Isolation, Cellulase Activity Test and Molecular Identification of Selected Cellulolytic Bacteria Indigenous Rice Bran

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ABSTRACT

Rice bran is the waste product of rice milling which is abundant in Indonesia, it can be used as a raw material for the manufacture of bioethanol by fermentation. Before being fermented, rice bran must be hydrolyzed into glucose by biomass degrading. This study was aimed to isolate indigenous cellulolytic bacteria from rice bran as producer of cellulolytic enzymes and resulted in 22 bacterial isolates that demonstrated cellulolytic activity being identified. Among them, BE 8 and BE 14 isolates showed the highest endoglucanase activity at 2.16 and 1.31 U/mL respectively. Identification of the 16S rDNA showed that BE 8 belongs to Bacillus subtilis *and BE 14 in* Bacillus cereus.

Keywords: *cellulase enzyme; rice bran; 16S rDNA method;* Bacillus subtillis; Bacillus cereus

ABSTRAK

Bekatul (Rice bran) merupakan limbah dari hasil penggilingan padi yang jumlahnya cukup melimpah di Indonesia sehingga bisa dimanfaatkan sebagai bahan baku untuk pembuatan bioetanol dengan cara fermentasi. Sebelum fermentasi maka bekatul harus dihidrolisis menjadi glukosa menggunakan enzim selulase. Tujuan dari studi ini untuk mengisolasi bakteri selulolitik indigenous bekatul sebagai penghasil enzim selulase sehingga diperoleh isolat yang stabil dan sesuai dengan substrat bekatul. Hasil isolasi dan skrining menunjukkan ada 25 isolat bakteri indigenous bekatul dan 22 isolat bakteri yang menunjukkan adanya aktivitas selulolitik. Pada uji aktivitas selulase dari 6 isolat terpilih menunjukkan bahwa isolat BE 8 dan BE 14 menunjukkan aktivitas endoglukanase tertinggi dengan nilai masing-masing 2,16 dan 1,31 U/mL. Berdasarkan identifikasi 16S rDNA menunjukkan bahwa isolat BE 8 adalah Bacillus subtilis *dan isolate BE 14 adalah* Bacillus cereus.

Kata Kunci: *enzim selulase; bekatul; metode 16S rDNA;* Bacillus subtillis*;* Bacillus cereus

INTRODUCTION

Bioethanol receives much attention as an alternative to petroleum-based energy sources. The advantages of bioethanol as compared to petroleum are that it has a higher octane number, higher flame speed, more combustible, and gives higher vaporization heat that provides a higher compression ratio and a shorter burn time in the internal combustion engine [1]. Over the period 1990 to 2020, CO_2 emissions from road traffic around the world will have increased by 92% [2]. The more complete combustion of Bioethanol fuel is due to it contains 35% of the oxygen that it will reduce particulate and NOx emissions [3].

Bioethanol production in Indonesia focuses on the use of starchy materials such as cassava and corn, and it is feared that this may potentially lead to the decrease of biodiversity through monoculture, and agricultural practices that damage the quality of lands [4], and create continuing problems of raw material supply and price fluctuations. These issues can be solved by diversifying the starchy raw materials used for bioethanol production by utilizing the waste products of rice milling, such as rice bran.

Indonesia is one of the largest rice producers and consumers in Asia. The rice production in 2017 is expected to have increased by 3.9% from the 72 million metric tons produced in 2016 [5]. The rice bran obtained from rice milling amounts to approximately 17% [6] or about 13 million tons available annually. Rice bran contains 25% carbohydrates, composed of 22–25% insoluble fibers (such as cellulose) and 0.1–1.5% soluble fiber, 16–19.5% protein, 17–20% fat, and 7–8% minerals [7]. The high level of carbohydrates, in

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particular cellulose, in rice bran can serve as a potential source of raw materials in the manufacture of bioethanol [8].

