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Heterologous Expression of *Chaetomium thermophilum* Xylanase 11-A (CtX 11-A) Gene

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Abstract. *Chaetomium* has a potential source of xylanase and cellulase enzymes, both of which are required in the treatment of fibre in the poultry feed. The titre of the enzymes needs to be enhanced by using recombinant DNA technology for fulfilling the requirement of the industries. Efforts are made to construct prokaryotic and eukaryotic expression cassettes that can be cloned under specific strong promoters i.e., T7 and AOX1, respectively, and the enhancer elements to get the maximum gene expression. In the present study BL21 *E. coli* and GS115 *Pichia pastoris* strains are used as model organisms to express the CtX 11-A gene in the presence of 1 mM IPTG and 100% methanol upto final concentration of 0.5. In case of BL21 expression, the maximum xylanase activity was observed after 1.5 h in the presence of 1% xylose, which was 2.302 U/ml and after 7 h in the presence of 0.5% lactose, was 1.708 U/ml. However, in *Pichia pastoris* the maximum production of xylanase was 2.904 and 0.006 U/ml as compared to control 0.484 and 0.06 U/ml, respectively.

Keywords: thermophilic fungi, *Cheatomium thermophilum* xylanase A (CtXA), cloning and gene expression, *Escherichia coli*, *Pichia pastoris*

Introduction

Among the most abundant hemicelluloses in plant cell wall polysaccharides, xylanase has many important applications in various industries such as foods, chemicals, paper, fuels etc., (Boettner *et al.*, 2002; Christov and Prior, 1996; Alam *et al.*, 1994; Coughtan and Hazlewood, 1993; Wong and Saddler, 1992).

Xylanases are classified into two distinct families, F/10 and G/11, of the glycoside hydrolases (Henrissat and Bairoch, 1993). A number of xylanase-producing fungi and bacteria have been isolated from a variety of sources, which have a close relation with the characterization of the produced xylanases. Sinha *et al*. (2004) isolated an extremely thermostable xylanase from a thermophilic eubacterium. Collins *et al.* (2002) isolated a cold active xylanase from the antarctic bacterium *Pseudoalteromonas haloplanktis*. *Bacillus* sp., strain AR-009, an alkaliphile from an alkaline soda lake, produced two alkaliphilic xylanases (Emami *et al.*, 2002). In addition, an acidophilic xylanase was isolated from *Penicillium* sp. 40, which was screened from an acidic soil (Kimura *et al.*, 2000).

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 filamentous fungus that produces thermostable xylanase (Latif *et al*., 1995) It is frequently found in soil, air and plant debris and produces endoxylanase, Xyn11-A. Enhanced enzyme production can be achieved by isolation, characterization, cloning and expression of the genes under specific strong promoters and enhancer elements.

In recent years, several industrial yeasts have been developed as recombinant host systems for the commercial production of heterologous proteins. These organisms combine ease of genetic manipulation with the ability to perform many eukaryotic post-translational modifications (Lin Cereghino *et al*., 2002). One of the most commonly used systems is the methylotrophic yeast *Pichia pastoris*, in which expression is driven by one of the strongest known regulated promoters, the alcohol oxidase I (AOX1) promoter, which is induced by methanol and repressed by other carbon sources such as glucose, glycerol, and ethanol (Lin Cereghino and Cregg, 2000). Another important feature of this system is its ability to achieve extremely high cell densities, enabling efficient protein production and secretion (Pickford and O'Leary, 2004; Sreekrishna *et al.*, 1997).

At the National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan, there is a large collection of thermophilic fungi (Latif *et al*., 1994), which have

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potential applications in the poultry feed industry and paper and pulp industry. Wong and Saddler (1992) demonstrated that *C. thermophile* shows large amounts of extra cellular cellulase and xylanase activity when grown on cellulosic or lignocellulosic substrates as carbon sources. 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 prokaryotic and yeast model systems. In this context, the expression model systems BL21 (Novogen) and *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.

