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Genomic and Haplotype Comparison of Butanol Producing Bacteria Based on 16S rDNA

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ABSTRACT

High butanol demand for transportation fuel triggers butanol production development. Exploration of butanol-producing bacteria using genomic comparison and biogeography will help to develop butanol industry. The objectives of this research were butanol production, genome comparison and haplotype analysis of butanol-producing bacteria from Ranu Pani Lake sediment using 16S rDNA sequences. The highest butanol concentrations were showed by *Paenibacillus polymyxa* RP 2.2 isolate (10.34 $g.L^{-1}$), followed by *Bacillus methylotrophicus* RP 3.2 and *B. methylotrophicus* RP 7.2 isolate (10.11 $g.L^{-1}$ and 9.63 $g.L^{-1}$) respectively. *Paenibacillus polymyxa* RP 2.2 showed similarity in nucleotide composition (ATGC) with *B. methylotrophicus* RP 3.2, *B. methylotrophicus* RP 7.2, *P. polymyxa* CR1, *Bacillus amyloliquefaciens* NELB-12, and *Paenibacillus polymyxa* WR-2. *Clostridium acetobutylicum* ATCC 824 showed similarity in nucleotide composition (ATGC) with *Clostridium saccharoperbutylacetonicum* N1-4, and *Clostridium saccharobutylicum* Ox29. The lowest G+C content was *C. saccharobutylicum* Ox29 (51.35%), and the highest was *B. methylotrophicus* RP 7.2 (55.33%). Conserved region of 16S rDNA (1044 bp) were consisted of 17 conserved sequences. The number of Parsimony Informative Site (PIS) was 319 spot and single tone was 48 spot. We found in this study that all of butanol-producing bacterial DNA sequences have clustered to 8 haplotypes. Based on the origin of sample, there were three haplotype groups. Bacteria from group A were could produce butanol 8.9-10.34 $g.L^{-1}$, group B 9.2-14.2 $g.L^{-1}$ and group C was could produce butanol 0.47 $g.L^{-1}$. The haplotype analysis of bacteria based on 16S rDNA sequences in this study could predict capability of butanol production.

Keywords: *16S rDNA, bacteria, butanol, haplotype*

INTRODUCTION

Petroleum limitation gives effect to the development of bio-fuel to fulfill transportation demand. Butanol and ethanol are alternative energy that was highly recommended for transportation. Butanol has higher energy density, lower heating value (LHV), higher hydrophobicity and lower evaporation than ethanol [1]. Butanol can be produced by *Clostridium* [2], *Bacillus*, and *Paenibacillus* [3; 4].

Clostridium acetobutylicum, Clostridium beijerinckii, and *Clostridium saccharoperbutylacetonicum,* are *Clostridia* species that could produce butanol [5]. The number of *Bacillus* and *Paenibacillus* that could produce butanol, there are *Bacillus* sp. 15, *Bacillus amyloliquefaciens* NELB-12 [4; 6], and *Paenibacillus polymyxa* CR1 [3].

Microbial biogeography is essential to predict metabolism and other activity that will give an advantage to human [7]. However, there have not been any analysis on relationship between butanol production and microbial biogeography. The objective of this study is to compare butanol production capability, profile nucleotide composition and analyse haplotype based on 16S rDNA sequences.

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MATERIALS AND METHODS

Collection of the samples

Samples were collected from 5 locations of Ranu Pani edge, Indonesia. Samples stored in ice boxes. Bacterial reference sequences accessed from Genebank NCBI (http://www.ncbi.nlm.nih.gov/). Information on butanol production capability was obtained from some references (Table 1).

Tabel 1. PCR composition material

No.	Solution	Volume (µL)	Concentration
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	Temperature (^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

Isolation of bacteria

Twenty five grams of sediment from Ranu Pani Lake was suspended in 225 ml sterile aquadest. Suspension was diluted serially until 10-6 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 liter, pH 6,5 standardized using 1 N NaOH and sterilized at 115°C, 15 minutes) [8] and incubated at 27°C for 48 hours.

Butanol production

Two and half mililiter of bacterial culture in Thioglicolate medium (was incubated at 30°C, 48 hours) 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. Butanol was measured at the end of fermentation using Gas Chromatography (GC).

16S rDNA isolation and amplification

DNA was isolated from the highest butanol producing isolates using a modified method of Ausubel et al. [9]. Sequences of 16S rDNA were amplified using a couple of primer (27F: 5'-AGAGTTTGATCMTGGCTC-3'; 1492R: 5'-GGTTACCTTGTTACGACTT-3') [10]. Material composition and reaction condition of PCR are shown in Table 1 and Table 2 [11].

