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Cloning, Gene Expression and Characterization of Xylanase Coding Gene from Mesophilic Filamentous Fungus *Trichoderma reesei* (Hypocreaceae) in *E.coli*

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This research paper describes an efficient bacterial transformation system for production of Xylanase enzyme from *Xyn2* gene collected from *Trichoderma reesei*. Xylanase coding gene was collected from Genbank and artificially synthesized and cloned into *E. coli* cells. Artificial nucleotide gene sequence showed that the 840 long DNA fragment of *Xyn2* gene had open reading frames encoding polypeptides of 229 amino acid residue. The *Xyn2* gene made by two exon, first one start from 59 and end in 348bp and second one tart from 411 and end in 810bp and one intron present in the sequence, it start with 349 and end in 410 bp. Xylanase encoding gene-*Xyn2* of *Trichoderma reesei* ligated into the pUC19 vector and numbered as GS57308 PUC19-*Xyn2* system. The ligation mixture contained *Xyn2*-pUC19 DNA was then transformed into *E. coli* BL 21by $CaCl_2$ method. The potent positive clone was identified by Blue -White colony screening, Congo red staining and RBB–Xylan assay. The positive clone of *E.coli* containing the *Xyn2*-pUC 19 gene was cultured for production Xylanase enzyme. The crude enzyme extract was identified and purified by SDS PAGE electrophorosois with coomasive blue staining. The Zymogram assay was performed for the qualitatively testing the presence of xylanase in the crude extract. The cloned xylanase Gene2 from *T.reesei* in *E.coli* showed highest enzyme activity of 176 Uml^{-1}. The cloned *Xyn2* gene for xylanase enzyme from *T.reesei* into *E.coli* could be a model system for gene expression, secretion, and purification of xylanase enzyme and it can be utilized for large-scale production of xylanase2 from *E. coli* transformant.

Key words: pUC19 vector; transformant; Zymogram; RBB-Xylan assay; congo red.

The growing public concern regarding environmental impact of pollutants from paper and pulp industry was the strong driving force behind the endeavors leading to novel bleaching practices. Phenolic chlorinated chemicals and polychlorinated biphenyls produced during conservative pulp bleaching methodology arise from residual lignin present in wood pulp. The majority of the chloroaromatic compounds released at the time of the pulp bleaching process is noxious and accumulates in the biotic and abiotic components of the ecosystem. (Subramaniyan and Prema, 2000). The enzyme xylanase are speedy becoming a chief group of industrial enzymes finding significant function in paper and pulp industry. It is used in paper and pulp industries as the hydrolysis of xylan facilitates release of lignin from paper pulp and reduces the level of usage of chlorine as the bleaching agent (Shoham *et al.*, 1992).

The importance of xylanases is not bound to the paper and pulp industry and there are other industries with equal significance of applicability in the area of applications in the area of xylanases in the area of clarification of fruit juices,

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bioconversion of lingo-cellulosic material and agro-wastes to fermented products,, development in uniformity of beer and the digestibility of animal feed materials (Wong *et al.*, 1988).

Numerous microbes including bacteria and fungi have been reported to be gladly hydrolysing xylans by synthesizing â-xylosidases (EC.3.2.1.37) and 1, 4-â-D endoxylanases (E.C.3.2.18). Xylanase activity and FPase activity from *Trichoderma viride* were reported (Gomes *et al.*,. 1992). The *T. reesei* was also known to produce higher xylanase activity is 960 IU/ml and cellulase activity is 9.6 IU/ml (Wong *et al.*, 1988) *Schizophillum commune* is also one of the high xylanase producers with a xylanase activity, CMCase activity of 65.3 U/ml and FPase activity (Copa-Patino *et al.*, 1993).One of the white rot fungi, *Phanerochaete chrysosporium* a potent plant cell wall degrading fungus, produced a xylanase in the culture medium, but it also produced high amounts of cellulose activity measuring about 12% of maximum xylanase activity (Steiner *et al.*, 1987) .

Remarkably high enzymatic activity of fungi was received great impetus by the researchers to express and characterize the genes encoding hemi-cellulolytic enzymes in *E.coli*. Xylanase genes of *XynA* (Gilbert *et al.*, 1992), *XynB* (Black *et al.*, 1994), *Xxyn3* (Durand *et al.*, 1996) , *XynC* (Liu *et al.*, 1999), *Xyn11A* and *Xyn11B* (Huang *et al.*, 2005) from *Neocallimastix spp*. were investigated well in the extensive manner.

Endo-1, 4-beta-xylanase genes from *Verticillium dahliae* (Zhang *et al.*, 2008), Xyn A4 genes from *Alicyclobacillus species* (Bai *et al.*, 2010*), Xyn C* genes from *Cellvibrio mixtus* (Fontes et at 2000), *XynA* genes from *Bacillus species* BP-7 (Gallardo *et al.*, 2004), *Xyn* 11A genes from *Lentinula edodes* (Lee *et al.*, 2005) was already cloned. In present investigation aimed to synthesis the enzyme Xylanase through *Xyn2* gene collected from *Trichoderma reesei* and transferred into *E.coli* through pUC19 vector system for large scale production of xylanase.

