SCREENING AND IDENTIFICATION OF POTENTIAL GREEN POLYLACTIC ACID (GPLA)-DEGRADING BACTERIA ISOLATED FROM BIOFERTILIZER SAMPLES

By

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ABSTRACT

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KEE WEI XUAN

Plastic pollution has emerged as a global issue that caused negative impact to the environment and human health. This has led to the development of biodegradable plastic, such as polylactic acid (PLA) to reduce the plastic hazards. Green polylactic acid (GPLA) is originated from the PLA family which is derived from renewable sources with an enhanced biodegradability. Biofertilizer is frequently used in the agricultural sector to improve plant growth and crop yields. It contains microbial agents which possess potential in plastic biodegradation. In this project, bacterial isolates from biofertilizer samples were screened for GPLA degradation using clear zone assay and identified by 16S rDNA sequencing. The bacterial isolates were incubated on M9 screening media supplemented with different composition of GPLA powder at 30°C for a week. Coomassie Brilliant Blue solution was employed to visualise the formation of clear zone. Bacterial isolates that demonstrated zone of clearance were selected to perform genomic DNA extraction using phenol chloroform method, followed

by PCR amplification of the 16S rDNA encoding gene and sequence analysis. The sequencing results were compared to the database of NCBI using BLAST programme to determine the identity of the potential GPLA degraders. The results showed that two bacterial isolates were able to produce clear zone on 0.2, 0.4, and 0.6% GPLA screening plates. Based on the 16S rDNA sequence analysis, the bacterial isolates were identified as *Brucella intermedia* and *Stenotrophomonas maltophilia* strain W1-2. Further evaluation of GPLA degradation by *B. intermedia* and *S. maltophilia* is required to understand the biodegradation mechanisms. This study provided insights in the application of microorganisms in the biofertilizer for plastic waste management.

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DECLARATION

I hereby declare that this project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

(KEE WEI XUAN)

FACULTY OF SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date: 4 April 2024

PERMISSION SHEET

It is hereby certified that **KEE WEI XUAN** (ID No.: **20ADB01565**) has completed this final year project entitled ""**SCREENING AND IDENTIFICATION OF POTENTIAL GREEN POLYLACTIC ACID (GPLA)-DEGRADING BACTERIA ISOLATED FROM BIOFERTILIZER SAMPLES**" under the supervision of Dr. Nor Ismaliza Binti Mohd Ismail from the Department of Biological Sciences, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project thesis in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

(KEE WEI XUAN)

APPROVAL SHEET

This final year project report entitled "**SCREENING AND IDENTIFICATION OF POTENTIAL GREEN POLYLACTIC ACID (GPLA)-DEGRADING BACTERIA ISOLATED FROM BIOFERTILIZER SAMPLES**" was prepared by KEE WEI XUAN and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biotechnology at Universiti Tunku Abdul Rahman.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
µL	Microlitre
µM	Micro molar
BLAST	Basic Local Alignment Search Tool
bp	Base pair
dH_2O	Distilled water
FTIR	Fourier Transform Infrared
GPLA	Green polylactic acid
LB	Luria Broth
LDPE	Low density polyethylene
min	Minutes
mM	Milli molar
NCBI	National Centre for Biotechnology Information
PCR	Polymerase chain reaction
PE-PU-A	Polyether polyurethane acrylate
PHA	Polyhydroxyablkanoates
PLA	Polylactic acid
PLLA	Poly (L-lactide) acid
PP	Polypropylene
PS	Polystyrene
ROP	Ring-opening polymerisation
s	Seconds
SEM	Scanning electron microscopy
TAE	Tris acetate EDTA
TE	Tris EDTA
U	Unit
UV	Ultraviolet
V	Voltage

CHAPTER 1

INTRODUCTION

Plastic pollution has become a global environmental crisis which has been causing adverse effects to human health, environment, and wildlife. Vast production of single-use plastic and improper plastic waste management has led to plastic accumulation and deposition in the environment which was about 70 million tons per year (Mehmood et al., 2023). These accumulated plastics will eventually break down into small plastic fragments known as microplastics due to the exposure of environmental stressors like UV radiation (He et al., 2023). Microplastics, which can be easily transported by water flows such as rainfall and by air, have been found everywhere in atmospheric, aquatic, and terrestrial ecosystems worldwide (He et al., 2023). Previously, microplastics pollution in terrestrial ecosystems was not actively researched due to the idea that microplastics were impenetrable into plant cell wall (Qadeer et al., 2021). However, it was proven wrong by the research conducted by Li et al. (2020), which showed that microplastics can be up taken by plants from the soil via crack-entry mode. It raises concerns of pollution in food production as it can be hazardous if the staple foods and grasses in agricultural fields are deposited with microplastics and consumed by farm animals and humans, causing health concerns (Qadeer et al., 2021). Plastics are widely applied in the agricultural sector, such as mulching film and plastic-coated seeds (United Nations

Environment Programme, 2022). Plastic mulching film is advantageous to agricultural systems as it acts as a barrier to defence against plant diseases, weeds growth, and harms from pests (Liang et al, 2024). These plastic products in the agriculture settings play a crucial role in increasing crop yields and satisfying the food demand of the growing population.

Frequent disposal of the plastic waste after every single growing season contributes to the increasing plastic waste accumulation. Improper disposal of the used mulching film such as burning in open air and burying in the ground can lead to environmental pollution and health concerns (Merino, Zych and Athanassiou, 2022). To address this issue, the development of mulching film and other plastic consumables in agriculture using sustainable and biodegradable materials has been introduced to help with the agricultural plastic waste management.

Polylactic acid (PLA) is one of the promising biodegradable materials to make the mulching film and other plastic consumables to degrade more rapidly under suitable conditions, making it less harmful to the environment. PLA is a biopolymer which can be produced from polymerisation of lactic acid that is derived from renewable natural resources like sugarcane and corn starch (de Albuquerque et al., 2021). This material provides advantage of biodegradability, while still offering the comparable characteristics to conventional plastics. Green polylactic acid (GPLA) is from the same family as the traditional PLA, but its derivation is only from renewable resources or waste materials, which has an

enhanced biodegradability and sustainability compared to the traditional PLA. GPLA can be turned into useful substrates such as monomers and oligomers for microorganisms through their depolymerase enzymes production to degrade GPLA (de Albuquerque et al., 2021). Potential GPLA-degrading microbes can be isolated from biofertilizer, which is widely used in the agricultural sector.

Biofertilizer contains plant growth-promoting bacteria that can synthesise plant growth regulators, which can enhance growth of crop plants and improve crop yields in agriculture (Hafeez et al., 2006). The microbial communities in the biofertilizer can perform nitrogen fixation, phosphate solubilisation, and cellulase activities, which can raise the microbial number and catalyse microbial mechanism that support nutrient assimilation by plant (Khosro and Yousef, 2012). Research have shown that plastic-degrading bacteria can secrete secondary metabolites that can trigger plastic biodegradation, as well as synthesise phytohormones to promote plant growth (Ikhwan and Nurcholis, 2020). By using the microbial communities present in biofertilizers, agricultural companies have the potential to use them as the agents for biodegradation of PLA plastic waste used in their plantation. This can save the cost and labour in managing agricultural plastic waste generated from their plantation operation.

This project aims to screen for GPLA-degrading bacteria isolated from biofertilizer samples and to identify the potential GPLA-degrading bacteria by employing molecular approaches, which are Polymerase Chain Reaction (PCR) and 16S rDNA sequence analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 Polylactic Acid (PLA) Polymer

Polylactic acid (PLA) is an aliphatic polyester which is largely manufactured due to its favourable properties such as strength and stiffness that are comparable with the traditional petrochemical-based plastics, but it can be easily degraded by microorganisms (Zaaba and Jaafar, 2020). It is known as a microorganism-derived biodegradable plastic in which its production involves microbial activity that utilises plant component like starch as the substrate to perform polymerisation (Lee, Rahmat and Rahman, 2012). Microorganisms ferment carbon sources from plant components into lactic acid molecules, which are the monomers to synthesise PLA polymer. Lactic acid is an organic acid existing in two isomers due to the presence of asymmetric carbon atoms in its structure. It can be produced from the fermentation of monosaccharides from food waste such as sweetcorn and potatoes by bacteria like *Lactobacillus* and *Bifidobacterium* (Figure 2.1) (Stefaniak and Masek, 2021).

Figure 2.1: Fermentation process by bacteria to produce lactic acid (Stefaniak and Masek, 2021).

The synthesis of polylactic acid from lactic acid monomers can be performed via three methods: direct condensation, azeotropic dehydrative condensation, and ring-opening polymerisation (ROP). Direct condensation involves oligomer formation from lactic acid by removal of water, followed by polymerisation into PLA in which dehydration happens simultaneously. Azeotropic dehydrative condensation has an improved mechanism to remove water by employing the azeotropic distillation technique with a high-boiling-point solvent to produce PLA with high molecular weight. Ring-opening polymerisation (ROP) starts with assemble of lactide from lactic acid through high-temperature dehydration under vacuum conditions, then proceeds with ring-opening polymerisation of the purified lactide into PLA (Stefaniak and Masek, 2021). Figure 2.2 shows the manufacture of PLA using the methods mentioned above.

