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Cloning of a Cellulase Gene from Indigenous Strain of *Bacillus* Species

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Abstract

The following study presents the cloning of a cellulase gene from indigenous strain of *Bacillus* sp. The genomic DNA of *Bacillus* sp was isolated and employed for the PCR amplification of the cellulase gene. For this, different sets of primers were designed following the available sequences of cellulase gene in NCBI data base. A PCR product of cellulase gene of 1.65 κb was successfully amplified. The amplified gene was cloned into pTZ57R/T cloning vector. The cloning of the gene was confirmed through blue white screening and colony PCR screening.

Keywords: DNA isolation**,** PCR amplification**,** Cloning, Cellulase gene, *Bacillus* sp.

Introduction

Lignocellulose is the most abundant plant cell wall component found in the biosphere and also the most voluminous waste produced by our society. Such cellulosic and lignocellulosic materials need to be degraded. This degradation may be brought about with the help of various enzymes especially cellulases. Moreover, conversion of this cellulosic material to glucose and eventually to bioethanol has a global significance (Lynd et al., 2008).

There are three components of cellulase i.e., exoglucanase, endoglucanase and β-glucosidase (Li et al., 2009), classified on the basis of their mode of action. Endoglucanases are found to randomly attack the amorphous regions of cellulose, which yield high degree of polymerization oligomers. Cellobiohydrolases are exoenzymes and hydrolyze crystalline cellulose, which further releases cellobiose. Both types of enzymes hydrolyze β-1, 4-glycosidic bonds. The β-D-glucosidase or cellobiase convert cellooligosaccharides and cellobiose to glucose (Dienes et al., 2004).

Cellulases have got applications in different industries such as food, feed, textile, pulp, paper, detergent and agriculture (Li et al., 2009). A major constraint in enzymatic saccharification of cellulosic materials for the production of fermentable sugars is low productivity and the cost of cellulases (Sukumaran et al., 2009). The combination of cellulosomes with novel production concepts could in future become necessary for economical conversion of cellulosic biomass to biofuels (Edward et al., 2007).

Different components of cellulose system have been studied and isolated from various species of bacteria such as *Clostridium*, *Cellomonas*, *Bacillus*, *Thermonospora*, *Ruminococcus*, *Bacteriodes*, *Erwinia*, and *Acetivibrio* (Woo and Kim, 2007). In this paper, we report PCR amplification and cloning of a cellulase gene into PTZ57R/T cloning vector from genomic DNA of an indigenous strain of *Bacillus* sp.

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Materials and Methods

Microorganisms

The test organism used in this experiment was *Bacillus subtilis* JS2004 that was acquired from Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan.

Media and Culture Conditions

Bacillus subtilis JS2004 was maintained on Luria Broth (LB) medium containing (g L^{-1}) NaCl 10, yeast extract 5 and peptone10 (Awan and Rehman, 2002). The optimum temperature and pH for the growth of strain was found 45 °C and 8.5 respectively. Freshly inoculated culture was overnight incubated at 45 °C and used for the isolation of genomic DNA.

DNA Isolation

The genomic DNA of *Bacillus subtilis* JS2004 was isolated following the method of Yamada et al. (2002). Overnight grown bacterial culture was resuspended in 5 mL of 0.1xSSC solution and centrifuged for five minutes at 14000 x g (4°C). The pellet was suspended in 0.01 M sodium phosphate buffer in 20% sucrose (pH 7.0) containing lysozyme (2.5 mg/mL) and incubated for 45 minutes at 37 °C. The sample was deproteinized by phenol chloroform extraction and the supernatant was precipitated in 2.5 volume of 100% cold ethanol overnight. The pellet was suspended in 100 µL of Tris EDTA buffer after

washing in 75% ethanol. The DNA concentration was determined spectrophotometrically. The samples of genomic DNA from *Bacillus* sp. were treated with DNAse free RNAse A (Fermentas) following manufacturer's protocol.

PCR conditions

Different set of primers were employed for the amplification of the cellulase genes from the genomic DNA of indigenous *Bacillus* sp. Following set of primer yielded successful amplification of a cellulase gene. This set of primer was designed following the NCBI gene sequence (Accession number NP241469.1).

Both forward and reverse primers were used with final concentrations of 0.25 µM for preparation of PCR mixture and different annealing temperatures were set in accordance with T_m of respective primers during the PCR reactions.

Ligation and Transformation

The amplified gene of cellulase was purified from the agarose gel after electrophoresis using Qiagen gel extraction kit and ligated into the pTZ57R/T cloning vector (Fermentas) using DNA ligase and ligation buffer.

Transformation of the ligated gene in the vector was performed by heat shock method (Sambrook and Russel, 2001). The colonies having clones of cellulases gene were screened by blue/white screening. Due to IPTG induction, white colored colonies containing cellulase gene were produced after overnight incubation at 37 ˚C. The positive transformants were re-plated on IPTG-Xgal-LB-agar-ampicillin plates and subjected to colony PCR.

