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Bioprospecting of Halophilic Bacteria *Staphylococcus haemolyticus* Strain Stp-Griv-024 as Biosurfactant Producer and Its Potential Application for Microbial Enhanced Oil Recovery

Izzul Islam[1], Cut Nanda Sari[2], Ali Budhi Kusuma[1,3], Ika Kurniasari[1], Lisnawati[1], Rini Surya Ningsih[1] and Elisa Rosani[1]

[1]Department of Biotechnology, Faculty of Lifescience and Technology, Universitas Teknologi Sumbawa
Raya Olat Maras Batu Alang Street Pernek, Sumbawa, Indonesia

[2]LEMIGAS R & D Centre for Oil and Gas Technology
Ciledug Raya Street Kav. 109, Cipulir, Kebayoran Lama, South Jakarta, Indonesia

[3]Archi Biotech
Kerato Street No.11, Sumbawa, West Nusa Tenggara, Indonesia

Corresponding author: izzul.islam@uts.ac.id

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ABSTRACT - The use of halophilic bacteria to produce effective and stable biosurfactants in the Microbial Enhanced Oil Recovery (MEOR) process is getting much attention from researchers. A diversity of halophilic bacteria that produce biosurfactants can be found in areas with intense oil exposure, such as the waters of Bima Bay, which is closed and are one of the main sea transportation routes in eastern Indonesia. This research aims to isolate potential local halophilic bacteria in producing biosurfactants to degrade hydrocarbons. The research methodology included bacterial isolation, gram staining, hemolysis test, total petroleum hydrocarbon (TPH) analysis, emulsification, and phylogenetic analysis of the 16S rRNA marker gene. STP-GRIV-024 was successfully isolated using Kish, Halophilic, Soil extract, and Oatmeal agar media, with the highest enumeration results found on Kish media supplemented with 3% (w/v) NaCl. Microscopic morphological characterization using Gram staining showed results as a Gram-positive group with round colony shapes, smooth circular edges, sloping and white. This isolate grew in the 7-15% (w/v) NaCl range and was classified as moderately halophilic. TPH analysis showed that concentration and incubation time influenced hydrocarbon degradation activity. On day 10, the concentrations of T1 (1%), T2 (3%), T3 (5%), and T4 (7%) showed a decrease in TPH of 1.96%, 0.51%, 0.25 %, and 0.15% respectively. 16S rRNA sequencing identified the isolate as closely related to *Staphylococcus haemolyticus* strain MTCC3383T, with a DNA sequence similarity of 99.9%. These findings provide an important foundation for further development in applying halophilic bacteria in MEOR practices to increase the efficiency of sustainable and environmentally friendly oil production.

Keywords: biosurfactants, halophile bacteria, *staphylococcus haemolyticus*, microbial enhanced oil recovery, bioremediation.

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INTRODUCTION

The search for sustainable and environmentally friendly alternative materials in various industrial applications has led researchers to explore the diversity of microbes that inhabit extreme environments (Chandra et al. 2020; Sari et al. 2016). Among the groups of microbial extremophiles, halophilic bacteria have emerged as promising candidates as sources of diverse and abundant new bioactive molecules (Selvarajan et al. 2017). Halophiles, microorganisms survive in highly saline environments and thrive in elevated saline environments such as salt flats (Hazzouri, K. M. et al. 2022), hypersaline lakes (Naghoni et al. 2017), and salt mines (Cycil et al. 2020). These extreme habitats provide unique physicochemical and geochemical conditions where microorganisms have to thrive under the high salinity pressures that prevail in this biological niche. Halophilic bacteria, in particular, have evolved extraordinary strategies to maintain cell integrity and function under extreme osmotic conditions (Dutta & Bandopadhyay 2022). The complex interactions between cellular mechanisms in these microorganisms have become a focal point of scientific investigation, and researchers are uncovering their potential contribution to various industrial processes.

One group of compounds that have received significant attention is biosurfactants, biologically derived surface-active substances with a wide range of applications ranging from environmental bioremediation to enhancing oil recovery by reducing the interfacial tension between oil and water, thus facilitating the mobilization of trapped hydrocarbons (Putri et al. 2020; Sari et al. 2019; Sari, C. N & Lubnah, L. 2017). Identifying and characterizing the biosurfactant potential of halophilic bacteria is a promising avenue for pursuing sustainable biotechnological solutions.

