VIETNAM ACADEMY OF SCIENCE AND TECHNOLOGY

INSTITUTE OF PHYSICS

The 8th Academic Conference on Natural Science for Young Scientists Master & PhD. Students from ASEAN Countries

Vinh City, Vietnam. August 27-30, 2023

PROCEEDINGS

Publishing House for Science and Technology

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ISBN: 978- 604- 357- 225-4

Publishing House for Science and Technology - 2023

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ISOLATION AND SELECTION OF SOME MICROBIAL STRAINS PRODUCING BIOSURFACTANT FOR DRILLING MUD TREATMENT IN THE PETROLEUM INDUSTRY

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Abstract: Drilling mud is generated in conducting exploratory drilling and mine development, consisting of a mixture of soil and rock contaminated with oil, chemicals, and drilling fluid. It is very difficult and expensive to treat this waste source, so the biosurfactant produced by microorganisms is considered a highly effective biological treatment method. The aim of this study was conducted to select some microbial strains with high biosurfactant production capacity from samples of soil, water, and mud drilling in some coastal Vietnam. The microbial strains were identified by API - 20NE and API - 50 CHB test kits combined with a Bergey taxonomy key. The results showed that 3 microbial strains with high biossurfactant production capacity had been identified namely *Oligella ureolytica*, *Stenotrophomonas maltophilia* and *Paenibacillus mancerans*, similarity reached 91, 99.99, and 91%, respectively. Strain M150 from Department of Petroleum Microbiology - Institute of Biotechnology classified by 16S rRNA as name *Brevibacterium celere*. This strain was considered as a potential producer strain at fermentation conditions pH = 7.5, temperature 30°C, concentration NaCl 1%, carbon source is DO oil and emulsifying ability reached 73%.

Keywords: *Biosurfactants, Drilling mud, M150, Microoganism, Petroleum industry.*

I. INTRODUCTION

The oil and gas industry are an important sector in the economic development strategy of the country. The reserves and potentials of oil and gas in Vietnam are estimated about 4,600 million tons and distributed mainly in the continental shelf. Only in the past 2022, the oil and gas industry has achieved a revenue of 931.2 trillion VND, equivalent to 9.8% of the country's GDP, contributing 9.5% to the national budget [1]. Besides the great benefits, activities in the oil and gas industry also cause serious ecological pollution to both water, soil, and air environments. According to statistics of the International Association of Oil Tankers, Vietnam is one of the three countries (along with China and the United States) with the highest number of oil spills in the 39 countries listed. During the period from 2005 to 2014, there were an average of 10 or more incidents per year. Since 1992, there are 190 oil spills occurred in Vietnam with 37 cases offshore accounting for 19%, 88 cases inshore accounting for 47% and 65 cases on land accounting for 34% [1].

Moreover, the oil and gas exploration drilling activities take place regularly to the amount of big drilling mud disposing into environment. These waste resources have the potential for environmental pollution in which the oil-based drilling mud is highest. Drilling mud is created when conducting exploratory drilling and mines development, it is a mixture of soil and rock contaminated with oil and chemicals in the drilling fluid [2, 4]. The more oil-based drilling

fluids are used, the more pollution is greater. According to the law, this source of waste must be brought to the shore for treatment. However, this treatment process is difficult and expensive, so contractors are often neglected. Currently, the inner oil and gas industry has only applied conventional physico-chemical measures to reduce harmful effects, without thoroughly treating them before being discharged into the environment. The current urgent requirement is to handle them effectively, simply, and economically.

One of the highly effective biological treatment methods is the use of biosurfactants produced by microorganisms. Biosurfactant is a bipolar compound that allows the dissolution of insoluble substances in water, creating an emulsion that helps microorganisms better contact the oil and easily decomposes the contaminated oil [2-4]. On the other hand, biosurfactants are easily biodegradable, non-toxic and can be produced from cheap substrate sources such as industrial waste, thereby completely solving the pollution caused by these waste sources. However, there are only a few studies on biosurfactants, their research direction and application are still limited. Therefore, this study was conducted to isolate and select some microbial strains with height biosurfactant production capacity from samples of soil, water, and mud drilling for drilling mud treatment in the petroleum industry.