Colony PCR

In order to confirm that cellulase gene was successfully inserted in the pTZ57R/T cloning vector, colony PCR was performed. The composition and programming of PCR reaction was same as mentioned above, with exception that a single colony from the plates was picked using autoclaved tip and dipped in PCR tube containing the reaction ingredients. This colony was used as a template during the colony PCR. The results of the colony PCR were observed on agarose gel electrophoresis. The colonies showing positive result after colony PCR were employed for the further culturing and isolation of plasmid DNA.

Miniprep and restriction analysis

For the isolation of plasmid DNA from successfully transformed colonies, LB-ampicillin medium (5 mL) was inoculated by colonies and incubated overnight at 37 ˚C in an orbital shaker at 220 rpm. After incubation, 1.5 mL of the culture was transferred to an eppendorf tube, centrifuged and the pellet was resuspended in 100 µL of an ice cold GTE solution (50 mM glucose; 10 mM EDTA; 25 mM Tris Cl, pH 8) followed by alkaline lysis miniprep method (Sambrook and Russel, 2001).

pTZ57R/T plasmid was digested with *Bam*HI and *Eco*RI using approprtiate buffer (Fermentas). The reaction was carried out at 37 ˚C for one hour. The result of digestion was observed by agarose gel electrophoresis.

Results and Discussion

Growth of *Bacillus* sp.

In our experiment, Lauria Bronsted (LB) medium proved to be a good source for the culture growth of the *Bacillus subtilis* JS2004. Peptone and yeast extracts were used as nitrogen source in the growth medium (Awan and Rehman, 2004). Yeast extract has also been used as nitrogenous ingredient for the growth of *Bacillus* sp. by Bo et al. (2010) and Gurdeep et al. (2010).

Isolation of genomic DNA

Different methods have been reported for the genomic DNA isolation of bacterial species. The method of Yamada et al. (2002) for the isolation of genomic DNA from *Bacillus subtilis* JS2004 was followed. While Chun et al. (2010) isolated the genomic DNA of *Paenibacillus* species using the method described by Srivastava et al. (1981). We got sufficient concentration of genomic DNA (0.495 mg/mL $\pm$ 0.155) from the culture of *Bacillus* sp. which was confirmed by running the samples on agarose gel (Fig 1).

Amplification of Cellulase gene

Among 10 sets of primers tried during the study, the best amplification was observed by the primer set CelBF (forward primer; **5′CCATGGATCATGAGG ATGTGAAAACTC**) and CelBR (reverse primer; **5-′CTCGAGTGAATTGGTTGTCTGAGCTG**). This set of primer was designed following the sequence of cellulase gene from *Bacillus* species as reported by Takami and Horikoshi (1999). Among rest of sets of primers, sufficient amplification of cellulase gene could not be achieved as some set of primers showed non specific amplification of gene while a few others amplified DNA fragments of very minute base pair size (~300). The amplification of 1.65 kb gene of cellulase by primer set CelBF & CelBR is shown in Figure 2.

Cloning of Cellulase gene

We used *DH5α* strain of *E. coli* for the cloning of cellulase gene. This strain proved to be an efficient strain and we successfully cloned a cellulase gene from local isolate of *Bacillus* species. Earlier, cellulase genes have been cloned, expressed and characterized from various species of the bacteria (Feng et al., 2011; Chun et al., 2010; Gurdeep et al., 2010). A cellulase and β-1,4-endoglucanase genes were isolated and cloned from genomic DNA library

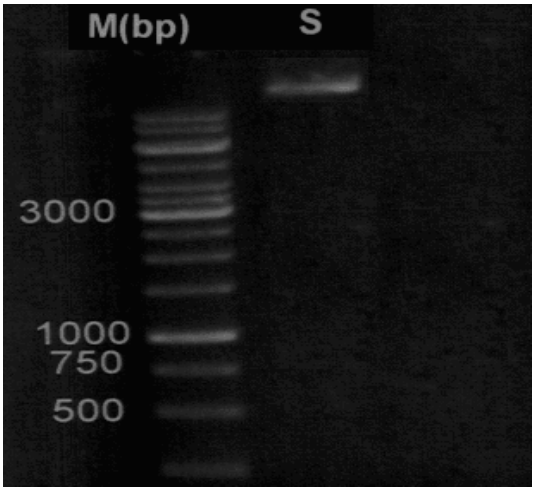

Fig 1 Genomic DNA of *Bacillus* sp.

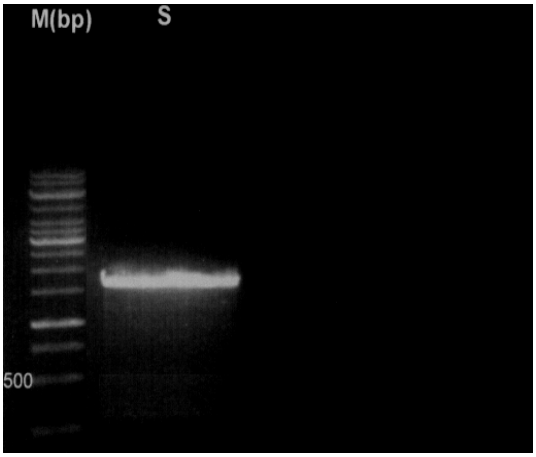

Fig 2 Amplification of cellulases gene from *Bacillus sp.* Lane I: 1 kb DNA ladder (Fermentas) Lane II: amplified cellulase gene using CelBF & CelBR primers at annealing temperature of 52˚C.

of alkaliphilic *Bacillus agaradhaerans* and endophytic *Bacillus* by Rees et al. (2003) and Lima et al. (2005), respectively.

