

Cloning and Expression of Endoglucanase Gene from Thermophilic Bacteria *Bacillus* sp. RP1

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An endoglucanase gene belonging to glycoside hydrolase family 5, had been isolated from *Bacillus* sp. RP1 and cloned into *Escherichia coli*. The cloned gene comprised the promoter, coding sequence, and terminator of the gene. This gene encoded a protein with 499 amino acid residues (predicted molecular weight=55.2 kDa) with a typical *Bacillus* signal peptide. The recombinant endoglucanase (EG) had optimum activity at pH 5.0 and 50 °C. The recombinant EG was expressed in the extracellular, intracellular, and periplasmic fractions with the highest total activity (60.15%) in the intracellular fraction, measured at three hours after isopropyl- β -D thiogalactopyranoside (IPTG) induction. Three hours after the addition of 1% carboxymethyl cellulose (CMC), there was a two-fold increase in intracellular EG specific activity compared to the uninduced cells. Three hours after the addition of 1 mM IPTG, 1% glucose, 1% galactose, or 1% cellobiose the intracellular EG specific activity decreased compared to the uninduced cells.

Key words: *Bacillus* sp. RP1, native promoter, native signal peptide, recombinant endoglucanase

Gen endoglukanase yang termasuk famili 5 glikosida hidrolase telah diisolasi dari *Bacillus* sp. RP1 dan diklon ke *Escherichia coli*. Gen yang telah diklon mencakup promotor, daerah pengkode, dan terminator dari gen. Gen ini mengkode protein yang terdiri dari 499 asam amino (perkiraan berat molekul 55,2 kDa) dengan peptida sinyal khas *Bacillus*. Endoglukanase (EG) rekombinan ini mempunyai aktivitas optimum pada pH 5.0 dan suhu 50 °C. EG rekombinan ini terdapat di dalam sel, di periplasma dan di luar sel, dengan aktivitas total terbanyak di dalam sel (60.15%), yang diukur tiga jam setelah induksi dengan *isopropyl- β -D thiogalactopyranoside* (IPTG). Tiga jam setelah penambahan 1% *carboxymethyl cellulose* (CMC) terjadi peningkatan aktivitas spesifik EG intraseluler, dua kali lebih tinggi dibandingkan sebelum diinduksi. Tiga jam setelah penambahan 1 mM IPTG, 1% glukosa, 1% galaktosa, atau 1% sellobiosa terjadi penurunan aktivitas spesifik EG intraseluler dibandingkan sebelum diinduksi.

Kata kunci: *Bacillus* sp. RP1, endoglukanase rekombinan, peptida sinyal asli, promotor asli

The increased demand for fossil fuel while it is running low has raised efforts to explore and utilize alternative energy sources. The use of feed stocks for biofuel production may cause problems in food security (Actionaid 2013). Cellulose is the most abundant biomass in nature and is not used as food source. The content of cellulose in plant reaches 35-50% of plant dry weight (Lynd *et al.* 2002).

Cellulases consist of three types of enzymes: (1) endoglucanase (EG, E.C.3.2.1.4), breaks internal bonds and opens the polysaccharide chains, (2) exoglucanase (E.C.3.2.1.9), breaks 2-4 units from the ends of the exposed chains produced by EG resulting in tetrasaccharides or disaccharides, such as cellobiose,

(3) β -glucosidase (E.C.3.2.1.21), hydrolyses cellobiose into glucose. The production of cellulase is mainly regulated at the transcriptional level, by induction and catabolite repression (glucose). Generally, there is a low level of constitutive expression of the cellulases. Hydrolysis products of cellulose or their derivatives will induce expression of the cellulase genes (Aro *et al.* 2005). Simple sugars that are not hydrolysis products of cellulose can also induce cellulase expression. For example lactose can induce expression of cellobiohydrolase I gene from *Trichoderma reesei* (Messner and Kubicek 1991) and EG genes from *Bacillus brevis* (Singh and Kumar 1998) and *Bacillus* sp. MTCC 10046 (Sadhu *et al.* 2014).

