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Article

Bacteria producing hopanoids from crude oils: The route for using microorganism as crude oil and sediments marker

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ABSTRACT

The presence of specific geochemical hopanoids has been recently observed in some living organisms. In this work, bacteria from crude oils reservoir (a part of mature sediments) has been isolated and identified. Their hopanoids have been screened. Hopanoids are only detected in bacteria from the depth reservoir (high temperature and pressure), namely Kawengan and Bangko reservoir. Bacteria from Kawengan crude oils produce an α,β-methy hopane, a compound that usually serve as a main marker for sediment maturation. Bacteria from Bangko crude oils posses several ordinary biohopanoids and one geochemical hopanoid, an acid hopanoid with a double bound at C-17 and C-21. According to 16S rRNA data, the two bacteria are respectively close to *Streptomyces* species and *Alicyclobacillus* species.

| Hopanoids | Bacteria | Sediment marker |

1. Introduction

Hopanoids were first detected in geological material in crude oils and sediments by the group of Prof. Ourisson from University of Louis Pasteur, France, in 1969. The study was soon followed by others groups around the world. Many of them focused their research on the structure diversity of the sedimentary hopanoids and others on the archeological origin of the hopanoids.

The peak of the researches occurred in 1974. In this year, the former group (Ourisson's group) stated a correlation between the presence of hopanoids stereoisomers and the sediment maturation. They showed that the less stable hopanoids, assigned as ββ-hopanoids, were only found in living organism and young (immature) sediments. Whilst, the most stable stereoisomers, αβ-hopanoids are only detected in old (mature) sediments, never in living organisms. Based on these facts, they proposed a theory of the stereoisomers transformations that αβ-hopanoids are derived from ββ-hopanoids stereoisomers during psycho-chemicals processes [1]. Hence, the αβ-hopanoids were then widely utilized as a marker for recognizing mature sediments (including crude oils).

After the brilliant research, the study of geological hopanoids is relatively stagnant. Most of the studies concerned the correlation between sediments by means hopanoid markers at specific area (e.g., at Pacific alongside the Japanese Islands). In contrast, researches on biological hopanoid increased. One of the best results was also performed by the same group. Based on the discovery an extended hopanoid, named

bacteriohopanetetrols, in prokaryotes, they proposed that the geological hopanoids are of microbial origin [2]. The research led then to revolutionary theory for isoprenoids biosynthesis: a novel pathway for isoprenoids biosynthesis [3]. This topic became a famous topic for most in triterpenoids chemistry. Among the interest are the correlation between the pathway and pathogenesis of bacteria [4] and the reconstruction of the pathway [5, 6, 7].

The geological hopanoids was revisited after the research of Poralla's group from Germany. They showed that some cyclic triterpenes which is predicted to be not synthesized by living organisms are found in bacteria *Alicyclobacillus acidocaldarius* [8]. This phenomenon indicate that the bacteria have the capacity to synthesize cyclic triterpenes in which the structures are similar than those of non-biological hopanoids. The proof of this hypothesis was given by our unexpected result. Here, the α,β-hopanoids which is claimed as a specific geological hopanoids can also be identified in *Frankia*, a nitrogen-fixation bacteria, in a significant quantity [9]. The bacteria itself reveals as the best bacteria producing hopanoids which has been ever analyzed [10].

Both experiments, Poralla's and our experiment, suggested a new mechanism for squalene cyclization, the main step of hopanoid biosynthesis in living organism, apart of the old Rudzizca rule. However, the suggestion is not yet experimentally confirmed. A set of experiment will be needed to detect any intermediate in the proposed mechanism.

The appearance of α,β-hopanoids in *Frankia* must influence the theory of sediment maturation. Microbial activities must be considered as one of the main factors apart from psycho-chemical processes in transformation of hopanoid compounds. In other word, it can be proposed that some geological fossil resources contain microorganisms producing hopanoid.

In this report, we show the result of our experiment on crude oils taken from four reservoir of petrol. Two reservoirs are the deep reservoirs (located at Kawengan, Center of Java and Bangko, Jambi, Indonesia). The two others are the ground surface reservoir. The two deep reservoir contain bacteria which are capable to synthesize hopanoids. Whereas the two others gave a negative results.

2. Experimental

Isolation of bacteria from crude oil reservoir

Crude oil sample (2 % w/v) was incubated for 7 days at 50 °C in oil enriched (2 %) NB (Nutrient Broth) Difco media. The culture was agitated on 120 rpm. Microbial cells resulted from the culture was then diluted with physiological salt (NaCl 0.85 %) by the factor of 10^{-1} to 10^{-6}. One ml of each suspension was streaked on petri-dishes containing oil enriched (2 %) NA (Nutrient Agar) Difco media. The cultures were leaved for 3 days at 40 °C. Each different colony was transferred to the fresh NA media. Each pure culture was prepared to be identified.

