MICROBIOLOGY INDONESIA
ISSN 1978-3477, eISSN 2087-8587
Vol.8, No.4, December 2014, p 147-160

Available online at
http://jurnal.permi.or.id/index.php/mionline
DOI: 10.5454/mi.8.4.2

Cloning, Sequencing, and Expression of the Gene Encoding a Family 9 Cellulase from *Bacillus licheniformis* F11 in *Escherichia coli* and *Bacillus megaterium*, and Characterization of the Recombinant Enzymes

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A gene encoding cellulase belonging to the glycosyl hydrolase family 9 along with its native promoter was isolated from *Bacillus licheniformis* F11, cloned in *Escherichia coli* DH5 α and subcloned by transconjugation to *Bacillus megaterium* MS941. Functionality of the encoded protein was proven both in heterologous hosts, *E. coli* and *B. megaterium*. In the latter, the gene product was found in the extracellular fraction expressing a high specific activity; whereas in *E. coli* the protein was not secreted into the medium, and rather, showed a lower specific activity. The optimum temperature of the recombinant enzyme expressed in the hosts range from 65-75 ºC; whereas the optimum pH is 6. The recombinant enzyme was stable between 50-60 ºC and in a broad pH range (pH 5 - 9). Addition of Ca^{2+} and Fe^{3+} enhanced the enzyme activity, whereas EDTA and Cu^{2+} had the opposite effect. Lichenin, rather than carboxyl methyl cellulose, is the preferred substrate.

Key words: *Bacillus licheniformis*, *Bacillus megaterium,* cloning, *E. coli*, expression, family 9 cellulase, transconjugation

Sebuah gen penyandi selulase (glikosil hidrolase keluarga protein 9) bersama dengan promotor aslinya diisolasi dari *Bacillus licheniformis* F11 dan dieskpresikan pada *Escherichia coli* DH5 α dan disubklon secara konjugasi ke *Bacillus megaterium* MS941. Protein yang dikodekan oleh gen tersebut terbukti dapat berfungsi dengan baik di kedua host heterolog, *E. coli* dan *B. megaterium*. Pada *B. megaterium*, produk gen disekresikan dalam fraksi ekstraseluler (supernatatan) dengan aktivitas spesifik yang tinggi; sedangkan pada *E. coli* produk gen (enzim) ditemukan dalam fraksi intraseluler dan memiliki aktivitas spesifik yang lebih rendah. Suhu optimal enzim rekombinan di kedua inang berkisar 65 - 75 ºC; sedangkan pH optimum adalah 6. Enzim rekombinan ini stabil pada kisaran suhu 50 - 60 ºC dan dalam kisaran pH yang luas (pH 5 sampai 9). Penambahan Ca^{2+} dan Fe^{3+} meningkatkan aktivitas enzim, sedangkan EDTA dan Cu^{2+} memiliki efek sebaliknya. Lichenin adalah substrat yang paling disukai oleh selulase rekombinan ini.

Kata kunci: *Bacillus licheniformis*, *Bacillus megaterium, E. coli*, ekspresi, kloning, selulase keluarga 9, transkonjugasi

Cellulose is nature's most abundant polysaccharide consisting of β-1, 4-linked glucose units. As the major constituent of plant cell walls, the polymer represents the most important resource for production of bioethanol and other fine chemicals (Lynd *et al.* 2005; Chandel *et al.* 2012). The use of cellulolytic enzymes rather than acid hydrolysis ensures environmentally friendly glucose formation, a prerequisite of a number of biotechnological applications including the above mentioned ones.

With respect to the catalyzed reaction, there are three types of cellulolytic enzymes: (i) endocellulases (EC 3.2.1.4), also known as glucanases, which randomly cleaves internal bonds at amorphous sites, thereby generating new chain ends; (ii) exocellulases (EC 3.2.1.91), which cleaves cellulose two to four units from the ends of the exposed chains, releasing oligosaccharides such as cellotetraose or cellobiose, and (iii) cellobiases (EC 3.2.1.21) or beta-glucosidases, which splits the above oligosaccharides into monosaccharides (Zhang *et al.* 2006).

The demand for cellulases steadily rises due to their usefulness for food processing, in the textile and pulp and paper industry, as feed additives, and, as mentioned previously, for lignocellulose based bioethanol production (Maki *et al.* 2009). Endo-1,4-β-glucanases (1, 4-β-D-glucano-glucohydrolases; E.C. 3.2.1.4) are rather widespread. By randomly hydrolyzing internal β-1, 4-D-glycosidic bonds, they decrease the polymer length, concomitantly increasing the concentration of reducing sugars (Onsori *et al.* 2005). Although currently commercialized cellulases are predominantly produced by fungi (Maki *et al.* 2009; Chandel *et al.*

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2012), studies about bacterial cellulases are quite frequently performed on *Bacillus subtilis*, *Paenibacillus*, *Clostridium cellulolyticum*, *Thermobifida fusca*, and *Clostridium thermocellum* (Singhania *et al.* 2010). The investigation of cellulase genes from *Bacillus* encoding family 5 enzymes was most often performed by cloning and expression in *E. coli* without the respective native promoter (Bischoff *et al.* 2005; Qiao *et al.* 2009; Jung *et al.* 2010). Compared to cellulase family 5, the cellulase family 9 is less studied. Cellulase family 9 (GH9) is known to have both endocellulolytic and exocellulolytic activities and it also shows synergism with both endocellulases and exocellulases (Qi *et al.* 2008). There was a report describing that a single GH9 cellulase is essential for microbial cellulose degradation, and that GH9 alone can perform cellulose degradation (Tolonen *et al.* 2009; Wilson 2009). Cloning of family 9 members from bacteria other than *Bacillus*, such as *Thermobifida halotolerans,* had been reported (Zhang *et al.* 2011); also, Liu *et al.* (2004) reported the cloning of cellulase genes (family 12 and family 9) from *Bacillus licheniformis* GNXII in *E. coli* using pET expression system (again without the respective promoter). However, no information available regarding the characteristics of the recombinant enzymes.

In this study, a cellulase gene of the glycosyl hydrolase family 9 along with its original promoter was PCR-amplified from *Bacillus licheniformis* F11, which was previously isolated from Indonesian shrimp waste (Waldeck *et al.* 2006). The obtained fragment was subcloned into a conjugative *E.coli-Bacillus* shuttle vector allowing expression not only in *E. coli* but also in *Bacillus megaterium* MS941 (Wittchen and Meinhardt 1995). The key properties of the recombinant enzymes present in the different hosts were determined.

