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Isolation and Molecular Cloning of Cellulase Gene from Bovine Rumen Bacteria

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ABSTRACT

Cellulases are the enzymes that hydrolyze cellulosic biomass and are produced by the microorganisms that grow over cellulosic matters. The objective of this research was to isolate and clone cellulase gene from cellulose-degrading bacteria of bovine rumen. Cellulose-degrading bacteria was isolated from rumen fluid using a selective medium. Total RNA was isolated from selected colony having cellulose degrading activity and was used as a template for cDNA construction using reverse transcriptase polymerase chain reaction (RT-PCR) technique. The resulted cDNA was employed as a template for PCR amplification of cellulase gene using specific primers. The cellulase gene candidate obtained was cloned into the pGEM-T-Easy vector followed by determination of its nucleotide sequence. The sequence was then aligned with sequences of cellulase genes from GenBank. Results showed that a number of isolates of rumen bacteria exhibit cellulase activity and the CR-8 isolate was selected for further analysis. The successful isolation of total RNA from CR-8 was indicated by the presence of two intense bands of ribosomal RNA (23S and 16S). The reverse transcription process was successful and the amplification of cellulase gene using the specific primers F1 and R1 resulted in a DNA fragment of 1900 bp as a candidate of cellulase gene. The fragment was successfully cloned into the pGEM-T-Easy vector, and the resulted recombinant plasmid was successfully introduced into the E. coli cells. Nucleotide sequence analysis suggested that the cloned gene is cellulase gene and shares 99% homology with the endo-1,6-beta-glucanase of T. harzianum.

Keywords*: cellulase, rumen bacteria, cloning, GenBank, bioinformatic.*

1. INTRODUCTION

Cellulose, a polymer of glucose with β(1-4) linkage is the main component of plant materials and also widely distributed on earth. Cellulose builds up plant cell wall, together with other material such as hemicellulose, lignin, etc. Consists of glucose, cellulose is one potential big source of ethanol fuel expected to replace fossil fuel. In the United States, ethanol recently has been produced from corn (Rendleman and Shapouri 2007). The use of corn for ethanol production, however, can interfere with the food security as it is one of the food sources for human being. On the other hand, utilization of cellulose as an energy source is considered not to harm food security. Cellulose can be obtained from biomass, dead plants, and other non-crops plants. One of the challenges in cellulose utilization is to break down cellulose into its glucose monomer in order to facilitate the efficient use of cellulose for maximum product yield.

Cellulase refers to a group of enzymes that act together to hydrolyze cellulose and is comprised of exoglucanase, endoglucanase and β-glucosidase (cellulase complex). In detail, it subdivided into β-1,4-endoglucanases (EG I, II, III and V), β-1,4-cellobiohydrolases (CBH I and II), xylanases (XYN I and II), β-glucosidase, a-L-arabinofuranosidase, acetyl xylan esterase, β-mannanase and α-glucuronidase (Lenting and Warmoeskerken 2001). Cellulase molecules generally have a similar structure, via the catalytic domain, cellulose binding domain and the connecting bridge (linker). As a result, cellulose can be degraded into glucose with this enzyme in synergistic action. A large number of bacteria, fungi and actinomycetes are known to degrade cellulose (Nagaraju *et al.* 2009). In addition to the cellulase hydrolysis of cellulose into glucose and other active ingredients, plant cell contents improve the cellulose extraction rate by increasing the permeability of plant cell walls. Cellulose, therefore, is widely used as plant-based raw materials for industrial and agricultural production. Cellulase not only benefit the agricultural industry, but also other area of industry can gain benefit from using cellulase such as textile industry like Jeans. Cellulase can used as biostoning agent to give uniformed aged look to Denim Fabric, thus eliminate environment pollution when using acid as biostoning agent in traditional method (Bai *et al*. 2012)

