ISOLATION AND CHARACTERISATION OF POLYLACTIC ACID-DEGRADING ACTINOBACTERIA

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ABSTRACT

Polylactic acid (PLA) is one of the biodegradable plastics available in the market where the monomers lactic acids are joined together by ester bonds. Actinobacteria is a bacterial group with high G+C mol% and capability to produce various kinds of useful secondary substrates due to their unique metabolic pathways. In this study, soil samples from a contaminated landfill site and a non-contaminated soil at camp site were collected and screened for actinobacteria that have the ability to degrade PLA. PLA-emulsified medium, which contains only PLA as carbon source, was used as selective isolation medium to isolate PLA degraders from soil samples. Isolated strains were characterized by using polyphasic method. A total of 235 strains of PLA degrading actinobacteria were isolated from ten soil samples. Among these 235 strains, PLA degraders were found from different genera such as *Agromyces, Rhodococcus, Micromonospora, Mycobacterium* and *Streptomyces*, contrary to previous reports. In addition, two novel strains, which are members of *Agromyces* and *Mycobacterium*, were identified. Relative enzyme activity of isolated strains was checked and most of the strains have relative enzyme activity from 1.00 to 3.00 toward both low and high molecular weight PLA. Crude enzyme was extracted from strains P253 and P256, which had a relative enzyme activity higher than 3.00 for both low and high molecular weight PLA. Molecular weight of PLA-degrading enzyme was determined by using SDS PAGE and zymography method. The PLA-degrading enzyme from strains P253 and P256 were found to have molecular weight ranging from 10-35kDa and 35-40kDa, respectively.

ABSTRAK

Polylactic acid (PLA) merupakan sejenis plastik yang boleh diurai secara semula jadi. Polimer ini adalah diperbuat daripada unit-unit asas iaitu acid laktik. Actinobacteria adalah kumpulan bakteria yang mempunyai kandungan G+C yang tinggi dalam DNA dan juga keupayaan untuk menghasilkan substat yang berguna. Oleh itu, beberapa sampel tanah daripada dua lokasi yang berlainan, iaitu tapak pelupusan yang tercemar dan tapak perkhemahan yang tidak dicemari oleh sampah plastik, telah dikumpul supaya actinobacteria yang berupaya untuk mengurai PLA dapat ditemui. Agar PLA yang hanya mempunyai PLA sebagai sumber karbon telah digunakan dalam proses isolasi. Ciri-ciri strain telah dikaji dan dicatatkan. Sejumlah 235 strain actinobacteria telah ditemui daripada sepuluh sampel-sampel tanah dengan menggunakan agar PLA, di mana mereka adalah daripada genera *Agromyces, Rhodococcus, Micromonospora, Mycobacterium* dan juga *Streptomyces*. Selain daripada ini, dua strain daripada 235 strain ini merupakan strain yang baru ditemui. Mereka adalah dari genera *Agromyces* dan *Mycobacterium*. Aktiviti enzim relatif untuk setiap strain telah dikaji dan kebanyakan strain mempunyai nilai aktiviti enzim relatif di antara 1.00 kepada 3.00 terhadap PLA yang mempunyai berat molekul yang rendah dan juga yang tinggi. Selepas itu, berat molekul bagi enzim penguraian PLA telah dikaji dengan menggunakan SDS PAGE. Enzim mentah telah diestrakkan daripada strain P253 dan P256. Ini adalah kerana dua-dua strain ini mempunyai nilai activiti enzim relatif yang tinggi. Berat molekul enzim penguraian PLA daripada strain P253 adalah di antara 10-35kDa, manakala berat molekul enzim penguraian PLA daripada strain P256 adalah di antara 35-40kDa.

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TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

All strains do not decompose adenine, xanthine or urea. All strains can grow in the presence of 2% NaCl but not at 10% NaCl. All strains can grow at 28^{o}C and 37^{o}C. All strains showed positive reaction in catalase test.

