Identification of Cellulase-Producing Bacteria from Soil in Nasinuan Forest, Kantarawichai District, Mahasarakham Province

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Abstract

This study aimed to isolate and identify bacteria that can produce cellulase enzyme from Nasinuan forest, Kantarawichai District, Mahasarakham Province, Thailand. Five bacterial isolate representatives out of 82 isolates with cellulase-producing capacity on 1% carboxymethyl cellulose (CMC) agar were chosen for strain identification. Five bacteria were gram-positive, rod shaped with similar colony morphologies and identified as *Bacillus* spp. using 16S rRNA gene analysis. The highest halo:colony ratios on CMC agar were in the order: *Bacillus subtilis* 1.1CL4 > *B. licheniformis* 1.1CL3 = *B. licheniformis* 1.2CL3 > *B. licheniformis* 1.1CL1 > *B. subtilis* 1.2CL2. Their closest relatives were Bacillus spp. found in Sudan, Saudi Arabia, India and Pakistan. These cellulase–producing bacteria can be applied for use in a wide variety of industries such as dessert production, beverage production, textile production and dairy industry.

Keywords: Carboxymethyl Cellulose, Cellulase, Bacteria, Soil, Industry

Introduction

Cellulose is regarded as the most abundant biomass on Earth and the most dominating agricultural waste [1]. This cellulosic biomass is a renewable resource with great potential for bioconversion to value-added bio-products. Cellulose is commonly degraded by an enzyme called cellulase. This enzyme is produced by several microorganisms namely bacteria and fungi [2]. Components of cellulase systems were initially classified based on their mode of catalytic activity and have more recently been classified based on structural characteristics [3]. Three major types of their enzymatic activities are found: (1) endoglucanases or 1,4-β-D-glucan-4-glucanohydrolases (EC 3.2.1.4) randomly hydrolyses at internal amorphous sites in the cellulosic polysaccharide chain, resulting in oligosaccharides of different lengths and consequently new chain ends, (2) exoglucanases including 1,4-β-D-glucan glucanohydrolases (cellodextrinases) (EC 3.2.1.74) and 1,4-β-D-glucanfcellobiohydrolases (cello-biohydrolases) cut in a possessive manner on the reducing or non-reducing ends of cellulosic polysaccharide chains, generating either glucose (glucanohydrolases) or cellobiose (cellobiohydrolase) as main products (EC 3.2.1.91) and (3) β-glucosidases or β-glucoside glucohydrolases (EC 3.2.1.21) can hydrolyse microcrystalline cellulose [4].

Cellulase production from bacteria has been considered more advantageous than that from fungi due to higher bacterial growth rate and are often more effective biocatalysts [5]. They are likely to be less inhibited by the presence of materials that have already been hydrolysed (feedback inhibition) [6]. Members of the genera *Cellulomonas*, *Clostridium*, *Bacillus*, *Thermomonospora*, *Ruminococcus*, *Bacteriodes*, *Erwinia*, *Acetovibrio*, *Microbispora* and *Streptomyces* are some bacteria reported to produce cellulases [7].

To date, a number of cellulase-producing bacteria has been documented, however, no study on cellulase-producing bacteria from soil in the Nasinuan Community Forest, Kantharawichai District, Mahasarakham Province, Thailand is documented. This forest seems to be rich in microorganisms and plant biodiversity that can be useful in the production of industrial enzymes including cellulase. This is first report to identify cellulase-producing isolated from Nasinuan Forest. These bacterial cellulases can be applied to various industries including pulp and paper, textile, bioethanol, wine and brewery, food processing, animal feed, and agriculture in the future.

Materials and methods

Collection of soil samples

The soil samples were collected from Nasinuan Community Forest, Kantarawichai District, Mahasarakham Province, Thailand (Figure 1). They were collected from 4 location points randomly in one area (20 rai out of 120 rai). The samples were kept in room temperature till further analysis. Soil samples were suspended in water and recorded for pH value and electroconductivity which corresponds to soil salinity.

