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Full Length Research Paper

Molecular Cloning and Sequencing of *xylanase* gene from Penicillium chrysogenum

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The filamentous fungus, *Penicillium chrysogenum,* is able to grow on xylan as a sole carbon source. Under these conditions, high levels of a xylanase (XYLP) are secreted into the medium. Xylans are the principle non-starch polysaccharides of wheat, and top surface of wheat in poultry diets can increase the intestinal viscosity, that is obstacle the absorption of nutrients and circulation. In this study, we clonned *xylanase* gene from *Penicillium chrysogenum* in pGEM vector and the construct was transformed into TOP10 *E. coli* cells after purification and characterization of the enzyme. Genomic sequencing was done by using oligodeoxyribonucleotides derived from partial amino acid (aa) sequences of the purified enzyme. Sequencing confirmed that *xylanase* was succesfuly cloned and the length of *xylanase* was 661 bp; BLAST search showed that the sequence of *xylanase* gene of the *Penicillium chrysogenum has* 97% homology with other records existing in GenBank. The aa sequence of XYLP shows considerable homology to high-M(r) acidic xylanases (Xln) and cellulases from different bacteria, yeasts and fungi.

Keywords: Xylanase; Gene cloning and expression; Sequence analysis *Penicillium chrysogenum.*

INTRODUCTION

Filamentous fungi are saprophytic organisms secreting a wide array and high level of proteins involved in the breakdown and recycling of complex polymers from both plants and animal tissues. These characteristics and the existence of a well-established technology for large-scale fermentation of these organisms advanced their industrial application in secretion of heterologous proteins. Although *Penicillium chrysogenum* is of significant industrial importance and has the generally recognized as safe status, only preliminary attempts have been made to utilize this fungus as a host for homologous and heterologous protein production and secretion.

Xylans constitute the main polymeric component of the hemicellulose fraction of plant cell walls. It is covalently and no covalently attached to cellulose, lignin, pectin and other polysaccharides to maintain cell wall integrity (Ahmed et al., 2009). They have been isolated from different sources and exhibit considerable variation in composition and structure, and hence the xylans represent a considerable reservoir of fixed carbon in nature. In general, xylan is composed of a backbone structure of β-1, 4-linked D-xylose residues with a-L-arabinofuranose and glucuronic acid side chains, most of which are acetylated. Biodegradation of

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these xylans involves the action of several hydrolytic enzymes, including two major xylanolytic enzymes, endoxylanase (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8) and β-xylosidase (1,4-β-D-xylan xylohydrolase; EC 3.2.1.37) (Caprita et al., 2010). Endo-1,4-β-D-xylanases (EC 3.2.1.8) are responsible for random cleavage of the xylan backbone and hence have broad industrial significance because it may be possible to use them in the pulp and paper production and ramie degumming (Jeya et al., 2010). During the process of ramie degumming, for example, application of xylanases, combined with pectinase, have been an ideal alternative to the environment-polluting alkaline degradation for the production of high-quality fibers Numerous xylanases have been isolated and characterized from various microorganisms, including fungi, bacteria and yeasts, and some xylanase genes have been cloned and expressed in *Escherichia coli* (Cazemier et al., 1999, Li and Ljungdahl, 1996 and Srivastava and Mukherjee, 2001). However, the xylanases currently used have limitations such as low purity and efficiency, which limits their applicability in different areas such as pulp bleaching, ramie degumming and food processing. Screening and designing of new xylanases with higher efficiency and specificity via genetic manipulation has become a major focus of researchers in this field.

The arabinoxylan found in the cell walls of grains has an anti-nutrient effect on poultry. When such components are present in soluble form, they may raise the viscosity of the ingested feed, interfering with the mobility and absorption of other components. If *xylanase* be added to feed containing maize and sorghum, both of which are low viscosity foods, it may improve the digestion of nutrients in the initial part of the digestive tract, resulting in a better use of energy. The joint action of the rest of the enzymes listed produces a more digestible food mixture (Polizeli *et al*., 2005). In this study, we clonned and sequenced *xylanase* gene from *Penicillium chrysogenum* in pGEM vector and the construct was transformed into TOP10 *E. coli* cells after purification and characterization of the enzyme.

MATERIALS AND METHODS

Bacterial strains Vectors and culture conditions

Penicillium chrysogenum was used as the source of chromosomal DNA. *E. coli* strain JM109 was used as the hosts for DNA manipulation. The plasmid pGEM was used for cloning, DNA sequencing and expression. The *E. coli* strains carrying recombinant plasmids were cultivated in LB broth. For plating, 1.6% agar was added to LB broth. For selection of recombinants, 100 µg/ml of ampicillin, 1 mM isopropyl-β-thiogalactopyranoside (IPTG) and 40-µg/ml 5-bromo-4-chloro-3-indoly-β-D-galactopyranoside (X-Gal) was added to agar gel.

