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Polyphasic Identification of a Thermophilic Bacterium from Geyser of Cisolok, Indonesia

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Abstract. A thermophilic bacterium strain UICC B-76 was isolated from litter in geyser of Cisolok, West Java, Indonesia by using The International Streptomyces Project (ISP1) medium. The strain UICC B-76 was able to hydrolyze various substrates such as casein, cellulose, gelatin, starch, and xylan, at 50 ^{o}C, and decolorize Remazol Brilliant Blue R (RBBR) at 50 ^{o}C, an indicator for ligninolytic degradation ability. These abilities of strain UICC B-76 suggest its potential for industrial applications. The aim of this study was to identify strain UICC B-76 based on phenotypic and genotypic characterizations. Strain UICC B-76 was Gram-positive, spore-forming, aerobic and facultative anaerobic, and rod-shaped with flagella. The strain grew at 30 ^{o}C to 60 ^{o}C (optimum 50 ^{o}C) and pH 7.0 but can not withstand temperature of 70 ^{o}C as well as pH 4.0 and 9.0. Strain UICC B-76 could not grow in more than 1% NaCl. This strain has menaquinone 7 (MK-7) as the major quinone system; cell wall fatty acids consisted of Iso-C15:0 (68.56%), Iso-C17:0 (12.48%), and Anteiso-C15:0 (8.45%); and the main amino acids of the cell wall was meso-diaminopimelic acid (meso-DAP). Phylogenetic analysis based on the full sequence of 16S rRNA gene (1450 nt) indicated that strain UICC B-76 belongs to the genus *Brevibacillus* (Family *Paenibacillaceae*, Order *Bacillales*, Class *Bacilli*, Phylum *Firmicutes*). Strain UICC B-76 was closely related with type strains of *Brevibacillus parabrevis* and those of *B. agri*, however, strain UICC B-76 was separated from both species. The 16S rDNA sequence similarity of strain UICC B-76 showed relatively low sequence homology value, less than 99%, to the type of strains *B. parabrevis* (ranged between 98.13% and 98.20%) and *B. agri* (98.13%). Moreover, the G+C content of strain UICC B-76 (55 mol%) differs with *B. parabrevis* (51.8-52.2 mol%) and *B. agri* (53.5 mol%). This result indicated that strain UICC B-76 may represent a new species of *Brevibacillus*. Hybridization of DNA among strain UICC B-76, *B. parabrevis* and *B. agri* is required to clarify the proposal of a new species.

INTRODUCTION

Due to the growing market and the potential applications of thermostable enzymes, there is an increasing interest in the isolation of new bacterial isolates as enzyme producers with novel properties for industrial applications. Thermophilic microorganisms in Indonesia were poorly explored. Whereas, some regions and hot springs, including geyser in Indonesia, which rarely explored, is likely to yield such isolates. The diversity of thermophilic bacteria in Cisolok geyser was reported by Myung-Ji *et al*. [1], however, there was no information on enzyme-producing thermophilic bacteria isolated from this habitat. We have isolated many bacterial isolates from litters of Cisolok geyser in October 2013 and screened these isolates. We obtained one potential strain, UICC B-76, which was able to hydrolyze various substrates such as casein, cellulose, gelatin, starch, xylan, and to decolorize Remazol Brilliant Blue R (RBBR) as indicator for ligninolytic degradation, at 50 ^{o}C. The ability of strain UICC B-76 to hydrolyze various substrates at 50 ^{o}C suggests its potential for industrial applications. The aim of this research was to obtain

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information on genotypic and phenotypic characters of enzyme-producing thermophilic bacterium from Cisolok geyser, Indonesia.

MATERIALS AND METHODS

Microbial isolate. The sampling was conducted in October 2013. Bacterial isolate LC2-23 was obtained from litter in Cisolok geyser, Pelabuhan Ratu, West Java, Indonesia. The isolate was deposited in the Universitas Indonesia Culture Collection (UICC), Center of Excellence for Indigenous Biological Resources-Genome Studies, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok, Indonesia, and designated as strain UICC B-76.

Growth medium. General morphological, cultural, physiological, and biochemical properties of the bacterial isolate were examined in International Streptomycetes Project (ISP) medium, and determined by the methods employed for taxonomic characterization. Additional nutrient media and characteristic substances were used.

Determination of the effects of pH, temperature, and NaCl on bacterial isolates growth. The strain UICC B-76 was cultivated in ISP 3 medium to determine the effect of pH, temperature and NaCl on bacterial isolates growth. Temperature, pH and NaCl concentration tests were carried out at 20-60 ^{o}C (pH 7.0), pH 4.5-10.5 (50 ^{o}C) and 0-3% NaCl (50 ^{o}C, pH 7.0). Anaerobic growth was observed in Anaerobox using ISP 1 medium [2]. Air was replaced with N_2 gas. Assimilation of various sole carbon sources was assessed using API20NE Kit (Bioméreux). Hydrolysis of cellulose, xylan, chitin, starch, gelatin and casein was evaluated on isolation plates supplemented with each substrate at a concentration of 1% (w/v). Hydrolysis of the substrate was indicated by the presence of a clear zone around the colony. Catalase activity was determined by bubble production in a 3% hydrogen peroxide solution.