To utilize cellulase in the industrial section, the enzyme has to be produced and isolated. One of the strategies that can be employed is cloning and expression of the cellulase gene in an efficient host cells such as *Eschericia coli*. Prior to cloning for expression, the gene must be isolated and characterized. Cellulase gene can be isolated from cellulase-degrading bacteria or fungi that can be found in nature as free organism or forming mutualistic symbiosis such as in rumen of many livestock. Although cellulase from bacteria or fungi has been widely studied, the performance of the enzymes from different sources may be different, depending on the type and condition of plants used for livestock feed. It is therefore considered to be necessary to study cellulase from local species in Indonesia. In the present study, cellulose-degrading bacteria from bovine rumen was selected as a source of cellulase gene. Bovine has ability to digest and thrive on diets high in fiber but low in protein, thanks to their possession of cellulolytic bacteria, highly fibrolytic fungi and protozoa (Chen *et al.* 2008: Lee *et al.* 2004). The isolated gene can be further cloned and expressed in particular microbial host cells for large scale production of cellulase.

2. MATERIALS AND METHODS

Isolation of Rumen Bacteria

Rumen bacteria was obtained from slaughter house located in Bubulak Bogor. Guts from freshly bovine rumen were taken and kept in an air isolated container to keep the anaerobic environment. The guts were squeezed to obtained rumen extract. The extract was stored in a sterile bottle and the temperature was kept 37°C. Confirmation of the bacteria that produce cellulase were performed by streaking extract on the cellulose Congo-Red agar (CCRA, Hendricks *et al*. 1995), with modified composition: KH_2PO_4 0.5 g, $MgSO_4$ 0.25 g, Congo-Red 0.2 g, agar 7.5 g, gelatin 2 g, carboxymethyl cellulose 1.88 g; distilled water 1 L and at pH 6.8 – 7.2 (Gupta *et al.* 2012). The use of Congo-Red as an indicator for cellulose degradation in agar medium provides the basis for a rapid and sensitive screening test for cellulolytic bacteria. Colonies that show discoloration of Congo-Red after 1 day of incubation were taken as positive cellulose-degrading bacterial colonies. The cellulolytic colonies were cultured on standard Luria Broth media at 37°C, 150 rpm for one night. Then the bacteria were stored on refrigerator for future experiments.

Cellulase Activity Assay

To determine cellulase activity from isolated rumen bacteria, simple DNS method was used. DNS method measuring the glucose concentration that produced from cellulose breakdown by cellulase enzyme. Bacterial culture (50 µL) was mixed with 950 µL citric buffer and 1% CMC. CMC were used as it is the best carbon sources to induce cellulase production (Ahmad *et al*. 2003: Dashtban *et al*. 2011). The solution was homogenized by vortexing and incubated for 10 minutes at 50°C. The reaction was halted by entering the solution to boiling water for 15 minutes. DNS soluton was added to the mixture, then heated at 100°C followed by absorbance reading

at 540 nm. As a standard, glucose solution was used with glucose-aquadest, CMC and DNS ratio 1:1:1. Glucose concentration ranged from 100, 200, 400, 800, to 1600 ppm.

RNA Isolation and cDNA Synthesis

To obtain pure RNA from bacterial isolate, *peqGOLD Bacterial RNA Kit* (catalogue no. 12-6850-01) from *peqlab* was used. Isolation and purification process was in accordance with the manual supplied. At the end, 30 µL of RNA solution was obtained. To verify the presence of RNA, gel electrophoresis was performed with gel density 1% (0.6 g agarose dilluted with 60 mL TBE 0.5x). Following RNA verification, cDNA synthesis was carried out using *Superscript*^{tm} *First-Strand* from *Invitrogen* (catalogue no. 11904-018). This step resulted in cDNA solution with a total volume of 20 µL.

Primer Design

Primer design for cellulase gene amplification was carried out using sequences of cellulase genes available in the gene database (NCBI nucleotide database) as template. Sequences of cellulase genes were searched from NCBI nucleotide database. One of organism that has been studied thoroughly for its cellulase activity was *Trichoderma reesei*, a cellulolytic filamentous fungus that are beneficial to industry due to their ability to secrete large amount of cellulases and hemi cellulases (Oinonen 2004). Primer design was based on *T. reesei* endoglucanase template. For designing primer, criteria to construct good primer from Rozen & Skaletsky (1999) were followed.