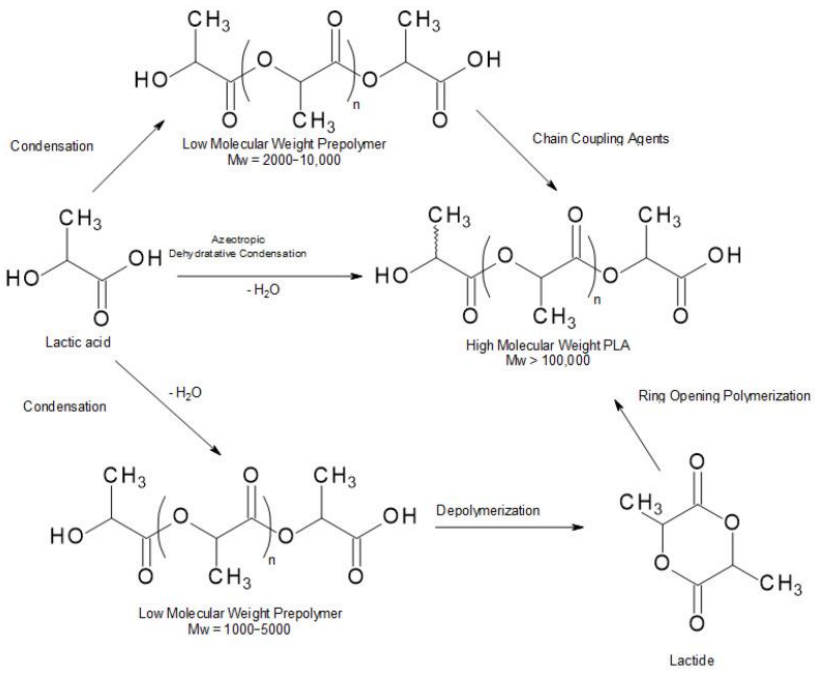


Figure 2.2: Synthesis of PLA from lactic acid via direct condensation, azeotropic dehydrative condensation, and ring-opening polymerisation (ROP) (Stefaniak and Masek, 2021).

2.2 Biodegradation of PLA Polymer

Biodegradation involves the hydrolysis of organic matter by living organisms aerobically or anaerobically (Shah et al., 2008). This process is dependent on multiple factors such as surface structures, molecular weight of the polymer, and substrate availability, resulting in physical or chemical changes of the polymer (Mohanan et al., 2020). Biodegradable polymers are more easily degraded by microbes compared to conventional plastics as most microbes can produce depolymerases that target biodegradable polymers. When comparing to other biodegradable polymers such as polyhydroxyalkanoates (PHA), there are lesser microbes that can degrade PLA as it is not the natural substrate for microbes (Viljakainen and Hug, 2021).

Microbial degradation of PLA is initiated when the potential PLA-degrading microbes attach to the PLA surface. The microbes will secrete extracellular PLA-degrading enzymes such as serine proteases, esterase, and cutinases, which adsorb to the PLA surface and perform hydrolysis of the PLA structure. These enzymes catalyse the cleavage of ester bonds that link between the lactic acid monomers, resulting in the production of lactic acid oligomers and monomers. These monomers become the substrates of the microbes to perform metabolism for their growth. Biodegradation of PLA starts at the surface level and spread into the internal structure, which is assisted by water diffusion that promotes microbial growth and enzymes secretion within the polymer (Zaaba and Jaafar, 2020).

Several studies have revealed that some PLA-degrading enzymes secreted by microbes can hydrolyse PLA polymers. Proteinase K from *Tritirachium album* is the first enzyme reported by (Williams, 1981) that demonstrated hydrolytic activity of poly (L-lactide) (PLLA). The development of modern molecular approaches such as sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and 16S rDNA sequencing have allowed the researchers to purify and identify extracellular PLA-degrading enzymes from microbes (Qi, Ren and Wang, 2017). Most PLA-degrading enzymes are proteases, only a few of them are lipase and cutinase (Qi, Ren and Wang, 2017). Hanphakphoom et al. (2014) have purified proteases from *Laceyella sacchari* LP175, which demonstrated PLA degradation after incubated at 50°C for a week. The purified proteases possessed the highest specificity to PLLA substrate and exhibited the ability of cleaving ester and peptide bonds (Hanphakphoom et al., 2014). Some researchers also detected novel PLA-degrading enzymes that did not show hydrolytic activity relative to protease, lipase, and esterase. Liang et al. (2016) have discovered that *Pseudomonas tamsuii* possessed novel PLA-degrading enzymes, as it did not exhibit protease, nattokinase, lipase, and esterase activity when the enzyme was incubated in respective substrates to perform degradation assays. Further study of the novel enzymes is necessary to reveal the enzyme family to understand the possibility of PLA-degrading enzymes.

2.3 Potential PLA-Degrading Microorganisms

Up to 2020, at least 94 microorganisms, of which 63 were bacteria species and 31 were fungal species, were identified as PLA-degrading microorganisms. Most

of the PLA degraders were from the family *Pseudonocardiaceae*, which were reported by 11 publications, and a total of 25 bacterial species from this family were identified (Gambarini et al., 2021). Other bacteria also possess PLA degradability, such as *Bacillus, Strenotrophomonas,* and *Pseudomonas*, in which *Bacillus brevis* was the first bacteria reported to degrade PLA. The main source of the PLA-degrading microorganisms is soil. Other sources can be wastewater, compost, and activated sludge (Qi, Ren and Wang, 2017).

PLA degradability of 20 *Pseudonocardia* strains isolated from rhizosphere soil samples were tested by incubating the isolates in basal medium broth containing 0.1%(w/v) gelatine and 50 mg PLA films at 30°C for 8 days. Among the isolates, *Pseudonocardia alni* AS4.1531 demonstrated the highest percentage of PLA film degradation (71.5%) after 4 days of incubation. Its PLA degradability was further proved by the raising of lactic acid accumulated in the basal medium broth, resulting in the decrease of pH value. Rough surface and holes formation on the surface of the PLA film was observed under Scanning Electron Microscope (SEM), indicating that the film was hydrolysed by the isolate (Konkit et al., 2012).

PLA-degrading bacteria were also isolated from compost containing mixture of dairy manure and straws, which were identified as *Chryseobacterium* sp., *Sphingobacterium* sp., and *Pseudomonas aeruginosa.* M9 minimal medium broth with sterilised PLA films was used as the enrichment medium to isolate the potential bacteria by incubating the compost sample at 30°C in a shaking

incubator. Six consecutive transfers to fresh M9 medium with new PLA films was performed every 7 days, resulting in a total of 42 days incubation. This was to isolate potential PLA-degrading bacteria which were able to utilise PLA as the sole carbon source and grow. Spread plate was carried out to spread out the final enrichment on the low nutrient agar R2A and incubated for a week to obtain colonies with different morphology. Individual PLA degradation assay for each isolate was conducted and showed that the isolates were able to attach to the PLA film and form biofilm. Among the isolates, *Pseudomonas aeruginosa* had the highest growth rate which doubling in approximately 4-5 hours. They demonstrated the same degradation rate when incubated in buffered and unbuffered conditions, indicating that pH had minimal effect on their PLA degradability (Satti et al., 2017).

2.4 Screening of Potential Plastic-Degrading Microorganisms

2.4.1 M9 Screening Media

M9 medium is a frequently used synthetic medium for culturing *Escherichia coli* and other bacteria strains. It is often employed to determine the limiting factor for the desired strain and assess the microbial physiological activity in minimal nutritional environments. The nutritional contents of the M9 medium are sulphate ($MgSO_4$), phosphate (KH_2PO_4 and Na_2HPO_4), nitrogen (NH_4Cl), and other additives like thiamine, which is in minimal nutrient for bacterial growth. Therefore, a sole carbon source, such as glycerol and glucose, is often added to

the medium (Soma et al., 2023). Researchers can modify the M9 medium composition and supplement with a specific carbon source according to the objective of their project. To study PLA degradation of a bacterial isolate, it is suitable to use this medium to screen for PLA degradation activity by evaluating their ability to degrade and utilise PLA as the sole carbon source for their growth. It can be achieved through the addition of PLA powder into the M9 agar medium during media preparation. Consistent distribution of the PLA powder in the M9 agar is important to make sure the bacterial isolate can use the PLA evenly.

There are various publications that employed M9 medium to study plastic polymer biodegradation by potential microbes. Kim et al. (2017) prepared PLA screening media using M9 supplemented with phosphate buffer, mineral, and vitamin solution. PLA dissolved in chloroform was added onto the M9 agar and let sit in a fume hood to evaporate the chloroform and solidify. The modification of the M9 composition allowed PLA-degrading bacteria to use the PLA as the sole carbon source and grow under a stable pH. Other bacterial strains that cannot degrade PLA will grow slower or cannot grow due to the minimal nutritional environment of the M9 screening media. Howard et al. (2023) modified the M9 minimal medium broth by supplementing with polyester substrate as the sole carbon source and high NaCl concentration same as sea water to introduce selective pressures to the bacteria so that they can switch their metabolism network to use polyester substrate as the primary carbon source.

