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

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The various thermophilic fungi like *Chaetomium thermophile* has potential to secrete xylanase and cellulase enzymes. In the present study eukaryotic expression system of *Pichia pastoris* (yeast) was used to express xylanase gene. The xylanase (Xyn 11-A) gene was isolated from C. *thermophile* strain NIBGE-1. Primers were designed to amplify the gene, ligated into *P. pastoris* pPIC3.5K vector, the resultant recombinant clone pSSZ810 was transformed into the genome of *P. pastoris* GS115 strain through electroporation. Transformants were selected on yeast peptone dextrose medium (YPD) plates containing antibiotic geneticin (100 mg/mL) upto final concentration of 0.75 mg/mL. The maximum activity of xylanase 2.04 U/mL after incubation of 2 hrs at 50ºC was observed in the presence of 100% methanol inducer upto final concentration of 30µL (0.5%) as compared to control. HPLC analysis represented high peak of xylose as compared to control. SDS-PAGE indicated approx. 28 kDa protein of expressed xylanase gene.

Keywords : Thermophilic fungi, Xylanase, Cloning and gene expression, *Pichia pastoris*

1. Introduction

Xylanase enzyme that degrade xylan has many important applications in various industries such as the conversion of lignocellulosic material to fuels and chemicals [1] and the processing of hemi-cellulose to paper [2], nutritional improvement of foods, increasing animal feed digestibility [3], biobleaching of pulp in the pulp and paper industry, biopulping [4], production of ethanol, methane 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 production of thermostable cellulases and xylanases from thermophilic fungi is an important industrial source for hemi-cellulases such as glucanases, xylanases, galactanases, mannases, galactomanases and pentosanases. *C. thermophilum* is a thermophilic fungus that produces thermostable xylanase [7]. *Chaetomium* is a filamentous fungus frequently found in soil, air and plant debris and belongs to the kingdom Fungi and phylum Ascomycota. The major inducible endo-xylanase secreted by these fungi is Xyn11- A. Enhanced enzyme production can be achieved by isolation, characterization, cloning and expression of the genes under specific strong promoters and enhancer elements.

P. pastoris is the methylotrophic eukaryotic yeast, has been developed to be an outstanding host for the production of foreign proteins since its alcohol oxidase *AOXI* promoter was isolated and cloned; its transformation was first reported in 1985 [8, 9]. Compared to other eukaryotic expression systems, *Pichia* offers many advantages, because it does not have the endotoxin problem associated with bacteria, nor the viral contamination problem of proteins produced in animal cell culture. Furthermore, *P. pastoris* can utilize methanol as a carbon source in the absence of glucose. In *P. pastoris* expression system, the methanol-induced alcohol oxidase (*AOX1*) promoter was used, which controls the gene that codes for the expression of alcohol oxidase, the enzyme which catalyzes the first step in the metabolism of methanol. This promoter has been characterized and incorporated into a series of *P. pastoris* expression vectors. Since the proteins produced in *P. pastoris* are typically folded correctly and secreted into the medium, the fermentation of genetically engineered *P. pastoris*

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provides an excellent alternative to *E. coli* expression systems. A number of proteins have been produced using this system, including tetanus toxin fragment, Bordatella pertussis pertactin, human serum albumin and lysozyme. [10, 11].

The isolation and purification of a foreign protein product is done by growing *P. pastoris* on a simple mineral media and it does not secrete high amounts of endogenous protein. Therefore, the heterologous protein secreted into the culture is relatively pure and purification is easier to accomplish [12]. Secretion of the foreign protein is accomplished by recombining a signal sequence in front of the desired foreign gene when it is inserted into the host DNA.

In the past the methylotrophic yeast *P. pastoris* has developed into a highly successful system for the production of a variety of heterologous proteins. The increasing popularity of this particular expression system can be attributed to several factors, most importantly: (1) the simplicity of techniques needed for the molecular genetic manipulation of *P. pastoris* and their similarity to those of *Saccharomyces cerevisiae*, one of the most well-characterized experimental systems in modern biology; (2) the ability of *P. pastoris* to produce foreign proteins at high levels, either intracellularly or extracellularly; (3) the capability of performing many eukaryotic post-translational modifications, such as glycosylation, disulfide bond formation and proteolytic processing; and (4) the availability of the expression system as a commercially available kit [13].

