
**UNIVERSITI PUTRA MALAYSIA**

# *DEVELOPMENT OF NOVEL BACTERIAL CONSORTIUM FOR BIODEGRADATION OF PARAFFIN WAX IN CRUDE OIL*

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# DEVELOPMENT OF NOVEL BACTERIAL CONSORTIUM FOR BIODEGRADATION OF PARAFFIN WAX IN CRUDE OIL

By

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**October 2020**

**Chairman : Professor Raja Noor Zaliha Raja Abd Rahman, D. Eng**
**Faculty : Biotechnology and Biomolecular Sciences**

The deposition of paraffin wax in crude oil is a problem faced by the oil and gas industry during the extraction, transportation, and refining of crude oil. Millions of dollars were spent annually to control wax deposition in the pipeline. As an alternative to prevent wax deposition, this study aimed to develop a novel bacterial consortium for the biodegradation of paraffin wax in crude oil. Eleven paraffin wax degrading bacteria were screened at 70 °C using crude oil B and these isolates showed the ability to degrade the crude oil. The medium for bacterial growth and degradation was optimized with yeast extract alone to enhance the biodegradation of crude oil. The 16S rRNA gene identification of isolates revealed that these isolates are *Geobacillus kaustophilus* strain N3A7, *Geobacillus kaustophilus* strain NFA23, *Geobacillus kaustophilus* strain DFY1, *Parageobacillus caldoxylosilyticus* strain DFY3, *Parageobacillus caldoxylosilyticus* strain AZ72, *Geobacillus jurassicus* strain MK7, *Anoxybacillus geothermalis* strain D9 (previously identified), *Geobacillus thermocatenulatus* strain T7, *Geobacillus stearothermophilus* strain SA36, *Geobacillus stearothermophilus* strain AD24, and *Geobacillus stearothermophilus* strain AD11. Gas chromatography-mass spectrometry (GCMS) analysis of residual hydrocarbon showed more than 70% biodegradation efficiency by strains AD11, DFY1, AD24, N3A7, and MK7 compared to other strains. The degradative alkane monooxygenase enzyme activity of strain AD24 was the highest. Strains N3A7, MK7, DFY1, AD11, and AD24 (high degraders) also demonstrated relatively higher alcohol dehydrogenase, lipase, and esterase activity compared to other strains. Five consortia were developed based on the biodegradation efficiency of 11 bacterial strains. Consortium 3 (77.8%) showed the highest biodegradation with more long-chain alkane degraded throughout the incubation compared to other consortia. The degradation of long-chain alkane (paraffin wax) by all consortia also caused an increase of short-chain alkanes contents. Consortium 3 also exhibited higher alkane monooxygenase, alcohol dehydrogenase, lipase, and esterase activity compared to other consortia. The dominant bacteria in the

consortia were identified by denaturing gradient gel electrophoresis (DGGE). The results showed that *P. caldoxylosilyticus* strain DFY3, *P. caldoxylosilyticus* strain AZ72, and *G. kaustophilus* strain N3A7 were dominant in consortium 1, consortium 2, and consortium 3, respectively throughout the incubation. In consortium 4, *P. caldoxylosilyticus* strain DFY3, *G. thermocatenulatus* strain T7, and *G. stearothermophilus* strain SA36 were dominant on different days of incubation. Consortium 5 also showed the dominance of *G. kaustophilus* sp. and *G. stearothermophilus* sp. on different days of incubation. In conclusion, the bacterial consortia showed improvement in degrading more long-chain hydrocarbons while increasing some short-chain hydrocarbons. This improvement could lower oil viscosity and reduce oil production loss.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