MATERIALS AND METHODS

Strains, Plasmids, and Media. The bacterial strain originally used as host for obtaining and maintaining the recombinant plasmid was *E. coli* DH5 α. *E. coli* S17-1 served as the donor in conjugation experiments, and *Bacillus megaterium* MS941 as the recipient. The plasmid used was pBBRE194, an *E.coli-Bacillus* conjugative shuttle vector constructed from pE194 and pBBR MCS3 (Meinhardt Laboratory, Muenster University, Germany). The mobilizable vector carries two origins of replication as well as two antibiotic resistant genes (erythromycin and tetracycline). LB medium or LB supplemented with carboxyl methyl cellulose (CMC) and tetracycline (12.5 μg mL^{-1}) or erythromycin (5 μg mL^{-1}) was used to select the transformants. Genomic DNA of *B. licheniformis* F11 (Waldeck *et al.* 2006) served as the source of the cellulase gene.

DNA Extraction and Primers Design. *B. licheniformis* F11 was cultivated as previously described (Waldeck *et al.* 2006). The chromosomal DNA was extracted essentially as described in Helianti *et al.* (2010). All genetic experiments were performed according to the protocols in Sambrook and Russel (2001). The cellulase gene was amplified by using a pair of oligonucleotides, 5'GGGGTACCGGGC TGTCAGATCTGTTGACAATAAATAAAC -3' as the forward and 5'-CCGCTCGAGTTAGTA ACCGGGCTCATGTCCGAAAACGAG-3' as the reverse primer. Concomitantly designed *Kpn*I and *Pst*I restriction sites, which were used for cloning, are underlined. Primers were designed manually by retrieving and analyzing the genome of *B. licheniformis* DSM13 (NC_006322.1). The promoter regions were predicted using the promoter prediction server (http://www.fruitfly.org/seq_tools/promoter.html).

Polymerase Chain Reaction (PCR). After the initial 3-min hot start at 95 °C, the mixture was subjected to 25 cycles, each consisting of 20 s at 98 °C, 15 s at 71 °C, and 90 s at 72 °C, followed by 5 min at 72 °C to complete the elongation. The thermal cycler from Eppendorf (Germany) and the High Fidelity phusion DNA polymerase (NEB, UK) were used. The amplified fragment was purified using the Geneaid PCR Clean Up^{R} Kit (Geneaid, China), cut with *Kpn* I and *Pst* I and subsequently ligated into pBBRE194, which was linearized by the same restriction enzymes. The ligation mixture was used to transform *E. coli* DH5 α.

DNA Sequencing. Sequencing was performed with fluorescence –labeled dideoxynucleotides (Big Dye Terminator v3.1 kit, Applied Biosystems, Foster City, USA) and the ABI Prism 3730 capillary DNA Sequencer (Applied Biosystems, Foster City, USA). Sequencing was performed using the PCR primers and two internal primers (5'-TCGGCAA ACGGAGTATATGC-3' and 5'-AGAGTAAGA AGAATCTGTCG-3'). The primers used in the amplification of the cellulase gene and its promoter were designed based on the *Bacillus licheniformis* DSM13 genome sequence (http://www.ncbi.nlm.nih.gov) (Veith *et al.* 2004). The sequencing results were analyzed using Genetyx Software (Sci-Ed Software, North Carolina, USA).

Bacterial Conjugation. The recombinant plasmid pBBRE194-*cel9* was isolated from recombinant *E. coli* DH5 α and used to transform *E. coli* S17-1, which would serve as the donor for the conjugative transfer. The bacterial conjugation from *E. coli* S17-1 to *B. megaterium* MS941 was conducted based on the protocol developed by Aquino de Muro and Priest (2000), which was optimized by Richhardt *et al.* (2010) (Fig 1B). For conjugation process, *B. megaterium* MS941 cells were initially grown in LB broth without antibiotics, whereas *E. coli* S17-1 containing the recombinant plasmid pBBRE194-*cel9* was cultivated at 30 ºC overnight in LB medium with tetracycline. Two 250-ml Erlenmeyer flasks, each containing 50 ml of LB medium, was subsequently inoculated with either1 ml of the overnight *B. megaterium* or *E. coli* culture. The cultures were then grown at 30 ºC until OD_{600nm} reached 0.6-0.8. The cells were then harvested by centrifugation (3000 x g, 4 ºC) and washed twice in 15 ml holding buffer (12. 5 mM KH_2PO_4, 12.5 mM K_2HPO_4, 1 mM $MgSO_4$, pH 7.2), pelleted by centrifugation and, after resuspension in holding buffer, mixed with the donor cells. Using syringe and filter, the mixture was then compressed on a sterile nitrocellulose 0.45 µm filter to ensure close contact of donor and recipient cells. The filter was placed on sporulation agar for 48 h at 30 ºC (Schaeffer *et al.* 1965) with the side containing cells facing upwards. Counter selection of *Bacillus* transconjugants against *E. coli* was performed by pasteurization, where cells were collected from the filter by suspending in 900 µL holding buffer and then incubated for 20 min at 80 ºC, before subsequently spread on LB agar plates containing erythromycin. Transconjugants were further analyzed with respect to their cellulase activity and plasmid verification by restriction enzyme analysis. The procedure of cloning and transformation is shown schematically in Fig 1B.

Zymogram and SDS/PAGE Analyses. The molecular mass was determined by zymogram analysis using 10% polyacrylamide gels (PAGE), containing 0.1% sodium dodecyl sulfate (SDS), and 0.05% CMC for clear zone detection. PageRuler Protein ladder with molecular weight ranging from 10 to 200 kDa (Fermentas, Germany) was used as standard. The part of the gel with the protein marker was stained with Coomassie Brilliant Blue (CBB), the remaining part of the gel was used for zymogram analysis. The zymogram assay was conducted based on a previous report (Sunna *et al.* 1997). Congo Red, NaCl, and HCl were added sequentially to detect the clearing zone.

Enzyme Preparations from Recombinant *E. coli* DH5 α. A single recombinant colony containing pBBRE194-*cel9* was used to inoculate 5 mL media (LB and LB containing CMC and tetracycline) and grown overnight. The culture was then transferred into an Erlenmeyer flask with 50 ml medium and shaken for 24 h, 150 rpm at 37 °C in a Kühner Shaker (Kühner, Switzerland). In 6 h intervals, the cell density was determined and the cellulase activity in both supernatant (obtained by pelleting the cells) and intracellular fraction were measured. To obtain the latter, cell pellet was resuspended in 5 ml of 50 mM phosphate buffer containing 1 mM of 2-mercaptoethanol. Cells were disrupted by ultrasonication according to the previously reported method (Helianti *et al.* 2008) and the debris was removed by centrifugation to obtain the crude enzyme extract serving as the intracellular fraction. As a control, a single recombinant *E. coli* colony containing empty pBBRE194 was used as inoculum and subjected to the same procedure.