Bacillus species have long been used for the production of various industrial enzymes such as

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cellulase (Sukumaran *et al.* 2005). Cellulases belong to Glycoside Hydrolase (GH) enzymes and many bacterial EGs belong to GH family 5. *Bacillus* sp. RP1, a cellulolytic thermophilic bacterium, produced seven CMCases, six FPases, and four xylanases extracellularly (Puspitasari and Moeis 2008). In this study, we focused on its endoglucanase. The objectives of this study were to isolate one of its EG gene, determine the optimum pH and temperature of the recombinant enzyme, investigate its subcellular location, and determine the influence of the addition of specific inducers on gene expression through its native promoter.

MATERIALS AND METHODS

Bacterial Strain and Plasmids. *Bacillus* sp. RP1 was isolated from a hot spring in Cimanggu (West Java, Indonesia) (Puspitasari and Moeis 2008) and was used as source for genomic DNA. *Escherichia coli* strains used as host cells for cloning were DH5 α and TOP10. The plasmid used for cloning was pGEM[®]-T Easy (Promega).

DNA Isolation. A colony of *Bacillus* sp. RP1 was inoculated into 3 mL of LB media and grown at 41 °C, 150 rpm for 16 h. DNA was then isolated by Wang method as described in Doi and McGloughlin (1992). The cells were incubated in lysis buffer (0.15 M NaCl, 0.1M EDTA pH 8.0, 0.5 mg mL⁻¹ lysozyme), extracted with chloroform/isoamyl alcohol (24:1) and DNA was precipitated with isopropanol. The DNA pellet was washed with 70 % ethanol, centrifuged and dissolved in 200 μ L water.

Cloning of the EG Gene. PCR was performed using reagents from Fermentas on a Gene Amp PCR System 2400 (Applied Biosystem). The primers used to amplify the partial EG gene were designed based on the consensus sequences of *Bacillus* spp. EG genes from GH5 family as described by Rachim (2008) (Table 1). Touched down PCR was performed: 94 °C, 3 min; 5 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1

min; 10 cycles of 94 °C for 30 s, 55 to 45 °C at 1 °C interval for 30 s, and 72 °C for 1 min; 10 cycles of 94 °C for 30 s, 45 °C for 30 s, and 72 °C for 1 min. The PCR product was purified after electrophoresis using GeneAid preparation purification kit, ligated to pGEM[®]-T Easy (Promega). Transformation of *E. coli* DH5 α (partial gene) was performed by the CaCl₂ heat shock method and recombinant clones were selected by blue-white screening (Sambrook and Russell 2001). DNA was sequenced by Sanger method in Macrogen Inc., Korea.

To clone the complete gene, primers were designed based on the sequencing results of the partial EG gene, so that the PCR product would include the regulatory region and terminator. Primers were designed based on conserved sequences from six *Bacillus subtilis* strains (Acc. No. AY044252.1, X04689.1, D01057.1, AL009126.3, Z29076.1, and X67044.1) and one *Bacillus* sp. (Acc. No. AF045482.1). The sequence of the forward primer was GTTTGCATTCAGTTCA GTTGTC and reverse primer was TCAGTATTTTCATC CACAACGCA. The initial PCR condition was denaturation at 94 °C for 3 min followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 55.9 °C for 30 s, elongation at 72 °C for 2 min and final elongation at 72 °C for 7 min. The PCR product was gel purified and cloned into *E. coli* DH5 α Top 10. The cloning and sequencing methods used were the same as previously described.

Nucleotide sequence analysis. Sequencing results were analyzed using programs BioEdit (Hall 1999), VecScreen (<https://www.ncbi.nlm.nih.gov/tools/vecscreen/>), BLAST (Altschul *et al.* 1990) (<http://blast.ncbi.nlm.nih.gov/Blast/>). The *in silico* translated product of the EG gene was characterized using online programs from NCBI (www.ncbi.nlm.nih.gov), ExPasy Proteomic Server (Gasteiger *et al.* 2003) (<http://expasy.org/>), and InterProScan (Zdobnov and Apweiler 2001) (<http://www.ebi.ac.uk/Tools/pfa/iprscan/>) to know the domain and motif generated.