Cellulose is a polymer of glucose linked by β-1,4 glucosidase bond with a crystalline structure that is stabilized by intermolecular and intramolecular hydrogen bonds [9]. Cellulase enzymes can degrade the crystalline structure into glucose that can be used as raw material for bioethanol. Cellulase enzymes can be produced by microorganisms such as fungi and bacteria. Cellulase-producing bacteria have a high growth rate, meaning for them to degrade cellulose is shorter than for fungi [10]. Bacteria also secrete smaller cellulases than fungi which therefore diffuse more quickly into plant tissues that contain cellulose [11], their rate of growth is faster, they adapt successfully to extreme environments and it is easier to perform genetic engineering on them [12].

The cellulase enzyme is a multienzyme system comprising the complexes endo-β-1,4-glucanase (CMCase; EG; EC 3.2.1.4), exo-β-1,4-glucanase (cellobiohydrolase; CBH; EC 3.2.1.91), and β-1,4 glucosidase (GH; EC 3.2.1.21). The enzyme system works in synergy to degrade cellulose substrate. Some cellulose-producing fungi generate incomplete cellulase systems which affects the cellulose`s performance in cellulose degradation. *Trichoderma reesei* produces endo-β-1,4-glucanase and exo-β-1,4-glucanase up to 80% but β-glucosidase is lower than the main product of the hydrolysis instead of glucose and cellobiose [13]. *Aspergillus niger* is one of the commonly used for hydrolyzing cellulose. This microorganism produces high amounts of β-glucosidase but low amounts of endo-β-1,4-glucanase and exo-β-1,4 glucanase [14]. The *Bacillus circulans* has levels of high activity in terms of CMCase (endo-β-1,4-glucanase), avicelase (exo-β-1,4-glucanase) and β-glucosidase [15]. Therefore, this study aimed to isolation, identify cellulolytic bacteria from rice bran as cellulase producers, with the expectation that a complete and efficient cellulolytic system would be obtained from the cellulose hydrolysis of rice bran.

EXPERIMENTAL SECTION

Materials

Rice bran from Sierang type rice was collected from local rice milling in the city of Malang, Indonesia. All media for growth were purchased from Difco, chemicals including metallic, reduction agents from Sigma Aldrich product, materials for biochemical testing of microorganisms use Microbact 12A/E-24E kits from Oxoid Ltd. products, and molecular identification using a primer from Genetica Science.

Instrumentation

Enzyme activity was measured using UV-Visible spectrophotometer by Varian, amplification of DNA using PCR by Intron, and sequencing analysis was performed using a dye terminator dideoxy method by Sanger Singapore.

Procedure

Isolation and screening of cellulolytic bacteria

Twenty-five grams of rice bran that had been lest to decompose for one month was added to an Erlenmeyer flask containing 225 mL of 0.85% NaCl solution and was incubated for two weeks at room temperature. After incubation, a sample of 1 mL was diluted up to 10^6 times with 0.85% NaCl in a Petri dish containing *CYPE-agar* medium at room temperature for 24–48 h. *CYPE-agar* consisted of 1% CMC, 0.5% peptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4$, 0.5% NaCl, and 2% agar at pH 7.

The morphology of each colony was observed and then transferred to CYPE-agar slant for purification. The pure isolates obtained were tested for their ability to produce cellulolytic enzymes using *Congo red* [16]. Colonies of pure isolates flow were treated with 0.1% *Congo red* for 15 min then washed three times using 1 M NaCl. The diameter of the clear zone and colony formed were measured, and cellulase activity was determined from the cellulolytic index. The cellulolytic index is the ratio between the diameter of the clear zone and the diameter of the colony. Isolates with large cellulolytic index ability were selected and used for further treatment.

Growth curve of bacteria

For each selected isolates one colony was transferred into 50 mL of CYPE-broth medium and incubated at 120 rpm for 24 h. Thirty milliliters of each inoculum was transferred to 300 mL similar medium. Samples of 5 mL were removed every 4 h until the stationary phase, and absorbance was measured at 600 nm.

