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ISOLATION AND CHARACTERIZATION OF HALOTOLERANT AEROBIC BACTERIA FROM OIL RESERVOIR

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Abstract. Several halotolerant bacteria were isolated from brine samples from Semangkok oil reservoir. Biochemical and morphological characterization of the bacteria were carried out. These bacteria are gram positive spore formers and have been identified as belonging to the genus *Bacillus*. Most of the isolates could grow in medium containing kerosene as sole carbon source and energy and tolerate NaCl concentration up to 15%. Interfacial tension and surface tension tests showed that the bacteria were capable of producing biosurfactant. Six out of nine were able to produce exopolysaccharide. We believe these isolate could be appointed as future biopolymer producer especially for microbial enhanced oil recovery (MEOR).

Keywords: Bacteria, Characterization, Isolation, Surfactant, Exopolysaccharides.

Abstrak. Beberapa bakteria halotoleran telah berjaya dipencilkan dari pada sampel air masin telaga minyak Semangkok di mana ujian biokimia dan morfologi telah dijalankan bagi bakteria ini. Hampir semua bakteria yang dipencilkan tergolong di dalam genus *Bacillus*. Sebahagian besar daripada pencilan berkeupayaan untuk hidup di dalam medium pertumbuhan yang mengandungi kerosen sebagai sumber karbon utama dan tenaga dan berkeupayaan untuk hidup di dalam medium dengan kepekatan NaCl diantara 10-15%. Ujian ketegangan antara permukaan (IFT) menunjukkan pencilan menghasilkan biosurfaktan. Enam daripada sembilan pencilan menghasilkan eksopolisakarida di dalam medium pertumbuhan yang mungkin penting di dalam MEOR.

Kata kunci: Bakteria, pencirian, pengasingan, Surfaktan, Eksopolisakarida

1.0 INTRODUCTION

The presence of indigenous microorganisms in oil reservoir have been reported since 1940s. Viable bacteria have been detected in brine and oil samples from petroleum reservoirs. Nonsporulating halophilic bacteria were isolated by Gervetz *et. al* [1] from oil brines. The bacteria belong to the genera of *Methanohalophilus*, *Desulfovibrio* and possibly *Thermatoga*. Microorganisms involved in hypersaline ecosystem were assigned into four specific groups [2]. They are fermentative bacteria, homoacetogenic bacteria, sulfate-reducing bacteria and methanogenic bacteria. However heterotrophic aerobic bacteria such as *Bacillus* sp., *Mycobacterium* sp.,

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Curtobacterium sp., *Cellulomonas* sp., *Rhodococcus* sp. and *Pseudomonas* sp. have been isolated from oil storage caverns in northern Germany at the depth of 1500m [3].

The isolation of microorganisms in oil reservoir is an important element in the Microbial Enhancement of Oil Recovery (MEOR). MEOR involves the application of microorganisms and their metabolic products and this can be achieved by several ways [4]. One of them involves products such as polymer, solvents and emulsifiers or surfactants produced in fermenters and then injected into a reservoir or the indigenous microorganisms can be injected directly along with nutrients into the oil reservoir to aid in oil recovery. Biosurfactant and exopolysaccharide (EPS) are two metabolic products important in MEOR. Biosurfactant is a complex biopolymer compounds excreted by microbial cells grown on certain hydrocarbons or other substrates such as carbohydrates [5]. Biosurfactants have the potential in mobi-lizing heavy crude oil, transporting petroleum in pipelines, managing oil spills, controlling oil pollution and cleaning oil sludge from oil storage facilities. Exopolysaccharide is a polymer produced by the microorganisms outside the cell as slime layers or capsules. In MEOR, the addition of biopolymer improves the sweep efficiency and the displacement process by increasing the viscosity of the displacing phase [6].

In this work, the isolation and characterization of microorganisms from local oil fields were carried out. The potential of these isolates in producing biosurfactant and exopolysaccharides were examined by measuring the interfacial tension, surface tension and viscosity of the culture medium.

2.0 METHODOLOGY

2.1 Formation Water Samples

Formation waters were taken from Semangkok oil reservoir (well A12 and well A13) in the East Coast of Peninsular Malaysia. Well temperature measured ranged from 53°C to 62°C with pH between 8.0–8.8. Samples were collected from well head of selected oil wells into sterilized container. The samples were stored at 4°C until required.

2.2 Media and Growth

The enrichment medium used to isolate the microorganisms was modified from Magaritis *et. al* [5], Ramsay *et. al* [7] and Bock *et. al* [3] based on the results of the formation water analysis (data not shown). The modified mineral salts medium contained: 5–8 g/l NaCl, 0.1 g/l KCl, 1.0 g/l NH_4NO_3, 0.5 g/l K_2HPO_4, 0.5 g/l KH_2PO_4, 0.5 g/l $MgSO_4.6H_2O$, 0.1 g/l $CaCl_2$, 1.0 g/l yeast extract, 1.0 g/l glucose, 20 ml/L kerosene, and 1 ml/L of trace minerals solution [1]. The final pH of the medium was adjusted to 6.5–6.8.