A

B

Figure 1 Location of soil collection. (A) Map of Mahasarakham Province in Thailand where Nasinuan Community Forest is located. (B) Zone 1,2,3 of half of the 120-rai forest where soil samples were collected.

Bacteria isolation and screening for cellulase activity

Soil sample (10 g) was suspended in 90 mL of sterile 0.85% NaCl solution. The suspensions (100 µL) were spread on CarboxyMethyl Cellulose (CMC) agar containing (in g/100 ml); 0.5 CL, 0.1 $NaNO_3$, 0.1 KCl, 0.05 g $MgSO_4$, 0.05 yeast extract, 0.1 glucose and 1.5% agar pH 7.0 and incubated at 37 ºC for 3 days. To visualize the hydrolysis zone, the plates were flooded with an aqueous solution of 0.1% congo red for 15 min and washed with 1 M NaCl [8]. Diameter of the clear zone around colonies on CL agar was then measured. The Halo: Colony value was calculated following a method of Edi-Premono [9]. Subsequently, pure isolates were obtained through several passages of streaking and inoculated into general bacterial medium (in g/L); 10.0 yeast extract, 5.0 tryptone and 5.0 NaCl overnight at 37 ºC for the next experiment. The morphological properties of each bacterial isolate including shape, size, colony characteristics (color, shape, surface, elevation and edge) and Gram staining results were recorded.

RAPD-PCR analysis

The cellulase-positive isolates were cultivated in general bacteria medium at 37 ºC for 24 h. After centrifugation at 10,000g for 5 min, the bacterial cell pellets were extracted for genomic DNAs using Bacterial Genomic DNA extraction kit (Vivantis, Malaysia). The M13V (5'-GTTTTC-CCA-GTC-ACG-AC-3') was used. RAPD reactions were performed in 25 µL mixtures composed of genomic DNA 5 ng, 2x Master Mix Buffer A (Vivantis, Malaysia), 500 mM KCl, 100 mM Tris-Cl (pH 8.3), 0.1% TritonTMX100, 0.2 mM dNTP, 3.5 mM $MgCl_2$, 0.75 U Taq Polymerase and the final volume was adjusted with sterile nuclease-free water to 25 µL. PCR thermocycler (Thermo Scientific Hybaid Px2) was programmed as follows: (1) initial denaturation for 1 min at 94 ºC for 3 cycles; (2) denaturation at 94 ºC for 3 min; annealing at 40 ºC for 5 min, and extension at 72 ºC for 5 min for 32 cycles; (3) denaturation at 94 ºC for 2 min; annealing at 60 ºC for 3 min, and extension at 72 ºC for 5 min for 1 cycle. Samples were held at 4 ºC till further analysis. The PCR products were detected on 0.8% agarose gel to categorize the isolates using RAPD patterns. Representative bacterial isolates from each RAPD pattern were chosen for bacterial identification using 16S rRNA gene analysis.

