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Research Article

Cloning and sequencing of *xylanase* gene from *Bacillus subtilis*

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Abstract

Xylans are the principle non-starch polysaccharides of wheat in poultry diets which can increase the intestinal viscosity and decrease the nutrient absorption. *Xylanases* catalyze the hydrolysis of xylans. The aim of present study was to clone *xylanase* gene in pGEM vector and sequencing of this gene from the *Bacillus subtilis*. Genomic DNA from *B. subtilis* was isolated and amplified by PCR using *Xylanas*e specific PCR primers. Then *xylanase* was cloned by T/A cloning technique and transformed into TOP10 *E. coli* cells. Finally, after sequencing, *xylanase* sequence similarity was checked using nucleotide BLAST analysis. The results of present study showed that *xylanase* was successfully cloned in pGEM vector. Sequencing confirmed that *xylanase* was cloned and the length of *xylanase* was 661 bp. BLAST search showed that the sequence of *xylanase* gene of the *B. subtilis* has 99% homology with other records existing in GenBank.

Keywords: *Xylanase*; *Bacillus subtilis*; cloning and sequencing; probiotic; poultry diets

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Introduction

Bacillus subtilis has been a model for Gram-positive bacteria for more than a century (Barbe et al., 2009). This bacterium is a Gram positive, aerobic, spore-forming soil bacterium ubiquitous in the environment. The beneficial effects of *B. subtilis* spores on the balance of the intestinal micro flora are the rationale for its general use as a probiotic preparation in the treatment or prevention of intestinal disorders (Oggioni et al., 1998; Ghafari et al., 2014).

Macromolecules of monosaccharides linked by glycosidic bonds are polysaccharides. Non-starch polysaccharides (NSP) are chiefly non-α-glucan polysaccharides of the plant cell wall. NSP are an in accordant group of polysaccharides (Caprita et al., 2010). The major constituent of hemicellulose and the second most abundant polysaccharide in plant cell walls is xylan. Xylan is a complex polymer consisting of b-1,4-linked xylosyl residues, which can be acetylated or can have covalently linked arabinosyl and glucuronic acid side-groups. Complete enzymatic conversion of xylan into monomeric sugars requires the concerted action of several enzymes including endo-1,4-b-xylanase, b-xylosidase, a-L-arabinofuranosidase, a-glucuronidase and a number of esterases. Sequences of xylan-degrading enzymes, classified as members of different glycosyl hydrolase families, have been reported for a large number of fungi and prokaryotes (Mirzaie et al., 2012).

Most of the anti-nutritive activities of NSP which directly affect broiler performance have been attributed directly to soluble polysaccharides. A majority of polysaccharides when dissolved in water give viscous solutions. Increases in digesta viscosity associated with wheat arabinoxylan were noted in poultry. Hemicellulose

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is composed of xylan as a major component that constitutes about 20-40% of total plant biomass. Endo-β-1,4 xylanases catalyze the hydrolysis of the backbone of xylan to produce xylooligo-saccharides, which in turn can be converted to xylose by β-xylosidase (Caprita et al., 2010). Based on amino acid sequence homologies and hydrophobic cluster analysis, *xylanases* have been grouped mainly into two families of glycosyl hydrolyses (I) family for GH 10 and (II) family G or GH 11 (Kishishita et al., 2014). However, other glycoside hydrolase families, 5, 7, 8, and 43, have also been found to contain distinct catalytic domains with a demonstrated endo-1,4-β-*xylanase* activity (Kishishita et al., 2014). The optimum pH for xylan hydrolysis is around 5 for most fungal *xylanases*, where as pH optima of bacterial *xylanases* are generally slightly higher (Yu et al., 1987). The *xylanase* and cellulase together with pectinase account for about 20% of the world enzyme market. Filamentous fungi have been used for more than 50 years in the production of industrial enzymes (Dalboge, 1997). They are widely used as *xylanase* producers and are generally considered as more potent *xylanase* producers than bacteria and yeasts, which secrete much higher amount of xylanolytic enzymes into the medium than bacteria or yeast (Ahmed et al., 2009). These enzymes are produced mainly by microorganisms and take part in the breakdown of plant cell walls, along with other enzymes that hydrolyze polysaccharides, and also digest xylan during the germination of some seeds. Microbial sources and filamentous fungi are especially interesting as they secrete these enzymes into the medium and their *xylanase* level is very much higher than those found in yeasts and bacteria (Jeya et al., 2009). *Xylanase* gene has been isolated from microorganisms of various genera and expressed in *E. coli*. In bacteria, *xylanas*e is not only produced at lower activity levels than in fungi, but are also restricted to the intracellular or periplasmic fractions (Ahmed et al., 2009). Furthermore, enzymes expressed in bacteria are not subjected to post-translation modifications, such as glycosylation (Bernier et al., 1983).

The arabinoxylan found in the cell walls of grains has an anti-nutrient effect in 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 (Mirzaie et al., 2012). 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). Previous studies on *xylanase* gene were performed only for the purpose of cloning and sequencing, but present study was done with the aim of *xylanase* gene isolation from *B. subtilis* and cloning of this useful gene (pGEM-xylB) for transferring into other probiotic bacteria such as *Lactobacillus acidophilus*.

Materials and Methods

Bacterial strains and culture conditions

B. subtilis was used as a source of chromosomal DNA. Standard strain of *B. subtilis* bacteria was prepared in the Department of Microbiology Pasteur Institute of Iran and was grown at 37°C in Luria-Bertani broth (LB broth) and LB agar plates. Medium was supplemented with ampicillin (100 mg/ml). Grown colonies were biochemically confirmed as *B. subtilis* positive.

