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Phylogenetic Identification of Cellulolitic and Butanol production bacteria based on 16S rDNA from Ranu Pani Lake, East Java, Indonesia

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Abstract: Global trend today is how to develop alternative fuel to transportation; one of them is butanol production from cellulose. The objective of this study was to determine of bacterial density and diversity, cellulolytic activity, butanol production and genetic relation from Ranu Pani Lake sediment based on 16S rDNA sequences. The method in this study included sample collection, isolation, enumeration and characterization, cellulolytic test (semi quantitative and quantitative), butanol production, DNA isolation, 16S amplification, sequencing and data analysis. Highest cell density is RP 2.1 (3.6×10^3 CFU.g^{-1}) and bacterial diversity is 0.854. RP 3.2 and RP 7.1 isolate has highest semi-quantitative cellulolytic test (1005.84 and 992.42 mm^2). Quantitative cellulolytic test was showed RP 2.2 isolate have highest total cellulolytic activity (0.224 ± 0.01 FPU.ml^{-1}) and resulted 1.21 mg.ml^{-1} reducing sugar concentrations. High butanol concentration was resulted by RP 2.2 (10.34 ± 0.002 g.l^{-1} after 3 days fermentation), RP 3.2 and RP 7.2 (10.113 ± 0.012 g.l^{-1} after 5 days and 9.627 ± 0.006 g.l^{-1} after 3 days fermentation). RP 2.2 was identical with *Paenibacillus polymyxa* DYJL38 (99.6 %), whereas RP 3.2 was identical with *Bacillus* sp. X15, *Bacillus* sp. NG06 and *Bacillus methylotrophicus* LZ043 (99.8 %). RP 7.2 was identical with *Bacillus methylotrophicus* JF38, *Bacillus methylotrophicus* -Y48, *Bacillus methylotrophicus* R13, *Bacillus methylotrophicus* SDS11 and *Bacillus amyloliquefaciens* subsp. *plantarum* L12 (99.8 %).
Keywords: butanol, cellulolytic, phylogenetic, Ranu Pani Lake, 16S rDNA.

Introduction

Global production of petroleum has decreased during the last decade, but requirement and price has increased[1]. One of solution is bio-fuel, especially butanol. Butanol has high energy density, low heating value (LHV), high hydrophobicity and low evaporation[2].

Butanol can be produce using sugar, starch and cellulose[3]. Sugar and starch was cause competition with food consumption and have high price (at 2013, sugar Rp 13.388, starch Rp 9.128 per kilograms). Cellulose has low price (Rp 1.217 per kilograms) and abundant[4]. Cellulose biomass could be obtained from forest, field, household waste[5], and wood[6]. One of microbe that can produce butanol from biomass are *Clostridium*[7], *Bacillus*[8,9]. *Clostridium*, *Bacillus* and *Paenibacillus* could be isolated from soil, agricultural waste, compost, gastrointestinal tract[10] and Lake sedimentation from agriculture activity[8,11,12].

Genus *Clostridium* that have potency for butanol production using cellulose are *C. acetobutylicum* (using household waste), *C. beijerinckii* (wheat straw, wheat peel, corn fibre, barley straw and switch grass), *C. saccharoperbutylacetonicum* (grain peel), and *C. acetobutylicum* (cassava bagass)[10]. The most *Clostridium* that been used for butanol production is *C. Acetobutylicum*[8]. Genus *Bacillus* that can use cellulose to produce butanol is *Bacillus* sp. 15 and *Bacillus amyloliquefaciens* NELB-12[8,13]. Genus *Paenibacillus* that can use cellulose to produce butanol is *Paenibacillus polymyxa* CR1[9].

Ranu Pani Lake is the Lake include in Lumajang District. Ranu Pani Lake has been sedimentation from around agricultural activity and human activity[14] and not yet to microbial exploration, especially bacteria. Ranu Pani Lake is potential resource for isolation of bacteria that has ability for produce butanol from biomass. 16S rDNA is the most popular marker for bacterial taxonomic and phylogenetic analysis[15]. This research aimed to explore amount of indigenous bacteria species that have potency for cellulolytic and butanol production, both.