PCR Amplification of Cellulase Gene

PCR amplification of DNA fragment harbouring cellulase gene was carried out using specific primers designed primer for amplification of the cellulase coding sequence. PCR kit was from *Invitrogen*, *Platinum Taq DNA Polymerase* (catalogue no. 10966-018). PCR was performed using PCR ESCO APBIO. PCR Mix was composed of (1x): 2.5 µL *Taq* Polymerase Buffer ($MgCl_2$ included), 1 µL dNTP, 1 µL forward primer, 1 µL reverse primer, 13.5 µL molecular water, and 1 µL *Taq Polymerase*. cDNA was dilluted with molecular water (1:4 µL), and mixed with PCR reagent (total volume 25 µL). The reaction was set to 94^{o}C for denaturation, 50^{o}C for annealing, and 72^{o}C for elongation, and repeated in 35 times cycle. The PCR product was analyzed using gel electrophoresis 1% and *1 Kb Plus DNA Ladder, Invitrogen* (catalogue no. 10787-018) was used as marker to determine the size of DNA fragment.

DNA Recovery

The amplified DNA fragment verified with gel electrophoresis was extracted using *Purelink*^{TM} *Quick Gel Extraction Kit* from Invitrogen (catalogue no. K2100-12). Four tubes of PCR reaction (25 µL each) were combined to obtain 100 µL of PCR product to be separated using gel electrophoresis. For 100 µL DNA solution separated, the weight of the DNA containing-gel extracted was 221.3 mg. The volume of *wash buffer* (L3) from the kit was 663.9 µL. The DNA extraction was conducted following the manual supplied with the kit. The DNA was filtered by *extraction column* and then recovered with *recovery tube*. Total volume of purified DNA was 30 µL.

Ligation and Transformation

Competent cells, *Eschericia coli* strain XL1-Blue were selected to clone the interest DNA. *E.coli* was cultured on *Super Optimal Broth* (SOB) media supplemented with tetracycline. Cold temperature and DMSO treatment was applied to *E. coli* in order to make the cell competent. Ligation reagent which is composed of 5 µL buffer, 3.5 µL insert DNA, 1 µL T4 ligase and 0.5 µL pGEM-T-Easy vector (Promega) was freshly prepared. The DNA solution was mixed with the ligation reagent followed by incubation for 1 hour at room temperature. Afterward, the ligation product was mixed with competent cell (200 µL) at cold temperature. The mixture was then subjected to heat shock to facilitate plasmid introduction to the competent cells. After heat shock, the cells were quickly moved into ice. In order to restore cell condition, LB + glucose were added and followed by incubation for 2 hours at 37^{o}C. To select positive clones that harbour recombinant DNA, the mixture was spread on petri dish containing Luria Agar (LA + Ampicilin [100 mg/mL] + IPTG [0,8M 2 g/8 mL] + X-Gal [20 mg/mL]). The Petri dish was incubated for one night at 37^{o}C. Colony that white in colour

indicated the transformation was successful. White colony were taken, and PCR colony with M13 primers was performed. PCR colony with M13 primers is designed to amplify the insert DNA within the vector (pGEM-T-Easy). DNA plasmids isolated from the positive clones were sent to a company named 1st Base for nucleotide sequencing.

DNA Sequence Analysis

The DNA sequences obtained were analyzed confirm the successful isolation of the full length of the cellulase gene. DNA sequence analysis was carried out using DNASTAR v7.1 and Bioeditprograms. To assemble the DNA fragments, editseq program was used to open the sequences file (*.seq) from 1st Base, and to convert them to a readable format for seqman program analysis. After loading all fragments to seqman worktable, they were analyzed using assemble command in seqman. The consensus of all DNA fragment (contig) was exported to Fasta format (*.fas). Finally, using BLAST tool (blastx) from NCBI (https://blast.ncbi.nlm.nih.gov/), the confirmation isolation of full length of cellulase gene was obtained.

