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Bacterial Response after Exposure with Pure Metabolite Produced by *Streptomyces* sp. BL225 Isolated from Batanta Island's Leaf Litter

ARIF NURKANTO[1]*, ANDRIA AGUSTA[1], WELLYZAR SJAMSURIDZAL[2], AND HEDDY JULISTIONO[1]

[1]*Research Center for Biology, Indonesia Institute of Sciences (LIPI), Jalan Raya Bogor Km 46, Cibinong 16911, West Java, Indonesia;*
[2]*Department of Biology, Faculty Mathematics and Natural Science, Universitas Indonesia, Depok 16424, Indonesia*

The objective of this research was to investigate bacterial response after treatment with active metabolite produced by *Streptomyces* sp. isolated from Batanta Island. Minimum inhibitory concentration (MIC) values of four clinically tested bacteria (*Escherichia coli, Bacillus subtilis, Staphylococcus aureus,* and *Micrococcus luteus*) were successfully determined in this research using microdilution method. Leakages of nucleic acids and proteins from the tested microbes were detected using UV/VIS spectrophotometry method at 260 and 280 nm. Uracil leakage was analyzed using HPLC. Morphological changes of the bacterial cells were observed using scanning electron microscope (SEM). A *Streptomyces* isolate BL225 was identified based on the 16S rRNA gene sequence (1500 bp). When tested agains microbes, the MICs values of this compound were between 16-64 µg mL^{-1}. The results indicated leakages of protein, nucleic acid and uracil from *E. coli* and *B. subtilis* cells after treatment with pure metabolite isolated from BL225. Treatment using metabolite from BL225 also caused morphological changes and damages of the target bacterial cell. BL225 had been identified as a strain that has closed relation to *Streptomyces badius* (98.9%).

Key words : Batanta Island, nucleic acid, protein, SEM, *Streptomyces*, uracil leak

Tujuan penelitian ini adalah untuk mengetahui respon bakteri setelah pemaparan dengan metabolit aktif dari *Streptomyces* sp. yang diisolasi dari Pulau Batanta. Nilai konsentrasi hambat minimum (KHM) terhadap empat isolat klinik (*Escherichia coli, Bacillus subtilis, Staphylococcus aureus,* dan *Micrococcus luteus*) telah berhasil ditentukan dalam penelitian ini dengan menggunakan metode mikrodilusi. Kebocoran asam nukleat dan protein dari bakteri uji dideteksi dengan menggunakan metode spektrofotometri UV/VIS pada panjang gelombang 260 dan 280 nm. Kebocoran urasil dianalisa menggunakan HPLC. Perubahan morfologi sel bakteri uji diamati dengan menggunakan mikroskop elektron payar (SEM). Isolat *Streptomyces* sp. BL225 telah diidentifikasi berdasarkan sekuen gen 16S rRNA (1500 bp). Nilai MIC dari senyawa aktif produksi *Streptomyces* sp. BL225 terhadap bakteri uji berkisar antara 16-64 µg mL^{-1}. Hasil penelitian mengindikasikan adanya kebocoran protein, asam nukleat, dan urasil pada sel bakteri *E. coli* dan *B. subtilis* setelah perlakuan dengan metabolit murni BL225. Perlakuan dengan metabolit BL225 juga menyebabkan perubahan morfologi dan kerusakan pada sel bakteri. Isolat BL225 teridentifikasi sebagai strain yang mempunyai kekerabatan tinggi dengan *Streptomyces badius* (98,9%).

Kata kunci : asam nukleat, kebocoran urasil, Pulau Batanta, protein, SEM, *Streptomyces*

Diseases caused by microbial infection have been increasing progressively in the past decades (Anand *et al.* 2008; WHO 2011). Some of the reasons behind the increasing number of cases of the diseases are the growing number of new pathogenic microbes and the development of resistance in infectious microorganism. The confounding fact is instantly followed by the ever growing need for drugs. However, the number and variety of drugs available in the market are barely adequate for the new emerging diseases and resistant pathogen (Phoebe *et al.* 2001; Donaldio *et al.* 2010; Pozzi *et al.* 2011). Thus, it is crucial to search for potential new antimicrobial compounds to cope with the problems. This research was aimed to obtain general antimicrobial compounds which were focused on natural products. These natural products can be isolated from plants, animals, and microorganisms (Newman *et al.* 2000; Dewick 2002; Newman and Cragg 2004).

Microorganisms, especially actinomycetes, are potential sources of natural products. Actinomycetes are distributed in soil and leaf litter, and produce many important bioactive compounds with commercial values, including antibiotics (Takizawa *et al*. 1993). *Streptomyces* is the largest group of actinomycetes with potential biological activities and antibiotic (Omura *et al*. 2001; Bentley *et al*. 2002).

Antimicrobial screening of two hundred organic

*Corresponding author; Phone: +62-21-8765066, Fax: +62-21-8765062, E-mail: arif.nurkanto@lipi.go.id

and water solvent extracts derived from Raja Ampat's actinomycetes showed that 43% isolates had antimicrobial activities against bacteria and yeast (Nurkanto *et al*. 2012). Isolate capable of producing bioactive compound with the highest antibacterial activities was selected and used in this research. The major bioactive compound from actinomycetes BL225, the selected isolate, was isolated from broth fermentation. Pure bioactive compound was used to study bacterial responses of both Gram negative and Gram positive bacteria.

Bacterial responses to exposure towards various antibiotic substances are relatively diverse . One of the factors influencing these responses is the mechanism of action of the antibacterial compounds. Through study of bacterial cell reaction to antibiotic exposure, the mechanism of action of the specific substance against targeted bacteria could be revealed (Kohanski *et al*. 2010). Some of the relatively easily observed cell responses are excreted cell products or components such as protein, nucleic acid, and ions. Changes in cell morphology are also important parameter in studying cell response (Harold and Thomas 1996).

The objectives of this research are to determinate MIC value on some clinical bacterial and to study the mechanism of the action of newly discovered antibacterial compound. The mechanism of action was studied by observing protein and nucleic acid leakages and their effects to cell morphology. Taxonomical status of the isolated actinomycetes was also studied in this research.

MATERIAL AND METHODS

Sources of Isolate. Actinomycetes used as metabolite sources were isolated from litter sample in Batanta Island, Raja Ampat, West Papua using SDS-YE method (Hayakawa and Nanomora 1987). We had previously isolated more than one hundred actinomycetes (unpublished result). Our previous study focusing on the screening of antimicrobial activity from metabolites produced by these isolates (Nurkanto *et al*. 2012) had demonstrated that isolate BL225 had the highest antimicrobial activity, and thus selected to produce active compound.

