BIOREMEDIATION OF CANOLA COOKING OIL USING COLD-ADAPTED ANTARCTIC BACTERIAL COMMUNITY FROM SOILS

By

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Faculty : Biotechnology and Biomolecular Sciences

Hydrocarbons can cause pollution to Antarctic terrestrial and aquatic ecosystems, both through accidental release and the discharge of waste cooking oil in greywater. Such pollutants can persist for long periods in cold environments. In this study, using mixed native Antarctic bacterial communities, several environmental factors influencing biodegradation of waste canola oil (WCO) and canola oil (CO) were optimised using established one-factor-at-a-time (OFAT) and response surface methodology (RSM) approaches. Secondary mathematical equations were chosen for kinetic analyses and the effect of co-contaminant heavy metals on WCO biodegradation was discussed. The toxicity of different heavy metals in 1 ppm of concentration to the WCO-degrading bacteria was evaluated and further analysed using half-maximal inhibition concentration (IC_{50}) and effective concentration (EC_{50}) tests. Next, biosurfactant production was optimised using RSM after preliminary screening processes. The bacterial community was then investigated through metagenomics analysis and so with lipase-producing bacteria was identified using Sanger sequencing. As for the result, an Antarctic soil bacterial community (reference BS14) was confirmed to biodegrade canola oil. Using OFAT, the most effective microbial community examined was able to degrade 94.42% and 86.83% (from an initial concentration of 0.5% (v/v)) of WCO and CO, respectively, within 7 days. Using RSM, 94.99% and 79.77% degradation of WCO and CO was achieved in 6 days. Mathematical modelling demonstrated that the best-fitting model was the Haldane model for both WCO and CO degradation. Kinetic parameters including the maximum degradation rate (*μmax*) were obtained, which were 0.365 and 0.307 min^{-1} for WCO and CO degradation, respectively. As for heavy metal evaluation, the IC_{50} values of Ag and Hg for WCO degradation were 0.47 and 0.54 ppm, respectively. Meanwhile, Cr, As and Pb were well-tolerated and induced bacterial growth and WCO degradation, resulting in the EC_{50} values of 3.00, 23.80, and 28.98 ppm, respectively. Next, all preliminary screenings for biosurfactants were positive, where biosurfactant concentrations produced up to 13.44 and 14.06 mg/mL in the presence of WCO and CO, respectively, after optimisation. The bacterial

community in non-treated media were originated from Proteobacteria and Firmicutes families. High proportions of bacterial families in WCO and CO treated media were *Pseudomonadaceae* (98.27%), followed by *Carnobacteriaceae* (1.70%). Among all the bacterial strains identified in the metagenomic analysis, it was confirmed that a group of *Pseudomonas* and *Carnobacterium* strains were responsible for biodegrading WCO and CO. Hence, this study offers a novel insight into the potential of the BS14 Antarctic bacterial community for canola oil bioremediation in Antarctica due to its ability to degrade WCO and CO effectively and produce biosurfactant at the same time. Plus, the BS14 community was able to tolerate heavy metals while biodegrading WCO in low-temperature conditions, which is a crucial aspect in biodegrading oil due to the co-contamination of oil and heavy metals that can occur simultaneously, and at the same time it can be applied in heavy metal-contaminated areas.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

