ISOLATION AND CHARACTERIZATION OF CELLULOSE-DEGRADING BACTERIA FROM SOILS IN SAMUT SONGKHRAM PROVINCE, THAILAND

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Abstract—Eight microorganisms capable of cellulose degradation were isolated from soil of waste disposal site in Samut Songkhram province, Thailand. These isolates divided to three groups based on Gram stain and morphological properties including gram-negative with rod shaped (group I), gram-negative with cocci shaped (group II), and gram positive with rod shaped (group III). The 16S rRNA gene sequencing identified the isolates BK1-4, BK1-6 and BK2-14 as a Pseudomonas aeruginosa species. The isolates BNL1-4, BNL1-15 and BNL2-19 were identified as Pseudomonas stutzeri. While, isolates BK3-2 and BNL2-14 were identified as Bacillus anthracis and Enterobacter cloacae, respectively

Index Terms- 16S rDNA, cellulose-degrading bacteria, Characterization, Thailand.

I. INTRODUCTION

Cellulose is a linear polysaccharide of glucose residues with β-1, 4-glycosidic linkages. Abundant availability of cellulose makes it an attractive raw material for producing many industrially important commodity products. Sadly, much of the cellulosic waste is often disposed of by biomass burning, which is not restricted to developing countries alone, but is considered a global phenomenon. With the help of cellulolytic system, cellulose can be converted to glucose which is a multiutility product, in a much cheaper and biologically favourable process [1]. Cellulolysis is basically the biological process controlled
and processed by the enzymes of cellulase system. Cellulase enzyme system comprises three classes of soluble extracellular enzymes: 1, 4-β-endoglucanase, 1, 4-β-exoglu canase, and β-glucosidase (β-D-glucoside glucohydrolase or cellobiase). Endoglucanase is responsible for random cleavage of β-1, 4-glycosidic bonds along a cellulose chain. Exoglucanase is necessary for cleavage of the nonreducing end of a cellulose chain and splitting of the elementary fibrils from the crystalline cellulose, and β-1, 4-glucosidase hydrolyses cellobiose and water-soluble cellodextrin to glucose [2, 3]. Only the synergy of the above three enzymes makes the complete cellulose hydrolysis to glucose [4]–[6] or a thorough mineralization to H_2O and CO_2 possible. Microorganisms are important in conversion of lignocellulose wastes into valuable products like biofuels produced by fermentation [7]. Successful bioconversion of cellulosic materials mainly depends on the nature of cellulose, sources of cellulolytic enzyme and optimal conditions for catalytic activity and production of enzymes [8]. Cellulose quality, temperature, aeration, carbon sources, incubation period, medium additives, pH of the medium and presence of inducers are important parameters for the optimized production of cellulase enzymes [9]. Cellulase is the enzyme that hydrolyse the β-1, 4-glycosidic bonds in the polymer to release glucose units [10]. Cellulases are among the industrially important hydrolytic enzymes and are of great significance in present day biotechnology. Cellulases are widely used in the food, feed, textile and pulp industries [11]. The bioconversion of cellulosic materials is now a subject of intensive research as a contribution to the development of large scale conversion process beneficial to mankind [12].

The microbial population of soils is made up of five major groups including bacteria, actinomycetes, fungi, algae and protozoa, and among these groups, bacteria are the most abundant group [13] and the most important microbe for decomposing waste. Worldwide interest in microbiological decomposition of cellulose is still as strong as when it started more than three decades ago. Cellulose degrading bacteria have been isolated by Han and Srinivasan [14], Kauffmann et al [15], Steward and Leatherwood [16] based on enrichment culture technique utilizing different sources of carbon [17]. Researchers have strong interests in cellulases because of their applications in industries of starch processing, grain alcohol fermentation, malting and brewing, extraction of fruit and vegetable juices, pulp and paper industry, and textile industry [18], [19].

The aim of the present work was to isolate, identify and
characterize cellulose-degrading bacteria from soil of waste disposal site in Samut Songkhram province, Thailand by using enrichment cultures and 16S rDNA sequence analysis.

II. MATERIALS AND METHODS

A. Soil sampling

A total 5 soil samples were collected from waste disposal site in Samut Songkhram province, Thailand. The samples were collected in sterile plastic bag and

stored at 4°C until they were used. The collected samples were brought to the laboratory for isolation and characterization of soil bacteria.