Table C.1: Pictures of representative strain from each colour group

LIST OF SYMBOLS AND ABBREVIATIONS

$(NH_4)_2SO_4$	ammonium sulphate
µl	microliter
bp	base pair
DAP	diaminopimelic acid
DNA	deoxyribonucleic acid
g	gravity
HCl	hydrochloric acid
ISP2	International *Streptomyces* Project 2
K_2HPO_4	di-potassium hydrogen phosphate
kDa	kilo Dalton
KH_2PO_4	potassium di-hydrogen phosphate
LAB	lactic acid bacteria
$MgSO_4.7H_2O$	magnesium sulphate
Mw	molecular weight
N	normality
PAGE	polyacrylamide gel electrophoresis
PCL	polycaprolactone

PCR	polymerase chain reaction
PGA	polyglycolic acid
PHA	polyhydroxyalkanoate
PHB	poly-3-hydroxybutyrate
PLA	polylactic acid
psi	pound-force per square inch
PVA	polyvinyl alcohol
REI	relative enzyme activity
rpm	rotation per minute
rRNA	ribosomal ribonucleic acid
SB	sodium borate
SDS	sodium dodecyl sulphate
V	voltage
v/v	volume per volume
w/v	weight per volume

LIST OF APPENDICES

CHAPTER 1

1.0 Introduction

Polylactic acid (PLA) or polylactide is one of the biodegradable plastics that belongs to the family of aliphatic polyester. It is now commercially available in the market for various uses. For example it is widely used in medical applications such as surgical suture, orthopedic implant device, cosmetic surgery products and drug delivery system. Besides, it is also used to produce packaging materials, and nets and non-woven products for agriculture field and fishery. PLA has comparable strength to polyethylene or polystyrene, high transparency, weather resistance, heat resistance and workability compare to other biodegradable plastics (Matsumura, 2008). Production cost of PLA can be lower since it is produced from polymerization of lactic acid, which is a renewable resource. Therefore, PLA has high potential and market value for various applications. However, can PLA be degraded by microorganisms instead of accumulated in the environment?

In this study, PLA-degrading actinobacteria were isolated from soil samples and characterized. Soil samples were collected from two different places, a contaminated landfill site in Gopeng, Perak and a non-contaminated camp site in Gurun, Kedah. Landfill site was chosen as a sampling site because large number of plastic wastes had been thrown and left there for a long period. Therefore, the hypothesis is that microorganisms with plastic-degrading and hydrolytic ability could be found there because they may need this ability to absorb nutrient necessary for their survival. Another soil sample was collected from a non-polluted camp site on Gunung Jerai, Gurun, Kedah to check whether PLA-degrading actinobacteria could be ubiquitous in different kinds of environment.

PLA-degrading actinobacteria were isolated from soil samples using PLA-emulsified medium. Isolated strains were purified and their Gram reaction was determined by using the Gram staining method. In addition, cell and colony morphology of all the strains were also observed and recorded. Only Gram positive strains were selected for further tests and were considered as tentative actinobacteria. After that, the colour of their aerial mycelia, substrate mycelia and diffusible pigment were also observed and recorded after they were grown on ISP2 for 7 days at 28^{o}C. Then, catalase and oxidase tests were performed to allow more phenotypic traits or characteristics to be compared among the strains. The ability of strains to degrade PLA was also examined and recorded. All the data for phenotypic characteristics were collected and analyzed. A dendogram, which clusters similar strains together, was generated by using PAST software. From the dendogram, strains were randomly chosen from each cluster for identification based on their 16S rRNA gene sequence.

Besides characterization of isolated strains, PLA-degradation activity of strains was also studied in this project. Relative enzyme index (REI) for each strain was examined and a few of strains with high REI were chosen for further test. Crude enzyme was extracted, concentrated and molecular weight of the PLA-degrading enzymes was determined by using SDS-PAGE.

The objectives of this study are listed like below,

- To isolate and prepare an inventory of PLA-degrading actinobacteria from soil (landfill and undisturbed).
- To identify and characterize selected PLA-degrading actinobacteria.
- To extract PLA-degrading enzyme.