Hydrocarbon-degrading halophilic bacteria have a preference for living in environments with intense exposure to hydrocarbons (Kebede et al. 2021). The characteristics of Bima Bay, which is closed water and is one of the centers of sea transportation in eastern Indonesia, make it an ideal environment for hydrocarbon-degrading halophilic bacteria. Further investigation is needed to find potential halophilic bacterial candidates that are effective and unique in degrading hydrocarbons. Surfactant molecules from bacteria have unique advantages over chemical surfactants, such as biodegradability and low toxicity (Lima et al. 2011; Voulgaridou et al. 2021). In addition, the unique biochemical pathways used by halophiles for osmoregulation and stress response may contribute to the production of novel and efficient biosurfactants (Kindzierski et al. 2017; Yoo et al. 2023). Therefore, identifying halophilic bacteria as potential biosurfactant producers has significant implications for basic scientific understanding and applied biotechnological innovation. Understanding the ecological function of biosurfactants in halophilic environments is integral to appreciating the broader impact of microbial metabolites on ecosystem dynamics.

METHODOLOGY

Environmental Sample Collection

Sediment samples were taken from 10 different points at the Sanolo traditional solar saltern, Bima Regency (8°32’17”S 118°39’01.9” E; Figureure 1). A total of 330 mL samples were taken aseptically from the crystallization pond. The samples were put into a 50 ml falcon tube and then kept inside a cooler box before they were transported carefully to the Energy and Environmental Biotechnology laboratory, where they will be stored in a freezer up to -20 °C prior to being used for the experimental work.

Sample Pre-processing

Sample preparation was carried out using an air-dry technique. The semi-liquid soil sample taken from the primary sample is labeled and put into a beaker of 0.6 grams using a spatula. The beaker was then covered with aluminum foil, punctured, and dried at room temperature. The samples were dried for one week to reduce the water content of the material (Jiang et al. 2016)

Selective Isolation and Purification of Halophilic Bacteria

Halophilic bacteria were isolated from the highly saline sediment sample following the soil-sprinkle technique, as described by (Kusuma et al. 2020). This technique aims to isolate groups of fastidious (sensitive) microbes from environmental samples with a high level of dependence on several micronutrients at certain leve ls unavailable in commercial media (Idris 2016; Kusuma et al. 2020). A 0.6-gram sample that had been dried was then heated in the incubator at 80 °C for 20 minutes and then crushed using a sterile toothpick. Next, sprinkle

it on the surface of the agar media. The plates were incubated for 28 days until the substantive growth of halophilic bacteria was observed. The actively growing colonies were then picked using the sterile toothpicks and subjected for further purification purposes.

NaCl Tolerance Test

Determination of the optimal concentration of NaCl for the growth of halophilic bacteria using 100 µL of rejuvenated culture as a starter. The sample was then inoculated in 5 ml of Kish Broth growth medium with a composition (sugar and meat extract) containing various NaCl concentrations (w/v) of 7%, 10%, 15%, 20%, 25%, and 30%. The samples were then incubated at 37 ^{o}C for 7 - 14 days (Budiharjo et al. 2017). The NaCl test levels were made based on Irshad et al. (2014), which reported that "low" level halophilic growth optimally in the range of 2 - 5%, "medium" growth optimally in the range of 5 -20% , and "high" growth optimally in the range 20 - 30%. Positive results are indicated by changes in the color of the culture results or the level of culture turbidity, which is given on a scale of (+++) as "excellent growth", (++) as "good growth", (+) as "growth" and (-) as "no growth".

Figure 1
Sampling location. Sanolo solar saltern, the site is indicated by red square box and the red pin.

Micromorphology Characterization Using Gram Staining

Micromorphological of the isolate was characterized using the Gram staining procedure on a day-old active culture on the agar plate (Tripathi, N. & Sapra, A. 2023) 24-hour subculture. A smear of halophilic bacterial isolate was made by placing one drop of distilled water on the surface of the slide and then adding one dose of the isolate to the slide. The sample is then dried by fixing it to place the preparation on a glass object. The violet crystal is then dropped onto a glass slide and left for about 30 seconds. The crystal violet solution was then rinsed at the end of the slide using distilled water. The next step is to rinse the object glass with iodine and leave it for 30 seconds, then rinse it with distilled water and 95% ethanol for 30 seconds. The sample was then dripped with safranin for 30 seconds and rinsed again with distilled water. The sample was then observed under a light microscope with 1500x magnification.