At NIBGE, there is a large collection of thermophilic fungi [7] which have potential applications in the poultry feed industry and paper and pulp industry. *C. thermophile* shows large amounts of extra cellular cellulase and xylanase activity when grown on cellulosic or lignocellulosic substrates as carbon sources [14]. In the present studies efforts were made to isolate xylanase (Xyn 11-A) gene from *C. thermophile* strain NIBGE-1 and clone the xylanase gene in yeast model system. In this context the expression model system of *P. pastoris* is selected (Invitrogen, USA). *P. pastoris* has many advantages of higher eukaryotic expression systems such as protein processing, protein folding, and post-translational modification. It is faster, easier, and less expensive to use than other eukaryotic expression systems such as baculovirus or mammalian tissue culture, and generally gives higher expression levels.

2. Materials and Methods

2.1. Isolation and amplification of Xylanase (Xyn 11-A) gene from Chaetomium thermophile

The xylanase (Xyn 11-A) gene was isolated from *Chaetomium thermophile* strain NIBGE-1 and a set of Specific primers Xyn 11-A (F) 5´- GGC GAT AGC TAG CAT GGT CAA CTT CTC AAC TCTC -3´(34 mers) and Xyn 11-A (R) 5´- GGA AGG GCC CGC ACT GCA TGC TTG TTA GC -3´were designed to amplify an 860 bp DNA fragment based on the reported sequence from Genebank nucleotide sequence database accession no. AJ508931. This fragment was cloned into T/A cloning vector pTZ57R (MBI Fermentas) and cloned was sequenced from Microsynth GmbH, Switzerland. The sequence was submitted to Genebank and assigned accession no. AY366479 [15]. 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 5'-AGA CTC GAG TCG AAC CCC GGT ATC GAC -3' 27 mers and P_4 5'-CTG CTC GAG GCG CTG GAA ATG TTT TGT TGG -3' 30 mers.

2.2. Cloning and transformation of xylanase gene into E. coli TOP10F' strain

The intron-less 810 bp fragment of xylanase gene and pPIC3.5K *P. pastoris* vector was digested with *Eco*RI and *Not*I restriction enzymes, placed at 37 ºC for 1 hr. Digestion was run on 0.5% agarose gel, targeted fragments were eluted from the gel by using extraction Kit (MBI, Fermentas). Eluted fragments were ligated and transformed into heat shocked competent cells of *E. coli* TOP10F' strain [16]. Colonies were selected randomly from overnight grown *E. Coli* in LB agar medium containing ampicillin (100 mg/mL). Plasmid isolation was done by miniprep method (MBI, Fermentas). The resultant recombinant clone was confirmed through combination of different restriction enzymes i.e. *Eco*RI, *Not*I and *Kpn*I.

2.3. Transformation and screening of resultant recombinant clone into Pichia pastoris

The resultant recombinant vector pSSZ810 was linearized with *Not*I restriction enzyme, transferred in the cuvette and electric shock was given at 2.0 kvolts for integration in the genomic DNA of *P. pastoris*. Then immediately 1ml 1M sorbitol was added and placed the cuvettes on shaker at 30°C. After 2 hour the medium was spread on the different concentrations of geneticin YPD agar medium plates for the selection of transformants. These concentrations were 0.25, 0.5, 0.75, 1.0 and 1.25 mg/mL respectively, transferred these plates to incubator at 30 °C until colonies appeared. Colonies were picked from 0.75 mg/mL geneticin plate and cultured into YPD liquid medium without antibiotic, incubated at 30 °C till the time that cell density reaches upto (OD_{600} =1.0). The transformants were confirmed through PCR analysis by using set of primers. The sample was prepared for PCR reaction under denaturation 94 °C for 5 min, annealing at 60 °C for 1 min, extension 72 °C for 1 min and 35 no. of cycles, as 10µl of *P. pastoris* culture into 1.5 ml microcentrifuge tube, added 5µl zymolyase enzyme and incubated at 30°C for 10 minutes. The sample was frozen at -70 °C for 10 minutes.