Materials and Methods

Prokaryotic expression system. ***Isolation and amplification of Xyn 11-A gene.*** Xylanase gene (860 bps) was isolated from *Chaetomium thermophilum* NIBGE-1 strain by designing a set of 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′ based on the reported sequence from Gene bank nucleotide database accession no. AJ508931. This fragment was cloned into T/A cloning vector pTZ57R (MBI Fermentas) and the cloned was sequenced from Microsynth GmbH, Switzerland. The sequence was submitted to Gene bank and assigned accession No. 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 along with 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. The desired gene was named as Xyn 11-A, and the resultant construct was named as pSWXyn11-A(a) as indicated in Fig. 1.

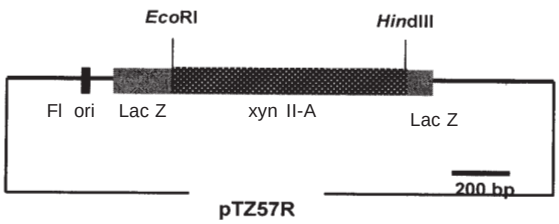


Fig. 1. Recombinant clone pSWXyn11-A(a) with restriction sites.

Cloning and Expression of Xyn 11-A in BL21 E. coli strain. Xyn 11-A gene, (810 bps), from pSWXyn11-A(a) and pET32a (+) was restricted with *Eco*RI and *Hin*d III, respectively. The eluted fragments were ligated for overnight at 16 °C and transformed into 10b *E. coli* strain through heat shock at 42 °C. The transformants were selected on Laurie bertini (LB) medium supplemented with 100 mg/ml ampicillin. The resultant construct pSWXyn11-A(b) was confirmed with the combination of different restriction enzymes *Eco*RI, *Hin*d III, *Xho* I and *Xba* I, respectively. The recombinant clone pSWXyn11-A(b) as indicated in Fig. 2, was transformed into heat shock BL21 competent cells and the transformants were verified through PCR analysis. The colony was cultured into broth LB medium supplemented with 100 mg/ml ampicillin and incubated at 37 °C for overnight. Next day, 2 ml of overnight culture was transferred to 50 ml (100 mg/ml) ampicillin LB broth medium and again incubated for 3 h at 37 °C. After three hours of growth, 70 µl of 1 mM IPTG inducer was added and the cells were again incubated at 37 °C for 3 h. Samples were collected every 30 min, cells were pelleted down and given the 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 for 10 min and supernatant was used for further analysis.

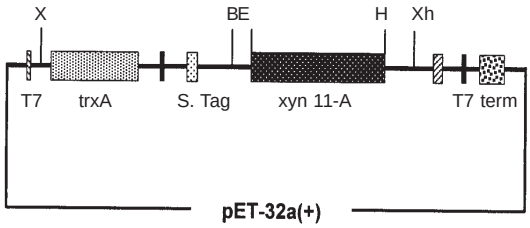


Fig. 2. Recombinant clone pSWXyn11-A(b) with restriction sites. E: *Eco* RI; H: *Hind* III; B: *Bam*HI; X: *XbaI*; Xh: *XhoI*; T7: T7 promoter; T7 term: T7 terminator.

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.

Western blot analysis. Protein, 15 µg, 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.

Inoculum preparation for induction of xylanase gene by using different carbon substrates. Different soluble carbon substrates were used at 0.5% and 1% level to induce the

xylanase gene expression in Dubose Salt (DS) media. These carbon sources were xylose, glucose, cellubiose and lactose. Colonies were picked and cultured in the test tubes containing 3 ml DS broth media with 100 mg/ml ampicillin (antibiotic) and xylose, glucose, cellubiose and lactose at 0.5% and 1% were used as carbon sources. Next day 1 ml culture was transferred to 2 ml DS broth media with 100 mg/ml ampicillin, and grown for 3 h on a shaker at 37 °C. After 3 h growth, 4.7 ml of (1m M) IPTG was added to act as inducer. Cultures were further grown on a shaker at 37 °C for one and a half hour and for 7 h. Medium containing growth was centrifuged at 13,500 rpm for 5 min. The xylanase activity from *E. coli* was assayed against oat spelt xylan method as described by Tuncer *et al*. (1999). Two ml of above reaction mixture containing 1 ml of cell lysate, 0.5 ml of 1 % oat spelt xylan and 0.5 ml of citrate phosphate buffer (pH 6) was incubated at 40 °C for 2 h at 60 rpm. The reducing sugar was determined by dinitrosalicylic acid (DNS) procedure.