Hemolysis Test

The hemolysis blood test was employed as the first step in screening bacteria capable of biosurfactant production (Gagelidze et al. 2016). The pure isolate was inoculated on 1.0 ml of blood agar medium streaked in a petri dish. The bacterial culture was then incubated for 48 hours at 37°C. Growing colonies are observed by observing the presence of a clear zone around the colony.

Total Petroleum Hydrocarbon Measurement (TPH)

TPH testing refers to (Zulkifliani et al. 2018). The degradation process was carried out at room temperature and observed every three days, namely on days 0, 3, 5, 7, and day 10. The process uses the gravimetric method for each sample, carrying out four repetitions of TPH measurements on soil samples. TPH concentration was measured by weighing 10 grams of soil sample and then adding Na_2SO_4 to purify the oil and water in the soil. The 250 ml boiling flask used is first put into the oven and weighed to determine the initial weight of the flask. The soil sample in the lead extract was then put into a soxhlet apparatus for the extraction process, adding 200 ml of n-hexane as a solvent. The boiling flask is heated using a reflux tool until the extraction results in clear lead, which takes approximately 6 hours to obtain oil from the sample. The resulting extract is then filtered by adding silica powder to filter paper. The boiling flask was then evaporated using a rotary evaporator to separate the n-hexane solvent and left in the oven at 70 °C. The flask was taken at a temperature of 40 °C and then placed in a desiccator for 1 hour, and the final weight was weighed.

Biosurfactant Emulsification

Biosurfactant emulsification was carried out to determine the ability of biosurfactants to dissolve crude oil (Ashish & Debnath (Das) 2018). The liquid media obtained from the TPH test results on days 0 to 10 is mixed with crude oil in a ratio of 1:1 v/v. The sample was then taken using a pipette, placed in a 15 ml test tube, and centrifuged at 3500 rpm for ± 15 minutes.

Phylogenetic Study Using 16S rRNA Gene Sequencing Analysis

The phylogenetic relationship of the bacterial isolate was elucidated following the molecular analysis using 16S rRNA gene marker sequencing (Clarridge J. E. 2004). The isolate was grown on the Kish broth agar at room temperature for 7 days. The genomic DNA was then extracted from the pooled fresh biomass as suggested by (Wilson 2001). The amplification of the 16S rRNA gene fragment was done following the cycling condition as follows: 96 °C for 4 minutes, followed by 30 cycles at 94 °C for 30 seconds, 57 °C for 30 seconds, and at 72 °C 1 minute, and a final extension step at 72 °C for 10 minutes (dos Santos et al. 2019). The expected fragment length of the 16S rRNA gene sequence is approximately 1,550 bp and consists of conserved regions. DNA was amplified by PCR using primers 27F 5'(AGAGTTTGA TCMTGGCTCAG)3' and 1492R 5'(TACGGYTACCTTGTTACGACTT)3'. The amplified DNA was purified according to the manufacturer's protocol (Macrogen Company, Singapore). 16S rRNA gene sequencing was carried out using Sanger sequencing technology with primers 785F 5' (GGATTAGATACCCTG GTA) 3' and 907R 5' (CCGTCAATTCMTTTRAGTTT)3' (Valenzuela-Heredia et al. 2020).

Table 1
Treatments and abbrevation in the TPH measurement

Treatment	Description	Abbreviations used
1	use 1 % of bacterial culture	T1
2	use 3 % of bacterial culture	T2
3	use 5 % of bacterial culture	T3
4	use 7 % of bacterial culture	T4

RESULT AND DISCUSSION

Morphological Characteristics

The morphology of the isolate was obtained based on research parameters according to (Reang et al. 2022).

Figure 2
Colonial morphology of isolate STP-GRIV-024 grow on Kish media after 2 days at 27 °C.

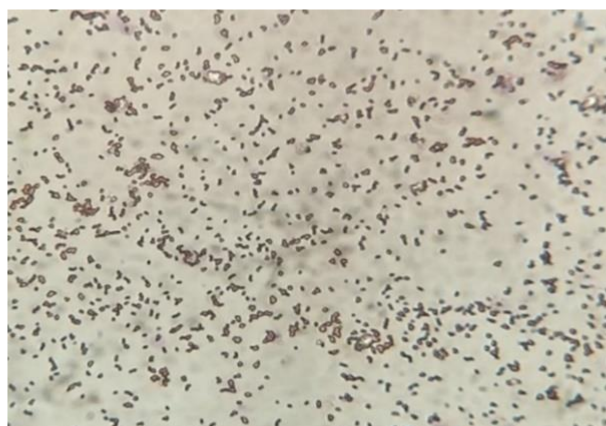

Figure 3
Cellular micromorphological feature of the isolate STP-GRIV-024 following the gram staining procedure observed at 1500x magnification.

