Isolation, Screening and Characterization of Soluble Exopolymer-Producing Bacteria For Enhanced Oil Recovery

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Abstract

Various types of bacteria isolated from samples of water-oil and palm oil mill effluents (POME) were screened for soluble exopolymers which has potential use in microbial enhanced oil recovery (MEOR) applications. The samples were collected from several Malaysian oil reservoirs and Sedenak palm oil mill. Based on the chemical analyses of the water-oil samples, 6 types of media (HAA, HAG, RAA, RAG, MMS and RGM1) were chosen to initiate bacterial growth. Amongst the 52 pure cultures obtained (44 strains from water-oil samples and 8 strains from POME), 19 strains were stable and able to grow rapidly within 24 hours. These strains were further grown in MM medium in order to enhance exopolymer production. Of these, 5 strains were selected based on the presence of slimy colonies and solubility of the exopolymers produced by the bacteria; T221a exhibited the highest production of soluble exopolymers and was the most heat stable strain. T221a was identified as Bacillus licheniformis via biochemical tests and 16S rDNA analyses.

Keywords: Microbial Enhanced Oil Recovery (MEOR), Soluble Exopolymer-Producing Bacteria, *Bacillus licheniformis*.

1. Introduction

The demand of petroleum in the world fuel economy has tremendously increased over the years. However, conventional oil recovery techniques only produced one third of the original oil in place (1). Among the factors affecting the unrecovered oil left in place is differing permeability regions in an oil reservoir. This occurs when the injected fluid preferentially moves through the more permeable regions and the oil entrapped in the less permeable regions will be bypassed. Therefore, the success of an enhanced oil recovery process ultimately depends on the sweep efficiency, that is, the fraction of the oil reservoir volume contacted by the recovery fluid (2). A method of improving sweep efficiency, thus increasing oil recovery rates is selective plugging (3).

Previous studies had shown that the *in situ* application of the microbial selective plugging process which required microorganisms to grow and produce exopolymers under the environmental conditions that existed in the oil reservoir does result in improving sweep efficiency. Many oil reservoirs are anaerobic and have high temperatures.

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However, little were known about bacteria that could grow and produced polymers under these conditions (2). In this paper, the isolation of bacteria were reported from samples of water-oil and palm oil mill effluents (POME) cultivated at temperatures ranging from 50°C to 100°C in media containing the major components of the aqueous phase of water-oil samples. Since the use of soluble polymers was reportedly successful in enhancing oil recovery (1, 4), therefore in this study only soluble exopolymer-producing bacteria were further screened. The best potential microbe for MEOR application purposes was characterized via biochemical tests and 16S rDNA sequence analyses.

2. Materials and Methods

2.1. Samples

Samples of water-oil and POME were used as microbial sources. The water-oil samples were obtained from selected Malaysian oil reservoirs in Terengganu (Tiong A27, Malong A9, Dulang Western D20L) and Sarawak (Baram G85L, Betty 28L, West Lutong 14L, Alab and Samarang) by Petronas Carigali Sdn. Bhd**.** Whereas, samples of POME were collected at Sedenak Palm Oil Mill, Johor. *In situ* pH of the samples ranged from 6.0 to 9.0.

2.2. Sample Analyses

Atomic Absorption Spectrometer (AAS) and HACH DR4000 spectrophotometer were used to characterize the components of the aqueous phase of only the water-oil samples to determine the suitable compositions for growth media, thus simulating the reservoirs condition. The NaCl concentrations of the samples were determined using hand refractometer (ATAGO, Type 500).

2.3. Media

Based on the chemical analyses of the formation water, enrichment media used for bacterial isolation were modified mineral salts medium (MMS), *Hyperthermophilic archaea* medium (HAG) (5), modified *Hyperthermophilic archaea* medium (HAA), ravot medium (RAG) (6), modified ravot medium (RAA) and modified reinforced clostridial medium (RGM1). The MMS which was modified from Grula *et al.*, (7) contained the following, in grams per liter: KCl, 0.1; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; NaCl, 18.0; peptone, 2.0; yeast extract, 1.0; glucose, 20.0; $MgSO_4.7H_2O$, 0.5; K_2HPO_4, 0.5 and NH_4NO_3, 1.0. The RGM1 was modified from Gibbs and Freame (8) with the addition of the following compositions in grams per liter: NH_4NO_3, 2.0 and NaCl, 5.0. The HAA and RAA were modified from HAG and RAG, respectively by replacing glucose with acetate.