II. MATERIAL AND METHOD

2.1 Setup the experiments

Thirty samples of water, soil and drilling mud in this study were taken on the coastal Thua Thien Hue, Cat Ba, and Vung Tau. The water and soil samples were taken at oil contaminated areas; the drilling mud was taken at crude oil drilling field. 1 gram or 1 mL of sample was put in a test tube or a conical flask containing 9 mL Gost mineral medium 1%, supplemented with 5% DO oil and 1% seed rate. The Gost mineral medium has the following chemical ingredients: Na_2HPO_4 = 0.7 $g.L^{-1}$; KH_2PO_4 = 0.3 $g.L^{-1}$; KNO_3 = 3 $g.L^{-1}$; $MgSO_4$ = 0.4 $g.L^{-1}$ and tap water 1L. Shaking the solution at pH = 7.5, v = 200 rpm and continuously monitored for 5 days. Observe the change of turbidity, the oil status in the medium. If any strains are found capable of decomposing oil, they are transferred to agar plates to keep and propagate for further experiments [4].

2.2 Collect of microbial strains

The selected strains were kept on Gost mineral medium supplemented with DO oil with glycerin at ratio 1:1. The strain was propagated on slanted agar aerobic in test tube, and then inoculated into Gost medium supplemented with 5% DO oil for further experiments.

2.3 Methods of observing bacterial cell and colony morphology

Colony morphology was observed by whisking the strains on aerobic medium, incubating at 30°C after 24 h. Cell morphology was observed by Gram staining test after 24 h and retested with KOH to increase accuracy - take some cell biomass on a slide, drop of 3% KOH solution, use a culture stick to destroy and mix well the cells. If the fluid is viscous the bacteria are Gram-negative, opposite, the bacteria are Gram-positive [4].

2.4 Naming by biochemical standard kit

ISBN: 978- 604- 357- 225-4

The API - 20 NE Biochemistry Standard Kit of Biomerieux uses 20 biochemical tests to identify Gram-negative microorganisms. The API - 50 CHB test kits use 50 biochemical tests to identify spore-forming Gram-positive microbial strains, including reactions with known substances, test the reactions and compare with the taxonomy key to find the unknown species name [4].

2.5 Evaluate the biosurfactant producing capacity by Pruthi method

The capacity for producing biosurfactant was evaluated by the emulsification index E24 [4]. The emulsification index E24 characterizes the emulsification ability of metabolic products produced by microorganisms in xylene solvent after 24 h at 4°C. Take 1 ml of culture solution, centrifuge to remove cells, add 1 ml of xylene into the test tube and voltex for 1 min at 2000 rpm. Samples were kept at 4°C for 24 h and then measured the height of emulsion column. The emulsion stability was determined and calculated after 24 h according to formula as follow [5]:

E24 = (Emulsion layer (height)/Solution (height)) x 100% (Height: mm)

2.6 Statistical analysis

The collected data in this study were statistics processed by the following software Excel 2010 with statistical significance of $\rho < 0.05$. The microbial strains were identified and determined the morphological characteristics by API - 20NE and API - 50 CHB test kits combined with a Bergey taxonomy key.

III. RESULT AND DISCUSSION

3.1 Results of selection of microbial strains

The result showed that 19 microbial strains were isolated from samples at the coastal Cat Ba, Thua Thien Hue, Vung Tau and 01 strain received from the Department of Petroleum Microbiology - Institute of Biotechnology have capacity for producing biosurfactant and decomposing oil. The strains were isolated including CB1C, CB2C, CB3C, CB4C, CB5C, CB6C, CB7C, CB8C, CB9C, CB10, NVD, CB P, CB F, PM 1b1-PM3b1, PM 5b1, PM 4b2, and Tb2. In which, there are three strains coded as CB 1C, NVD, and CB P with the highest oil degradation ability. Strain M150 from Department of Petroleum Microbiology - Institute of Biotechnology analyzed 16S rRNA sequence with name as *Brevibacterium celere*. The highest emulsification index E24 value of strains CB 1C, NVD, CB P and M150 after 5 days shaking oubation observed of 71, 52, 59 and 73%, respectively. These strains will be used for further experiments. The experimental results are shown in Table 1 and Fig. 1.