Direct observation of the colonial morphology of isolate STP-GRIV-024 showed circular colonies with smooth edges and white pigmentation (see Figureure 2). Further observation using Gram staining showed that the isolate was Gram-positive, cocci-shaped cells. This characteristic is essential in classifying bacteria and indicates that the bacteria are resistant to the decolorization step of the Gram staining procedure. In addition, microscopic examination shows a round, spherical or coccal morphology. The bacterial isolate observed showed characteristics similar to the *Staphylococcus* genus. The characteristic round shape and Gram-positive properties correspond to the characteristics of the *Staphylococcus* genus, which was isolated from the respiratory roots of the *Avicennia marina* plant (Yanti et al. 2022). The *Staphylococcus* genus is known to consist of cocci-shaped bacteria that form irregular groups. Their unique arrangement and Gram-positive staining properties distinguish them from other bacterial genera. According to (Ginting et al. 2018), the *Staphylococcus* genera have convex elevations, intact (flat) edges, smooth surfaces, and a milky white color.

Tolerance Profile Towards NaCl Exposure

NaCl resistance test data shows that halophilic microbes can grow in the 7-25% NaCl range. Growth was inhibited at a NaCl concentration of 30%. Optimal growth of isolate STP-GRIV-024 occurred in the NaCl range of 7-15% (w/v). According to Irshad et al. (2014), the STP-GRIV-024 isolate is of the "moderate" level of halophilic type. Halophilic microbes can adapt to high salt concentrations by accumulating organic substances in their cytoplasm (Budiharjo et al. 2017).

Table 2
NaCl variation test

Siderite Volume (%)	Sw								
	1,00	0,90	0,80	0,70	0,60	0,50	0,40	0,30	0,2
0,00	10,60	19,30	25,10	27,00	27,90	28,80	29,60	30,00	30,4
0,45	8,50	13,50	15,50	16,40	17,10	17,40	17,80	18,10	18,4
0,90	9,20	9,30	14,00	14,90	14,40	14,40	15,00	15,30	15,9
1,34	10,00	10,20	13,80	14,40	14,70	14,90	15,10	15,20	15,3
1,79	5,50	5,90	8,40	8,90	9,30	13,60	13,90	13,90	13,9
2,24	3,28	3,44	4,40	6,20	7,10	9,50	9,80	10,10	10,5
3,36	3,70	4,20	5,90	6,10	6,30	6,40	6,60	6,70	6,8
4,48	2,54	2,90	6,00	7,40	7,52	7,70	8,50	8,70	9,3
5,60	4,40	4,50	7,50	8,30	8,50	8,60	8,70	8,80	8,9

Differences in microbial growth abilities are caused by differences in adaptation methods between low, moderate, and extreme halophilic. Halophiles can balance osmotic pressure against the effects of environments with high salt levels (Roeβler & Müller 2002). High osmotic pressure will denature halophilic microbial cells, causing them to shrink and lose fluid so that their activity is hampered (DasSarma & DasSarma 2017). these microbes can accumulate salts and osmolytes (organic molecules) in their cytoplasm to prevent fluid loss from the cells and avoid denaturation (Roeβler & Müller 2002)

Hemolysis Test

According to the findings of the bacterial test conducted with blood agar (Figureure 5), the bacterial colony exhibited a change in color, and a distinct zone formed around it. Bacterial colonies that possess the ability to lyse all red blood cells and generate a distinct transparent area can produce biosurfactants (Walter, V. et al. 2010).

The formation of the clear zone suggests a contact between bacterial cells and red blood cells, resulting in the degradation of the blood medium. Biosurfactants possess both hydrophilic (water-attracting) and hydrophobic (water-repelling) regions. The hydrophobic component of the biosurfactant can integrate into the lipid bilayer of the red blood cell membrane, while the hydrophilic component interacts with the adjacent water molecules. This contact disrupts the integrity of the cell membrane, resulting in enhanced permeability. Hemolysis results from a damaged cell membrane, which leads to the leakage of the red blood cell's contents, including hemoglobin, into the surrounding environment. (Zaragoza et al. 2010).