Enzyme Preparation from Recombinant *B. megaterium* MS941. Procedures were essentially performed as previously explained. However, both LB and LB- CMC media containing erythromycin, instead of tetracycline, were used. As a control, a single recombinant *B. megaterium* colony containing empty pBBRE194 was used as inoculum and subjected to the same procedure.

Partial Purification of the Recombinant Enzymes. To measure the effect of Cu^{2+}, SDS, and Tween 80 on the cellulase activity, partially purified enzyme was used. The purification procedure was as follows: crude enzymes from *E. coli* and *B. megaterium* fermented in LB-CMC were concentrated by membrane filtration (Millipore membrane with 10 kDa cut off). The concentrated enzymes were then subjected to Q-sepharose column (1 mL packed column). The target cellulase was eluted by 0-1 M NaCl in 20 mM phosphate buffer with 0.5 mL min^{-1} flow rate. The fractions with cellulase activity were pooled. The buffer was replaced with 20 mM phosphate buffer by membrane filtration, then the enzyme was subjected to assay as described above.

Enzymatic Activity Assay . The cellulase activity was measured (each sample in triplicate) by the method from Sanchez-Torres *et al.* (1996) using dinitrosalicylic acid to quantify the reducing sugars. D-glucose was used as standard. 100 µL of 1% CMC was mixed with equal volume of the enzyme preparation in 0.4 M phosphate buffer at pH 7. The

mixture was incubated at 50 °C for 10 min, after which, 3 ml of DNS reagent (1% dinitrosalicylic acid, 0.2% phenol, 0.05% sodium sulfite, and 1% sodium hydroxide, 20% (w/v) potassium sodium tartrate) (Miller 1959) was added. To stop the reaction, the mixture was boiled (100 °C for 5 min). After addition of 2 ml water, samples were centrifuged to obtain clear supernatants. For each sample, the absorbance at 520 nm was measured at the indicated pH and temperature , however, the enzymes were added following the addition of DNS. The protein concentration was determined by Bradford method using bovine serum albumin (BSA) as standard (Bradford 1976). One unit (U) of activity is defined as the amount of enzyme that produces 1µmol glucose per minute.

Effect of pH and Temperature on Cellulase Activity. The effect of temperature on cellulase activity was measured (each sample in triplicate) at temperature range between 30-80 °C, pH 7 in phosphate buffer. The effect of the pH on the cellulase activity was measured (each sample in triplicates) in a range between 5-10 at 60 °C using 50 mM of the following buffers; citrate buffer (for pH 5, 6), sodium phosphate buffer (for pH 6-8), Tris-HCl buffer (pH 8-9), and Glycin-NaOH buffer (pH 9-11).

pH and Temperature Stability. For checking the temperature (in)stability, the enzyme was preincubated without substrates at 50, 60, 70, and 80 ºC, for 20, 40, and 60 min, respectively. Subsequently, the activity was determined at 60 ºC pH 7. To check the influence of pH on the stability, the enzyme preparations without substrates were preincubated at pH 5, 6, 7, 8, and 9 at 60 ºC for 20, 40, and 60 min, respectively, and then the activity was determined at 60 ºC at the respective pH.

Effects of Various Additives and Substrates on Cellulase Activity. Effects of additives on cellulase activity were examined by adding various metal ions (Ca^{2+}, Cu^{2+}, Fe^{3+}, Zn^{2+}, and Mg^{2+}), chelator and detergents (EDTA, SDS, triton x-100, Tween 80) and substrates (birchwood xylan, oatspelt xylan, beechwood xylan, lichenin, corncobs, empty bunch oil palm, bagasse, filter paper). A mixture with CMC1% as substrate without additives was used as a control.

RESULTS

Cloning and Expression in *E. coli* and *B. megaterium*. We successfully amplified the specific 1.9 kb DNA fragment using primers designed based on the cellulase family 9 from *B.licheniformis* DSM13, with *B. licheniformis* F11 chromosomal DNA as template, and then cloned this specific fragment in pBBRE194 vector. The cloned fragment, including the putative glucanase family 9 gene (*cel9*), contains an upstream non-coding region of 304 bp with a potential promoter (bold face in Fig 1A) and a 1965 bp encoding the predicted glucanase (Fig 1A). The sequence is available at GenBank under the accession number KC663680. The *cel9* gene was expressed in *E. coli* DH5 α as well as in *Bacillus megaterium* MS941from the shuttle plasmid as can already be seen from the clearing halos around the positive colonies in LB medium containing CMC (Fig 2). The calculated molar mass of the native protein without its signal peptide is 69.91 kDa, which agrees with the result of the zymogram analysis (Fig 3).

Concerning the growth curves of the recombinant *E. coli* and *B. megaterium* strains harboring pBBRE194-*cel9* (in LB and LB medium containing CMC), there was no significant difference with respect to the cell densities (Fig 4). The cellulase activity of the recombinant *B. megaterium* was found exclusively in the culture medium. Intracellular cellulase activity was not detected. Up to 12 hours of growth, no enzyme activity was detected in the supernatant of the recombinant *E. coli*. However, there was some activity in the supernatant after 18 and 24 h (data not shown). The finding of extracellular activity after prolonged cultivation agrees with the plate assay, where old cells lysed and the enzyme was set free, that the activity could be measured in the supernatant. After 24 h of cultivation, the specific activities of the *E. coli* intracellular recombinant enzyme in LB and LB-CMC reached 4.3±0.1 and 2.6±0.1 U mg^{-1}, respectively, whereas the specific activities of the *B. megaterium* extracellular form were 54.8±1.5 and 78.8±4.7 U mg^{-1}, respectively (Fig 4). When *E. coli* and *B. megaterium* containing empty pBBRE194 plasmid were cultivated in LB medium, we could not detect any cellulase activity produce by any recombinant cells (Fig 4A). However, in LB-CMC medium *E. coli* and *B. megaterium* showed a faint intrinsic cellulase activity of 0.14 and 0.11 U mg^{-1}, respectively (Fig 4B). Recombinant *E. coli* and *B. megaterium* harboring empty pBBRE194 could not grow well in LB – CMC medium. The cell density only reached OD600 0.5 in the saturated condition (Fig 4B).