CHAPTER 2

2.0 Literature review

2.1 Characterization and identification of Actinobacteria

Actinobacteria is a group of Gram positive bacteria with high G+C mol% in their DNA. In the past, they have been classified as fungi due to the fact that they share some similarity in morphological characteristics with fungi. Their morphological characteristics vary from species to species or even individuals in the same species. For example, they may exist as coccoid, rod-coccoid, fragmented hyphal form or filamentous. Moreover, due to the fact that they are diverse in their metabolic and physiological characteristics, they are well known in the production of various useful secondary metabolites. Examples of secondary metabolites from actinobacteria are antibiotics such as streptomycin and rifamycin (Kim *et al.*, 2006), and enzymes like chitinase (Kawase *et al.*, 2004), lignocelluloses-degrading enzyme (McCarthy, 1987), and PLA-degrading enzyme (Tokiwa & Jarerat, 2004). Perhaps because of their special metabolic and physiological characteristics, they are capable to survive in various kinds of habitats and conditions which include some harsh environments such as the polluted or contaminated and low-nutrient environment. Another reason for their high survival rate in various environments is their capability in spore formation when the conditions become unfavorable to them. Therefore, actinobacteria are very useful in bioremediation due to the fact that their survival rate in the environment is relatively higher than other bacteria. Members of genera *Agromyces, Micromonospora, Mycobacterium, Rhodococcus* and *Streptomyces* were isolated in this study. Therefore, the following paragraphs are to introduce the general characteristics and applications of members of these several genera.

Agromyces is a genus that contained in the family Microbacteriaceae. Members of *Agromyces* generally have opaque, paste, entire colonies which usually penetrate into agar and are without mycelia. Their hyphae will branch and break up into diphtheroid and rod-like, irregular, non-motile fragments. Moreover, they are aerobic to microaerophilic, mesophilic, and show variable reaction in catalase and oxidase test for different species. They have been used in various applications such as waste-gas treatment and pollutants degradation (Bendinger *et al.*, 1990), aniline degradation (Aoki *et al.*, 1983), and oxidizing steroids (Arnaudi, 1954).

Species of *Micromonospora* have general characteristics such as having raised and folded colony with areas of different colors and their colonies are completely covered by mucoid, black mass of spores. They can survive in aerobic and anaerobic conditions such as deep sea sediment for a very long period. They are able to degrade biopolymers (Erikson, 1940) and lignin (McCarthy & Broda, 1984).

Generally, mycobacterium can be separated into two main groups based on their growth rate, which are, slow grower and fast grower. Most of the slow growers are associated with human and animal diseases while most of the fast growers are saprophytes found in the environment. Environmental mycobacteria are categorized as the non-medical mycobacterium. Some of the typical characteristics of mycobacterium: they are aerobic, acid fast, form curved or straight non-motile rod, their mycelium will fragmented into either rod or cocoid elements, and having wall chemotype IV, that is, their cell wall contain *meso*-diaminopimelic acid. Many species form whitish or cream-coloured colonies and for those that contain carotenoid pigments form bright yellow or orange colonies. Mycobacteria are found to have various catabolic or degradation activities for examples, the ability to degrade the morpholine (Cech *et al.*, 1988) and polycyclic aromatic

hydrocarbons such as pyrene (Heitkamp *et al.*, 1988) and phenanthrene (Guerin & Jones, 1988), simple molecules such as methanol and methylamines (Kato *et al.*, 1988; Urakami & Yano, 1989). In 1965, Jones and Jenkins had shown the abundance of mycobacterium in soils (Jones & Jenkins, 1965). Mycobacteria are able to survive in a wide range of environmental conditions and also utilize different kind of growth substrates.

Members of the genus *Rhodococcus* are aerobic, catalase positive, Gram positive to Gram variable, and non-motile in nature. They form rods to extensively branched substrate mycelia. Their colonies morphology varies among different species, i.e. they can be rough, smooth or mucoid, and have colours like buff, cream, yellow, orange or red. Moreover, they also have wall chemotype IV just like mycobacterium. Rhodococci had been found to have the ability to degrade various compounds such as phenol (Straube *et al.*, 1990), oligocarbophily (Tarnok, 1976), petroleum (Nesterenko *et al.*, 1978), humic acid (Cross *et al.*, 1976), lignin-related compounds (Eggeling & Sahm, 1980; Eggeling & Sahm, 1981; Rast *et al.*, 1980), pesticides (Nagy *et al.*, 1995), acrylamide (Arai *et al.*, 1981), aromatic hydrocarbons (Raymond *et al.*, 1971), and haloalkanes (Kesseler *et al.*, 1996).