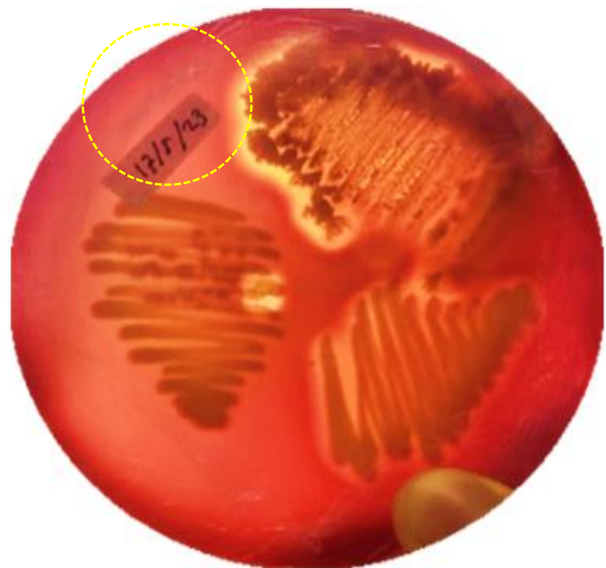

Figure 4
Hemolysis result of STP-GRIV-024

Total Petroleum Hydrocarbon (TPH) Measurement

The TPH results experienced a significant influence based on time and bacterial concentration. After the third day of the bioremediation process with different isolate concentrations, there was a significant decrease for all treatments, and the results obtained were T1 = 1.21%, T2 = 1.32%, T3 = 1.78%, and T4 = 1.61 %. However, in the TPH data on day 7, the TPH value at T1 experienced an increasing trend with a TPH value of 1.9%. This result was constant until day ten compared to other treatments, namely T2, T3, and T4, which experienced a decreasing trend with the final TPH value, respectively 0.51%, 0.25%, and 0.15% (Figureure 5).

The addition of biosurfactant liquid produced by bacteria will reduce the surface tension between oil and water by forming a microemulsion so that the biodegradation process will be more effective. Also, bacteria will find it easier to break down complex hydrocarbon as an energy source and carbon into shorter-chain carbon and various organic compounds such as alkanes (Xu et al. 2018), alcohols, ketones, fatty acids, aldehydes (Pandolfo et al. 2023), alkenes (Wu et al. 2018), CO_2 and H_2O (Sakshi & Haritash 2020).

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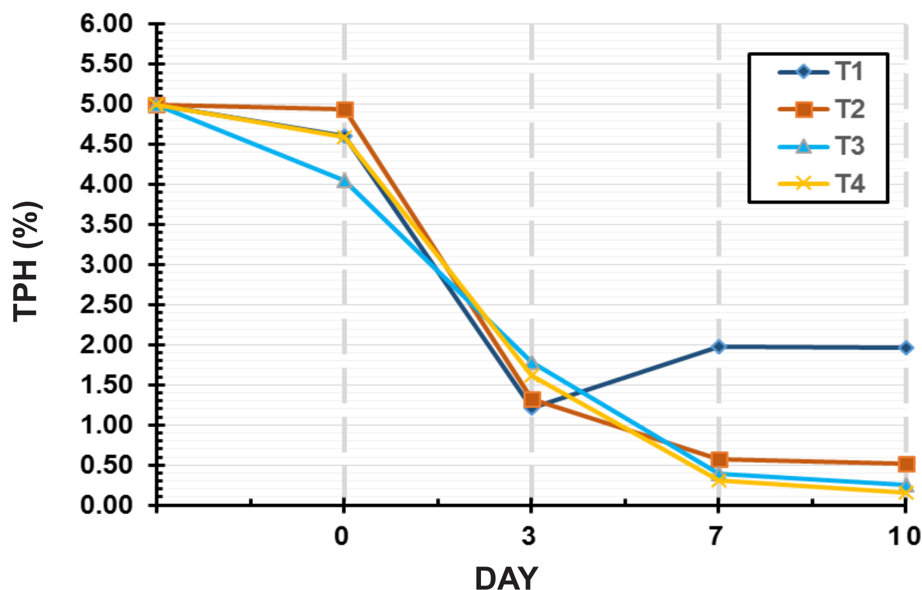

Figure 5
TPH concentration. Information: T1= treatment use 1% of bacterial culture; T2= treatment use 3% of bacterial culture T3= treatment use 5% of bacterial culture T4= treatment use 7% of bacterial culture.

