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POTENTIAL AND SYNERGY EVALUATION OF INDIGENOUS BIOSURFACTANT-PRODUCING BACTERIA FROM ABANDONED OIL WELLS FOR MICROBIAL ENHANCED OIL RECOVERY (MEOR) TECHNOLOGY DEVELOPMENT

Brian Saputra Manurung[1], Windy Natalia Nusaly[2], Abdul Mahid Ukratalo[3], Monalisa Pertiwi Jeriska Taihuttu[4], Fuadiska Salamena[5]
[1,2,3,4,5]Department of Biology, Faculty of Science and Technology, Universitas Pattimura, Ambon 97233, Indonesia

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Correspondence
Brian Saputra Manurung
e-mail:
brianmanurung75@gmail.com

ABSTRACT

Petroleum is a non-renewable natural resource that continues to be used in many applications. However, the availability of crude oil ready for use is decreasing due to the low productivity of oil wells, one of which is caused by its high viscosity. This needs to be resolved to meet the demand for crude oil. This study aims to determine the potential of eight petrophilic bacteria in producing biosurfactants and to determine their interaction to be applied to Microbial Enhanced Oil Recovery technology. The research design in this study was a Factorial Randomized Group Design with two types of treatments: 8 types of bacteria and 4 different temperatures. The experimental units were 32, with 2 replications of each, resulting in 64 experimental units. The observation variables in this study were the diameter of the clear zone and the interaction of bacteria. Clear zone diameter was analyzed using Analysis of Variance, then continued with Duncan's New Multiple Range Test at 5%. This study showed the formation of clear zones as an indicator of the production of biosurfactants influenced by temperature. *Brevundimonas diminuta* and *P. peli* have superior potential in forming clear zones in hemolytic assay than other bacteria, with clear zone diameters of 26.805 mm and 26.040 mm, respectively, at 44°C of incubation. Three bacteria have a high percentage of synergy in this study of 50%, which synergized with 4 other types of bacteria.

Keywords: Biosurfactants, Microbial Enhanced Oil Recovery, Petrophilic Bacteria, Potential Evaluation, Synergy Evaluation

INTRODUCTION

Crude oil is the world's most traded and strategic commodity (Norouzi & Fani, 2021). Hydrocarbons can be classified as aromatic (mono and polycyclic), asphaltenes (phenols, fatty acids, ketones, esters, and porphyrins), resins (pyridines, quinolines, carbazoles, sulfoxides, and amides), and saturates (branched and straight-chained) (Imam *et al.,* 2019).

The demand for crude oil has risen due to its use in many industries (Ali *et al*.,

2019). To meet this increasing demand, it is essential to ensure a sufficient supply of petroleum. Petroleum remains a key energy source for many years. Many petroleum companies are still facing the challenge of finding effective methods to recover heavy oil from maturing on-shore and off-shore oil fields to meet the global needs of petroleum (Al-Wahaibi *et al*., 2016).

In response to this challenge, the Indonesian government approved BUMD Petro Muba and BUMD Blora Patra Energi in 2020 to reactivate approximately 832 abandoned oil wells in the regions of Musi Banyuasin and Blora (Extractive Industries Transparency Initiative Indonesia, 2021). Reactivating these old wells presents a promising alternative to meet petroleum demand. However, one significant challenge is the viscosity of the crude oil found in these wells. High viscosity of crude oil is one challenge in its production and processing (Yarmola *et al*., 2023). Temperature is one of the important elements in biosurfactant production (Fardami *et al*., 2022). Therefore, this research aims to determine the best temperature for producing biosurfactant for each bacterium.

Oil recovery technology can be done in three stages. Primary and secondary oil recovery methods rely on physical and mechanical processes (Patel *et al*., 2015). Use of gas pressure and natural reservoir forces as primary recovery methods can only recover one-third of the oil in the reservoir, and the remaining 35-55% of oil inside the reservoirs (Safdel *et al*., 2017), thus requiring other alternatives to recover the trapped oil. MEOR is a tertiary oil recovery method that involves the application of microorganisms and their metabolic product to facilitate the oil recovery process (Wu *et al*., 2022).