Last but not least, members of genus *Streptomyces* which are generally known as streptomycetes are Gram positive, aerobic and catalase positive actinobacteria. They use form long and highly branched mycelia with minimal fragmentation and there are non-motile spores which are attached to the end of their mycelia. Their colonies can be discrete and lichenoid, leathery or butyrous. At the early stage of their life cycle, their colonies are relatively smooth in surface, however, after they have developed their aerial mycelium, their colonies become floccose, granular, powdery or velvety. Besides, they also tend to produce various pigments which are responsible for their colourful aerial and substrate mycelia and also diffusible pigments throughout their life cycle. Streptomycetes are well

known for their ability in producing antibiotics. Furthermore, they are also known to have the ability to degrade various recalcitrant substances such as chitin, cellulose, and lignin.

2.2 Examples of actinobacterial PLA degrader

Amycolatopsis sp. was first discovered as PLA-degrading bacteria by Pranamuda *et al.*, that is, *Amycolatopsis* sp. strain HT-32 (Pranamuda *et al.*, 1997). Then, PLA-degrading activity was found to be widely distributed in this particular genus (Chomchoei *et al.*, 2011; Nakamura *et al.*, 2001; Pranamuda & Tokiwa, 1999; Pranamuda *et al.*, 2001; Tokiwa *et al.*, 1999). PLA-degrading enzyme from *Amycolatopsis* sp. strain K104-1 has been isolated, purified, and characterized (Nakamura *et al.*, 2001). The molecular weight of the isolated enzyme is 24kDa and it has similar properties to alkaline serine protease.

Other than genus *Amycolatopsis*, PLA-degrading activity among other genera was studied by Jarerat and his group. Strains from the genera *Lentzea* and *Kibdelosporangium* that are, *Lentzea waywayandensis* and *Kibdelosporangium aridum*, were also found to possess the ability to degrade PLA. They have the ability to reduce the weight of PLA film by more than 90% and also assimilate the degradation product (Jarerat & Tokiwa, 2003; Jarerat *et al.*, 2003).

Besides, members of genera *Paecilomyces*, *Thermomonospora*, and *Thermopolyspora* are likely to have the capability to degrade PLA. This is because their gene sequences were found in the aerobic compost for PLA degradation by using molecular ecological techniques (Sangwan & Wu, 2008). Most recently, *Pseudonocardia alni* was found capable to degrade PLA too (Konkit *et al.*, 2012).

Moreover, PLA-degrading strains from several other genera like *Actinomadura keratinilytica*, *Micronomospora echinospora*, *Micromonospora viridifaciens*, *Nonomuraea terrinata*, *Nonomuraea fastidiosa*, *Laceyella sacchari*, and *Thermoactinomyces vulgalis* were isolated from soil samples (Sukkhum *et al.*, 2009). An enzyme was isolated and purified from *Actinomadura keratinilytica*. This enzyme has molecular weight of 30kDa and similar properties as serine protease (Sukkhum *et al.*, 2009).

2.3 Polylactic acid (PLA)

Biodegradable plastic is biopolymer that synthesized from renewable resources and can be biodegraded. Nowadays, biodegradable plastics have been used as alternative or replacement to petrochemical plastics due to its biodegradable characteristic. Generally, they can be synthesized biologically or chemically using biological materials (Demirbas, 2007).

Biodegradable plastics are mostly made from polyester because it is cheaper compare to other biopolymers such as polyamino acid. Polyesters can either be synthesized chemically or biologically. Examples of chemically synthesized polyesters are polyglycolic acid (PGA), polylactic acid (PLA), polycaprolactone (PCL), and polyvinyl alcohol (PVA). While biologically synthesized polyesters include polyhydroxyalkanoate (Teeraphatpornchai *et al.*, 2003) and poly-3-hydroxybutyrate (PHB) (Demirbas, 2007).