Medium for enhancement of the exopolymer production (MM) was modified from Wei (9). MM contained the following compositions in grams per liter: KH_2PO_4, 0.5; K_2HPO4, 0.5; yeast extract, 3.0; NaCl, 40.0; cysteine HCl, 0.5; $NaHCO_3$, 10.0; NH_4NO_3, 2.0 and sucrose, 100.0. All media were supplemented with 10 mL per liter (v/v) of filter-sterilized trace element and 10 mL per liter (v/v) vitamin solution (10). The final pH of media was adjusted to approximately pH 7.0 prior to sterilization at 121°C, 15 kPa for 15 minutes. 2% (w/v) agar was used as a solidifying agent. However, it was replaced by gelrite for cultures incubated at temperatures above 60°C.

Media preparation was based on the desired growth condition. Anaerobic growth was prepared using the Hungate technique where cultures were grown in rubber-stoppered and steel cap serum bottles (11, 12). However, cultures grown on solid media in plates were incubated in anaerobic jars (OXOID). Resazurin solution was added as indicator to anaerobiosis (13).

2.4. Bacterial Isolation

Initial bacterial isolation were carried out anaerobically where 1 mL of samples were inoculated on solid media through pour plate technique and 10% (v/v) of samples were inoculated in 50 mL liquid enrichment media. These cultures were incubated for 5 to 7 days at their respective temperatures which were selected based on their *in situ* reservoir temperature (Table 1). Bacterial survivability was observed via wet mount technique (16), whereas growth in liquid media was measured spectrophotometrically at 660 nm (2). Grown cultures from solid enrichment media were selected and transferred several times on the same media (9). Pure cultures were obtained by serial dilution of liquid enrichment media (14). Techniques used for bacterial isolation on solid media were rolling bottle, shake agar, pour plate, spread plate and dilution streak agar (11,12,13,14,15). Sterile syringe flushed with O_2-free gas was used for sampling of the anaerobic cultures (2). The pure cultures were confirmed based on gram staining of the cells and their colony morphologies. Isolated pure cultures were tested for their stability and ability to grow rapidly within 24 hours. Besides testing the ability of the selected cultures to grow at both anaerobic and aerobic conditions, their optimum incubation temperatures (37°C, 50°C, 60°C, 70°C) were also studied.

2.5. Screening for Soluble Exopolymers

Pure isolates obtained were screened for exopolymer producers based on the ability of isolates to produce slimy colonies on solid media (2, 17). The exopolymer production of selected strains were enhanced in MM medium supplemented with trace element solution (10 mL/L, v/v). Prior to screening for soluble exopolymer producers, exopolymer test was carried out. The test was done according to procedures described in Kim and Fogler (18): cultures (5 mL) were taken and centrifuged (6,000 rpm) for 20 minutes at 4°C to separate soluble and insoluble components. After centrifugation, the supernatant (soluble exopolymers and metabolic byproducts) was kept for the soluble exopolymer test. The pellet collected which consisted of insoluble exopolymers and bacterial cells was rinsed with deionized water before

Table 1: Incubation Temperatures for Bacterial Isolation

Sample	*In situ* Temperature (^oC)	Isolation Temperature (^oC)
Dulang Western D20L	93	70-100
Malong A9	104	70-100
Tiong A27	106	70-100
Baram G85L	79	60-80
Betty 28L	89	60-80
West Lutong 14L	77	60-80
Alab	71	50-60
Samarang	64	50-60
POME	45	50-70

centrifuging (6,000 rpm) for 20 minutes at 4^{o}C. After discarding the supernatant, KOH solution (10%, w/v) was added to dissolve the insoluble exopolymer, while keeping the cells intact. The cells and dissolved insoluble exopolymers were separated by centrifugation (6,000 rpm) for 20 minutes at 4^{o}C and the supernatant (insoluble exopolymer dissolved in KOH) was collected for the insoluble exopolymer test. The respective supernatant fractions were treated by adding two volumes of chilled (4^{o}C) ethanol (99.8%) to one volume of supernatant to precipitate out both types of exopolymers (soluble and insoluble) before being left overnight at 4^{o}C. Observations were recorded and samples producing soluble exopolymers were selected.

The soluble exopolymer-producing bacteria were further selected based on the highest exopolymer production and the best heat stable strain. The exopolymer production was observed within 24 hours of incubation at 50^{o}C via product dry weight analyses, whereas heat stability of the strains were determined via thermogravimetric analyses (Perkin Elmer thermogravimetric analyzer model TGA 7) (19, 20). Exopolymer extraction by chilled (4^{o}C) ethanol precipitation was carried out prior to both analyses.