Cellulase production by bacteria indigenous of rice bran

Selected cellulolytic bacteria isolates were incubated on CYPE-broth at room temperature with shaking at 120 rpm. The time of cellulase enzyme production was adjusted to a logarithmic growth phase of the growth curve result. The acquired culture was then centrifuged at l0000 rpm for 10 min at 4 °C temperature. The resulting supernatant was then used to test the cellulolytic ability and then identify the species of bacteria.

Cellulase activity assay

Endo-β-1,4-glucanase. Endo-β-1,4-glucanase activity was determined by incubation a mixture of 1% CMC in 1800 µL 20 mM phosphate buffer (pH 7.0) with 200 µL of crude extract of cellulase enzymes at 50 °C. After 30 min of reaction, 2 mL of 3,5-dinitrosalicylic acid (DNS) was added and boiled in water bath for 5 min to stop the reaction [17]. The sample was then cooled to room temperature and its absorbance was measured at 530 nm wavelength. One unit of endo-β-1,4-glucanase activity was defined as the amount of enzyme that could hydrolyze CMC and released 1 µg of glucose equivalent per minute reaction at a 50 °C [17].

Exo-β-1,4-glucanase. Exo-β-1,4-glucanase activity was determined by incubation of 1% avicel in 1800 µL 20 mM phosphate buffer (pH 7.0) with 200 µL of the enzyme at 50 °C temperature. After 30 min of reaction, 2 mL of 3,5-dinitrosalicylic acid (DNS) was added and boiled in water bath for 5 min to stop the reaction. The sample was then cooled to room temperature and its absorbance was measured at 530 nm wavelength. One unit of exo-β-1,4-glucanase activity was defined as the amount of enzyme that could hydrolyze avicel and released 1 µg of glucose per minute.

β-glucosidase. Beta-glucosidase activity was determined by hydrolyzing 1% cellobiose substrate in 1800 µL 20 mM phosphate buffer (pH 7.0) with 100 µL of crude extract of enzymes at 50 °C. After 30 min of reaction, 2 mL of 3,5-dinitrosalicylic acid (DNS) was added and boiled in water bath for five min to stop the reaction. The sample was then cooled to room temperature and its absorbance was measured at 530 nm wavelength. One unit of β-glucosidase activity was defined as the amount of enzyme that could hydrolyze cellobiose and released 1 µmol of glucose per minute.

Morphological and biochemical characterization of cellulolytic bacteria

Colony characterization was carried out, included gram staining and endospore function, and macroscopic observations of shape, form, elevation, margin, color, inside the structure. Biochemistry test using Microbact 12E method.

Identification of bacteria using 16S rDNA

The isolation of chromosomal DNA used a commercial kit. Furthermore, the 16S rDNA gene amplification process was carried out using PCR. The total volume in the PCR was 25 µL, containing PCR reaction mixture (DNA polymerase, dNTPs, reaction buffer, gel loading buffer), DNA template 2 µL, primer F (forward) and R (reverse) each 1 µL (10 pmol/mL), and 16 µL of distilled water. Primers used were 28F (5`-AGA GTT TGA TCA TGG CTC AG-3`), 651F (5`-AAT TAC TGG GCG TAA AG-3`) and 1495R (3`-TAC GGC TAC CTT GTT ACG A-5`) with PCR parameter: 94 °C pre-separation temperature for 4 min, 94 °C separation temperature for 45 sec, 52 °C annealing temperature for 45 min and of 72 °C polymerization temperature for two minutes over 35 cycles. The results obtained were then visualized by 1% agarose gel electrophoresis. PCR products were sequenced before then being purified using the PCR Clean-Up System. Determination of the 16S rDNA gene sequences was calculated using Sanger dideoxy dye terminator in Singapore. Results of sequences done contig with Bioedit software. Sequences were analyzed with the results contig BlastN program (Basic Local Alignment and Search Tool for Nucleotides) (www.ncbi.nlm.nih.gov) to determine the similarity with other sequences in GenBank and the phylogenetic tree created with MEGA 6.