2.4.2 Coomassie Blue Staining Solution

Clear zone assay is widely applied to detect plastic-degrading microorganisms. 56.4% of the research on plastic-degrading microorganisms implemented this method to screen for plastic degradation activity by measuring the halo zone formation around the isolates (Gambarini et al., 2021). Coomassie Blue stain provides visualisation of clear zone by binding to polymers or proteins with amine moieties to determine plastic degradation activity by the potential bacteria. The dye-amine complex can produce blue colour with high intensity in low substrate concentration condition, making it an effective agent for clear zone assay to detect enzyme activity within a short period of time. This assay only requires an incubation time of minimum one day to show the result, which can save time compared to those require days or weeks of incubation(Howard and Hilliard, 1999). Clear zone is formed when the bacteria secrete depolymerase to hydrolyse the plastic polymer into water soluble substances (Kumar Gupta, Devi and Rana, 2016). Clear zone will be observed within a blue background as no formation of the dye-amine complex to produce blue colouration around the bacterial colonies, indicating the presence of enzyme activity.

Howard and Hilliard (1999) proposed the clear zone visualisation procedure to verify the polyurethane degradation activity by bacteria, which was started with the treatment of the agar plates with 0.1% (w/v) Coomassie blue R-250 in 40% (v/v) methanol and 10% (v/v) acetic acid for 20 minutes. Then, the Coomassie blue solution was poured off, followed by treating with destain solution containing 40% (v/v) methanol and 10% (v/v) acetic acid for 20 minutes. Clear

zone formation was observed within a blue background, indicating polyurethane degradation.

This method was also employed by various researchers to study plastic degradability of bacterial isolates. Rana and Rana (2020) applied the clear zone method using Coomassie blue in their research to screen for polyethylene glycol degradation by microbes isolated from soil samples. The bacterial isolates demonstrated different degree of clear zone formation which implied that the isolates produced different concentration of enzymes to degrade the plastic. Nademo, Shibeshi and Gemeda (2023) detected low density polyethylene degradation activity by bacteria isolated from Koshe solid waste disposal area by observing the zone of clearance after staining with Coomassie Brilliant Blue solution.

CHAPTER 3

MATERIALS AND METHODS

3.1 List of Materials and Apparatus

Table 3.1 and Table 3.2 show the list of equipment, consumables, and apparatus with their manufacturers.

Table 3.1: Equipment, consumables, and apparatus used with their respective manufacturers.

Equipment/Consumables/Apparatus	Manufacturers
-20°C freezer	Liebherr
30°C Incubator	Memmert
4°C Chiller	Remi Laboratory Refrigerators
Analytical balance	Gemmy Industrial Adventure Pro
Autoclave machine	Hirayama Hiclave HVE-50
Beaker (50 mL, 100 mL, 250 mL, 500 mL, 1000 mL)	Schott, Duran
Bunsen burner	BUMEX, GQ
Centrifuge tubes (50 mL, 15 mL, 1.5 mL)	Beckman Coulter
Conical flask (100 mL, 250 mL)	BUMEX, GQ
Gel imaging system	Biorad
Gel tank	Major Science

Grinder	RT-08
Laminar flow cabinet	ESCO
Measuring cylinder	BUMEX, GQ
Micropipette (P-10, P-100, P-1000)	Eppendorf
Micropipette tips (P-10, P-100, P-1000)	Axygen Scientific
Microwave	Samsung
NanoDrop Microvolume UV-Vis Spectrophotometer	Thermo Fisher Scientific
Parafilm	Bemis
PCR tubes	Beckman Coulter
Petri dish	Plastilab
pH meter	Sartorious
Power supply	Major Science MP-250V
Schott bottle (50 mL, 100 mL, 250 mL, 500 mL, 1000 mL)	Schott Duran ®
Shaking incubator	Copens Scientific (M) Sdn Bhd
Spectrophotometer	Thermo Scientific GENESYS 10S UV-Vis
Syringe filter 0.22 μm	Membrane Solution
Thermal cycler	Biorad
Vacuum concentrator	Eppendorf concentrator plus
Vortex mixer	Gemmy Industrial

Table 3.2: Chemicals used with their respective manufacturers.

Chemicals	**Manufacturers**
1492R primer	1st BASE
27F primer	1st BASE
6x loading dye	Biotechrabbit
95% ethanol	Rank Synergy and Chemical Industries (Malaya)
99.9% Absolute ethanol	Chem Soln
Acetic acid	Systerm ChemAR
Agar powder	Chemiz
Agarose powder	1st BASE
Calcium chloride anhydrous	QRec
Chloroform	QRec
Coomassie Brilliant Blue R-250	Thermo Scientific
DNA ladder 100bp	Thermo Scientific
PCR reagents	1st BASE
GelRed® nucleic acid gel stain	Biotium
LB agar powder	MERCK
LB broth powder	Friendermann Schmidt
M9 minimal medium salt	amresco
Methanol	ECOCHEM
Phenol-chloroform-isoamyl alcohol	Sigma-Alderich
Proteinase K	Novagen
RNase	Thermo Scientific
SDS	amresco

Tris EDTA	Sigma-Aldrich
Yeast extract	MERCK

3.2 Project Outline

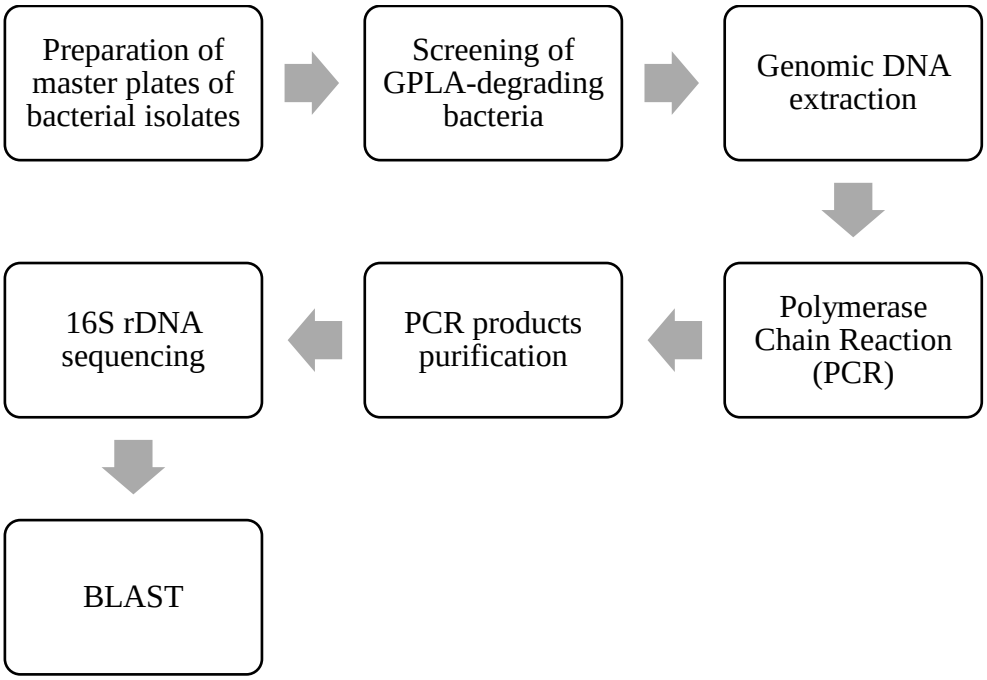


Figure 3: The flow of the project.

3.3 Preparation of GPLA Samples

GPLA provided by supervisor were cut into smaller pieces and were homogenised using grinder. The homogenised GPLA were easier to be incorporated into the screening media.

3.4 Preparation of Master Plates of Each Unknown Bacterial Isolates from Biofertilizer Samples

LB agar plates were prepared. Each bacterial isolate was streaked from the glycerol stock onto the LB agar plates using an inoculating loop. The LB agar plates were incubated in a 30°C incubator for 24 hours. After incubation, single colony of each plate was transferred to a new LB agar plate and incubated at 30°C for 24 hours to produce pure culture plates. The colony morphologies of each bacterial isolate, including colony size, colour, texture, elevation, form, and margin, were observed and recorded for future identification of the bacterial isolates.

3.5 Preparation of Screening Media

M9 screening media were prepared by weighing 11.3 g/L of M9 minimal medium salt, 1% yeast extract, and 1.8% agar powder, adding into a Schott bottle. The homogenised GPLA was weighed and incorporated into the Schott bottle to prepare M9 screening media with 0.2%, 0.4%, and 0.6% of GPLA. The mixture was then sent for autoclaving. The autoclaved M9 medium was poured into the petri dish and sat for 20 minutes to solidify.

3.6 Screening of GPLA-degrading bacteria using M9 medium

Bacterial suspension of each isolate were prepared. Two loopful of bacterial isolate were inoculated into a centrifuge tube containing 5 mL of LB broth and

incubated in a shaking incubator at 30°C, 200 rpm for 24 hours. After incubation, optical density of each bacterial suspension was measured using a spectrophotometer at wavelength of 600 nm. It was made sure that the optical density was in a range of 0.500 to 0.600 to ensure the consistency of the isolates. One µL of bacterial suspension was transferred to the M9 screening agar plates with different GPLA composition and was streaked using a sterile inoculating loop. Then, the agar plates were incubated at 30°C for one week.