BIOREMEDIASI MINYAK MASAK KANOLA MENGGUNAKAN KOMUNITI ADAPTASI SEJUK BAKTERIA ANTARTIK DARIPADA TANAH

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Hidrokarbon boleh menyebabkan pencemaran kepada ekosistem daratan dan akuatik Antartika, sama ada melalui pelepasan secara tidak sengaja dan pelepasan sisa minyak masak dalam air kelabu. Bahan pencemar sedemikian boleh menetap secara berterusan untuk jangka masa yang panjang dalam persekitaran yang sejuk. Dalam kajian ini, campuran komuniti asli bakteria Antartika digunakan, beberapa faktor persekitaran yang mempengaruhi penguraian sisa minyak kanola (WCO) dan minyak kanola (CO) dioptimumkan menggunakan pendekatan metodologi lazim satu-faktor-pada-satu-masa (OFAT) dan metodologi statistikal gerak balas permukaan (RSM). Persamaan matematik sekunder dipilih bagi analisis kinetik, dan juga kesan logam berat yang dicemarkan pada biodegradasi WCO telah dibincangkan. Ketoksikan pelbagai logam berat dalam kepekatan 1 ppm kepada bakteria yang menguraikan WCO dinilai dan dianalisis dengan lebih lanjut menggunakan separuh kepekatan perencatan maksimum (IC_{50}) dan ujian kepekatan berkesanan (EC_{50}). Seterusnya, pengeluaran biosurfaktan dioptimumkan menggunakan RSM selepas proses pemeriksaan awal. Komuniti bakteria kemudiannya disiasat melalui analisis metagenomi dan juga bakteria yang menghasilkan lipase telah dikenalpasti menggunakan penjujukan Sanger. Hasilnya, komuniti bakteria tanah Antartika (rujukan BS14) disahkan mampu mengurai minyak kanola. Dengan menggunakan OFAT, komuniti mikroorganisma yang paling berkesan yang diperiksa dapat menurunkan 94.42 dan 86.83% (dari kepekatan awal 0.5% (v/v)) WCO dan CO, masing-masing, dalam masa 7 hari. Menggunakan RSM, 94.99 dan 79.77% penguraian WCO dan CO dapat dicapai dalam masa 6 hari. Pemodelan matematik menunjukkan bahawa model yang paling sesuai adalah model Haldane untuk kedua-dua penguraian WCO dan CO. Parameter kinetik termasuk kadar penguraian maksimum (*μmax*) yang diperolehi, di mana masing-masing 0.365 dan 0.307 min^{-1} untuk WCO dan CO. Bagi penilaian logam berat, nilai IC_{50} bagi Ag dan Hg

semasa penguraian WCO adalah 0.47 dan 0.54 ppm. Sementara itu, Cr, As, dan Pb mampu bertoleransi dengan baik dan mendorong pertumbuhan bakteria dan penguraian WCO, ini menjadikan nilai EC_{50} masing-masing sebanyak 3.00, 23.80, dan 28.98 ppm. Seterusnya, semua saringan awal untuk biosurfaktan adalah positif, di mana kepekatan biosurfactant menghasilkan sehingga 13.44 dan 14.06 Komuniti bakteria dalam media yang tidak dirawat berasal dari keluarga *Proteobacteria* dan Firmicutes. Peratusan keluarga bakteria yang tinggi dalam media yang dirawat WCO dan CO adalah *Pseudomonadaceae* (98.27%), diikuti oleh *Carnobacteriaceae* (1.70%). Di antara semua strain bakteria yang dikenal pasti dalam analisis metagenomik, ia telah disahkan sekumpulan *Pseudomonas* dan strain *Carnobacterium* bertanggungjawab untuk biodegrade WCO dan CO. Oleh itu, kajian ini menawarkan gambaran baru mengenai potensi komuniti bakteria Antartika BS14 untuk bioremediasi minyak kanola di Antartika kerana keupayaannya untuk mengurai WCO dan CO dengan berkesan dan menghasilkan biosurfaktan pada masa yang sama. Selain itu, komuniti BS14 dapat bertoleransi dengan logam berat semasa penguraian WCO dalam keadaan suhu rendah, yang merupakan aspek penting dalam proses penguraian minyak kerana pencemaran minyak dan logam berat boleh berlaku serentak, dan pada masa yang sama ia boleh digunakan di kawasan yang tercemar dengan logam berat.