Expression and Recombinant Enzyme Preparation. *E. coli* TOP10 expressing recombinant EG was inoculated into 10 mL of LB broth containing 100 μ g mL⁻¹ of ampicillin at 37 °C for 16 h. After that, 0.1 mL of overnight culture was inoculated into 10 mL of new LB broth containing 100 μ g mL⁻¹ ampicillin and grown at 37 °C until the optical density (OD) reached 0.5 at 600 nm. Then, appropriate inducers were added into the culture broth. The culture was incubated at 37 °C for 3 and 6 h after induction. The fractionation of extracellular, periplasmic, and intracellular EG were

Table 1 Primer sequences used to isolate partial endoglucanase gene (Rachim 2008)

| Primer | Sequences |
|------------|------------------------------|
| Forward F1 | 5'-TRTATGTCATYATTGAYTGGCA-3' |
| Forward F2 | 5'-AYGAAATTGCRAAYGARCC-3' |
| Reverse R | 5'-CCCCAYTCHSTMACRAAWA-3' |

* R(A/G), Y(C/T), H(A/C/T), S(C/G), M(A/C), W(A/T)

carried out by the method of Yamabhai *et al.* (2008). Extracellular fraction was obtained from the supernatant by centrifugation. The periplasmic fraction was released by osmotic shock. The cells were resuspended in cold (4 °C) spheroplast buffer (100 mM Tris-Cl, pH 8.0, 0.5 mM EDTA, 0.5 mM sucrose, and 20 µg ml⁻¹ phenylmethylsulfonyl fluoride). After incubation for 5 min on ice, the bacterial cells were collected by centrifugation and resuspended in ice-cold sterile water supplemented with 1 mM MgCl₂ and incubated on ice for 5 min with frequent shaking. The supernatant was then collected by centrifugation as the periplasmic fraction. The precipitated cells were washed once with lysis buffer (50 mM Tris-Cl + 0.5 mM EDTA), resuspended in lysis buffer and sonicated (Bransonic 32, 150 Watt), for 30 s on and 30 s off repeatedly for 5 times at 4 °C. The cell debris was centrifuged and the supernatant was collected as the intracellular fraction. For *E. coli* TOP10 containing pGEM-T Easy without DNA insertion, only intracellular fraction was taken.

EG activity assay and total protein estimation.

EG activity was measured by incubating 0.05 mL of enzyme solution with 0.15 mL of 1% (w/v) CMC, prepared in 50 mM phosphate citrate buffer (pH 5) for 1 h at 50 °C. The release of reducing sugar was measured by ferricyanide alkali method (Walker and Harmon 1996) using a calibration curve for D-glucose. One unit of EG activity was defined as the amount of enzyme which produced one µmol of reducing sugar (glucose equivalent) per minute under the assay condition. Protein content in the supernatant was estimated by Bradford method (Bradford 1976) using Bovine Serum Albumin (BSA) as standard.

RESULTS

Characterization of the EG Gene of *Bacillus sp.*

RP1. Two clones containing the partial EG genes were obtained, named *egA* (406 bp) and *egB* (286 bp). Pairwise alignment of the sequences of both clones using software BioEdit showed that *egB* was part of *egA*. Sequence analysis of the partial genes using BLAST N showed that *egA* and *egB* had high similarities with EG genes from *Bacillus subtilis* strains, reaching 98% identity in nucleotide sequence.