Identification of the pure culture

Unknown bacteria was identified by Gram and Spore staining methods by physiological test, by morphological observation and 16s r-RNA sequencing. Physiological test involves catalase test, oxydase test and motility test. In catalase test, two drops of H_2O_2 3 % was put on glass-plate and mixed by one ose of the culture. Positive result was observed when the reaction produces the bubbles of oxygen. In oxydase test, bacteria colony was emerged by dimetil-p-fenildiamin hydrochloride (1 %). Positive result was marked by the subsequent alteration of colony's color (from pink to black). Lastly, in mobility test, the colony of the pure culture was grown on the tube containing SIM media and then incubated for 24 h at 40 °C. Sequencing of 16s r-RNA was performed by Laboratory of PPAU Bioteknologi ITB Bandung.

Identification of hopanoids of isolated bacteria

The identification was performed on predicted colonies of crude oils reservoir which are capable to produce hopanoids and on *Bacillus polymyxa* which has been isolated by Megga group from ITB Bandung.

Each different colony was grown on the oil enriched NB media (1 L) for 11 h at 50 °C and 120 rpm. Cells was harvested by centrifugation (6000 rpm, 30 minute, 4 °C) and followed by lyophilization (12 h, -84 °C, 0.001 atm). Lyophilized cells were then treated by Hopanoids Screening Degradation Methods as follow: Lyophilized cells (0.1 g) were extracted by 50 ml chloform-methanol (2:1, v/v) at 50 °C. The solvent was evaporated and the residue was reacted with H_5IO_6 (100 mg) for 1 h in the mixture of 3 ml THF-water (4:1, v/v). The reaction was stopped by the addition of 10 ml of aquadest. Product of the reaction was extracted with 30 ml hexane. The later solvent was then evaporated and the residue was reduced by $NaBH_4$ (100 mg) in ethanol solution (3 ml) for 1 h 30 minute at room temperature. The reaction was quenched by KH_2PO_4 100 mM (7 ml) and extracted with 30 ml hexane. Shorted alcoholic hopanoids was obtained after evaporating of hexane. The later product was then acetylated with Ac_2O/pyridine (1:1, v/v, 0.1 ml) for the night. Acetylated product was obtained after evaporating the mixture of Ac_2O/pyridine by means nitrogen flow. Fatty acids, phospholipides and other triglycerides were separated by small column containing silica gel Merck G-60 using CH_2Cl2 as a eluent. The presence of hopanoids was detected by TLC (Silica Gel of Merck 60 PF_{254}, eluent : cyclohexane/EtOAc (9:1, v/v), staining reagent, the alcoholic solution of berberin chloride). The number of hopanoid compounds was analyzed by GC method, with the condition: Unknown structure of the derivative hopanoids were identified by GCMS methods (EI, 70 eV) according to the profile of hopane specific fragments [1].

3. Results and Discussion

Bacteria from Kawengan reservoirs

Reservoir from Kawengan, Cepu, yields two different colonies of bacteria. Characteristic of the colonies and their cells are figured in Table 1. It can be seen that the two colonies have a similar characteristics. They can only be distinguished by the form of colony's extremity and colony's surface. The differences should be used for recognizing the specific species.

According to Bergey's Manual of Determinative Microbiology, all of the colony's character is *Bacillus* characters. The presence of catalase and oxydase mean that the two colonies posses strictly respiratory metabolism. The motility reflects that the ability of the bacteria in moving as a response of environmental change.

Table 1 Characteristic of Colonies from Kawengan Reservoir

Parameter	Colony-1	Colony-2
form of colony	disorder	disorder
Color	White	white
form of colony's extremity	Wave	plate
colony's surface	not smooth	bright
form of cell	Rod	rod
Gram's staining	Positive	positive
endospore	central/ellipse	central/ellipse
activity of catalase	Positive	positive
activity of oxydase	Positive	positive
Motility	Positive	positive

However, according to the result of the 16s r-RNA analysis, there is no species of *Bacillus* from NCBI data having 100 % of sequence r-RNA similarity. This reflects that the two colonies are the new species of *Bacillus*, so that they are signed as *Bacillus* sp(1) and *Bacillus* sp(2), or the completely different genus.

Preliminary TLC test on lipid extract of the two *Bacillus* reveals that the two *Bacillus* produce triterpenoid compounds. Triterpenoids from *Bacillus* sp. (1) lay on five spots (R_f = 0.13, 0.30, 0.43, 0.53 and 0.74). Triterpenoids from *Bacillus* sp (2) give three spots (R_f = 0.25, 0.65 and 0.74).