Production of Active Substance by Fermentation. Actinomycetes isolate BL225 was inoculated into a 5000 mL flask containing 2000 mL of Actino Medium No. 1 (Daigo, Japan) (with composition per liter: 5 g polypeptone, 3 g yeast extract, at pH 7.2). The flask was incubated at 28 °C for 7 d in shaker incubator.

Extraction and Purification Bioactive Compound. Broth culture was extracted using ethyl acetate and methanol (4:1) solvent. These organic solvents were mixed thoroughly by shaking and left to stand for 1 h. The two layers, the organic and water layers, were separated. The organic layer was concentrated by evaporation under vacuum. Dry extract of supernatant and biomass were purified using column chromatography (Merck, 60-120 mesh). Dichloromethane and methanol 20:1 (v/v) mixture was used as eluting solvent. Two milliliters of crude extract was added on silica gel column surface and the extract was adsorbed on top of the silica gel. Eight fractions were collected and tested for their antimicrobial activities (Usha *et al*. 2010). The purification of the active fraction from the column was confirmed using TLC GF_{254} (Merck) and HPLC (Shimadzu RF-10AXL). Column Ascentis ® C18 (Supelco, USA) 5 µm 4.6 x 150 mm for HPLC was used in this reserach. The volume injected was 100 µL per injection under conditions of average pressure of 1.350 psi in 254 nm wavelength, and the flow rate was 1 mL min^{-1} where the mobile phase was methanol : water (8:2) and time period was 25 min.

Determination of Minimum Inhibitory Concentration (MIC). Calculation of MIC value from pure extract produced by *Streptomyces* sp. BL225 against four bacterial srain was performed by Micro dilution method (Rahman *et al.* 2005). The pure extract was tested against *Escherichia coli* (LIPIMC 186 = NBRC 3301), *Bacillus subtilis* (LIPIMC 187 = NBRC 3134 = ATCC 6633), *Micrococcus luteus* (LIPIMC 76=NBRC 13867=ATCC 10240), and *Staphylococcus aureus* (LIPIMC 114 = NBRC 12732 = ATCC 6538P = DSM 1790).

Compounds (BL225 metabolite and chloramphenicol) were diluted in 50% DMSO and filtered with 0.22 µm cellulose membrane. Compounds were prepared one step higher or two times concentration than the final dilution range required, to compensate for the additional of an equal volume of inoculum. After added with microbial inoculum, the compounds' final concentrations were 0.5, 1, 2, 4, 8, 16, 32, 64, and 128 µg mL^{-1}. Bacterial test suspensions (1-5 x 10^7 cell mL^{-1}) were inoculated into microplate before incubation at 37 °C for 12 h. After the incubation period had elapsed microbes should have grown in all wells of the antibiotic free control plate. MIC were defined as the lowest concentration of compound at which there is no visible growth of the organism.

Cell Leakage Test. Cell leakage test was performed on two bacterial species, *E. coli* and *B. subtilis*, as representation of Gram negative and Gram positive bacteria. Methods used based on Miksusanti *et*

al. (2008) with some modification. Cell leakage test was conducted using several approaches, analyses of protein and nucleic acid leakages, uracil leakage, and cell morphology observation using Scanning Electron Microscope. These analyses were performed simultaneously. The first step in cell leakaege test is preparation of experimental microoraganisms. Microbes were cultivated in Mueller Hinton media (Difco) for 24 h at 37 °C with shaking. 0.5 mL of 0.1% tween 80 was added to about 10 mL cell suspension, and then mixed. The mixture was then centrifuged at 3500 rpm speed for 20 min in cold temperature (4 °C). Supernatant was discarded and the pellet was washed twice with phosphate buffer (pH 7). Pure extract was then added at concentrations 1MIC and 2MIC to the cell suspension in phosphate buffer. Only filtered 50% DMSO solution (without compound) was added to the negative control. Chloramphenicol solution was used as comparison control. Samples were then incubated in shaker incubator for 24 h at 37 °C. The suspension was centrifuged for 15 min. Afterward, supernatant and pellet were separated. This was then followed by analyses of protein, nucleic acid, and uracil leakages. Cell pellet was observed using SEM.

Protein and nucleic acid leakages were analysed by measuring absorbance using UV/VIS spectrophotometer at 260 and 280 nm wavelength. Uracil leakage was detected using HPLC. HPLC (Shimadzu, Japan) analyses were performed using acetonitrile: aquabidest (1:9) as solvent. One mL samples was injected. The column used was puresil 5m C18 4.6 x 150 mm. The flow rate used was 1 mL min^{-1} and column pressure was 1350 psi with 254 nm wavelength.

SEM Preparation. Bacteria was treated accordingly with BL225 compound at sub- and supra-MICs (1 MIC and 2 MIC) for 12 h at 37 °C in phosphate buffer. Untreated controls were also prepared in this experiment. After being fixed for 1 h with 2% glutaraldehydeand washed with cocodylate buffer pH 7.2, the bacteria was then post-fixed with 1% OsO_4. Samples were dehydrated with graded ethanol series (30, 50, 70, 80, 90, and 100%), with 10 min incubation after treatment with each ethanol concentration. Bacterial cells were observed using SEM (JOEL, JSM-5310LV). Electron images were taken at low electron energies (20 kV).

Actinomycetes identification. Actinomycete isolate was identified based on its 16S rRNA gene sequence. The isolate was cultured in 5 mL yeast starch broth medium (yeast extracts 2 g L^{-1}, soluble starch 15 g L^{-1} pH 7.2) (Miyadoh 2001). Pellet was collected during log phase growth. Chromosomal DNA was isolated using Pitcher *et al.* (1989) method. Twenty to 100 ng genomic DNA was used as a templete for polymerase chain reaction (PCR) amplification of an approximately 1500 base segment. The PCR primers used were 20F (5'-GATTTTGATCCTGGCTCAG-3') and 1500R (5'-GTTACCTTGTTACGACTT-3') (Suriyachadkun *et al*. 2010). PCR product was purified using Hiraishi *et al*. (1995) method. The purified PCR product was sequenced using an ABI 3130 genetic analyzer with BigDye terminator version 3.1 sequencing method. For completed sequence result, we used 6 primers. The sequence of the primers were 520F (5'-GTGCCAGCAGCCGCGG-3'), 920R (5'-CCGTCAATTCATTTGAGTTT-3'), 520R (5'-ACCGCGGCTGCTGGC-3'), 920F (5'-AAACTCAAATGAATTGACGG-3'), 20F (5'-GATTTTGATCCTGGCTCAG-3') and 1500R (5'-GTTACCTTGTTACGACTT-3') (Yukphan *et al*. 2004; Suriyachadkun *et al*. 2010; Techaoei *et al*. 2011). Full sequence analysis was conducted using reference strains from the Ribosomal Database project-II (http://www.rdp.cme.msu.edu), which were chosen based on high similarity rank with the strains in this study. Approximately 1400 bases were included in the phylogenetic analysis, which was performed with the Clustal X version 1.83 and NJ Plot computer program (Thomson *et al*. 1997; Felsenstein 1985).

