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Characterization of *Bacillus Licheniformis* Strain Ta62bi as Potential Selective Plugging Agent for Enchanced Oil Recovery

Fareh Nunizawati Daud[a], Mohd Nazrin Johari[a], Arifah Bahar[b], Ahmad Kamal Idris[c], Adibah Yahya[a*]

[a]*Department of Industrial Biotechnology, Faculty of Biosciences and Bioengineering, Universiti Teknologi Malaysia, 81300 UTM Johor Bharu, Johor, Malaysia*
[b]*Department of Mathematics, Faculty of Science, Universiti Teknologi Malaysia, 81300 UTM Johor Bharu, Johor, Malaysia*
[c]*Department of Petroleum Engineering, Faculty of Petroleum Renewable Energy Engineering, Universiti Teknologi Malaysia, 81300 UTM Johor Bahru, Johor, Malaysia*

*Corresponding author: adibah@fbb.utm.my

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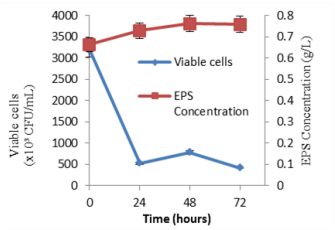
Graphical abstract



Abstract

Bacterial plugging agents for microbial enhanced oil recovery were peviously studied using non-hydrocarbon substrate. They lacked the ability to survive and form stable plug at high concentration of hydrocarbon compounds. As an alternative, hydrocarbon was used as substrate to determine the bacterial potential as plugging agent. In this study, *Bacillus licheniformis* Ta62bi was used to study the potential of the bacteria as plugging agent in polycyclic aromatic hydrocarbon (PAH)-rich condition. Three responses (growth, exopolysaccharides (EPS) and PAH consumption) were analyzed. The survivability pattern was observed at 72 hours. From the analysis, pyrene was the best PAH compared to naphthalene. It was based on increment of 214% (415 CFU/mL) in growth and 30% (0.759 g/L) in EPS production. However, the consumption of soluble PAH (0.002 to 0.015 mg/L) was low. The assimilation of hydrocarbon by potential bacterial plugging agent is the only means of survival. Otherwise, it would degrade to a great extent the oil components that would lead to the reduction of the oil quality. Next, a two-level factorial design was conducted to analyze the effects of different concentration of pyrene (0.1 to 10 g/L) and temperatures (27°C to 50°C) to the responses. The results showed that both factors significantly affect the responses ($P < 0.05$). Both factors inhibited growth of bacterium Ta62bi. As the PAH concentration was increased, the EPS production and PAH consumption was also found to increase at 27˚C. At 50°C, there was an increase in the EPS production but not in the PAH consumption. Therefore, EPS might be implied to having an important role in the tolerance of the TA62bi strain towards hydrocarbon. The findings will be further used in future research as a model to predict and control enhanced oil recovery plugging mechanism.

Keywords: Plugging agent; *Bacillus licheniformis*; enhanced oil recovery; polycyclic aromatic hydrocarbon

Abstrak

Bakteria, agen plak dalam aplikasi mikrob untuk peningkatan penghasilan minyak telah dikaji sebelum ini menggunakan substrat bukan hidrokarbon. Bakteria agen plak didapati tidak berupaya untuk bertahan dan membentuk plak yang stabil dalam hidrokarbon berkepekatan tinggi. Sebagai alternatif, hidrokarbon digunakan sebagai substrat untuk menentukan potensi bakeria sebagai agen plak. Dalam kajian ini, *Bacillus licheniformis* Ta62bi telah dikaji keupayaannya sebagai agen plak dalam keadaan medium kaya dengan hidrokarbon polisilik aromatik (PAH). Tiga jenis tindak balas (pertumbuhan, eksopolisakarida (EPS) dan pengambilan PAH larut) telah dianalisis. Corak kemandirian telah diperhatikan pada jam ke-72. Daripada hasil analisis, pyrene adalah jenis polisilik aromatik yang terbaik jika dibandingkan dengan naphthalene. Ini berdasarkan peningkatan pertumbuhan sebanyak 214% (415 CFU/mL) dan 30% (0.759 g/L) bagi penghasilan EPS. Sebaliknya pengambilan PAH larut (0.002 to 0.015 mg/L) adalah rendah. Asimilasi substrat oleh bakteria sebagai agen plak adalah satu-satunya cara hidup dalam hidrokarbon. Jika tidak, bakteria akan mendegradasi komponen minyak kepada suatu tahap yang agak tinggi sehingga menyebabkan kualiti minyak merosot. Kemudian, rekabentuk faktor dua peringkat dilaksanakan untuk mengkaji kesan perbezaan kepekatan pyrene (0.1 to 10 g/L) dan suhu (27°C to 50°C) kepada hasil tindak balas. Keputusan menunjukkan kedua-dua faktor memberikan kesan yang signifikan ($P < 0.05$). Kedua-dua faktor merencatkan pertumbuhan bakteria Ta62bi. Apabila kepekatan PAH meningkat, penghasilan EPS dan pengambilan PAH juga meningkat pada suhu 27˚C. Suhu 50°C pula meningkatkan penghasilan EPS tetapi pengambilan PAH menurun. Oleh itu, penghasilan EPS mungkin boleh di implikasi mempunyai peranan penting bagi strain TA62bi untuk dayatahan dalam hidrokarbon. Penemuan kajian akan digunakan untuk kajian pada masa hadapan sebagai model untuk meramal dan mengawal mekanisma pembentukan plak bagi peningkatan penghasilan minyak.