After one week incubation, the agar plates were stained for 20 minutes with Coomassie blue stain, which consisted of 0.1% (w/v) of Coomassie Brillant Blue R-250, 40% (v/v) methanol, and 10% (v/v) acetic acid, to visualise the formation of clear zone. After 20 minutes, the staining solution was poured off. Destain solution containing 40% (v/v) methanol and 10% (v/v) acetic acid was added to the agar plates and sat for 20 minutes. Colour changes and clear zone formation were observed. Formation of clear zone around the colony indicates that the bacteria show plastic degradability. Bacterial isolates that showed clear zone formation were selected for identification.

3.7 DNA Extraction using Phenol Chloroform Isoamyl Alcohol Method

1M Tris buffer stock was prepared by dissolving 121.14 g of Tris base in 1000 mL dH_2O, adjusted to pH 8.0. Lysis buffer was prepared by adding 1% SDS and 25 mM EDTA to 50 mM Tris buffer. Tris-EDTA (TE) buffer was prepared by mixing 100 mM EDTA, 100 mM Tris buffer, and 100 mL dH_2O, adjusted to pH

8.0. Proteinase K was prepared by dissolving 3mM $CaCl_2$ in 200 mM Tris buffer, Then, 0.02 g of proteinase K powder into 500 µL of Tris-$CaCl_2$ buffer. The mixture was sterilised using 0.2 µm syringe filter, followed by adding 100% glycerol until a final volume of 1 mL.

Bacterial suspension was prepared by inoculating two loopful of the potential GPLA-degrading bacteria isolate from fresh bacterial culture into a 1.5 mL microcentrifuge tube containing 1 mL of LB broth. Then, the tube was incubated in a shaking incubator at 30°C, 200 rpm for 3 hours. After incubation, 500 µL of the bacterial suspension was transferred to a new 1.5 mL microcentrifuge tube containing 500 µL lysis buffer, then the mixture was vortexed. 5 µL of commercial RNase was added and incubated at 37°C for 10 minutes. Then, 5 µL of proteinase K was added, followed by addition of 500 µL phenol:chloroform:isoamyl alcohol solution. The mixture was subjected to centrifugation at 13000 rpm for 10 minutes. The aqueous phase of the mixture was transferred to a new microcentrifuge tube, followed by addition of an equal volume of chloroform. The mixture was inverted to mix and centrifuged at 13000 rpm for 10 minutes. This process was repeated twice to retain more DNA in the aqueous phase. After that, an equal volume of chilled 99.9% absolute ethanol was added to the aqueous phase in a new tube and inverted to mix. The tubes were then incubated at -20°C for 30 minutes and subjected to centrifugation at 13000 for 20 minutes. The supernatant was discarded, then the pellet was washed with 70% ethanol and centrifugation at 13000 rpm for 10 minutes. The remaining ethanol was pipetted out from the tube and dried at a vacuum concentrator at 60°C for 10 minutes. The DNA sample was resuspended in 20 µL of TE buffer.

The concentration and purity of the DNA sample was determined using Nanodrop 1000. The DNA samples were stored at -20°C for future use.

3.8 PCR Amplification

Polymerase Chain Reaction (PCR) was performed to amplify the extracted DNA of the potential GPLA-degrading bacteria. The details of forward and reverse primers used in the reaction are shown in Table 3.3. Master mix for the PCR reaction was prepared and their respective volume and concentration are shown in Table 3.4. All the PCR reagents were thawed in an ice box and spun down. Each PCR reagents were transferred to a 1.5 mL microcentrifuge tube to prepare the master mix, then it was aliquoted into PCR tubes. 1 µL of each DNA sample was added into their respective PCR tubes. Negative control was prepared in which the DNA sample was replaced by 1 µL of autoclaved distilled water. The PCR parameters were recorded in Table 3.5.

Table 3.3: Primer sequences and their expected band size.

Primer	Primer Sequences (5' to 3')	Expected Band Size
27F	AGAGTTTGATCCTGGCTCAG	1500 bp
1492R	GGTTACCTTGTTACGACTT	1500 bp

Table 3.4: Concentration and volume of each PCR reagents for one reaction.

Reagent	Initial Concentration	Final Concentration	Volume
Taq Buffer (Mg^{2+} plus)	10x	1x	2.5 µL
dNTP Mix	10 mM each	0.2 mM each	0.5 µL
Taq DNA polymerase	5 U/µL	0.05 U/µL	0.25 µL
27F	10 µM	0.4 µM	1.0 µL
1492R	10 µM	0.4 µM	1.0 µL
DNA template	~200 ng/µL	~8 ng/µL	1.0 µL
Autoclaved dH_2O	-	-	18.75 µL
Total volume			25.0 µL

Table 3.5: PCR parameters.

Condition	Temperature (°C)	Time	Number of Cycles
Initial denaturation	95	3 min	1
Denaturation	95	30 s	30
Annealing	50	45 s	
Extension	72	2 min 30 s	
Final extension	72	5 min	1

3.9 Agarose Gel Electrophoresis and PCR Products Purification

After amplification, the PCR products were collected. Agarose gel electrophoresis was carried out to determine the presence of the 1500 bp band. 1% (w/v) agarose gel was prepared by adding 0.25 g agarose powder to 25 mL of 1X TAE buffer, then heated for 2-3 minutes in a microwave to dissolve all the powder. After the mixture was cooled down to 50°C, 1 µL of GelRed stain was added. The mixture was swirled to mix and poured to the gel cast to solidify. Then, the gel was placed in a gel tank filled with 1X TAE buffer until it covered the gel. DNA ladder 100bp, which acted as a DNA molecular marker, was loaded into the first well of the gel. 6x loading dye was added to each DNA sample and mixed well before loading the DNA sample to the following well. The gel was run at 95V for 45 minutes, followed by gel viewing under UV transilluminator. The image of the gel was taken and recorded.

Purification of the PCR products was carried out using PrimeWay PCR Purification Kit (1st BASE). The procedure followed the manual provided by the 1st BASE manufacturer. After purification, agarose gel electrophoresis was performed to check the presence of the 1500 bp band, followed by determination of the purified DNA concentration and purity using Nanodrop 1000.

3.10 16S rDNA Sequencing and Data Analysis

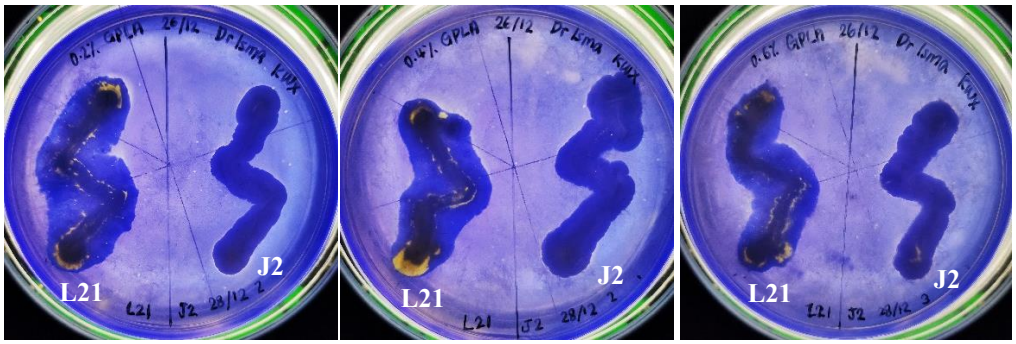
The purified PCR products were sent to Apical Scientific Sdn. Bhd. for 16S rDNA sequencing. The sequencing result was analysed using BioEdit (Biological sequence alignment editor) application and BLASTN program on the NCBI website. The sequences were compared with database online and the obtained alignments were recorded.

CHAPTER 4

RESULTS

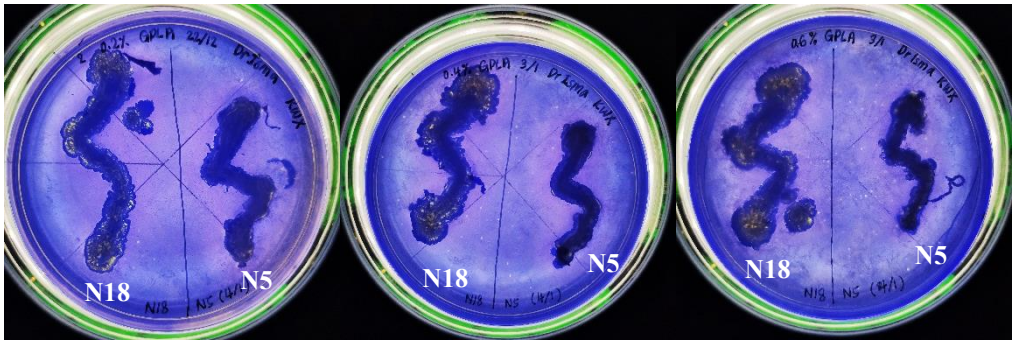
4.1 Screening of GPLA-degrading Bacteria

Unknown bacterial isolates J2, L21, N5, and N18 were inoculated onto M9 plates with GPLA composition of 0.2%, 0.4%, and 0.6% and incubated for a week. After a week, the bacterial isolates were screened for GPLA degradation by observing the formation of clear zone after staining with 0.1% Coomassie Brillant Blue R-250. Clear zone formation indicates that the bacteria can degrade the GPLA. Among the four isolates, only J2 and L21 showed clear zone formation around their colony on 0.2%, 0.4%, and 0.6% GPLA composition (Figure 4.1). The thickness of the clear zone was about the same at different GPLA composition, indicating that J2 and L21 showed the same degradation activity when incubated on M9 with different GPLA composition. No clear zone was observed for N5 and N18 (Figure 4.2).