Table 3. *The emulsifying capacity of microbial strain generating biosurfactants isolated from coastal Vietnam.*

No	Strains code	Day 1		Day 2		Day 3		Day 4		Day 5	
		E0 (%)	E24 (%)	E0 (%)	E24 (%)	E0 (%)	E24 (%)	E0 (%)	E24 (%)	E0 (%)	E24 (%)
1	CB 1C	0	0	0	0	24	24	54	54	71	**71**
2	CB 2C	0	0	0	0	0	0	19	19	26	26
3	CB 3C	0	0	0	0	0	0	27	27	36	36

4	CB 4C	55	55	44	44	50	50	37	37	36	36
5	CB 5C	0	0	0	0	0	0	58	58	46	46
6	CB 6C	37	37	33	33	33	33	36	36	33	33
7	CB 7C	11	11	10	10	15	15	34	34	50	50
8	CB 8C	0	0	0	0	0	0	46	46	52	52
9	CB 9C	0	0	0	0	18.5	18.5	31	31	22	22
10	CB 10C	0	0	0	0	26	26	52	52	18.5	18.5
11	NVD	0	0	0	0	50	50	50	50	52	**52**
12	CB P	0	0	25	25	36	36	54	54	59	**59**
13	CB F	10	10	18.5	18.5	18.5	18.5	60	60	37	37
14	PM 1b1	0	0	0	0	0	0	24	20	0	0
15	PM 2b1	0	0	0	0	38	38	17	17	52	52
16	PM 3b1	0	0	0	0	0	0	11.5	11.5	14	14
17	PM 4b2	10	0	48	48	41	41	40	40	38	38
18	PM 5b1	0	0	0	0	0	0	36	36	36	36
19	Tb 2	36	36	63	63	50	50	50	50	50	50
20	M150	35	35	50	50	65	65	69	69	73	**73**

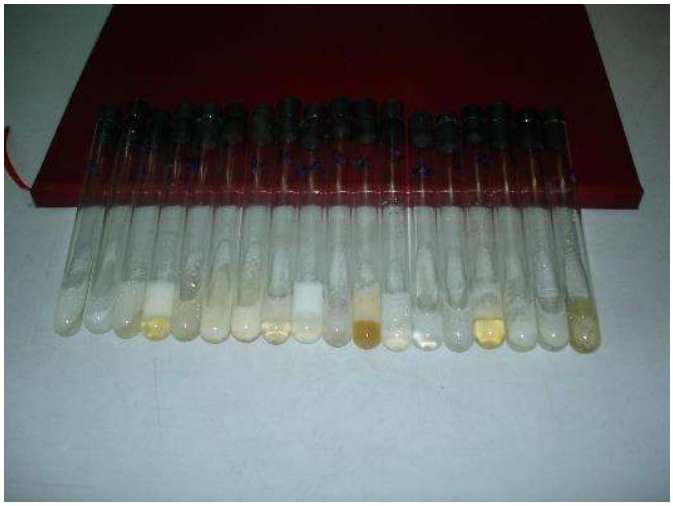

Fig. 1. *Emulsifying ability with xylene of microbial strains.*

3.2 *Morphological* characteristics of colonies and cells

From the ressult in 3.1 section, the microbial samples with E24 value > 50% were collected to culture for morphological characteristics of colonies and cells. The microbial samples were cultured on aerobic agar petri plate supplement 1% NaCl in conditions incubation 30°C. After 24 h, the samples were observed the colony morphology, motility ability and carried out the Gram stain to determine cell morphology. The classification results are shown in Table 2. The M150 strain with the highest E24 value was selected to observe the morphological characteristics of cell and colony. The results are shown in Fig. 2 and 3 below.