The complete EG gene was named *egC* (submitted to NCBI with Acc. No. KJ652953). Using ORF Finder program, the coding sequence of *egC* was found to be 1497 nucleotides at position 651 – 2147, producing a protein consisting of 499 amino acids. The promoter

sequence of *egC* was TACAAT (Pribnow box) and TAGACG (-35 consensus sequence) and the Shine Dalgarno was AGGAGG (Fig 1). Two inverted repeats were found in the sequence which could be terminator sequences. One was downstream the *egC* coding sequence and the other was upstream the *egC* promoter, which could be the terminator of a gene located upstream the EG gene (Fig 2).

Amino acid sequence analysis revealed that EgC had high amino acid sequence homology (97-100%) with EGs of *B. subtilis* strains. At the N terminus there was a signal peptide consisting of 29 amino acid residues (MKRSISIFITCLLITLLTMGGMIAS PASA). This was followed by a cellulase domain which had a motif for glycoside hydrolase family 5 (VIYEIANEPN). On the C terminus there was a Carbohydrate Binding Module (CBM3).

Subcellular Location of Recombinant Enzyme in *E. coli*. The optimum EG activity in the crude extract was obtained at pH 5 and 50 °C (Fig 3). The majority of the EG activity was in the intracellular fraction, both at 3 h (60.15%) and 6 h (66.58%) after IPTG induction. Three hours after IPTG induction, EG mainly accumulated in the cytoplasm and some were secreted into the periplasm (1.92%) and out of the cell (37.93%). The total activity of EG in all fractions after six hours induction was higher than after three hours induction. Six hours after induction, the activity in the periplasmic fraction increased to 6.56% and the extracellular fraction decreased to 26.86% compared to three hours after induction (data not shown). Since most of the proteins were present in the cytoplasmic fraction, the effect of specific inducers on EG activities were analysed in this fraction.

Effect of the Addition of Specific Inducers on EG expression. The specific activity of EG in non-induced cells after 6 h incubation (equivalent to 3 h after induction) was 23.5 U mg⁻¹ and the activity after 9 h incubation (equivalent to 6 hours after induction) was 2.1 U mg⁻¹. There were about two-fold increases in EG specific activities three hours after the additions of 1% CMC and 1% CMC+ 1 mM IPTG compared to non-induced cells. At six hours after induction, the increases in EG specific activities after addition of 1% CMC, 1% CMC+ 1 mM IPTG and 1 mM IPTG were six-fold (13.1 U mg⁻¹), 12-fold (24.7 U mg⁻¹) and 10-fold (20.3 mg⁻¹) respectively compared to non-induced cells (2.1 U mg⁻¹). Additions of 1% glucose, 1% galactose, 1% cellobiose and 1 mM IPTG (specifically three hours after induction) gave EG activities that were either similar or lower than non-induced cells (Fig 4).



Fig 1 Positions of promoter (-35, -10), transcription start site (TSS), ribosome binding site (RBS), start codon (START), stop codon (STOP) and terminator (REPEAT REGION) of the cloned endoglucanase gene (*egC*) of *Bacillus* sp. RP1.zone.