Data analysis

Data obtained from the study were repeated three times and analyzed using an analysis of variance (ANOVA) test with 0.5% confidence interval to examine the effect of treatment. If there was an influence, the analysis was then followed by a Tukey test with 0.5% confidence interval to discover which treatment would be influential or significantly different from other treatments.

RESULT AND DISCUSSION

Isolation and Screening for Cellulolytic Bacteria

This study obtained 22 bacterial isolates that were indigenous in rice bran with the ability to produce the cellulolytic enzymes when they were growing on CYPE agar plates. The capability of producing cellulase was demonstrated by the cellulolytic index obtained from the ratio/comparison between the diameter of the clear zone and the diameter of cell colonies using the *Congo red* method at cell concentrations (Optical Density = 0.4) or cell number 0.62 x 10^7 cfu.

There were six bacterial isolates with the highest cellulase ability (cellulolytic index of over 0.5): BE 5, BE 8, BE 14, B 3, B 5 and BT 2 (Fig. 1). Qualitatively, Isolate BE 5 had the highest cellulolytic index of all the isolates, and produced an 11.4 mm clear zone and a cellulolytic index is 1.78.

The results are similar to research which reported that cellulolytic from rice bran featured 12 mm clear zones [18]. Cellulolytic bacterial Isolates from soil produced 43 mm clear zone [19]. Clear zone resulting from this research were smaller in size, possibly caused by the different sources of rice bran and the amount of inoculum.