Chemotaxonomy characterization. The Guanine and Cytosine (G+C) content of the genomic DNA was determined by HPLC according to Tamaoka and Komagata [3]. Analysis of menaquinone was performed according toYokota *et al*. [4]. Amino acids in the cell wall were determined by TLC according to Harper and Davis [5]. Cell wall sugars were analyzed by TLC according to Hasegawa *et al*. [6]. Analysis of cellular fatty acids of strain UICC B-76 was performed according to the instructions of the Microbial Identification System (Sherlock version 2.1, TSBA40 xxx, MIDI) [7].

Amplification and sequence of 16S rRNA gene. The 16S rRNA gene of strain UICC B-76 was amplified with primers 27F (59-AGAGTTTGATCATGGCTCGA-39; positions 8-27 of the *Escherichia coli* 16S rRNA gene) and 1494 (59-GGCTACCTTGTTACGACTT-39; 1510–1494). The PCR reactions were carried out as follows: initial denaturation at 94 ^{o}C for 2 min followed by 30 cycles at 94 ^{o}C for 1 min, annealing at 52 ^{o}C for 1 min and 72 ^{o}C for 1 min with a final extension cycle of 72 ^{o}C for 2 min. Purified PCR products were sequenced by DNA automated sequencer.

Construction of phylogenetic tree. The 16S rRNA gene sequences of strain UICC B-76 and type strains were compared with those obtained from GenBank (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). *Brevibacillus parabrevis* and *B. agri* were used as type strains. Multiple alignments of the sequences were performed using Clustal X [8]. Phylogenetic trees were constructed using the neighbor-joining [9] with bootstrap values based on 1000 replications [10].

RESULTS AND DISCUSSION

The results from morphological and physiological characterization showed that strain UICC B-76 has creamy to whitish colony, colony elevation was raised, colony texture was butyrous, and margin of colony irregular. This strain was Gram-positive, spore-forming (ellipsoidal endospores, subterminal), aerobic and facultative anaerobic, and rod-shaped with flagella. The strain grew at 30-60 ^{o}C but not at 70 ^{o}C (optimum 50 ^{o}C). *In situ* measurement of pH and temperature of Cisolok geyser showed that the geyser has normal pH of 7 and temperature ranges from 80 ^{o}C to 90 ^{o}C at the water source. Strain UICC B-76 was able to grow at 50-60 ^{o}C, which indicates that it was thermophilic. The ability to form endospores enables this strain to survive at high temperature. According to Brown [11], thermophilic microorganisms could grow above temperature 50 ^{o}C. The strain grew at pH 7.0, but not at pH 4.0 and 9.0. Strain UICC B-76 assimilates various carbon sources, and was positive for D-glucose, D-mannitol, maltose, N-acetyl-glucosamine, potassium gluconate, and malic acid. It was negative for L-arabinose, D-mannose, capric acid, adipatic acid, trisodium citrate, and phenilacetic acid. Strain UICC B-76 could not grow in more than 1% NaCl. The chemotaxonomical characterization showed that the G+C content of this strain was 55 mol%, menaquinone 7 (MK-7) was the major quinone system, cell wall fatty acids consisted of Iso-C15:0 (68.56%), Iso-

C17:0 (12.48%), and Anteiso-C15:0 (8.45%); and the main amino acids of the cell wall was meso-diaminopimelic acid (*meso*-DAP), glycine, lysine, serine, glutamic acid, alanine, and glucosamine.

Nearly-complete 16S rDNA sequence (1450 nt long) was obtained for strain UICC B-76 and compared to 16S rRNA nucleotide sequences in GenBank. Sequence Homology search by BLAST program indicated that strain UICC B-76 belongs to the genus *Brevibacillus* (Family *Paenibacillaceae*, Order *Bacillales*, Class *Bacilli*, Phylum *Firmicutes*). Phylogenetically, strain UICC B-76 was closely related to *B. parabrevis* and *B. agri* among the members of the genus *Brevibacillus*. The morphological, physiological, and chemotaxonomical characters of strain UICC B-76 were also in agreement with the description of the genus *Brevibacillus* as proposed by Shida *et al.* [12]. Members of this genus produce oval endospores in swollen sporangia, grow in an optimum temperature range of 30-48 ^{o}C and contain menaquinone-7 (MK-7) as the major respiratory quinone. They possess anteiso-C15:0 and iso-C15:0 or just iso-C15:0 as the major cellular fatty acid and exhibit DNA G+C contents of 42.8 to 57.4 mol% [12].