16S rRNA gene sequencing and phylogenetic analysis

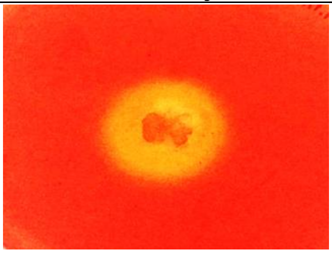
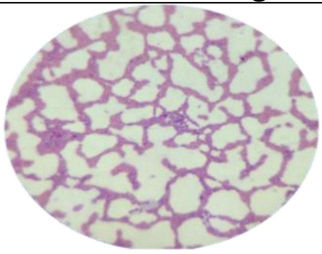
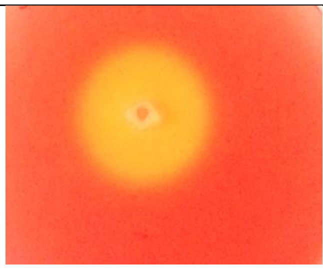
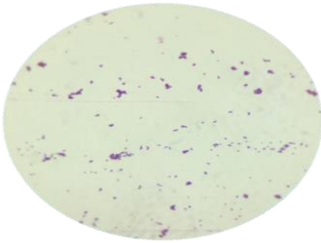
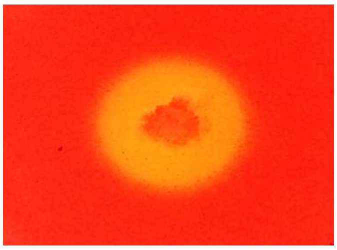
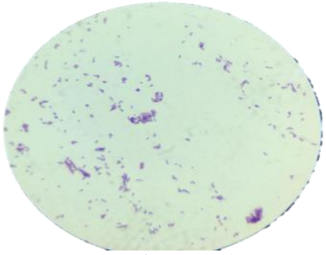
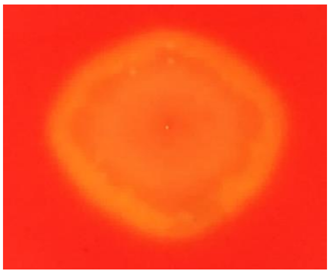
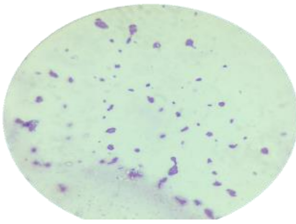
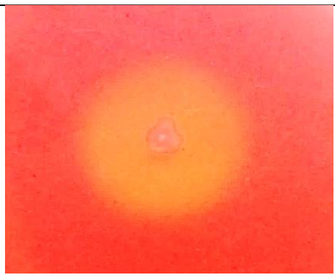
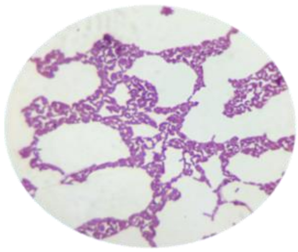
Pure bacterial isolates were identified using genomic DNAs obtained from the above method and universal primers: forward primer 27F 5'-GAGAGTTTGATYCTGGCTCAG-3' and reverse primer 1492R 5'AAGGAGGTGATCCARCCGCA -3'. In 25 µL PCR mixture, it was composed of genomic DNA 0.5 ng, 2X Master Mix (One PCR) of 100 mM Tris-HCl (pH 9.1), 0.1% TritonTMX-100, 200 mM dNTP, 1.5 mM $MgCl_2$, 0.005 U Taq DNA Polymerase and 0.2 µM forward and reverse primer with volume adjustment with nuclease-free water. PCR thermocycler (Thermo Scientific Hybaid Px2) was programmed as follows: (1) initial denaturation for 2 min at 94 ºC for 1 cycle; (2) denaturation at 94 ºC for 45 s; annealing at 54 ºC for 45 s, and extension at 72 ºC for 1 min for 32 cycles; (3) final extension at 72 ºC for 7 min. Samples were held at 4 ºC till further analysis. The PCR products of 16S rRNAs (1,500 bp) were detected on 0.8% agarose gel, purified using the PCR product purification kit (Vivantis, Malaysia), sent to First Base Co. Ltd. (Malaysia) for DNA sequencing. The 16S rRNA gene sequences were then compared with others available in GenBank using BLASTN program (Basic Local Alignment Search Tools) [10]. The Phylogenetic Tree was constructed using Muscle method for sequence alignment and neighbor-joining method using MEGA7 with 1,000 replicates of bootstrap values [11].

Results and discussion

Bacterial cellulase activity

In this study, 82 cellulase-positive isolates (1.1CL 1,3,4 and 1.2CL 2,3) showed clear zones on 1% CMC agar with different halo : colony ratios. The colonies showing discoloration of Congo red were taken as positive cellulose-degrading bacterial colonies [12]. Five bacterial representative strains showed similar colony morphologies and appeared to be Gram-positive and rod-shaped (Table 1). The result showed halo : colony ratios ranging from 1.2 to 2.8 (Table 1). This finding was very similar to that of Hatami et al. [13] and these 82 strains were subjected to RAPD analysis in order to categorize bacterial isolates based on RAPD patterns prior to strain identification using 16S rRNA gene sequencing.

