

Short Communication: Biochemistry Analysis and Molecular Approach to Identify the Cultured Bacterial from Ex-Tin Mining Lakes

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ABSTRACT

There are two methods to identify the bacterial characteristic, namely biochemical analysis and the 16S ribosomal ribonucleic acid gene (16S rRNA) sequencing analysis. The research aimed to identify the cultured bacterial from ex-tin mining lakes by biochemistry analysis and molecular approach. Nine bacterial were cultured and isolated in nutrient agar and then biochemically characterized by microbact™ 12A and 24E (Oxoid) identification kits. In addition, molecular analysis by 16S rRNA gene was sequenced primer 1492R and primer 27F. Based on biochemistry analysis, these bacterial were identified as belonging to species of *Bacillus amyloliquefaciens*; *Enterobacter gergoviae*; *Enterobacter aerogenes*; *Enterobacter agglomerans*; and *Nitrobacter* spp. The sequence analysis in gene bank of NCBI indicated that these species had similarity with *Klebsiella variicola* strain F2R9 (Accession NR_025635.1); *Enterobacter cloacae* subsp. *dissolvens* strain LMG 2683 (Accession NR_044978.1); *Serratia marcescens* strain NBRC 102204 (Accession NR_114043.1); *Bacillus marisflavi* strain TF-11 (Accession NR_118437.1); *Falsibacillus pallidus* strain CW 7 (Accession NR_116287.1); *Klebsiella pneumoniae* strain DSM 30104 (Accession NR_117683.1); and *Nitrobacter winogradskyi* strain Nb-255 (Accession NR_074324.1). However, phylogenetic tree was constructed by Neighbor-Joining Test showed the cultured bacterial were not in the same clade and also with *Salmonella enterica* subsp. *enterica* strain LT2 (Accession NR_074910.1); *Bacillus amyloliquefaciens* strain BCRC 11601; and *Escherichia coli* strain NBRC 102203 (Accession NR_114042.1) as in group species and *Micrococcus luteus* strain NCTC 2665 (Accession NR_075062.2); *Chloroflexus islandicus* strain isl-2 (Accession NR_148571.2); *Flavobacterium gondwanense* (Accession M92278.1); and *Cytophaga aurantiaca* strain JM110 (Accession MN758870.1) as their out group.

Keywords: bacterial, biochemistry, ex-tin mining lakes; molecular; 16S rRNA gene

ABSTRAK

Terdapat dua metode untuk mengidentifikasi karakteristik bakteri, yaitu analisis biokimia dan analisis sekuensing gen 16S ribosomal ribonucleic acid (16S rRNA). Karakterisasi bakteri telah dilakukan melalui analisis morfologi dan biokimia dan dikonfirmasi melalui pendekatan molekuler menggunakan sekuensing gen 16S ribosomal ribonucleic acid (16S rRNA). Penelitian ini bertujuan untuk mengidentifikasi bakteri yang dapat dikultur dari danau pascatambang timah melalui analisis biokimia dan pendekatan molekuler. Sembilan bakteri berhasil dikultur dan diisolasi di media nutrient agar dan kemudian secara biokimia dikarakterisasi menggunakan microbact™ 12A and 24E (Oxoid) identification kits. Lebih lanjut, analisis molekuler menggunakan gen 16S rRNA dilakukan sekuensing dengan primer 1492R dan primer 27F. Berdasarkan analisis biokimia, bakteri-bakteri tersebut termasuk ke dalam spesies *Bacillus amyloliquefaciens*; *Enterobacter gergoviae*; *Enterobacter aerogenes*; *Enterobacter agglomerans*; dan *Nitrobacter* spp. Analisis blasting pada *gene bank* di NCBI mengindikasikan bahwa spesies-spesies tersebut memiliki kemiripan atau similaritas dengan *Klebsiella variicola* strain F2R9 (Accession NR_025635.1); *Enterobacter cloacae* subsp. *dissolvens* strain LMG 2683 (Accession NR_044978.1); *Serratia marcescens* strain NBRC 102204 (Accession NR_114043.1); *Bacillus marisflavi* strain TF-11 (Accession NR_118437.1); *Falsibacillus pallidus* strain CW 7 (Accession NR_116287.1); *Klebsiella pneumoniae* strain DSM 30104 (Accession NR_117683.1); dan *Nitrobacter winogradskyi* strain Nb-255 (Accession NR_074324.1). Namun, pohon filogenetik yang dikonstruksikan dengan Neighbor-Joining Test menunjukkan bahwa bakteri yang dikultur tersebut tidak berada pada *clade* dan juga dengan *Salmonella enterica* subsp. *enterica* strain LT2 (Accession NR_074910.1); *Bacillus amyloliquefaciens* strain BCRC 11601; dan *Escherichia coli* strain NBRC 102203 (Accession NR_114042.1) yang digunakan sebagai spesies *in group species* maupun *Micrococcus luteus* strain NCTC 2665 (Accession NR_075062.2); *Chloroflexus islandicus* strain isl-2 (Accession NR_148571.2); *Flavobacterium gondwanense* (Accession M92278.1); dan *Cytophaga aurantiaca* strain JM110 (Accession MN758870.1) sebagai *out group*nya.