Hence, the results suggest that the promoter is recognized in *E. coli*. Indeed, the proposed promoter (bold face in Fig 1A) and the spacing of both motifs clearly agree with the *E. coli* -35 and -10 consensus

A

-35 -10

gggctgtcagatctg**ttgaca**ataaataaacaatcatgt**tagaat**ttccaaaatataacacttcgtttggaatgtgctgtctattagattt
ctactctcataacttagtttattgaacaaataaactaagttacttatcaaattcctcgcttgcagtcgtgtgctgatttaatgtgcaatcaat
atcttcggtttttcaactttggccttgttttgttcgccggcaaatctaaaaggaggtgagcatgttgtagacagatggagttgcttgatctt
aacgaacatgatgg***ggaggaa***gcagtac

```
          10        20        30        40        50        60
   gtgaaacagaaagtatttttaaaaatgaaagcgctttgtttggcacttttagtgatcttc
    M  K  Q  K  V  F  L  K  M  K  A  L  C  L  A  L  L  V  I  F

          70        80        90       100       110       120
   tctatgagcatagcgtcgttttcagaaaagacccgtgcagcttctgctgaagaatatcct
    S  M  S  I  A  S  F  S  E  K  T  R  A  A  S  A  E  E  Y  P

         130       140       150       160       170       180
   cataattatgctgaactgctgcaaaagtctttgttattttatgaagcacagcgctcggga
    H  N  Y  A  E  L  L  Q  K  S  L  L  F  Y  E  A  Q  R  S  G

         190       200       210       220       230       240
   agacttccggaaaacagccggctgaattggagaggagactccgggcttgaggacggaaaa
    R  L  P  E  N  S  R  L  N  W  R  G  D  S  G  L  E  D  G  K

         250       260       270       280       290       300
   gacgttggcctcgatttaacgggagggtggtatgatgccggcgaccacgtgaagttcggt
    D  V  G  L  D  L  T  G  G  W  Y  D  A  G  D  H  V  K  F  G

         310       320       330       340       350       360
   ctgccgatggcttattctgccgcaatcctgtcatggtcggtctatgagtaccgagatgcc
    L  P  M  A  Y  S  A  A  I  L  S  W  S  V  Y  E  Y  R  D  A

         370       380       390       400       410       420
   tacaaagaatcgggtcagcttgatgcggcgctggacaatattaaatgggcgacagactac
    Y  K  E  S  G  Q  L  D  A  A  L  D  N  I  K  W  A  T  D  Y

         430       440       450       460       470       480
   tttcttaaagcccatacggctccttatgaattgtggggccaagtcggaaatggcgctcta
    F  L  K  A  H  T  A  P  Y  E  L  W  G  Q  V  G  N  G  A  L

         490       500       510       520       530       540
   gaccacgcatggtgggggccggccgaagtaatgccgatgaagcgccctgcctataagatc
    D  H  A  W  W  G  P  A  E  V  M  P  M  K  R  P  A  Y  K  I

         550       560       570       580       590       600
   gatgccggctgtccggggtcagaccttgctggtggtacagccgcagcgctagcatcagca
    D  A  G  C  P  G  S  D  L  A  G  G  T  A  A  A  L  A  S  A

         610       620       630       640       650       660
   tcaattattttcaagccgacagattcttcttactctgaaaaattactggctcatgccaag
    S  I  I  F  K  P  T  D  S  S  Y  S  E  K  L  L  A  H  A  K

         670       680       690       700       710       720
   caattgtatgattttgccgaccgctaccgcggcaaatattcagactgcattacagacgca
    Q  L  Y  D  F  A  D  R  Y  R  G  K  Y  S  D  C  I  T  D  A

         730       740       750       760       770       780
   cagcaatattataattcgtggagcgggtataaagatgaactgacatggggagctgtctgg
    Q  Q  Y  Y  N  S  W  S  G  Y  K  D  E  L  T  W  G  A  V  W

         790       800       810       820       830       840
   ctctacttggcaacagaagaacaacaatatttggataaagcccttgcttcggtctcagat
    L  Y  L  A  T  E  E  Q  Q  Y  L  D  K  A  L  A  S  V  S  D
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       850       860       870       880       890       900
tggggcgatcccgcaaactggccttaccgctggacgctttcctgggatgacgtcacttac
 W  G  D  P  A  N  W  P  Y  R  W  T  L  S  W  D  D  V  T  Y

       910       920       930       940       950       960
ggagcacagctgctgctcgctcgtctgacaaacgattcccgttttgtcaaatctgtcgaa
 G  A  Q  L  L  L  A  R  L  T  N  D  S  R  F  V  K  S  V  E

       970       980       990      1000      1010      1020
cgcaatcttgattattggtcgacaggctacagtcataatggaagcatagaacggatcacg
 R  N  L  D  Y  W  S  T  G  Y  S  H  N  G  S  I  E  R  I  T

      1030      1040      1050      1060      1070      1080
tatacgccgggcggtttggcctggcttgagcagtggggatcattgcgatacgcttcgaat
 Y  T  P  G  G  L  A  W  L  E  Q  W  G  S  L  R  Y  A  S  N

      1090      1100      1110      1120      1130      1140
gccgcttttctcgctttcgtttattccgattgggtggatacagaaaaagcgaaaagatat
 A  A  F  L  A  F  V  Y  S  D  W  V  D  T  E  K  A  K  R  Y

      1150      1160      1170      1180      1190      1200
cgggattttgctgttcggcaaacggagtatatgctaggagataatccgcagcagcgaagc
 R  D  F  A  V  R  Q  T  E  Y  M  L  G  D  N  P  Q  Q  R  S

      1210      1220      1230      1240      1250      1260
tttgtcgttggatacggtaaaaatccgccgaaacatccgcatcaccgtacagcacacggt
 F  V  V  G  Y  G  K  N  P  P  K  H  P  H  H  R  T  A  H  G

      1270      1280      1290      1300      1310      1320
tcatgggccaatcagatgaatgtgcctgaaaaccatcgccataccctatacggcgcatta
 S  W  A  N  Q  M  N  V  P  E  N  H  R  H  T  L  Y  G  A  L

      1330      1340      1350      1360      1370      1380
gtcggcggtccgggaagggacgattcgtaccgagatgacataacagattatgcgtcaaac
 V  G  G  P  G  R  D  D  S  Y  R  D  D  I  T  D  Y  A  S  N

      1390      1400      1410      1420      1430      1440
gaagttgcgatcgattataatgccgcttttaccggcaacgtagcgaaaatgtttcagctg
 E  V  A  I  D  Y  N  A  A  F  T  G  N  V  A  K  M  F  Q  L

      1450      1460      1470      1480      1490      1500
ttcgggaaaggccatgttccgctgcctgattttccggagaaggaaacacctgaggacgaa
 F  G  K  G  H  V  P  L  P  D  F  P  E  K  E  T  P  E  D  E

      1510      1520      1530      1540      1550      1560
tattttgcagaggcatcaatcaacagctccggaaacagctatactgaaatccgggcgcag
 Y  F  A  E  A  S  I  N  S  S  G  N  S  Y  T  E  I  R  A  Q

      1570      1580      1590      1600      1610      1620
ctcaataaccgttcgggatggccggcaaagaaaaccgatcaattgtctttccgctactac
 L  N  N  R  S  G  W  P  A  K  K  T  D  Q  L  S  F  R  Y  Y

      1630      1640      1650      1660      1670      1680
gttgacttgacggaagctgtagaagcgggatattccgccgaagatataaaagtcacagcc
 V  D  L  T  E  A  V  E  A  G  Y  S  A  E  D  I  K  V  T  A

      1690      1700      1710      1720      1730      1740
ggctataacgaaggggcctcggtatcagagctgaagccgcatgacgcttcaaagcacatt
 G  Y  N  E  G  A  S  V  S  E  L  K  P  H  D  A  S  K  H  I
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      1750      1760      1770      1780      1790      1800
tactatacagaagtcagcttcagcggggttttgatttatccaggcggtcaatccgcccat
 Y   Y   T   E   V   S   F   S   G   V   L   I   Y   P   G   G   Q   S   A   H

      1810      1820      1830      1840      1850      1860
aaaaaagaagtgcagttccgcctttcggcaccagacggaacgtctttttggaacccggaa
 K   K   E   V   Q   F   R   L   S   A   P   D   G   T   S   F   W   N   P   E

      1870      1880      1890      1900      1910      1920
aatgaccactggtatcagggtctgtcacatgcgcttctgaagacgcggtatattccaacg
 N   D   H   W   Y   Q   G   L   S   H   A   L   L   K   T   R   Y   I   P   T

      1930      1940      1950      1960
gccgccggccagcggctcgttttcggacatgagcccggttactaa
 A   A   G   Q   R   L   V   F   G   H   E   P   G   Y   *
```