Table 1 Characteristics of five cellulase-producing bacterial representative strains

Strain	Colony morphology	Halo : colony ratio	Gram staining
B. licheniformis 1.1CL1	Irregular shape Flat structure Curled margin Cloudy white color	 2.0	 G^+, rod
B. licheniformis 1.1CL3	Irregular shape Flat structure Curled margin Cloudy white color	 2.6	 G^+, rod
B. subtilis 1.1CL4	Irregular shape Flat structure Curled margin Cloudy white color	 2.8	 G^+, rod
B. subtilis 1.2CL2	Irregular shape Flat structure Curled margin Cloudy white color	 1.2	 G^+, rod
B. licheniformis 1.2CL3	Irregular shape Flat structure Curled margin Cloudy white color	 2.6	 G^+, rod

RAPD-PCR patterns

RAPD patterns of PCR products from 82 isolates showed 5 distinct patterns (Figure 2) and thus we selected one isolate with the highest halo : colony ratio from each distinct RAPD pattern for strain identification using 16S rRNA gene analysis.

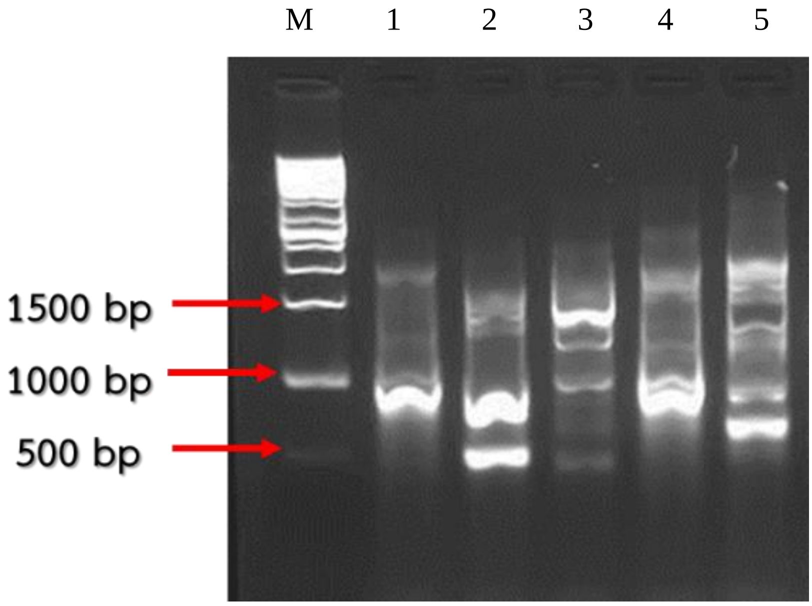


Figure 2 RAPD patterns. M = DNA marker; 1 = *B. subtilis* 1.1CL4; 2 = *B. licheniformis* 1.1CL3; 3 = *B. licheniformis* 1.2CL3; 4 = *B. licheniformis* 1.1CL1; 5 = *B. subtilis* 1.2CL2.

Bacterial strain identification

PCR products of 16S rRNA genes from 5 cellulase-producing representative isolates were approximately 1,500 bp (Figure 3) and identified by BLAST search on GenBank database.