RESULTS

Pure compound derived from *Streptomyces* sp. BL225 was investigated and checked (Fig 1) and was used in our experiments. The compound's structure is still currently being investigated using LCMS and NMR. The chemical structure and elucidation study of this active compound will be published separately. In this article, we focused on the evaluation of the pure compound to determine MICs and cell leakages.

Examination of Minimum Inhibitor Concentration value. MIC values of BL 225 pure extract differed from one tested microbe to another (between 16-64 µg mL^{-1}). Compared to chloramphenicol, MIC values of the extract were relatively higher (Table 1).

Nucleic Acid and Protein Leak Analyses. Generally, the microbes treated with the pure extract showed considerable changes. The level of nucleic acid leakage of the tested microbes increased after treatment with 1 and 2 MIC. This level of leakage was higher compared to the negative and positive controls. The pattern of protein leakage level was similar to the

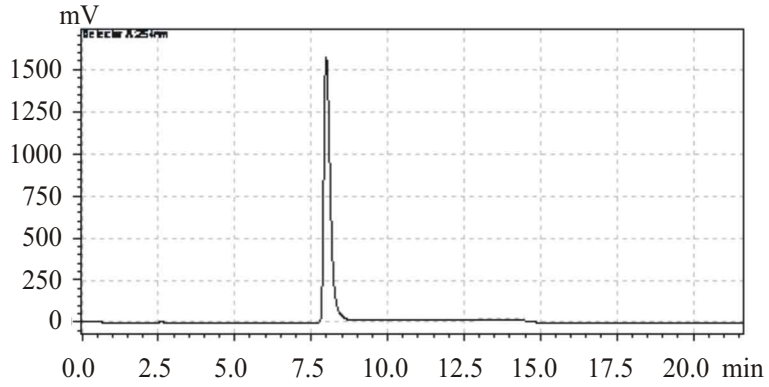

Fig 1 Pure active compound produced by BL225 isolate after purification by column chromatography. This compound was detected by HPLC using methanol: water (8:2) as mobile phase and time period was 25 min.

Table 1 MIC values of pure metabolite produced by BL225 isolate against some bacteria, incubation temperature 37 °C for 12 h

Bacterial tested	Metabolite (µg mL^{-1})	Chloramphenicol (µg mL^{-1})
E. coli	64	16
M. luteus	64	16
S. aureus	16	16
B. subtillis	16	8

one of nucleic acid leakage. Treatment with pure extract produced higher protein leakage than the negative control, but still lower than the positive control (chloramphenicol treated cell) (Fig 2 and 3).

The leakage profiles of *E. coli* and *B. subtilis* showed similar pattern although the values were different. In *E. coli,* the nucleic acid leakage was highest at 2 MIC, which was 38.01 µg mL^{-1}. The negative control showed the lowest nucleic acid leakage with 16.56 µg mL^{-1}. Chloramphenicol treated cells showed the highest protein leakage with 995.85 µg mL^{-1}. The lowest protein leakage was observed in the negative control with 278.14 µg mL^{-1}. *B. subtilis* showed the highest nucleic acid leakage level at 2 MIC with 10.08 µg mL^{-1} and the lowest in the negative control cell with 3.92 µg mL^{-1}. The highest protein leakage was in observed in chloramphenicol treated cell with 282.16 µg mL^{-1}, and the lowest was in negative control cell with 97.44 µg mL^{-1}.

Analyses of Uracil Leakage. To observe uracil leakage, HPLC grade uracil standard (Sigma) was used at concentration 0.31-5 ppm (Fig 4). Uracil standard was detected at retention time about 2.7 mins. HPLC analyses indicated several peaks with different retention time. Uracil leakage was detected on every treatment (control cell, treatment with 1 and 2 MIC, and treatment with cholramphenicol) (Fig 5 and 6). Generally, all treatments of *E. coli* and *B. subtilis* increased uracil leakage relative to the negative control. Uracil leakage in *B. subtilis* when treated with extract at 1 MIC and 2 MIC, and chloramphenicol were 2.274 ppm, 2.784 ppm, and 3.152 ppm respectively (Table 2). Those values were higher compared to the negative control, which showed uracil leakage of only 1.483 ppm. Similar result occurred in *E. coli*, where uracil leakage was higher in treated cell than in cell without treatment (negative control cell). In *E. coli* uracil leakage in bacterial cells treated with 1 and 2 MIC, and with chloramphenicol were 8.169, 17.784, and 18.158 ppm respectively (Table 2). Uracil leakage in *B. subtilis* negative control was 0.092 ppm.

Cell Morphology Observation using SEM. SEM observation on *E. coli* showed diverse cells' morphology (Fig 7). The negative control cells seemed to be all intact. The positive control cells showed significant changes in morphology. The cells were clearly damaged and rounder in form. Treatment with 1 MIC and 2 MIC extracts also changed the cells' morphology. A big hole was observed in each cell (Fig 7C and 7D). Treatment with 2 MIC had apparently damaged the cells causing the cells' death.