ISBN: 978- 604- 357- 225-4

Table 2. *The morphological characteristics of some microbial strains.*

Strain code	Colony characteristic	Cell morphology	Gram type
CB 1C	Opalescent, slightly brown; neat edges, wet smooth, indistinct center; round colonies, ϕ = 1 - 3mm	Cylinder shape, motionless, dispersion.	-
CB 8C	***CB 8 C1:*** Milk white; evenly rounded; wet smooth; there is a protrusion in the middle; neat edge with three concentric rings above colonies surface, ϕ = 3mm	Oval shape, motionless, single bonded, dispersed.	+
	CB 8 C2: Opalescent brown, flat shape, wet smooth, neat edge, ϕ = 2mm	Oval shape, motionless, single bonded, dispersion.	+
PM 2b1	***PM 2b11:*** Milk white, evenly rounded; wet smooth, sticky, neat edge, ϕ = 3mm	Oval shape, motionless, single bonded some time paired bonded, dispersion.	+
	PM 2b12: Opalescent, neat edge, wet smooth, flattened.	Cylinder shape, motionless, dispersion.	+
CB F	***CB F1:*** Milk white, evenly rounded; wet smooth; neat edge, center in the middle, ϕ = 2mm	Round and oval shape, motionless, dispersion.	+
	CB F2: Opalescent brown; wet smooth; flattened; There are three concentric rings concave, slightly convex edge, ϕ = 3mm	Cylinder shape, motion, dispersion	+
CB P	***CB P1:*** Opalescent brown, flat shape, wet smooth, convex center, neat edge, ϕ = 4 - 5mm	Oval shape, motion, single bonded, dispersion	-
	CB P2: Opalescent, round, wet smooth, neat edge, convex center, ϕ = 1 - 2mm	Oval shape, paired, dispersion	+
NVD	***NVD 1:*** Slight yellow, wet smooth, sticky, flattened center, neat edge, ϕ = 1 - 4 mm	Short oval shape, motionless, dispersed, single bonded	+
	NVD 2: Opalescent, round, wet smooth, neat edge, convex center, ϕ = 1 - 2mm	Cylinder shape, motionless, dispersion.	+
M150	Carrot yellow, round, wet smooth, convex center, neat edge, ϕ = 1 - 3 mm	Short, slightly curved rod, motion	+

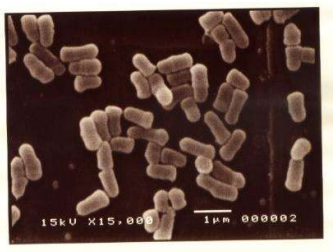

Fig. 2. *The morphological characteristic of cell M150 strain.*

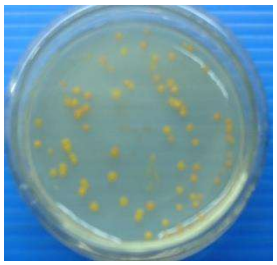

Fig. 3. *The morphological characteristic of colony M150 strain.*

The classification results showed that most of the microorganism isolated belong to Gram-positive group (13/15 strains), only two strains were Gram-negative species. The four strains with the highest E24 index will be selected for identification name in the next experiments.

3.3 Classification of some microbial strains by biochemical kit

The nature of the test kits is based on the biochemical reactions in the test wells, compared with the classification table to determine the microbial species name. The results of the biochemical reactions of the selected strains are presented in Tables 4 and 5.

Table 4. *The result test by API-20NE kit.*

No.	Test	CB 1C strain	CB P strain	No.	Test	CB 1C strain	CB P strain
1.	NO_3	-	-	12.	MAN	-	-
2.	TRP	-	-	13.	NAG	-	+
3.	GLU	-	-	14.	MAL	+	+
4.	ADH	-	-	15.	GNT	-	-
5.	URE	+	-	16.	CAP	-	-
6.	ESC	-	+	17.	ADI	-	-
7.	GEL	-	+	18.	MLT	+	+
8.	PNPG	-	-	19.	CIT	+	+
9.	GLU	+	+	20.	PAC	-	-
10.	ARA	+	-	21.	OX	-	-
11.	MNE	-	+				

