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Molecular Cloning of Glycoside Hydrolase Family 9 Cellulase Gene from Buffalo Rumen

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***Abstract*—Glycosyl hydrolase family 9 (GHF9) is an enzyme that acts upon β-1-4 linked glucose. This study aims to isolate a cellulase gene of glycosyl hydrolase family 9 (GHF9) from metagenomic DNA in the ruminal fluid of buffalo (*Bubalus bubalis*) by polymerase chain reaction (PCR) technique using the degenerated primers, Cel9_F1 and Cel9_R1. The amplicon of approximate 1,200 base pair and cloned into pTZ57R/T vector and then transformed into *Escherichia coli* DH5α host strain. Two clones were verified for nucleotide analysis, namely cel9_A and cel9_B. The clone named cel9_A and cel9_B were confirmed to encode a polypeptides which shared an amino acid maximal identity of 40% and 49% to the GHF9 cellulase from *Clostridium thermocellum* ATCC27405 (YP_001036864) and *Ruminococcus* sp. 18P13(CBL17554), respectively. This result indicated that the isolate cellulase gene fragments are new members of the cellulase GHF9. Further identification of the sequence termini will be performed using Genome walking approach to complete the full length encoding gene for bacterial expression.**

Keywords-cellulase, glycosyl hydrolase, buffalo rumen, genome walking

I. INTRODUCTION

Cellulose is the most abundant polymer on the earth. It is homopolymer of β-1-4 linked glucose residues. Cellulose can be biodegraded by the cellulose degrading enzyme called "cellulase" [1]. Cellulase are classified into 14 glycoside hydrolase families (GHF) (GHF5, 6, 7, 8, 9, 10, 12, 26, 44, 45, 48, 51, 61 and 74), and 5 of these families have been reported in animals (GHF5, 6, 9, 10 and 45) [2]. Cellulose decomposition requires multiple enzymes. There are two basic types of cellulose [3]: Endocellulase (EC 3.2.1.4) which attacks regions of low crystallinity in the cellulose fiber creating free chain ends, and exocellulase or cellobiohydrolase (EC 3.2.1.91) which degrades the molecule further by removing the cellobiose unit from the chain ends. β-glucosodase or cellobiose (EC 3.2.1.21), in a similar manner hydrolyzes cellobiose to produce glucose [4].

Our study was aimed at analyzing the sequences of GHF9 cellulase from bacteria in rumen fluid, designing a phylogenetic tree based on obtained data, and determining the relationships between the GHF9 sequences and the GHF9 cellulase genes reported by the GenBank.

II. MATERAL AND METHODS

A. DNA extraction

Rumen fluid samples were isolated from buffalos (*Bubalus bubalis*) at the Department of Agricultural Biotechnology, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok. Metagenomic DNA extraction was applied adopting the method of Tajima *et al.* (1999) [5]. The rumen fluid was mixed with an extraction buffer (200 mM Tris-HCl pH 8.0, 100mM EDTA pH 8.0, 0.2% SDS and 20 mg/ml proteinase K). This suspension was mixed, incubated at 65°C for 30 min and immediately frozen at -20°C for 60 min for 5 cycle. After extraction with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) solution, nucleic acids were precipitated by the addition of 5.3M NaCl and 1 volume of isopropanol. The DNA pellet was washed with 70% ethanol, dried and dissolved in distilled water. The DNA solution was store at -20 °C.

B. Cloning of GHF9 cellulase

A fragment of cellulase family 9 gene was amplified by PCR using the primers Cel9_F1 and Cel9_R1 [6]. The PCR conditions were as follows: primary denaturation at 94 °C for 3 min, denaturation at 94 °C for 45 s, annealing at 55 °C for 1 min, and extension at 72 °C 2.30 min, the number of cycle was 30, and the final extension was 72 °C for 10 min.

The master mix contained 2.5 µl of 10X PCR Buffer, 0.5 µl of dNTPs (10mM), 1 µl of Cel9_F1 primer and 1 µl of Cel9_R1 primer (both at a concentration of 10mM), 0.5 µl of Taq DNApolymerase (5 units/µl), and 18 µl of PCR water. The volume of the DNA(100 ng) was 1-2 µl. PCR products were separated by electrophoresis in 1% agarose gel purified using QIAquick Gel Extraction Kit (Qiagen, Germany).