Blue/White Screening

The blue/white screening method showed successful isolation of positive transformants containing cellulase gene. A large number of positive (white) colonies were observed on the LB agar plates showing high efficiency of ligation (Fig 3). One recombinant plasmid designated as pTZ57-cell was selected for further analysis. We used 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) as substrate for the growth of transformants of cellulase gene on LB-ampicillin plates while Setlow et al. (2004) employed 4 – methylumbelliferyl – phosphor – β - d-glucopyranoside for the screening of glucosidase gene of *Bacillus subtilis*.

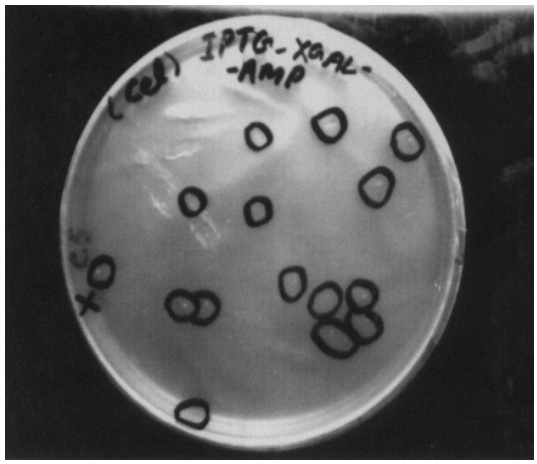

Fig 3 Blue/white screening of clones transformed with cellulase gene on IPTG-Xgal-Ampicillin agar plates. The final concentration of ampicillin was maintained at 50 µg/mL

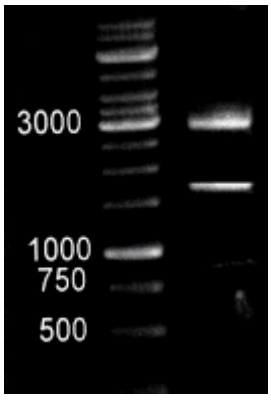

Fig 4 Restriction digestion analysis of Vector. `Lane I: 1 Kb DNA ladder (Fermentas) Lane II: Vector & gene insert

The screening method of Liu et al. (2004) is similar to our method regarding cloning of cellulase genes as they screened two novel genes designated as cel12A and cel9A encoding an endoglucanase from a thermo philic bacteria *Bacillus* licheniformis GXN151, on the basis of plates showing the activity of carboxyl methyl cellulase. Whereas Chun et al. (2010) used congo red method for the screening of cellulase positive clones. The method of blue/white screening for determination of cellulase activity has been preferred by the researchers than congo red screening method. Because positive clones can not show hydrolytic activity in congo red method due to inframe cloning of cellulase gene whereas blue/white screening is based on alpha complementation and the positive clones with in frame gene clone can also be screened.

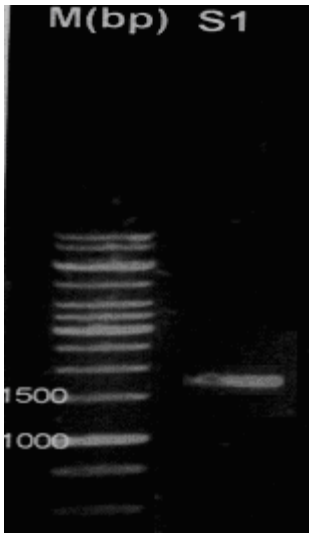

Fig 5 Colony PCR; A single colony was picked from the plates and used as template in the PCR reaction; Lane I: I Kb DNA ladder (Fermentas) Lane II: 1.65 Kb amplified cellulase gene

Restriction analysis and Colony PCR

The restriction digestion of the recombinant plasmid was performed followed by Colony PCR. The results of restriction analysis and colony PCR are shown in Figure 4 and 5 respectively. These results showed that inserts of 1.65 kb gene was successfully cloned into pTZ57R/T vector. The amplified size of gene fragments by colony PCR was also the same as expected (1.65 kb) which further confirmed cloning of the cellulase gene from *Bacillus subtilis* JS2004.

Conclusion

To our knowledge, no similar study on this local strain of *Bacillus* sp. has been performed. Optimal conditions for the cloning of cellulase by *B. subtilis* JS2004 were established in this study. The cellulase gene was successfully amplified from genomic DNA of the *Bacillus* strain and cloned into pTZ57R/T cloning vector.

Our findings show that it is possible to use this strain of *Bacillus* species for isolation and cloning of different cellulase genes. The results of this study may further be employed for characterization of a recombinant cellulase enzyme after its expression in a suitable host because novel cellulases with better efficiency are needed in industry.

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