Pichia pastoris* (yeast expression system). *Cloning and transformation of xylanase gene into E. coli TOP10F' strain. The intron-less 810 bp fragment of xylanase gene from pSWXyn11-A(b) and pPIC3.5K *P. pastoris* vector were digested with *Eco*RI and *Not*I restriction enzymes and placed at 37 ºC for 1 h. Digestion was run on 0.5 % agarose gel and the 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 (Li *et al*., 2005). 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.

Transformation and screening of resultant recombinant clone into Pichia pastoris. The resultant recombinant vector pSWXyn11-A(c) (Fig. 3) was linearized with *Not*I restriction enzyme, transferred to the cuvette and electric shock was given at 2.0 kvolts for integration in the genomic DNA of *P. pastoris*. Then immediately 1ml of 1M sorbitol was added and cuvettes were placed on shaker at 30 °C. After 2 h the medium was spread on the 0.75 mg/ml concentration of geneticin YPD agar medium plates for the selection of transformants. These plates were incubated at 30 °C until colonies appeared. Colonies were picked from 0.75 mg/ml geneticin plate and cultured into YPD liquid medium without antibiotic and incubated at 30 °C till the time that cell density reached up to ($OD_{600=}$1.0). The transformants were confirmed through PCR analysis by using set of primers P3 and P4. The

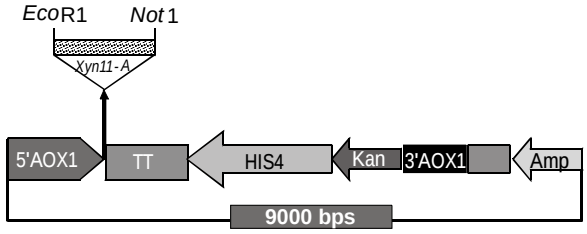


Fig. 3. Recombinant clone pSWXyn11-A(c) with restriction sites.

sample was prepared for PCR reaction under denaturation at 94 °C for 5 min, annealing at 60 °C for 1 min, extension to 72 °C for 1 min and 35 cycles, as 10 µ litre of *P. pastoris* culture into 1.5 ml microcentrifuge tube, 5 µ litre zymolyase enzyme was added and incubated at 30 °C for 10 min. The sample was frozen at -70 °C for 10 min.

Optimization of P. pastoris growth for xylanase assay and SDS-PAGE analysis. *P. pastoris* transformants having pSWXyn11-A(c) were picked from 0.75 mg/ml geneticin YPD agar media plates along with non-transformant GS115 as control and cultured into YPD broth media without antibiotic at 30 °C (OD_{600} = 1.0). Cells were harvested by centrifugation at 3000 rpm for 5 min at room temperature. Supernatant was discarded and cell pellets were resuspended into 25 ml minimal glycerol medium (MGM) and 0.02% 10 X dextrose in a 100 ml flask. Cultures were placed at 28-30 °C in a shaking incubator (150-200 rpm) until growth reached the log phase. Once the cells are in log phase, they can be induced for xylanase expression. One ml culture was taken before each induction of 100 % methanol to a final concentration of 0.5% in 25 ml MG medium. Induced culture was collected at different time intervals i.e. 24, 48, 72, 96 and 120 h. One ml of culture was transferred to 1.5 microcentrifuge tubes. These samples were used to analyze expression levels and determine the optimal time from post-induction to harvest. Cells were centrifuged at 13,500 rpm at room temperature for 2-3 min. For intracellular and secreted expression, both supernatant and pellet were stored at -70 °C until ready for protein assay.