Material and Method

Collection of the Samples

Samples were collected from 5 locations and so far 1 m from Ranu Pani Edge. Samples stored in ice box. The pH was determined after re-suspending and homogenizing 1 g sample for 20 min in 10 ml distilled water using pHmeter. Dissolved oxygen and conductivity was determined using DOmeter and conductivitimeter.

Isolation, Enumeration and Characterization of Bacteria

Amount of 25 g sediment from Ranu Pani Lake was suspended in 225 ml sterile aquadest. Suspense was serial diluted until 10-6. 0,1 ml every serial dilution was transfer to petri dish and added 9 ml Tryptone Yeast Extract Acetate (TYA) agar medium (6 g bacto tryptone (Bacto); 2 g yeast extract (Bacto); 3 ml acetic acid; 0.5 g KH_2PO_4; 0.3 g $MgSO_4.7H_2O$; 0.01 g $FeSO_4.7H_2O$; 20 g glucose and 20 g agar (Bacto) per litter, pH 6,5 standardized using 1 N NaOH and sterilized at 115 ˚C, 15 minute)[16] and incubated under anaerobic condition using Gas Generating Kit Anaerobic System BR0038B (Oxoid) in anaerobic jar at 27 °C for 48 hours.

After incubation, cell counted using Total Plate Count (TPC) (Equation 1), colony and cell were characterized based on Bergey's Manual of Determinative Bacteriology[17]. Data was analyzed significance test using One Way ANOVA (α=0.2) (SPSS 16 for Windows).

$$\text{Cell amount } (\text{CFU}.g^{-1}) = \text{coloni counted} \times \frac{1}{\text{dilution}} \times \frac{1}{0},1 \quad \ldots (1)$$

Cellulase Activity Measurement

Pure culture were inoculated to 10 ml Thioglicolate medium (15 g enzymatic digest of casein; 5 g yeast extract; 5.5 g dextrose; 0.5 g L-cystine; 2.5 g NaCl; 0.5 g $C_2H_4O_2S$; 0,001 resazurin; 0.75 g agar (Bacto) per litter) sterile and incubated at 30 °C, 3 days in Anaerobic Jar (added Gas Generating Kit Anaerobic System BR0038B, Oxoid). 100 µl culture transfer to well (diameter 8,45 mm) at Carboxymethylcellulose (CMC) agar medium (1 g KH_2PO_4; 1 g KCl; 0.5 g $MgSO_4.7H_2O$; 0.5 g NaCl; 0.01 g $FeSO_4.7H_2O$; 0.01 g $MnSO_4.H_2O$; 0.3 g NH_4NO_3, 10 g CMC; 18 g agar per litter) sterilized and incubated at 30 °C, 3 days under anaerobic condition (Ghose, 1987). After incubation, clear zone measured using callipers.

Cellulase activity measured based on Ghose (1987)[18]. Pure culture were inoculated to 10 ml Thioglicolate medium transferred to 10 ml basal mineral salt medium (BSM) (2 g $NaNO_3$; 1 g K_2HPO_4; 0.5 g KCl; 0.5 g $MgSO_4.7H_2O$; 2 g peptone; 10 g filter paper Whatmann no 1 per litter) sterilized and incubated at 27 °C, 3 days under anaerobic condition. After incubation, 10 ml culture was centrifuge at 14,000 rpm, 10 minute, 4 °C.

Amount of 2,5 ml supernatant was transferred to test tube and added 5 ml 0.05 M phosphate buffer pH 8[19]. Then was added 10 mg filter paper Whatmann no 1 and incubated at water bath 50 °C, 1 hour. Reducing sugar was measured using *3,5-dinitrosalicylic* (DNS) method[20]. Cellulase activity measured using Equation 2[18].

Data was analyzed significance test using One Way ANOVA (α=0.05) (SPSS 16 for Windows). Best five cellulase activity isolate was used to butanol production.

$$\text{Cellulase activity}\,(\text{FPU.ml}^{-1}) = \text{mg glucose} \times 0,185 \quad \ldots (2)$$

Butanol Production

One oose culture bacteria was inoculated to 10 ml Thioglicolate medium and incubated at 30 °C, 48 hours. After incubation, 2,5 ml culture was transferred to 22,5 ml modified TYA medium pH 6,5 (without glucose, added 1 % filter paper Whatmann no 1) and incubated at 30 °C, 7 days under anaerobic condition using Generating Kit Anaerobic System BR0038B (Oxoid). Amount of cell, acidity, temperature, reducing sugar and butanol concentration was measured every 2 days. Amount of cell was counted using Petroff-Hauser method, whereas acidity and temperature was measured using pHmeter and thermometer. Reducing sugar concentration was measured using DNS method[20]. Butanol was measured using Gas Chromatography (GC).