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Khadijah Zahri, 2023

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LIST OF ABBREVIATIONS

α	Alpha
β	Beta
%	Percent
% v/v	Percent volume per volume
% w/v	Percent weight per volume
~	Approximately
±	Plus minus
×	Times
× g	Times gravity
°C	Degree celsius
µ	Micro
µg	Microgram
µL	Microlitre
µM	Micromolar
µmax	Maximum specific rate
µV	Microvolt
$(NH_4)_2SO_4$	Ammonium sulphate
16S rRNA	16S ribosomal ribonucleic acid
3D	Three dimensional
A_{260}/A_{230}	Absorbance ratios at 260 and 230 nanometres
A_{260}/A_{280}	Absorbance ratios at 260 and 280 nanometres
Adj R^2	Adjusted R squared
AF	Accuracy factor

Ag	Argentum or Silver
AGE	Agarose gel electrophoresis
Al	Aluminium
AICc	Corrected Akaike information criterion
ANOVA	Analysis of variance
As	Arsenic
BE	Biodegradation efficiency
BF	Bias factor
BLASTn	Basic local alignment search tool
bp	Base pair
CCD	Central composite design
CCS	Circular consensus sequencing
Cd	Cadmium
Co	Cobalt
Cr	Chromium
Cu	Copper
CV	Coefficient of variation
d	Day
df	Degree of freedom
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
E_{24}	Emulsification index after 24 hours
EB	Elution buffer
EC_{50}	Half-maximal effective concentration

et al.	and others
exp	Exponential
F	Fischer value
FAMEs	Fatty acid methyl esters
Fe	Iron
FID	Flame ionization detector
g	Gram
g/L	Gram per litre
GC	Gas chromatography
gDNA	Genomic deoxyribonucleic acid
h	hour
HCl	Hycrochloric acid
Hg	Mercury
HMM	Hidden Markov model
IC_{50}	Half-maximal inhibitory concentration
IDH	Intermediate disturbance hypothesis
kb	Kilobase
kg	Kilogram
K_i	Inhibition constant
K_s	Substrate half-saturation constant
KNO_3	Potassium nitrate
L	Litre
m	metre
MATH	Microbial adhesion to hydrocarbon

MEGA	Molecular Evolutionary Genetics Analysis
mg	Milligram
mg/L	Milligram per litre
Mg_2SO_4	Magnesium sulphate
min	Minute
min^{-1}	Per minute
mL	Millilitre
MSM	Minimal salt medium
MTP	Microbiome taxanomic profile
n	Exponent representing the impact of the substrate to *μmax*
NA	Nutrient agar
NaCl	Sodium chloride
$Na_2HPO_4.2H_2O$	Sodium phosphate dibasic dihydrate
$NaNO_3$	Sodium nitrate
NaOH	Sodium hydroxide
NB	Nutrient broth
NCBI	National Centre for Biotechnology Information
nt	Nucleotide
NGS	Next-generation sequencing
NH_4Cl	Ammonium chloride
NH_4NO_3	Ammonium nitrate
Ni	Nickel
nm	Nanometer
OFAT	One-factor-at-a-time
OD_{600nm}	Optical density at wavelength 600 nanometer

p	Probability value
PacBio	Pacific Biosciences
PBD	Plackett-Burman design
Pb	Lead
pH	-log H^+ concentration
PCR	Polymerase chain reaction
pg	Picogram
p-NPP	Para-nitrophenyl phosphate
ppm	Part per million
Pred R^2	Predicted R squared
QC	Quality control
R^2	R squared
RMSE	Root mean square error
rpm	Revolutions per minute
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RSM	Response surface methodology
S	Substrate concentration constant
SD	Standard deviation
sec	seconds
SEM	Standard error mean
S_m	Critical substrate concentration constant
SMRT®	Single-molecule-real-time technology
SSE	Sum of square error

sp.	Species
sy.x	Standard error of estimate
U/mg	Unit per milligram
UV	Ultraviolet
UV-Vis	Ultraviolet-Visible
V	Volts
Zn	Zinc