Kata kunci: bakteri, biokimia, danau pascatambang timah, molekuler, gen 16S rRNA

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1. Introduction

Tin mining activity in Bangka Belitung Archipelago Province produces a water in a mined land looked like a lake, called kolong. The waters have poor characteristics such as low dissolved oxygen (DO) (Ashraf et al. 2011), cation exchange capacity (CEC), poor nutrient and organic component (Oktavia et al. 2014), acidic pH, and also heavy metals contamination (Kurniawan et al. 2019). Tin mining activities can also cause damage to the ecology, include distraction and alteration of microorganisms' ecology and functional stability of microbial community (Kurniawan et al. 2018; Kurniawan, 2016; Li et al. 2014). The ecological damage due to mining activities causes ecosystem imbalance and also changes in the diversity of microorganisms (Lad & Samant, 2015; Giri et al. 2014; Singh et al. 2013; Ashraf et al. 2010; Vyas and Pancholi, 2009; Fan et al. 2002).

The microorganisms' capability to responds the ecosystem changes quickly can be utilized as an indicator to understanding the changes of water quality (Lau & Lennon, 2012; Moscatelli et al., 2005; Niemi & McDonald, 2004; Paerl et al., 2003). This is used to link the relationship between environmental changes to the microbial diversity because they have role in biogeochemical and biotransformation cycles in the biosphere (Gadd, 2010; Prosser et al., 2007). It is important for understanding the microbial community diversity, structures, dynamics, and functional. The existence and biochemical characteristics of microorganisms in an environment can be known by growing them in synthetic medium and identification by 16S rRNA gene. This gene sequence is a biological marker that is widely studied to explain the existence of microbe in an environment, molecular evolution for

taxonomic classification, and microbial phylogenetic analysis. The 16S rRNA gene has a hypervariable region so that it can be used to identify microbes (Yang et al. 2016; Lozupone and Knight, 2008).

This research aimed to identify the diversity of microbes, especially the cultured bacterial from ex-tin mining lakes in Bangka Regency, Indonesia. Identification of bacterial by molecular approach showed name of species based on the gene, besides biochemical characteristics. This research showed the potential of bacterial as a bioindicator and their role in the ecosystem with their characteristics of biochemistry. Further, their capability as bioremediator can be elaborated to remediate and recover the waters quality of abandoned tin mining lakes.

2. Method

2.1. Study area

The research stations were located in Bangka Regency, Bangka Belitung Archipelago Province, Indonesia. The research areas were encoded as Station A (lake < 1 year), Station B (lake 5-10 years), and Station C (lake > 15 years). The coordinates of Station A were 01°59' S in points 36,0"; 36,2"; 36,4"; 36,5"; 36,6" and 106°06' E in points 36.5"; 36.9"; 37.3"; 37.4"; 37.5". The coordinates of Station B were 01°59' S in points 41.3"; 41.4"; 41.5"; 42.4"; 42.5" and 106°06' E in points 39.2"; 39.5"; 41.4"; 42.7"; 43.1". The coordinates of Station C were 01°55' S in points 40.9"; 58.9"; 59.1"; 59.2"; 59.5" and 106°06' E in points 19.5"; 19.7"; 19.9"; 22.4"; 29.2" (Figure 1) (Kurniawan et al. 2018).