B. subtilis negative control showed intact cells with rod shape (Fig 8). There were no changes observed in the cell morphology. Treatment with chloramphenicol, as positive control, did not seem to disrupt the cells or change the cells' morphology. However, treatment with

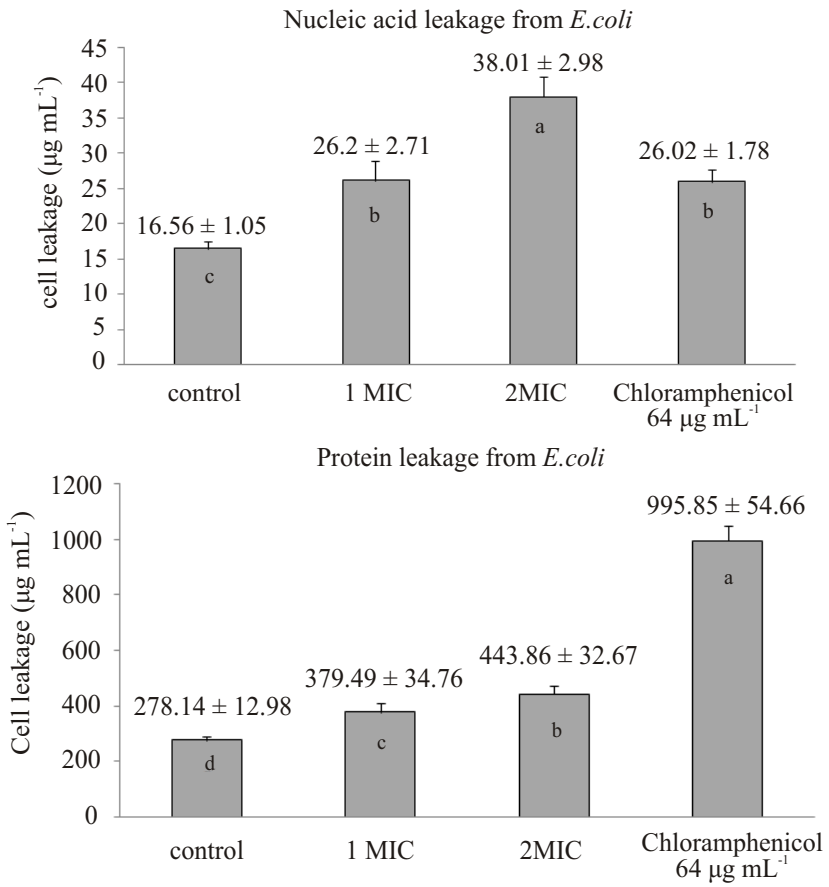

Fig 2 The leakages of nucleic acid and protein (µg mL^{-1}) in *E. coli* after treatment with metabolite from BL225 isolate, incubated at 37 °C for 12 h. Control: without compound. Values represent the mean ± standard deviation (SD) of three measurements. a,b,c,d: Means within a selected graph are significantly difference based on LSD-test at p ≤0.05.

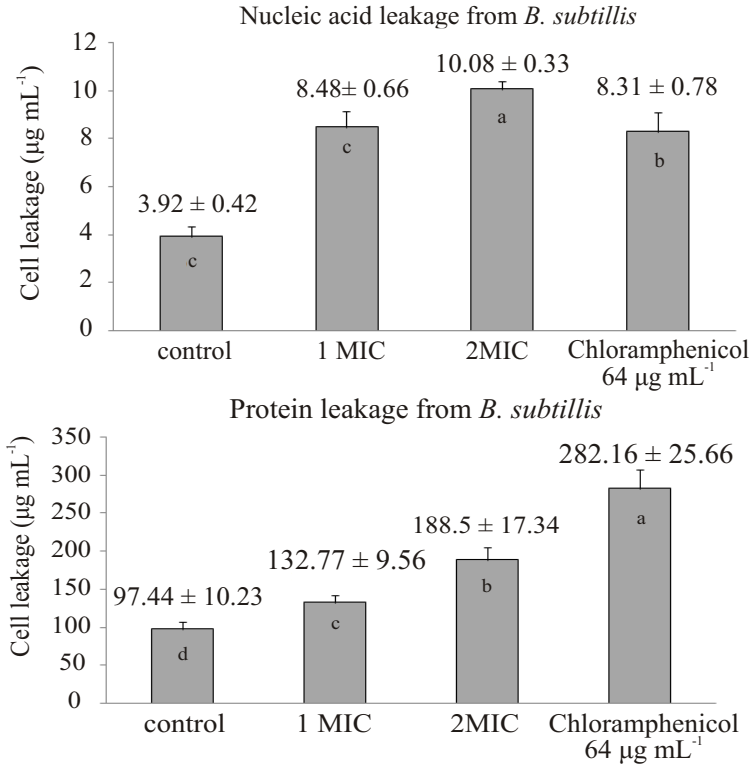

Fig 3 The leakages of nucleic acid and protein (µg mL^{-1}) in *B. subtilis* after treatment with metabolite from BL225 isolate, incubated at 37 °C for 12 h. Control : without compound. Values represent the mean ± standard deviation (SD) of three measurements. a,b,c,d: Means within a selected graph are significantly difference based on LSD-test at p ≤0.05.