Microbial metabolic products, such as gas, can increase the pressure in the reservoir and reduce oil viscosity by dissolving it. In addition, microbial metabolic products in the form of acids can dissolve carbonates, while biopolymers can increase the viscosity of water in water flooding activities (Paryoto *et al*., 2021). Various microbes and their other metabolic products have also been applied to MEOR technology. Some of these microbes include *Bacillus* sp., *Leuconostoc* sp., *Xanthomonas* sp., with microbial products in the form of biomass that can be used for selective plugging and wettability alteration; *Acinetobacter* sp., *Bacillus sp*., *Pseudomonas* sp., which produce surfactants that play a role in the emulsification and de-emulsification process through reducing interfacial tension; *Bacillus* sp., *Brevibacterium* sp., *Xanthomonas* sp. can produce polymers that contribute to injectivity profile and viscosity modification, and selective plugging; *Clostridium* sp., *Klebsiella* sp., and *Zymomonas* sp., can ease the rock dissolution process and reduce oil viscosity with the solvent produced; *Clostridium* sp., and Enterobacter sp., can enhance permeability and emulsification through the acids they produce. Additionally, *Clostridium* sp., *Enterobacter* sp., and *Methanobacterium* sp. contribute to the Microbial Enhanced Oil Recovery (MEOR) process by increasing pressure and reducing both viscosity and interfacial tension (Budiharjo *et al*., 2017).

Using biosurfactants as a metabolite product of microorganisms can help to cope with the high viscosity challenge. While chemicals are commonly used in oil recovery, they can lead to environmental issues, such as the formation of harmful residues. In contrast, MEOR technology, which relies on microbes and their by-products, offers a more environmentally friendly and cost-effective alternative for extracting crude oil (Patel *et al*., 2015). Thus, Microbial Enhanced Oil Recovery (MEOR) is one of the alternatives to recover the trapped oil.

Surfactant is an amphiphilic compound composed of a hydrophilic and a

hydrophobic part, which is in the form of a fatty acid linked to the polar head, therefore increasing the bioavailability and solubility of the compounds (Otzen, 2017). Surfactant produced by microorganisms is called biosurfactant. Biosurfactant is one of the metabolites produced by microbes. Biosurfactants offer several advantages over synthetic surfactants, particularly in emulsification, and their production depends on the type of microorganisms used and the conditions under which they are produced (Gudiña *et al*., 2015).

The ability of the consortium to degrade petroleum is higher than a single strain. This was also observed by Chen *et al*. (2023), five strains of bacteria showed the degradation rate was 31.54% higher compared to a single strain in a 6% (m/v) petroleum concentration after 16 days of co-culture. Similarly, Zobaer *et al*. (2024) isolated *Bacillus* strain JR3 and *Acinetobacter* strain JR7 from oil-polluted soil. Both of these strains possess strong emulsification abilities, making them suitable for enhanced oil recovery. Additionally, *Pseudomonas* and *Alcanivorax* in consortia exhibited good ability in petroleum degradation (Muangchinda *et al*., 2018). The ability of bacteria to live and work together in consortia can be evaluated by synergy evaluation.

Studies on potency and synergy evaluation have been conducted in many places in Indonesia and worldwide. However, the researchers are interested in examining petrophilic bacteria in the Angit River Village, Babat Toman, South Sumatra, to determine the characteristics and potential of indigenous isolates in abandoned wells over there to help the continuity of petroleum availability. In this study, we evaluated the biosurfactant production capabilities of eight bacterial strains using a hemolytic assay. We also examined the potential synergy between these strains for the crude oil recovery from abandoned wells. The synergy evaluation is critical to determine the ability of each bacterium to live and work together in a consortium to accelerate petroleum degradation, because interaction among bacteria can be synergistic or antagonistic. So that oil degradation can work efficiently.