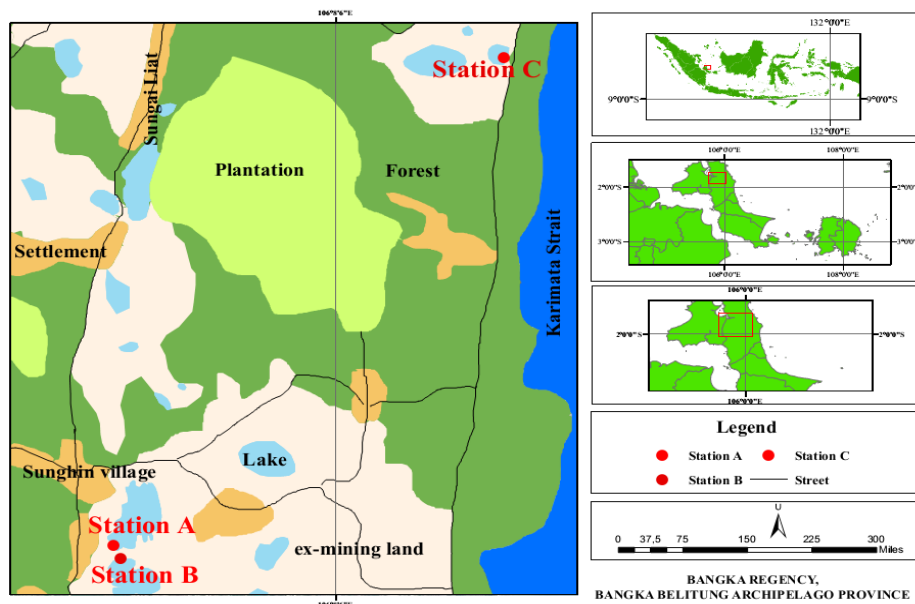


Figure 1 Research location along with the research stations in Bangka Regency, Bangka Belitung Province Archipelago. Station (A) was ex-tin mining lake in < 1 year; Station (B) 5-10 years; Station (C) > 15 years

2.2. Identification of the cultured bacterial

The bacterial of ex-tin mining lakes were isolated by nutrient agar (NA) and showed nine bacterial isolates (Table 1) that were prepared for biochemistry analysis. The biochemical characteristics of cultured bacterial isolates were identified by microbact™ 12A and 24E (Oxoid) identification kits (Osuntokun et al. 2018). While, molecular analysis was done by 16S rRNA gene analysis with primer 1492R (5'GGTTACCTTGTTACGACTT3') as reverse primer and primer 27F (5'GAGTTTGATCAT GGCTCAG3') as forward primer for Polymerase Chain Reaction (PCR). The DNA template was prepared from an individual colony of each species of the cultured bacterial and then the amplification of the 16S rRNA gene was carried out by PCR. The denaturation process in PCR was occurred at 94 °C (2 min), annealing process at 94 °C (1 min) with 35 cycles, and a final extension at 72 °C (3 min) (Senthilraj et al. 2016). The product quality of PCR was visualized by 0.80% agarose gel with amount of DNA ladder loaded per lane 0.1 µg, 1 kb DNA ladder (bp), and volume of sample loaded per lane was 1 µL.