B

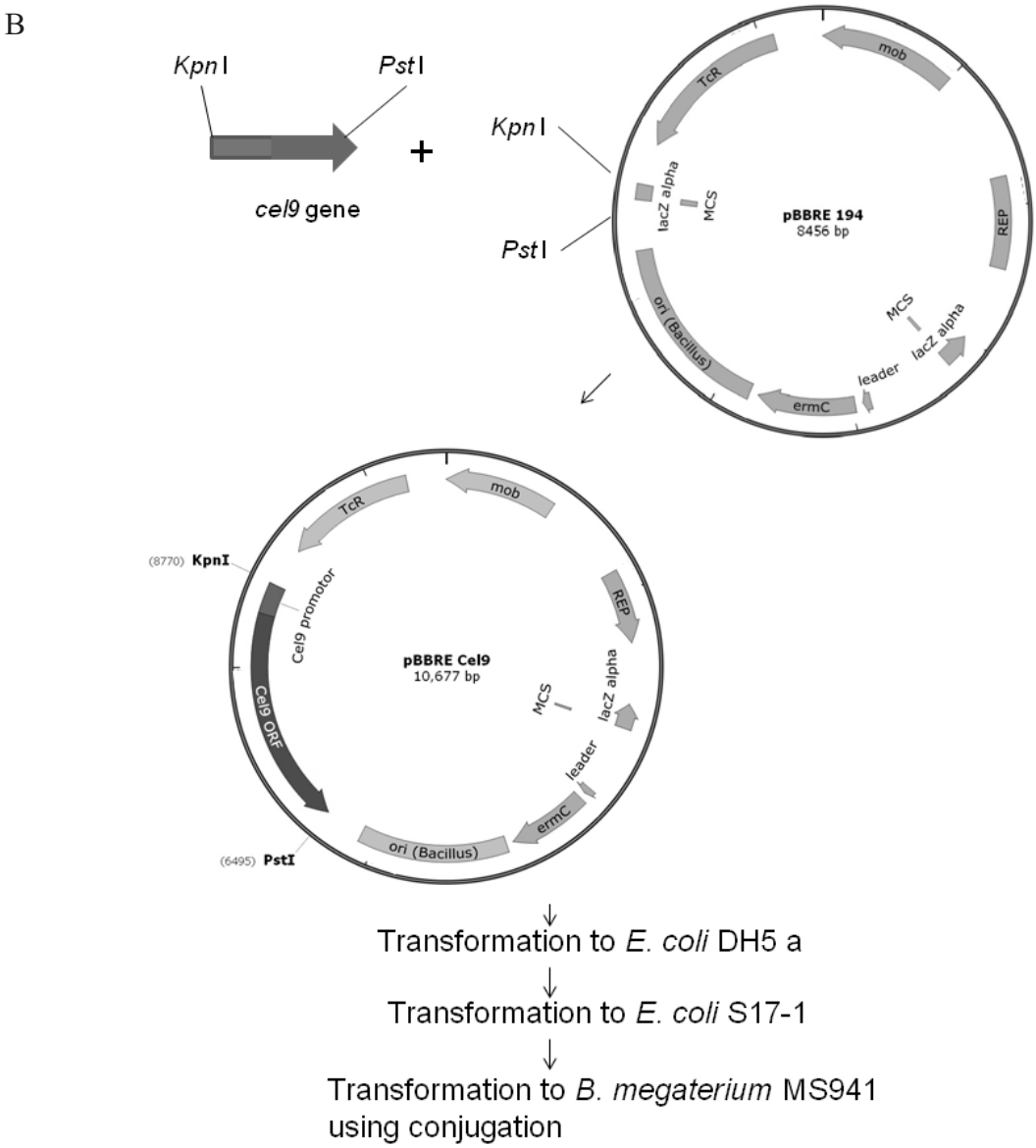

Fig 1 (A) DNA and the predicted protein sequences of the cellulase family 9 gene of *Bacillus licheniformis* F11. The putative promoter (with its -35 and -10 region, respectively) is highlighted in bold face. The putative Shine/Dalgarno sequence or ribosome binding site, as well as the corresponding start codon (gtg), are in italics. Amino acids are given in the one letter codes. Underlined amino acids refer to the predicted signal peptide. The translational stop is marked with an asterisk. (B) Schematic representation of the cloning procedure. The noncoding region of the cloned fragment is given in blue. The coding region, including the promoter, is in red (the figure not drawn to scale).