DNA Extraction

Genomic DNA from *B. subtilis* was isolated by the method of DNA extraction kit (DNP™, CinnaGen, Iran) according to the manufacturer's protocols.

Amplification of xylanase gene (xylB)

Xylanase gene was amplified using Taq DNA polymerase (Roche applied science) and the primers specific for the coding region of *xylB*. Primers were designed according to the published sequence for *xylB* of the *B. subtilis* (accession number: NC_018520.1 and Gene ID: 13522035). Primer sequences were as following: the forward primer was *xylB*-F: 5'-GTGCACGTTCATAAAAGGAGGAAG- 3 and the reverse primer was *xylB*-R: 5'- GCCCAAGCTTGGGT TATTTTCCGCTT -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°C 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 *B. subtilis*

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. *XbaI*/*KpnI* (Promega) restriction analysis 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

Analysis of PCR products for the presence of the *xylB* gene of *B. subtilis* on 1 % agarose gel revealed a 661-bp fragment (Fig. 1). After cloning of the *xylB* gene recombinant plasmids of pGEM-*xylB* were isolated from transformed cells using a plasmid extraction kit (Bioneer Co., Korea), and restriction digestion with *XbaI* and *KpnI*, and agarose gel electrophoresis were used to confirm the gene cloning, yielding fragments of 661 bp and 3015 bp (Fig. 2). Finally, the nucleotide sequence obtained from a fragment of the *xylB* gene of *B. subtilis* was found to be *B. subtilis*-specific based on BLAST searches. The sequence was 99% identical to others published in the GenBank database (accession number: NC_018520.1 and Gene ID: 13522035).

Discussion

When birds are fed 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. 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 hydrolyze NSP, reduce the viscosity of gut contents, and result in improved 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).

Bernier in 1983, isolated a gene coding for *xylanase* synthesis in *B. subtilis* by direct shotgun cloning using *E. coli* as a host (Bernier et al., 1983). By revealing the importance of *xylanase* several studies were performed about this enzyme. Jeya et al. (2009) conducted cloning and expression of GH11 *xylanase* gene from *Aspergillus fumigatus* MKU1 in *Pichia pastoris*. Hwang et al. (2010) conducted cloning of *xylanase*, KRICT PX1 from the strain *Paenibacillus sp*. HPL-001. Wang et al. (2011) conducted direct cloning, expression and enzyme characterization a novel cold-active *xylanase* gene (*Xyn*GR40). Lin et al. (2013) successfully conducted cloning and expression of a

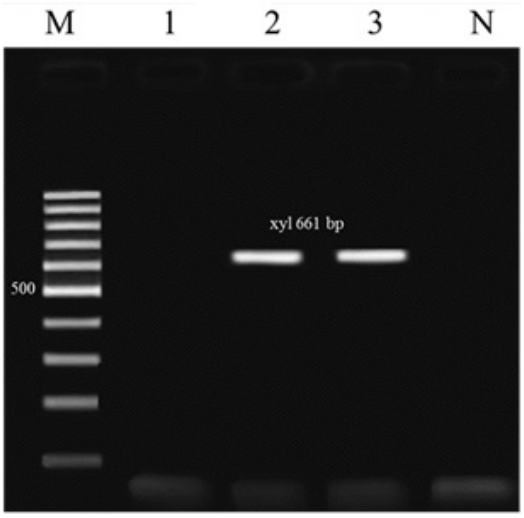

Fig. 1: Agarose gel electrophoresis of PCR amplification products for detection of *xylB* gene from *B. subtilis* (Lane M is 100-bp DNA molecular marker, Lane 1 is negative sample, lanes 2 and 3 are positive samples and lane N is negative control)

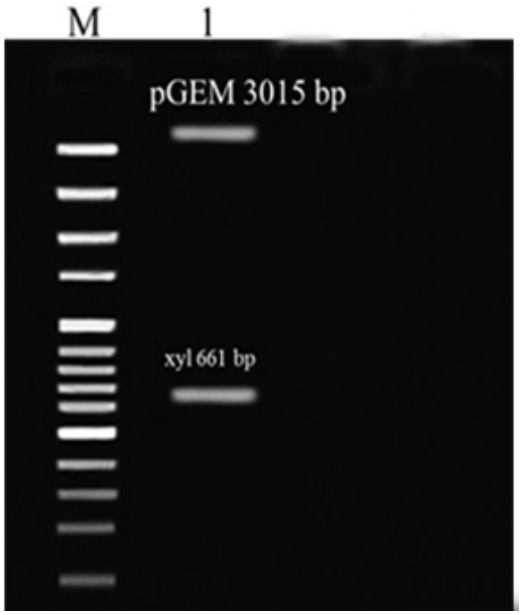

Fig. 2: Analysis of pGEM-*xylB* recombinant vector using *XbaI* and *KpnI* restriction endonuclease enzyme (Lane M is Fermentas 100-bp DNA molecular marker, lane 1 is *xylB* (661bp) and also pGEM vector without *xylB* (3015bp).

thermostable *xylanase* from *Bacillus halodurans* C-125 (C-125 *xylanase* A). Recently, Kishishita et al. (2014) conducted cloning and expression of cellulose-inducible endo-β-1, 4-*xylanase* (*Xyl*10A) from the mesophilic fungus Acremonium cellulolyticus. The present study was done with the aim of *xylanase* gene isolation from *B. subtilis* and cloning of this useful gene

(pGEM-xylB) to be used for transfer of *xylanase* gene to other probiotic bacteria such as *Lactobacillus acidophilus*.

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