MATERIALS AND METHODS

Chemicals

All the chemicals related to gene analysis were purchased from Sigma- Aldrich, India, other chemicals from Merck, India and microbiological medium from Hi-Media, India. MilliQ ultra purified water was used for all the experiments. The oligonucleotide DNA was synthesized by Epoch Life science Inc, USA.

Gene selection

The gene sequence of *Xyn*2 of *Trichoderma reesei* were collected from Genbank database by NCBI BLAST service based on similarity search. The selected sequences was displayed from the database and sequence was collected in the form of FASTA format and gene sequence used for artificial gene synthesis

Gene synthesis

Artificial gene construction is the process of synthesizing a gene in vitro without the need for initial template DNA samples. The oligonucleotide synthesis was developed (Beaucage S.L. and Caruthers M.H. 1981) and the method was known as phosphoramidite method or the solid phase method. DNA synthesis cycle involves four steps such as Detritylation, Coupling, Capping and Oxidation. The oligonucleotide synthesis is done by using a solid support of control pour glass (CPG) of 50 nm or 100 nm size to which dNTPs are attached covalently. The selected gene sequence from Genbank was synthesized by Epoch Life science Inc, USA. The size of the DNA sequence was confirmed with DNA Agarose electrophoresis.

Plasmid vector

The 2694 bp pUC19 has SmaI, BamHI, EcoRI, HindIII, and BamHI restriction sites were purchased from Epoch Life sciences and used for cloned the Xyn2 gene (Fig 1). The size of the DNA sequence was confirmed with DNA Agarose electrophoresis.

Vector Dephosphorylation

pUC19 plasmids was dephosphorylated for the purpose of to reduce the probability of self relegation. The plasmid pUC19 was incubated with BamHI enzyme and mixed with 2.5 U Phosphatase, Tris Buffer contain at 1 x concentration of 100mM NaCl, 50mM Tris-HCl, 10mM MgCl2, 1mM DTT, pH 7.9@25°C at 37ºC for 1.5 hours and then deactivated at 65ºC for 20 minutes.

Ligation Reaction for Xyn2 with vector

The Xyn2 gene was ligated into digested, dephosphorylated, and purified pUC19 vector. The each ligation reaction carried out by 30 fmol pUC19, 90 fmol *Xyn 2* gene, 1 µL T4 DNA Ligase, 1X Ligase

Buffer, and dH_2O. All the ligation reactions occurred at room temperature for the duration of 16 hours. A control ligation reaction carried out with pUC19 plasmid vector without any gene. Then the ligated sequence of Xyn2- pUC19 was confirmed with Agarose gel electrophoresis.

Culture conditions for *E.coli*

The LB medium was prepared by dissolving 10 g of Tryptone, 5 g of Yeast Extract and 10 g of NaCl in 1 L of distilled water. For medium add agar to a final concentration of 1.5%. Heat the mixture to boiling to dissolve agar and sterilize by autoclaving at 15 psi, from 121°C for 15 minutes (Gerhardt *et al.*, 1994; Sambrook and Russel, 2001).

Preparation of TB ($CaCl_2$) solution

10 mM Pipes, 15 mM $CaCl_2$, 55 mM $MnCl_2$, 250 mM KCl, 2.205 g/l of $CaCl_2.2H_2O$, 3.021 g/l of PIPES, 10.885 g/l of $MnCl_2.4H_2O$ 18.637 g/l of KCl, were mixed. All the components except for $MnCl_2$ were mixed and the pH was adjusted to 6.7 with KOH solution. Then, $MnCl_2$ was dissolved, the solution was sterilized by filtration through a utrafilteration unit and stored in refrigerator at 4ºC, all chemicals were added as solids, always kept in cool temperature.

Preparation of competent cells

A 10 µl stock of an *E. coli* strain containing no plasmids was allowed to thaw at room temperature and added to 40 ml of liquid SOC medium. The culture was incubated at 37ºC for one hr, then transferred to an incubator-shaker, at 200 rpm , at 37ºC, shaking for 2-3 hrs until an OD600 of 0.2-0.4 was reached.

Then the cells were pelleted by centrifugation at 8000 rpm for 1 min at 4ºC, then resuspended in one-half volume (20 ml) of sterile cold TB ($CaCl_2$) solution, and incubated on ice for 25 minuts. After that continue the centrifugation step as above, the resulting cell pellet was resuspended in one-tenth volume (4 ml) of sterile cold TB ($CaCl_2$) solution to yield the final competent cell suspension. The competent cells from the research can be stored at 4ºC for up to 3 days.

Preparation of competent cells for glycerol stocks

Transfer 1.6 ml of the competent *E.coli* cell suspension to sterile cryo-storage tubes, and add 0.4 ml of sterile 100% glycerol to give a final concentration of 20% glycerol, and then mix together. The glycerol stocks are placed at -4ºC for future use.

Bacterial transformation

Ligation product of *Xyn2*-pUC19 was transformed into *E. coli* competent cells using the heat shock method. Ligation products was mixed gently with thawed competent *E.coli* cells, and the mixture was incubated for 30 minutes on ice, 2 minutes at 42ºC, and 2 minutes on ice again. LB broth was added to a total volume of 1 ml and the culture was incubated at 37ºC for 1 hour with shaking before plating. The control for ligation experiment made by purified pUC19 was added. Transformants were at first plated on LB agar containing 100 µg/ml ampicillin, 800 µg X-gal, and 400 µg IPTG.