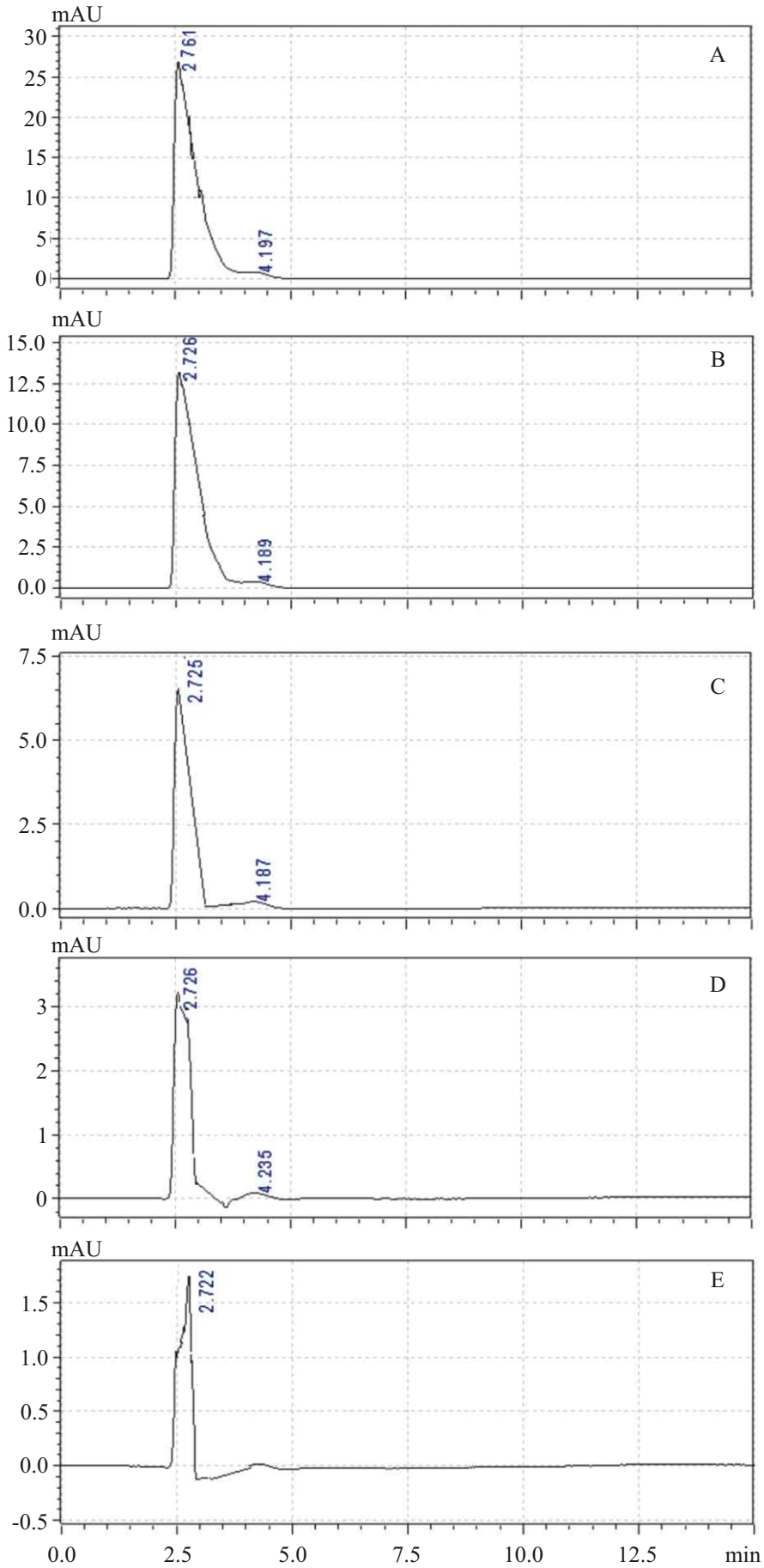

Fig 4 Uracil standard 5 ppm (A), 2.5 ppm (B), 1.25 ppm (C), 0.63 (D), and 0.31 ppm (E) on HPLC analysis.

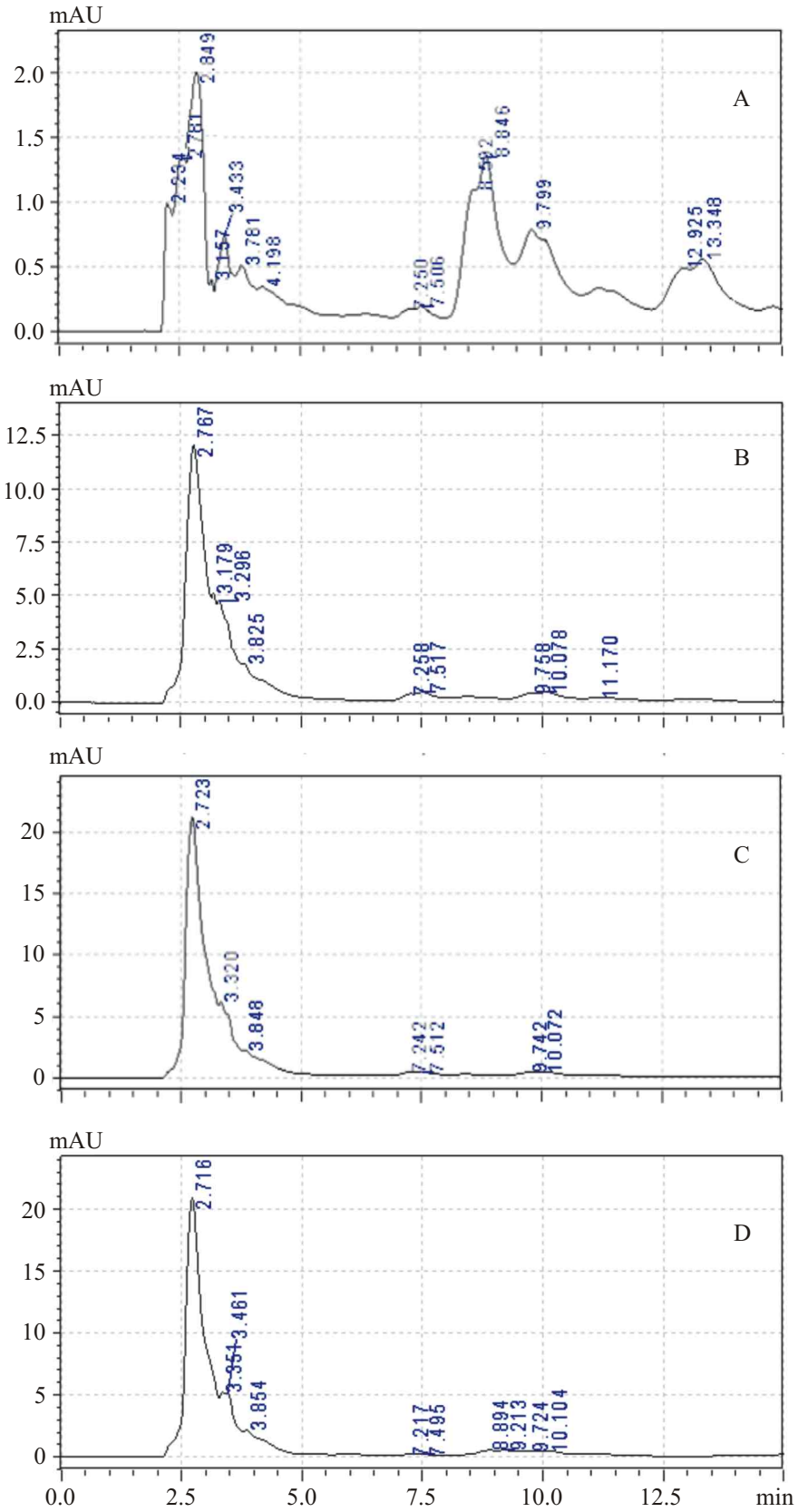

Fig 5 HPLC analyses of uracil leakage on *E. coli* after treatment with metabolite from BL225 isolate. A: cell control; B: 1 MIC; C: 2 MIC; and D: chloramphenicol.

pure extract at 1 and 2 MIC had caused morphological changes, indicating cell damage. There seemed to be some holes formed in the cell wall (Fig 8C and 8D).