Purified PCR product was cloned into the pTZ57R/T vector in the InsTAclone PCR Cloning Kit (Fermentas, USA) and transformed by heat-shock technique into *E. coli* DH5α. The white colonies were checked by size screening analysis [7], after that recombinant plasmids were isolated by alkaline lysis method [8] and rechecked by restriction enzyme double digestion. The obtained recombinant plasmids were subjected to further nucleotide sequencing.

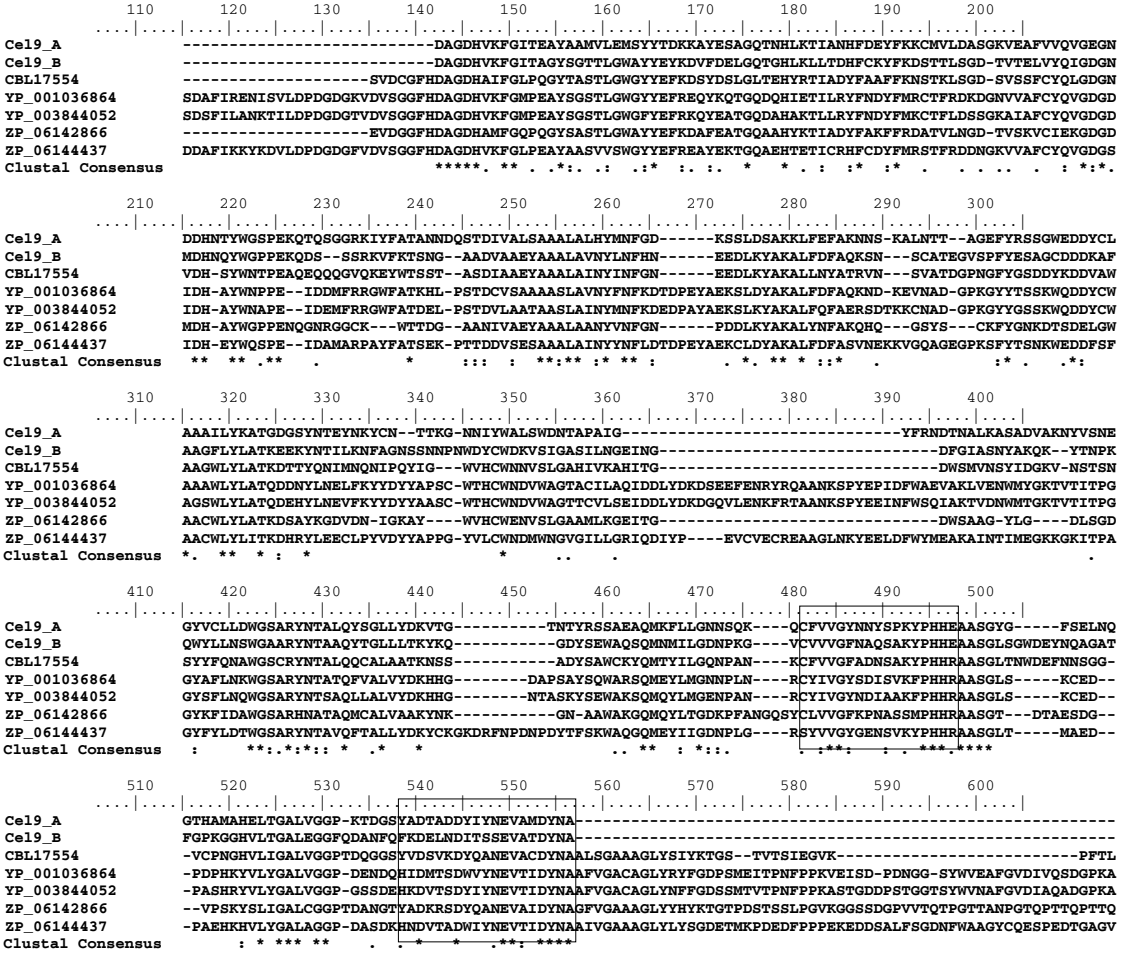

Figure 1. Amino acid sequence alignment of Cel9_A, Cel9_B and GHF9 cellulase from GenBank database. Protein numbers are indicated in front of genus of bacteria. Stars indicate identical residues. Dots indicate highly conserved residues. The frame indicates consensus region of GHF9.

C. Sequence Alignment and Phylogenetic Analysis

The sequences were identified in the GenBank by Blast searches with a variety of seed sequences previously identified as GHF9 genes. The sequences were initially aligned using ClustalW [9]. A phylogenetic analysis and construction of phylogenetic trees were conducted using BioEdit software version 7.0.4.1 [10]. Trees were calculated by the neighbor-joining method on the stipulation that gaps were absent. The statistical probability of branching out was determined by bootstrap analysis.