Blue white colony screening

Colonies of potential clones of *Xyn2* gene were grown overnight in LB media containing 100 µg/ml ampicillin at 37ºC.

Agarose gel electrophoresis

The *Xyn* 2 gene , pUG 19 plasmid, ligated *Syn2* gene with pUG 19 vector were analyzed on 0.8% agarose gel. The DNA was dissolved in Trisborate buffer (89 mM Tris base, 2.5 mM disodium EDTA, and 8.9 mM boric acid). A dye solution consisting of bromophenol blue (0.07%), and glycerol (33%) in water was added at 5 µl per sample to DNA samples prior to electrophoresis. Electrophoresis was carried out in a gel apparatus. The electrophoresis for DNA was carried out at 120 V, 60 mA, for two hrs or until the dye neared the bottom of the gel. The gel was then placed in a solution of ethidium bromide in water (0.4 µg/ml) and stained for 15 min. The DNA band was analyzed with facilitate of Gel Documentation system.

Screening of Transformants by Congo red staining

The alkaline Xylan agar medium was used for screening transformant for the cloned xylanase gene into *E.coli*. The medium contained Larchwood xylan (10.0 g/L), Yeast extract (5.0g/L), Peptone (5.0 g/L), K_2HPO_4 (1.0 g/L), $MgSO_4 \cdot 7H_2O$ (0.2 g/L) and add the agar as solidifying agent. Sterilize the medium in autoclave at 121°C for 15 minutes and after autoclaving, adjust pH to 10.0 with sterilized 10% Na_2CO_3 solution and transferred into petridish for transformant screening. The transformant of *Xyn2*-pUC19 plasmid containing *E.coli* cells was inoculated into alkaline Xylan agar

plate. Screening of the xylanase-positive clones was done by the plates was made to Congo red staining. The colonies harboring xylanase activity showed clear zones on the plates.

Remazol Brilliant Blue R"D-Xylan (RBB-xylan) agar mediated Dye diffusion assay

Xylanolysis basal medium (XBM) (g/L) prepared by adding Ammonium tartrate (5g/ L), Yeast Extract (0.1g/L), KH_2P0_4 (1 g/L), $CaCI_2.2H_20$ (0.001 g/L), $MgS0_4.7H_20$ (0.5 g/L). The basal medium described here may be conveniently stored as a 10 x sterilized stock. Prepare XBM medium supplemented with 1.6 % w/v agar and transfer into culture flask. Autoclave and allow to solidify the medium. The prepared separately the 1% w/v RBB-xylan and 1.6 % w/v agar and autoclave and cool until viscous. Gently mix the agar prepared in step 2 and then carefully aliquot 0.1 rnl aseptically on to the surface of the solidified agar as an overlay. Inoculate with transformant bacteria and retain uninoculated bottles as controls. Incubate at 25 C in darkness and examine daily for 10 days. Migration of the dye into the clear nearby colony by dye decolorization indicates Xylanolysis.

Production of xylanase

A single colony was isolated from the stock culture of *Xyn2*-pUC 19 plasmid containing *E.coli* culture and inoculated, into 5 ml of Luria–Bertani (LB) medium. The culture was incubated overnight at 37 C on a rotary shaker at 300 rpm. After incubation period that, one ml of overnight culture of *E.coli* cells were inoculated into 100 ml of fresh Luria–Bertani (LB) medium and incubated at 37 C on a rotary shaker at 300 rpm until the A600 nm reached 0.8 -1.0. Then, added 0.1 mM of isopropyl-d-1-thiogalactopyranoside (IPTG) into the culture for releases the tetrameric repressor from the *lac* operator in an allosteric manner, thereby allowing the transcription of *Xyn2* gene in the *lac* operon. The *E. coli* culture was subsequently incubated for another 24 h at 20 C for production of Xylanase.

Isolation of Xylanase Enzyme

After incubation period, the culture was under centrifuged at 7000 rpm for 10 min. The supernatant was decanted and the cell pellet was collected for enzyme extraction. The cell pellets were crushed with the aluminia powder using a mortar and a pestle at 4 C for 30 min with 100 mM PMSF (phenylmethanesulfonyl fluoride) dissolved in isopropyl alcohol. The PMSF is a serine protease inhibitor commonly used in the preparation of cell lysates to block the activity of protease. Then the mixture was suspended in 1× phosphate buffer and cell debris was removed by centrifugation at 9500 rpm for 30 min. The clear supernatant was collected and it containing the *Xyn2* gene product. This purified xylanase enzyme was used for all subsequent enzyme assays.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed for separation xylanase enxyme from extract using a 5% stacking gel and 10% separating gel under standard condition (Laemmli, U. K. 1970). The separating gel (10%) was prepared by adding the 2 ml ddH_2O, 1.67 ml 30% acrylamide/Bis, 1.25 ml 1.5 M Tris (pH 8.8), 25 µl 20% SDS , 25 µl 10% ammonium persulfate (make it fresh), 2.5 µl TEMED. Add a small layer of isopropanol to the top of the gel prior to polymerization to straighten the level of the gel. The stacking gel (5%) was prepared by adding (total volume= 3 ml) 2.088 ml dH_2O, 0.506 ml 30% acrylamide/Bis, 0.375 ml 1 M Tris (pH 6.8), 15 µl 20% (w/v) SDS, 15 µl 10% ammonium persulfate and 1.5 µl TEMED.