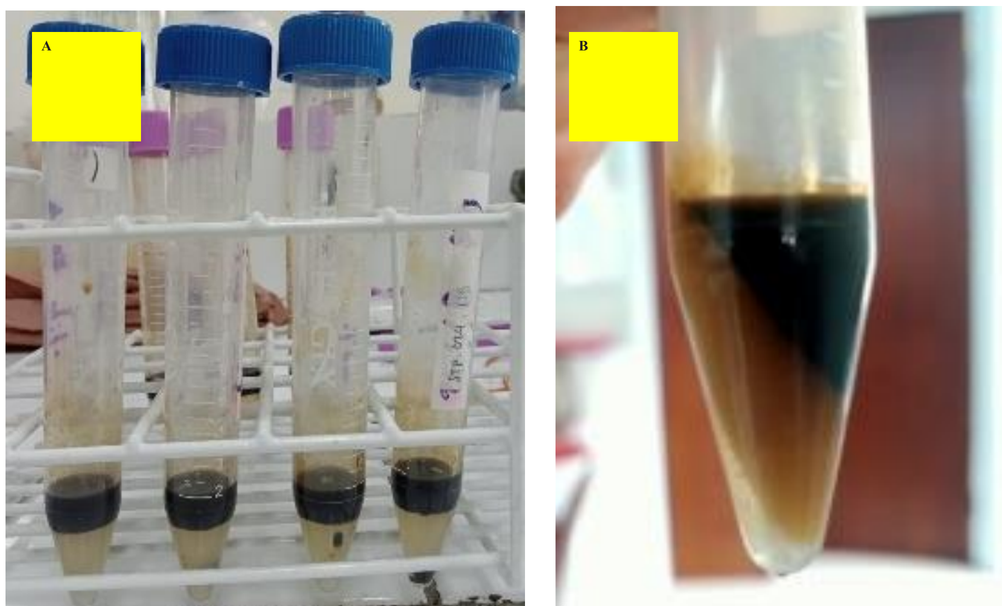

Figure 6
Biosurfactant emulsification: (A) before the emulsification process; (B) After the emulsification process

Biosurfactant Emulsification

The biosurfactant emulsification ability test showed three layers: bacterial culture, oil, and crude oil (Figure 7). Emulsification occurs due to biosurfactant compounds produced by bacteria that can separate crude oil, biosurfactants, and water. Biosurfactants can form micelles that can reduce surface and interfacial tension to optimize compounds that are less soluble in water. The structure and components of the biosurfactant influence the emulsifying capacity of bacteria (Satpute et al. 2010). Biosurfactants generally consist of sugars, amino acids, fatty acids, and functional groups such as carboxylic acids. Glycolipid compounds

(rhamnolipids, sophorolipids, and trehalolipids) are biosurfactants of various sugars bound to hydroxy fatty acids (Awaludin & Sari 2017). In contrast, lipopeptides (surfactants, iturin, and fengycin) consist of cycloheptapeptides with amino acids bound to various fatty acid chains. This structure causes the biosurfactant to be amphiphilic and soluble in polar and non-polar solvents. Due to this amphiphilic structure, biosurfactants can also reduce the surface and interfacial tension of two different phases and form emulsions. This emulsifying ability is an important step that facilitates the acceptance of hydrophobic substrates into cells (Liu et al. 2013; Setiani, N. A. et al. 2019).

Phylogenetic Analysis Using 16S rRNA Marker Gene Sequencing

Phylogenetic analysis using molecular data such as DNA or protein can describe evolutionary relationships between species. The phylogenetic tree was constructed using the neighbor-joining tree method. The neighbor-joining tree method has rows that, when combined, will provide the best estimate of the nearest branch's length to reflect the distance between rows. The phylogenetic tree was tested statistically using the bootstrap method with 1000 replications. The greater the bootstrap value, the higher the confidence in the reconstructed tree topology (Dharmayanti 2011; Pangestika et al. 2015).

The 16S rRNA gene from the isolate was successfully sequenced with a sequence length of 1476 bp (Table 3). Further identification using the EZ Biocloud web server showed that the isolate STP-GRIV-024 is most closely related to S. haemolyticus, S. borealis, and S. pragensis, as depicted from corresponding sequence similarity values of 100 %, 99.86 %, and 99.32 %, respectively. In turn, phylogenetic reconstruction substantiated the evidence of the close evolutionary relationship between isolate STP-GRIV-024 and S. haemolyticus. The relationship is supported by a high bootstrap value of 100% and consistent tree branch topology when constructed using three different tree-making algorithms, including Neighbour-Joining (NJ), Maximum-Likelihood (ML), and Maximum Parsimony (MP). Macrococcus equipercicus was used as the out-group. Table 3 showed that the halophilic bacterial isolate sample was most closely related to the species *Staphylococcus haemolyticus* MTCC3383^{T} with a sequence similarity value (99,90%). The Staphylococcus genus is a facultative anaerobic bacteria that does not form spores or move and is often found in aquatic environments (Fischetti et al. 2019). *Staphylococcus haemolyticus* is a Gram-positive cocci, and *Staphylococcus haemolyticus* was also found in tuna from the results of 16S rRNA sequence analysis with 99% homology (Dewi et al. 2015). *Staphylococcus haemolyticus* is genetically close to S. aureus and S. epidermidis, showing an average nucleotide identity value of 75% (Lamers et al. 2012). S. haemolyticus bacteria are pathogenic bacteria that can cause diseases related to the central nervous system, septicemia, peritonitis, wounds, and bone and joint infections (Eltwisy et al. 2020, 2022). In addition, S. haemolyticus has the potential to produce biosurfactants to determine its effect on bacterial growth and biofilm formation (Rossi et al. 2016)