Taxonomical Status of Actinomycetes Bl225. BL225 isolate was identified based on 16S rRNA gene sequence. After contig assembly from sequences

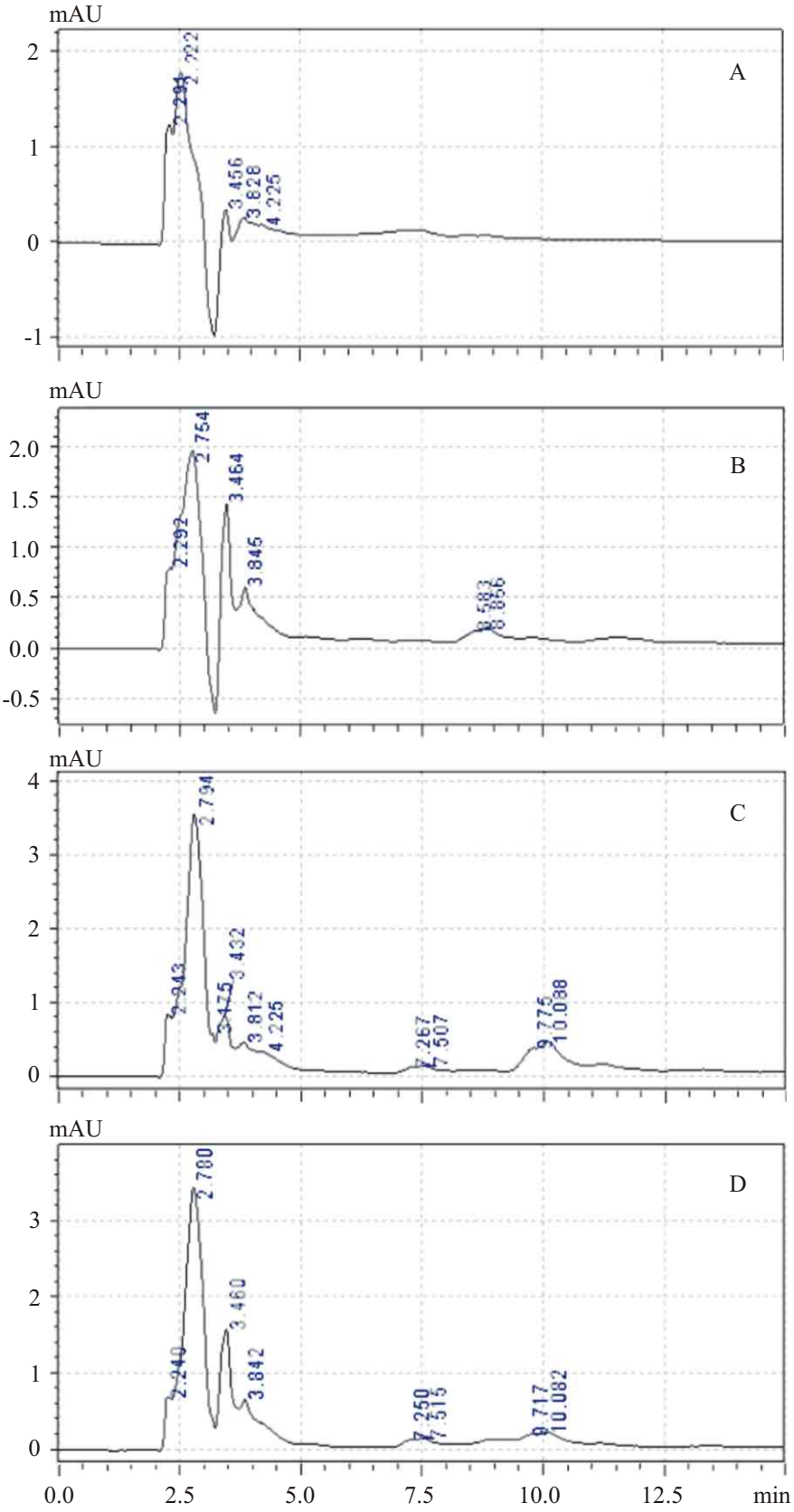

Fig 6 HPLC analyses of uracil leakage on *B. subtilis* after treatment with metabolite from BL225 isolate. A: cell control; B: 1MIC; C : 2MIC; and D : chloramphenicol.

derived from 6 primers, we obtained 1391 bases of nucleotide for complete sequence analysis of this gene. BLAST result showed that BL225 had high similarity to *S. badius* (T) NRRL B-2567 and *Streptomyces parvus* (T) NRRL B-1455T with 98.9 % and 98.7 % homology, respectively. Phylogenetic analysis (Fig 9)

Table 2 Uracil concentration in medium after treatment, incubated at 37 °C for 12 h.

Microbes	Treatment	Uracil concentration (ppm)
E. coli	Control	0.092 ± 0.002^{d}
	1 MIC	8.169 ± 0.026^{c}
	2 MIC	17.784 ± 0.054^{b}
	Chloramphenicol	18.158 ± 0.055^{a}
B. subtilis	Control	1.483 ± 0.006^{c}
	1 MIC	2.274 ± 0.009^{b}
	2 MIC	2.784 ± 0.100^{b}
	Chloramphenicol	3.152 ± 0.011^{a}

Control: without compound; a,b,c,d: values are significantly different

demonstrated that BL225 was monophyletic with *S. badius* and *S. parvus* with bootstrap value more than 900. The 16S rRNA sequence was submitted in NCBI gene bank with accession number KF146316. The isolate was also deposited in Indoesia Culture Collection (InaCC) at Research Center for Biology, LIPI, Indonesia.

DISCUSSION

The MIC values were used for standard activity of the active compound. MICs are defined as the lowest concentration of an antimicrobial agent that can inhibit the visible growth of a microorganism after overnight incubation. Higher the MIC value indicates weaker