16S rDNA purification and sequencing

Purification and sequencing of 16S rDNA sequence performed in First Base, Malaysia. Sequences were submitted to NCBI (KT036393, KT036394, and KT036395).

16S rDNA profiling

16S rDNA was profiled (nucleic acid composition, pattern and conserve sequences) using *Bioedit* and *MEGA 6.06* for *Windows*.

Haplotype

Sequences of 16S rDNA bacteria were analysis haplotype using *DNAsp* 5 and *Haplotype Network* 4.6.1.3 for *Windows*.

RESULTS AND DISCUSSION

Butanol production

There are 13 isolates of bacteria cultured from Ranu Pani Lake sediment. The highest butanol concentrations were resulted by *P. polymyxa* RP 2.2 isolate (10.34 $g.L^{-1}$, in 3 days fermentation) and then *Bacillus methylotrophicus* RP 3.2 and *B. methylotrophicus* RP 7.2 isolate (10.11 $g.L^{-1}$, in 5 days and 9.63 $g.L^{-1}$, in 3 days fermentation) using filter paper substrate. On the other hand, Table 4 shows that *B. amyloliquefaciens* NELB-12 could produce 8.9 $g.L^{-1}$ butanol using 30 $g.L^{-1}$ starches [4]. Using CMC *C. Saccharoperbutylacetonicum* N1-4 could produce 0.47 $g.L^{-1}$ butanol [12] whereas *C. acetobutylicum* ATCC 824 and *Clostridium saccharobutylicum* Ox29 could produce 14.2 $g.L^{-1}$ (using 80 $g.L^{-1}$ glucose) and 9.2 $g.L^{-1}$ (using 60 $g.L^{-1}$ glucose) butanol [13, 14]. There is no information on butanol production capability for *Paenibacillus*. Some references only refer those potential [15, 16].

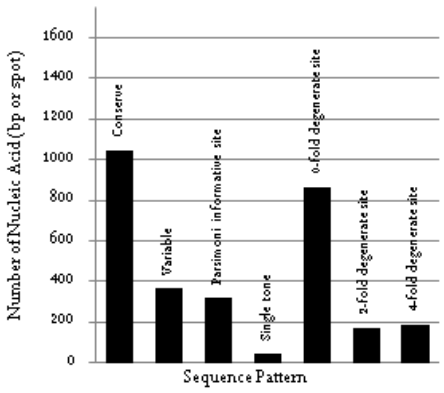

Figure 1. Butanol-producing bacterial 16S rDNA pattern

Table 3. Butanol-Producing bacterial identity

No	Bacterial Species	Source	Accession Number	References
1	*Paenibacillus polymyxa* RP 2.2	East Java, Indonesia	KT036393	This study
2	*Bacillus methylotrophicus* RP 3.2	East Java, Indonesia	KT036394	This study
3	*Bacillus methylotrophicus* RP 7.2	East Java, Indonesia	KT036395	This study
4	*Paenibacillus polymyxa* CR1	Ontario, Canada	KF620436	Eastman *et al.*, 2014
5	*Bacillus amyloliquefaciens* NELB-12	Beijing, China	KF418240	El-Hadi *et al.*, 2014
6	*Clostridium acetobutylicum* ATCC 824	Pennsylvania	U16166	Bayer *et al.*, 2008 Al-Shorgani *et al.*, 2011
7	*Clostridium saccharoperbutylacetonicum* N1-4	Pennsylvania	U16122	Bayer *et al.*, 2008 Al-Shorgani *et al.*, 2011
8	*Clostridium saccharobutylicum* Ox29	Freising, Germany	AM998793	Bayer *et al.*, 2008
9	*Paenibacillus polymyxa* WR-2	Jiangsu, China	KF224925	Eastman *et al.*, 2014

Table 4. Nucleic acid composition and butanol production of butanol-producing bacteria