2.3. Sequence analysis

Sequence analysis was carried out by First Base Agent. Sequence alignments were analyzed by Program BioEdit and then were compared with bacterial genes in National Center for Biotechnology Information (NCBI) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

The phylogenetic tree was constructed with Neighbor-Joining Test in Program Mega 6.06. The phylogenetic tree for sequences of samples was constructed and compared with *Klebsiella variicola* strain F2R9 (Accession NR_025635.1); *Enterobacter cloacae subsp. dissolvens* strain LMG 2683 (Accession NR_044978.1); *Serratia marcescens* strain NBRC 102204 (Accession NR_114043.1); *Bacillus marisflavi* strain TF-11 (Accession NR_118437.1); *Falsibacillus pallidus* strain CW 7 (Accession NR_116287.1); *Klebsiella pneumoniae* strain DSM 30104 (Accession NR_117683.1); *Nitrobacter winogradskyi* strain Nb-255 (Accession NR_074324.1); *Salmonella enterica subsp. enterica* strain LT2 (Accession NR_074910.1); *Bacillus amyloliquefaciens* strain BCRC 11601 (Accession NR_116022.1); and *Escherichia coli* strain NBRC 102203 (Accession NR_114042.1) as in group species of Phylum Proteobacteria and Firmicutes of Kingdom Bacteria. While, out group species for the phylogenetic tree were *Micrococcus luteus* strain NCTC 2665 (Accession NR_075062.2) from Phylum Actinobacteria; *Chloroflexus islandicus* strain isl-2 (Accession NR_148571.2) from Phylum Chloroflexi;

Flavobacterium gondwanense (Accession M92278.1) from Phylum Bacteroidetes; and *Cytophaga aurantiaca* strain JM110 (Accession MN758870.1) from Phylum Cytophagia.

3. Result

Mustikasari The biochemistry analysis showed some characteristics of nine cultured bacterial (bac 1, bac 2, and bac 3 from Station A; bac 4, bac 5, and bac 6 from Station B; and bac 7, bac 8, and bac 9 from Station C). Kurniawan et al. (2018) have reported some biochemical characteristics of these cultured bacterial such as gram, oxidase, motility, ornithin, glucosa, indole, Voges-Proskauer (V-P), citrate, and catalase. The other properties of biochemistry was investigated (Table 1) and these characteristics indicated bacterial of *Bacillus amyloliquefaciens*; *Enterobacter gergoviae*; *E. aerogenes*; *E. agglomerans*; and *Nitrobacter* spp.

These cultured bacterial was isolated and identified their DNA with PCR. The product of PCR showed the DNA quality and estimation of base pair (bp) of the 16S rRNA gene were about 1,400-1,500 bp (Figure 2).

The sequence analysis produced profile of sequences and then they were blasted in NCBI. The sequence analysis of 16S rRNA gene showed that the name of bacterial species did not represent the results of biochemistry analysis, there were differences species of these bacterial, although the blast of NCBI website showed that the cultured bacterial had high (90-100 %) similarity with strains were used as in group species. The research evidence revealed species name which analyzed by biochemistry approach to the cultured bacterial were different with blasting investigation in gene bank of NCBI.

The phylogenetic tree (Figure 3) was constructed by involving bacterial of in group species and out group species. All of the cultured bacterial were not in the same clade with in group species such as *K. variicola* strain F2R9; *E. cloacae subsp. dissolvens* strain LMG 2683; *S. marcescens* strain NBRC 102204; *B. marisflavi* strain TF-11; *F. pallidus* strain CW 7; *K. pneumoniae* strain DSM 30104; *N. winogradskyi* strain Nb-255; *S. enterica subsp. enterica* strain LT2; *B. amyloliquefaciens* strain BCRC 11601; and *E. coli* strain NBRC 102203. Further, the phylogenetic tree showed sequences of the cultured bacterial were also different form their outgroup species such as *M. luteus* strain NCTC 2665 from Phylum Actinobacteria; *C. islandicus* strain isl-2 from Phylum Chloroflexi; *F. gondwanense* from Phylum Bacteroidetes; and *C. aurantiaca* strain JM110 from Phylum Cytophagia.