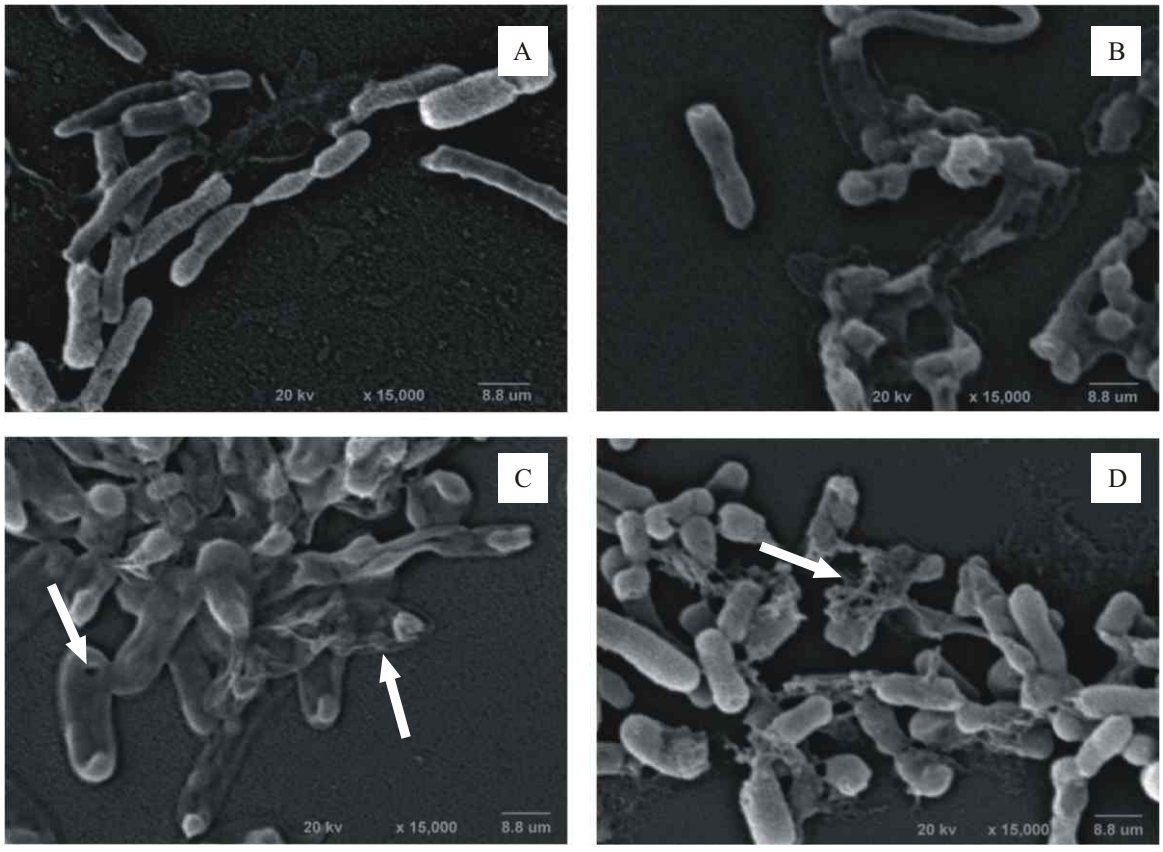

Fig 7 Morphology of *E. coli* using SEM observation (A: control; B: Cloramphenicol; C: 1 MIC; D: 2 MIC), incubated at 37 °C for 12 h. Holes are indicated by white arrows.

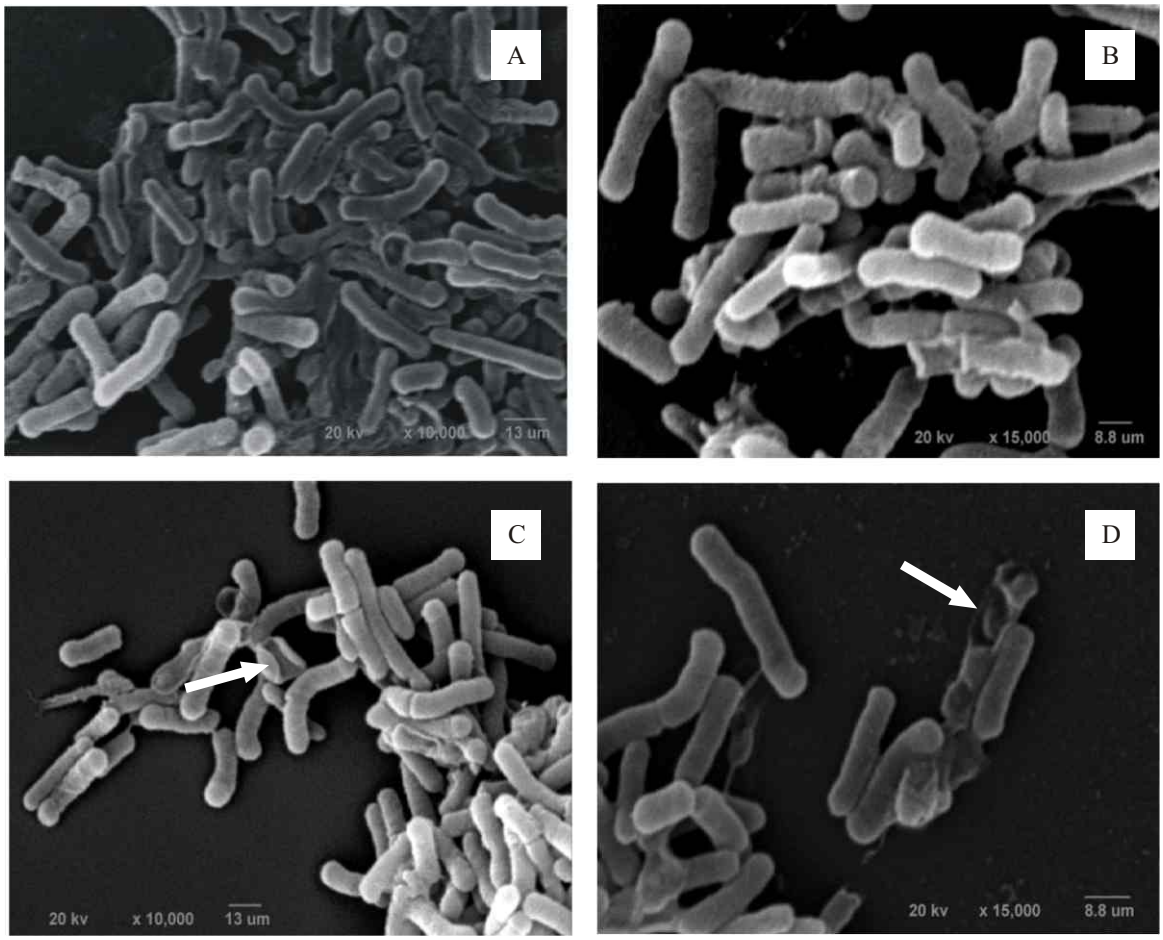

Fig 8 Morphology of *B. subtilis* using SEM observation (A: control; B: Cloramphenicol; C:1 MIC; D : 2 MIC), incubated at 37 °C for 12 h. Holes are indicated by white arrows.

