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Identification and Characterization of Biosurfactant Producing Bacteria *Arthrobacter* sp. P2(1)

Fatimah[1,2]*, Suharjono[2], Tri Ardyati[2], Ni'matuzahroh[1], Afaf Baktir[3] and Ahmad Thontowi[4]

[1]Department of Biology, Faculty of Science and Technology, University of Airlangga, Indonesia.
[2]Biology Doctoral Program, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Indonesia.
[3]Department of Chemistry, Faculty of Science and Technology, University of Airlangga, Indonesia
[4] Research Center for Biotechnology, LIPI, Cibinong, Indonesia

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The aims of this research were to identify and characterize bacteria isolated from petroleum contaminated soil, P2(1) isolate. This isolate was able to grow on glucose containing medium and produce biosurfactant. This isolate was identified by morphological, physiological and biochemical characteristics and phylogenetic analysis by 16S rDNA sequences. Morphological characteristics observed were colony and cell morphology. Physiological characteristics were observed by microbact kit 12A, 12B. Phylogenetic tree was constructed by comparing 16S rDNA sequences. The results showed that P2(1) isolate was classified into Genus *Arthrobacter* based on some characteristics, Gram negative in young culture and Gram positive in old culture, rod coccus cycle, and has cream colony on Nutrient Agar medium. The phylogenetic analysis of 16S rDNA sequences showed that P2(1) isolate has similarity with *Arthrobacter* sp. XBGRY2 (HQ891968.1) with 95% similarity. It can be concluded that the isolate was identified as *Arthrobacter* sp. P2(1) (KU361211).

Key words: *Arthrobacter* sp. P2(1), characteristics of isolate, phylogenetic analysis, biosurfactant.

One of the factors affecting biodegradation of oil waste in the ground and water by microbes is the low solubility of oil in water. Hydrophobicity of oil would reduce cell contact of microbes with substrate (oil). The Efforts to increase oil solubility, among others, by adding a biosurfactant. Biosurfactants or microbial surfactants are surface-active biomolecules produced by a variety of microorganisms when grown on water miscible or oil substrate[1]. Biosurfactant are amphiphilic compounds produced on living surfaces, mostly on microbial cell surfaces. This compound is excreted extracellularly and contain hydrophobic and hydrophilic moieties that confer the ability to accumulate between fluid phases, thus reducing surface and interfacial tension at the surface and interface respectively[2]. Biosurfactants are important to enhance oil recovery, environmental bioremediation, food processing and pharmaceuticals owing to their unique properties such as higher biodegradability and lower toxicity. Interest in the production of biosurfactants has steadily increased during the past decade[3].

Biosurfactants producing strains is one of the factors affecting the production of

* To whom all correspondence should be addressed.
E-mail: fatimahyusuf25@yahoo.com

biosurfactants. Efforts in the discovery of new biosurfactant producing microbes has been done by many researchers with various methods.The findings focused on obtaining new strains with great production capability and high yield.

Indonesia with a very high biodiversity has a great opportunity to develop local production of microbial surfactants. Researcher before successful[4] in exploring oil degrading microbes from petroleum contaminated soil at oil drilling in Wonocolo village, Kedewan district, Bojonegoro regency, and Pertamina Depo, Tanjung Perak Surabaya, East Java, Indonesia. Thirteen bacteria isolates and three yeasts have been isolated. Further[5], tested the ability of these isolates to produce biosurfactant by measuring the surface tension of culture supernatant and emulsification activity assay using a variety of hydrocarbon (kerosene, diesel, edible oil, and crude oil). The results showed that there were ten isolates, included P2 (1) isolate (that was used in this research), able to decrease surface tension and emulsify several hydrocarbons. P2(1) isolate could decrease surface tension of supernatant from 72 mN/m into 43mN/m. This isolate could emulsify diesel oil until 31.2%. P2(1) isolate can produce biosurfactant when grown on several substrates hydrocarbons such as diesel oil, palm oil, kerosene and crude oil. P2(1) isolate is one of the potential candidates for developing biosurfactant producer. The biosurfactant produced has properties as bioemulsifier and surface active agent[4, 5]. These properties are characteristics of a biosurfactant that can be used in various industrial applications and environmental protection technology (bioremediation). The objectives of this research were to identify and characterize P2(1) isolate based on morphological characteristics, biochemical assay, and phylogenetic analysis using 16S rDNA as a molecular marker.