Thermogravimetric analyses was conducted by weighing 10 mg of exopolymer samples. The weighed exopolymers were placed in an analytical balance and furnace was programmed for a linear rise in temperature with time. The samples were weighed continuously with rise of temperature. The temperature was raised at a constant rate of 20^{o}C per minute and heated from 30^{o}C to 900^{o}C. Nitrogen gas was used throughout the analyses to prevent oxidation of the samples. The measurement of weight loss versus temperature was produced on a thermograph. Temperature at material weight loss of approximately 5% was taken as the exopolymer's temperature stability (19, 20).

2.6. Strain Characterization

T221a was characterized via biochemical tests which were carried out based on a tentative identification described by Laskin and Lechevalier (21). 16S rDNA sequence analyses were performed to further confirm the characterization result produced via biochemical tests. In this analyses, genomic DNA extraction of T221a, polymerase chain reaction (PCR) amplification of the 16S rDNA and sequencing of PCR products were done as described by Margarita et al. (5) accept that universal primers were used for DNA amplification and sequencing reactions. The 16S rDNA sequence was then referred to the gene bank data centre using BLAST system to identify the genus and species of the T221a.

3. Results

3.1. Sample Analyses and Media Selection

Chemical analyses of the formation water were performed to determine major chemical compounds present [22]. However, only the aqueous phase of the water-oil samples was analyzed for the purpose of determining nutrient requirements and trace elements for simulation of the reservoir condition. The most abundant cation was Na^+ (440-10300 ppm), followed by K^+ (60-150 ppm). Whereas, NO_3^- was found as the most abundant anion which varied from 7 ppm to 17 ppm compared to PO_4^{3-} (0.3-0.6 ppm). As for the NaCl concentrations, it ranged between 14000 ppm to 32000 ppm.

Based on the analyses of the aqueous phase of water-oil samples, 6 different types of enrichment media (HAG, HAA, RAG, RAA, MMS and RGM1) were used to initiate bacterial growth. MM medium which also contained the major components of the formation water was used to enhance exopolymer production of selected pure cultures. Trace elements and vitamin solutions were used to support growth.

3.2. Bacterial isolation

A total of 52 strains of bacteria were isolated as pure cultures; 7 strains from Dulang Western D20L, 4 strains from Malong A9, 25 strains from Tiong A27, 6 strains from Betty 28L, 2 strains from Baram G85L and 8 strains from POME. All strains were isolated under anaerobic condition and survived at their respective temperatures except at 100^{o}C due to Mailard reaction which occurred in media containing glucose. Amongst the isolated bacteria, 20 strains were stable and able to grow rapidly within 24 hours. The selected strain's optimum incubation temperature was at 50^{o}C and showed the ability to grow well at both aerobic and anaerobic condition with optical densities of 0.5 to 2.0 within 24 hours. Consequently, all the forthcoming experiments were carried out at 50^{o}C.

3.3. Screening for Soluble Exopolymer Producers

Out of 20 selected strains, 11 strains were determined as exopolymer producers. These strains were then cultivated in MM medium to enhance exopolymer production prior to screening for soluble exopolymer-producing bacteria. Through exopolymer tests (18), 5 strains (TA62bi, T221a, B160, M4B80 and P) were identified producing soluble exopolymers. Amongst the 5 strains, T221a showed the highest heat stability with a weight loss of 5% at 140^{o}C (Table 2) and the highest production of soluble exopolymers (13.57 g/L) (Figure 1).

Table 2: Exopolymers Heat Stability

Strain	Weight loss (%)	Temperature (T^{o}C)
TA62bi	5	112
T221a	5	140
B160	5	76
M4B80	5	100
P	5	120

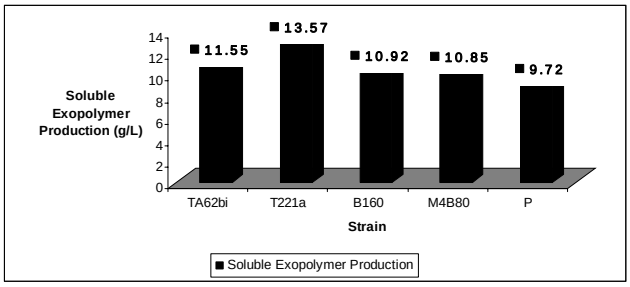

Figure 1: Optimum Soluble Exopolymer Production

3.4. Characterization of T221a

The morphological characteristics of T221a as a gram-positive, motile, rod-shaped, spore-forming bacterium was describe in Bergey's manual as a bacteria that may belong to the genus of *Bacillus* (23). Based on the biochemical tests carried out, T221a showed production of acetoin, enzyme catalase and enzyme gelatinase. T221a also was able to hydrolyse starch and utilize citrate. The positive results in fermentation-oxidation tests determined T221a as a facultative anaerobic bacteria. Following positive results of these tests, ability of T221a to grow in 7% (w/v) NaCl assigned it as *Bacillus licheniformis* (21). Phenotypic characterization via 16S rDNA sequence analyses confirmed T221a as *Bacillus licheniformis* with 98% similarity to the sequence in the gene bank data.