MATERIALS AND METHODS

Tools and materials

Autoclave (GEA YX-24 LM), stirring rod (Pyrex), beaker glass (Pyrex), bunsen, petri dish (Pyrex), erlenmeyer (Pyrex), measuring beaker (Pyrex), measuring cylinder (Pyrex), Hausser Scientific™ Bright-Line Hemocytometer (Catalog #100-1181, Hausser Catalog #3100), hotplate (IKA C-MAG HS 10), incubator (Memmert Incubator), calipers (Vernier Caliper, MTA-57961632), inoculating loop (Single Nichrome Wire, Aluminum Handle Mfr #A2Z-ZR148, Zoro #G6282815), object glass (Sail Brand, Cat. 7101), cover glass (OneLab), digital camera (Sony ZV-1), laboratory cotton (OneMed), paper disc (HiMedia No. Cat.: SD067-1PK), magnetic stirrer, micropipette (Eppendorf), microscope (Olympus CX23 Binocular microscope), analytical balance (NEW Radwag AS 220.R2 PLUS), tweezers, test tubes (Pyrex), tips (Eppendorf), vortex (SWIRLEX – Vortex Shaker E11190), sterile distilled water, *Bacillus firmus, Brevundimonas diminuta, Burkholderia glumae, P. acidovorans, P. aeruginosa, P. citronellolis, P. fluorescens,* and *P. peli*, 5% sheep Blood Agar plate (provided by BTKL Palembang), Nutrient Agar medium (NA) (Oxoid CM0003), and Nutrient Broth medium (NB) (Oxoid CM0001).

Preparation of Petrophilic Bacteria

Bacillus firmus, Brevundimonas diminuta, Burkholderia glumae, P. acidovorans, P. aeruginosa, P. citronellolis, P. fluorescens, and *P. peli* that have been isolated by Laini *et al*.,

(2014) from abandoned wells in Angit River Village, Babat Toman District, Musi Banyuasin Regency, South Sumatra. All bacteria were sent to SITH ITB for molecular identification. Each petrophilic bacterium was inoculated on the NA (Nutrient Agar) slant agar medium aseptically and then incubated at 37°C for 24-48 hours. Stock culture and working culture were ready to be used (Benson, 2001).

Petrophilic Bacterial Inoculum Preparation and Counting

One loop of bacterium from the Nutrient Agar (NA) slant was taken to make a petrophilic bacterial inoculum, put into a test tube containing 10 mL of Nutrient Broth (NB) medium, vortexed, and incubated at 37°C for 24 hours. One drop of inoculum from the liquid culture was then placed in the counting chamber, observed, and the number of bacterial cells was counted under an Olympus CX23 Binocular microscope. The bacterial inoculum used was an inoculum with a bacterial cell density of $\pm 10^6$ cells/mL.

Hemolytic Activity

The hemolytic test of the eight petrophilic bacteria was carried out with the Kirby-Bauer Method (diffusion method) by placing a sterile paper disc with a diameter of 6 mm (HiMedia No. Cat.: SD067-1PK) on the 5% sheep blood agar plate and then dripped with 5 µL of bacterial inoculum to be tested for potential (Benson, 2001), then incubated in four different temperatures: 30°C, 37°C, 44°C, 51°C for 24-48 hours.

Hemolytic activity, characterized by the formation of a clear zone around the bacterial colony, can be an indicator that the bacteria have the potential to produce biosurfactants (Carrillo *et al*., 1996). The diameter of the clear zone formed was measured using a caliper. The diameter of the clear zone was analyzed using ANOVA at a 5% significance level, followed by Duncan's New Multiple Range Test (DNMRT) at the 5% significance level. Data analysis was carried out using Statistic 7.0 software.