MATERIALS AND METHODS

Bacterial Isolate

P2(1) isolate was obtained from microbiology laboratory's collection of Biology Department in Airlangga University and stored in Airlangga University Culture Collections. This isolate was isolated from petroleum contaminated soil at oil drilling in Wonocolo village, Kedewan district, Bojonegoro regency, East Java, Indonesia.

Morphological Characterization

Morphological characteristics of bacterial colony and cell were observed under microscope.

Physiological Characterization

Physiological characteristics of bacteria were observed using microbact identification kit 12A, 12B (Oxoid) and analyzed by software of Microbact 2000 program.

Culture Characterization

Bacteria was grown in 20 ml Nutrient Broth medium with various condition of temperature 20-50°C, pH 5-8, and salinity (1- 4%). These cultures were incubated in orbital shaker (GFL, 3015) with 150 rpm for 24 h. Bacterial growth was monitored by measuring turbidity at 450 nm wave length using spectrophotometer (Thermoscientific, Genesys 20).

Genomic DNA Extraction

Genomic DNA was isolated using protocol of Sigma's GenElute Bacterial Genomic DNA Kit. DNA obtained was amplified using PCR. PCR reaction (50 µl volume) consisted of 25 µl Q5 High Fidelity 2x Master Mix, 2.5 µl (10 µM) 27f (forward), 2.5 µl (10 µM) 1492r (reverse), 2 µl DNA template (30.67 ng/µl), and 18 µl nuclease free water. The PCR conditions consisted of initial denaturation (98°C, 30 s), 35 cycles of 98°C (10 s), 65°C (20 s), and 72°C (45 s), with a final extension for 2 minutes. PCR product was detected using electrophoresis (Biorad gel electrophoresis) in 1% agarose gel, 100 volts, 40 minutes and visualized using gel illuminator.

DNA Sequencing and Sequence Analysis

PCR product was purified using GenElute™ Gel Extraction Kit Sigma Aldrich, further it was sequenced using Big Dye® Terminator v3.1 Cycle Sequencing Kit. The sequence of 16S rDNA was analyzed using an automatic machine DNA sequencer (3730*xl* DNA Analyzer, Thermo Fisher Scientific). Then, sequence of 16S rDNA was compared with database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using BLAST program[6]. Sequence alignment was conducted using ClustalW program[7], while phylogenetic tree was constructed using Neighbor Joining plot, Mega 6.06[8].

RESULTS AND DISCUSSION

Morphological Characteristics

Based on morphological colony, P2(1) isolate has circular shape, convex, cream colour, shiny, 2-4 mm in diameter (Fig. 1-A; 1-B), while cell characteristics were rod-coccus cycle shaped, Gram negative at 16 h incubation (Fig.1-C), Gram positive at 3 days incubation (Fig. 1-D).

Physiological Characteristics

Based on physiological characteristics using microbact kit (Table 1), P2(1) isolate has probability 67.61% similar to lead-*Acinetobacter faecalis* type II. The probability is very low, so this isolate has not been identified.

The Genus *Arthrobacter* isolated from soil and appeared as Gram negative rods in young cultures and as Gram positive cocci in older cultures[9]. In addition to their morphological characteristic, members of Genus *Arthrobacter* are originally described as highly aerobic bacteria, nutritionally non exacting, and capable of liquefying gelatin slowly[10]. However, this identification based only on growth cycle and Gram staining, it may be mistaken with other genera such as *Brevibacterium* and *Rhodococcus*[11]. So it needs another characteristic to ensure the result.