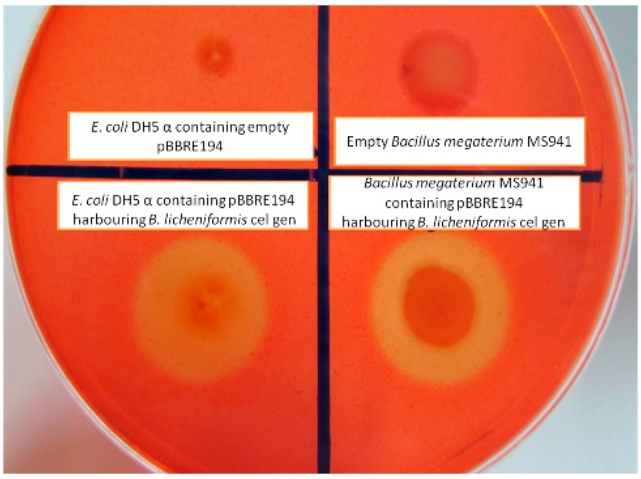

Fig 2 The colonies of recombinant *E. coli* and *Bacillus megaterium* on LB agar medium containing CMC 1% stained with Congo Red.

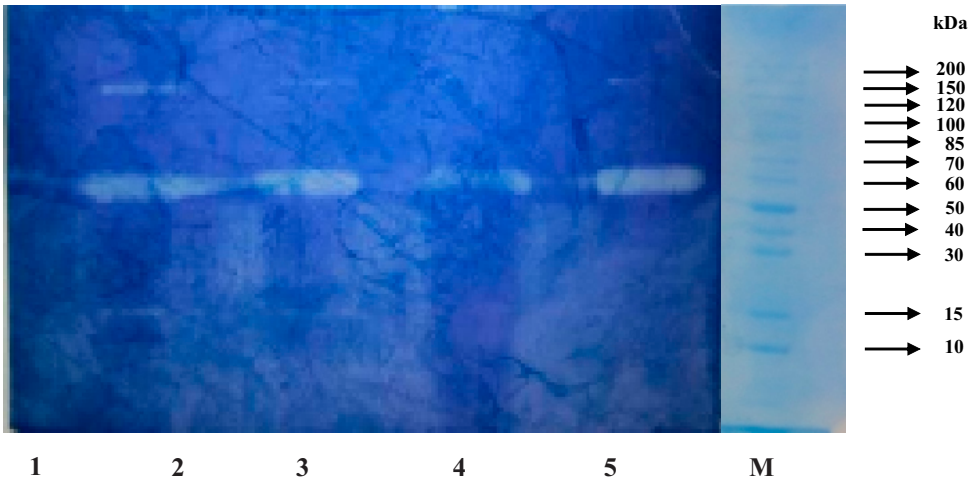

Fig 3 The zymogram of the protein crude extract. The protein marker is PageRuler protein ladder, the ladder is shown in figure. 1) Negative control (supernatant recombinant *Bacillus megaterium* MS941 with empty vector); 2) Supernatant of recombinant *Bacillus megaterium* MS941 (pBBRE194 -*cel9*) cultivated in LB-CMC; 3) Supernatant of recombinant *B. megaterium* MS941 (pBBRE194 -*cel9*) cultivated in LB; 4) Sonication extract of recombinant *E. coli* containing (pBBRE194 -*cel9*) cultivated in LB-CMC; 5) Sonication extract of recombinant *E. coli* (pBBRE194 -*cel9*) cultivated in LB.

sequences. The promoter is probably constitutive, since in medium without the substrate, the gene was expressed well. The signal peptide is probably also recognized in the Gram negative host but secretion is solely possible through the plasma membrane, thereby delivering (and capturing) the recombinant protein to the periplasmic space.

Characterization of the Recombinant Gene Product from Two Hosts. The properties (pH and temperature profiles) of the gene products expressed in the different hosts in different media were, as expected, rather similar. The activity of the recombinant cellulases was optimal at pH 6 (data not shown) and 65-75 ºC (Fig 5), the enzymes were stable at 50 and 60 ºC at pH 5-9 (Fig 6). The stability at 50 and 60 ºC in a wide pH range (5-9) of the recombinant extracellular enzyme expressed by *B. megaterium* MS941 is a distinct character of cellulase family 9.

Most of the tested metal ions, except for Cu^{2+}, enhanced or had no negative effected on the activity of the crude extract of recombinant cellulases. When we investigated the crude extract of the enzymes further, we found that Cu^{2+}, SDS, and Tween 80 had different effects on cellulase activity of the crude extract. Cu^{2+}, SDS, and Tween 80 increased the cellulase activity of the intracellular fraction/crude extract of the recombinant *E. coli*, while decreasing the cellulase activity of the supernatant of recombinant *B. megaterium* (data not shown). To clarify, we conducted partial purification. After purification of the crude