CHAPTER 1

INTRODUCTION

Cooking oils are used for culinary purposes around the world, including at the science stations in Antarctica. Canola oil and canola margarine are the usual dietary fats in most Antarctic stations (Matheson et al., 1996) for food preparation. The percentage of edible vegetable oils (such as palm oil, soybean oil, canola oil, sunflower oil, and peanut oil) allocated to food uses has increased by about 76% from 2014 to 2022 globally (Shahbandeh, 2023). Awogbemi et al. (2019) also mentioned that the largest vegetable oils are used for frying and cooking in households, restaurants, and fast-food outlets. The increasing global consumption and demand every year and along with it can lead to high production of waste cooking oil from the kitchen in different sectors. Therefore, the possibility of oil spills incident or accidents due to improper waste management of cooking oils can occur.

Generally, oil pollution could lead to negative environmental impacts, including acute toxicity and mechanical injury towards organisms. Acute toxicity is a measure of the amount of volatile compounds in the waste cooking oil that readily dissolve into water and are capable of killing plants and animals by poisoning (Department of Ecology State of Washington, 2016). The composition of cooking oils allows it to change their structure and properties when heated above certain temperatures (Kumar et al., 2012a). Lipid oxidation causes the production of aldehydes, epoxides, hydroxyketones and dicarboxylic compounds that would react with amino acids and produce acrylamide, which is a carcinogenic compound (Choe and Min, 2007). Meanwhile, deep-fat frying process can produce dangerous volatile compounds such as toluene, benzene and hexylbenzene (Kochhar, 2016). At the same time, cooking oil pollution can have physical effects on wildlife, such as coating, disruption of the interlocking barbule structure of sea birds' feathers, elimination of waterproofing properties, which can cause hypothermia, and increased risk of predation and dehydration. Furthermore, cooking oils have a high persistence and tend to foul habitats for longer periods and create a long-term toxicity threat the organisms, as stated by Department of Ecology of Washington (2016).

To ensure the protection of the Antarctic environment, the Antarctic Treaty adopted the Protocol on Environmental Protection to the Antarctic Treaty in 1991. According to the Annex IV of the protocol, the discharge of oil and oily waste into the sea is prohibited, where the waste cooking oils are supposed to be stored in waste containers to be shipped out of the Antarctic. However, this practice will increase the fuel consumption of the ships, which is a waste of resources. Therefore, alternatives to shipping out the waste cooking oil should be investigated. One solution to this problem is through the bioremediation of the cooking oils by using microorganisms as suggested in this study. Bioremediation is potentially more effective and eco-friendly compared to chemical or physical remediation. Bioremediation using microbes is more practical, because it mainly

relies on the enzymes involved in microbial metabolic pathways. Biodegradation of cooking oils begins with the breakdown of the complex molecule by enzymes produced by microbes, primarily with various types of lipolytic enzymes (Alkhatib et al., 2015).

Heavy metals pollution in Antarctica becoming more common and is caused by from natural sources and anthropogenic activities from the research stations in the continent. Elevated levels of heavy metals such as copper (Cu), lead (Pb) and mercury (Hg) have been detected in Antarctica. These metals are associated with hydrocarbons contaminants, along with other types of heavy metals such as barium (Ba), beryllium (Be), cadmium (Cd), cobalt (Co) and nickel (Ni). The presence of these heavy metals in the environment could affect the growth and degradation of hydrocarbons, since they could destroy proteins and inhibit enzyme actions. According to the Alnuaimi et al. (2012), metal ions can bind directly to the sulfhydryl groups of proteins/enzymes and lead to protein inactivation and denaturation. Also, some of the heavy metals could be deposited in the cell and eventually suppress cell division and damage the cell structure (Jung et al., 2008).