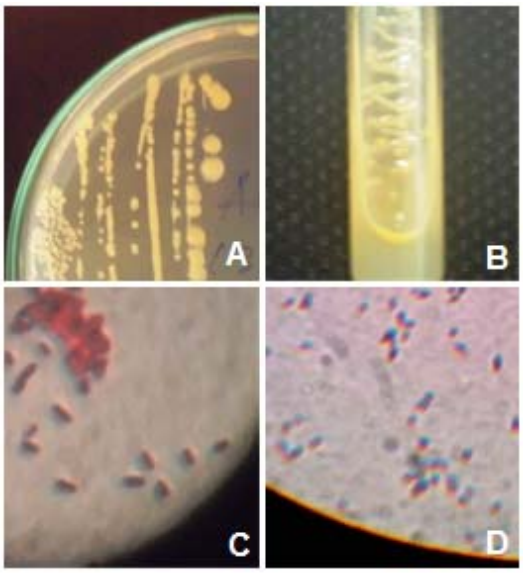

Fig. 1. Morphological characteristics of colony and cell of P2(1) isolate. (A: colony on Nutrient Agar plate, B: colony on Nutrient Agar slant, C: rod shape, Gram negative, 16 h on Nutrien Broth, D: coccoid shape, Gram positive, 3 days on Nutrient Broth), M=1000x.

Culture Characteristics

Figure 2-A showed that P2 (1) isolate grown on medium with range of pH 5-8. The optimum growth was showed at pH 8 with a generation time is 66 minutes/generation. Meanwhile, the growth of bacteria was inhibited at pH 5-6, with a generation time of 120 and 168 minutes respectively. The P2 (1) isolate grown well in the range of 1-4% salinity. Its optimal growth was observed at 1% salinity for 60 minutes in generation time (Figure 2-B). It also survived in the range of 30-40°C, with an optimum temperature at 40°C.This Bacteria could not grow at temperature 20 °C and 50 °C (Figure 2-C).

In contrast to animals or plants, the determination of species in prokaryotes is not easy. Parameters that can easily be used for other groups of organisms are difficult to apply in prokaryotes. Prokaryotes have a special character, microscopic and has a relatively simple structure[12]. Morphological characteristics are rarely used to

Table 1. Physiology characteristics of P2(1) Isolate

No.	Characteristics	Observations
1.	OXI (Oxidase)	+
2.	MOT (Motility)	+
3.	NIT (Nitrate Reduction)	-
4.	LYS (Lysine Decarboxylase)	-
5.	ORN (Ornithine Decarboxyl)	-
6.	H_2S (H_2S Production)	-
7.	GLU (Acid from Glucose)	-
8.	MAN (Acid from Mannitol)	-
9.	XYL (Acid from Xylose)	-
10.	ONP (ONPG)	-
11.	IND (Indole)	-
12.	UR (Urea Hydrolysis)	-
13.	VP (Voges Proskauer)	-
14.	CIT (Citrate Utilization)	-
15.	TDA (Tryptophan Deaminase)	-
16.	GEL (Gelatin Liquefaction)	-
17.	MAL (Malonate Inhibition)	-
18.	INO (Acid from Inositol)	-
19.	SOR (Acid from Sorbitol)	-
20.	RHA (Acid from Sorbitol)	-
21.	SUC (Acid from Sucrose)	+
22.	LAC (Acid from Lactose)	-
23.	ARA (Acid from Arabinose)	-
24.	ADO (Acid from Adonitol)	-
25.	RAF (Acid from Raffinose)	-
26.	SAL (Acid from Salicin)	-
27.	ARG (Arginin Dihydrolase)	-

characterize prokaryotes because of the simplicity of cell structure[13].

In contrast to the morphology, physiology of prokaryotes group is very complex and diverse. Physiological phenotypes are commonly used as parameter for determining species of prokaryotes. Metabolic pathway can describe the evolution distance of prokaryotes groups. However, this approach is difficult to do because it requires cultured bacteria. It is estimated that only about 1% of all prokaryotes in nature that can be cultured in laboratory. Genomics approach is more accurate for species determination. This approach allows species identification of cultured bacteria. Among the various techniques used[14], 16S rDNA analysis is widely used[14,15]. 16S rDNA is important component in protein synthesis because its function is stable, widely distributed in the cell, and stored on a broad range organisms. According to these reasons, 16S rDNA analysis for bacteria identification become precise option supported with availability of database and amplification using PCR[16].