Enrichment and isolation of cellulose-degrading bacteria

10 g of soil sample was weighed and added into 250 ml of nutrient broth medium (NB). These cultures were incubated for 24 h. in a shaker incubator at 37°C at 100 rpm. Streaking plate method was used for obtaining pure bacterial strain. The bacterial cultures were streaked on the cellulose Congo-Red agar media with the following composition: KH_2PO_4 0.5 g, $MgSO_4$ 0.25 g, cellulose 2 g, agar 15 g, Congo-Red 0.2 g, and gelatin 2 g; distilled water 1 L and at pH 6.8–7.2 [1]. The plates were incubated at 37°C for 24-48 h. Quantitative assay using Congo red Dilution assay [20] was performed where zone of clearance was observed visually by staining plate with 0.1% congo red for 15 minute and destained with 1 M NaCl. Colonies showing discoloration of Congo-Red were taken as positive cellulose-degrading bacterial colonies [21], and only these were taken for further study.

B. Physiological and biochemical characterization

Eight bacterial isolates, BK1-4, BK1-6, BK2-14, BK3-2, BNL1-4, BNL1-15, BNL2-14 and BNL2-19 were isolated. Morphological and biochemical properties of the isolate were identified, evaluated, and compared, as described in Bergey's Manual of Systematic Bacteriology [22]. The parameters investigated included Gram's reaction, Indole production, Methyl Red (MR) test, Voges- Proskauer (V-P) reaction, citrate utilization, catalase production, motility and Triple Sugar Iron (TSI) test.

D. PCR amplification of 16S rRNA gene

Pure culture of the target bacteria was grown overnight in liquid NB medium for the isolation of genomic DNA using a method described by Jin-Long et al [23]. The extracted DNA was used as template for PCR amplification with 16S rRNA primers (27F Primer: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R Primer: 5'-GGTTACCTTGTT ACGACTT-3'). The reaction mixture of 50 µl consisted of genomic DNA, 0.2 µl of 5 U of *Taq* DNA polymerase, 5 µl of 10X PCR amplification buffer (0.5 M KCl, 0.1 M Tris-HCl, pH 9.0 and 1% Triton X-100), 1 µl of 10 mM dNTPs, 2 µl of 10 pmoles each of the two primers and 2.0 µl of 25 mM $MgCl_2$. The amplification program consisted of initial denaturation at 94°C for 5min, proceeded by 35 cycles each consisting of a denaturation step at 94ºC for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min followed by a final extension step at 72ºC for 15 min. 10 µl of the reaction mixture was then analyzed by submarine gel electrophoresis using 1.0 % agarose with SYBR® Safe DNA gel stain (Thermo Fisher, USA.) and gel electrophoresis using 1.0 % agarose with ethidium bromide at 8V/cm and the reaction product was visualized under Gel doc/UV trans-illuminator.

E. 16S rRNA Sequencing and Data Analysis

The 16S rDNA amplified PCR product was used for the sequencing. The purified products were then sent to First BASE Laboratories Sdn Bhd**.,** (Malaysia) for sequencing in BigDye® Terminator v3.1 (Applied Biosystems) sequencer. The sequences were then aligned and compared with other 16S rRNA sequences in the GenBank using the NCBI Basic Local alignment search tools (BLASTn) program (http://www.ncbi.nlm.nih.gov/BLAST). Sequences were analyzed and concatenated by using DNASTAR (DNASTAR, Inc., Madison, Wis.) A multiple-sequence alignment was prepared by using CLUSTAL W [24], and phylogenetic trees were constructed by using the neighbor-joining method.

III. RESULTS

C. Isolation and Characterization of cellulose-degrading bacteria

Eight isolates of cellulose-degrading bacteria were isolated for soil samples in Samut Songkhram province, Thailand and named BK1-4, BK1-6, BK2-14, BK3-2, BNL1-4, BNL1-15, BNL2-14 and BNL2-19. Light microscopy showed isolates BK1-6, BK2-14 and BNL1-4 were gram negative and rod shaped. Isolates BK1-4 and BNL2-14 were gram negative and cocci shaped, whereas BK3-2, BNL1-15 and BNL2-19 were gram positive and rod shaped. The

biochemical properties of eight isolates including motility, Indole production, Methyl Red (MR) test, Voges-Proskauer (V-P) reaction, citrate utilization, catalase production and Triple Sugar Iron (TSI) test were shown in Table 1.

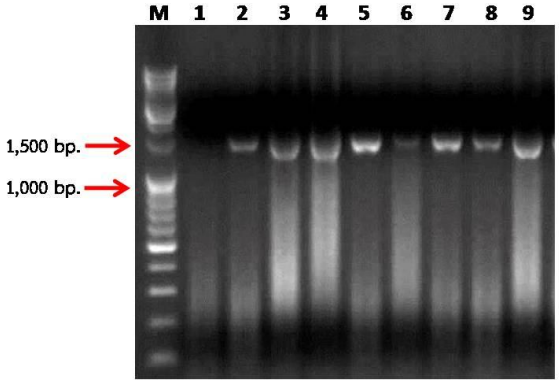


Fig. 1 Electrophoresis on 1% agarose gel of universal PCR products. Lanes: M, molecular ladder; 1, , negative control ; 2, BK1-4; 3, BK1-6; 4, BK2-14; 5, BK3-2; 6, BNL1-4; 7, BNL1-15; 8, BNL2-14; 9, BNL2-19. The numbers to the left are molecular sizes in base pairs.