2.3 Microbial Isolation and Characterization

Formation water was initially inoculated into the liquid enrichment medium and incubated for 5 to 7 days at 45°C, 50°C and 60°C. Following that, colonies were picked and re-streaked on the same agar medium to obtain pure cultures. Isolated pure cultures were differentiated by colony and cell morphology, as well as physiology and biochemical tests. The isolates were grown in enrichment medium containing kerosene as the sole carbon source in hydrocarbon degradation test.

2.4 Metabolic Product

Metabolic product tests were carried out in 500-ml Erlenmeyer flasks containing modified enrichment medium. The flasks were incubated aerobically on a rotary shaker (200 rpm) at optimal growth temperature. Samples were taken every four hourly and the optical density measured at 540 nm [8–9] using the UV-1100 spectrophotometer.The samples were then centrifuged at 10,000g for 10 min at 4°C [10] to pellet the cells and the supernatant collected.

2.4.1 Biosurfactants Production Test

Biosurfactants production was detected by measuring the surface activity [surface tension or interfacial tension (IFT)] of the growth medium. Surface activity was measured with CDS-DuNouy ring tensiometer. Surface tension was measured by placing the platinum-iridium ring just below the surface of supernatant solution, which then raised through the surface of the aqueous solution until the ring broke. The amount of force required for breaking through the surface layer was recorded in dynes/cm.

The same apparatus and procedure were used for measuring IFT between supernatant solution and iso-octane phase that acted as hydrocarbon phase. The platinum-iridium ring was placed below the surface of supernatant solution, followed by the addition of iso-octane to the layer of the sample. The ring was raised until it broke through the surface of sample and hydrocarbon phase. The amount of force was also recorded in dynes/cm. All measurements were done at room temperature.

2.4.2 Biopolymers Production Test

Biopolymers production was detected by measuring the viscosity of growth medium. The viscosity of supernatant solution was measured using a glass capillary viscometer (Ubbelohde viscometer) at 25°C in a refrigerated bath and circulator (Hetofrig CB11e). The sample broth was first equilibrated in a refrigerated bath for 2 minutes before viscosity was measured. The efflux time for the meniscus to pass the marked distances was measured. Kinematic viscosity (mm^2/s) and dynamic viscosity (mpa.s or cP) were calculated as follows:

$$\text{Kinematic viscosity, } \mu_K = t_e \times K_{viscometer} \quad (1)$$

$$\text{Dynamic viscosity, } \mu_D = \mu_K \times \rho_{sample} \quad (2)$$

where t_e = efflux time (s), $K_{viscometer}$ = viscometer constant, 0.0961 mm^2/s^2), and ρ_{sample} = density of sample (g/ml)

3.0 RESULTS AND DISCUSSION

3.1 Isolation and Characterization of Microorganisms

Nine possible strains of bacteria have been isolated from oil brine samples from Semangkok oil field. All strains were grown on the modified enrichment medium [3, 5, 7] containing kerosene as carbon and energy source under aerobic condition. The isolated microorganisms were stained gram-positive and formed oval-shaped endospore. All strains were found to be rod-shaped, some grew in short chain and some in long chains. Previous researchers [3, 6, 11] reported that most of the microorganisms from hypersaline environment are rod shaped. Anaerobic and catalase tests show that all strains were able to grow under anaerobic condition and are catalase positive. These findings are in good agreement with previous studies which reported that aerobic microorganisms present in well saline are predominated by gram-positive, facultative and spore forming rod bacteria [12]. Based on the biochemical and morphological tests (Table 1) done according to the Bergey's manual [13], all strains belonged to the genus *Bacillus*.

All strains could grow at temperature ranging from 37°C to 65°C, however optimum temperature varied from 45°C to 50°C (Table 2). *Bacillus* sp. S12A, S13 and S17A could grow in a medium containing NaCl up to 15%. *Bacillus* sp. S1 and S9B can tolerate NaCl concentration up to 12% while strains S4A, S7, S19 and S21A can tolerate only 10% of NaCl concentration as shown in Table 2. All of these isolates were able to grow over a broad pH ranging from pH 5 to pH 12. However, these isolates grew well between pH 6-9. Growth declined as the pH of the medium increase or decrease. Most reservoir microorganisms have been reported to grow well in pH ranging from 4.5–8.5 [1].