Preparation of samples for xylanase assay and SDS-PAGE. The activity of xylanase was determined by the method described by Tuncer *et al*. (1999) against oat spelt xylan. The sample was prepared for both SDS-PAGE and xylanase assay as thawed cell pellets and quickly placed on ice. Pellets, were dissolved in 1 ml distilled water and 100 µ litre breaking buffer and an equal volume of acid washed glass beads (size 0.5mm) was added, vortexed for 30 sec., incubated on ice for 30 sec (repeated for several times) than centrifuged at 13,500 rpm for 10 min. Clear supernatant was transferred to a fresh 1.5 ml microcentrifuge tube, and 50 µ litre SDS-PAGE loading dye

was added for SDS-PAGE analysis and boiled for 10 min at 100 °C in a dry bath; 10-20 µ litre sample per well was loaded into SDS-PAGE gel whereas other used for xylanase assay and the rest were stored at -20 °C for use in future. Electrophoresis was performed using a discontinuous buffer system, for the analysis and separation of proteins. Developer solution was added to enhance and bands were visualized during silver staining of SDS-PAGE of xylanase Xyn11-A protein in *P. pastoris*.

Results and Discussion

Results in prokaryotic expression system. ***Confirmation of cloning of Xylanase into pET vector*.** The resultant recombinant clone pSWXyn11-A(a) was confirmed through digestion as shown in Fig. 1. After digestion, the xylanase gene of approx. 810 bp was cloned in the pET expression vector, and finally the recombinant clone pSWXyn11-A(b) was transformed into *E. coli* strain BL21 for bacterial expression. To confirm the cloning of intron-less Xyn11-A gene in pSWXyn11-A(b), *Eco*RI and *Hin*dIII restriction enzymes were used. Digestion with *Hin*dIII and *Eco*R1 produced approx., 900 bp fragment. Similarly, digestion with *Hin*dIII and *Xho*1 which produced a correct sized fragment of approx. 600 bp. Confirmation of pSW Xyn 11-A(b) was also made with *Hin*dIII and *Xba*1 restriction enzymes which produced approx., 800 and 600 bp fragments.

Confirmation of transformants BL21 having pSWXyn 11-A(b). After confirmation of recombinant clone pSWXyn11-A(b) through combination of different restriction enzymes, pSWXyn11-A(b) and pET 32a(+) vector was transformed into *E. coli* BL21 strain by heat shock transformation method. The target gene from pSWXyn11-A(b) was confirmed through PCR analysis by using set of Xyn 11-A specific primers P_1 and P_2. The amplification of 810 bp fragment confirmed the transformation of recombinant vector in *E. coli* strain BL21.

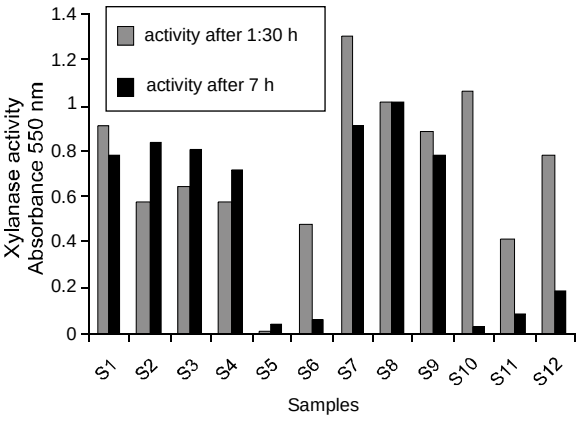
Estimation of the xylanase gene expression in the form of fusion protein was carried out using xylanase assay. The xylanase gene expression in *E. coli* strain BL21 was induced by IPTG (1mM) inducer in LB broth media as indicated in Table 1 and shown in Fig. 4. The activity of xylanase by DNS method was obtained in U/ml. The maximum activity of xylanase gene U/ml was obtained after incubation of 2 h at 40 °C. The maximum and minimum production of xylanase was 4.62 and 3.99 U/ml, respectively, as compared to the control (4.19 and 3.91 U/ml, respectively).

Effect of different carbon sources on xylanase production. Effect of different carbon sources was observed on the xylanase gene expression, which was carried out through xylanase assay. The xylanase activity in *E. coli* BL21 strain in DS liquid media containing ampicillin (100 mg/ml) and different carbon sources i.e., xylose, glucose, cellobiose and lactose, was carried out after 1.5 and 7 h, (Table 2, Fig. 4). Maximum activity of xylanase (U/ml) in the presence of carbon sources was obtained after incubation for 10 min at 40 °C. The maximum xylanase activity after 1.5 h, observed in the presence of 1% xylose, was 2.302 U/ml whereas minimum activity, observed in

Table 1. Xylanase activity in cell lysate of *E. coli* (BL21) harboring the pET expression vector with intron-less xylanase fragment.