Difference of 16S rDNA sequences is used to determine evolution distance, so it can be used as a good evolutionary chronometer. 16S rDNA has a few areas which consist of nucleotide base are varied and relatively conserved. Variation of conserved sequences is useful for constructing universal phylogenetic tree because it reflect the

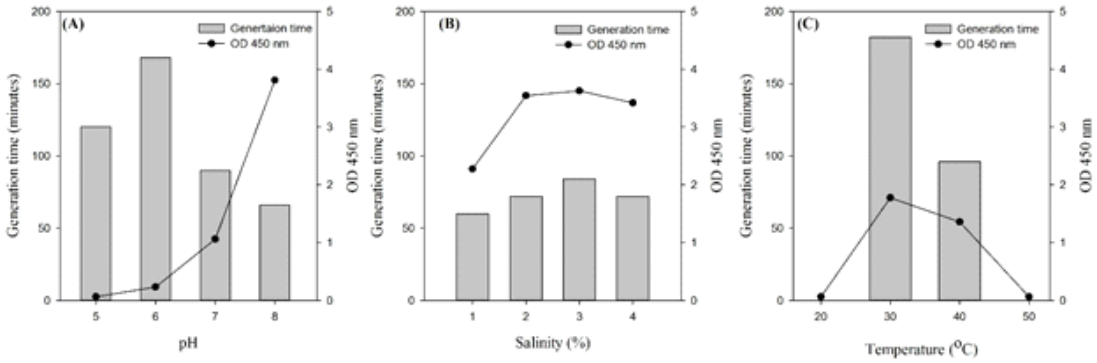

Fig. 2. Generation time and Optical Density (OD) of P2(1) isolate on Nutrient Broth medium with variation of pH (A), salinity (B), and temperature (C)

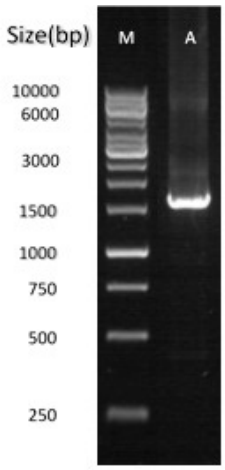

Fig. 3. Electrophoregram of 16S rDNA in 0.8 % agarose gel. M= 1kb DNA Ladder (bp) ; A= 16S rDNA of P2(1) isolate

chronology of the earth evolution. It can be used to track the diversity and determine strains within a species. If 16S rDNA sequence show low degree of similarity between two taxa, description of a new taxon can be done without DNA-DNA hybridization[14]. The similarity of sequence which less than 97%, it can be considered as a different species.

Definition of prokaryotes species is based on the genetic and phenotypic parameters to describe relatedness in phylogeny. Phylogenetic species concept is more suitable to be applied in prokaryotes than biological species because this group reproduce asexually. Definition of prokaryotes species widely accepted is restricted to isolate with high degree of similarity in many independent characters, especially if it is coherent in genomic[12].

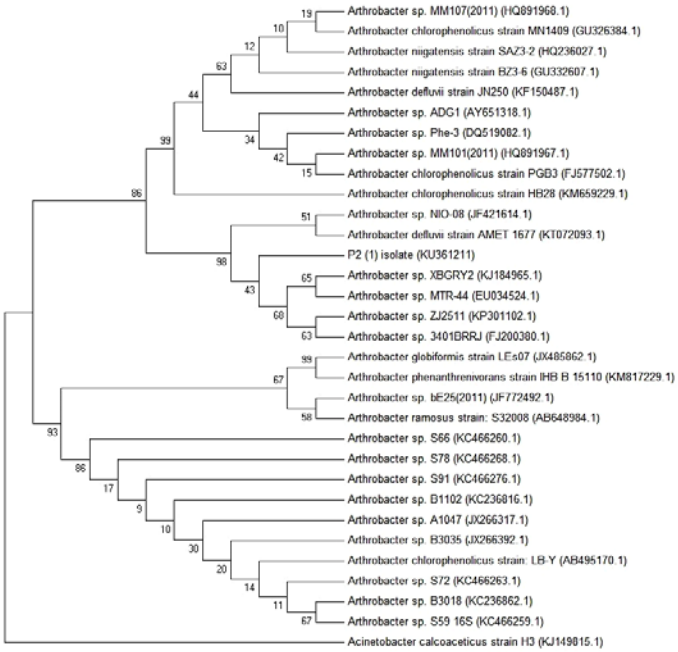

Fig. 4. Phylogenetic tree based on nucleotide sequences alignment of partial 16S rDNA of P2(1) isolate. *Acinetobacter calcoaceticus* strain H3 (KJ149815.1) was used as out group species.