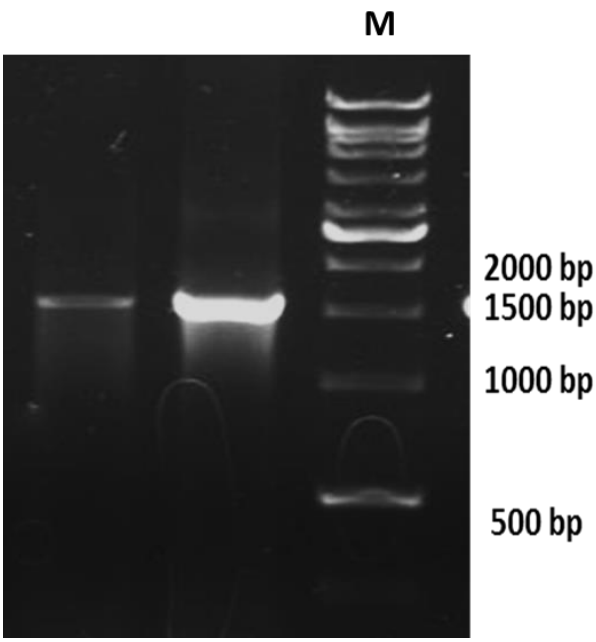


Figure 3 PCR products of 16S rRNA genes approximately 1,500 bp on agarose gel.

The BLAST results displayed that these five cellulase-positive representative isolates belong to the Genus *Bacillus*, namely *B. subtilis* 1.1CL4*, B. licheniformis* 1.1CL3*, B. licheniformis* 1.2CL3*, B. licheniformis* 1.1CL1 and *B. subtilis* 1.2CL2 (Table 2). Their closest relatives were *Bacillus* spp. found in Sudan, Saudi Arabia, India and Pakistan with a range of 94-98%. Similarly, the previous study found 57 bacterial isolates from soil in Botanical garden in India identified as *Bacillus* spp. [14].

Table 2 CMC-degrading bacterial strains identified by 16S rRNA analysis

Isolate	Closest relative*	Accession No.*	%Identity*	Origin*
1.1CL1	*Bacillus licheniformis* CMF1Ph	KX424373.1	98	Fish gut, India
1.1CL3	*Bacillus licheniformis* TM13	KC857623.1	96	Saudi Arabia
1.1CL4	*Bacillus subtilis* SA4	KY285264.1	94	Soil, Sudan
1.2CL2	*Bacillus subtilis* SA9	KY285265.1	99	Soil, Sudan
1.2CL3	*Bacillus licheniformis* MA-42	KX426642.1	97	Soil, Pakistan

*Based on results from BLAST search (www.ncbi.nlm.nih.gov/pubmed)

The 16S rRNA sequencing made it possible to identify and distinguish closely related bacterial species [14]. Therefore, we used the present molecular identification technique which is more accurate than API kit [15]. In the previous report, it was found that *B. subtilis* was able to use cellulosic waste as a carbon source. Moreover, our finding is similar to Tabo and Monsalud [16] who also reported the occurrence of *B. cereus*, *B. licheniformis* and *B. pumilus* with cellulase-producing capacity from Philippines mangrove soil.

The phylogenetic tree of five bacterial strains and two reference strains using MEGA 7.0 showed that *B. licheniformis* 1.1CL1 was evolutionarily different from the other four Bacillus srains (Figure 3). *B. licheniformis* 1.2CL3 evolved similarly to *B. licheniformis* WJB11 (NCBI accession no. KU877628.1) isolated from paddy field manure in China. However, *B. licheniformis* 1.1CL3 was more closely related to *B. subtilis* than the other two *B. licheniformis* strains already mentioned above. *B. subtilis* 1.1CL4 and *B. subtilis* 1.2CL2 were closely related and evolutionarily similar to *B. subtilis* SCB-1 (NCBI accession no. MF893335.1) isolated from sugarcane leaf in India.