Kata kunci: Agen plak; *Bacillus licheniformis*; penghasilan minyak tetingkat; hidrokarbon polisilik aromatik

■1.0 INTRODUCTION

Microbial enhanced oil recovery (MEOR) is one of the tertiary methods supposed to increase oil recovery. This current investigation is focused on the potential use of bacteria via profile modification of water channeling in oil wells and is also called as selective plugging method. Formation of microbial could plugs the water channels, diverts the water flow from the high to the low permeability zone where the residual oil is trapped. A common type of carbon source used by the bacterial plugging agent study is cheap and non-hydrocarbon substrate such as molasses and starches (Kim and Fogler, 2000). However, the selected bacterial agent is lack of ability to survive and form plug effectively in high concentration of hydrocarbon compound. Alternatively is, to select potential bacterial plugging using hydrocarbon as substrate.

Bacterial survival and bacterial strain-specific mechanisms such as production of exopolysaccharides (EPS) may be shown in medium supplemented with polycyclic aromatic hydrocarbon (PAH), (Chakraborty *et al*. 2010). The EPS may act as anchor to hydrophobic surfaces as well as to accumulate recalcitrant substrate such as PAH in biofilm formation. On the other hand, the EPS is responsible for the structural integrity in biogranules. Nevertheless both formations like biofilm and biogranules are used in selectively plug the high permeability zone (Hamme 2003). In this study, *Bacillus licheniformis* strain Ta62bi was used to determine its potential as plugging agent in medium supplemented with either naphthalene or pyrene as rich polycyclic aromatic model. Three responses (growth, EPS production and PAH consumption) were statistically analyzed. Comprehension of bacterial physiological changes that assists in the survival of the organism as well as in predicting in its behavior is important for microbial enhanced oil recovery applications.

■2.0 EXPERIMENTAL

2.1 Growth Condition

Strain TA62bi was locally isolated and kept as culture collection at Industrial Biotechnology Department, Faculty of Bioscience and Bioengineering, UTM. The strain was isolated Malaysian oil reservoir and proven to produce exopolymer that act as biosurfactant and/or bioemulsifier. For biomass production, all bacteria were grown in nutrient broth. All experiments were carried out by introducing the harvested bacterium at 37°C with shaking at 150 rpm in a chemically defined medium (CDM) described by Bushnell and Haas, 1941. Either pyrene or naphthalene was used as substrate at the concentration of 1 g/L and 10 g/L, respectively.

2.2 Biochemical and Molecular Identification

Biochemical tests were performed in order to characterize and identify the strain relationship with other known bacterial genus. All biochemical analysis was carried out following the standard method. Results from the biochemical analysis were used to find the closest match with known bacterial genus and to assign the bacterial signature according to Bergey's Manual (Madigan *et al* 2009). The molecular identification of the strain was performed by 16 S rDNA analysis. For PCR amplification of 16S rRNA genes, genomic DNA isolated by Genomic DNA Isolation kit (PROMEGA) was used as template. 16S rDNA sequences were amplified using the primers, FD1 5'-AGAGTTTGATCCTGGCTCAG-3' and RD1 5'-AAGGAGGTGATCGAGCC-3'. Hot-start PCR at 94˚C for 5 minutes was followed by 34 cycles as follows: 94˚C for 5 minutes, 55˚C for 30 seconds, 72˚C for 1 minutes and a final chain elongation step at 72˚C for 10 minutes. The amplified fragment was sent to 1st Base Sdn Bhd to sequence it. Taxonomic analysis was conducted by the Genbank BLAST program. For phylogenetic and molecular evolutionary analysis, the MEGA version 4.0 was used (Kumar *et. al*, 2004). The resultant tree topology was evaluated by bootstrap analysis of the neighbour-joining method based on 1000 resamplings.

2.3 Determination of Bacterial Growth

Growth of the bacteria was determined by means of viable cell count (Madigan *et al.* 2009).