0.2% 0.4% 0.6%

Figure 4.1: Clear zone formed by isolates L21 and J2 on M9 plates supplemented with 0.2%, 0.4% and 0.6% GPLA after treated with 0.1% Coomassie Brillant Blue R-250.



0.2% 0.4% 0.6%

Figure 4.2: Isolates N18 and N5 on M9 plates supplemented with 0.2%, 0.4% and 0.6% GPLA after treated with 0.1% Coomassie Brillant Blue R-250.

4.2 Identification of Potential GPLA-degrading Bacteria

4.2.1 Genomic DNA Extraction

Bacterial isolates J2 and L21 that showed clear zone formation were selected to proceed with genomic DNA extraction using phenol chloroform isoamyl alcohol method. The nucleic acid concentration and purity of the extracted DNA of J2 and L21 were shown in Table 4.1. The purity (A_{260}/A_{280}) of J2 and L21 were close to the ideal range of 1.8 – 2.0. The extracted DNA was utilized for PCR amplification.

4.2.2 PCR Amplification

PCR amplification of the extracted DNA of J2 and L21 was performed using the universal primer (27F and 1492R). The PCR products showed approximately 1500 bp band after visualising the agarose gel, indicating that the 16S rDNA sequence was present, and the amplification of the targeted DNA was successful. The gel image of the PCR products is shown in Figure 4.3.

Table 4.1: The nucleic acid concentration and purity of the extracted DNA of J2 and L21.

Bacterial isolate	Concentration (ng/µL)	A_{260}	A_{280}	A_{260}/A_{280}	A_{260}/A_{230}
J2	215.4	4.309	2.046	2.11	1.98
L21	73.3	1.466	0.747	1.96	1.15

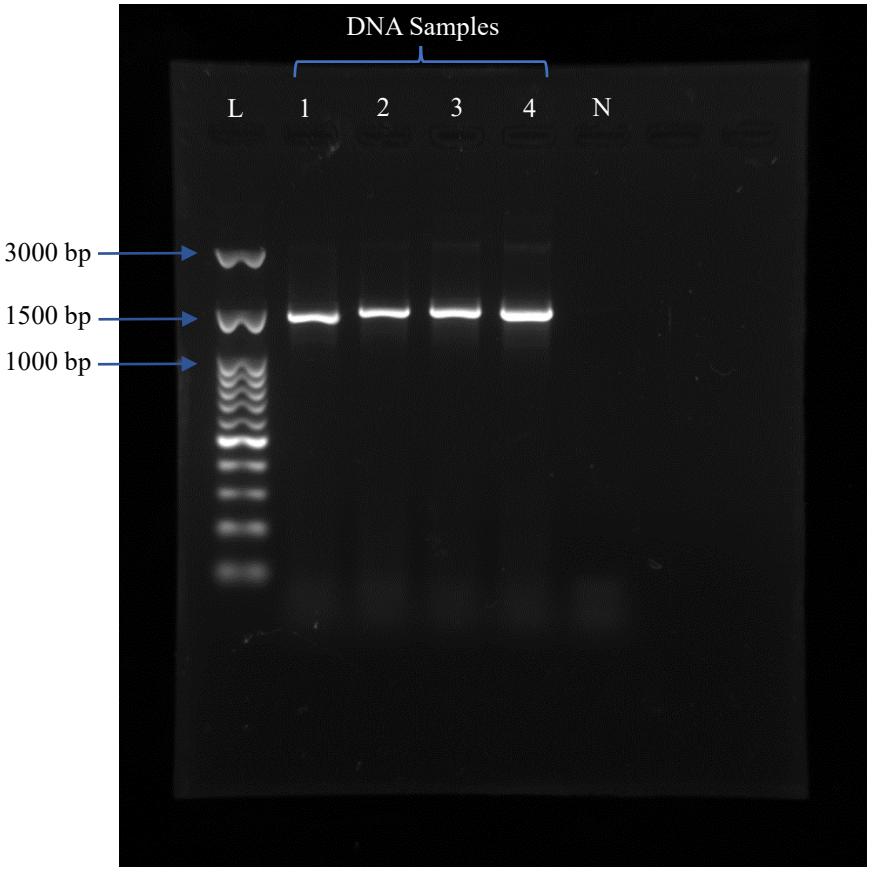


Figure 4.3: Gel image of the PCR products. Lane L represents the 100bp DNA ladder. Lane 1 and 3 are DNA sample of J2. Lanes 2 and 4 are DNA sample of L21. Lane N represents the negative control without DNA sample.

4.2.3 Purification of The PCR Products

Purification of the PCR products was carried out to remove unwanted reaction components. The presence of the 1500 bp band was visualised using agarose gel electrophoresis. The gel image of the purified PCR products is shown in Figure 4.4. The concentration and purity of the purified PCR products was determined using Nanodrop 1000, and it is shown in Table 4.2.

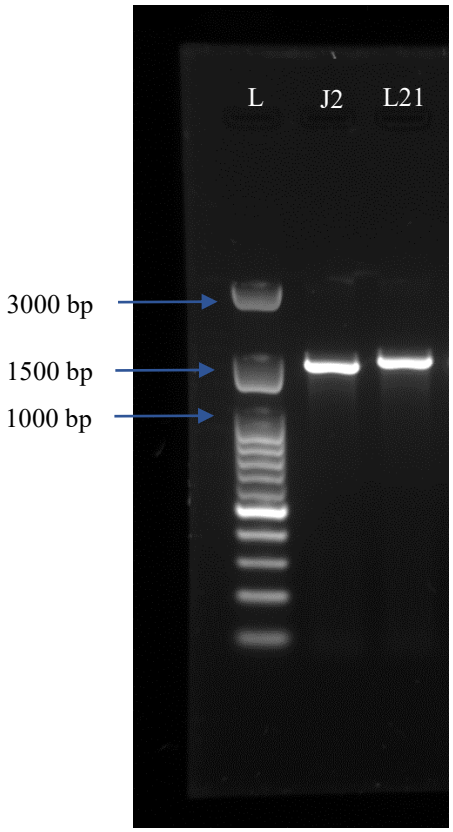


Figure 4.4: Gel image of the purified PCR products. Lane J2 and L21 are the DNA samples of the bacterial isolates after purification. Lane L represents the 100bp DNA ladder.

Table 4.2: The nucleic acid concentration and purity of the purified PCR products.

Bacterial isolate	Concentration (ng/µL)	A_{260}	A_{280}	A_{260}/A_{280}	A_{260}/A_{230}
J2	114.0	2.281	1.211	1.88	1.30
L21	90.8	1.816	0.946	1.92	0.52

4.3 16S rDNA Sequencing and BLASTN Analysis

The purified PCR products of J2 and L21 were sent for sequencing. The PCR products were sequenced using 27F and 1492R primers. Consensus sequences of J2 and L21 were generated from the resultant forward and reverse sequences using BioEdit application. The sequences were compared against the sequences in the database of NCBI using BLASTN program. The consensus sequences of J2 and L21 are shown in Table 4.3. Figure 4.5 and Figure 4.6 are the alignment result of J2 and L21 respectively.

Table 4.3: The consensus sequences of bacterial isolate J2.

Bacterial Isolate	Consensus sequence
J2	>J2_con AGTAACGCGTGGGAACGTACCATTTGCTACGGAATAACTCA GGGAAACTTGTGCTAATACCGTATGTGCCCGAAAGGGGAAA GATTTATCGGCAAATGATCGGCCCGCGTTGGATTAGCTAGTT GGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGT CTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCC CAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAAT GGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAA GGCCCTAGGGTTGTAAAGCTCTTTCACCGGTGAAGATAATG ACGGTAACCGGAGAAGAAGCCCCGGCTAACTTCGTGCCAG CAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGATTT ACTGGGCGTAAAGCGCACGTAGGCGGGCTAATAAGTCAGG GGTGAAATCCCGGGGCTCAACCCCGGAACTGCCTTTGATAC TGTTAGTCTTGAGTATGGTAGAGGTGAGTGGAATTCCGAGT GTAGAGGTGAAATTCGTAGATATTCGGAGGAACACCAGTGG CGAAGGCGGCTCACTGGACCATTACTGACGCTGAGGTGCG AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC ACGCCGTAAACGATGAATGTTAGCCGTTGGGGAGTTTACTC TTCGGTGGCGCAGCTAACGCATTARACATTCCGCCTGGGGA GTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGG CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAAC GCGCAGAACCTTACCAGCCCTTGACATCCCGATCGCGGTTA GTGGAGACACTATCCTTCAGTTCGGCTGGATCGGAGACAGG TGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGG TTAAGTCCCGCAACGAGCGCAACCCTCGCCCTTAGTTGCCA GCATTCAGTTGGGCACTCTAAGGGGACTGCCGGTGATAAGC CGAGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCT TACGGGCTGGGCTACACACGTGCTACAATGGTGGTGACAGT GGGCAGCGAGCACGCGAGTGTGAGCTAATCTCCAAAAGCC ATCTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAG TTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAA TACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGG G

Table 4.4: The consensus sequences of bacterial isolate L21.