2.4. Optimization of Pichia pastoris growth for xylanase assay HPLC and SDS-PAGE analysis

P. pastoris transformants having pSSZ810 were picked from 0.75 mg/ml geneticin YPD agar media plates alongwith non-transformant GS115 as a control and cultured into YPD broth media without antibiotic at 30 ℃ (OD_{600} = 1.0). Harvested cells by centrifugation at 3000 rpm for 5 minute at room temperature. Discarded supernatant and resuspended cell pellet into 25 ml minimal glycerol medium (MGM) and 0.02% 10 X Dextrose in a 100 ml flask. Cultures were placed at 28-30 ℃ in a shaking incubator (150 – 200 rpm) until growth was reached log phase. Once the cells are in log phase, they can be induced for xylanase expression. Take 1 ml culture before each induction of 100 % methanol to a final concentration of 30 µl in 25 ml MG medium. Induced culture was collected at different time intervals i.e. 24, 48, 72, 96 and 120 hrs, respectively. Transfered 1 ml of expression culture into 1.5 microcentrifuge tubes. These samples were used to analyze expression levels and determine the optimal time from post-induction to harvest. Centrifuged cells at 13,500 rpm at room temperature for 2-3 minutes. For intracellular and secreted expression, both supernatant and pellet was stored at -70 °C until ready for protein assay.

2.5. Preparation of samples for xylanase assay, HPLC and SDS-PAGE

The activity of xylanase was determined by the method described by [17] against oat spelt xylan. The sample was prepared for both SDS-PAGE and xylanase assay as thawed cells pellets quickly and placed on ice. Dissolved pellet in 1 ml distilled water and 100 µl Breaking buffer. Added an equal volume of acid washed glass beads (size 0.5mm). Vortex for 30 seconds, incubated on ice for 30 seconds (repeated for several times) then centrifuged at 13,500 rpm for 10 minutes. Transferred clear supernatant to a fresh 1.5 microcentrifuge tube, added 50 µl SDS-PAGE loading dye for SDS-PAGE analysis and boiled for 10 minutes at 100°C in a dry bath. Loaded 10-20 µl sample per well into SDS-PAGE gel whereas other used for xylanase assay and remaining were stored at -20°C for use in future. Electrophoresis was performed as described by using a discontinuous buffer system, for the analysis and separation of proteins. Developer solution was added to enhance AND Visualized the bands during silver staining of SDS-PAGE of xylanase Xyn 11-A protein in *P. pastoris*.

For HPLC system refractive index detector and analyzed the calibration standards, and samples by using a HPLC Biorad Aminex HPX-87H column. The other different conditions used for HPLC to detect protein were illustrated as, Sample volume: 20 µl, dependent on sample concentration and detector limits, Mobile phase: 0.001 N sulfuric acid, 0.2 µm filtered and degassed, Flow rate: 0.6 ml/ minute, Column temperature: 45 – 65 °C, Detector: refractive index, Run time: 12 minutes.

3. Results and Discussion

3.1. Confirmation of recombinant clone pSSZ810 through restriction analysis and transformation into GS115 Pichia pastoris strain

Xylanase gene was digested with *Eco*RI and *Not*I enzymes and cloned at the same sites in pPIC3.5K. The recombinant clone pSSZ810 (Fig.1) was confirmed through digestion analysis. Upon