Synergy Evaluation of Biosurfactant-Producing Bacteria

The synergy evaluation of biosurfactant-producing bacteria was conducted by the pour plate method (Prescott *et al*., 1999) and the Kirby-Bauer method (diffusion method) (Benson, 2001). 1 mL of 1st bacterial inoculum was put into a sterile Petri dish aseptically, then 15 mL of Nutrient Agar (NA) medium was added and homogenized. Once solid, a sterile 6 mm diameter paper disc (HiMedia No Cat.: SD067-1PK) was placed on top of the Nutrient Agar (NA) medium, and 5 µL of another bacterial inoculum was placed on the paper disc. The same thing is done for other bacteria, such as 1st bacterial isolate, so that synergistic and antagonistic bacteria can be known. Petri dishes containing cultures were then incubated at 37°C for 24 hours, and observations were made of the growth of each bacterium, whether it inhibited the growth of other bacterial isolates or not. Synergistic isolates are indicated by the formation of no clear zone. Interactions between bacteria can be determined by calculating the percentage of synergy using the formula: Number of bacterial species that can synergize/Total number of test bacterial species x 100% (Hardestyariki and Yudono, 2021).

RESULTS AND DISCUSSION

The eight types of petrophilic bacteria used in this study have different potential in forming a clear zone as an indicator of biosurfactant production at four temperatures:

30°C, 37°C, 44°C, and 51°C. The higher the temperature, the lower the potential of the eight bacteria to form a clear zone. This can be seen from the average diameters of the clear zones formed (Figure 1).

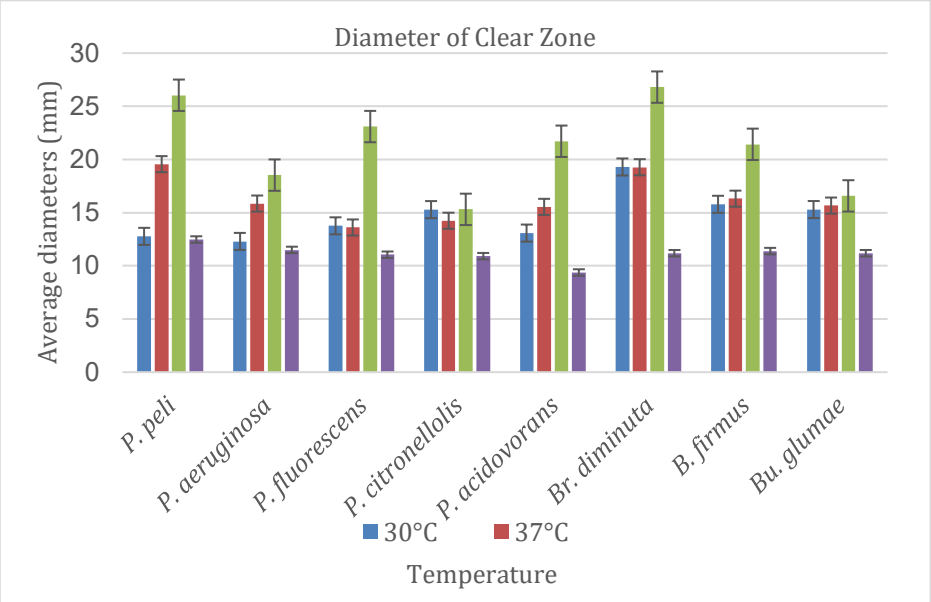

Figure 1. Graph of average clear zone diameters of the eight petrophilic bacteria after incubation at four different temperatures

The test of potential in forming a clear zone as an indicator of biosurfactant production in this study was conducted by a hemolytic test. The clear zone formed around the bacterial colony is an indication of biosurfactant production through surface-active molecules produced (Jemil *et al*., 2016). Measurement of clear zone diameter is a qualitative method that is often used as an indicator of the ability of bacteria to produce biosurfactants (Rajesh *et al*., 2017). The clear zone formed around the bacterial colony is due to the hemolytic activity of bacteria against red blood cells in the blood agar medium. The larger clear zone formed around the colony is caused by the increasing number of lysed red blood cells.