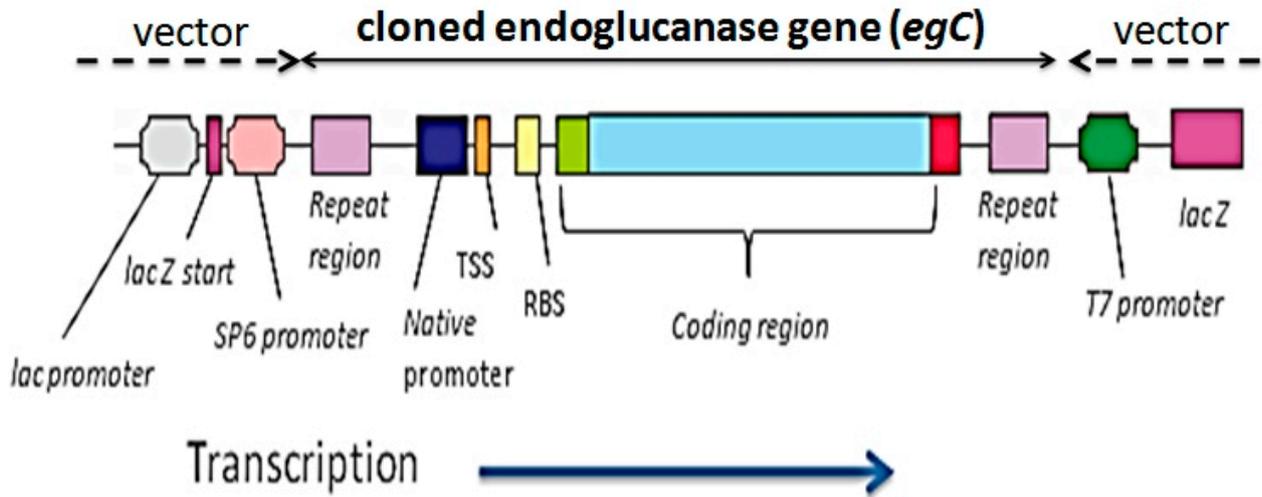


Fig 2 Full length endoglucanase gene of *Bacillus* sp. RP1 with its regulatory elements that was inserted into pGEM-T Easy plasmid and cloned into *E. coli* TOP10. Repeat regions = terminators; TSS = Transcription Start Site; RBS = Ribosomal Binding Site.

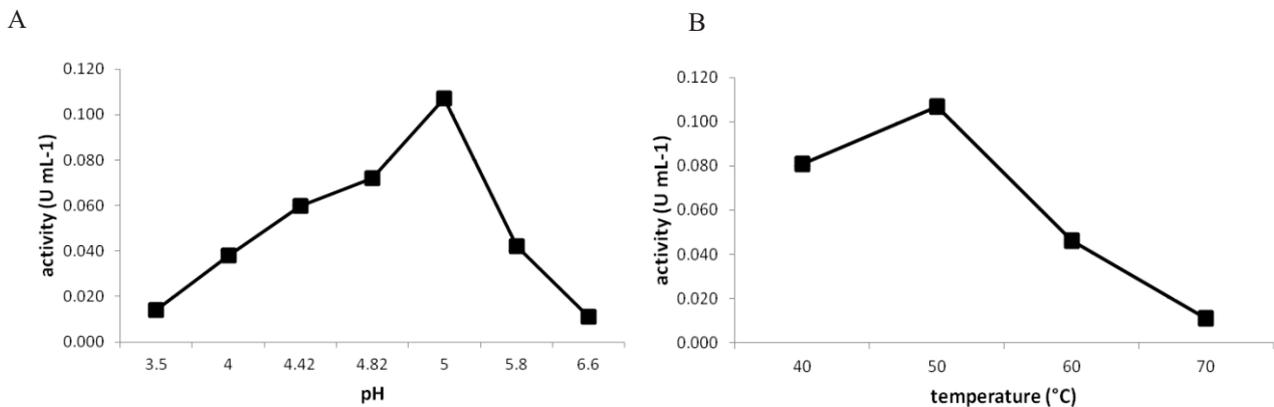


Fig 3 Effect of pH (A) and temperature (B) on the *Bacillus* sp. RP1 recombinant EG activity