Time of induction*	Enzyme activity U/ml **		
	Sample 1	Sample 2	Sample 3 (control)
At zero h	3.19	3.74	3.44
After 30 min	4.62	3.40	3.81
After 60 min	4.03	3.80	4.19
After 90 min	3.95	3.99	3.92
After 120 min	4.19	3.94	3.75

***** = induction with IPTG (1 mM); ** = Hl sample 1 and 2: pSWXyn11-A(b); sample 3: pET 32a(+)



S 1: (0.5% xylose + DS media + xylanase gene)
S 2: (0.5% glucose + DS media + xylanase gene)
S 3: (0.5% cellubiose + DS media + xylanase gene)
S 4: (0.5% lactose + DS media + xylanase gene)
S 5: (without carbon source + DS media + pET)
S 6: (without carbon source + DS media + xylanase gene)
S 7: (1% xylose + DS media + xylanase gene)
S 8: (1% glucose + DS media + xylanase gene)
S 9: (1% cellubiose + DS media + xylanase gene)
S 10: (1% lactose + DS media + xylanase gene)
S 11: (LB media + pET)
S 12: (LB media + xylanase gene).

Fig. 4. Xylanase activity in cell lysate of *E. coli* (BL21) in the presence of different carbon sources

Table 2. Xylanase activity in cell lysate of *E. coli* (BL21) in the presence of different carbon sources.

E. coli strain transformed with vector	Medium used	Carbon source	Enzyme activity U/ml with vector	
			After one and half h	After 7 h
pSSZ810 (b)	DS*	0.5 % xylose	1.375	0.575
pSSZ810 (b)	DS	0.5 % glucose	-0.454	1.139
pSSZ810 (b)	DS	0.5 % cellubiose	0.515	1.539
pSSZ810 (b)	DS	0.5 % lactose	0.860	1.708
PET 32a (+) control	DS	without carbon source	-0.303	0.139
pSSZ810 (b)	DS	without carbon source	2.787	0.236
pSSZ810 (b)	DS	1 % xylose	2.302	0133
pSSZ810 (b)	DS	1 % glucose	1.363	1.369
pSSZ810 (b)	DS	1 % cellubiose	-0.136	-1.424
pSSZ810 (b)	DS	1 % lactose	0.412	-5.853
PET 32a (+) control	LB**	without carbon source	2.411	0.488
pSSZ810 (b)	LB	without carbon source	2.696	-0.983

* = first media; ** = second media

the presence of 1 % cellobiose, was -0.136 U/ml. On the other hand the maximum activity of xylanase after 7 h, observed in the presence of 0.5 % lactose, was 1.708 U/ml, whereas, minimum activity, in the presence of 1 % lactose, was -5.853 U/ml.

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 kDa protein on SDS-PAGE. The amount of recombinant protein increased with different induction times as indicated in Fig. 5.

Western blotting of recombinant xyalnase. Western blot analysis was carried out by using polyclonal antibodies raised against fusion protein.

Results of *Pichia pastoris* (yeast expression system). ***Confirmation of recombinant clone pSWXyn11-A(c) 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. Upon digestion with *Eco*RI and *Not*I, recombinant clone pSWXyn11-A(c) produced (approx.) 810 bp fragment along with vector backbone of pPIC3.5 K of 9000 bp. pSSZ810 recombinant clone has two *Kpn* I restriction sites, an internal and the other *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.

The purified and linearized fragment of pSWXyn11-A(c) 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. 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 0.75 mg/ml geneticin. The integration of linearized fragment pSWXyn11-A(c) having xylanase gene in the genome of *P. pastoris* was confirmed through PCR amplification by using xylanase specific primers.