2.4 Determination of PAH Consumption

The amount of PAH residual in the supernatant portion was extracted by using hexane (Wilczynska *et al.* 1984). The absorbance was read at 220 nm and 335 nm for naphthalene and pyrene, respectively. The consumption of PAH was determined by comparing the changes between the available residual with absorbance of the control flask.

2.5 Exopolymers Assay

The amount of exopolysaccharides (EPS) produced by the bacteria was measured using phenol sulphuric acid method of Dubois *et al.* (1956). Glucose was used as standard.

2.6 Statistical Analysis

Statistical analysis was done by factorial ANOVA in determining factors affecting responses such as growth, EPS productions and PAH consumption. The conducted analysis was done using Design Expert version 6.0 (Montgomery, 1991).

■3.0 RESULTS AND DISCUSSION

The biochemical test shows that the Ta62bi was categorized *as Bacillus* sp. It was observed as a single rod-shaped, motile cells, facultative anaerobes, produce exopolymer and sporulating.The molecular identification i.e 16S rDNA analysis confirmed it as *Bacillus licheniformis* strain Ta62bi as it found to be the closest homologue of *Bacillus licheniformis* (GenBank Accession number: JX485829) as shown in Figure 1. The sequence obtained in this study was deposited in the EMBL database. The accession number for the 16S rRNA gene sequence of strain Ta62bi is KC108680.

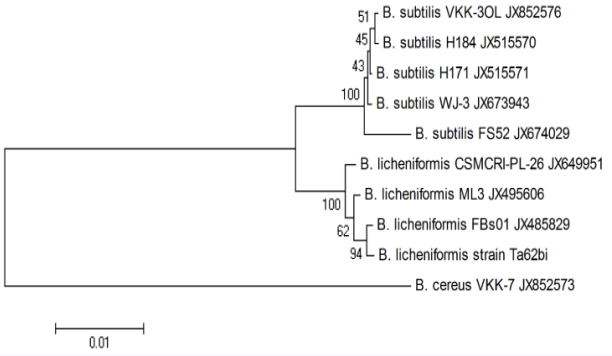


Figure 1 Phylogenetic location of strain Ta62bi among *Bacillus* species. *Bacillus cereus* JX852573 was used as an outgroup. The scale bar indicates the number of substitution per nucleotide position

Figure 2 shows growth pattern of Ta62bi in relation to exopolysaccharides (EPS) production and pyrene consumption. The adaptation pattern of the cells' survival towards the hydrocarbon-rich condition environment was shown in the figure. The increment of EPS production was drastically may be as a survival mechanism in toxic condition. Interestingly, biofilm was formed after three days of fermentation which may be has a relation of maximum EPS production. The sampling point (72 hours) was selected since the bacterial growth was survived with maximum EPS production and minimum consumption of pyrene. The microbial assimilation of hydrocarbon only means of survival will secure the crude oil quality (Brown 2010). Thus, 72 hours of sampling point was chosen in the next factorial design experiment.

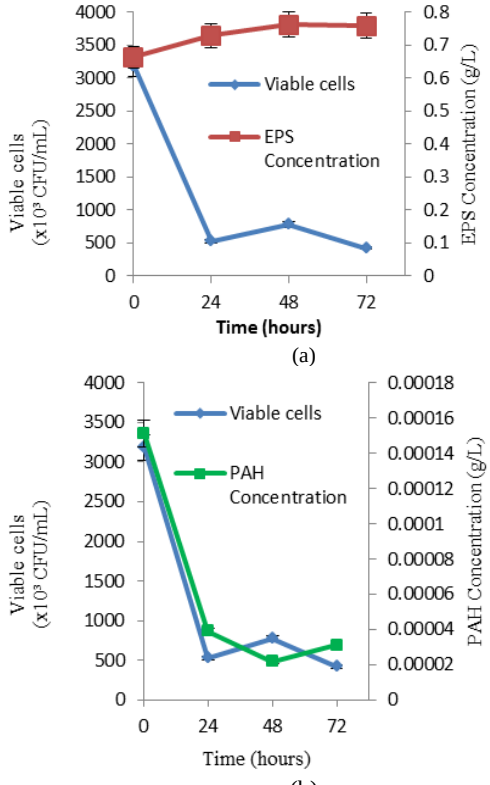


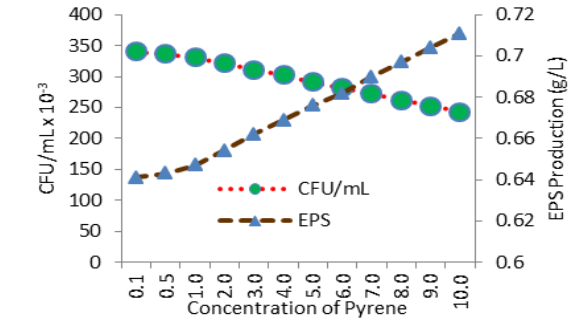
Figure 2 Growth pattern of Ta62bi in relation to (a) EPS production and (b) PAH consumption in BH medium supplemented with 10g/L of pyrene