III. RESULTS AND DISCUSSION

PCR amplification of GHF9 cellulase on metagenomic DNA from buffalo ruminal fluid was conducted. The sample fragment was composed of 1,200 base pairs and amplified with the Cel9_F1 and Cel9_R1 primers. The insertion of the fragment into the pTZ57R/T vector transformed the vector into *E.coli* DH5α host strain. From this transformation, two clones named cel9_A and cel9_B were collected. After sequencing, the encoded polypeptides of the 2 clones were compared with proteins registered in the GenBank database. All clones showed the similarity with the known GHF9 cellulase; however, cel9_A and cel9_B sequences were found to be identical. All sequences contained the consensus region of GHF9: [SYV]-X-[LIVMFY]-[STV]-X(2)-GX-[NKR]-X(4)-[PLIVM]-H-X-R and [FYW]-X-D-X(4)-[FYW]-X(3)-E-X-[STA]-X(3)-N-[STA], are shown in Figure 1. Clones cel9_A and cel9_B shared the maximal identity of 40% and 49% to the GHF9 cellulase from *Clostridium thermocellum* ATCC27405(YP_001036864) and *Ruminococcus* sp. 18P13(CBL17554), respectively. The

amino acid sequences alignment of the 2 fragments and the GHF9 cellulase from GenBank database are shown in Figure 1.

A phylogenetic analysis was carried out using the amino acid sequences of GHF9 cellulase from the GenBank. As a result, the GHF9 cellulase indicated that cel9_A and cel9_B are located in different clusters

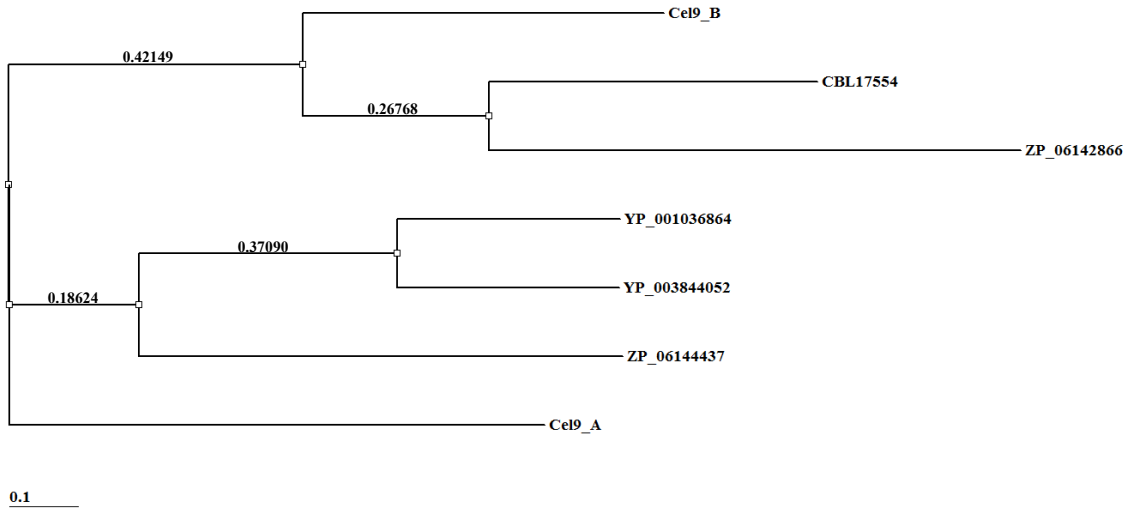

Figure 2. Phylogenetic tree of Cel9_A, Cel9_B and GHF9 cellulase from GenBank database using BioEdit software version 7.0.4.1. Protein numbers areindicated in front of genus of bacteria. Bootstrap test are shown on each branch.

(Figure 2), thus confirming that the partial sequences were new members of the GHF9 cellulase. Further identification of the sequence termini will be performed using Genome walking approach to complete the full length encoding gene for bacterial expression.

IV. CONCLUSION

Two partial GHF9 cellulase genes were isolated from metagenomic DNA of bacteria in buffalo ruminal fluid. The phylogenetic analysis confirmed the two partial genes as new members of the GHF9 cellulase. The complete full length sequences of the genes are under further investigation.

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