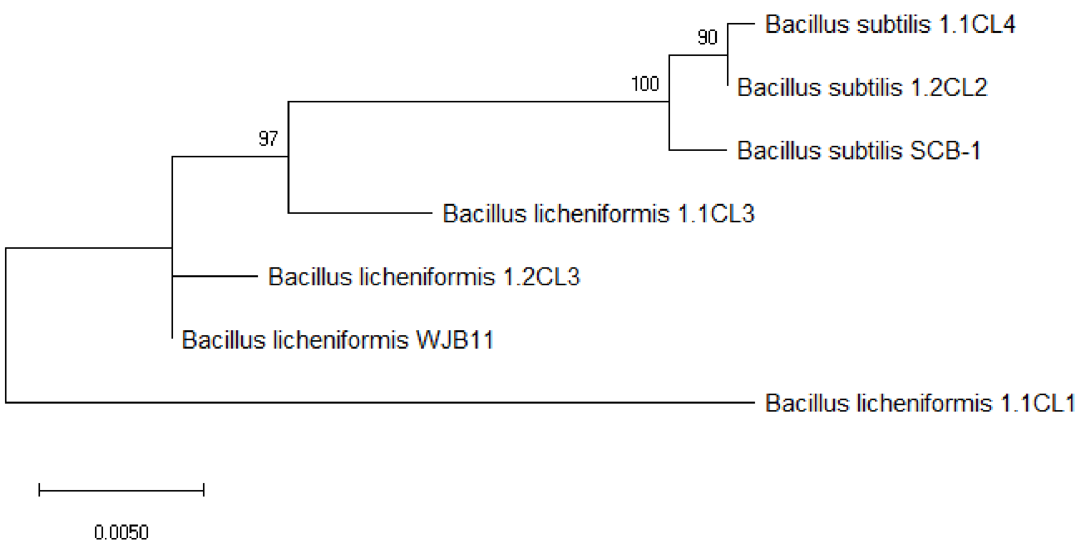


Figure 3 Phylogenetic tree of five bacterial strains and two reference strains using MEGA 7.0.

Several factors have an effect on enzyme activity such as pH, temperature, inoculum size, fermentation process, substrate concentration or nutrient sources. In this present study, the soils sample were collected from soil in Nasinuan Community Forest, Kantarawichai District, Mahasarakham Province, Thailand. We found that soil sample at the locations 1.1 and 1.2 where five cellulase-positive bacteria existed had pH values at 7 and 8, respectively which is in a good agreement with the optimum of pH values of 5 - 9 that promote the bacteria growth. The surrounding environment at locations 1.1 and 1.2 showed the growth of local trees *Azadirachta indica* and *Senna siamea*; *Terminalia chebula* and anthill, respectively (Table 3). Electroconductivity of soil at location 1.1 exhibited the values of 3.62 which corresponded to 0.15% salt (low salinity), however, at location 1.2 exhibited the values of 1.99 which corresponded to <0.1% salt (no salinity). It was noticeable that cellulase-producing bacteria are likely to be associated with plantation areas where cellulose is abundant such as those trees and anthill that are already mentioned.

Table 3 Descriptive details of the soil collection sites

Location of soil collection	Surrounding area	Electroconductivity (μS/cm)
1.1 Coordinates N 16 20 17.945, E 103 12 14.731	*Azadirachta indica* and *Senna siamea* are grown around this area  	3.62 Low saline (0.15% salt)
1.2 Coordinates N 16 20 17.945, E 103 12 14.731	Found *Terminalia chebula* and anthill 	1.99 No saline (<0.1% salt)

Cellulolytic bacteria are responsible for much of the cellulose degradation in soils. Aerobic bacteria are capable of producing numerous extra-cellular enzymes with binding modules for various cellulose structures, however anaerobic bacteria possess a unique extracellular multi enzyme complex, called cellulase [17]. *Bacillus* spp. found in our study are facultative anaerobes and seemed to exhibit cellulase activity.

Mainly efficient cellulase activities are observed in fungi but there is increasing interest in cellulase production by bacteria because bacteria show higher growth rate as compared to fungi and thus has a better potential to be used for cellulase production in large scale [17].

Conclusions

This is the first report of identifying 5 cellulase-producing bacterial isolates from soil collected in Nasinuan Community Forest. All were identified as *Bacillus* spp. showing gram-positive and rod shaped. In general, *Bacillus* is the most commonly found as cellulase-producing bacteria in soil. Further studies are in progress to obtain high yield of cellulase enzyme with high specific activity. These bacteria can be applied in various industrial processes.