16S rDNA Isolation and Amplification

Isolate of bacteria was inoculated to Thioglicolate agar medium and incubated at 48 hour, 30 °C. Five millilitres sterile aquadest was added to culture and homogenize until colony was diluted. Two millilitres of diluted culture was centrifuged at 4250 rpm, 15 min, 4 °C. Pellet was re-suspended with 567 µl TE buffer (Tris 10mM, EDTA 1mM pH 8.0). Ten microgram per-millilitre lysozyme was added and incubated at 37 ͦC, 1 hour. After incubation, 30 µl Sodium dodecyl sulfate (SDS) 10 %, 10 µl proteinase-K, 100 µl NaCl 5 M, 80 µl CTAB/NaCl was added and incubated at 55 ºC, 1 hour. The next steps were referring to Ausubel *et al.* (1995)[21]. Sequences of 16S rDNA was amplified using 2 primer (27F: 5'-AGAGTTTGATCMTGGCTC-3'; 1492R: 5'-GGTTACCTTGTTACGACTT-3')[22]. Material composition and reaction condition of PCR are shown in Table 1 and Table 2[23].

Tabel 1. PCR composition material

No.	Solution	Volume (µl)	Conc.
1	ddH_2O	6	
2	PCR mix (*i-Taq*^{TM})	15	
3	Primer Forward	3	30 pmol
4	Primer Reverse	3	30 pmol
5	DNA *template*	3	<1 µg
Total		30	

Table 2. Reaction condition for PCR

No.	Reaction	Temp. (^oC)	Time (min)
1	Initial denature	94	4
2	35 cycles : Denature	94	1
	Annealing	55	1
	Extension	72	1
3	Final extension	72	5

16S rDNA Purification and Sequencing

Purification and Sequencing of 16S rDNA sequence performed in First base, Malaysia. Sequences was submited to NCBI (KT036393, KT036394, KT036395).

Data Analysis

Data of isolation, cellulolytic and butanol productivity was analysis using One way ANOVA SPSS 16. DNA sequences of bacteria isolates and reference isolates were collected from GenBank

(http://www.ncbi.nlm.nih.gov) and presented in FASTA format. Analysis of DNA sequences and construction of phylogeny tree were done using MEGA ver. 6.06 programs[24].

Result

Density and diversity of Bacteria

After isolation, a total of 13 isolate that could grow in the TYA medium (RP 1, RP 2.2, RP 2.2, RP 3.1, RP 3.2, RP 4, RP 5, RP 6, RP 7.1, RP 7.2, RP 8, RP 10 and RP 14). Isolate RP 2.1 and RP 2.2 have highest cell density (1.8 x 10^3 CFU.g^{-1}). Simpson diversity index (D) of bacteria is 0.854. Ranu Pani Lake has acidity and temperature is 7.76 ± 1.41 and 19.86 ± 0.69 °C, whereas dissolved oxygen and conductivity are 5.39 ± 0.7 ppm and 41.42 ± 8.25 µs.cm^{-1}.

Cellulolytic activity of Bacteria

Mostly of isolate from Ranu Pani Lake sediment have potency for cellulolytic activity (Figure 1). Highest semi quantitative cellulolytic (CMC degradation) activity showed by isolate RP 3.2 and RP 7.1 (1005.84 and 992.42 mm^2) (Figure 1A) and clear zone diameter (include well) are 36.7 and 36.53 mm. Although highest quantitative cellulolytic (total cellulolytic) activity showed by isolate RP 2.2 (0.224 ± 0.01 FPU.ml^{-1}) and released the number of reducing sugar 1.21 mg.ml^{-1}. The other isolates that have high cellulolytic activity are RP 7.2 (0.178 ± 0.006 FPU.ml^{-1}), RP 3.2 (0.133 ± 0.001 FPU.ml^{-1}), RP 8 (0.121 ± 0.005 FPU.ml^{-1}) and RP 5 (0.07 ± 0.001 FPU.ml^{-1}).