D. Identification of cellulose-degrading bacteria

16S rDNA was amplified and sequenced by using their respective primers (Fig. 1). A total of 1,318 bp of the 16S rRNA gene was sequenced and used for the identification of isolated bacterial strain. The BLAST result showed that the 16S rDNA sequence

of isolates BK1-4, BK1-6 and BK2-14 have 99%, 100% and 97.5% sequence identity with *Pseudomonas aeruginosa*, respectively. Isolates BK3-2 showed highest similarity with *Bacillus anthracis* (90.2%), whereas BNL1-4, BNL1-15 and BNL2-19 showed 99.2%, 97.8% and 99.1%, respectively with *Pseudomonas stutzeri*. Isolates BNL2-14 was found closest related to *Enterobacter cloacae* with 99.5% similarity.

C. Phylogenetic Relationships

Further sequence analysis revealed a relatively distant phylogenetic relationship of BK1-4, BK1-6, BK2-14, BK3-2, BNL1-4, BNL1-15, BNL2-14 and BNL2-19 with various other bacteria in NCBI database (Fig. 2). Isolates were grouped into five phylogenetic branches or well-supported main clusters, with some subclusters. In the first phylogenetic branch isolates BK1-4, BK1-6 and BK2-14 were clustered with *Pseudomonas aeruginosa*, whereas the second phylogenetic branch clustered BNL1-4, BNL1-15 and BNL2-19 with *Pseudomonas stutzeri*. Isolates BNL2-14 was placed to the same branch with *Enterobacter cloacae*. Finally, the BK3-2 isolates positioned in the same cluster with *Bacillus anthracis.*

Table I

Biochemical characterization of the cellulose-degrading bacteria isolated from soils in Samut Songkhram province.

Isolates	Indole	MR Test	VP Test	Citrate Utilization	Catalase Test	Motility Test	TSI Test
BK1 - 4	-	-	-	+	+	-	N/N
BK1 - 6	-	+	-	+	+	+	A/A, G, H_2S
BK2 - 14	-	+	+	+	+	+	A/A
BK3 - 2	-	+	-	+	+	+	A/A, H_2S
BNL1 - 4	-	+	-	-	+	-	N/N
BNL1 - 15	-	+	-	+	+	+	A/A, G, H_2S
BNL2-14	+	+	+	+	+	+	A/A, G
BNL2-19	+	+	+	+	+	+	A/A, G

Based on morphological characterization, sequence comparisons and phylogenetic analyses, isolates BK1-4, BK1-6 and BK2-14 were identified as a members of *Pseudomonas aeruginosa* and named *Pseudomonas aeruginosa* strain BK1-4, strain BK1-6 and strain BK2-14, respectively. Isolates BK3-2 was identified as a new strains of *Bacillus anthracis* and named *Bacillus anthracis* strain BK3-2. BNL1-4, BNL1-15 and BNL2-19 isolates were identified as a members of *Pseudomonas stutzeri*, named *Pseudomonas stutzeri* strain BNL1-4, strain BNL1-15 and strain BNL2-19, respectively. While, isolates BNL2-14 was identified as a strain of *Enterobacter cloacae* and named *Enterobacter cloacae* strain BNL2-14. Further characteri- zation of this strain may lead to a better affiliation in the future.

CONCLUSIONS

From the present study it can be concluded that all the eight cellulose-degrading bacteria isolated from soil in Samut Songkhram province of Thailand could efficiently degrade cellulose in the medium. They were classified into 4 bacterial species including *Pseudomonas aeruginosa*, *Pseudomonas stutzeri*, *Enterobacter cloacae* and *Bacillus anthracis.*

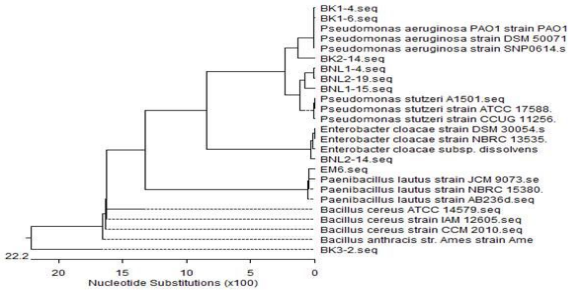


Fig. 2 Neighbor-joining tree based on the 16S rDNA sequences of the cellulose-degrading isolates (BK1-4, BK1-6, BK2-14, BK3-2, BNL1-4, BNL1-15, BNL2-14 and BNL2-19) with related 16S rDNA sequences found in GenBank database.

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