Bacterial Isolate	Consensus sequence
L21	>L21_con AGAGCTTGCTCTCTGGGTGGCGAGTGGCGGACGGGTGAGG AATACATCGGAATCTACTCTGTCGTGGGGGATAACGTAGGG AAACTTACGCTAATACCGCATACGACCTACGGGTGAAAGCA GGGGACCTTCGGGCCTTGCGCGATTGAATGAGCCGATGTCG GATTAGCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACG ATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAAC TGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGG AATATTGGACAATGGGCGCAAGCCTGATCCAGCCATACCGC GTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTG GGAAAGAAATCCAGCTGGCTAATACCCGGTTGGGATGACGG TACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCC GCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGG GCGTAAAGCGTGCGTAGGTGGTCGTTTAAGTCCGTTGTGAA AGCCCTGGGCTCAACCTGGGAACTGCAGTGGATACTGGGC GACTAGAATGTGGTAGAGGGTAGCGGAATTCCTGGTGTAGC AGTGAAATGCGTAGAGATCAGGAGGAACATCCATGGCGAA GGCAGCTACCTGGACCAACATTGACACTGAGGCACGAAAG CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGC CCTAAACGATGCGAACTGGATGTTGGGTGCAATTTGGCACG CAGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGT ACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCC CGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGC GAAGAACCTTACCTGGCCTTGACATGTCGAGAACTTTCCAG AGATGGATTGGTGCCTTCGGGAACTCGAACACAGGTGCTGC ATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT CCCGCAACGAGCGCAACCCTTGTCCTTAGTTGCCAGCACGT AATGGTGGGAACTCTAAGGAGACCGCCGGTGACAAACCGG AGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGG CCAGGGCTACACACGTACTACAATGGTAGGGACAGAGGGCT GCAAGCCGGCGACGGTAAGCCAATCCCAGAAACCCTATCTC AGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGA ATCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGT TCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTT G

Brucella intermedia 16S ribosomal RNA gene, partial sequence

Sequence ID: MK344317.1 Length: 1421 Number of Matches: 1

Range 1: 78 to 1342 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
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Query  1     AGTAACGCGTGGGAACGTACCATTTGCTACGGAATAACTCAGGGAAACTTGTGCTAATAC  60
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Sbjct  78    AGTAACGCGTGGGAACGTACCATTTGCTACGGAATAACTCAGGGAAACTTGTGCTAATAC  137

Query  61    CGTATGTGCCCGAAAGGGGAAAGATTTATCGGCAAATGATCGGCCCGCGTTGGATTAGCT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  138   CGTATGTGCCCGAAAGGGGAAAGATTTATCGGCAAATGATCGGCCCGCGTTGGATTAGCT  197

Query  121   AGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  198   AGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCA  257

Query  181   GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG  240
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Sbjct  258   GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG  317

Query  241   GACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGT  300
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Sbjct  318   GACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGT  377

Query  301   AAAGCTCTTTCACCGGTGAAGATAATGACGGTAACCGGAGAAGAAGCCCCGGCTAACTTC  360
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Sbjct  378   AAAGCTCTTTCACCGGTGAAGATAATGACGGTAACCGGAGAAGAAGCCCCGGCTAACTTC  437

Query  361   GTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGATTTACTGGGCGTAAA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  438   GTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGATTTACTGGGCGTAAA  497

Query  421   GCGCACGTAGGCGGGCTAATAAGTCAGGGGTGAAATCCCGGGGCTCAACCCCGGAACTGC  480
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Sbjct  498   GCGCACGTAGGCGGGCTAATAAGTCAGGGGTGAAATCCCGGGGCTCAACCCCGGAACTGC  557

Query  481   CTTTGATACTGTTAGTCTTGAGTATGGTAGAGGTGAGTGGAATTCCGAGTGTAGAGGTGA  540
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Sbjct  558   CTTTGATACTGTTAGTCTTGAGTATGGTAGAGGTGAGTGGAATTCCGAGTGTAGAGGTGA  617

Query  541   AATTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGACCATTACTGAC  600
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Sbjct  618   AATTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGACCATTACTGAC  677

Query  601   GCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA  660
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Query  721   ATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCA  780
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Sbjct  798   ATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCA  857

Query  781   CAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCCCTTGAC  840
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Sbjct  858   CAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCCCTTGAC  917

Query  841   ATCCCGATCGCGGTTAGTGGAGACACTATCCTTCAGTTCGGCTGGATCGGAGACAGGTGC  900
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Sbjct  918   ATCCCGATCGCGGTTAGTGGAGACACTATCCTTCAGTTCGGCTGGATCGGAGACAGGTGC  977

Query  901   TGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  978   TGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA  1037

Query  961   CCCTCGCCCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGGGACTGCCGGTGATAAGC  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1038  CCCTCGCCCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGGGACTGCCGGTGATAAGC  1097

Query  1021  CGAGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTACGGGCTGGGCTACACACG  1080
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1098  CGAGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTACGGGCTGGGCTACACACG  1157

Query  1081  TGCTACAATGGTGGTGACAGTGGGCAGCGAGCACGCGAGTGTGAGCTAATCTCCAAAAGC  1140
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Sbjct  1158  TGCTACAATGGTGGTGACAGTGGGCAGCGAGCACGCGAGTGTGAGCTAATCTCCAAAAGC  1217

Query  1141  CATCTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGTTGGAATCGCTAGTAATC  1200
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Sbjct  1218  CATCTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGTTGGAATCGCTAGTAATC  1277

Query  1201  GCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACC  1260
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Sbjct  1278  GCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACC  1337

Query  1261  ATGGG  1265
             |||||
Sbjct  1338  ATGGG  1342
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Figure 4.5: Alignment of bacterial isolate J2 consensus sequence with *Brucella intermedia* (Accession number: MK344317.1). The alignment result showed 100% query coverage, 1264/1265 (99.92%) identities, 2333 score in bits, 0.0 E-value, and 0/1265 (0%) gaps.

Stenotrophomonas maltophilia strain W1-2 16S ribosomal RNA gene, partial sequence

Sequence ID: MG905249.1 Length: 1412 Number of Matches: 1

Range 1: 23 to 1368 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
2453 bits(1328)	0.0	1341/1347(99%)	2/1347(0%)	Plus/Plus

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Query  1     AGAGCTTGC-TCTCTGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACTC  59
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Sbjct  23    AGAGC-TGCTTCTCTGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACTT  81

Query  60    TGTCGTGGGGGATAACGTAGGGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAA  119
             | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  82    TTTCGTGGGGGATAACGTAGGGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAA  141

Query  120   GCAGGGGACCTTCGGGCCTTGCGCGATTGAATGAGCCGATGTCGGATTAGCTAGTTGGCG  179
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Sbjct  142   GCAGGGGATCTTCGGACCTTGCGCGATTGAATGAGCCGATGTCGGATTAGCTAGTTGGCG  201

Query  180   GGGTAAAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACT  239
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Sbjct  202   GGGTAAAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACT  261

Query  240   GGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGG  299
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  262   GGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGG  321

Query  300   GCGCAAGCCTGATCCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCT  359
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  322   GCGCAAGCCTGATCCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCT  381

Query  360   TTTGTTGGGAAAGAAATCCAGCTGGCTAATACCCGGTTGGGATGACGGTACCCAAAGAAT  419
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  382   TTTGTTGGGAAAGAAATCCAGCTGGCTAATACCCGGTTGGGATGACGGTACCCAAAGAAT  441

Query  420   AAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCG  479
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  442   AAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCG  501

Query  480   GAATTACTGGGCGTAAAGCGTGCGTAGGTGGTCGTTTAAGTCCGTTGTGAAAGCCCTGGG  539
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Sbjct  502   GAATTACTGGGCGTAAAGCGTGCGTAGGTGGTCGTTTAAGTCCGTTGTGAAAGCCCTGGG  561

Query  540   CTCAACCTGGGAACTGCAGTGGATACTGGGCGACTAGAATGTGGTAGAGGGTAGCGGAAT  599
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Sbjct  562   CTCAACCTGGGAACTGCAGTGGATACTGGGCGACTAGAATGTGGTAGAGGGTAGCGGAAT  621

Query  600   TCCTGGTGTAGCAGTGAAATGCGTAGAGATCAGGAGGAACATCCATGGCGAAGGCAGCTA  659
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  622   TCCTGGTGTAGCAGTGAAATGCGTAGAGATCAGGAGGAACATCCATGGCGAAGGCAGCTA  681

Query  660   CCTGGACCAACATTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCC  719
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  682   CCTGGACCAACATTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCC  741

Query  720   TGGTAGTCCACGCCCTAAACGATGCGAACTGGATGTTGGGTGCAATTTGGCACGCAGTAT  779
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Sbjct  742   TGGTAGTCCACGCCCTAAACGATGCGAACTGGATGTTGGGTGCAATTTGGCACGCAGTAT  801

Query  780   CGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAG  839
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Sbjct  802   CGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAG  861

Query  840   GAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAG  899
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Sbjct  862   GAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAG  921

Query  900   AACCTTACCTGGCCTTGACATGTCGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAA  959
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Sbjct  922   AACCTTACCTGGCCTTGACATGTCGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAA  981

Query  960   CTCGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTC  1019
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Sbjct  982   CTCGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTC  1041