Based on the results of analysis from the EzBiocloud web server (Table 4), the genotype characteristics of isolate STP-GRIV-024 and Staphylococcus borealis were in second place with a similarity of 99.86%. Staphylococcus borealis was discovered for the first time in human skin and blood (Pain, M. et al. 2020). Meanwhile, in third place is the species Staphylococcus pragensis (Madhaiyan et al. 2020; Švec, P. et al. 2015), with a similarity of 99.32%. Staphylococcus borealis

Table 3
Result from 16S rRNA gene sequencing analyses for Isolate STP-GRIV-024

Isolate Code	Reference Name	Hit Strain	Name	Similarity (%)	Accession Number	Length (bp)
STP-GRIV-024	*Staphylococcus haemolyticus*	MTC	3383 (T)	99.90	LILF010000 56	1,476

Tabel 4
Blast result of STP-GRIV-024 on webserver ezBiocloud

SN	Str.	Sim. (%)	*MN*
S.haemolyticus	MTCC3383 (T)	100	0/1472
S. borealis	MT586030 (T)	99.86	2/1472
S. pragensis	NRL/St 12/356 (T)	99.32	10/1471

Information: SN = Species name; MN = mismatch nucleotide; Sim. = Similarity; Str = Strain

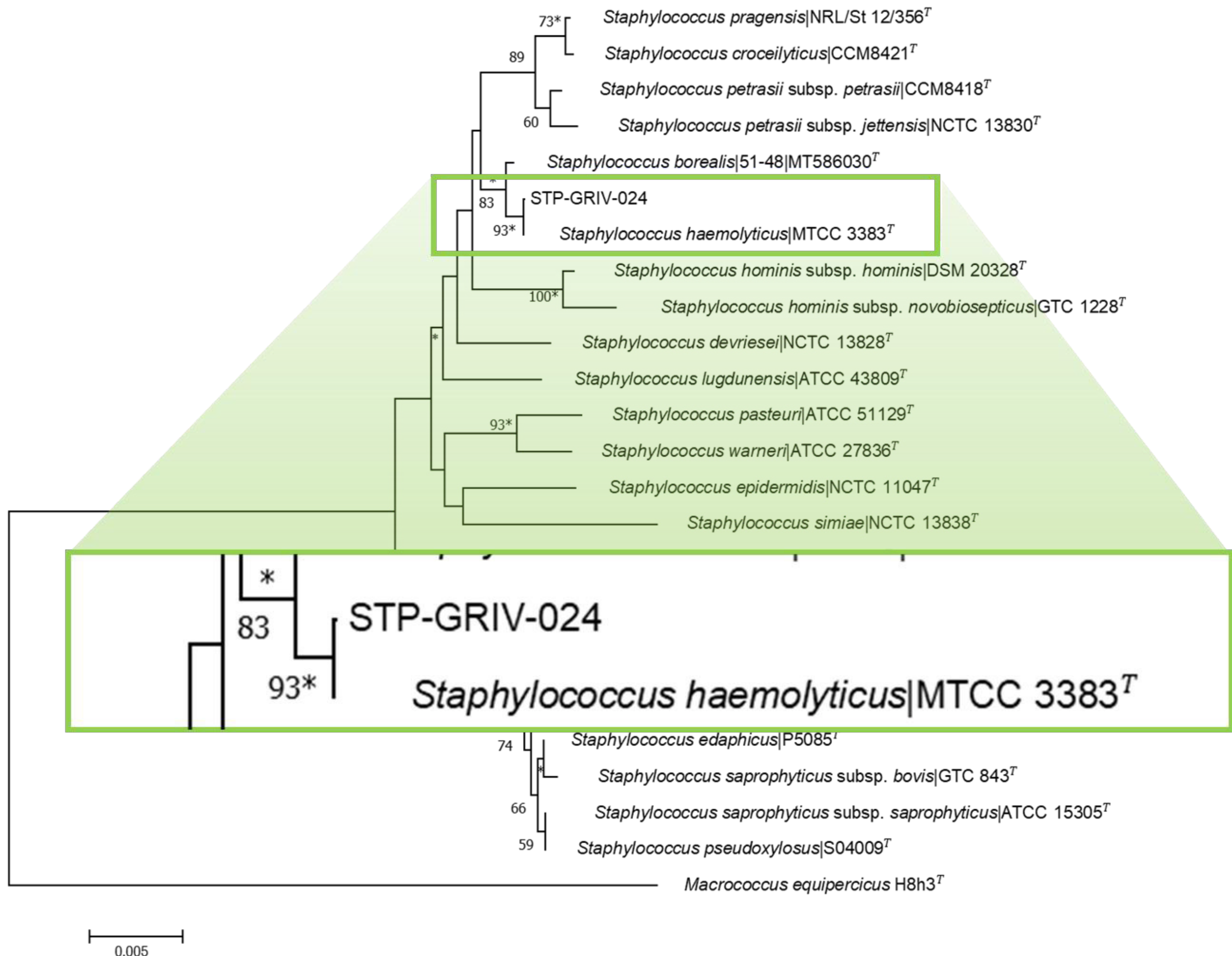

Figure 7
Phylogenetic Tree of Isolate STP-GRIV-024 constructed using Neighbour Joining Algorithm using Asterisk Symbols (*) on branches. (asterisk symbols are consistent codes with phylogenetic trees reconstructed using the maximum likelihood and maximum parsimony algorithms on the GGDC webserver).

and Staphylococcus pragensis. Figureure 7. shows the relationship of 25 species of STP-GRIV-024 isolates that form branches with *Staphylococcus haemolyticus* MTCC3383T with a bootstrap value of 93 and followed by a bootstrap value of 83 for the species *Staphylococcus borealis* MT586030T. In the same clade *Staphylococcus petrasii* subsp. Jettensis has a reasonably low bootstrap value, namely 60. Phylogenetic analysis using the out-group *Macrococcus equipercicus* shows that 25 Staphylococcus species combined with a bootstrap value not too far from the percentage of 70%. Bootstrap analysis with a 70% or higher value indicates reliable grouping (Hillis & Bull 1993).

CONCLUSION