The ability of the isolates to grow on kerosene as sole carbon source and energy was also tested (Table 2). We observed that growth for strain S21A was abundant in medium containing kerosene. All the other strains were also capable of growing on kerosene containing medium except for strain S7. Although most of reservoir microorganisms have been reported to have the ability to utilize hydrocarbon [12], some do not oxidize higher hydrocarbons but can only degrade low molecular-weight hydrocarbon such as gases [7]. The ability to grow on hydrocarbons is widespread for bacteria such as *Pseudomonas*, *Alcaligenes*, *Micrococcus*, *Norcadia*, *Bacillus*, *Rhodococcus* and *Proteus*. All these strains could also utilize a variety of carbohydrate

Table 1 Biochemical Test of the Isolates

Test \ Isolates	S1	S4A	S7	S9B	S12A	S13	S17A	S19	S21A
Gram stain	+	+	+	+	+	+	+	+	+
Spore stain	+	+	+	+	+	+	+	+	+
Motility	–	–	–	+	+	–	–	+	–
Anaerobic test	+	+	+	+	+	+	+	+	+
Carbohydrate catabolism									
OF (Fermentative)	+	+	+	+	+	+	+	+	+
Starch	+	+	+	+	+	+	+	+	+
Polysaccharides	+	+	+	+	+	+	+	+	+
Carbohydrate fermentation									
Methyl Red	–	–	–	–	–	–	–	–	–
Voges Proskauer	+	+	+	+	+	+	+	+	+
Gas from glucose	–	–	–	–	–	–	–	–	–
Citrate	–	–	–	–	–	–	–	–	–
Protein catabolism									
Urease	–	–	–	–	–	–	–	–	–
Gelatin	–	–	–	–	–	–	–	–	–
Casein	–	+	–	–	+	+	+	+	–
H_2S	–	–	–	–	–	–	–	–	–
Indole	–	–	–	–	–	–	–	–	–
Respiration									
Catalase	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	–
Nitrate reduction	+	+	+	–	+	+	+	+	+

Table 2 Physiological Characterization of Isolates from Semangkok Reservoir

Strain	Group	Optimum temperature (°C)	Growth in NaCl (%w/v)	Growth in kerosene	pH of culture
S1	Group I *Bacillus* sp.	45	0-12	++	5-12
S4A		45	0-10	++	5-12
S7		45-50	0-10	–	5-12
S9B		45-50	0-12	++	5-12
S12A		45	0-15	+	5-12
S13		50	0-15	++	5-12
S17A		45	0-15	+	5-12
S19		50	0-10	+	5-12
S21A		45	0-10	+++	5-12

sources such as glucose, sucrose, fructose, maltose, xylose, mannitol, salicin, glycerol, D-sorbitol, lactose, except for dulcitol in which no growth was observed.

The presence of biosurfactant produced by the bacteria have been shown to reduce the surface tension and interfacial tension (IFT) of the medium. Table 3 shows that all isolates can reduce the IFT of the medium ranging from 12.07 – 27.27 dynes/cm. Hydrocarbon degrading microorganisms can synthesize biosurfactant and the release and function of biosurfactant are often related to hydrocarbon uptake [14]. In this study, we observed that *Bacillus* sp. S17A, S19A and S21A were capable of reducing the IFT of the medium up to 25 dynes/cm or more. Therefore, our own values are in agreement with values obtained by other researchers [4, 6] which is around 19-25.8 dynes/cm. Figure 1 shows the reduction if IFT and ST of the culture medium due to the production of biosurfactant by *Bacillus* sp. S17A. The biosurfactant production is directly proportional to cell growth. As cell growth increases, surface tension and IFT decreases. The surface tension and interfacial tension became constant and increased slightly during late stationary phase. Similar findings were also obtained for other surfactant-producing bacteria [15].

Table 3 Exopolysaccharide and Biosurfactant Production. Initial Viscosity, ST and IFT are 1.01 cp, 71.10 dynes/cm and 67.17 dynes/cm, respectively.

Group	Strain	Final Viscosity (cP)	Final IFT (Dynes/cm)	Total reduction of IFT (Dynes/cm)	Final ST (Dynes/cm)	Total reduction of ST (Dynes/cm)
I *Bacillus* sp.	S1	3.51	47.27	19.92	61.88	9.22
	S4A	–	47.92	19.27	62.7	8.4
	S7	3.42	54.72	12.47	65.85	5.25
	S9B	3.43	48.25	18.94	64.23	6.87
	S12A	–	55.12	12.07	68.98	2.12
	S13	3.76	47.27	19.92	63.55	7.55
	S17A	3.38	39.92	27.27	51.7	19.4
	S19	3.54	41.92	25.27	48.45	22.65
	S21A	–	41.97	25.22	49.75	21.35