**PENGHASILAN KONSORTIUM BAKTERIA UNTUK BIODEGRADASI LILIN PARAFIN DALAM MINYAK MENTAH**

Oleh

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Pemendapan lilin parafin dalam minyak mentah merupakan masalah yang dihadapi dalam industri minyak dan gas semasa proses pengekstrakan, pengangkutan, dan penapisan minyak mentah. Jutaan wang diperuntukkan setiap tahun untuk mengawal pemendapan lilin dalam saluran paip. Sebagai alternatif, kajian ini bermatlamat untuk menghasilkan konsortium bakteria untuk biodegradasi lilin parafin dalam minyak mentah. Sebelas bakteria pemakan lilin parafin telah disaring pada suhu 70 ºC menggunakan minyak mentah B dan 11 bakteria menunjukkan potensi untuk memakan minyak mentah. Medium untuk pertumbuhan bakteria dan biodegradasi telah dioptimumkan dengan ekstrak yis untuk meningkatkan biodegradasi minyak mentah. Identifikasi 16S rRNA menunjukkan bahawa isolat tersebut adalah *Geobacillus kaustophilus* strain N3A7, *Geobacillus kaustophilus* strain NFA23, *Geobacillus kaustophilus* strain DFY1, *Parageobacillus caldoxylosilyticus* strain DFY3, *Parageobacillus caldoxylosilyticus* strain AZ72, *Geobacillus jurassicus* strain MK7, *Anoxybacillus geothermalis* strain D9 (telah dikenalpasti), *Geobacillus thermocatenulatus* strain T7, *Geobacillus stearothermophilus* strain SA36, *Geobacillus stearothermophilus* strain AD24, dan *Geobacillus stearothermophilus* strain AD11. Analisa kromatografi gas-spektrometri jisim (GCMS) menunjukan lebih daripada 70% kecekapan biodegradasi bagi strain-strain AD11, DFY1, AD24, N3A7, dan MK7 berbanding strain lain. Strain AD24 menunjukkan aktiviti enzim degradasi alkana monooxygenase tertinggi. Strain N3A7, strain MK7, strain DFY1, strain AD11, dan strain AD24 (pengurai terbaik) juga menunjukkan kadar aktiviti alkohol dehidrogenase, lipase, dan esterase yang tinggi. Lima konsortium bakteria telah dihasilkan berdasarkan kecekapan biodegradasi 11 bakteria. Konsortium 3 (77.8%) menunjukkan biodegradasi tertinggi serta lebih banyak pengurangan alkana berantai panjang berbanding konsortium lain. Biodegradasi alkana berantai panjang (lilin parafin) oleh semua konsortia juga mengakibatkan peningkatan komposisi alkana berantai pendek. Konsortium 3 menunjukkan aktiviti enzim degradasi alkana monooxygenase, alkohol dehidrogenase, lipase, dan esterase yang tinggi

berbanding konsortium lain. Bakteria yang dominan dalam konsortia telah dikenalpasti melalui *denaturing gradient gel electrophoresis* (DGGE). Hasil kajian menunjukkan *P. caldoxylosilyticus* strain DFY3, *P. caldoxylosilyticus* strain AZ72, dan *G. kaustophilus* strain N3A7, masing-masing lebih dominan dalam konsortium 1, konsortium 2, dan konsortium 3 semasa inkubasi. Konsortium 4 menunjukkan dominasi bakteria pada hari berlainan bagi *P. caldoxylosilyticus* strain DFY3, *G. thermocatenulatus* strain T7, dan *G. stearothermophilus* strain SA36. Konsortium 5 juga menunjukkan dominasi *G. kaustophilus* sp. and *G. stearothermophilus* sp. pada hari berlainan. Sebagai kesimpulan, bakteria konsortia menunjukkan penambahbaikan degradasi hidrokarbon berantai panjang serta berupaya meningkatkan komposisi hidrokarbon berantai pendek. Penambahbaikan ini dapat mengurangkan kepekatan minyak serta kadar kerugian minyak yang dihasilkan.

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## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF APPENDICES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| ADH | Alcohol dehydrogenase |
| AMO | Alkane monooxygenase |
| APS | Ammonium persulphate |
| bp | Base pair |
| BE% | Biodegradation efficiency percentage |
| β | Beta |
| °C | Degree celcius |
| DNA | Deoxyribonucleic acid |
| DGGE | Denaturing gradient gel electrophoresis |
| GCMS | Gas chromatography mass spectrometry |
| $g^{-l}$ | Gram per liter |
| g | Gram |
| × *g* | Gravity |
| kb | Kilobase |
| L | Litre |
| µL | Microliter |
| µm | Micrometer |
| µmoles | Micromoles |
| mM | Milimoles |
| MBH | Modified Bushnell and Haas |
| M | Molar |
| NADH | Nicotinamide adenine dinucleotide hydrogen |
| $NAD^+$ | Nicotinamide adenine dinucleotide |
| OWM | Ocean water medium |
| $A_{600nm}$ | Optical density at wavelength 600 nanometer |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| sp. | Species |
| TEMED | N, N, N, N- Tetramethylenediamide |
| TAE buffer | Tris-acetate-EDTA buffer |
| U/mL | Unit per mililiter |
| V | Volt |
| v/v | Volume per volume |
| w/v | Weight per volume |