DNA Manipulations and PCR Amplification of xylanase gene (xylB)

Genomic DNA was isolated from *Penicillium chrysogenum* cells by a modification of the Marmur method (Marmur, 1961). Cells were harvested in mid-exponential growth phase by centrifugation (8000*g* for 10 min at 4 °C) and suspended in TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]). Cells were treated with lysozyme (1 mg/ml) for 20 min and then treated with 0.5% sodium dodecyl sulfate (SDS) and proteinase K (100 mg/ml) at 37 °C for 1 h, subsequently 10% CTAB at 65 °C for 20 min. The lysate was twice extracted with chloroform-isoamyl alcohol (24:1); DNA was precipitated with 0.6 volumes of isopropanol and harvested by centrifugation (10,000*g* for 15 min at 4 °C) and re suspended in TE buffer.

Digestion of DNA with restriction endonucleases, separation of fragments by agarose gel electrophoresis, ligation of DNA fragments, transformation of *E. coli* with plasmidic DNA and extraction of recombinant DNA were all performed as described by Sambrook et al. (1989). DNA fragments were recovered from low-melting agarose gels using the Gel Extraction Kit of Takara.

Xylanase gene was amplified using Taq DNA polymerase (Roche applied science) and the primers specific for the coding region of *xylB*. Primer sequences were the following: the forward primer was *xylB*-F: 5'-GTGCACGTTCATAAAAGGAGGAAG- 3 and the reverse primer was *xylB*-R: 5'-GCCCAAGCTTGGGTTATTTTCCGCTT -3. Restriction enzyme sites of *Xba*I and *KpnI* (underlined nucleotides in the above sequences) were integrated into the 5' end of primers *xylB* -F and *xylB* -R, respectively. PCR reactions were performed in a total volume of 25 µL containing 100 ng of template DNA, 1µM of each primers, 2 mM MgCl2, 5 µL of 10X PCR buffer, 200 µM dNTPs and 1 unit of Taq DNA polymerase (Roche applied science). The following conditions were applied: initial denaturation The PCR product was at 95°C for 5 minutes, followed by 30 cycles; denaturation at 94°C for 1 min, annealing at 61°C for 1 min, elongation at 72° for 1 min. The program was followed by final elongation at 72°C for 5 minutes. The PCR-amplified products were detected in 1% ethidium bromide (EtBr)-stained agarose gel electrophoresis. After electrophoresis, images were obtained in UVIdoc gel documentation systems (UK).

Cloning and sequencing of the xylB gene of Penicillium chrysogenum.

First, the PCR-amplified products from *xylB* gene were extracted from an agarose gel using a DNA extraction gel kit (Bioneer Co., Korea) according to the manufacturer's protocol. Then, gel-purified products were cloned in pGEM-T Easy Vector (Invitrogen, San Diego, CA) using a T/A cloning technique. *Xbal*/*KpnI* (Promega) restriction analysis

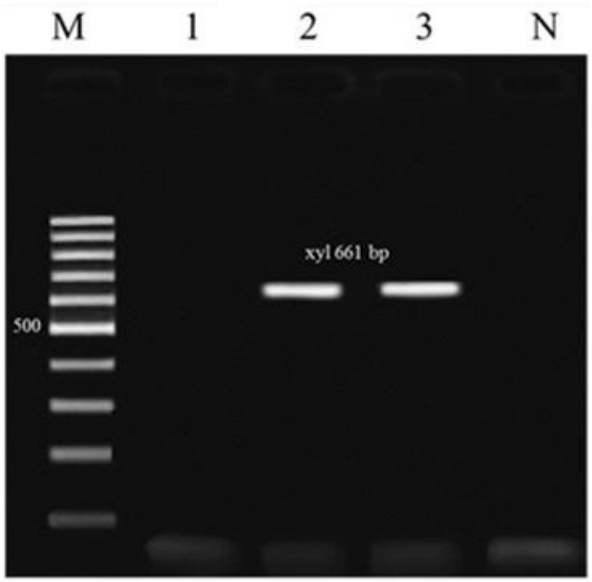

Figure 1. Agarose gel electrophoresis of PCR amplification products for detection of *xylB* gene from *Penicillium chrysogenum* (Lane M is 100-bp DNA molecular marker, Lane 1 is negative sample, lanes 2 and 3 are positive samples and lane N is negative control).

was used to confirm the gene cloning. Finally, the sequencing from cloned fragments was done and sequence similarity was checked using nucleotide BLAST analysis at http://www.ncbi.nlm.nih.gov/blast/Blast.cgi.