Table 5. *The result test by API - 50 CHB kit.*

No.	Test	Substrate	Result	No.	Test	Substrate	Result
0		Control		25	ESC	Esculin ferric citrate	+
1	GLY	Glycerol	+	26	SAL	Salicin	+
2	ERY	Erythritol	-	27	CEL	D - celiobiose	+
3	DARA	D - arabinose	-	28	MAL	D - maltose	+
4	LARA	L - arabinose	-	29	LAC	D - lactose	+
5	RIB	D - ribose	+	30	MEL	D - melibiose	+
6	DXYL	D - xylose	-	31	SAC	D - saccharose	+
7	LXYL	L - xylose	-	32	TRE	D - trehalose	+
8	ADO	D - adonitol	-	33	INU	Inulin	-
9	MDX	Methyl - β,D - xylopyranoside	-	34	MLZ	D - melezitose	+
10	GAL	D - galactose	+	35	RAF	D - Raffinose	+
11	GLU	D - glucose	+	36	AMD	Amidon (starch)	+
12	FRU	D - fructose	+	37	GLYG	Glycogen	+
13	MNE	D - mannose	-	38	XLT	Xylitol	-
14	SBE	L -sorbose	-	39	GEN	Gentiobiose	+
15	RHA	L - rhamnose	-	40	TUR	D - turanose	+
16	DUL	Dulcitol	-	41	LYX	D - lyxose	-
17	INO	Inositol	+	42	TAG	D - tagatose	-
18	MAN	D - mannitol	+	43	DFUC	D - fucose	-
19	SOR	D - sorbitol	-	44	LFUC	L - fucose	-
20	MDM	Methyl - αD - mannopyranoside	-	45	DARL	D - arabitol	-
21	MDG	Methyl - αD - glucopyranoside	+	46	LARL	L - arabitol	-
22	NAG	N - acetyl glucosamine	+	47	GNT	Potassium gluconate	+
23	AMY	Amygdalin	+	48	2KG	Potassium 2 – keto gluconate	-

ISBN: 978- 604- 357- 225-4

No.	Test	Substrate	Result	No.	Test	Substrate	Result
24	ARB	Arbutin	+	49	5KG	Potassium 5 – keto gluconate	-
25	ESC	Esculin ferric citrate	+				

Where: +: positive reaction/-: negative reaction

The result of using the API - 20 NE biochemical test kit for the Gram-negative strain CB 1C and CB P showed that in the 21 biochemical test wells have 06 and 08 wells respectively were positive reaction, changed the color of stock solution in the well. Those are numbered wells for CB 1C, and CB P as follows 5, 9, 10, 14, 18, 19 and 6, 7, 9, 11, 13, 14, 18, 19, respectively. The result of using the API - 50 CHB biochemical test kit for the spore-forming Gram-positive strain NVD showed that in the 50 biochemical test wells have 26 wells were positive reaction, changed the color of stock solution in the well. Those are numbered wells: 1, 5, 10, 11, 12, 17, 18, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 34, 35, 36, 37, 39, 40, 47.

Based on Bergey's taxonomy key, the names of microbial strains identified namely as follows: CB 1C, CB P and NVD strains belong to the *Oligella ureolytica*; *Stenotrophomonas maltophilia* and *Paenibacillus mancerans* species with similarity reaching respectively 91, 99,99, and 91%. M150 strain has been classified by 16S rRNA (Fig. 7) as *Brevibacterium celere*. The classification results are shown in Figures 4, 5, 6 and 7.

Fig. 4. *The isolation result strain CB 1C by API-20NE biochemical kit.*

Fig. 5. *The isolation result strain CB P by API-20NE biochemical kit.*

Fig. 6. *The isolation result strain NVD by API-50CHB biochemical kit.*

This result is consistent with results obtained for biosurfactant-producing capacity of microbial strains by Shah et al., (2016), Barakat et al., (2017), Joshi et al., (2014),… [6-8]. According to Shah et al., (2016), the biossurfactant produced by *Pseudomonas aeruginosa* can decompose the crude oil [6]. The result showed that the biosurfactant was supplemented with 1% crude oil (TAPIS) as the carbon source, the emulsification index E24 of Rhamnolipids to TAPIS was maximum value of 42% [6]. Barakat et al., (2017) had isolated 21 oil-spilled seawater samples from Shalateen, Red Sea, Egypt [7]. The result obtained that two strains SH20 and SH24 with the highest emulsification index (E24) 57 and 56%, respectively. The classification results by 16S rRNA were identified as *Bacillus amyloliquefaciens* SH20 and *Bacillus thuringiensis* SH24 [7]. The fact that biosurfactant can reduce surface tension of both aqueous solutions and hydrocarbon mixtures [8]. Therefore, Joshi et al., (2014) indicated that