Gudiña *et al*. (2015) CMC is defined as the concentration of amphiphilic compounds at which micelles are initiated in a solution. Critical Micelle Concentration (CMC) is an important characteristic of biosurfactants, where, after reaching CMC, the surface tension is relatively constant due to saturation of the interface with surfactants (Jemil *et al*., 2016). Biosurfactant has important roles in recovering trapped oil by reducing surface and interfacial tension. Ali *et al*. (2019) found that a biosurfactant reduced surface tension from 72.0 to 26.21 ± 0.3 and interfacial tension by 0.26 ± 0.1 $mN.m^{-1}$ against crude oil.

The formation of clear zones around bacterial colonies is closely related to the decrease in surface tension and interfacial tension. The larger diameter of the clear zone indicates a higher potential of bacteria to produce biosurfactants. The larger the clear zone formed, the greater the reduction in surface and interfacial tension that occurs around the bacterial colony. The different potential of the eight petrophilic bacteria in forming clear zones is influenced by temperature. Marajan *et al*. (2018) proved that temperature affects the production of biosurfactants produced by *Bacillus subtilis* and *Bacillus tequilensis* as well as the efficiency of reducing surface tension. Poshala (2020) also explained that biosurfactant production is strongly influenced by temperature.

Based on the results of the Analysis of Variance, the p-value = 0.000000 ($p < 0.05$) was obtained at the α level of 5%, this indicates that each bacterial species and temperature have a significant effect on the diameter of the clear zone formed (Table 1), followed by the Duncan New Multiple Range Test α0.05. The results of Duncan's New Multiple Range Test $\alpha_{0.05}$ influence of temperature and bacterial species on the diameter of the clear zone (Table 2).

Table 1. ANOVA results of the Factorial Randomized Group Design of Clear Zone's Diameter

No	Source	dF	SS	MS	F-value	Probability
1	Intercept	1	16012.69	16012.69	12412.94*	0.000000
2	Replication	1	1.00	1.00	0.77*	0.000000
3	Species	7	177.82	25.40	19.69*	0.000000
4	Temperature	3	835.81	278.60	215.97*	0.000000
5	Species*Temperature	21	212.61	10.12	7.85*	0.000000
6	Error	31	40.10	1.29	1.00*	0.000000
7	Total	63	1267.35			

Notes: dF: degree of freedom, SS: sum of squares, MS: mean squares, *: significant at α < 0,05

Table 2. Average diameters of clear zones of eight petrophilic bacteria at four temperatures

No	Bacteria \ Temperature	30°C	37°C	44°C	51°C
1	*P. peli*	12.770^{bcde}	19.560^{jk}	26.040^{m}	12.475^{bcd}
2	*P. aeruginosa*	12.295^{bcd}	15,855^{gh}	18.530^{ij}	11.495^{abc}
3	*P. fluorescens*	13.760^{cdefg}	13.605^{bcdefg}	23.095^{l}	11.045^{ab}
4	*P. citronellolis*	15.285^{efgh}	14.240^{defgh}	15.310^{efgh}	10.910^{ab}
5	*P. acidovorans*	13.080^{bcdef}	15.545^{fgh}	21.715^{kl}	9.375^{a}
6	*Br. diminuta*	19.295^{jk}	19.270^{jk}	26.805^{m}	11.185^{abc}
7	*B. firmus*	15.785^{gh}	16.315^{ghi}	21.425^{kl}	11.385^{abc}
8	*Bu. glumae*	15.290^{efgh}	15.665^{fgh}	16.575^{hi}	11.185^{abc}

Notes: 1. Average diameters of clear zones in mm; 2. Numbers followed by the same lowercase letter indicate not significantly different according to Duncan New Multiple Range Test $\alpha_{0.05}$.