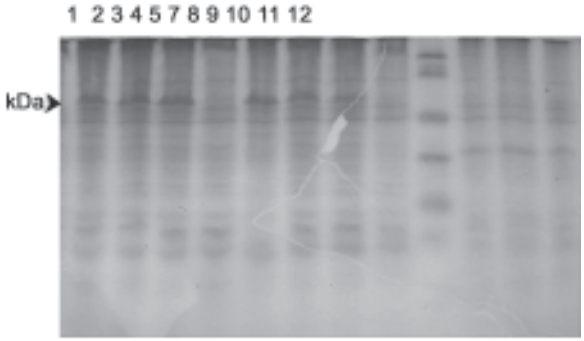


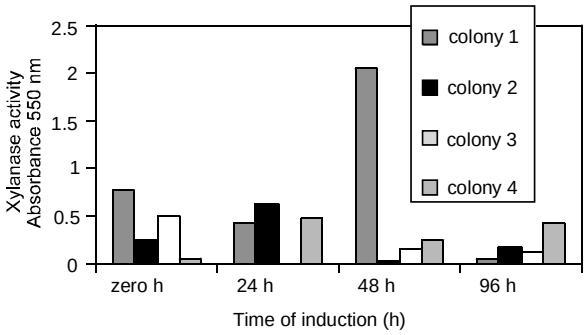
Fig. 5. SDS-PAGE analysis of total protein isolated from bacterial strain BL21 transformed with pSWXyn11-A(b) and pET 32a(+).
Lane 1-8: pSWXyn11-A(b) transformed BL21.
Lanes 10-12: pET 32a(+) transformed BL21
From right to left in lane 12, 8 and 4: protein induced after zero minute,
Lane 11, 7 and 3: protein induced after 30 minutes,
Lane 10, 6 and 2: protein induced after 60 minutes,
Lane 5 and 1: protein induced after 90 minutes.
9: represents prestained protein marker #SM0441 118 k Da (MBI Fermentas).

Xylanase assay of pSWXyn11-A(c) in GS115 *Pichia pastoris* strain. Production of xylanase protein by recombinant *P. pastoris* GS115 strain was induced by 100 % methanol up to a final concentration of 0.5 % in minimal glycerol media (MGM) broth as indicated in Table 3. The maximum and the minimum production of xylanase was 2.04 and 0.006 U/ml as compared to the control, 0.484 and 0.06 U/ml, respectively. Li *et al*. (1993) 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 48 h of induction (Fig.6).

Table 3. Xylanase activity in *Pichia pastoris* GS115 strain

Time of induction*	Xylanase activity (U/ml)**			
	Sample 1	Sample 2	Sample 3	Sample 4
At zero h	0.769	0.242	0.5	0.06
After 24 h	0.424	0.632	0.006	0.484
After 48 h	2.04	0.014	0.151	0.242
After 96 h	0.06	0.175	0.133	0.436

* = 100 % methanol inducer upto 0.5% final concentration; ** = sample 1-3: transformants having pSSZ810(c) xylanase gene; sample 4: non-transformant *Pichia pastoris* GS115 strain



Colony 1: having pSWXyn11-A(c) at zero, 24, 48 and 96 h
Colony 2: having pSWXyn11-A(c) at zero, 24, 48 and 96 h
Colony 3: having pSWXyn11-A(c) at zero, 24, 48 and 96 h
Colony 4: without pPIC3.5k at zero, 34, 48 and 96 h

Fig.6. Xylanase production by*Pichia pastoris* strain GS115 at different time period

SDS-PAGE (silver staining) of pSWXyn11-A(c) in GS115 *P. pastoris* strain.The intensity of desirable protein (approx.) 28 kDa increased with increase in time after 100 % methanol up to a final concentration of 0.5 % induction. The maximum activity of pSWXyn11-A(c) having xylanase protein was observed after 96 h of induction period whereas no band was observed in case of non-transformant *P. pastoris* GS115.

Conclusion

Hyperactivity of this xylanase versus those from other sources may be due to the presence of glycine rich C-terminal region which is absent in other fungi.However, for determination of its exact function further investigations are required. The maximum xylanase activity was observed in the presence of 100% methanol inducer and, after incubation for 2 h at 50 °C, is lower as compared to 2 mg/ml, observed in the previous studies (Faber *et al*., 1995)

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