RESULTS

The PCR product was obtained using total *Penicillium chrysogenum* chromosomal DNA as PCR template, and primers *xylB*-F and *xylB*-R.The PCR product was about 661 bp and no other nonspecific bands were found (Figure. 1). The PCR product was digested with restriction enzymes *Xba*I and *KpnI* and recovered from the agrose gel, and ligated to pGEM, and recombinant plasmids were transformed into *E. coli* JM109, then and agarose gel electrophoresis were used to confirm the gene cloning, yielding fragments of 661 bp and 3015 bp (Figure. 2) Positive clones, screened by ampicillin, IPTG and X-Gal and detected by the presence of clear zones around colonies suggesting xylan hydrolysis, were obtained. Finally, the nucleotide sequence obtained from a fragment of the *xylB* gene of *Penicillium chrysogenum* was found to be *Penicillium chrysogenum* -specific based on BLAST searches.

DISCUSSION

When birds are fed with wheat-based diets, the presence of NSP can give rise to highly viscous conditions in the small intestine and decrease contact between digestive enzymes and substrates, hence depressing nutrient absorption and broiler performance. In addition to the direct effects of NSP on nutrient digestion, there appear to be secondary effects attribution. Enzyme inclusion decreased the size of the digestive organs and the gastrointestinal tract to some extent. The benefits of exogenous enzyme supplementation to NSP-rich diets are well documented. These enzymes can partially hydrolyzed NSP, reduce the viscosity of gut contents, and result in improvements in nutrient absorption. Several studies have also demonstrated that enzyme treatment can affect the intestinal morphology in birds fed barley-based diets or decrease the small intestinal fermentation attributed to high NSP diets (Wang *et al*., 2005).

By revealing the importance of *xylanase* several studies were performed about this enzyme. In 2009, Jeya *et al*. conducted cloning and expression of GH11 *xylanase* gene from *Aspergillus fumigatus* MKU1 in *Pichia pastoris* (Jeya *et al*., 2009). In 2010, Hwang *et al*. conducted cloning of a *xylanase*, KRICT PX1 from the strain *Paenibacillus sp*.

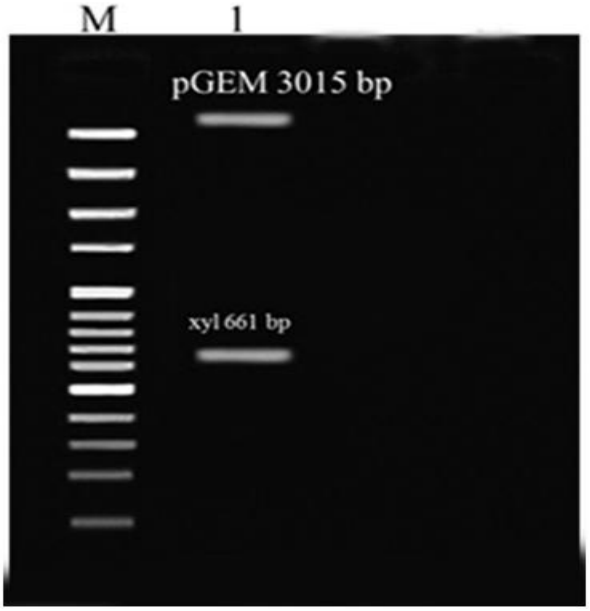

Figure 2. Analysis of pGEM-*xylB* recombinant vector using *XbaI* and *KpnI* restriction endonuclease enzyme (Lane M is Fermentas 100-bp DNA molecular marker, lane 1 is *xylB* (661bp) and also pGEM vector without *xylB* (3015bp).

HPL-001 (Hwang *et al*., 2010). In 2011, Wang *et al*. conducted direct cloning, expression and enzyme characterization a novel cold-active *xylanase* gene (*Xyn*GR40) (Wang *et al*., 2011). In 2013, Lin *et al*. successfully conducted cloning and expression of a thermostable *xylanase* from *Bacillus halodurans* C-125 (C-125 *xylanase* A) (Lin *et al*., 2013). In 2014 Kishishita *et al*. conducted cloning and expression of cellulose-inducible endo-β-1, 4-*xylanase* (*Xyl*10A) from the mesophilic fungus Acremonium cellulolyticus (Kishishita *et al*., 2014). In present study, we did the cloning and sequencing of *xylB* gene of *Penicillium chrysogenum* successfully, which pGEM-*xylB* plasmid that was generated in this study is ready for sub-cloning and production of *xylanase* enzyme in fungi that used as probiotics in poultry diets. Therefore, we suggest use of prepared construct (pGEM-*xylB*) in present study for sub-cloning and finally production of genetic manipulation of the fungus as probiotic and their use in poultry diets.

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