Two bacteria, *Brevundimonas diminuta* and *P. peli*, have superior potential in forming clear zones with clear zone diameters of 26.805 mm and 26.040 mm at 44°C (Table 1, Figure 2). This indicates that the appropriate temperature to form a clear zone is an indicator of biosurfactant production for both bacteria in this experiment at 44°C. The formation of the clear zone is closely related to the ability of bacteria to produce biosurfactants in the surrounding environment and cause red blood cell lysis on the Blood Agar medium. Rajesh *et al*. (2017) bacteria that produce biosurfactants can cause the lysis of erythrocytes and show a clear zone around the colony as a result.

Figure 2. Clear zones formed. (A) *Brevundimonas diminuta* and (B) *P. peli* at a temperature of 44°C

The results of Duncan's New Multiple Range Test α0.05 (Table 1) show that there are three phenomena observed in the formation of clear zones that are closely related to the four temperatures of 30 °C, 37 °C, 44 °C, and 51 °C in this study. The three phenomena are the most suitable temperature, the suitable temperature, and the less suitable temperature in clear zone formation. The most appropriate temperature for the formation of clear zones based on the results obtained from the Duncan New Multiple Range Test α0.05 results above is 44 °C. Five petrophilic bacteria, namely *Bacillus firmus*, *P. acidovorans, P. fluorescens, P. peli,* and *Brevundimonas diminuta*, show good potential to form clear zones at 44°C. The best clear zone formation potential occurs at a temperature classified as mesophyll, which is 44°C in this research. Suitable temperature has an important role in bioremediation because it affects the growth and metabolic rate, diversity of microorganisms, which help the biodegradation process (Wartell *et al*., 2021).

The diameter of the clear zone formed at 30°C and 37°C is relatively large, but the diameter of the clear zone formed is not as large as the diameter of the clear zone formed at 44°C. However, it is larger when compared to the clear diameter formed at 51°C. The least suitable temperature tested to determine the potential of the eight bacteria in the production of biosurfactants in this study was 51°C. The clear zone diameter formed at 51°C ranged from 9.375 mm to 11.495 mm. The eight petrophilic bacteria used as test bacteria decreased their potential to form clear zones at 51°C. This is in line with the research of Parthipan *et al.* (2017) that the highest biosurfactant production and emulsification activity by *Bacillus subtilis* A1 was found at a treatment temperature of 40°C and decreased at 50°C and 60°C. Each bacterium has a different optimal growth temperature that can affect its ability to produce biosurfactants and its emulsification activity.

Hydrocarbon compounds can be degraded by bacteria with the aid of the degrading enzymes they produce. An experiment by Parthipan *et al*. (2017), biosurfactant and enzyme production by *B. subtilis* A1 can increase the biodegradation efficiency of hydrocarbon compounds in crude oil. The consortium of *Kocuria flava* and *Rhodococcus pyridinivorans* degraded 61.32%, 64.72%, and 66.64% of 10 mg/L phenanthrene, anthracene, and fluorene, respectively, for a 15-day treatment period with help of catabolic enzyme activity such as: Catechol 2,3-dioxygenase (C23O), dehydrogenase, and peroxidase which play important role in metabolism of phenanthrene, anthracene, and fluorene (Sakshi *et al*., 2021).

Besides being influenced by temperature, biosurfactant production is also influenced by genetics. The ability to produce biosurfactants is affected by the genes owned by the bacteria themselves. However, biosurfactant production can be increased, one of which is through the process of mutagenesis. A study by Bouassida *et al*. (2018) stated that random mutagenesis on *B. subtilis* can heighten the production of biosurfactants, where *B. subtilis* M2 can produce biosurfactants twice as much as compared to the B. subtilis wild-type strain due to the mutations in certain genes affecting the ability of bacteria to produce biosurfactants.

The synergistic relationship of the eight petrophilic bacteria (*P. peli, P. aeruginosa, P. fluorescens, P. citronellolis, Brevundimonas diminuta, Bacillus firmus, Burkholderia glumae,* and *P. acidovorans*) (Table 3).