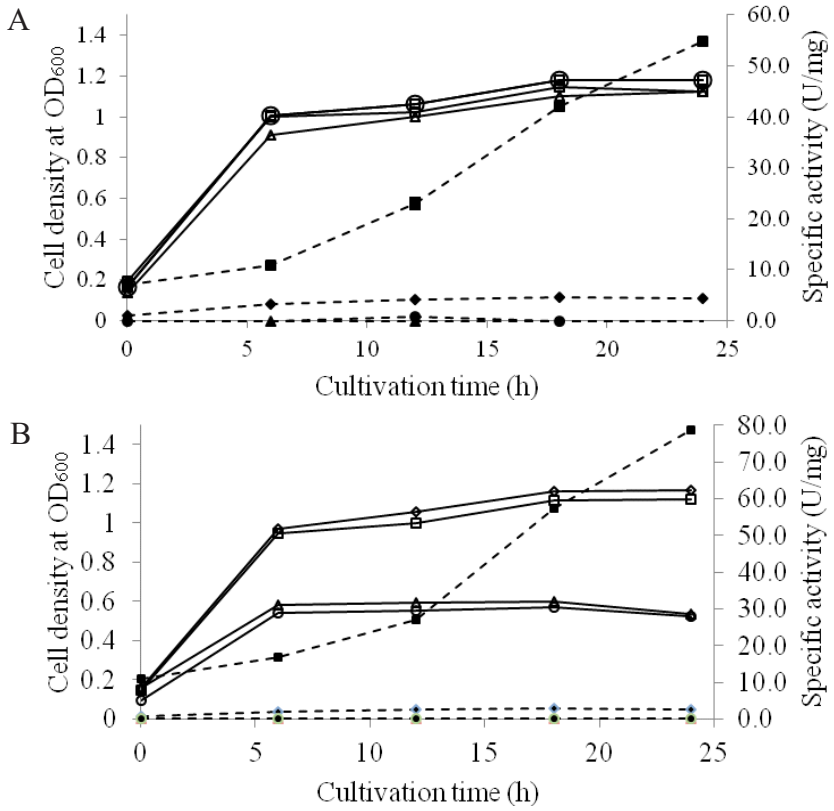

Fig 4 The growth of recombinant *Bacillus megaterium* MS941 and recombinant *E. coli* containing pBBRE194 -*cel9* and their cellulase productivity cultivated in LB (A) and LB-CMC medium (B). The solid line with symbols□: cell density of recombinant *B. megaterium* (pBBRE194 -*cel9*) ;◇: cell density of recombinant *E.coli* (pBBRE194 -*cel9*); △: cell density of recombinant *B. megaterium* (empty pBBRE194); ○: cell density of recombinant *E. coli* (empty pBBRE194). The dotted line with symbols ■: specific activity of supernatant of recombinant *B. megaterium* (pBBRE194 -*cel9*); ◆ : specific activity of intracellular fraction of recombinant *E. coli* (pBBRE194 -*cel9*); ▲ : specific activity of supernatant of recombinant *B. megaterium* (empty pBBRE194); ● : specific activity of intracellular fraction of recombinant *E. coli* (empty pBBRE194).

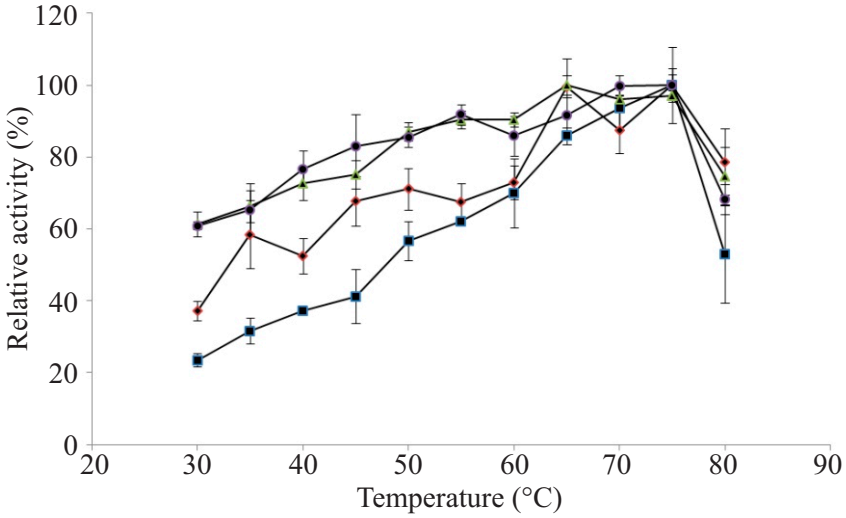

Fig 5 Effect of temperature on activity of recombinant cellulase produced in *E. coli* and *B. megaterium* in LB and LB-CMC, respectively. For this temperature profile, cellulase activity was measured at indicated temperature with temperature range 30-80 °C for 10 min at pH 7 using phosphate buffer. Each sample was in triplicates. The error bars showed the standard deviation of three values of independent experiment. The symbols ■ : are the supernatant of recombinant *Bacillus megaterium* MS941 (pBBRE194 -*cel9*) cultivated in LB; ◆ : supernatant of recombinant *Bacillus megaterium* MS941 (pBBRE194 -*cel9*) cultivated in LB-CMC; ▲ : sonication extract of recombinant *E. coli* containing (pBBRE194 -*cel9*) cultivated in LB; ● : sonication extract of recombinant *E. coli* containing (pBBRE194 -*cel9*) cultivated in LB-CMC.

extract , the specific activity of the enzymes produced by *E. coli* and *B. megaterium* cultivated in LB-CMC increased to 23.94 and 298 U mg^{-1}, respectively. Using the partially purified enzymes, it was confirmed that Cu^{2+} and Tween 80 reduced the activity significantly, whereas SDS only moderately decreased the activity (Table 1). The crude extract of recombinant cellulase displayed substrate preferences, where the specificity against lichenin was the highest in each case (Table 1).

DISCUSSION

Bacillus licheniformis F11 is known to harbor a cellulase family 5 gene and, indeed, displayed cellulase activity (Waldeck *et al.* 2006). However, there was no information related to the cellulase family 9 gene. Furthermore, compared to cellulase family 5, the cellulase family 9 is less studied. Cellulases family 9 is potential for many applications since they are known to have both endocellulolytic and exocellulolytic activities on processing cellulose, and also show synergism with both endocellulases and exocellulases (Qi *et al.* 2008). The cellulase family 9 gene we cloned had 99% similarity to the gene of *B. licheniformis* ATCC14580 or DSM13 (Veith *et al.* 2004; Rey *et al.* 2004). When we conducted BLAST analysis against GenBank data base, the results showed that there were only a few DNA sequence information retrieved for this cellulase family 9 gene. The analysis of amino acid sequences deduced from the genes indicated that the

Table 1 Effect of additives and different substrates on recombinant cellulase activity

	Relative activity (%)[a]	Relative activity (%)[b]
Control	100	100
Metal ions (5mM)		
CaCl2	120.2±3.6	118.4±3.6
CuSO4[c]	41.3±5.3	34.6±2.9
FeCl3	114.7±4.1	128.9±5.3
ZnCl2	103.8±9.5	128.9±9.5
MgCl2	108.9±8.0	104.7±2.6
Detergent (0.25%)		
SDS[c]	195.8±5.1	198.9±3.4
triton x-100	100.7±2.4	114.0±10.0
Tween 80[c]	82.2±4.4	78.2±3.8
Chelator (10 mM)		
EDTA 10 mM	57.9±8.6	53.2±3.1
Substrate (1%)		
Birchwood	0±3.6	12.1±6.0
Oatspelt	29.4±5.9	0±5.4
Beechwood	39.6±9.4	21.1±9.4
Lichenin	123.0±7.7	126.8±5.7
Corncobs	1.3±6.8	40.2±9.5
Empty bunch oil palm	32.3±8.8	24.2±10.0
Bagasse	26.0±10	42.9±10

*a: Supernatant of recombinant *Bacillus megaterium* MS941 (pBBRE194 -*cel9*) cultivated in LB-CMC; b: Sonication extract of recombinant *E. coli* (pBBRE194 -*cel9*) cultivated in LB-CMC; c: Partially purified recombinant cellulase samples were used.
*A mixture solution without additional substance and CMC1% as substrate were used as control

enzyme consisted of a catalytic domain belonging to glycosyl hydrolase family 9, a linker domain, and a carbohydrate binding module family 3 from N-terminal to C-terminal as Liu *et al.* reported (2004).