Acknowledgements

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References

[1] Tomme PR, Warren R and Gilkes NR. Cellulose Hydrolysis by Bacteria and Fungi. *Advances in Microbial Physiology*. 1995; 37, 1-8.

[2] Bahkali AH. Influence of various carbohydrates on xylanase production by *Verticillium tricopus*. *Bioresource Technology*. 1996; 53 (3), 265-268.

[3] Henrissat B, Teeri TT, and Warren A. Scheme for designating enzymes that hydrolyse the polysaccharides in the cell walls of plants. *FEBS Lett*. 1998; 425, 352-354.

[4] Teeri TT. Crystalline cellulose degradation: New insight into the function of cellobiohydrolases. *Trends in Biotechnology*. 1997; 15 (5), 160-167.

[5] Sethi S, Datta A, Gupta BL and Gupta S. Optimization of Cellulase Production from Bacteria Isolated from Soil. *International Scholarly Research Notices Biotechnology*. 2013; 1–7.

[6] Acharya S and Chaudhary A. Optimization of fermentation conditions for cellulases production by *Bacillus licheniformis* MVS1 and *Bacillus* sp. MVS3 isolated from Indian hot spring. *Brazilian Archives of Biology and Technology.* 2012; 55 (4), 1-7.

[7] Saratale RG, Saratale GD, Chang JS, Govindwar SP. Decolorization and biodegradation of reactive dyes and dye wastewater by a developed bacterial consortium. *Biodegradation*. 2010; 21, 999–1015.

[8] Apun K, Jong BC and Salle MA. Screening and isolation of a cellulolytic and amylolytic *Bacillus* from sago pith waste. *The Journal of General and Applied Microbiology*. 2000; 46 (5), 263–267.

[9] Edi-Premono M, Moawad MA and Vleck PLG. Effect of phosphate solubilizing *Pseudomonas putida* on the growth of maize and its survival in the rhizosphere. *Indonesian Journal of Crop Science*. 1996; 11, 13-23.

[10] Altschul SF, Gish W, Miller W, Myers E W and Lipman DJ. Basic local alignment search tool. *Journal of Molecular Biology*. 1990; 215 (3), 403–410.

[11] Tamura K and Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution*. 1993; 10, 512-526.

[12] Lu WJ, Wang HT, Nie YF, Wang ZC, Huang DY, Qiu XY and Chen JC. Effect of inoculating flower stalks and vegetable waste with ligno-cellulolytic microorganisms on the composting process. *Journal of Environmental Science and Health, Part B*. 2004; 39(5-6), 871-87.

[13] Hatami S, Alikhani HA, Besharati H, Salehrastin N, Afrousheh M and Yazdani ZJ. Investigation on Aerobic Cellulolytic Bacteria in Some of North Forest and Farming Soils. *American-Eurasian Journal of Agricultural & Environmental Sciences*. 2008; 3(5),

[14] Basavaraj I, Shivasharan CT and Kaliwal BB. Isolation and Characterization of Cellulase producing bacteria from Soil. *International Journal of Current Microbiology and Applied Sciences.* 2014; 3 (5), 59-69.

[15] Rahna K, Rathnan DJ and Balasaravanan T. Isolation, screening, identification and optimized production of extracellular Cellulase from *Bacillus subtilis* using cellulosic waste as carbon source. *Journal of Microbiology, Biotechnology and Food Sciences*. 2013; 2 (6), 2383-2386.

[16] Tabo NSC and Monsalud RG. Characterization and Identification of High Cellulase-Producing Bacterial Strains from Philippine Mangroves. *Philippine Journal of Systematic Biology*. 2010; 4, 13–20.

[17] Maki ML, Broere M, Tin Leung K and Wensheng Q. Characterization of some efficient cellulases producing bacteria isolated from paper mill sludges and organic fertilizers. *International Journal of Biochemistry and Molecular Biology*. 2011; 2 (2), 146-154.