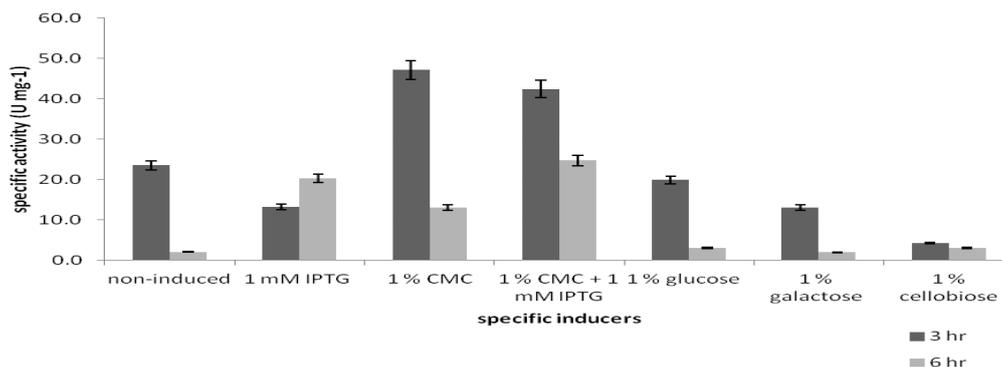


Fig 4 Specific activity of Intracellular EG with addition of specific inducers (non-induced, 1 mM IPTG, 1 % CMC, 1 % CMC + 1 mM IPTG, 1 % glucose, 1 % galactose, and 1 % cellobiose) at 3 hours (dark gray column) and 6 hours (light gray column) after induction. The error bars are based on three experimental replicates.

DISCUSSION

Optimum pH and Temperatures of *Bacillus* sp.

EGs. The optimum pH and temperature of EgC was the same as the crude extract of extracellular EG of *Bacillus* sp. RP1, i.e. at pH 5 and 50 °C (Puspitasari and Moeis 2008). This is different from the optimum conditions of other *Bacillus* EGs. There was a wide range of optimum pHs (4-10) and temperatures (40-65 °C) for EGs in *Bacillus* spp. The optimum conditions for EG activities were pH 4 and 40 °C for *Bacillus thuringiensis* var. *israelensis* and *Bacillus thuringiensis* var. *thompsoni* (Lin *et al.*, 2012), pH 5 and 60 °C for *Bacillus* sp. M9 (Bajaj *et al.* 2009), pH 7 and 50 °C for *Bacillus* sp. (Sadhu *et al.* 2014), pH 9 - 10 and 50 °C for *Bacillus* sp. (Nizamudeen and Bajaj 2009), pH 6-8 and 65 °C for *Bacillus subtilis* LH (Zhao *et al.* 2012).

Secretion of EgC. There was an increase in total activity of the cell compartments along with inducer exposure time. The signal peptide sequence of EgC was a typical signal peptide for *Bacillus subtilis*. This signal peptide consisted of hydrophilic amino acid such as lysine and arginine, followed by 18 hydrophobic amino acids (rich in leucine and isoleucine) and a short sequence before the cleavage site which contained several alanine residues (MacKay 1986). Typical *Bacillus* signal peptide could be recognized by *E. coli* secretion machinery (Yamabhai *et al.* 2008). This suggested that the mechanisms of protein secretion in Gram-negative and Gram-positive bacteria were highly conserved although there was a great difference in the structure of the cell wall and cell membrane (Yamabhai *et al.* 2008; Mergulhão *et al.* 2005). However, the *E. coli* protein secretion system could not secrete this recombinant protein through the

inner and the outer membranes efficiently. This was shown in this experiment by the fact that most of the activities were in the intracellular fractions. The intracellular EG specific activity was lower at six hours compared to three hours after the addition of the inducers, except after the addition of IPTG. IPTG cannot be metabolized by the cell, EG synthesis remained relatively constant. On the other hand, all the other inducers could be metabolized by the cell. This could decrease the rate of EG synthesis lower than the rate of secretion. Other *Bacillus* spp. may have different secretion efficiency. Secretion efficiency of recombinant enzyme depends on the inducer exposure time, the type of signal peptide, and the molecular mass of the enzyme (Yamabhai *et al.*, 2008). Expression of the cellulase gene from *B. subtilis* H12 in *E. coli* JM109 were mainly in the periplasm (80%) and none were found in the medium (Oh *et al.* 2008). On the other hand, EG of *B. subtilis* I15 that was cloned in *E. coli* BL21 was mostly extracellular (Yang *et al.* 2010).