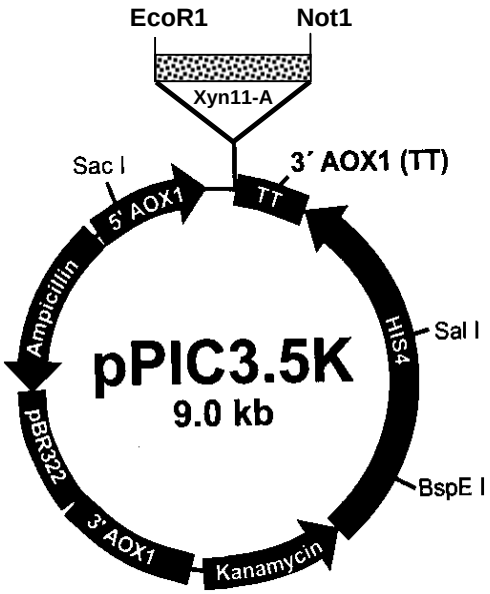


Figure 1. Recombinanat clone pSSZ810 having *Eco*RI and *Not*I restriction sites.

digestion with *Eco*RI and *Not*I, recombinant clone pSSZ810 produced (Approx) 810 bp fragment alongwith vector backbone of pPIC3.5K of 9000 bp (Fig. 2). pSSZ810 recombinant clone has two *Kpn*I restriction sites, an internal and another *Kpn*I site is located in the vector backbone, therefore; upon digestion with *Kpn*I it produced two fragments of sizes 500 and 300 bp, respectively as shown in Fig. 3.

Table 1. Xylanase activity in Pichia pastoris GS115 strain.

Time of induction*	Protein concentration (mg/mL)**			
	Sample 1	Sample 2	Sample 3	Sample 4
At zero hr.	0.234	0.159	0.177	0.196
After 24 hrs.	0.339	0.202	0.165	0.220
After 48 hrs.	0.394	0.195	0.174	0.304
After 96 hrs.	0.244	0.163	0.015	0.24

* 100% methanol inducer

** Sample 1-3 transformations having pSSZ810 xylanase gene Sample 4 transformant Pichia pastoris GS115 strain.

The purified and linearized fragment of pSSZ810 with *Not I* was transformed into *P. pastoris* strain GS115 through electroporation for integration into genomic DNA of *P. pastoris*. Concentration of geneticin was optimized for the selection of transformants from 30, 50, 70, 90, 110, and 130 µg/ml as shown in Figs. 4 to 6. Suitable concentration of geneticin antibiotic for selection of transformants was found to be 0.75 mg/mL. The transformants were grown on YPD agar media plates containing 0.75mg/ml geneticin antibiotic as

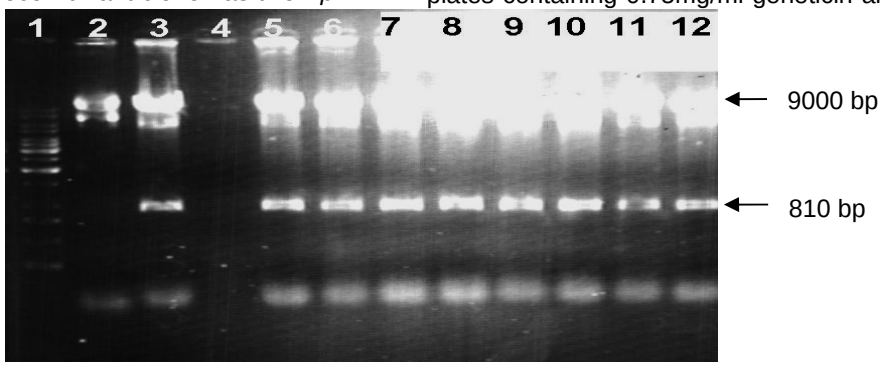


Figure 2. pSSZ810(c) was confirmed through restriction/digestion with *Eco*RI and *Not*I restriction enzymes. Lane 1: represents 1 kb DNA ladder, Lane 2- 12: selected colonies.