Remove the isopropanol layer by using filter paper. Rinse the top layer of the gel with ddH_2O and dry off as much of the water as possible by using filter paper. Poured 1x electrophoresis running buffer into the opening of the casting frame between the gel cassettes upto fill the wells of the gel. Slowly load the enzyme sample into well as well as load 10 µl of protein MW marker. The 10x running buffer prepared by adding the 30.3 g Tris-base, 144.0 g glycine, 10.0 g SDS into 800 ml ddH_2O and dissolved completely, then more ddH_2O up to 1 liter.

The enzyme bands were visualized by Coomasive brilliant blue staining. Fixed gel in 25% IPA, 10% HOAC in water for 30 - 60 minutes. Stained gel in 10% acetic acid in water, containing 60 mg/L of Coomasive Blue R-250. Bands will appear in 2 hrs. Destained gel in 10% acetic acid for 2 hours and store gels in 7% HOAC.

Assessment of Xylanase activity in SDS – Xylan - PAGE gel

For activity staining (Zymogram) of xylanase by SDS-Xylan-PAGE, SDS was removed

by washing the gel at room temperature in solution-A (50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2), isopropanol) for ne hr and solution-B (50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2)) for one hr, respectively. The gel was kept overnight in solution-C (50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2), 5 mM â-mercaptoethanol, 1 mM EDTA) at 4°C for renaturation of the enzyme. It was then sealed with film and incubated at 55°C for 4 h. After incubation, the gel was stained in 0.1% (w/v) Congo-red dye for one hr and washed with 1% (w/v) NaCl for 30 min to visualize the clear band of xylanase activity (Huang *et al.,* 2005).

Xylanase assay from crude enzyme extract

Xylanase activity was assayed according to the method of measuring the amount of reducing sugars ie xylose liberated from xylan using 3, 5-dinitrosalicylic acid (Hespell. and Whitehead 1990). The reaction mixture containing 490 µL of 1% birch wood xylan as substrate and 10 µL of enzyme extract was incubated at 55°C for 5 min. The reaction was terminated by adding 1.5 mL of 3, 5-dinitrosalicylic acid reagent. A control was run simultaneously that contained all the reagents but the reaction was terminated prior to the addition of enzyme by adding DNS. All the test tubes were placed in a boiling water bath for 10 min followed by cooling in ice cold water for 10min. The absorbance of the resulting colour was measured against the control at 540 nm in a spectrophotometer.

RESULTS AND DISCUSSION

Xylanase encoding gene - *Xyn2* of *Trichoderma reesei* as synthesized by artificially and a 840 long DNA fragment inserted into the pUC 19 vector and the new construction vector was numbered as GS57308 PUC19-*Xyn2* system and transformed into *E.coli* EC1000 successfully.

The Xyn2 gene base pairs were selected from Genbank for artificial gene synthesis. The *Xyn* 2 Gene sequences are collected from Genbank database from the *Trichoderma reesei* (Hypocreaceae) by BLAST np search. The Genbank ID for *Xyn2* is emb|X69574.1 and it located in the gene locus is X69574. Totally 840 base pairs present in the *Xyn2* gene (Table 1) and it produced Xylanases enzyme (Torronen *et al.,* 1992). The *Xyn 2* gene made by two exon, first one start from 59 and end in 348bp and second one tart from 411 and end in 810bp and one intron present in in the sequence, it start with 349 and end in 410 bp.

The gene *Xyn* 2 encoding the enzyme

Table 1. Xyn2 gene sequence of *Trichoderma reesei* from Genbank in FASTA format

>gi|396565|emb|X69574.1| T.reesei xyn2 gene, complete CDS
AGACAGCAAGCTCAACTGCATAGTATCGACTTCAAGGAAAACACGCACAAATAACATCATGGTTGCCTTT
TCCAGCCTCATCTGCGCTCTCACCAGCATCGCCAGTACTCTGGCGATGCCCACAGGCCTCGAGCCTGAGA
GCAGTGTCAACGTCACAGAGCGTGGCATGTACGACTTTGTTCTTGGAGCTCACAATGATCATCGCCGTCG
TGCTAGCATCAACTACGACCAAAACTACCAAACTGGCGGACAAGTCAGCTATTCGCCTTCCAACACTGGC
TTCTCAGTGAACTGGAACACTCAAGATGACTTTGTTGTGGGCGTTGGTTGGACGACTGGATCTTCTGCGT
AGGAGGACTCCTCATCATTCTGCACTTTGAAAGCATCTTCTGACCAAAAGCTTCTCTTAGTCCCATCAAC
TTTGGCGGCTCTTTTAGTGTCAACAGCGGAACTGGCCTGCTTTCCGTCTATGGCTGGAGCACCAACCCAC
TGGTTGAGTACTACATCATGGAGGACAACCACAACTACCCAGCACAGGGTACCGTCAAGGGAACCGTCAC
CAGCGACGGAGCCACTTACACCATCTGGGAGAATACCCGTGTCAACGAGCCTTCCATCCAGGGCACAGCG
ACCTTCAACCAGTACATTTCCGTGCGGAACTCGCCCAGGACCAGCGGAACTGTTACTGTGCAGAACCACT
TCAATGCTTGGGCCTCGCTTGGCCTGCACCTTGGGCAGATGAACTACCAGGTTGTCGCTGTCGAAGGCTG
GGGTGGTAGTGGTTCTGCCTCACAGAGTGTCAGCAACTAGGTTCTGTTGATGTTGACTTGGAGTGGATGA