4. Discussion

Enrichment media (HAG, HAA, RAG, RAA, MMS and RGM1) selected for the use of bacterial isolation contained among the major components (Na^{+}, K^{+}, NO_3^{-} and PO_4^{3-}) of the water-oil sample's aqueous phase. The presence of Na^{+} and K^{+} in the media was an advantage due to its importance in bacterial growth (24). Besides that, the media also contained carbon source, nitrogen source, phosphate source, water and various mineral nutrients which were reported as the basic nutritional requirements for bacterial cultivation (14). These variations of enrichment media were used for the purpose of isolating a larger diversity of bacteria. HAA was selected for the cultivation of *Hyperthermophillic archaea* (5). Whereas, RGM1 had been reported containing compounds suitable for the cultivation of *Clostridium sp* (7). As for RAA and MMS, Wei (9) described them as suitable media for the cultivation of oil reservoir bacteria. Modification of HAA and RAA by replacing glucose as its carbon source with acetate was done to prevent Mailard reaction which affected microbial growth (10). Mailard reaction occurred when media containing glucose were incubated at temperatures higher than 90^{o}C. Agar was replaced by gelrite as a solidifying agent for incubation temperatures more than 60^{o}C because agar's boiling point was at 60^{o}C, whereas gelrite maintained in a solid form at high temperatures up to approximately 100^{o}C (25). MM was used as a exopolymer enhancement medium because it contained all the major components (Na^{+}, K^{+}, NO_3^{-} and PO_4^{3-}) of the water-oil sample's aqueous phase in substances such as sucrose and sodium bicarbonate which reportedly enhanced exopolymer production (26, 27, 28).

Of the bacteria isolated, 20 strains were selected amongst the 52 strains based on their ability to grow rapidly within 24 hours and stability of the strains. Stability was addressed by Kim and Fogler (18) as an important factor for MEOR bacteria to sustain growth. This was to ensure that bacteria could survive for a long period of time. The selected strains were also determined as facultative anaerobic bacteria due to its capability to grow well in both aerobic and anaerobic condition. Out of these 20 strains, 11 strains were determined as exopolymer producing bacteria from the presence of slimy colonies on plate (2). These slime-forming bacteria had been reported to have a much greater effect on permeability reduction rather than the non-slimy bacteria (29).

Further screening determined 5 strains as soluble exopolymer-producing bacteria via exopolymer test. Amongst the strains, T221a was selected as the best soluble exopolymer-producing bacteria for MEOR applications due to its highest heat stability and highest production of soluble exopolymers. Heat stability of the soluble exopolymers was an important criteria for *in situ* MEOR applications due to its high reservoir temperature which ranged between 63.9^{o}C and 106.1^{o}C (Table 1). T221a was identified as *Bacillus licheniformis* via biochemical tests and 16S rDNA analyses. The characteristics of T221a as a spore former was an advantage due to the purpose of this isolation study which was to obtain soluble exopolymer-producing bacteria that could survive in the oil reservoirs. This was because spore-forming bacteria were reported to have greater survivability in extreme environmental conditions [30].

5. Conclusion

The information on the composition of the aqueous phase of water-oil samples, pH and the *in situ* reservoir temperatures was very important in selecting the appropriate media and condition to initiate bacterial growth, thus simulating the reservoir condition. Since the reservoir's temperature ranged between 64°C and 106°C, the survivability of cultures at high temperatures were also crucial. Besides that, the stability and ability of the isolated bacteria to grow rapidly within 24 hours were among the criteria of a potential enhanced oil recovery bacteria. Screening for soluble exopolymer producers via exopolymer test was essential. Throughout this study, T221a which was identified as *Bacillus licheniformis* produced the highest production of soluble exopolymers and had the highest heat stability amongst the other strains. Therefore, T221a was selected as the most potential soluble exopolymer producer for microbial selective plugging process in enhancing oil recovery.

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