Table 1. Biochemistry Characteristics of Bacterial by Microbact™ 12A and 24E

Biochemistry Characteristics	Research Stations								
	Station A			Station B			Station C		
	bac 1	bac 2	bac 3	bac 4	bac 5	bac 6	bac 7	bac 8	bac 9
Gram*	+	+	-	-	-	-	-	-	-
Spore	+	+	-	-	-	-	-	-	-
Oxidase*	+	+	-	+	-	+	+	+	-
Motility*	-	-	+	-	+	-	-	-	+
Nitrate	+	+	+	+	+	+	+	+	+
Lysin	-	-	+	-	+	-	-	-	+
Ornithin*	-	-	+	-	+	-	-	-	-
H ₂ S	-	-	+	-	-	-	-	-	-
Glucosa*	-	-	+	+	+	+	+	+	+
Mannitol	-	-	+	-	+	-	-	-	+
Xylose	-	-	+	+	+	+	+	+	+
ONPG	+	+	+	-	+	-	-	-	+
Indole*	-	-	-	-	-	-	-	-	+
Urease	-	-	+	-	-	-	-	-	+
V-P*	+	+	+	+	+	+	+	+	+
Citrate*	-	-	+	-	+	-	-	-	+
TDA	-	-	-	-	-	-	-	-	-
Gelatine	-	-	-	-	-	-	-	-	-
Malonate	-	-	-	-	-	-	-	-	-
Inositol	+	+	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-	-
Lactoss	-	-	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-
Salicin	-	-	-	-	-	-	-	-	-
Arginine	-	-	-	-	-	-	-	-	-
Catalase*	+	+	-	-	-	-	-	-	-
Coagulase	-	-	-	-	-	-	-	-	-
Haemolysis	Beta	Beta	Alpha	Alpha	Alpha	Alpha	Alpha	Alpha	Alpha
Sensitive novobiosin	No	No	No	No	No	No	No	No	No
Starch hydrolysis	-	-	-	-	-	-	-	-	-
Casein hydrolysis	+	+	-	-	-	-	-	-	-

Legend: Station A (lake < 1 year), Station B (lake 5-10 years), and Station C (lake > 15 years). The asterisk (*) and biochemistry analysis indicated species *Bacillus amyloliquefaciens* (bac 1); *Bacillus amyloliquefaciens* (bac 2); *Enterobacter gergoviae* (bac 3); *Nitrobacter* spp. (bac 4); *Enterobacter aerogenes* (bac 5); *Nitrobater* spp. (bac 6); *Nitrobater* spp. (bac 7); *Nitrobater* spp. (bac 8); and *Enterobacter agglomerans* (bac 9) (Kurniawan *et al.*, 2018).

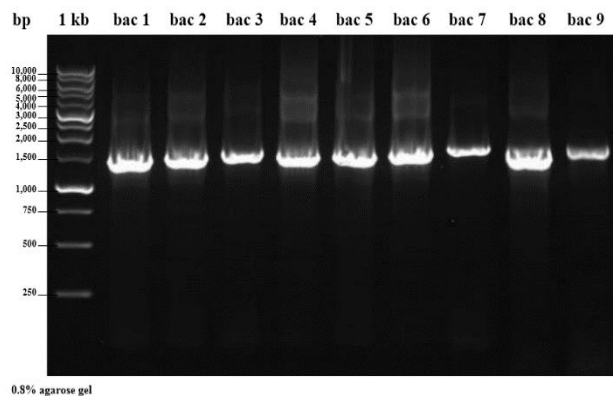


Figure 2 The PCR quality of nine cultured bacterial species for sequencing process



Figure 3 The phylogenetic tree showed that cultured bacterial were different from some species sequences database of NCBI. The biochemistry analysis indicated species *Bacillus amyloliquefaciens* (bac 1); *Bacillus amyloliquefaciens* (bac 2); *Enterobacter gergoviae* (bac 3); *Nitrobacter* spp. (bac 4); *Enterobacter aerogenes* (bac 5); *Nitrobacter* spp. (bac 6); *Nitrobacter* spp. (bac 7); *Nitrobacter* spp. (bac 8); and *Enterobacter agglomerans* (bac 9)