Table 2. Amino acid sequence of Endo beta-1,4-xylanase isotype 2 of *Trichoderma reesei* from Genbank in FASTA format

>gi|396566|emb|CAA49294.1| xylanase [Trichoderma reesei]
MVAFSSLICALTSIASTLAMPTGLEPESSVNVTERGMYDFVLGAHNDHRRRASINYDQNYQTGGQVSYSP
SNTGFSVNWNTQDDFVVGVGWTTGSSAPINFGGSFSVNSGTGLLSVYGWSTNPLVEYYIMEDNHNYPAQG
TVKGTVTSDGATYTIWENTRVNEPSIQGTATFNQYISVRNSPRTSGTVTVQNHFNAWASLGLHLGQMNYQ
VVAVEGWGGSGSASQSVSN

Xylanase (EC No.:3.2.1.8) comes under the Glycosyl hydrolases family. The enzyme made by 229 aminoacid residue (Table 2) and it functioned as Endohydrolysis of (1→4)-β-D-xylosidic linkages in xylans (Torronen *et al.*, 1992) .

The pUC 19 vector was selected for transformation of *Xyn2* gene into *E. coli*. The plasmid pUC19 is one of a series of plasmid cloning vectors and it is one of the most widely used vector molecules as the cells into which foreign DNA has been introduced, can be easily distinguished from the non-recombinants based on color differences of colonies on growth media (Störkle and Dominic (2007). pUC19 is a circular double stranded DNA and has 2694 base pairs (Fig.1). pUC19 cloning vectors contain a multiple cloning site at the *lacZ'* region, recombinant plasmids can be verified via blue/white colony screening using agar plates containing IPTG and X-Gal. Expression of target DNA is enabled by the presence of a *lac* promoter in the cloning vectors (Yanisch-Perron *et al.*, 1985).

The *Xyn* 2 gene is ligated successfully with pUC19 by T4 DNA ligase enzyme and verified with Agarose gel electrophoresis (Fig 2). Lane 1 was 1 kb plus DNA ladder, Lane 2 was loaded with *Xyn2* DNA, Lane 3 was loaded with pUC19, and Lane 4 was loaded with ligated SmaI fragments of *Xyn2* into pUC19. Lane 4 shows that Xyn 2/pUC19 fragments underwent complete ligation to generate new band and high molecular weight band of 3534base pairs was also observed. The ligated *Xyn2*/pUC19 DNA sequence are denoted as GS57308 PUC19-xyn2 system and it is used for transformation experiments. The location of xyn2 gene and other important genes on pUC19 plasmid and the partial restriction map for GS57308 PUC19-xyn2 system shown in Fig 1. The GS57308 PUC19-xyn2 map contains Xyn2 CDS gene in 419 -1258, *Lac z* misc in 256 -398, M13 in 1313 – 1329, *Amp R* gene in 2477 -3265, *Lac Z* gene in 239 -307, *Lac O* in 1335 - 1357 and *Lac* 1362 -1391.

The cloning experiments involved cloning *Xyn2* gene into pUC19 and screening the potent clone by Blue-White colony screening. The more numbers of white transformant colonies were observed on X-gal agar plates (Fig 3). White colonies not turned blue under prolonged incubation at 37ºC or prolonged storage at 4ºC.

β-xylosidase (xynB) gene from *Bacillus pumilus* along with β-xylanase-2 (xyn2) gene from

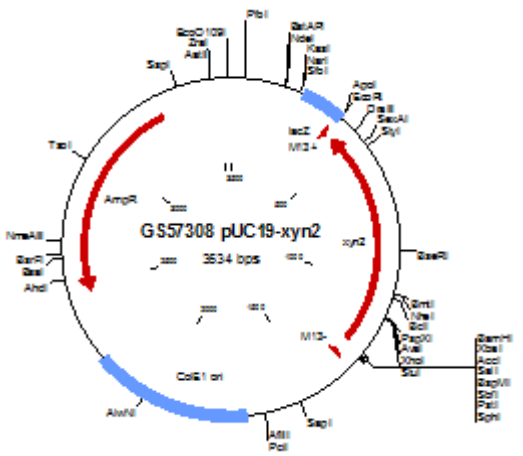

Fig. 1. The location of xyn2 gene on pUC19 plasmid and the partial restriction map for GS57308 PUC19-xyn2 system

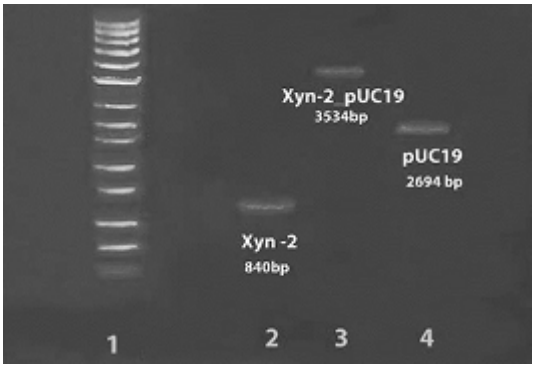

Fig. 2. Ligation experiment of SmaI fragments of Xyn2 into pUC19 by T4 DNA ligase