# CHAPTER 1

## INTRODUCTION

Petroleum crude oil is an essential commodity and a main source of energy worldwide. It consists of a mixture of alkanes (paraffin), aromatics, resins, and asphaltenes. Paraffins with chain lengths longer than $C_{18}$ are called waxes, and crude oil containing more than 50% of the wax composition is considered as waxy crude oil (El-Dalatony et al., 2019; Bai and Zhang, 2013). Waxy crude oil accounts for approximately 20% of the world's oil reserves (Lim et al., 2018; Kumar et al., 2015).

Therefore, waxy crude oil is a problem to the oil and gas industry in Malaysia because wax deposition in pipelines and on the surface of equipment could disrupt the flow of crude oil. Temperature and pressure reduction in the pipeline cause the wax crystal to form. Henceforth, reducing the oil's quality and quantity for oil recovery, refining, and transportation for downstream purposes. The wax deposit layer can limit the area in the pipe for oil to flow and decreased the flow rate of crude oil, which eventually will lead to production shutdown.

Conventional methods such as using chemical, mechanical, and thermal to control or remove some of the wax layers in the system exist. However, the disadvantages of using these techniques are toxicity issues (Ragunathan et al., 2020), formation damage (El-Dalatony et al., 2019), and expensive cost for long production tubes (Sakthipriya et al., 2017). Hence, microbial remediation has been sought as an alternative technique to reduce wax deposits due to their ability to degrade paraffin wax by producing enzymes that served as paraffin solvents. Alkane monooxygenase, alcohol dehydrogenase, lipase, and esterase are the *n*-alkane degrading enzymes produced by hydrocarbon-degrading microorganisms under aerobic conditions. These enzymes are present in the aerobic *n*-alkane degradation pathway and an important indicator in monitoring the biodegradation of crude oil (Kadri et al., 2018a; Ji et al., 2013). The operating temperature in the pipeline and petroleum reservoirs sometimes exceeds 50 ºC, therefore thermophilic hydrocarbon degraders are of particular importance.

Despite several successful studies on the microbial degradation of paraffin wax by a single bacterial strain at high temperature (Fan et al., 2020; Sun et al., 2015; Wenjie et al., 2012), it is believed that the biodegradation of paraffin wax requires more than one bacteria due to the broader enzyme activities that they have. The presence of multiple catabolic genes in a consortium could accelerate wax degradation efficiency (Xu et al., 2018). There is also a possibility that a species can transform toxic compounds that inhibit the degradation to a non-toxic derivative while allowing another bacteria to completely remove other compounds (Abatenh et al., 2017). Therefore, it can

be hypothesized that since bacteria can produce hydrocarbon-degrading enzymes to degrade crude oil, thus, by developing a thermophilic bacterial consortium it could be more effective in degrading paraffin wax in crude oil. This is because of the broader enzyme activities that they produced.

Hence, this study aims to develop a novel bacterial consortium for the biodegradation of paraffin wax in crude oil. The three major objectives of this study are:

1. To screen and identify thermostable crude oil-degrading bacteria;

2. To develop bacterial consortium able to reduce paraffin wax; and

3. To determine the biodegradation rate of paraffin wax in crude oil by bacterial consortia and the hydrocarbon-degrading enzymes they produced.

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(nom. corrig., formerly '*thermoglucosidasius*'); transfer of *Bacillus thermantarcticus* to the genus as *G. thermantarcticus* comb. nov.; proposal of *Caldibacillus debilis* gen. nov., comb. nov.; transfer of *G. tepidamans* to *Anoxybacillus* as *A. tepidamans* comb. nov.; and proposal of *Anoxybacillus caldiproteolyticus* sp. nov. *Journal of Systemic and Evolutionary Microbiology* 62: 1470-1485.

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## PUBLICATION

Adlan, N.A., Sabri, S., Masomian, M., Ali, M.S.M. and Rahman, R.N.Z.R.A. (2020). Microbial biodegradation of paraffin wax in Malaysian crude oil mediated by degradative enzymes. *Frontiers in Microbiol* 11: 565608.