Basal EG Activity. The promoter of *egC* was not identical to any of the constitutive promoters of *E. coli* (Shimada *et al.* 2014), nor *B. subtilis* (Radeck *et al.* 2013). However, EG activity was present in non-induced recombinant *E. coli*, suggesting low level of expression which was needed for the regulation of the cellulase synthesis (Aro *et al.* 2005; Lynd *et al.* 2002; Sukumaran *et al.* 2005). The LB medium that was used could have contributed to the relatively high basal activity. In *B. subtilis*, basal activities from promoters was higher when the bacteria were grown in LB medium compared to MOSE medium (MOPS-based chemically defined medium with succinate and glutamate) (Radeck *et al.* 2013). LB medium contains yeast extract and carbohydrates present in the yeast extract might have contributed to the low level of *egC*

expression.

Effect of the Addition of Inducers on EG activity. Since there was a possibility of inefficient secretion and a decrease in inducer concentrations affecting the results at six hours after induction, analysis of the effect of inducer on the recombinant EG were performed at three hours after induction. IPTG addition increased EG expression in *Bacillus* sp. RP1 (unpublished data) but decreased it in recombinant *E. coli*. IPTG is a synthetic molecule that is stable because it is not metabolized by the cell and can replace lactose as an inducer. No sequence similar to the *lac* operator in the regulatory region of *egC* was present, therefore a different regulatory protein would be involved. The molecular mechanisms of lactose induction of EG in *Bacillus* spp. are still unknown. Lactose had variable effects on EG expression of *Bacillus* species. Lactose induces EG gene expression in *Bacillus brevis* (Singh and Kumar 1998) and *Bacillus* sp. MTCC 10046 (Sadhu *et al.* 2014), but had no effect on *Bacillus* sp. X10-1-2 (Yan *et al.* 2013). Since IPTG could not increase EG activity from *egC* promoter in *E. coli*, the regulatory protein involved in *egC* induction by IPTG in *Bacillus* sp. RP1 might be absent in *E. coli*.

Among all the inducers tested three hours after induction, only CMC showed EG activities greater than without induction. CMC is a soluble cellulose derivative that can induce cellulase synthesis in many fungal and bacterial species. CMC is a macromolecule that could not enter the cell. Low level of EG synthesis would break down CMC to smaller molecules that could induce cellulase synthesis (Aro *et al.* 2005).

The addition of glucose decreased EG expression, however the EG activities after the additions of galactose and cellobiose were even lower. These sugars were usually known as cellulase inducers. Glucose as the final product of cellulose hydrolysis is known as a carbon catabolite repressor that can inhibit the production of cellulase (Sukumaran *et al.* 2005). On the other hand, Chundakkadu (1998) stated that the addition of glucose can be used as an inducer in the production of cellulase. Addition of galactose inhibited EG expression, however the mechanism is yet to be known. *E. coli* TOP10 used in this study had a mutation in *galK*, so it cannot metabolize galactose. Because the galactose was not metabolized by cells, the cells remained inhibited six hours after induction.

Cellobiose was known to be an inducer for many cellulase genes. At high concentrations, cellobiose was known to inhibit cellulase production (Kubicek and Penttila 1998). Cellobiose (two β -1,4-linked glucose

unit) can be hydrolyzed by β -glucosidase into glucose molecules. β -glucosidase produced by the host cell (*E. coli* has *bgl* operon to produce β -glucosidase) will help in the formation of inducers for EG synthesis (Aro *et al.* 2005) through the transglycosylation mechanism. β -glucosidase has an ability to transglycosylate cellobiose to sophorose (two β -1,2-linked glucose unit), which would induce cellulase synthesis. The effect of cellobiose addition on the EG expression occurs in the balance of hydrolysis and transglycosylation mechanism (Aro *et al.* 2005). Sophorose is a known inducer for fungal cellulases, but there are no report on the effect of sophorose inducing *Bacillus* cellulases.

A full length EG gene from *Bacillus* sp. RP1 was cloned in *E. coli*. No EG gene with the same sequence of *egC* has been characterized before. This protein has potential to be used in industrial applications.

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