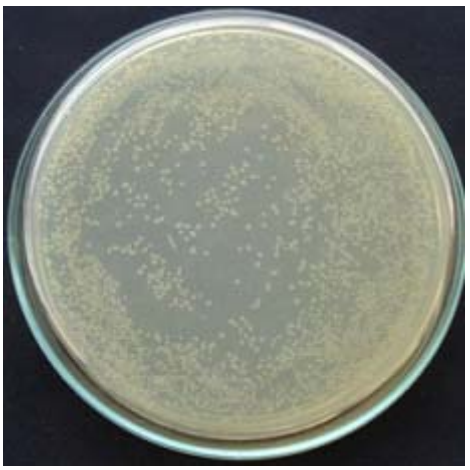

Fig. 3. Blue / White colony screening for GS57308 PUC19-Xyn2 gene Transformants of *E.coli*

Trichoderma reesei were successfully cloned and co-expressed in the yeast *Saccharomyces cerevisiae* (La-Grange *et al.,* 2000) .Genomic DNA from *Bacillus circulans* Teri-42 was cloned in *E. coli* DH5-alpha using plasmid pUC19, however, 14 fold increase in expression was observed in *B. subtilis* clone harbouring recombinant plasmid pBA7 (Panbangred *et al.,* 1985) .

Xylanase producing microbes are expected to be found at places where hemicellulosic compound is occurring. Using Xylan Agar media for differentiate the xylanase producing microbes from other microbes. The *Xyn2*-pUC19 inserted *E.coli* transformant is screened from non transformant by using Xylan Agar plate (Fig 4).

Screening of transformant bacterial strain by plate assay was done. For bacteria Xylan agar as a substrate xylanase were screened by inoculating the organism on the agar plate was observed for cleared zone around the colony by stained with Congo red. Positive xylanolytic isolates were detected based on the clear zones of hydrolysis. Bacterial strains, which produced distinct clearing zones around their colonies, were selected. The *Xyn2*-pUC19 gene transformant showed on xylanase enzymatic activity, clearing zones formation i.e. 53 mm around the single colony (Fig.5). The appearance of zone after staining with Congo red dye indicates that xylanase secreted by bacterial culture hydrolyzes the xylan backbone resulting in a mixture of xylo-oligosaccharide (La-Grange *et al.,* 2000; Gat *et al.,* 1994) .

Important method for selecting xylanase-expressing microorganisms from a various microbial population is necessary if screening techniques are to be well one. A preliminary search for recombinants on Xylan -Agar plate identified xylanase-positive clones containing DNA for Xyn-2. Further the xylanase positive clone expressing xylanase activity further identified by activity-

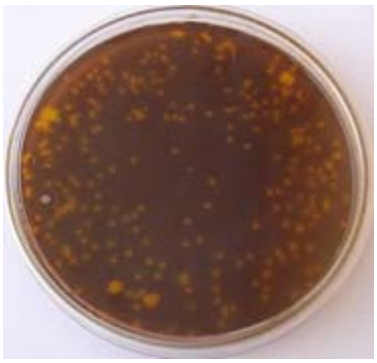

Fig. 4. Screening of transformant by Xylan mediated agar medium

Fig. 5. Screening of Xyn-2 gene inserted *E. coli* transformant by congo red staining

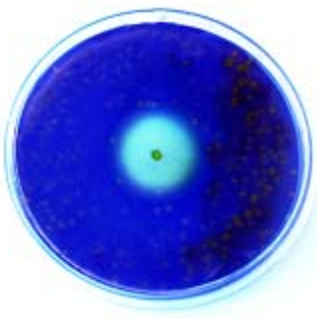

Fig. 6. RBB xylan mediated Screening of Xyn-2 gene inserted Ecoli transformant

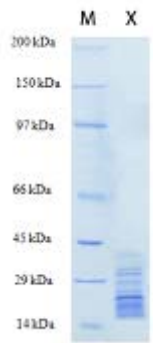

Fig. 7. SDS-PAGE analysis of crude extract of recombinant xylanase 2 isolated from *E. coli* (pUC19-Xyn2). Lane M, molecular mass markers (Bio-Rad); lane X, crude extract Xylanase of *E. coli*

based xylanase screening using RBB-Xylan plates (Fig 6). The reaction mechanism for screening of positive clones from non-transformants by positive reactions is made to degradation of the 4-0-methyl-Dglucourono-D-xylan is bound to the dye remazol brilliant blue to form the substrate RBB-xylan by The degradation of xylan by xylanase results in the release of bound dye from the substrate, the migration of which can be monitored in the agar medium (Durand *et al.,* 1996). The positive selected and screened recombinant DNA of xylanase-2 gene positive clones by the way of movement of Blue stain in the agar medium.

Both genes under common promoter and terminator sequences resulted in 25% increase in the amount of reducing sugar released from Birchwood xylan. *Bacillus sp*. strain NG-27 Xylanase (47 kDa) active at 70^0C and pH 8.4 was cloned in *E.coli* using shot gun library method (Gupta *et al.,* 1996). Xylanase gene from *Vibrio sp.* strain XY-214 was also manipulated by using the host *E.coli*. The 1383 bp long gene was responsible for 51,323 Da proteins (Araki *et al.,* 2000). Similarly the xylanase from *Paenibacillus sp*. was also cloned in *E.coli* (Lee *et al.,* 2000).