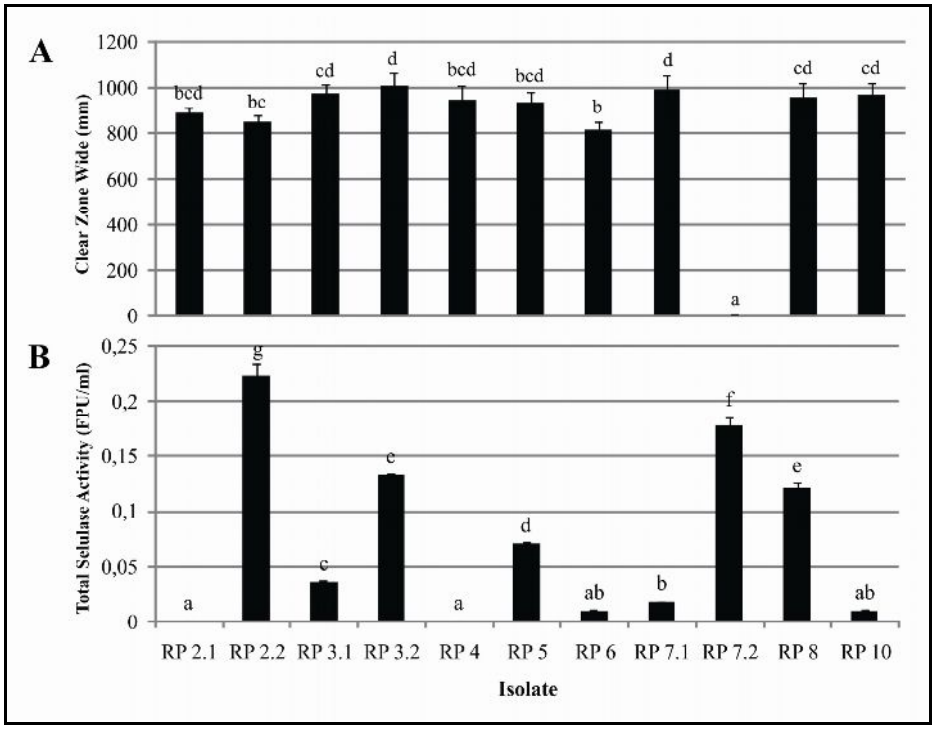

Fig. 1. Semi quantitative (A) and quantitative (B) test of cellulolytic activity of bacteria from Ranu Pani Lake sediment

Butanol Production of Bacteria

Acidity, cell density, butanol and reducing sugar concentrations were variably during fermentation process (Figure 2). Isolate RP 3.2, RP 7.2 and RP 8 was increase of cell density significantly ($p<0.05$), but isolate RP 2.2 and RP 5 only have increase phenomenon at some fermentation times. Medium acidity was increase during fermentation time. Reducing sugar and butanol concentration have positive correlation ($p<0.05$). Increase of reducing sugar concentration was allowed by increase of butanol concentration. Butanol concentration was increase and decrease during fermentation process. Highest butanol concentrations were

resulted by isolate RP 2.2 (10.34 ± 0.002 $g.l^{-1}$ after 3 days fermentation) and then isolate RP 3.2 and RP 7.2 (10.113 ± 0.012 $g.l^{-1}$ after 5 days and 9.627 ± 0.006 $g.l^{-1}$ after 3 days fermentation).

Genetic Relation Bacteria Isolate

Phylogenetic identification was showed that RP 2.2 include in *Paenibacillus* cluster whereas RP 3.2 dan RP 7.2 include in *Bacillus* cluster (Figure 3). RP 2.2 was identical with *Paenibacillus polymyxa* DYJL38 (99.6 %), whereas RP 3.2 was identical with *Bacillus* sp. X15, *Bacillus* sp. NG06 and *Bacillus methylotrophicus* LZ043 (99.8 %). RP 7.2 was identical with *Bacillus methylotrophicus* JF38, *Bacillus methylotrophicus* -Y48, *Bacillus methylotrophicus* R13, *Bacillus methylotrophicus* SDS11 and *Bacillus amyloliquefaciens* subsp. *plantarum* L12 (99.8 %) (Tabel 8).