As indicated in Table 2, all isolates tested for polysaccharide production on sucrose containing medium agar plate exhibit the presence of mucoid colony due to the presence of exopolysaccharide. However, only three strains that are S1, S7, S9B, S13, S17A and S19 were able to increase the viscosity of the liquid culture medium in shake flask after 16 hours of incubation. Figure 2 shows the production of

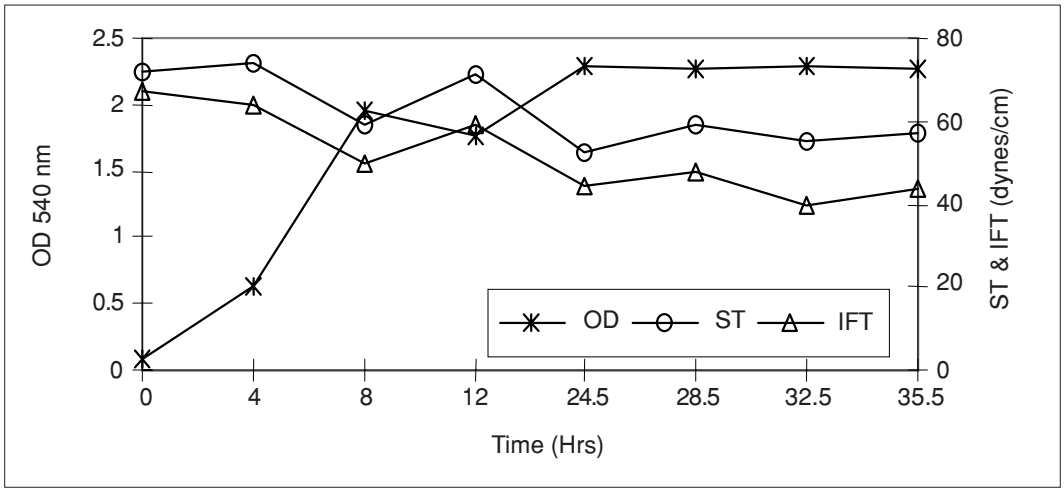

Figure 1 Growth versus Surface Tension and IFT Reduction by Strain S17A

exopolysaccharide from *Bacillus* sp. S13A of increasing the viscosity of the medium from initially 1.01 cP up to 3.76 cP, the highest observed. The medium viscosity increased largely during exponential growth of the bacteria. The viscosity was maintained for about 5 hours and decreased soon after. Similar patterns for exopolysaccharides production was also observed by Souw and Demian [15].

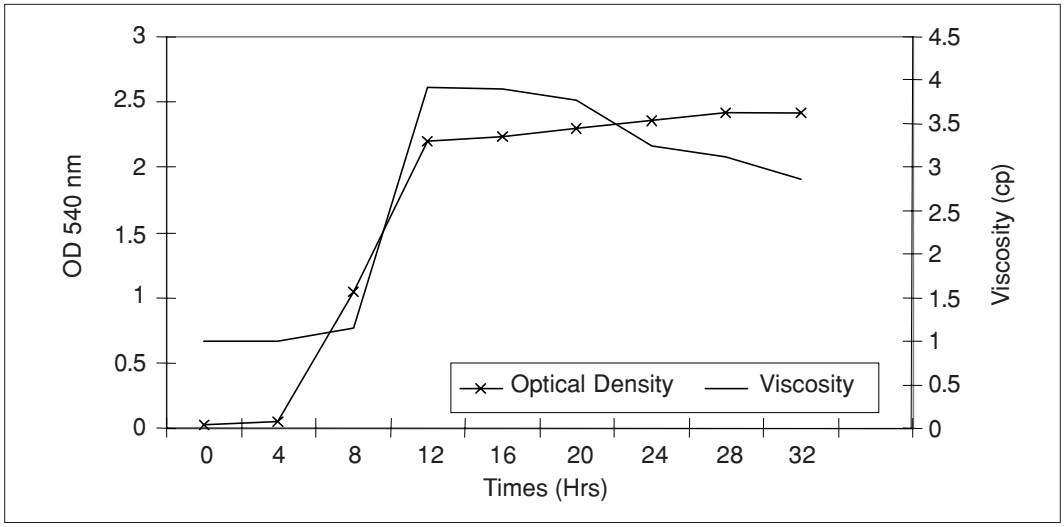

Figure 2 Growth and Viscosity by Aerobic Cultures of Strain S13

4.0 CONCLUSION

Nine bacterial strains from oil brines sample from Semangkok oil reservoir belongs to the genus *Bacillus* sp. The isolated bacteria had the ability to tolerate high

concentration of NaCl and could grow on a wide range of pH. *Bacillus* sp. S17A and S13 were the best exopolysaccharide and biosurfactant producers, respectively. The ability of these bacteria to grow in medium containing kerosene as the only carbon and energy source could provide an opportunity for further research and applications in bioremediation and MEOR.

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