The synergy percentage of the eight petrophilic bacteria ranges from low to high synergy percentage: *P. acidovorans, Burkholderia glumae, Bacillus firmus, P. aeruginosa, P. peli, Brevundimonas diminuta, P. citronellolis,* and *P. fluorescens*. The synergy evaluation results showed three phenomena of results, namely, the percentage of synergy classified

as high is 50%, the percentage of synergy classified as medium is 37.5% and 25%, and the percentage of synergy classified as low in this study is 12.5% (Table 2).

Table 3. Synergy evaluation among the eight petrophilic bacteria

No	Isolate	B.01	B.02	B.03	B.04	B.05	B.06	B.07	B.08	PS (%)
1	B.01	*	-	-	+	+	+	-	-	37,5
2	B.02	-	*	+	+	-	+	-	-	37,5
3	B.03	-	+	*	+	+	-	-	+	50
4	B.04	+	+	+	*	+	-	-	-	50
5	B.05	+	-	+	+	*	-	+	-	50
6	B.06	+	+	-	-	-	*	-	-	25
7	B.07	-	-	-	-	+	-	*	-	12,5
8	B.08	-	-	+	-	-	-	-	*	12,5

Note: Isolate B.01: *P. peli*; Isolate B.02: *P. aeruginosa*; Isolate B.03: *P. fluorescens*; Isolate B.04: *P. citronellolis*; Isolate B.05: *Brevundimonas diminuta*; Isolate B.06: *B. firmus*; Isolate B.07: *Burkholderia glumae*; Isolate B.08: *P. acidovorans*; The sign (+) indicates a synergistic interaction; The sign (-) indicates an antagonistic interaction; The sign (*) states the inoculated bacteria; PS: Percentage of Synergy

P. fluorescens, P. citronellolis, and *Brevundimonas* diminuta have a percentage of synergy with other bacteria of 50% (Table 3). The percentage of synergy is high when compared to the percentage of synergy of other bacteria. Table 3 shows that *P. fluorescens* can synergize with *P. aeruginosa, P. citronellolis, Brevundimonas diminuta,* and *P. acidovorans. P. citronellolis* bacteria can synergize with *P. peli, P. aeruginosa, P. fluorescens,* and *Brevundimonas diminuta*. *Brevundimonas diminuta* was found to synergize with *P. peli, P. fluorescens, P. citronellolis,* and *Burkholderia glumae*. The synergy results above show that bacteria belonging to the genus *Pseudomonas* sp can live together.

The synergy evaluation of several bacteria aims to determine the ability to work synergistically among bacteria in the process of hydrocarbon degradation. The process of hydrocarbon biodegradation can take place well and efficiently if it involves several bacteria in a consortium in several studies. Diverse metabolic processes and metabolite products from the consortium can enhance the crude oil degradation process when compared to a single culture (Rahmati *et al*., 2022).

Chen *et al*. (2024) also found a halotolerant bacterial consortium consisting of *Pseudoxanthomonas* sp. S1-2, *Bacillus* sp. S2-A, *Dietzia* sp. CN-3, and *Acinetobacter* sp. HC8-3S can degrade alkanes, cycloalkanes, branched alkanes, and aromatic hydrocarbons very well during 10 days of treatment. The study by Xia *et al.* (2019) consortium of three bacteria, namely *Serratia proteamaculans, Alcaligenes* sp*,* and *Rhodococcus erythropolis,* showed a significant increase in the crude oil degradation process to 85.26% during 15 days of treatment compared to the use of a single culture. This was reinforced by the detection of the alkane hydroxylase gene (alkB), which indicates the presence of crude oil-degrading enzymes.

Synergistic growth between bacteria is needed to support the process of hydrocarbon biodegradation efficiently and faster. Synergistic interaction can also facilitate the complete biodegradation (Omrani *et al*., 2018). Interaction among bacteria in a consortium is a complex phenomenon that remains unclear. Two hypotheses are thought to influence interactions between bacteria, some bacteria can produce enzymes that can degrade certain components of a compound more effectively, or other bacteria can produce stimulatory exudates or molecules that play a role in quorum sensing that

coordinate interactions between bacteria (Deng & Wang, 2016).