Bacterial Species	Nucleic acid composition						Butanol production ($g.L^1$)
	T	*C*	*A*	*G*	*%G+C*	*%A+T*	
Paenibacillus polymyxa RP 2.2	19.8	23.5	25.5	31.2	54,70	45.30	10.34
Bacillus methylotrophicus RP 3.2	19.9	23.7	25.0	31.4	55.14	44.86	10.11
Bacillus methylotrophicus RP 7.2	19.8	23.9	24.9	31.5	55.33	44.67	9.63
Paenibacillus polymyxa CR1	20.0	23.6	25.5	30.9	54.51	45.49	NR
Bacillus amyloliquefaciens NELB-12	19.9	23.7	24,9	31.5	55.25	44.75	8.9
Clostridium acetobutylicum ATCC 824	21.3	22.0	27.0	29.7	51.69	48.31	14.2
Clostridium saccharoperbutylacetonicum N1-4	21.5	21.6	26.5	30.4	52.01	47.99	0.47
Clostridium saccharobutylicum Ox29	21.6	21.4	27.0	29.9	51.35	48.65	9.2
Paenibacillus polymyxa WR-2	19.6	23.5	25.5	31,4	54.93	45.07	NR*

*NR: Number of reference

16S rDNA profiling

16S rDNA sequence composition were varied between all bacteria (Table 4). *P. polymyxa* RP 2.2 showed similarity in nucleotide composition (ATGC) with *B. methylotrophicus* RP 3.2, *B. methylotrophicus* RP 7.2, *P. polymyxa* CR1, *B. amyloliquefaciens* NELB-12, and *P. polymyxa* WR-2. *C. acetobutylicum* ATCC 824 showed similarity in nucleotide composition (ATGC) with *C. saccharoperbutylacetonicum* N1-4, and *C. saccharobutylicum* Ox29. G+C content is about 51.35-55.35%. The lowest G+C content was *C. saccharobutylicum* Ox29 (51.35%), and the highest was *B. methylotrophicus* RP 7.2 (55.33%).

The environment plays an active role in shaping GC content, such as surface water vs. soil, and bacteria living in aquatic conditions have an average low GC content (~34%), whereas soil-dwellers have an elevated high GC content (~61%) [17]. *P. polymyxa* RP 2.2, *B. methylotrophicus* RP 3.2, *B. methylotrophicus* RP 7.2 had 54-55% G+C content and isolated from sediment sample.

Sequences of 16S rDNA were formed some patterns, which are conserved region, variable region, Parsimony informative site (PIS), single tone, 0-fold degenerate site, 2-fold degenerate site and 4-fold degenerate site (Figure 1). Conserved region of 16S rDNA (1044 bp) were consisted of 17 conserved sequences. The number of Parsimony Informative Site (PIS) was 319 spot and single tone was 48 spot. The number of 0-fold degenerate site, 2-fold degenerate site, and 4-fold degenerate site as much as 865, 169 and 188. There are 17 conserved sequences (Table 5).

Table 5. Conserve region of butanol-producing bacterial sequences

No.	No. of base		Nucleic Base (5'-3')
	Start	End	
1	45	69	AGCGGCGGACGGGTGAGTAACACGT
2	235	261	CGATGCGTAGCCGACCTGAGAGGGTGA
3	289	313	CCAGACTCCTACGGGAGGCAGCAGT
4	348	369	AGCAACGCCGCGTGAGTGATGA
5	467	503	--CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
6	505	521	GGCAAGCGTTGTCCGGA
7	647	668	GTGTAGCGGTGAAATGCGTAGA
8	682	697	ACCAGTGGCGAAGGCG
9	729	743	GAAAGCGTGGGGAGC
10	745	771	AACAGGAT--TAGATACCCTGGTAGTC
11	773	789	CACGCCGTAAACGATGA
12	859	874	AGTACGGTCGCAAGA
13	937	966	--AATTCGAAGCAACGCGAAGAACCTTACC
14	1028	1100	GACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT CCCGCAACGAGCGCAACC
15	1165	1207	GGAGGAAGGTGGGGATGA-CGTCAAATCATCATGCCCCTTATG
16	1366	1386	CGCGGTGAATACGTTCCCGGG
17	1388	1412	CTTGTACACACCGCCCGTCACACCA