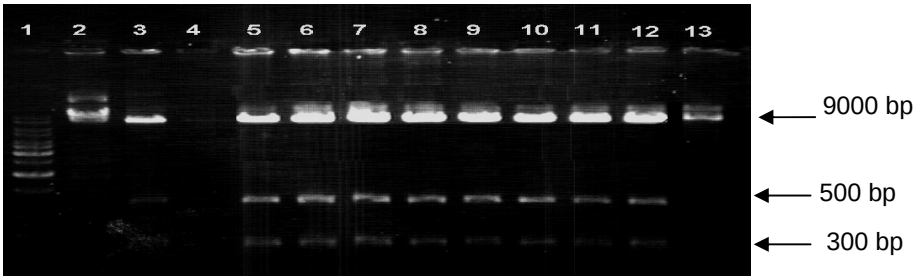
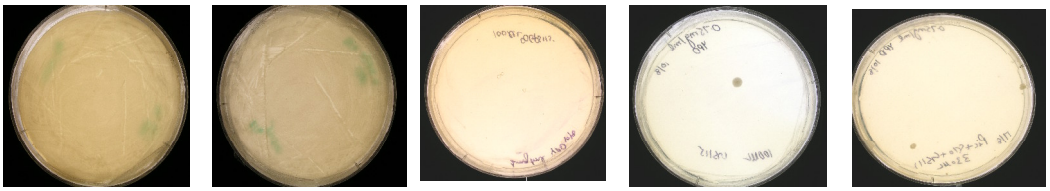


Figure 3. Confirmation of pSSZ810(c) with digestion through *Kpn* I enzyme. Lane 1 represents 1 kb DNA ladder, Lanes 2 -12 represents pSSZ810(c) with *Kpn* I

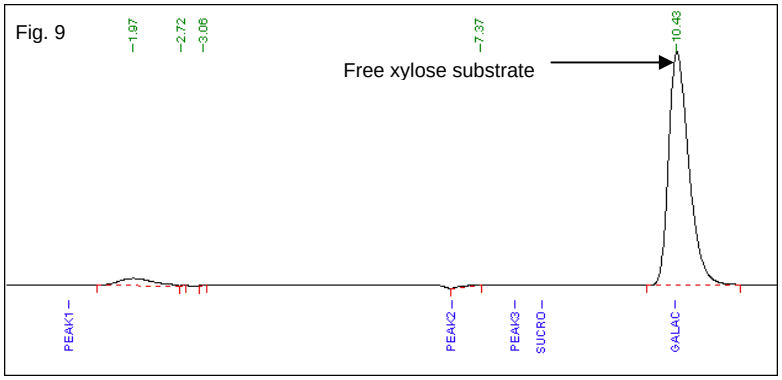
shown in Figs. 7 and 8. The integration of linearized fragment pSSZ810 having xylanase gene in the genome of *Pichia pastoris* was confirmed through PCR amplification by using xylanase specific primers.

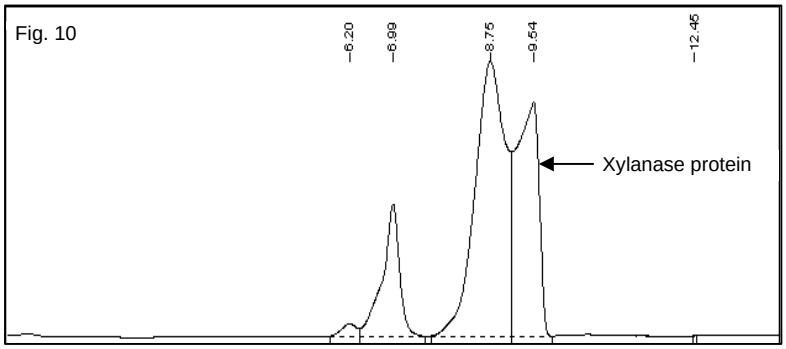
3.2. *Xylanase assay of pSSZ810 in GS115 Pichia pastoris strain*

