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