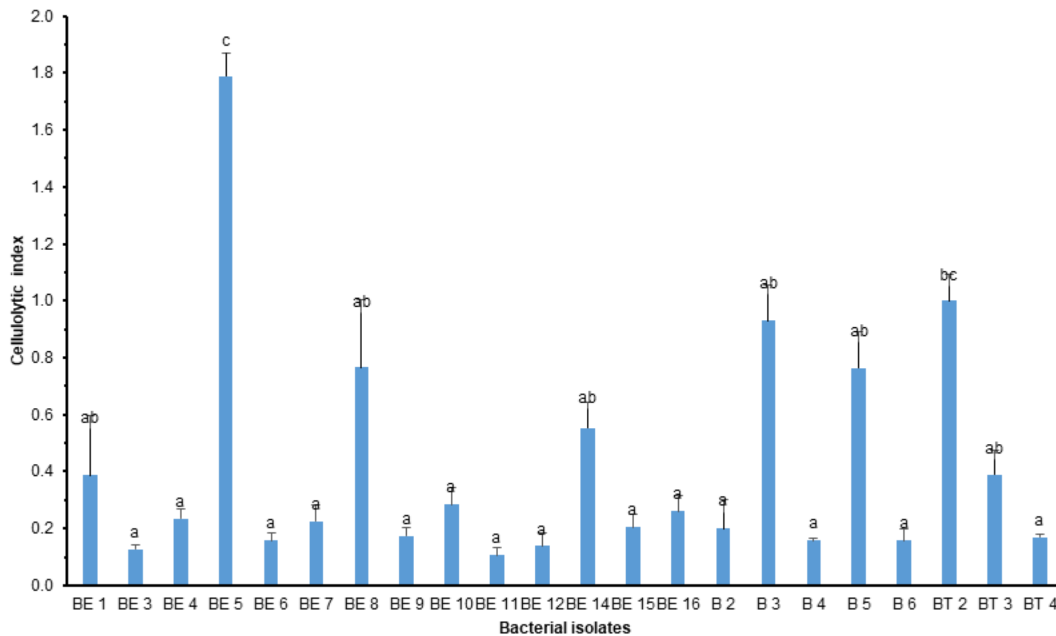

Fig 1. Cellulolytic index of bacterial isolates from rice bran

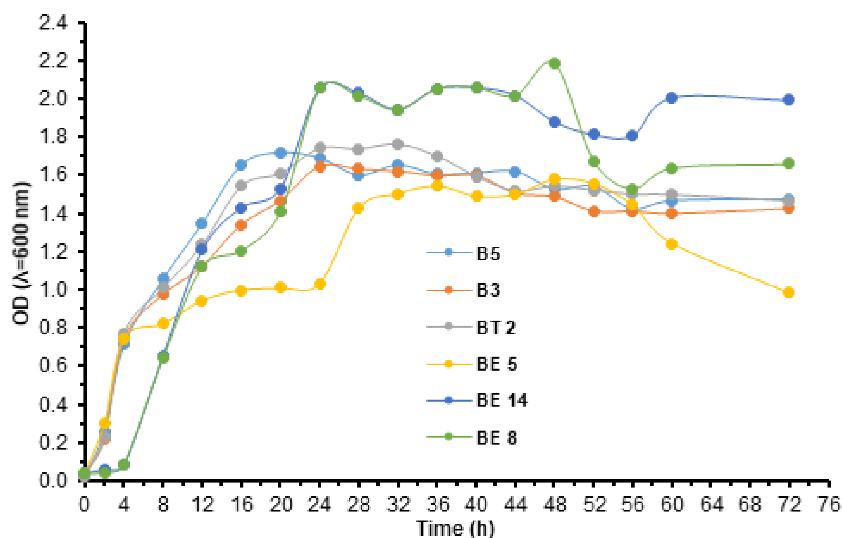

Fig 2. Growth curve of selected bacterial isolate

Growth Curve of Selected Bacterial Isolates

Analysis of the growth curve of each isolate was performed before testing cellulolytic activity, to determine the fast growth/ exponential phase. The most optimal production of endoglucanase occurred in the last phase of exponential growth. The observations were carried out from 0 to 72 h. Growth curves were plotted as absorbance vs time.

Endoglucanase production can be seen in the growth curve of cellulolytic bacteria. The increased of enzyme production was associated with the increased of cell growth, which indicated that cellulose was actively used by cellulolytic bacteria during the growth phase [20].

Based on Fig. 2, the following features can be seen: the adaptation phase occurred between 0 and 4 h and the exponential phase between 4 and 24 h. The stationary phase was from 24–48 h for isolate BE 8, 24–44 h for isolate BE 14, 24–72 h for BT 2, and 24–56 h for isolate BE 5, B 3 and B5. From these results it can be concluded that the best production of cellulase enzyme for all isolates is at 24 h.

The result of this study are similar to those of other researchers, in that the exponential phase of BY-2 *Bacillus subtilis* took 0–20 h, the stationary phase took

Table 1. Cellulase enzyme activity of indigenous isolate of rice bran

Isolates	Activity (U/mL)		
	endo-β-1,4-glucanase	exo-β-1,4-glucanase	β-glucosidase
BE 5	0.71^{a}	0.61^{a}	164.15^{c}
BE 8	2.16^{c}	0.64^{a}	163.66^{c}
BE 14	1.31^{b}	0.62^{a}	164.64^{c}
B 3	0.73^{a}	0.61^{a}	134.28^{a}
B 5	0.73^{a}	0.64^{a}	129.41^{a}
BT 2	0.72^{a}	0.61^{a}	139.74^{b}

Table 2. Morphological characterization of the colony from indigenous cellulolytic bacteria of rice bran

Test	Isolate	
	BE 8	BE 14
Gram	+	+
Shape	Bacill	Bacill
Endospore	+	+
Form	circular	Irregular
Elevation	raised	Undulate
Margin	entire	Entire
Color	beige	Yellow
Inside structure	opaque	transparent

20–39 h, and fermentation for the production of cellulase enzymes took 24 h [21]. The production of cellulase enzymes was conducted for 24 h [22]. The exponential phase of *Bacillus licheniformis* JK7 took place over 6–16 h [20]. Cell growth decline in the stationary phase, caused by the metabolites repression after hydrolysis into glucose and cellobiose. Cellulase enzymes used for further analysis were produced for 24 h with 0.12×10^{7} (OD = 0.4) cells and activity was tested based on the ability to hydrolyze a CMC substrate (endo-β-1,4-glucanase), avicel substrate (exo-β-1,4-glucanase) and cellobiose substrate (β-glucosidase).