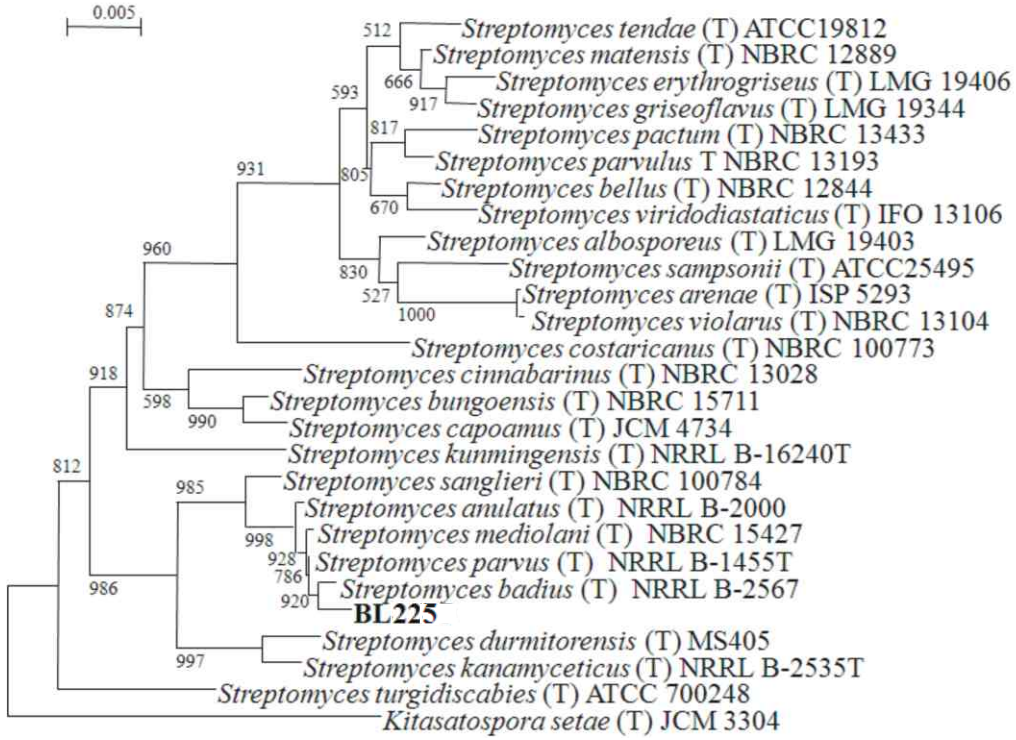

Fig 9 Phylogenetic tree analysis of BL225 based on 16S rDNA sequence and relationship to other *Streptomyces* constructed by NJ plot software with 1000 times bootstrap. The scale bar, the mean number of nucleotide substitutions per site.

antimicrobial activity, whereas lower MIC value indicates stronger antimicrobial activity.

The presence of proteins in the media indicated cell damage. Pure extract at 1 and 2 MIC, as well as chloramphenicol treatment caused secretion of high concentration of protein. The higher the extracellular concentration, the higher the level of cell damage. Nucleic acid was also detected in the medium. Pure extract-treatedand chloramphenicol-treated (positive control) cells showed higher extracellular concentration of nucleic acid compared to cells without treatment (negative control cells). Nucleic acid extracellular concentration also indicated the level of cell damage. Uracil is part of RNA material. If high amount of uracil were secreted, translation process would be disturbed and thus lead to the failure or inhibition of protein synthesis. Although MIC value of chloramphenicol was relatively lower compared to the pure extract, uracil leakage observed after treatment with the pure extract was higher compared to chloramphenicol treatment. It indicated that the BL225 pure extract was more effective in causing uracil leakage than chloramphenicol. It was widely known that chloramphenicol is a bacteriostatic antibiotic. Chloramphenicol stops bacterial growth by inhibiting protein synthesis. The antibiotic prevents protein chain elongation by inhibiting the peptidyl transferase activity on the bacterial ribosome. Chloramphenicol binds to 23S rRNA of the 50S ribosomal subunit, preventing peptide bond formation (Jardetzky 1963; Wolfe and Hahn 1965; Hahn *et al*. 1955).

Our results showed that cell leakages still occured in untreated cell, both *E. coli* and *B. subtilis.* However, cell leakages in untreated cell was less than treated cell. These phenomenon might be caused by some factors. Cells would be dead after a certain period of time because of nutrient deficiency. In our research, we cultivated cell for 12 h in buffer with no nutrient sources. Some cells might die naturally at this condition. Dead cells could release intracellular material such as protein, nucleic acid, and uracil. These materials were detected in our research.

SEM observation on *E coli* showed changes in morphology of the cell after treatment with the extract. Those cells underwent shrinkage, elongation, and produced protusions on the cell wall. According to Miksusanti (2008), protusion forming was caused by the inability of peptidoglycan to sustain intracellular pressure as the effect of given metabolite compound. Cytoplasm and membrane cell were leaking. Biosynthesis of cell wall also did not occur or disturbed by metabolite compound activities which was given. At 2MIC treatment, some cells were completely disrupted. Cell contents were ruptured. These processes indicated cell death was the result of the extract treatment. This statement is supported by the recorded protein, nucleic acid, and uracil leakage data. Nora *et al*. (2001) and Ronald and Chopra (1986) had previously mentioned that cellular mambrane rupture caused the loss of free amino acid, protein, nucleic acid, uracil, and K^+ ion and release cytoplasmic protein from bacteria.

Our result indicated similar nucleic acid, protein, uracil profiles and morphological changes after treatment. Active compound could attach to cell wall or cell membrane or insert in bacterial cells. Cells metabolism could be disturbed. Cell walls leaked and intracellular material were released. Protein and nucleic acid are intracellular materials, whereas uracil is a component of RNA. The higher the activityof the compound or extract used for treatment, the more intracellular material (nucleic acid, protein and uracil) were released out of the cell. Cellular leakage could be investigated using morphological observation. SEM observation clearly show us morphological differences between treated and untreated cells.

Holes formation in extract treated cells will affect the cells. These holes will disturb cellular metabolism and inhibit or even halt cell proliferation and growth. Our result clearly demonstrated that pure extract produced by isolate BL225 caused protein, nucleic acid, and uracil leakages and disrupted the cell. However, specific mechanisms of this disruption was unclear.

The results of identification and taxonomical studyindicated that BL225 belongs to *S. badius* because of its close homology. The closest species, *S. badius*, have antibiotic and antifungal activities (Debananda *et al*. 2011). However, up to now, commercial drugs derived from this species had never been discovered. Study on *S. badius* was limited only to antagonistic test on microbial pathogens.

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