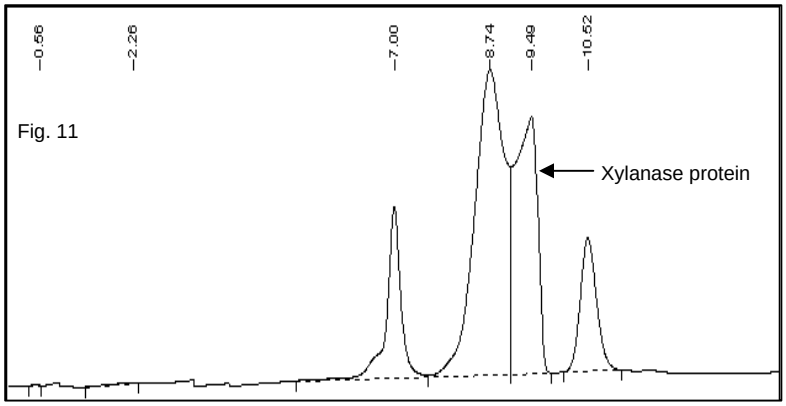
The production of the xylanase protein by recombinant *P. pastoris* GS115 strain was induced by 100 % methanol upto a final concentration of



Figures 4, 5, 6, 7 and 8 from left to right. Concentration of geneticin was optimized for the selection of transformants from 30, 50, 70, 90, 110, and 130 µg/ml.







Figures 9-11. HPLC results showed the peak of free xylose was greater in *Pichia pastoris* transformants having recombinant vector pSSZ810 as shown in Fig. 11 as compared to control in Fig 10. Fig. 9 represents free xylose peak.

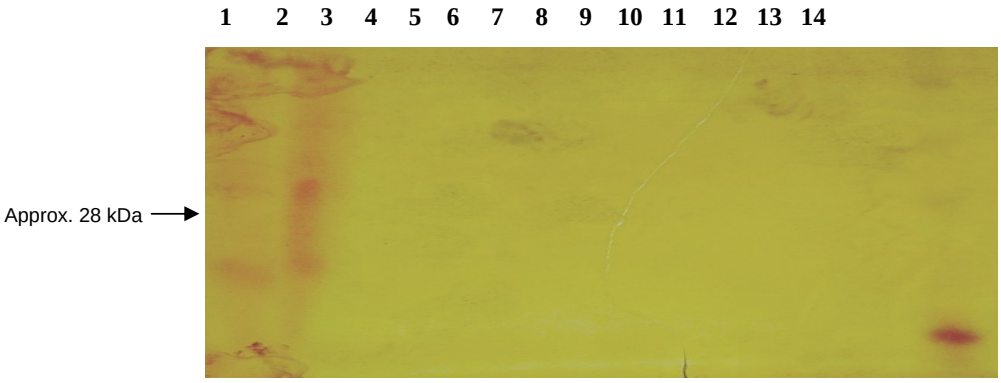


Figure 12. SDS-PAGE analysis of Xylanase 11-A gene in *Pichia pastoris* strain GS115.as indicated in lane 1 and 2.

0.5 % in Minimal Glycerol Media (MGM) broth as indicated in Table 1. The maximum and minimum production of xylanase was 2.04 and 0.006 U/mL as compared to control 0.484 and 0.06 U/mL, respectively. [18] determined the expression of Mmr-1 protein in *P. pastoris* by induction under the concentration of 0.5% methanol. The level of this recombinant protein was about 50 mg/mL which is greater than our expressed protein after 96 hrs of induction.

3.3. HPLC of pSSZ810 in GS115 Pichia pastoris strain

HPLC analysis was carried out to check the products formed from xylanase assay products by hydrolysis. The hydrolysis was carried out at 50°C for 2 hrs. The pattern of free xylose sugar released from enzyme alongwith standard concentration of 1% xylose as shown in Figure 9. The peak of free xylose was high in *Pichia pastoris* transformants having recombinant vector pSSZ810 as compared to control (Figs. 10 and 11).

3.4. SDS-PAGE (Silver staining) of pSSZ810 in GS115 Pichia pastoris strain

The intensity of desirable protein (approx) 28 kDa was increased with increase in time after 100 % methanol upto a final concentration of 0.5 % induction as indicated in (Fig. 12). The maximum activity of pSSZ810 having xylanase protein was observed at after 96 hrs of induction period whereas no band was observed in the case of non-transformant *P. pastoris* GS115.

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 [19]. 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.

The maximum xylanase activity was observed in the presence of 100% methanol inducer and after incubation of 2 hrs at 50℃ is lower as compared to 2mg/mL to previous studies [20].

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