microbial strain isolation from soil samples at petrol pumps and garages in Kalyan also have able produced biosurfactant [8]. To confirm the ability of isolates to produce biosurfactant, the surface tension and emulsification index (E24) test were conducted.

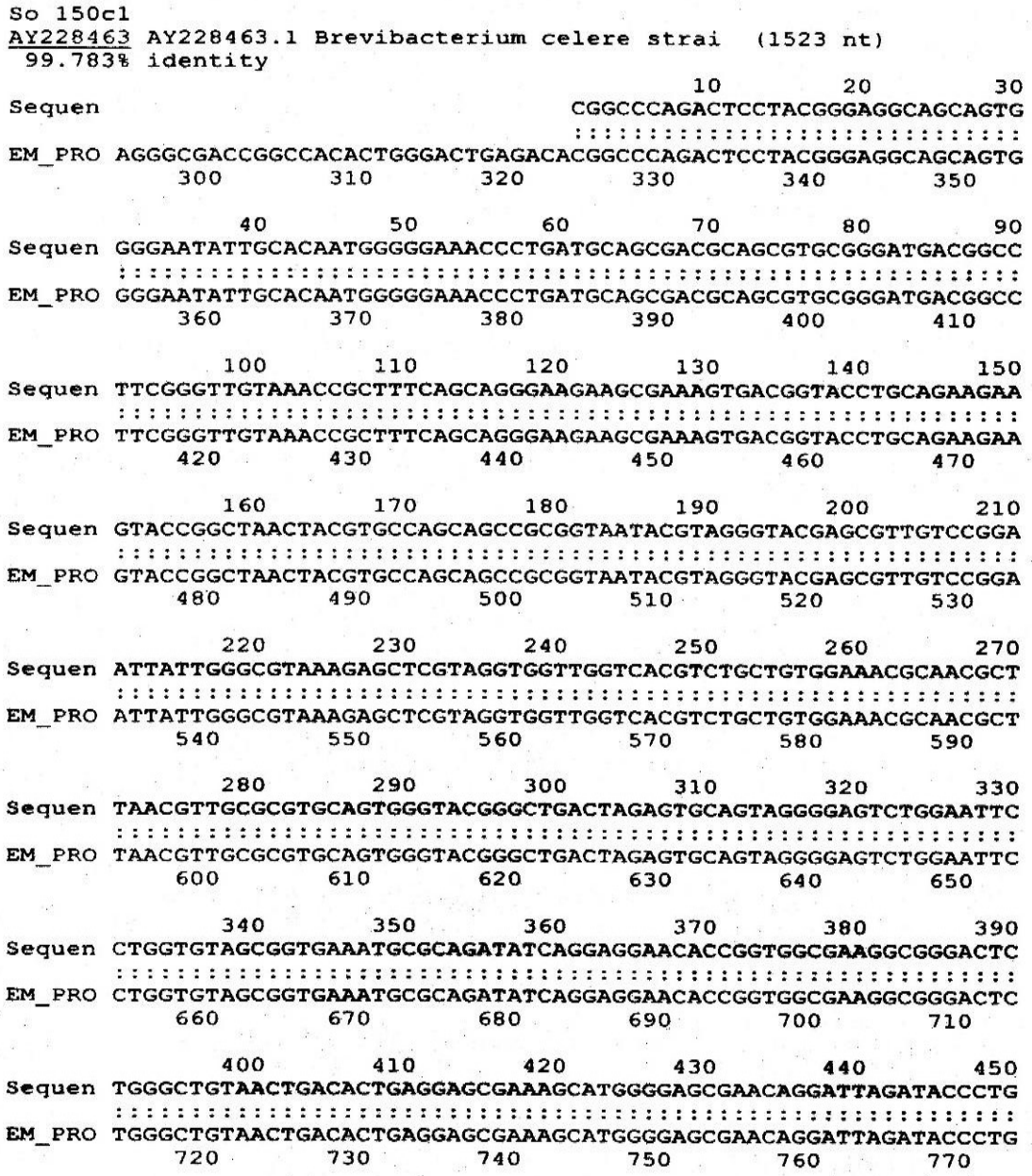

Fig. 7. *The taxon diagram 16SrARN of M150 strain.*

The isolation result indicated that a maximum biomass and biosurfactant yield of 1.72 and 0.68 $g.L^{-1}$, respectively. The identification result with 16s rRNA analysis showed that this strain identified as *Pseudomonas stutzeri*. The different microbial strains have the biosurfactant-producing capacity and degrading oil differently. The source of initial oil used in many studies is also very diverse, but mainly focus to use DO oil, saraline for lubricating the drill bit or increasing the pressure to push the oil. Therefore, the biosurfactant-producing strain

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would have great potentials in bioremediation activities, microbial enhanced oil recovery and drilling mud treatment in the petroleum industry.

IV. CONCLUSION

This study collected a total of 19 microbial strains from the coastal Cat Ba, Thua Thien Hue and Vung Tau including 04 strains with high surfactant capacity, coded as CB 1C, CB P, NVD, and M150. The emulsifying E24 index of four strains had calculated with values of 71, 52, 59, and 73%, respectively. The analysis result by biochemical kits API - 20NE and API - 50CHB showed that the taxonomic positions of 3 biosurfactant-producing strains were identified: CB 1C, CB P, and NVD belongs to *Oligella ureolytica*, *Stenotrophomonas maltophilia*, and *Stenotrophomonas maltophilia* species with similarity reaching respectively 91%, 99.9%, and 91 %. Strain M150 classified by 16S rRNA as name *Brevibacterium celere*. The biosurfactants produced by microbial strains isolated from the coastal Vietnam and especially *Brevebacterium celere* strain can be applied in drilling mud treatment and environmental pollution treatment.