Enzyme Activity Assay

The sixth isolates were later quantitatively tested for their ability to produce cellulase enzymes including, endo-β-1,4-glucanase, exo-β-1,4-glucanase, and β-glucosidase abilities.

The ability of an isolate to hydrolyze a CMC substrate or called endo-β-1,4-glucanase. The highest endoglucanase activity of BE 8 isolate was 2.16 U/mL. In term of the ability to hydrolyze avicel or exo-β-1,4-glucanase activity, on average all isolates had the same ability (no significant difference) but with lower activity i.e., 0.6 U/mL on average. β-glucosidase activity i.e. the ability to hydrolyze cellobiose, had the highest value among endo-β-1,4-glucanase and exo-β-1,4-glucanase activities. This is because the cellulase enzyme was easier to hydrolyze in the substrate with low molecular weight or shorter polymer.

The cellobiose substrate has a molecular structure that consists of 2 glucose monomers. Avicel is more crystalline than CMC, meaning that is was more difficult for cellulose enzyme to hydrolyze avicel CMC. This could be identified from the avicelase activity was lower than for CMCase. Among the factors that affect the rate of enzymatic hydrolysis, cellulose crystallinity was considered the most important.

Gundllapalli et al. [23] state that in the decrease of crystallinity, cellulose becomes more easily enzymatically hydrolyzed. The crystalline structure in cellulose substrate is due to the presence of inter and intramolecular hydrogen bonds. Based on the cellulase activity analysis, two isolates with the highest activity that was BE 8 and BE 14 isolate, and these were therefore then observed for bacterial colony morphology, biochemical test, and molecular identification.

Morphological and Biochemical Characterization of Cellulolytic Bacteria

The two isolates potentially identified as cellulolytic bacteria BE 8 and BE 14, were the cells characterized morphologically and biochemically using Microbact method 12E.

The resulting from Table 2, observations of colony morphological colony for both isolates indicated many similarities, in that they were gram positive, rod-shaped, and endosporic. The difference of both between the isolates lay in form, elevation, margin, color and inside the structure.

Based on the biochemical test (Table 3), it can be seen that isolate BE 8 was able to hydrolyze xylose, mannitol, and arabinose, while isolate BE 14 was able to hydrolyze glucose and maltose. The maximum growth temperature of isolate BE 8 at 45 °C, meanwhile isolate BE 14 could grow in temperature to 50 °C. Isolate BE 14 could not grow on 7% NaCl and both isolates were unable to reduce methylene blue. Based on the morphological criteria and the biochemical test indicated on Bergey`s manual it was demonstrated that isolates BE 8 and BE 14 were the same genus of *Bacillus*.

Molecular Identification of Cellulolytic Bacteria

Of 22 cellulolytic bacterial isolates obtained from rice bran, those selected with highest cellulase activity were BE 8 and BE 14. Molecular identification of cellulolytic bacterial isolates used 16S rDNA gene sequences because in this gene it was more stable and ubiquity toward bacteria that it was appropriate to analyze it at the molecular level [24]. The following are

Table 3. Biochemical characterization of indigenous cellulolytic bacteria of rice bran

Test	Isolate	
	BE8	BE14
Fermentation of sugars		
Glucose	-	+
Xylose	+	-
Mannitol	+	-
Lactose	-	-
Sucrose	-	-
Maltose	-	+
Arabinose	+	-
Temperature of growth		
25 °C	+	+
37 °C	+	+
40 °C	+	+
45 °C	+	-
55 °C	-	+
Growth at		
Nutrient broth	+	+
MCA	-	-
TSI	A/A,H2S-	A/A,H2S-
Citrate	-	-
INDOL	-	-
VP	-	-
NaCl 7%	+	-
Motilities	+	+
Starch hydrolysis	+	+
Casein hydrolysis	+	+
Penicillin	Sensitive	Sensitive
Beta-Hemolysis	+	+
Catalase	+	+
Oxidase	+	+
Reduction of Nitrate	+	+
Reduction of Methylene Blue	-	-

the result of phylogenetic analysis using Maximum Likelihood method (Fig. 3).