Discussion

High Simpson diversity index indicated low of species dominancy at Ranu Pani Lake sediment. High diversity show there is no species domination[25]. Some isolate has no significant with other, because any other condition at sampling site cause different species and live capability[26]. *Bacillus* have optimum temperature and acidity are 10-60 °C and 5-10[27], whereas *Paenibacillus* 10-37 °C and 7[12].

Ranu Pani Lake dissolved oxygen is 5.39 ± 0.7 ppm, this is aerobic condition because more than $mg.l^{-1}$ [28]. *Bacillus* and *Paenibacillus* can growth at aerobic and facultative anaerobic. Those genuses can produce endospore that residence to oxygen and other environment threat[27,12]. Ideal conductivity is 150-500 $\mu s.cm^{-1}$ [29].

Cellulolytic activity of RP 2.2, RP 7.2, RP 3.2, RP 8 and RP 5 are high potency because generally only can utilize 3.6 % or release 0.36 $mg.ml^{-1}$ of reducing sugar[30]. Genus *Bacillus* generally can produce total clear zone (include well) 20-27.5 mm using CMC substrate[31]. *Bacillus subtilis* only can produce clear zone 21 mm after 8 days incubation[32].

Increase of acidity during fermentation period was caused by bacterial acid consumption. Acetic and butyric acid was released by bacteria was consumed again and used to cell and butanol production. This is cause increase of pH fermentation medium[33]. *C. acetobutylicum* ATCC 824 have decrease of pH after 0-12 hours and increase after 48 hour of fermentation time[34]. *Bacillus* sp. 15 have increase of pH after 48 hour until 72 hour[13].

Tabel 3. Similarity bacteria based on 16s rDNA sequences

Bacterial isolate	Identical bacteria	Similarity
RP 2.2	*Paenibacillus polymyxa* DYJL38	99,6 %
RP 3.2	*Bacillus* sp. X15 *Bacillus* sp. NG06 *Bacillus methylotrophicus* LZ043	99,8 %
RP 7.2	*Bacillus methylotrophicus* JF38 *Bacillus methylotrophicus* -Y48 *Bacillus methylotrophicus* R13 *Bacillus methylotrophicus* SDS11 *Bacillus amyloliquefaciens* subsp. *plantarum* L12	99,8 %