4. Discussion

The biochemistry analysis served as preliminary characterization of bacterial and this identification test gives some information about morphology, physiology, chemistry, and what these microorganisms were able to do with their specific biochemical functions. While, molecular methods are always useful to identify microbes to the species or strain (Franco-Duarte et al. 2019; Bochner, 2009). The biochemical properties of nine cultured bacterial were included as species of *Bacillus amyloliquefaciens*; *Enterobacter gergoviae*; *E. aerogenes*; *E. agglomerans*; and *Nitrobacter* spp. However, they can't be justified enough as those species because the molecular analysis by 16S rRNA gene did not indicate them. Approximately, more than 1400 bp in length of the 16S rRNA genes of nine cultured bacterial were sequenced. Analysis of these sequences confirmed that species were most similar to biochemistry identification species. For examples bac 1 and bac 2 namely species of *Bacillus amyloliquefaciens* by biochemistry analysis showed the different clade with *B. amyloliquefaciens* strain BCRC 11601 (Accession NR_116022.1) in gene bank of NCBI.

The results of all 16S rRNA gene sequencing presented different group with the blasted species.

They were not in the same group or clade, although they had similarity blasting percentage > 90-100% with species of Phylum Proteobacteria and Firmicutes and also so different with the other bacterial from out group species of Phylum Actinobacteria, Chloroflexi, Bacteroidetes, and Cytophagia. The 16S rRNA gene sequence has about 1,550 bp in length and this gene has differentiation at the genus level of bacterial. This gene usually related to more than one individual which the similar sequences (Clarridge, 2004).

The 16S rRNA gene as genetic marker play an important role in identification process of bacterial, the discovery of novel species, taxonomy, and also to construct the bacterial phylogeny (Al Kaabi & Al Yassari, 2019; Manjul & Shirkot, 2018; Woo et al., 2008). Whatever the explanation of this discordance, the discrepancy between these two methods gave important information. Their biochemical characteristics can be explored and elaborated to be used as biological profile of cultured bacterial from ex-tin mining lake. They can be used for various purposes such as bioremediation of ex-tin mining waters ecosystem. It due to their capacity as bioremeditors of heavy metals, wastewater, and organic pollution in the environment (Li et al. 2019; Badiefar et al. 2015; Sonia

et al. 2015; Cardak & Altug, 2014; Raja et al. 2014; Amin et al. 2013; Ogot et al. 2013; Naggat et al. 2010). In spite of their biochemical characteristics indicated their roles, however the further researches are needed to verify them. Based on the analysis of 16S rRNA gene has proven the similarity and relationship of cultured bacterial gene with the other bacterial from gene bank. The results of this study have convinced that the cultured bacterial were different with blasting results. Nevertheless, the advanced researches are also needed to prove them as novel species of bacterial.

5. Conclusion

Thirty-three of biochemical properties from the cultured bacterial were used for identification of them include carbohydrate, amino acid, and lipid utilization or degradation, gram characteristic, motility, sulphuric activity, etc. In this study, species of bacterial from ex-tin mining lakes in Bangka Regency were isolated in NA and identified with microbact test kits. There were nine cultured species from ex-tin mining waters in this study. The biochemistry analysis showed bacterial were identified as belonging to species of *Bacillus amyloliquefaciens*; *Enterobacter gergoviae*; *E. aerogenes*; *E. agglomerans*; and *Nitrobacter* spp. However, phylogenetic tree was constructed by Neighbor-Joining Test showed the cultured bacterial were not in the same clade with the blasted species from gene bank of NCBI. Those bacterial were not similar with some bacterial of gene bank such as species from Genus *Bacillus*, *Enterobacter*, *Nitrobacter*, *Klebsiella*, *Serratia*, *Falsibacillus*, *Salmonella*, and *Escherichia*. They indicated the different clade with them and also with species of Genus *Micrococcus*, *Chloroflexus*, *Flavobacterium*, and *Cytophaga*. In this study, we indicate a new species bacterial were found, although this claim must be proven by further research.

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Conflict of Interest

The author declares that there is no conflict of interest in this publication.

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