Fig. 8. Zymogram of Xylanase 2 enzyme stained with Congo red

Fig. 9. Xylanase production from the Xyn2-pUC 19 mediated gene transfer *into E.coli* culture

Similarly, 1236 bp open reading frame of *Bacillus stearothermophilus* T-6 xylanse gene was cloned using *E. coli (*Gat *et al.,* 1994*)*. They also found that the â-xylosidase gene was present 10 kb downstream of the xylanase gene, but it was not a part of the same operon. *Clostridium thermocellum* xylanase gene was cloned in *B.subtilis* and constructed the vector pJX18 by inserting a Bam HI 1.6 kb DNA fragment of pCX18, which contained the xylanase structural gene (Jung K.H. and Pack M.Y. 1993).

The molecular weights of crude extract of xylanase extracted from *E.coli* transformant were estimated using the technique of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The results showed that more number of clear bands appeared when stained with coomasive blue, indicating the presence of two or more proteins in crude extract (Fig 7). Among the bands the 26 KDa is more prominent and it may be considered as Xylanase. These findings are in agreement with other investigators that reported the presence of xylanase with different molecular weights of xylanase produced by various microorganisms (Huang *et al.,* 2005; La-Grange 2000; Gat *et al.,* 1994) .

The Zymogram assay showing xylanase activity in SDS Xylan PAGE electrophoresis of crude cell extracts isolated from Xyn2-pUC19 gene expressed in *E.coli* transformant (Fig 8). Zymogram analysis of Xyn 2 expressed from pUC19 revealed a single protein band approximately 26 kDa in size was identified by compared with the standard. As mentioned by previous study, the mature *Xyn* 2 secreted by *Trichoderma virde* has a molecular weight of 26 kDa (Gerhardt *et al.,* 1994). However, the expressed recombinant *Xyn2* into *E .coli* in this study has a similar molecular weight than that of native enzyme (25 vs. 26 kDa), it will indicate that the process of glycosylation is performed by expression system very well. The molecular weight

of wild type of xylanase from the *Enterobacter* is bound be 43 kDa while xylanase isolated from *B.subtilis* had a high molecular weight of 340kDa. For xylanase production and measurement of their enzyme activities, the *Xyn2* transformant were cultured in flasks (Fig. 9) and the enzyme activities of the culture supernatant were measured. The culture supernatant of the *Xyn2* transformant showed strong xylanase activity was 176 Uml^{-1} by measured with release of reducing sugars. The cloned xylanase Gene from *Paenibacillus* sp. DG-22 in *E.coli* showed enzyme activity over a pH of 6 at 50°C. The optimal enzymatic activity of xylanase is 157.8 Uml^{-1}(Lee *et al.*, 2000).

CONCLUSIONS

The successful cloning of gene encoding xylanase enzyme will lead to the development of a new novel effective bleaching strategy to achieve significant reduction in the consumption of polluted resources like phenolic chlorinated chemicals. The results presented here suggest that cloned xylanase from *T .reesei* into *E .coli* could be a model system for gene expression, secretion, and purification of protein. Large-scale production of xylanase from *E. coli* transformant will also be useful for industrial applications. Further studies are anticipated involving the optimization of the experimental conditions necessary for achieving maximum gene expression for production of xylanase enzyme.

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REFERENCES

1. Araki T Hashikawa S and Morishita T. Cloning, sequencing, and expression in *Escherichia coli* of the new gene encoding β-1, 3-xylanase from a marine bacterium, *Vibrio* sp. strain XY-214, *Appl. Environ. Microbiol* 2000; **66**: 1741
2. Bai Y Wang J Zhang Z Yang P Shi P Luo H Meng K Huang H and Yao BA. New xylanase from thermo acidophilic *Alicyclobacillus* sp. A4 with broad-range pH activity and pH stability. *J Ind Microbiol Biotechnol*. 2010; **37**:187-94.
3. Beaucage SL and Caruthers MH., Deoxynucleoside phosphoramidites—A new class of key intermediates for deoxypolynucleotide synthesis". *Tetrahedron Letters* 1981; **22**: 1859-1862
4. Black GW Hazelwood GP Xue GP Orpin CG and Gilbert HJ., Xylanase B from *Neocallimastix patriciarum* contains a non-catalytic 455-residue linker sequence comprised of an octapeptide. *J Biochem* 1994; **299**: 560-569
5. Copa-Patino JL Kim YG and Broda P. Production and initial characterization of the xylan-degrading system of *Phanerochaete chrysosporium*, *Appl. Microbiol. Biotechnol.* 1993; **40**: 69
6. Durand R Rascle C and Fevre M. Molecular characterization of xyn3, a member of the endoxylanase multigene family of the rumen anaerobic fungus *Neocallimastix frontalis. Curr. Genet.* 1996; **30**: 531–540
7. Fontes CM Gilbert HJ Hazlewood GP Clarke JH Prates JA McKie VA Nagy T Fernandes TH and Ferreira LM. A novel Cellvibrio mixtus family 10 xylanase that is both intracellular and expressed under non-inducing conditions. *Microbiology.* 2000; **46**:1959-67.
8. Gallardo O Diaz P and Pastor FI. Cloning and characterization of xylanase A from the strain Bacillus sp. BP-7: comparison with alkaline pI-low molecular weight xylanases of family 11. *Curr Microbiol.* 2004; **48**:276-79.
9. Gat O Lapidot A Alchanati I Regueros C and Shoham Y. Cloning and DNA sequence of the gene coding for *Bacillus stearothermophilus* T-6 xylanase, *Appl. Environ. Microbiol* 1994; **60**: 1889
10. Gerhardt P Murray RGE Wood WA and Krieg NR, Methods for general and molecular Bacteriology American society for Microbiology, Washington, D.C. 1994.
11. Gilbert HJ Hazlewood GP Laurie JI Orpin CG and Xue GP. Homologous catalytic domains in a rumen fungal xylanase: evidence for gene duplication and prokaryotic origin. *Mol. Microbiol.* 1992; **6**: 2065-2072
12. Gomes I Gomes J Steiner W and Esterbauer H Production of cellulase and xylanase by a wild strain of *Trichoderma viride*, *Appl. Microbiol. Biotechnol* . 1992; **36**: 701
13. Gupta N Reddy VS Maiti S and Ghosh A. Cloning, expression, and sequence analysis of the gene encoding the alkali-stable, thermostable endoxylanase from alkalophilic, mesophilic *Bacillus sp. strain NG27, Appl. Environ.*