Query  1020  CCGCAACGAGCGCAACCCTTGTCCTTAGTTGCCAGCACGTAATGGTGGGAACTCTAAGGA  1079
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Query  1080  GACCGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACG  1139
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Sbjct  1102  GACCGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACG  1161

Query  1140  GCCAGGGCTACACACGTACTACAATGGTAGGGACAGAGGGCTGCAAGCCGGCGACGGTAA  1199
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Query  1200  GCCAATCCCAGAAACCCTATCTCAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGT  1259
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Sbjct  1222  GCCAATCCCAGAAACCCTATCTCAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGT  1281

Query  1260  CGGAATCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTA  1319
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Sbjct  1282  CGGAATCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTA  1341

Query  1320  CACACCGCCCGTCACACCATGGGAGTT  1346
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Sbjct  1342  CACACCGCCCGTCACACCATGGGAGTT  1368
```

Figure 4.6: Alignment of bacterial isolate L21 consensus sequence with *Stenotrophomonas maltophilia* strain W1-2 (Accession number: MG905249.1). The alignment result showed 99% query coverage, 1341/1347 (99.55%) identities, 2453 score in bits, 0.0 E-value, and 2/1347 (0%) gaps.

CHAPTER 5

DISCUSSION

5.1 Screening of Potential GPLA-degrading Bacteria

Bacterial isolates J2, L21, N5, and N18 were inoculated onto M9 agar supplemented with 1% yeast and different composition of GPLA (0.2%, 0.4%, 0.6%) and incubated for a week to screen for GPLA degrading ability. M9 medium was selected as the screening medium as it does not contain carbon sources, which allows the bacteria to use GPLA as the sole carbon source. 1% of yeast extract was included in the screening media to assist the growth of the bacterial isolates. This was because the one-week incubation time was quite short due to the time constraint of this project. Supplementing the M9 medium with 1% yeast extract can increase the growing rate of the bacterial isolates within the short incubation period. Consequently, the bacterial isolates were able to grow and produce the enzymes to degrade the GPLA in the screening medium within one week.

After one-week incubation, the M9 medium were stained with Coomassie Brillant Blue R-250 to visualise the clear zone formation. The clear zone formed around the colony indicates that the bacterial isolate can degrade the polymer. The mechanism usually involves the adherence of microbes onto the surface of the polymer, followed by secretion of extracellular enzymes by the microbes,

which hydrolyse the polymer into water-soluble molecules (Tokiwa et al., 2009). The Coomassie Blue dye cannot interact with the hydrolysed polymer, resulting in the formation of clear zone within a blue background (Howard and Hilliard, 1999). Bacteria that can secrete higher concentration of the enzymes targeting polymer degradation will show larger clear zone area.

Isolates J2 and L21 showed similar thickness of clear zone in 0.2%, 0.4%, and 0.6% GPLA composition after staining with Coomassie Brillant Blue. Therefore, 0.2% GPLA composition was chosen as the threshold for GPLA degradation by bacteria. It was the lowest composition that allowed the clear zone formation to screen for GPLA degradation activity, which can reduce the material cost in future study. However, the clear zone was not obvious, which was a very small area. This might be due to insufficient incubation time of the bacterial isolates on the M9 media, resulting in a lower concentration of enzymes secretion and slower degradation of the GPLA. Consequently, the bacteria only degraded a small area of GPLA and formed a thinner clear zone around the colony after one-week incubation. It also indicated that J2 and L21 had a lower rate in producing the extracellular enzymes to degrade the GPLA. Elongating the incubation period might increasing the area of clear zone formation as it can provide sufficient time for the bacteria to secrete a higher amount of enzymes to hydrolyse more GPLA surrounding the colony.

5.2 Identification of the Potential GPLA-degrading Bacteria

After comparing the consensus sequences to the database of NCBI using BLASTN program, J2 is identified as *Brucella intermedia* while L21 is identified as *Stenotrophomonas maltophilia* strain W1-2. Both alignments are high in query coverage, indicating that high percentage of the consensus sequence length is encompassed within alignment against the matched sequence, and it falls within the ideal query coverage of more than 70%. The high percent identity for both alignments shows that the nucleotides are highly identical with only 0/1265 and 2/1347 gaps for J2 and L21 respectively. The introduction of gaps indicates that insertions or deletions happen between the consensus sequence and the matched sequence. Both alignments exhibit 0.0 E-value, showing that they are high quality matches as the lower the E-value, the more significant the alignment.

5.3 Plastic Degradation Ability of *Brucella Intermedia*

Brucella is a gram-negative and aerobic proteobacterium, which possesses colony morphology of punctiform, smooth, circular, translucent, raised, and convex. The colony is non-pigmented and non-haemolytic. It is stained in pink and observed as coccoid rods under microscope (Adem and Duguma, 2020). *Brucella* was previously classified as *Ochrobactrum* and new names were proposed. For example, *Brucella intermedia* is the new name for *Ochrobactrum intermedium* (Lu et al., 2021). *Brucella intermedia* can be isolated from

environmental samples, such as plants and animals, soil, and water (Lu et al., 2021).

B. Intermedia was found in the BP6 bacterial community isolated from landfill, which demonstrated degradability of polyether polyurethane acrylate (PE-PU-A) copolymer, PE-PU foam, and other additives (Sánchez-Reyes et al., 2023). The BP6 bacterial community was able to use PE-PU-A copolymer and other additives present in a commercial PU coating (PolyLack) as the sole carbon source to grow. It was discovered that the bacterial cells adhered to the PE-PU-A copolymer and PE-PU foam surfaces and the appearance of the copolymer was changed compared to the control when observed under SEM after 20 days of incubation. The analysis of Fourier Transform Infrared (FTIR) revealed that the PE-PU-A copolymer and PE-PU foam underwent chemical changes as different functional groups of the copolymer were attacked by the bacterial community using oxidative and hydrolytic mechanisms, showing their biodegradative activity on the copolymer. The present of sequences homologous to genes that code for esterase was detected in the BP6 bacterial community (Sánchez-Reyes et al., 2023). This might indicate the ability of *B. Intermedia* to secrete esterase to cleave the ester bonds that link the lactic acid monomers of PLA to degrade PLA. As the plastic degradation activity was performed by a group of bacteria, *B. intermedia* might require interaction with other bacteria to perform plastic degradation. This might be one of the factors that caused the thin clear zone area by *B. intermedia* J2 in Figure 4.1 as it was inoculated individually at one side of the screening medium. There was minimal interaction of *B. intermedia* J2 with other bacteria, causing the low biodegradative activity of

GPLA and smaller clear zone area. *S. maltophilia* L21, which was inoculated on the opposite site of *B. intermedia* J2 on the same screening agar plate, might provide support to *B. intermedia* J2 for biodegradation of GPLA. However, further study of the combination of *B. intermedia* J2 and *S. maltophilia* is required to determine the interaction between the bacteria in GPLA biodegradation.

5.4 Plastic Degradation Ability of *Stenotrophomonas* sp.

Stenotrophomonas is a gram-negative, non-endospore forming bacteria. It exhibits straight or slightly curved rod shape with two or more polar flagella when observed under microscope. *Stenotrophomonas maltophilia* possess polar fimbriae for cell adherence and motility. It can produce flavin which is a yellow pigment that contribute to its yellowish colony on complex medium (Ghosh, Chatterjee, and Mandal, 2020). *S. maltophilia* can be isolated from water, such as lakes, rivers, bottled water, and sewage. It is known as a pathogen that causes chronic respiratory diseases, but it is also used for organic compounds degradation and bioremediation of water or soil (Said, Tirthani, and Lesho, 2023).

Researchers have successfully isolated *Stenotrophomonas* sp. from different environmental samples and tested the ability to degrade plastic polymers. It has been discovered that these bacteria can degrade polylactic acid (PLA), as well as other plastic polymers like polypropylene (PP), polystyrene (PS), and low-density polyethylene (LDPE). Table 5.1 showed the research about plastic

degradation by *Stenotrophomonas* sp. Most of the plastic-degrading *Stenotrophomonas* are *S. maltophilia* and *S. pavanii.*

Table 5.1: Research on plastic degradability of *Stenotrophomonas* sp.

Strains	Substrate	Source	References
Stenotrophomonas maltophilia ATCC 13637	PLA	Compost from a farm field	Jeon and Kim, 2013
Stenotrophomonas pavanii DSM 25135	PLA	Soils from sanitary landfill sites	Bubpachat et al., 2018
Stenotrophomonas maltophilia strain W1-2	PP	Seawater	Juliandi et al., 2020
Stenotrophomonas maltophilia	PS	Plastic-polluted rivers and soils	Xiang et al., 2023
Stenotrophomonas pavanii DSM 25135	LDPE	Waste dumpsite and drilling fluid	Dey et al., 2020
Stenotrophomonas sp.	LDPE	Solid-waste dumpsite	Nadeem et al., 2021

A study of PLA degradation by bacterial isolates from farm field compost samples was conducted by Jeon and Kim (2013), which successfully identified *Stenotrophomonas maltophilia* as the potential PLA-degrading bacteria. The *S. maltophilia* strain exhibited clear zone after incubation on enrichment medium containing 0.1% PLA as the sole carbon source at 37°C for a week. PLA biodegradation by *S. maltophilia* was analysed by incubating on mineral medium supplemented with PLA powder for 40 days and determining the molecular weight of the PLA powder. In the presence of *S. maltophilia*, the molecular

weight of PLA decreased more rapidly compared to that without *S. maltophilia* inoculation, suggesting that the degradation of PLA was assisted by extracellular enzymes secreted by *S. maltophilia.*