The general factorial result showed that overall analysis for both pyrene and naphthalene showedno significant difference ($P < 0.05$). However, maximum growth as well as EPS production, but minimum PAH consumption only being achieved by using pyrene as carbon source. The growth of the strain was very high which 124% higher in the flask supplied with pyrene compared to naphthalene (data was not shown). The EPS production for the strain grown in pyrene also 30% higher compared to naphthalene. Whereas the PAH consumption for the flask supplied with pyrene was very minimum which 99% lower than results of flasks supplied with naphthalene. Therefore, pyrene was used as carbon source in the next experiment using 2D factorial design at different concentration of pyrene (1 g/L and 10 g/L) and temperatures (27°C and 50°C).

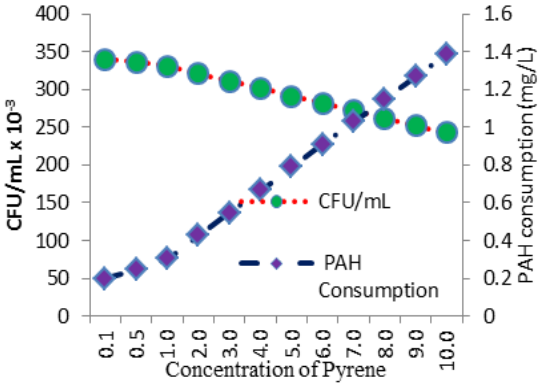
Figure 3 shows prediction of relationship between growth with either EPS production or PAH consumption, at varies concentration of pyrene (0.1 to 10.0 g/L) incubated at 27°C and 50 °C. The 2-level factorial design results showed only growth of the strain was affected significantly by both parameters (concentration of PAH and temperature) ($P < 0.05$). As the concentration of PAH and temperature increased, the growth was decreased notably ($P< 0.05$). Even though the viable cells in the medium suspension was nil at 50°C (Figure 3C and 3D) but there was thin layer of biofilm was formed on the flask wall. The thin layer was streaked onto plate and showed similar single isolated colonies as strain TA62bi colonies. This observation also indicated that higher temperature and pyrene affect the bacterial behavior by forming aggregates of cells adhere to the flask surface after 3 days of incubation.

4.0 CONCLUSION

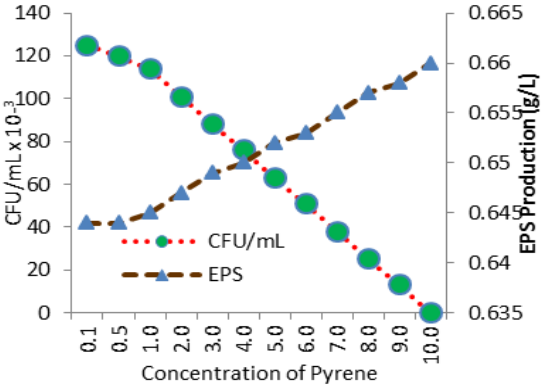
Bacillus licheniformis strain Ta62bi has the potential as plugging agent for microbial enhanced oil recovery application. Pyrene as the carbon substrate provided the polycyclic aromatic hydrocarbon (PAH)-rich condition that might induced the bacterium strain-specific mechanisms. The bacteria would most probably produced EPS to protect the cells from the hostile environment (at different temperatures and concentrations of pyrene).This also provided proof of the hyphothesis strategies for survival of the bacteria under stressful or unfavorable condition is initiated *via* formation of biofilm, aggregates or granules.



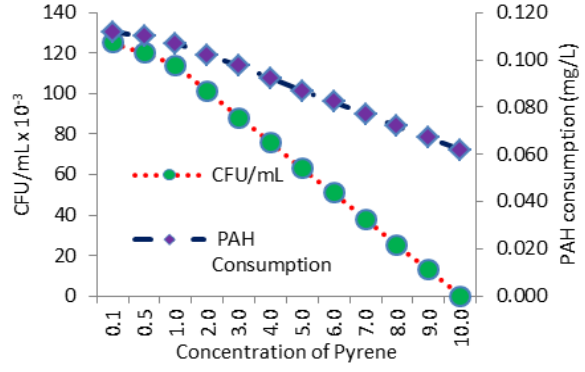
(a)



(b)



(c)



(d)

Figure 3 Prediction of relationship between growth with either EPS production (Left) or PAH consumption (Right), at varies concentration of pyrene (0.1 to 10.0 g/L) at 27°C (a and b) and 50 °C (c and d). The resultant was derived from 2-level factorial design

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