After degraded by periodic acid and sodium borohydrat, these triterpenoids are obtained in different R_f of the same TLC condition. Triterpenoids from *Bacillus* sp (1) are situated on R_f = 0.40, 0.72 and 0.94 and those of *Bacillus* sp(2) have found on R_f = 0.15, 0.76 and 0.86.

Three TLC spot of triterpenoids from *Bacillus* sp(1) produce four pairs peak on GC chromatogram. The four pairs have similar pattern. This indicates that the pairs are sterochemical relationship. The same phenomena have been observed on *Frankia* hopanoids (Rosa Putra *et al*., 2001). However, according to the MS spectra, no one of the peaks related to hopanoid compounds. One of the important fragment of hopanoids (m/e = 191 and its counterpart, 369) can not be seen on the spectra. The trace quantity of the compounds (0.2 mg/g dry cells) arise the difficulty for other spectroscopy analysis (e.g. NMR analysis).

In contrast, *Bacillus* sp(2) can synthesize surprisingly hopanoid compounds. Only the more polar fraction of TLC gives a peak on GC chromatogram. The others fraction are too small due to the trace quantity of the compounds. MS spectra showed three important fragment of hopane, m/e = 177, 191 and 205. This fragment relate to disruption of pentacyclic skeleton of methyl hopane. The proposed fragmentation of α,β-hopane is shown in Figure 1.

Considering the high intensity ratio of fragment 205 and fragment 191, it can be concluded that the hopanoid is a part of α,β-stereoisomer series. Unfortunately, the molecular ion of the hopane (m/e = 426) and pentacyclic fragment (m/e = 382) does not present due to their instability.

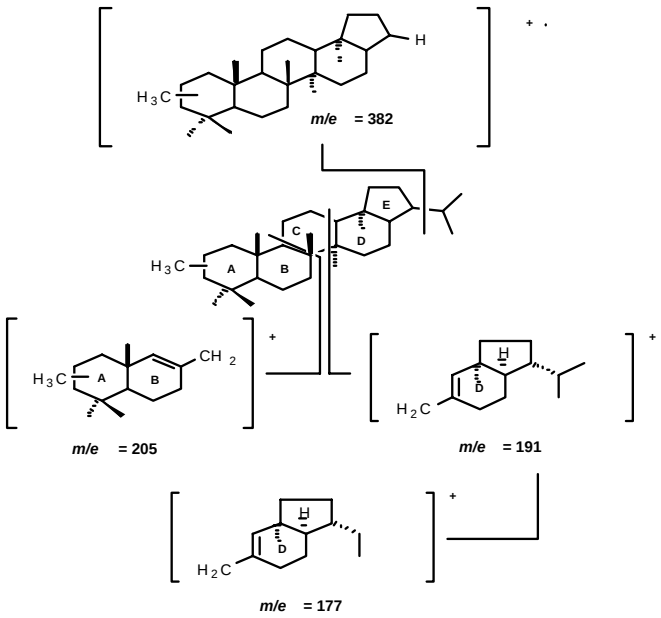

Figure 1 The proposed fragmentation of α,β-hopane.

The presence α,β-hopane in bacteria from Kawengan crude oils lead to two results. First result concerns the revisit of the type of bacteria. At present, no analyzed *Bacillus* produces detectable hopanoids. Thus, the organism may be different genus. The sequence of 16S r-RNA is also close to *Streptomyces* which is well confirmed as a source of hopanoids [11]. Second result is a first confirmation of the participation of bacteria in crude oils formation. The participation should recently occur so that α,β-hopane could no longer be considered as a main geochemical fossils.

Identification of bacteria producing hopanoids from ground surface reservoir

The next question is, whether the result mentioned in paragraph above can be generalized. To answer the question, we done two investigation on crude oils reservoir around Surabaya (Gresik and Wonokromo). The two reservoirs locate at the surface and no longer in service.

Crude oils from Wonokromo's reservoir contain four genera *of Staphylococcus, Micrococcus, Pseudomonas* and *Bacillu*s. Those from Gresik are *Pseudomonas* and *Bacillus*. The preliminary test of the extractable lipid of such culture shows that there are triterpenoids trace. However, no fragment in MS's spectra rely on hopanoid fragments. This indicates that the crude oils don't contain the bacteria producing hopanoids. In contrast, the crude oils itself contain a fraction of hopanoids [12].

Considering the fact, it can be deducted that bacteria producing hopanoids can not grow in atmospheric condition. It might be present when the reservoir was in the deep in which the temperature and pressure are high.

To confirm the hypothesis, a same experiment has been done on bacteria which are claimed as *Bacillus polymixa* [13]. This bacteria was selected due to its great tolerance in culture temperature and due to the same condition of its resources, crude oils reservoir from Bangko, Jambi, Indonesia.