Any attempt at bioremediation in Antarctica needs to use native Antarctic microorganisms to degrade the pollutants. This is because the introduction of non-native microorganisms is not permitted according to the Antarctic Treaty (Jesus et al*.,* 2015). The use of bacterial consortium in bioremediation is claimed to be more effective and to produce better degradation compared to single cultures. This may be due to the synergistic effect among the members of the consortium (Patowary et al., 2016). Several studies have shown that the use of bacterial consortium in degrading pollutants gave better results since the survival and stability of the bacteria are better in such communities (Piakong and Zaida, 2018). The action of the bacterial consortium is also faster than pure cultures due to their supportive activity and the interlinked relationships or group of interspecific semiosic links in biocoenosis (Skariyachan et al. 2018). Another compound that could generate and help the bioremediation of cooking oil is the biosurfactant. Biosurfactants are a useful tool in bioremediation that can act as emulsion-stabilisers (either as an emulsifier or demulsifier) and anti-adhesive agents through mobilisation and solubilisation mechanisms (Pacwa-Plociniczak et al., 2011). The biosurfactants' surface function is derived from their amphiphilic composition, via the water-soluble and water-insoluble portions of their molecules (Kubicki et al., 2019). Consequently, biosurfactants are able to increase the surface area of hydrocarbons by lowering the interfacial tension between the hydrophilic and hydrophobic parts and thus, could play a key role in the bioremediation process.

Soil microorganisms carry out important processes that are vital for life on our planet, including the cycling of carbon and other nutrients and sustaining the growth of other microorganisms. Molecular interactions between many microbial species and their environment strongly influence the fate of soil nutrients and

these interaction details are largely unknown (Jansson and Hofmockel, 2018). Metagenomics is the ecological study of microorganisms from the environment, in which the whole microbial population can be explored and analysed, including their structure and function, especially for the organisms that are difficult to culture (Amrane and Lagier; 2018). It is a relatively new method of discovering the microbial ecology and biogeography that has big impact on modern microbiology and has revolutionized our understanding of the whole world (National Academy of Sciences, 2007). Metagenomics offers genetic information on potentially novel biocatalysts or enzymes, genomic linkages between functional enzymes and the phylogeny of uncultured organisms, and evolutionary profiles of community sequence and function (Thomas et al., 2012).

In the process of breaking down the complex molecule of cooking oil, lipolytic enzymes are responsible to catalyse the cleavage of carboxyl ester bonds in acylglycerols (Alkhatib et al., 2015). Lipases modify oils through the hydrolysis reaction in the presence of water to produce fatty acids, or in another reaction, alcohol is displaced through a transesterification reaction to produce glycerols (Okino-Delgado et al, 2017). Then, β-oxidation degradation of fatty acids will take place, where they are broken down into their metabolites and serve as a main source of energy for the microorganisms.

The bacterial community from Antarctic soil samples was hypothesised to degrade canola cooking oils effectively after the optimisation process. Moreover, the finest degradation kinetic models of the canola oil-degrading bacterial community can be plotted using non-linear regression equations. This study also seeks the effect of bacteria activity in the presence of heavy metals. And the selected bacterial community could produce biosurfactants during the canola oil degradation process. Lastly, the bacterial community responsible for degrading WCO and CO can be identified at the end of the study.

Therefore, the present study was conducted with the following objectives:

1. To screen for the best waste canola oil (WCO)- and canola oil (CO)-degrading bacterial community from Antarctic soil samples
2. To optimise biodegradation of WCO and CO by the selected oil-degrading bacteria community using one-factor-at-a-time (OFAT) and response surface method (RSM) approaches
3. To determine the degradation kinetic models of the WCO- and CO-degrading bacterial community
4. To investigate the effect of heavy metals on bacterial growth and degradation of WCO
5. To examine the ability of biosurfactants production on degrading WCO and CO by the selected bacterial community
6. To identify the best WCO- and CO-degrading bacterial community that are responsible for the degradation of canola oil

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