Microbial consortia had higher degradation efficiency than axenic cultures because axenic culture needs more energy and resources, while in a consortium, each member has their role in metabolism, which supports each other (Lázaro-Mass *et al*., 2023). This is in line with Kebede *et al.* (2021), broad catabolic enzymes and genes in a consortium may accelerate the biodegradation process. The percentage of synergy was classified as moderate in *P. peli* (37.5%), *P. aeruginosa* (37.5%), and *Bacillus firmus* (25%). *P. peli* was able to synergize with *P. citronellolis, Brevundimonas diminuta,* and *Bacillus firmus. P. aeruginosa* can live together with *P. fluorescens, P. citronellolis,* and *Bacillus firmus* (Figure 3). *Bacillus firmus* can synergize with *P. peli* and *P. aeruginosa*. Bacterial consortia have a good capability to degrade hydrocarbons because single bacteria have limitations in using hydrocarbons as a substrate (Wu *et al.,* 2016). Research by Deng & Wang (2016) added that the complexity of the substrate can also affect interactions between bacteria.

Figure 3. Synergy evaluation of *P. aeruginosa* with 7 other petrophilic bacteria

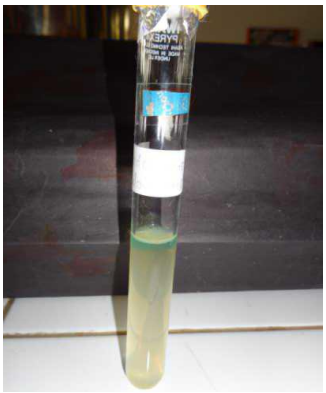

Figure 4. Blue pigment on the surface of NB medium produced by *P. acidovorans*

The percentage of synergy is relatively low in *Burkholderia glumae* and *P. acidovorans*, with a percentage of synergy of 12.5% each. *Burkholderia glumae* is only able to synergize with *Brevundimonas diminuta*. Like *Burkholderia glumae*, *P. acidovorans* can only synergize with *P. fluorescens* (Table 2). This low percentage of synergy could be influenced by toxins produced by these bacteria that can interfere with the growth of other bacteria. There is a blue liquid on the surface of the medium that has been inoculated with *P. acidovorans* after two days of incubation (Figure 4).

This blue liquid is thought to be a toxin pigment produced by the bacteria that inhibits the growth of other bacteria. Some members of *Pseudomonas* sp. are capable of producing toxin pigments. The production of toxin pigments is a part of the antagonistic

effect. Antagonistic effects among bacteria in a consortium can hinder the bioremediation efficiency (Chen *et al*., 2015).

Hardestyariki and Yudono (2021) also stated that interactions between bacteria that are antagonistic can cause competition between bacteria that can inhibit the growth and degradation process of pollutants to resulting in low or inhibited. Deveau *et al*. (2018) added that interaction among members in the consortium can be antagonistic due to the presence of inhibitory or toxic substances by members, which can hinder the growth and metabolism of other members. Members of consortia usually compete for nutrients, space, and many environmental resources.

CONCLUSIONS

The potential of the eight petrophilic bacteria to form clear zones at four temperatures showed different potentials. Two bacteria with superior potential in the hemolytic test are *Brevundimonas diminuta* and *P. peli* at 44°C, with clear zone diameters of 26.805 mm and 26.040 mm, respectively. Based on the results of the synergy of petrophilic bacteria that have been carried out, it is known that the high percentage of synergy in this study is *P. fluorescens, P. citronellolis*, and *Brevundimonas diminuta*, which is 50%, where the three types of bacteria can synergize with 4 other types of bacteria. Burkholderia glumae and P. acidovorans have a low synergy percentage of 12.5%, where these two bacteria are only able to synergize with one bacterium. The potential and synergy data in this study can be used as a reference and further developed to overcome the problems in abandoned wells of the oil industry, to reactivate abandoned wells, and increase the availability of oil.

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