Microbiol. 2000; **66**: 2631
14. Hespell RB and Whitehead TR., Physiology and genetics of xylan degradation by gastrointestinal tract bacteria. *J. Dairy Sci.* 1990; **73**: 3013-3022.
15. Huang YH Huang CT and Hseu RS. Effects of dockerin domains on *Neocallimastix frontalis* xylanases. *FEMS Microbiol. Lett*. 2005; **243**: 455-460.
16. Jung KH and Pack MY.Expression of a *Clostridium thermocellum* xylanase gene in *Bacillus subtilis , Biotech. Letts*. 1993; **15**: 115
17. Laemmli, UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**(5259): 680-685.
18. La-Grange DC Claeyssens M Pretorius IS and van-Zyl WH. Coexpression of the *Bacillus pumilus* beta-xylosidase (xynB) gene with the *Trichoderma reesei* beta-xylanase-2 (xyn2) gene in the yeast *Saccharomyces cerevisiae Appl. Microbiol. Biotechnol.* 2000; **54**: 195
19. Lee CC Wong DW and Robertson GH. Cloning and characterization of the xyn11A gene from *Lentinula edodes.Protein J.* 2005; **24**: 21-26.
20. Lee H J Shin D J Cho N C Kim H O Shin SY Im S Y Lee HB Chun S B and Bai S. Cloning, expression and nucleotide sequences of two xylanase genes from *Paenibacillus sp. Biotechnol. Lett* 2000; **22**: 387
21. Liu JH Selinger BL Tsai CF and Cheng KJ Characterization of a *Neocallimastix patriciarum* xylanase gene and its product. *Can. J. Microbiol.* 1999; **45**: 970-974
22. Panbangred W Fukusaki E Epifanio EC Shinmyo A and Okada H. Expression of a xylanase gene of *Bacillus pumilus* in *Escherichia coli* and *Bacillus subtilis, Appl. Microbiol. Biotechnol* 1985; **22**: 259
23. Sambrook J and Russel DW. Molecular Cloning: A Laboratory Manual, Volume 1. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York, 2001.
24. Shoham Y Schwartz Z Khasin A Gat O Zosim Z and Rosenberg E. Delignification of wood pulp by a thermostable xylanase from *Bacillus stearothermophilus* strain T-6. *Biodegradation* 1992; **3**: 207-218
25. Steiner W Lafferty RM Gomes I and Esterbauer H. Studies on a wild strain of *Schizophyllum commune*: Cellulase and xylanase production and formation of the extracellular Polysaccharide *Schizophyllan Biotechnol. Bioeng.* 1987; **30**: 169
26. Störkle and Dominic."Complex Formation of DNA with Oppositely Charged Polyelectrolytes of Different Chain Topology: Cylindrical Brushes and Dendrimers". *Macromolecules* 2007; **40**(22): 7998–8006
27. Subramaniyan S and Prema., Biotechnology of microbial Xylanases: Enzymology, Molecular Biology and Application. *Critical Reviews in Biotechnology* 2002; **22**: 33-46
28. Torronen A Mach RL Messner R Gonzalez R Kalkkinen N Harkki A. and Kubicek CP. The two major xylanases from *Trichoderma reesei:* characterization of both enzymes and genes. *Biotechnology* 1992; **10**: 1461-1465
29. Wong KKY Tan LUL and Saddler JN., Multiplicity of β-1,4-xylanase in microorganisms: Functions and applications, *Microbiol. Rev* . 1988 ; **52**: 305
30. Yanisch-Perron C Vieira J and Messing J. "Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors". *Gene* 1985; **33**(1): 103–119
31. Zhang G Rao B Ye J Ma L and Zhang. Molecular cloning and heterologous expression of a new xylanase gene from *Verticillium dahliae*. Wei Sheng Wu Xue Bao.; 2008; **48**:765-71.