Brevibacillus parabrevis was found in a breast abscess, and human blood. *Brevibacillus agri* has been isolated from a pharmaceuticals fermenter plant and its antibiotic raw product, from sterilized milk, a gelatin processing plant, clinical specimens, and a public water supply. Some species of *Brevibacillus* are thermophilic. *Brevibacillus levickii* was isolated from geothermal soil of the northwest slope of Mount Melbourne, a volcano in Antarctica. *Brevibacillus thermoruber* was isolated from mushroom compost [13]. *Brevibacillus agri* can grow at 45 ^{o}C [14].

The 16S rDNA sequence similarity of strain UICC B-76 to the type strains *Brevibacillus parabrevis* ranged between 98.13% and 98.20%, and 98.13%. to *B. agri*. This sequence homology value was relatively low, less than 99%. According to Janda and Abbot [15] strains within the same bacterial species have sequence homology of their 16S rRNA gene ≥ 99%. A phylogenetic tree was constructed by aligning 16S rRNA sequences of 45 type strains taken from GenBank, NCBI and sequence of the strain UICC B-76, and *Bacillus subtilis* was used as an outgroup (Fig. 1). Phylogenetic tree showed that strain UICC B-76 was clustered together with *B. parabrevis* and *B. agri*, however, its position was separated from type strains of *B. parabrevis* and those of *B. agri*. In addition to this, we found that strain UICC B-76 has the G+C content of 55 mol% which differed to both its closest species, i.e. the G+C content of *B. parabrevis* and *B. agri* were 51.8-52.2 mol%, and 53.5 mol%, respectively.

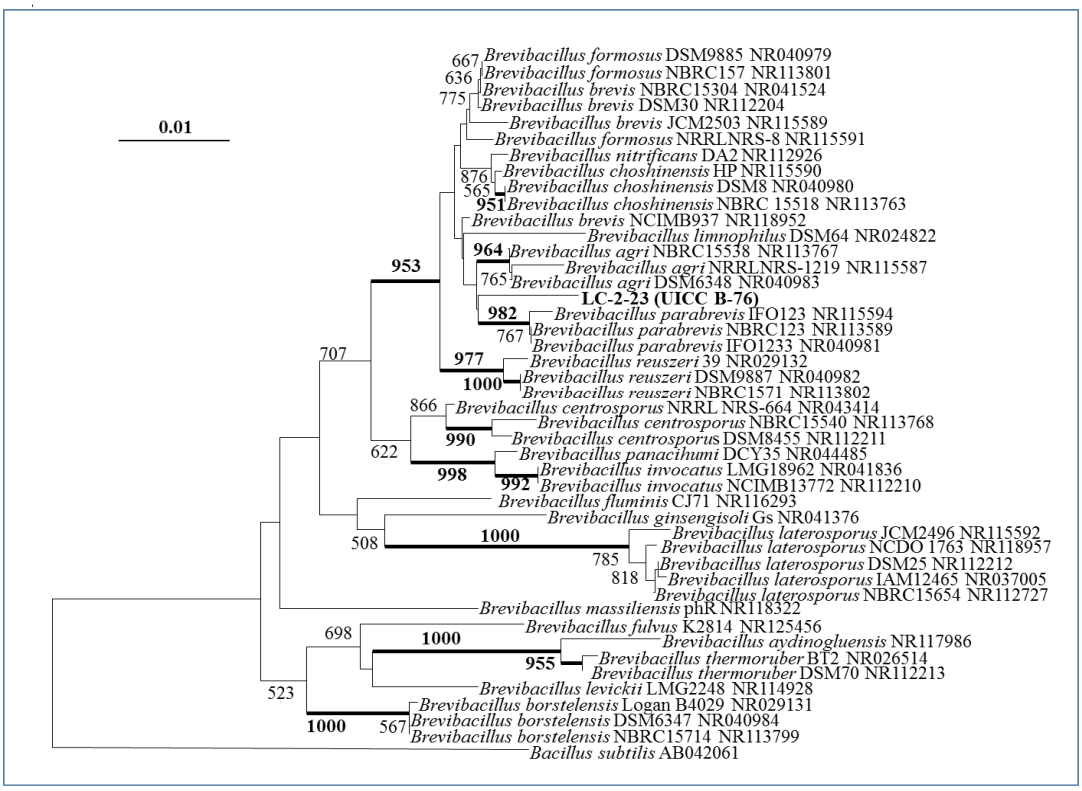

FIGURE 1. Neighbour-joining tree based on full sequence of 16S rRNA gene. Bootstrap values above 60% (based on 1000 replications) are represented at the branch points. *Bacillus subtilis* (AJ277905) was included as an outgroup. Bar, 0.01 substitutions per nucleotide position

In conclusion, based on chemotaxonomical difference in the mol% G+C content, the relatively low sequence homology to its closely related species, and its distinct phylogenetic position, we suggest that the strain UICC B-76 may represent a new species of *Brevibacillus*. Hybridization of DNA among strain UICC B-76, *B. parabrevis* and *B. agri* is required to clarify the proposal of a new species.

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