BE 8 Isolate was *Bacillus subtilis* with 99.9% similarity to the *Bacillus subtilis* 168 strain (Accession Nr 102783), *Bacillus subtilis* J5 (Accession CP018295.1) with 100% similarity and *Bacillus subtilis* ZJ2 (Accession KY121111.1) with 100% similarity. BE 14 isolate was 94.9% similarity of *Bacillus cereus* Se05 strain (Accession JN700108.1), *Bacillus cereus* strain 14759 (Accession NR 0745401), *Bacillus cereus* strain IIF4SW B1 (Accession KY218861.1), *Bacillus cereus* strain LC19 (Accession KY534425.1), *Bacillus cereus* strain MER 27 (Accession KT719607.1), strain *Bacillus cereus* XH05 (Accession KU986647) and strain *Bacillus cereus* Y3 (accession GQ462534).

This result was also similar to some studies of rice bran isolate i.e., genus *Bacillus* [18,25]. Most Bacillus groups had the ability to produce cellulase enzyme, such as *Bacillus subtilis* [26-27], *Bacillus licheniformis* [6],

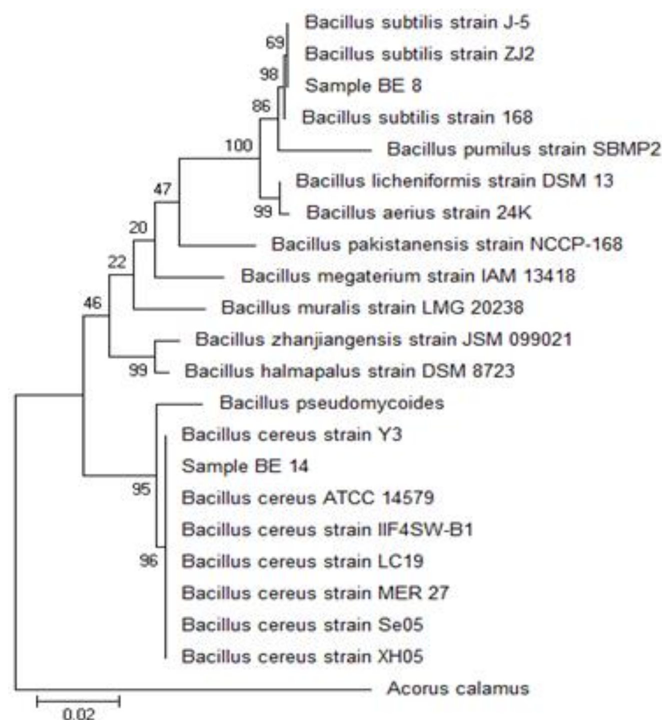

Fig 3. Molecular Phylogenetic analysis using Maximum Likelihood method

Bacillus circulans [18], *Bacillus cereus* [28], *Bacillus pumilus* [18], *Bacillus brevis* [29], *Bacillus firmus* [30], etc. *Bacillus* group is used extensively for industrial applications because of its ease of production and because it produces more stable extracellular enzyme [31].

There were two isolates potentially as cellulolytic bacteria -BE 8 and BE 14. The highest CMCase activity was performed by the BE 8 isolate .at 2.16 U/mL and BE 14 at. 1.31 U/mL. Avicelase activity was low for all isolates, while β-glucosidase activity was the highest. Based on molecular identification method, it appears that the BE 8 isolate was *Bacillus subtilis* and BE 14 isolate was *Bacillus cereus*.

CONCLUSION

There are two isolates potentially as cellulolytic bacteria -BE 8 and BE 14. The highest CMCase activity is performed by BE 8 isolate, at 2.16 U/mL. Based on molecular identification method, it has been shown that BE 8 isolate is *Bacillus subtilis* and BE 14 isolate is *Bacillus cereus*.

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