3. RESULTS

Bacterial Isolate with Cellulose Degrading-Activity

From a number of colonies cultured on several petri dishes with CCRA media, only one petri dish showed CCRA discoloration. The colony from this petri was named CR-8. The cellulase enzyme activity of the isolate was confirmed using DNS assay and high absorbance score at 540 nm wavelength.

Isolated RNA and Construced cDNA

Isolation of total RNA from CR-8 gave good results with absorbance ratio at λ260 to λ280 of 1.695. The gel electrophoresis assay showed a relatively good integrity of two distinct bands with high intensity (Figure 1A). cDNA was succesfully constructed using the total RNA isolated from CR-8 as a template.

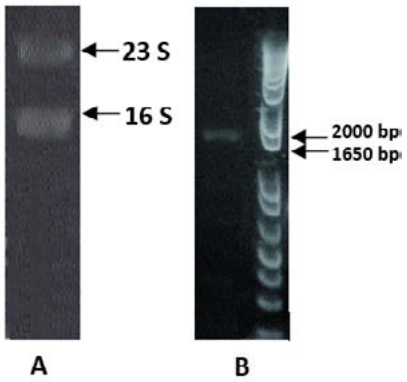

Figure 1. Profile of CR-8 electrophoresis. The figure shows total RNA with 23S and 16S ribosomal RNA bands (A); cDNA of CR-8 amplified by primer F1 (B).

Primer Design and Amplified Cellulase Gene

In the present study, two pairs of specific primers were designed for cellulase gene (Table 1). The PCR amplification using the F1 and R1 primers was successful and resulted in DNA fragment of 1900 bp (Figure 1B) as a cellulase gene candidate.

Clone of Cellulase Gene

The candidate of the cellulase gene was successfully inserted into the pGEM-T-Easy vector. The recombinant plasmids were successfully introduced into the *E. coli* cells marked by the presence of white colonies following host cell transformation and then confirmed by the results of the PCR colony.

Nucleotide Sequence and Assembly

The cloned fragment was sequenced by the 1st Base company and resulted in DNA sequences with around 1500 ~ 1800 bp length. The chromatograms of the essensial domains showed good quality with clean peaks and low noises interference. Following

Table 1 Primer constructed from *T.reesei* cellulase DNA sequence template.

Primer	Sequence (5' -> 3')	Tm (°C)
F1	AAG AGG ACC TCG ATA TGA TCT GGA CAC T	62.6
R1	TCA TCC CAC ATT CTA ATG CCT GTA GGT A	61.6
F2	ATG ATC TGG ACA CTC GCT CCC TTT GTG G	67.4
R2	CTA ATG CCT GTA GGT AGA TCC AAT ATC T	58.3

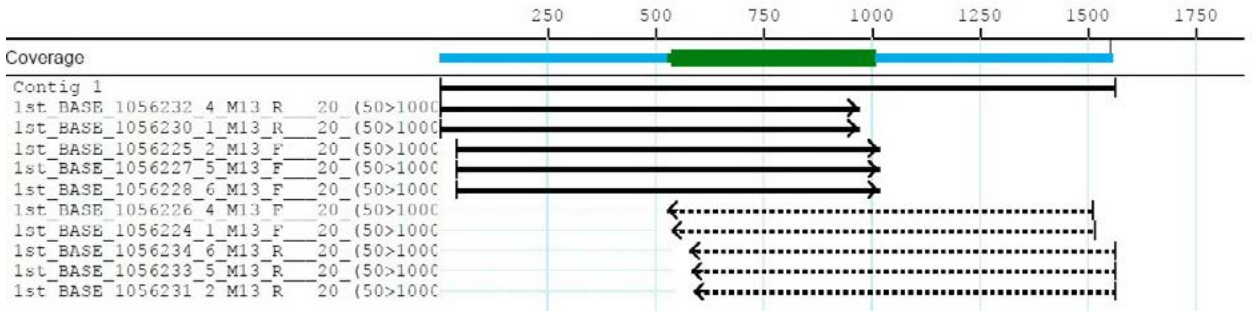

Figure 2. Contiguous sequence of DNA fragments

removal of sequence noises, the sequences were loaded in *seqman* program and the analysis of the whole sequences resulted in one overlapping sequence (Figure 2).