The results of this study show that Kish media with 3% NaCl supplementation has a higher success rate in isolating halophilic bacteria compared to the three other media, namely Halophilic, Soil Extract, and Oatmeal Agar Media. Microscopic characterization of the isolate and Gram staining showed that the bacterial isolate had Gram-positive characteristics with round, white colonies and smooth and rounded edges. The results of the Nacl sensitivity test confirmed that the isolate was moderately halophilic because it could survive at a Nacl concentration of around 7-15% (w/v). The initial potential test for the ability to produce biosurfactant using the Hemolysis test showed that the isolate had the potential to produce biosurfactant, this was indicated by the presence of a clear zone around the edge of the colony. In the TPH test results, concentration and incubation time influenced the level of degradation by the isolate. The isolate with an incubation period of 10 days and a concentration of 7% (T4) had the highest TPH degradation value with a final TPH value of 0.15% compared to other treatments, namely T1 (1%), T2 (3%), T3 (5%) with a TPH value of 1.96%, 0.51%, and 0.25%, respectively. Genetic confirmation using 16S rRNA sequencing identified that the isolate was close to *Staphylococcus haemolyticus* strain MTCC3383T with a similarity level of 99.99%. These results demonstrate the potential application of halophile bacteria for MEOR applications and biodegradation of hydrocarbon pollution in the environment. Further research is needed to find out in detail the types of metabolites and genomes in efforts to develop better biosurfactants.

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GLOSSARY OF TERMS

Symbol	Definitions	Unit
MEOR	Microbial enhanced oil Recovery	
TPH	Total petroleum Hydrocarbon	
DNA	Deoxyribonucleic acid	
16S rRNA	16S ribosomal ribonucleic Acid	
NaCl	Sodium cloride	
CO2	Carbon dioxide	
H2O	Dihydrogen oxide	
NJ	Neighbour-joining	
ML	Maximum-likelihood	
MP	Maximum parsimony	

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