Identification hopanoids of *'Bacillus polymixa'*

The first screening of its lipid extract by TLC method shows that the bacteria produce pentacyclic triterpenoids. Two spot in TLC plate can be observed at R_f = 0.10 and 0.22. After degraded by $H_5IO_6/NaBH_4$ method, the related hopanoid was still on the same R_f. This indicates that there is no great change in complex hopanoids structure during degradation process. The quantity of the hopanoids is 12 mg/g lyophilized cells.

All fraction of hopanoids are then injected to GC yielding the chromatogram. By this chromatogram, it is clear that the hopanoid fraction contain several compounds. All compounds are analyzed by GCMS. In fact, only the 3^{rd} peak (t_R = 28.6 minute), the 5^{th} peak (t_R = 29.0 minute), the 6^{th} peak (t_R = 30.1 minute), the 7^{th} peak (t_R = 32.4 minute) and the 8^{th} peak (t_R = 32.6 minute) produced the hopanoids fragmentation. It can be confirmed by the revelation of one, two or three characteristic fragment of hopanoids. The other peaks are the other pentacyclic terpenoids.

The most interesting compound is the 5^{th} peak. The pattern of its fragmentation drive to an acid hopanoid having one double bound at C-17 and C-21. The confirmation of its presence can be observed by the presence of fragments, m/e = 57, m/e = 175, m/e = 316 and m/e = 367. Due to the slight quantity and instability, the other peaks don't appear. However, one can be deduced that, the compound is a C_{35} acid or alcohol derivative. Fragmentation of hopen-35-ol ester is shown in Figure 2.

Acid hopanoids or its alcohol compounds are never found in living organism. Their presence in sediments has been used to support the theory of the nature of geochemical hopanoids: bacterial hopanoids, bacteriohopanetetrols (C_{35}), as geological hopanoids precursors [14, 15]. Elimination of hydroxyl groups in C_5 side chain occurs during psycho-chemical process in sediment maturation. Thus, the presence of such hopanoids in *Bacillus polymixa* must also imply on sediment maturation theory.

In addition of the interesting structure, it can be also observed the unusual position of the double bound. The same kind of structure has been proposed as an alternative intermediate in α,β-hopanoids formation in living organism [9]. Thus, the discovery serves as a first confirmation of the hypothetical intermediate.

Other compounds of the hopanoids fraction consist of ambiguously structure of hopanoids. However, most of them are α,β-hopanoids stereoisomers, the usual hopanoids present in living organisms.

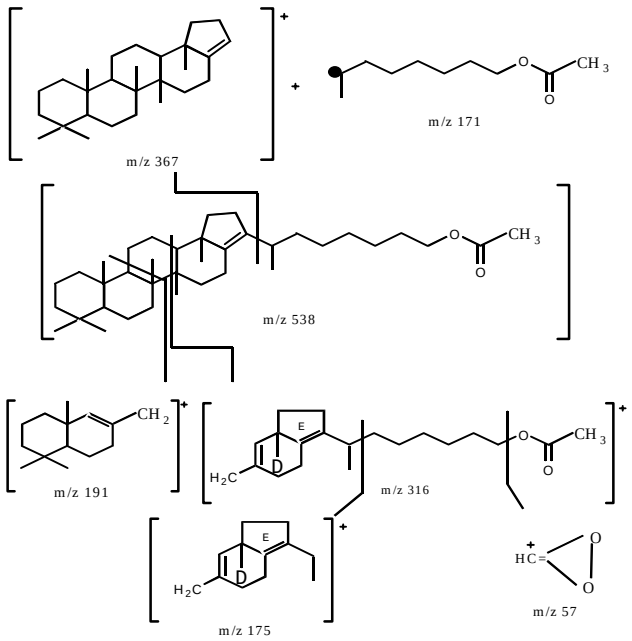

Figure 2 Fragmentation of hopen-35-ol ester.

The presence of hopanoids arise the question of the correct species of the organism. For such purpose, other analyze of 16s r-RNA has been conducted. The nucleotide pattern showed that '*Bacillus polymixa*' is very close to *Alicyclobacillus* species, the former organism for hopanoid investigation.

4. Conclusion

The research done arise some conclusion as pointed out by following statements:

a. Crude oils from the deep reservoir consist potentially of bacteria producing hopanoids.
b. Sedimentary hopanoids, a,b-methylhopane and hopen-35-ol ester, which are widely used as geochemical fossils are also founds in the bacteria living in the crude oils.
c. The appearance of sedimentary hopanoids in *Bacillus* suggest that the *Bacillus* might be a different species.
d. Crude oils from the ground surface reservoir might not contain bacteria producing hopanoids or the bacteria don't express their capacity in producing hopanoids.

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