The Gram-negative bacterium *E. coli* is the most frequently used organism for heterologous protein production since this bacterium is well known and its genetic manipulation methods are well established (Tempe 2006). There are several reports describing the cloning and expression of cellulase genes from Gram positive bacteria in *E. coli*. They reported that the protein was found solely intracellular (Bischoff *et al.* 2007; Qiao *et al.* 2009; Jung et al. 2010). In our study, a clearing halo around the *E. coli* colony was observed, although the enzyme is not expected to occur outside the outer membrane, since the cellulase activity in the supernatant was not significant (Fig 2). This might be due to cell lysis occuring when we overexpressed a protein in the periplasm of *E.coli*. This phenomenon had previously been observed, for example with glucanase overexpression in *Paenibacillus macerans* (Borriss *et al.* 1990).

Compared to other cellulase family 9 from other resources, this cellulase has several different properties. The cellulase of the *B. licheniformis* F11 family 9 is stable over a wide pH range (5-9) at 50 ºC and 60 ºC. To our understanding, this character is distinct from other cellulases family 9. It seems to be more thermo- and acidophilic than that of *Thermobifida halotolerans,* which has an optimum activity at 55 ºC and pH 8 (Zhang *et al.* 2011), and that of the cellulase family 9 enzyme from a German grassland soil metagenomic library with an optimal activity at 55 ºC and pH 7 (Nacke *et al.* 2012). All metal ions, except for Cu^{2+}, enhanced the activity of recombinant cellulases from both hosts. Consistent with the findings that metal ions enhanced the activity, EDTA was found to inhibit the protein, suggesting the cellulase being a metalloenzyme as for the cellulase family 9 from *Thermobifida halotolerants* (Zhang *et al.* 2011). Interesting results were also obtained with 0.25% SDS. SDS usually decreased the activity. However, in this recombinant cellulase, it had only slight moderate negative effect on the activity (Table 1). SDS was shown to positively affected the activity of recombinant *Bacillus lehanus* protease expressed in *E. coli* (Joshi and Satyanarayana 2013). SDS is also known to have dual interaction through binding as both a denaturant and a recovery reagent (Xiang *et al.* 2006).

From our experiments, we found that the intrinsic cellulase activity of *E. coli* and *B. megaterium* were extremely faint. Although, the genomic information demonstrated that *E.coli* str. K-12 substrain MG1655 (GenBank: U00096.3), which is the parent of *E. coli* DH5 α, had at least two families of glycosyl hydrolase gene (family 25 and 65), no data confirmed whether any of these glycosyl hydrolases was cellulase. On the other hand, the genomic information of *Bacillus megaterium* DSM319 (GenBank: CP001982.1; Wittchen and Meinhardt 1995, Eppinger *et al.* 2011), which is the parent of *B. megaterium* MS941, demonstrated that the strain had 3 families of glycosyl hydrolase; family 31, 5, and 18, which, especially family 5, was predicted to be cellulase. The reason why this intrinsic cellulase gene family 5 was not expressed well in the *B. megaterium* MS941 is still unclear. In other report, it was described that the newly isolated *B. megaterium* had cellulase activity (Shobharani *et al.* 2013).

The specific activities of the intracellular form of the *E. coli* recombinant cellulase cultivated in LB and LB-CMC were low; whereas, those of the *B. megaterium* extracellular form were high (Fig. 3). The recombinant *B. megaterium* expressed cellulase activity exclusively in the culture medium and no intracellular cellulase activity was detected. Such findings agree with the presence of a signal peptide for the general secretory pathway (Tjalsma *et al.* 2000). These results showed *B. megaterium* as the preferred host for production of secreted heterologous enzymes. Previously, we overexpressed the *Bacillus subtilis* AQ1 xylanase in *E. coli* and found significant extracellular enzyme activities (Helianti *et al.* 2010). The rather low extracellular cellulase activities found in this study may be due to the different promoter used to regulate the level of gene expression and different signal peptide that determine the protein allocation (Takemori *et al.* 2012).

In this study we used *B. megaterium* MS941 as *Bacillus* host. Due to the deletion of the *nprM* gene, *B. megaterium* MS941 had almost completely lost its extracellular proteolytic activities and additionally displays stable plasmid maintenance (Wittchen and Meinhardt 1995). Indeed, highly efficient expression of homologous and heterologous genes was reported in *B. megaterium* (Meinhardt *et al.* 1989, Biedendieck *et al.* 2011) and currently it is a rather popular host for a number of applications (Vary 2004). Since transformation of the species is difficult to perform (Vary *et al.* 2007), we have chosen the conjugational approach developed by Richhardt *et al.* (2010).

The presented work confirmed that the cellulase

gene from *B. licheniformis* was expressed succesfully in both *E. coli* and *B. megaterium*. However, the extracellular expression in *B. megaterium* can be more efficient in terms of enzyme recovery and downstream process (for example removing the cell disrupting step), since the gene product is secreted into the medium. The conjugative transformation protocol applied readily generated the respective transformants and thus again proved useful for genetic manipulation of *Bacillus* (Richhardt *et al.* 2010). Combining our approach with a *Bacillus* high copy number plasmid will probably facilitate efficient overexpression of foreign proteins via conjugative plasmid transfer.

ACKNOWLEDGMENT

The part of the work was funded by National Innovation System Research Grant of Incentive (InSINas) Program from Indonesian Ministry of Research and Technology No RT 2013-1363 granted to IH. The authors thank Prof. Meinhardt, University of Muenster, for the collaboration and for critically reading the manuscript. We thank Dr. Larsen for the construction of plasmid pBBRE194.

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