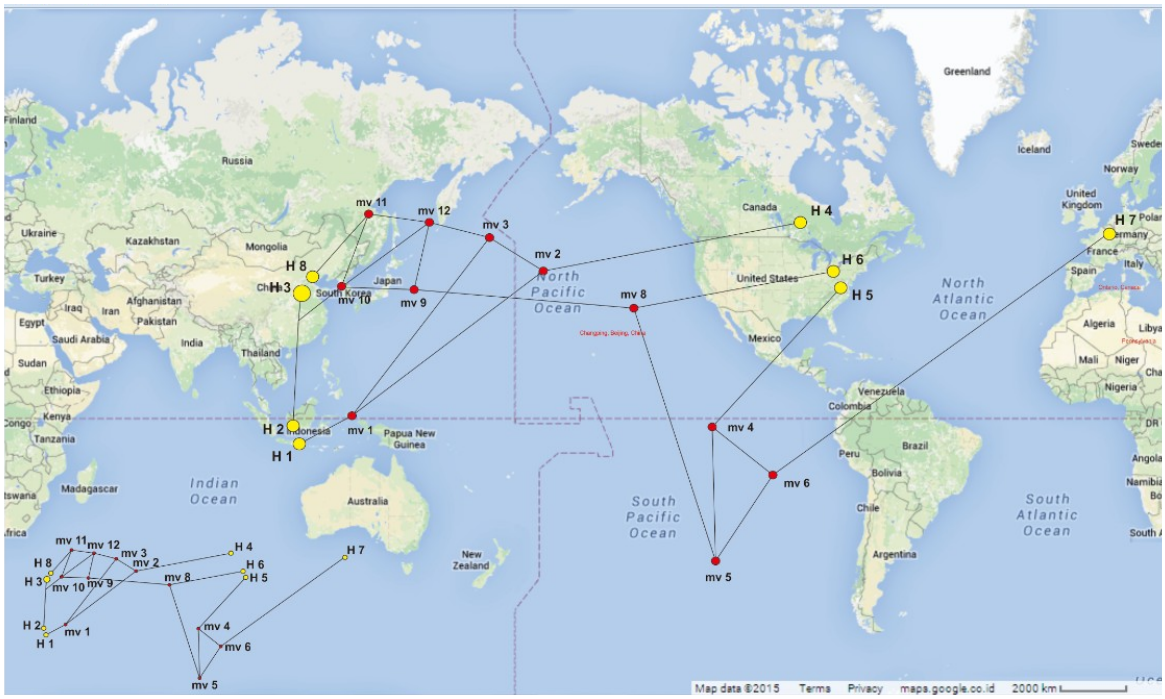

Figure 2. Haplotype network of butanol-producing bacteria (overlay with map)

Table 6. Haplotype of butanol-producing bacterial sequences

Haplotype	*Species of Bacteria*
hap 1	*Paenibacillus polymyxa* RP 2.2
hap 2	*Bacillus methylotrophicus* RP 3.2
hap 3	*Bacillus methylotrophicus* RP 7.2 & *Bacillus amyloliquefaciens* NELB-12
hap 4	*Paenibacillus polymyxa* CR1
hap 5	*Bacillus amyloliquefaciens* NELB-12
hap 6	*Clostridium acetobutylicum* ATCC 824
hap 7	*Clostridium saccharoperbutylacetonicum* N1-4
hap 8	*Paenibacillus polymyxa* WR-2

Haplotype of 16S rDNA

All sequences of butanol-producing bacteria were grouped to 8 haplotypes (Figure 2). Haplotype 3 is consisting of *B. amyloliquefaciens* NELB-12 and *B. methylotrophicus* RP 7.2. Haplotype 1 is *P. polymyxa* RP 2.2, haplotype 2 is *B. methylotrophicus* RP 3.2. Another species were grouped to other haplotypes (Table 6). Based on the origin of sample, there were three haplotype groups. Group A was isolated from Asia (hap 1, 2, 3 and 8), group B was isolated from America (hap 4, 5 and 6) and group C was isolated from Europe (Hap 7) (Figure 2). Bacteria from group A could produce butanol 8.9-10.34 $g.L^{-1}$, group B 9.2-14.2 $g.L^{-1}$ and group C could produce butanol 0.47 $g.L^{-1}$.

Distribution and difference of butanol-producing bacteria was caused by many factors. Bacteria are very influenced by environmental condition, such as temperature, altitude, humidity, gasses, salinity, biotic and other factors [18]. Distance of every ecosystems or micro-ecosystems gives significant of phenotype and genotype characteristics of bacteria [19].

Therefore, even under similar environmental conditions, microbial communities from different placeses might function differently. A better understanding of microbial biogeography is essential to predict such effects. It is also crucial in the search for novel pharma - ceutical and other compounds of industrial importance [7].

CONCLUSIONS

High butanol production were showed by *P. polymyxa* RP 2.2, *B. methylotrophicus* RP 3.2 and *B. methylotrophicus* RP 7.2 isolate. All isolates showed different nucleotid composition. All of butanol-producing bacterial DNA sequences have clustered to 8 haplotypes. The haplotype analysis of bacteria based on 16S rDNA sequences in this study could predict capability of butanol production.

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