Bubpachat, Sombatsompop and Prapagdee (2018) have isolated bacteria from soils and wastewater sludge and conducted PLA degradation assay of the bacterial isolates. One of the potential PLA-degrading bacteria was identified as *Stenotrophomonas pavanii,* which possessed protease and PLA-degrading enzymes activities. When the bacterial isolates were screened for protease activity on skim milk agar, *S. pavanii* showed the largest halo zone area when compared to other isolates, implying high enzymatic activity. Protease and PLA-degrading enzymes activity of *S. pavanii* were determined by using azocasein method and observing the turbidity of emulsified PLA after incubation of the bacteria in the Bifidus Selective Medium (BSM) broth with emulsified PLA. It was observed that protease concentration was proportional to bacterial growth, indicating the utilisation of PLA for protease production. PLA-degrading enzyme activity showed a similar pattern as the protease production. This study has also analysed the PLA biodegradability of *S. pavanii* by incubation of the bacteria in BSM containing PLA film to determine the biofilm formation on the film. By observing the film under SEM, presence of bacterial cells was observed on the surface of the PLA film after 8 days of incubation at 30°C, showing that the cells adhered to the PLA surface to use it for metabolism.

There is also research about degradation of other types of plastic polymer by *Stenotrophomonas* sp., which demonstrate the potential of *Stenotrophomonas* sp. to degrade a wide range of plastic polymer. Juliandi et al. (2020) have found out that *Stenotrophomonas maltophilia* strain W1-2 isolated from seawater samples was able to degrade polypropylene (PP) when it was incubated in mineral medium containing PP film for 30 days. Among the bacterial isolates from the seawater samples, *S. maltophilia* strain W1-2 showed 10.8% of PP weight reduction after 30-days incubation. Xiang et al. (2023) have discovered that *Stenotrophomonas maltophilia* isolated from plastic-polluted river and soil was able to degrade polystyrene (PS). It showed a higher degradation efficiency when combined with another isolate *Bacillus velezensis,* which was proven by a higher percentage of weight reduction (21.9%) when incubated on the basal salt medium (MSM) containing 0.1 g of PS microplastic for 30 days compared to the weight reduction caused by single bacterium incubation. It was suggested that the interspecific collaboration or the metabolites produced by the two bacteria increased the degradation efficiency (Xiang et al., 2023). Biodegradation of LDPE by *Stenotrophomonas* sp. was also verified by the biofilm formation on the LDPE beads observed under SEM (Dey et al., 2020) and 32% weight loss of LDPE after incubation on carbon free medium supplemented with LDPE (Nadeem et al., 2021).

All the findings have shown that *Stenotrophomonas* sp. is capable of secreting extracellular enzymes to degrade plastic polymers. It possesses high potential to be utilised in biodegradation of a broad range of plastic polymers and further study is recommended to optimise its biodegradation activities. In the present

study, the identity of L21 as *Stenotrophomonas maltophilia* strain W1-2 was 99.55% similar to the bacteria obtained by Juliandi et al. (2020), which demonstrated PP biodegradability. The macroscopic observations of the colony morphology between L21 and *S. maltophilia* strain W1-2 exhibit similarities, which is shown in Table 5.2.

Table 5.2: The comparison of macroscopic observations between bacterial isolate L21 and *Stenotrophomonas maltophilia* strain W1-2 obtained by Juliandi et al. (2020).

Macroscopic observation	**L21**	***Stenotrophomonas maltophilia* strain W1-2**
Colour	White	White
Texture	Smooth	Smooth
Elevation	Low convex	Raised
Form	Circular	Circular
Margin	Entire	Entire

Further identification of L21 via Gram staining and microscopic observations are needed to obtain the morphological characteristics of L21. Biochemical tests such as catalase, oxidase, and hydrogen sulphide can provide vital information for accurate identification of L21. There is no finding about PLA degradation by this strain. However, research by Jeon and Kim (2013) showed that *Stenotrophomonas maltophilia* strain LB 2-3 can degrade PLA. Since they are from the same species, they might possess the ability to encode enzymes that can degrade PLA.

5.5 Future Study

Analysis of enzymes production and activity by the potential PLA-degrading bacteria can obtain qualitative and quantitative data to monitor the degradation activity. It can be performed by providing respective substrates to test the enzymatic activity of protease, lipase, and esterase, which allows further analysis of the type and family of the depolymerase secreted by *B. intermedia* and *S. maltophilia* strain W1-2. Screening of protease activity can be carried out by inoculating potential PLA-degrading bacteria on skim milk agar. Formation of halo zones indicates the secretion of protease by the bacteria to digest the skim milk. Crude enzymes can be extracted from the medium broth inoculated with the potential bacteria to measure the crude enzyme concentration and enzymatic activity. Lipase activity can be determined by the enzyme assay using *p*-nitrophenyl containing compound as the substrate, such as *p*-nitrophenyl palmitate, *p*-nitrophenyl phosphate, and *p*-nitrophenyl decanoate. *p*-nitrophenyl containing compound contains ester bond, which is cleaved by the lipase enzyme to produce acid and *p*-nitrophenol with bright yellow colouration. The intensity of the yellow colour is measured using a spectrophotometer at 405 nm. The absorbance will increase over the time of reaction. A graph of absorbance against *p*-nitrophenol concentration can be obtained, which is utilised in determining the lipase activity (Sahonero-Canavesi et al., 2016).

Further analysis of PLA biodegradation by the bacterial isolates can be performed using weight reduction percentage, SEM, and FTIR. Weight reduction percentage provides quantitative data which can be used to compare

the biodegradation efficiency with other potential isolates and to give clues of the factors contributing to biodegradation activity. The weight of the PLA film is determined using analytical balance before and after the incubation with potential PLA-degrading bacteria. The percentage of weight reduction is calculated using the following equation:

$$\text{Percentage of weight reduction} = \frac{W_{\text{initial}} - W_{\text{final}}}{W_{\text{initial}}} \times 100$$

The weight reduction percentage data is then used to plot a graph of weight reduction percentage against incubation time to compare with different potential PLA-degrading bacterial isolates. It allows further study of the optimum conditions for PLA biodegradation by comparing their weight reduction percentages and graphs.

SEM visualises the physical PLA biodegradation activity by giving a 3D image which provides insights of the biodegradation mechanism by the bacterial isolates, such as bacterial cells adhesion to the PLA surfaces. SEM allows the study of the degree and pattern of biodegradation by the bacterial isolates (Kim et al., 2017). To perform SEM, the bacterial isolates are incubated on minimal medium with a PLA film as the sole carbon source. After incubation, the film with cells is treated with 2.5% glutaraldehyde and 50 M potassium phosphate buffer (pH 7.0) for overnight, then with 30% to 100% graded series of ethanol to induce sequential dehydration (Kim et al., 2017). Fixation on aluminium stubs and deposition of gold on the treated film is performed, then it can be observed under SEM (Kim et al., 2017).

Fourier Transform Infrared (FTIR) spectrophotometry is an advanced bioanalytical tool widely used to monitor the molecular structure and chemical composition of materials. It utilises vibrational spectroscopy which correlates the molecular structures of materials with the physical properties (Griffiths and Haseth, 2007). Measurement of the infrared spectrum provides qualitative, quantitative, and kinetic data which results in a wide range of applications (Griffiths and Haseth, 2007). In terms of PLA biodegradation by the bacterial isolates, FTIR is utilised to examine the chemical changes of the PLA film after degradation by the potential bacteria. The comparison between the biodegraded PLA and the control can be determined using the carbonyl index (%) to obtain the ratio of band intensity originating from the carbonyl group to the band intensity of the unchanged group. Carbonyl index is increased at the beginning of the biodegradation due to initiation of oxidation of the PLA polymers. In the subsequent biodegradation process, number of carbonyl bonds will be reduced due to PLA biodegradation, resulting in the reduction of carbonyl index (Janczak et al., 2018).

CHAPTER 6

CONCLUSIONS

Biofertilizer contains microbial agents that promote plant growth, which also possess potential in plastic degradation. In this study, bacterial isolates J2, L21, N5, and N18 from biofertilizer samples were screened for GPLA degradation activity. Among the isolates, J2 and L21 demonstrated clear zone formation on M9 agar media with 0.2, 0.4, 0.6% of GPLA composition after staining with Coomassie Blue solution. Through molecular approaches and BLAST application, J2 and L21 were identified as *Brucella intermedia* and *Stenotrophomonas maltophilia* strain W1-2, respectively. There is limited study of *B. intermedia* on PLA degradation and its degradability might be promoted by the interaction of *B. intermedia* with other potential bacterial strains. *S. maltophilia* had been shown to degrade various types of plastic and its degradation on PLA was also determined by other researchers. Further evaluation on their PLA degradation can be conducted through weight loss percentage determination, observation of PLA film under SEM, and FTIR analysis on the chemical properties. It is important to understand their mechanisms in PLA degradation as it can help develop sustainable plastic waste management especially in the agricultural sectors by utilising the double-potentiality of microbes present in biofertilizers to reduce their hazard to the environment and living organisms.

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