16S rDNA amplification result of P2(1) isolate showed that the length of sequences approximately 1500 bp (Figure 3). BioEdit program resulted the sequences was about 1401 bp. Based on BLAST analysis, 16S rDNA sequences of isolates P2(1) (KU361211) had 95% similarity to *Arthrobacter* sp. XBGRY2[17]. Sequence similarity of 97% classify two isolates in the same species. P2(1) isolate has less than 97% similarity with other species of Genus *Arthrobacter*, hence it is a different species. However, these allegations should be tested using several methods of identification. Further, phylogenetic tree was constructed to trace relatedness P2(1) isolate compared with other prokaryotes in the database (Figure 4)[18].

CONCLUSIONS

Based on morphological and physiological characteristics and phylogenetic analysis, P2(1) isolate which able to produce biosurfactant has 95 % similarity with *Arthrobacter* sp. XBGRY2 (HQ 891968.1). The isolate was classified into Genus *Arthrobacter* and identified as *Arthrobacter* sp. P2(1) (KU361211).

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REFERENCES

1. Mukherjee, S., Das, P., Sen, R. Towards commercial production of Microbial surfactants. *TRENDS in Microbiology*. 2006; **24**(11): 509-5015
2. Karanth, N.G.K., Deo, P.G., and Veenanadig, N.K. Microbial production of biosurfactants and their importance. *Current Science*, 1999; **77**: 116–123.
3. Muthusamy, K., Gopalakrishnan S, Ravi T.K., and Sivachidambaram P. *Current Science*, 2008; **94**: 6.
4. Ni'matuzahroh, Alami, N.H., Fatimah, Supriyanto, A., Affandi, M. Exploration of Polyaromatic Hydrocarbonoclastic Microbes From Oil Polluted Soil. *Proceeding* 10th Congress and International Conference of Indonesian Society for Microbiology, 2009; Surabaya.
5. Fatimah, Ni'matuzahroh, Alami, N.H.,

Supriyanto, A., Affandi, M. Screening of biosurfactant production of hidrocarbonoclastic microbes isolated from oil polluted soil. *Proceeding* 10th Congress and International Conference of Indonesian Society for Microbiology, 2009; Surabaya.

6. Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, 1997; **25**(17): 3389–3402.
7. Higgins, D.G., Sharp, P.M. CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. *Gene*, 1988; **73**: 237-244.
8. Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution*, 2013; **30**: 2725-2729.
9. Conn, H.J., A type of bacteria abundant in productive soils, but apparently lacking in certain soils of low productivity. *New York State Agricultural ExperimentalStation Technical Bulletin*, 1928; **138**: 3–26.
10. Conn, H.J., Dimmick, I. Soil bacteria similar in morphology to *Mycobacterium* and *Corynebacterium*. *Journal of Bacteriology*, 1947; **54**: 291–303.
11. Jones, D., and Keddie, R.M. The Prokaryotes. Springer New York, 2006; 945–960.
12. Rosello-Mora, R., and Amann, R. The species concepts for procaryotes. *FEMS Microbiology Review,* 2001; **25**: 39-67.
13. Pangastuti, A. Definisi Spesies Prokaryota Berdasarkan Urutan Basa Gen Penyandi 16S rRNA dan Gen Penyandi, *Biodiversitas*, 2006; **7**(3): 292-296
14. Stackebrandt, E., and Goebel, B.M. A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *International Jurnal of Systematic Bacteriology*, 1995; **44**: 846-849.
15. Bottger, E.C. Rapid determination of bacterial ribosomal RNA sequences by direct sequencing of enzymatically amplified DNA. *FEMS Microbiol. Lett.*, 1989; **65**: 171-176.
16. Pace, N. A molecular view of microbial diversity and the biosphere. *Science.*, 1997; **276**: 734-740.
17. Hagstrom, A., Pommier, T., Rohwer, F., Simu, K., Stolte, W., Svensson, D., and Zweife, U.L. Use of 16S ribosomal DNA for delineation of marine bacterioplankton species. *Appl. Environ. Microbiol.*, 2002; **68**: 3628-3633.
18. Schloss, P.D., and Handelsman, J. Status of the microbial census. *Microbiol. Mol. Biol. Rev.*, 2004; **68**: 686-691.