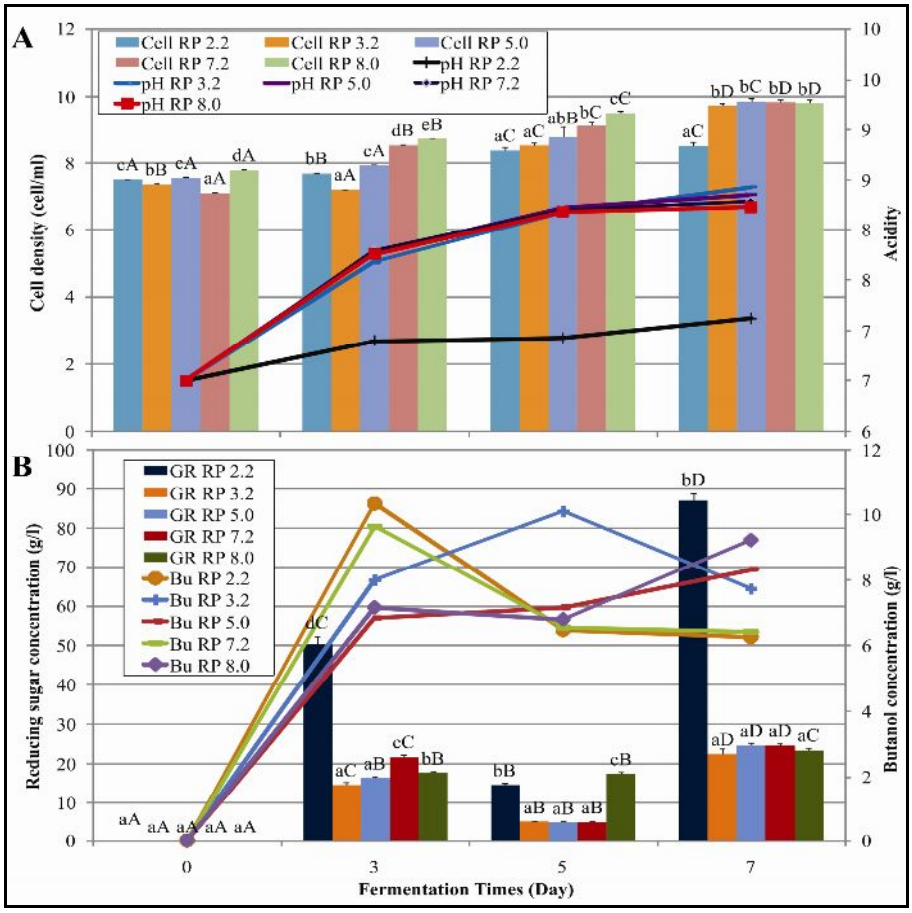

Fig. 2. Acidity, cell density (A), butanol and reducing sugar concentration (B) of bacteria from Ranu Pani Lake sediment.

Decrease of butanol concentration show at paddy straw fermentation by *C. beijerinckii* P260 at 24-72 hours[35]. *Bacillus methylotrophicus* can degradation of *n*-butanol and have degradation efficiency until 20% in basal medium[36].

All isolate was produced of butanol at high concentration. The other hand, *C. saccharoperbutylacetonicum* N1-4 only can produce 0.47 $g.l^{-1}$ butanol from CMC substrate[37]. At the other hand *C. saccharobutylicum* Ox29; *C. saccharobutylicum* Ox44 and *C. beijerinckii* TISTR 1461 can produce 9.2; $g.l^{-1}$ [38] and 7.98 $g.l^{-1}$ [39] butanol from 60 g/l glucose. Using 80 $g.l^{-1}$ glucose *C. acetobutylicum* ATCC 824 could produce 14.2 $g.l^{-1}$ butanol[34] *Bacillus* sp. 15 could produce 10.38 $g.l^{-1}$ butanol under aerob aerobic and microaerobic condition[13]. *B. amyloliquefaciens* NELB-12 could produce 8.9 $g.l^{-1}$ butanol under aerobic conditions[8]. *Paenibacillus* that could produce butanol are *Paenibacillus polymyxa* CR1[9] and *Paenibacillus polymyxa* WR-2[40].

Two species of *Bacillus* (*Bacillus amyloliquefaciens* and *Bacillus methylotrophicus*) similar with RP 7.2. Based on species concept, similarity value more than 99 % are the same species[41,42]. The similar case is purpose of taxonomic revision of *Bacillus amyloliquefaciens* subspesies *plantarum* FZB42T to *Bacillus methylotrophicus* because that strain include in *Bacillus methylotrophicus* claster[43].

Bacillus methylotrophicus only could growth under aerobic condition and could not produce butanol[44,45] but Rp 3.2 and Rp 7.2 isolate could growth under anaerobic condition and could produce butanol. *Bacillus* sp. 15 was isolate from Singapore could produce butanol at aerobic and microaerobic fermentation (10.38 $g.l^{-1}$)[13]. All isolate have negative result at catalase test, but *Bacillus methylotrophicus* and *Paenibacillus polymyxa* are positive[27,12].