Until now, there is only 1% of bioplastics in the market and PLA is one of them (Nampoothiri *et al.*, 2010). Initially, PLA was patented by DuPont in 1954 and this make the commercialization of PLA available. Thereafter, there are several companies which

have become active in PLA market also such as Cargill Inc., Teijin Ltd., and Shimadzu (Nampoothiri *et al.*, 2010).

PLA has high market value because of several reasons. First, it has comparable strength to polyethylene or polystyrene, high transparency, weather resistance, heat resistance and workability compare to other biodegradable plastics (Matsumura, 2008). Second, its production cost can be lowered since it is produced from polymerization of lactic acid, which is a renewable resource. Third, it seldom contaminated by microorganisms because it is degraded primarily by hydrolysis instead of microbial degradation. Therefore, it is commonly used in food packaging, mulching film and also medical application (Gross & Kalra, 2002). Up to date, PLA is the biodegradable plastic which has been developed furthest towards practical use in different fields such as covering materials in agriculture, fibers, earth retaining netting, weed-preventing bags and others (Matsumura, 2008).

Lactic acid, which is a naturally occurring organic acid, is the monomer for PLA. It can be produced either by chemical synthesis or fermentation. Lactic acid can be synthesized chemically through many different pathways such as degradation of sugar by using base, hydrolysis of lactonitrile by strong acid, oxidation of propylene glycol and etc.. However, increasing interest is shown to biotechnology production of lactic acid, that is through fermentation, because it is more economic and also environmental friendly compared to chemical synthesis. This is due to the fact that biotechnological method uses low cost renewable substrates instead of petrochemicals, low production temperature and low energy consumption. Moreover, high product specificity of biotechnology technique makes it the preferable method to produce lactic acid. There are two main groups of microorganism that involve in the fermentation to produce lactic acid, that are, lactic acid bacteria (LAB) and some filamentous fungus. The substrate or carbon source for the

microbial production of lactic acid can be pure sugar, sugar containing materials, or starchy materials. That means production cost can be lowered because cheaper substrates are being used. Recent studies even show that it is possible to combine the saccharification and fermentation steps to further cut down the production cost by avoiding high energy consuming biomass saccharification (Nampoothiri *et al.*, 2010).

Due to the presence of both hydroxyl and carboxyl end in lactic acid, polycondensation method can be used to polymerize lactic acid to PLA directly. However, this method can only produce low molecular weight PLA (Nampoothiri *et al.*, 2010).

In order to produce high molecular weight PLA, open-ring polymerization reaction can be used. In this reaction, lactic acid will be polymerized into high molecular weight polymer through two steps. A cyclic lactic acid dimer, which is an intermediate lactide, is formed in the first step through oligomerization reaction. There are three stereoforms of the intermediate lactide: L-lactide, D-lactide, and meso-lactide. Purified intermediate lactides are converted into high molecular weight PLA in the second step through open-ring polymerization reaction. This reaction is carried out by catalysts such as stannous octoate (Pranamuda *et al.* 2001) and tin chloride (Nampoothiri *et al.*, 2010).

Later, this open-ring polymerization process is modified by Cargill Dow LLC into a low-cost continuous process for PLA production, that is, PLA is synthesized in melt rather than in solution. That means, usage of high cost solvent, which is environmental unfriendly, is completely eliminated in this modified process (Gruber *et al.*, 1992; Gruber *et al.*, 1993; Gruber *et al.*, 1994).

2.4 PLA degradation and PLA-degrading enzyme

Degradation of PLA is mainly due to the scission of its ester bond and this process can be done either biologically or chemically (Nampoothiri *et al.*, 2010).

Biological degradation or biodegradation of PLA is carried out through the utilization of enzymes or microorganisms. Up to now, almost all the isolated PLA-degrading microorganisms belong to the class Actinobacteria. They are members of different genera such as *Amycolatopsis*, *Kibdelosporangium*, *Lentzea*, *Pseudonocardiacea*, and others. These PLA-degrading microorganisms may or may not utilize the degradation products as their carbon source. For those that do not utilize PLA as carbon source, PLA degradation is the result of co-degradation (Teeraphatpornchai *et al.*, 2003). This kind of PLA-degrading microorganism is preferred to be used in situ because they are not affected by the presence of other carbon sources.