The consensus sequence of all DNA fragment was 1522 bases. The contiguous sequence start from 71st base and stop at 1451st base, with a total of 1381 bases. Analysis of the contiguous sequence using BlastX from NCBI, followed with confirmation suggested that the contiguous sequence is a cellulase coding sequence marked by the presence of cellulase domain within the sequence (Figure 3) and the high similarity to sequence of the endo-1,6-beta-glucanase from *Trichoderma harzianum,* which is 99% in homology (Figure 4).

4. DISCUSSION

We report the successful isolation and molecular cloning of the full length of a cellulase gene of bovine rumen bacteria. The gene is of a great interest as it can be further cloned into an expression vector and then expressed in a particular host cells for cellulase production potentially useful for cellulose degradation. Cellulose degradation is one of the most challenging steps in the utilization of cellulose for bioenergy production.

In the process of isolation of rumen bacteria, the cattle guts were kept in an anaerobic chamber in order to mimic the environmental conditions inside the cattle rumen. Most bacteria in the bovine rumen are anaerobic due to no oxygen availability. Extract of guts were kept at body temperature to maintain the bacterial culture. Some cellulolytic bacteria that has been identified from bovine rumen is *Fibrobacter succinogenes, Ruminococcus albus* and *Ruminococcus flavefaciens* (Koike and Kobayashi 2001: Deng *et al*. 2007). The isolated *Fibrobacter* from the rumen indicated that there are two different species of *Fibrobacter, F. succinogenes* S85 and *F. intestinalis* NR9 with low similarity between them (Qi *et al.* 2004: Bera-Maillet *et al.* 2004). Aside from cellulolytic bacteria, the most abundant bacteria in rumen fluid were come from *Prevotella sp and Eubacterium sp,* followed with*, Ruminococcus sp, Clostridium sp, Roseburia sp* and other genera with the total of 25 genera known (Broadway *et al.* 2012).

On the other hand, Ozutsumi *et al.* (2005) found that the most abundant bacteria is *Bacteroides sp* and *Prevotella sp*. Wang *et al*. (2011) isolated and characterized cellulase enzyme from *Neocallimastix patriciarum*, one kind of fungus that live in bovine rumen. They isolated glycosil hydrolases (GH) produced by this anaerobic fungus. Their research indicate that both bacteria and fungus can form mutualism symbiotic with bovine in producing cellulase.

To specifically select the cellulose-degrading bacteria, the media used should contain cellulose as a source of energy. The Cellulose Congo-Red agar invented by Hendricks *et al*. (1995) serves as the basis for rapid selection of cellulose-degrading bacteria. The colonies showing discoloration of media suggest that they have ability to degrade cellulose. From a number of petri dishes prepared to culture the rumen