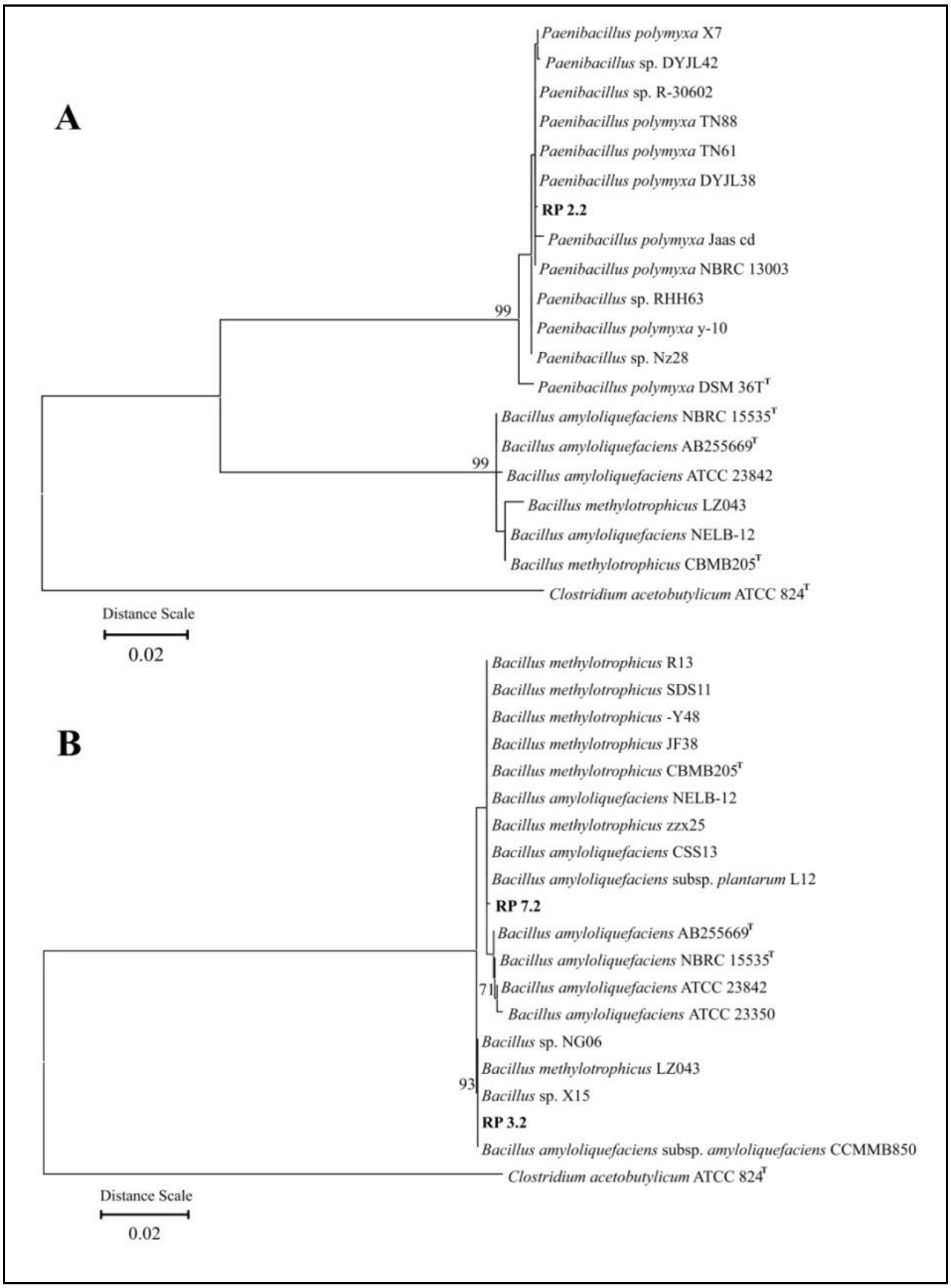

Fig. 3. Phylogenetic tree of RP 2.2, RP 2.3, RP 7.2 from Ranu Pani Lake sediment using *Maximum Likelihood* (Tamura-Nei)

Conclusion

Highest cell density is RP 2.1 (3.6 x 10^3 CFU.g^{-1}) and bacterial diversity is 0.854. RP 3.2 and RP 7.1 isolate has highest semi-quantitative cellulolytic test (1005.84 and 992.42 mm^2). Quantitative cellulolytic test was showed RP 2.2 isolate have highest total cellulolytic activity (0.224 ± 0.01 FPU.ml^{-1}) and resulted 1.21 mg.ml^{-1} reducing sugar concentrations. High butanol concentration was resulted by RP 2.2 (10.34 ± 0.002 g.l^{-1} after 3 days fermentation), RP 3.2 and RP 7.2 (10.113 ± 0.012 g.l^{-1} after 5 days and 9.627 ± 0.006 g.l^{-1} after 3 days fermentation). RP 2.2 was identical with *Paenibacillus polymyxa* DYJL38 (99.6 %), whereas RP 3.2 was identical with *Bacillus* sp. X15, *Bacillus* sp. NG06 and *Bacillus methylotrophicus* LZ043 (99.8 %). RP 7.2 was identical with *Bacillus methylotrophicus* JF38, *Bacillus methylotrophicus* -Y48, *Bacillus methylotrophicus* R13, *Bacillus methylotrophicus* SDS11 and *Bacillus amyloliquefaciens* subsp. *plantarum* L12 (99.8 %).

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