PLA-degrading enzymes generally belong to hydrolases such as esterase, lipase and protease, which are capable in catalyzing the hydrolysis of ester bond (Akoh *et al.*, 2004; Teeraphatpornchai *et al.*, 2003) that join the monomers of PLA together. For examples, protease and esterase were identified and purified as PLA depolymerase from *Amycolatopsis* sp. and *Bacillus* sp., respectively (Nakajima & Shigeno, 2006; Pranamuda *et al.*, 2001). Besides, up to date, proteinase K is the only enzyme that can degrade or hydrolyze high molecular weight PLA (Tsuji & Miyauchi, 2001). Hydrolases are useful in bioremediation of pollutants due to their low substrate specificity, which means that their activity will not be affected even when pollutants present as a mixture (Gianfreda *et al.*, 2006).

Two types of enzymes have been used in the biodegradation of PLA, which are, intracellular and extracellular enzymes. If intracellular enzymes are used to degrade or transform PLA, presence of whole microbial cells in the polluted site is required. On the other hand, in the case of extracellular enzymes, presence of whole cells may be required because of their specific metabolic pathway, or else, cell free enzyme alone is enough for the biodegradation process (Gianfreda *et al.*, 2006). Utilization of cell free enzymes in situ has several advantages over the utilization of whole cell, that are, there are no predators for the enzymes, they are not sensitive to the presence of inhibitor of cell metabolism and drastic change in pollutants' concentration, they are not affected by the presence of toxic pollutants, and they are able to withstand a wide range of environmental conditions, which are usually unfavorable to the microbial cells (Gianfreda *et al.*, 2006).

After the PLA-degrading enzyme is discovered, extracted and purified, it can be commercialized in granule, powder, tablet, or any other forms. Besides, other components such as stabilizer, excipient, pH adjustor, extender or binder may be added into the tablet for better performance of the enzyme (Nakajima & Shigeno, 2006).

For chemical degradation methods, pyrolysis and copyrolysis are two main choices. Pyrolysis is a process where heat is to degrade PLA while copyrolysis can be considered as an upgrading step of the pyrolysis. This is due to the fact that copyrolysis has several advantages over the pyrolysis process such as chemicals recovery, reduction of the wastes' volume and replacement of fossil fuels (Wang & Li, 2008). Other than pyrolysis and copyrolysis processes, acid and alkali are also use to hydrolyze the PLA.

All the mechanism has their advantages and disadvantages. For biodegradation of PLA which uses the enzymes and/or microorganisms, it is more environmental friendly and most

probably it is cheaper than chemical and physical processes. Besides that, monomers produced in biodegradation will have regular terminals, which allow the re-polymerization of the monomers, while monomers produced in chemical and physical processes have irregular terminals (Matsumura, 2008; Nakajima & Shigeno, 2006). This is because enzymes are specific towards their substrates while chemical processes that use acid or alkali are random in action. Hence, downstream process for enzyme depolymerization process is always simpler and cheaper compare to chemical recycling process, which requires additional purification step for the mixture of monomers (Nakajima & Shigeno, 2006). However, biodegradation of PLA in situ can be affected by factors like humidity, nutrient supply, pH, redox condition and temperature. These factors will either affect the enzyme or the growth of microorganisms or both (Nampoothiri *et al.*, 2010). On the other hand, chemical and physical degradation processes are more stable since they are not be affected by environmental conditions, however, both of them are high in energy consumption and produce large amount of carbon dioxide (Matsumura, 2008).

Despite the method that is used to degrade PLA, there are some general problems in PLA degradation. One of the problems is that plastic wastes are always mixed with different kinds of materials like domestic wastes (Nakajima & Shigeno, 2006). This will decreases the rate of biodegradation because PLA-degrading microorganisms, which supposed to use PLA as their carbon source, tend to use other organic materials as their carbon source rather than the PLA (Nakajima & Shigeno, 2006). Besides, PLA has lower biodegradability than other biodegradable plastics such as PCL or PHB, that is, PLA will only be degraded into water and carbon dioxide after several years depending on the environmental condition (Matsumura, 2008). It is well known that PLA is not degraded by microorganisms unless it is present in suitable conditions, which are, high temperature and high humidity. This is

due to the fact that PLA is degraded by a two-stage reaction. It is first hydrolyzed at high temperature and high humidity, which result in the drop of molecular weight, then only degraded by microorganisms (Matsumura, 2008).