REFERENCES

[1] BH. Diem, DTT. Phuong, and TP. Hung, Environment protection in oil and gas exploration and production activities, offshore Vietnam, *Journal of oil and gas*, **12**, 2022, pp. 45-49.

[2] LT. Hien, Lecture for master's in petroleum microbiology*, Science and Technology Publishing House,* 1997.

[3] JD. Desai, and IM. Banat, Microbial production of surfactants and their commercial potential. *Microbiol Mol Biol R*, **61**(1), 1997, pp. 47-64.

[4] LT. Hien, DV. Thang, NT. An, TC. Van, DT. Hoa, CHHBMSH-producing bacteria isolated from Nha Trang sea, *Journal of Marine Science and Technology*, **2**, 2004, pp. 2-13.

[5] M. S. Bami, P. Khazaeli, H. Forootanfar, G. Dehghannoudeh, and M. Ohadi, Isolation and identification of biosurfactant producing bacterial strain from saline soil samples in Iran; Evaluation of factors on biosurfactant production, *Jundishapur Journal of Natural Pharmaceutical Products,* **15** (4), 2020, pp. e96798.

[6] MUH. Shah, M. Sivapragasam, M. Moniruzzaman, and SB. Yusup, A comparison of recovery methods of rhamnolipids produced by *Pseudomonas aeruginosa, Procedia Engineering*, **148**, 2016, pp. 494-500.

[7] KM. Barakat, SWM. Hassan, and O. M. Darwesh, Biosurfactant production by haloalkaliphilic *Bacillus* strains isolated from red sea, Egypt*, The Egyptian Journal of Aquatic Research*, **43**(3), 2017, pp. 205-211.

[8] PA. Joshi, and DB. Shekhawat, Screening and isolation of biosurfactant producing bacteria from petroleum contaminated soil, *European Journal of Experimental Biology*, **4**(4), 2014, pp. 164-169.