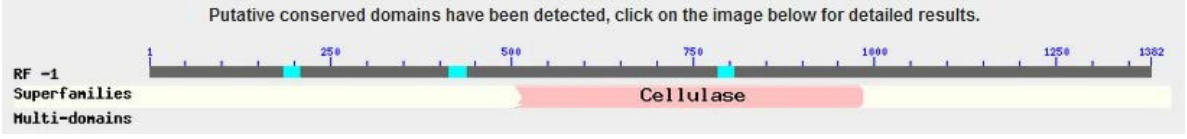

Figure 3. Conserved domain of cellulase from contiguous sequence

```
ALIGNMENTS
>emb|CAA55789.1| glucan endo-1,6-beta-glucanase [Trichoderma
harzianum]
Length=430

Score =   772 bits (1993),  Expect = 0.0, Method: Compositional
matrix adjust.
Identities = 370/372 (99%), Positives = 370/372 (99%), Gaps = 1/372
(0%)
Frame = -2

Query  GWLISEPWMMSNEWNNNMGCNNAASEFDCMRNNYMGSKRATGNTKFQNHYRDWINPATVQ
       GWLISEPWMMSNEWNNNMGCNNAASEFDCMRNNYMGSKRATGNTKFQNHYRDWINP TVQ
Sbjct  GWLISEPWMMSNEWNNNMGCNNAASEFDCMRNNYMGSKRATGNTKFQNHYRDWINPPTVQ

Query  SVHDVGLNTIRIPIGYWSYDAIVDTASEPFADGNLQLPYLDAVVQKAADLGIYVIIDLHG
       SVHDVGLNTIRIPIGYWSYDAIVDTASEPFADGNLQLPYLDAVVQKAADLGIYVIIDLHG
Sbjct  SVHDVGLNTIRIPIGYWSYDAIVDTASEPFADGNLQLPYLDAVVQKAADLGIYVIIDLHG

Query  APGGQQQDAFTGQNPNPAGFYNSYDYGRAEKWLSWMTNRIHTNPAYSTVGMIEVLNEPVS
       APGGQQQDAFTGQNPNPAGFYNSYDYGRAEKWLSWMTNRIHTNPAYSTVGMIEVLNEPVS
Sbjct  APGGQQQDAFTGQNPNPAGFYNSYDYGRAEKWLSWMTNRIHTNPAYSTVGMIEVLNEPVS

Query  RHDGGGRYPAPGQDPSMVQTYYPGALKAVRDAEAALNVPSNKKLHVQFMSSKWDSGDPRS
       RHDGGGRYPAPGQDPSMVQTYYPGALKAVRDAEAALNVPSNKKLHVQFMSSKWDSGDPRS
Sbjct  RHDGGGRYPAPGQDPSMVQTYYPGALKAVRDAEAALNVPSNKKLHVQFMSSKWDSGDPRS

Query  NAAVKNDPMVGFDDHNYIGFALSNTGDQYSLMHSACTDSRVVSGQDFAITGEWSMTSGAD
       NAAVKNDPMVGFDDHNYIGFALSNTGDQYSLMHSACTDSRVVSGQDFAITGEWSMTSGAD
Sbjct  NAAVKNDPMVGFDDHNYIGFALSNTGDQYSLMHSACTDSRVVSGQDFAITGEWSMTSGAD

Query  WHDGNFFTKFFTAQQQLYESPGMDGWIYWTWKTELNDPRWTYSYATYLNYIPTNAAAL-Q
       WHDGNFFTKFFTAQQQLYESPGMDGWIYWTWKTELNDPRWTYSYATYLNYIPTNAAAL Q
Sbjct  WHDGNFFTKFFTAQQQLYESPGMDGWIYWTWKTELNDPRWTYSYATYLNYIPTNAAALQQ

Query  QNVYQDVCSGYR
       QNVYQDVCSGYR
Sbjct  QNVYQDVCSGYR
```

Figure 4. High similarity of cellulase gene candidate with endo-1,6-beta-glucanase from *T. harzianum*

bacteria in the present study, only one petri dish showed a sign of excellent growth of colony with discoloration of CCRA medium denoted as CR-8. As most of microorganisms inside bovine rumen are anaerobic, oxygen exposure was avoided as it may potentially kill the targeted cellulose-degrading bacteria and leave the facultative anaerobic bacteria to survive.

RNA isolation of cellulose-degrading bacteria show two distinctive bands (Figure 2). Bacterial RNA, like RNA from other prokaryotes shows two distinct RNA bands representing the 50S (5S & 23S) and 30S (16S), that are subunits of 70S ribosomal RNA. The 23S and 16S subunits of ribosomal RNA have 2906 and 1542 nucleotides respectively. When gel electrophoresis shows two distinct bands from total RNA, it indicates that the isolation technique works well as the ribosomal RNAs are the abundant RNA molecules in the cells. The quality of the RNA isolated determines the success of the following process such as RT-PCR and mRNA purification.