CHAPTER 3

3.0 Methodology

3.1 Soil samples

Soil samples were collected from two different sites, that are, landfill site in Gopeng, Perak (N04^{o}23’35”, E101^{o}10’54”) and campsite on Gunung Jerai, Gurun, Kedah (N05^{o}48’21”, E100^{o}25’56”).

From the landfill site in Gopeng, a total of five soil samples with five replicates each (DS1A-E, DS2A-E, DS3A-E, DS4A-E and DS5A-E) were collected. These replicates of soil samples were to maximize the number of PLA-degrading actinobacteria isolated from each sampling point. While five soil samples (SP1-5) were collected from Gunung Jerai, which is an uncontaminated camp site. Soil samples were collected from both contaminated and non-contaminated site to determine the stimulation effect of plastics pollution on the PLA-degrading activity or PLA-degrading enzyme production from microorganisms.

Surface soil (1-3cm depth) was collected using clean spatula and kept in sterile falcon tubes. For each soil sample, replicates were collected within 50cm radius. Soil samples were stored in room temperature until for further use. Soil samples were used directly for pH determination and isolation without being air dried. This is because the air drying method is one of the selective or pretreatment methods which will limit the type of actinobacteria to be isolated from the soil samples.

For soil sample’s pH determination, soil sample (1g) was transferred into a sterile universal bottle with 9ml of ¼ strength Ringer’s solution. The mixture was mixed by vortex for half

an hour and left to stand for 30 minutes before pH was taken. pH was taken as mean average of three readings measured with pH meter.

To compare the effects of pretreatment, one soil sample (DS2D) was pretreated by mixing PLA film (5000Mw) into 1g of soil sample and incubated at 30^{o}C for 1 week prior to being used in isolation of PLA-degrading actinobacteria (Tomita *et al.*, 2004).

3.2 Culture media

Two main culture media, which were used in this study, were PLA-emulsified medium and International *Streptomyces* Project medium 2 (ISP2). Their formulation per liter was listed as below. Two types of antifungals, which are nystatin (50µg/ml) and cyclohexamide (50µg/ml), were added into the isolation medium to exclude or reduce the number of fungus in the isolation. The medium was prepared and heated to dissolve all the ingredients by using microwave. Then, the pH of the medium was checked and adjusted accordingly before sterilizing at 121^{o}C, 15psi for 20 minutes.

PLA-emulsified medium (modified from (Sukkhum *et al.*, 2009))

$(NH_4)_2SO_4$	4.00g
K_2HPO_4	2.00g
KH_2PO_4	1.00g
$MgSO_4.7H_2O$	0.50g
Yeast extract	0.10g
PLA (5000Mw/20000Mw, WAKO, Japan)	1.00g
Bromocresol purple	0.10g

Bacto agar	15.00g
pH	7.00

ISP2 (Shirling & Gottlieb, 1966)

Yeast extract	4.00g
Malt extract	10.00g
Glucose	4.00g
Bacto agar	15.00g
pH	7.00

3.3 Isolation of PLA-degrading actinobacteria

3.3.1 Preparation of PLA film (modified from (Jarerat *et al.*, 2003))

PLA powder (0.1g; 5000Mw; WAKO, Japan) was dissolved in 50ml of dichloromethane. After that, the solution was poured and dried in a petri dish. The PLA film formed was washed by using 70% ethanol and cut into small pieces.

3.3.2 Preparation of PLA emulsified medium (modified from Sukkhum *et al.*, 2009)

PLA suspension was prepared by dissolving 0.1% (w/v) of low molecular weight (5000Mw) or high molecular weight (20000Mw) PLA in 50ml of dichloromethane, followed by 1 hour of sonication in 100ml of distilled water with 1% (v/v) Tween 80. After emulsification, dichloromethane was removed by stirring the solution at 90^{o}C in fume hood. After that, medium was sterilized by autoclaving at 121^{o}C, 15psi for 20 minutes.