For amplification of DNA fragment harbouring the cellulase gene, two sets of primer were prepared to anticipate the existance of multiple reading frames. To eliminate ambiguity related to multiple reading frame, we compared several cellulase coding sequences from close distant species such as *Trichoderma virens, Trichoderma harzianum,* and other cellulose-degrading bacteria. Reading frames that appear mostly on those sequences were selected as the basis for designing specific primers. The melting point and GC percentage of the primer pairs were balanced each other to reach the appropriate annealing temperature. Amongst several PCR assays with various primer combinations, F1-R1, F1-R2, F2-R1 and F2-R2, only the F1-R1 pair succesfully amplified the cellulase gene. The others primer pairs failed to amplify the cellulase gene, possibly due to inability to form a stable double-stranded bond with template during the initial stage of the PCR process.

Introduction of DNA into XL1-Blue competent cells, was carried out using a common

procedure. The process was successfully done indicated by the emergence of white colonis following the incubation step. Those colonies were then tested with PCR colony using M13 forward and reverse primer (GTAAAACGACGGCCAG and CAGGAAACAGCTATGAC). Gel electrophoresis showed a distinct band of about 1900 bp corresponding to the insert from all colonies tested. All of the positive clones was sent to be sequenced by the 1st Base services.

Generally, the DNA sequences from 1st Base services showed good chromatogram profiles, with low noises and clean peaks. However, in some sequences, the signal strength was slightly low. This could be caused by insufficient amount of DNA template, contaminants, or inefficient primer binding (Kingdon *et al*. 2012). Multiple DNA sequences of cellulase genes were subjected to alignment using CLUSTAL program that is used to carry out multiple sequence alignment of DNA fragments. To be run in this program, the sequences must be freed from "noise" that usually present in both ends. DNA sequences with incorrect direction must be reversed prior to alignment (Chenna *et al*. 2003). Confirming sequence direction and aligning multiple sequences can be achieved with *seqman* DNASTAR Suite program. *Seqman* lists the sequences with their direction and analyze the whole sequence to generate the consensus sequence of all fragments (Figure 3). The overlapping sequences generated consensus sequence around 1500 bp and was confirmed by the BlastX as a cellulase gene. The highest similarity found in gene databank was to the endo-1,6-beta-glucanase from *T. harzianum*. The predicted enzyme consists of 430 amino acids, with bits score of 722 and E-value of 0.0, suggested that the sequence has high similarity with the one in the GenBank (Claverie & Notredame 2007: Davidson and Blaxter 2005). As comparison, another glycosyl hidrolase family (GHF) that isolated from buffalo rumen (*Bubalus bubalis*) has size around 1200 bp although it is not the full length gene (Rungrattanakasin *et al*. 2011). Another glicosyl hidrolase family gene that successfully isolated from rumen is GHF 45 from *Fibrobacter succinogenes*, unfortunately the isolated gene lack several important sequences (Park JS *et al.* 2007) The endo-1,6-beta-glucanase gene available in the GenBank was of Cruz *et al*. (1995). The enzyme was suspected to play a role related to their mycoparasitism activity. The endo-1,6-glucanase was reported to be able to hydrolize fungal cell walls made of chitin and glucan (1,3 and 1,6 linkage). This enzyme also reported to show higher activity in mycoparasitic fungi than in nonmycoparasitic ones (Cruz *et al*. 1995). High activity glucan hydrolysis may give benefit in bovine rumen to break down cellulose polymer faster than other cellulase types .

In conclusion, gene encoding cellulase can be isolated from rumen bacteria using common isolation procedures. To facilitate further analysis and uses, the gene of interest can be cloned and amplified in *E. coli* host cells. Sequence analysis showed that the gene isolated from bovine rumen bacteria in the present study is a cellulase gene with homology reached 99% with the endo-1,6-beta-glucanase from *T. harzianum*.

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