3.3.3 Isolation procedures (Cappuccino, 2005)

Soil samples were diluted through the serial dilution method before spread plating was performed. 1g of soil sample was suspended in 9ml of ¼ strength Ringer's solution to form a 10^{-1} soil suspension. 1ml of the 10^{-1} soil suspension was transferred into another 9ml of Ringer's solution to form 10^{-2} soil suspension. This step was repeated until 10^{-4} dilution. Then, 100µl of the soil suspension from each dilution were inoculated onto the surfaces of selective isolation plates. The soil suspensions were then spread by using a glass rod spreader (Cappuccino, 2005). Each dilution was spread in duplicate plates. All plates were incubated at 28^{o}C for 7 days or more until yellow colour or zone was observed on the isolation medium that is, PLA-emulsified medium. The yellow zone is an indicator of PLA degradation activity carried out by PLA degrader.

3.4 Characterization

3.4.1 Gram staining (Cappuccino, 2005)

Bacterial smear was prepared on glass slide and heat fixed. The fixed slide was flooded with crystal violet and allowed to remain for 1 minute. After that, the crystal violet was rinsed off with distilled water. Then the slide was flooded with iodine and allowed to remain for 1 minute. After 1 minute, the iodine was rinsed off with distilled water. The slide was then flooded with acetone for 3 seconds. The acetone was rinsed off with distilled water. For the counterstain step, the slide was flooded with safranin for 30 seconds. After that, the safranin was rinsed off with distilled water. The slide was blot dried with bibulous paper and observed under light microscope.

The Gram reaction and cell morphology of isolates were observed under the light microscope and recorded.

3.4.2 Colour grouping

All the presumptive actinobacterial strains were grown on ISP2 medium for 7 days. The colour of aerial mycelia, substrate mycelia and diffusible pigment for each strain were observed and recorded according to National Bureau of Standard Colour Name Chart (Appendix E, page 80).

Medium ISP2 instead of ISP3 was chosen as medium for colour grouping because ISP2 medium can support the growth of all types of actinobacteria while ISP3 can only support the growth of streptomycetes.

3.4.3 Oxidase test (Cappuccino, 2005)

Reagent solution was prepared by dissolving 1% (w/v) of N, N, N', N'-tetramethyl-p-phenylenediamine in distilled water. Filter paper was soaked with fresh prepared reagent solution. Fresh growth colony was scraped by using sterile toothpick and rubbed onto the filter paper. Blue colour examined within 10 seconds was recorded as a positive result.

3.4.4 Catalase test (Cappuccino, 2005)

Hydrogen peroxide (3%; v/v) was prepared. Approximately 0.2ml of hydrogen peroxide solution was placed on a clean glass slide. Presumptive actinobacteria colony was carefully

picked by using sterile toothpick. The colony was rubbed within the hydrogen peroxide solution. Vigorous bubbling observed within 10 seconds was recorded as positive result.

3.4.5 Analysis of cell wall composition by using thin layer chromatography (Lechevalier & Lechevalier, 1980)

Two loopful of actinobacterial cell were placed in 250µl of 6N hydrochloric acid (HCl). Actinobacterial cells were homogenized by vortexing. This mixture was autoclaved for 20 minutes at 121^{o}C, 15psi. After that, the mixture was centrifuged at 10,000rpm to 13,000rpm for 15 minutes. The supernatant was removed into a new eppendorf tube and kept at 100^{o}C to evaporate and concentrate it. The residue formed was resuspended in 30µl sterile distilled water and repeat this step for another time. After evaporate for second time, the residue was resuspended in 10µl sterile distilled water for spotting purpose. The cell extracts were spotted on TLC cellulose plate (No. 1.05552.0001, Merck) for 20 times. A standard, 2,6-diaminopimelic acid (0.01%; D1377, Sigma-Aldrich), was applied together with the samples. Mobile phase which consists of methanol, sterile distilled water, 6N HCl and pyridine was developed in the proportion of 80:26:4:10 (v/v), respectively. For visualization, cellulose plate was sprayed with 0.2% (w/v) ninhydrin in acetone and dryed in 80^{o}C for 5 to 7 minutes. A strain that has LL-DAP spot indicates that it is a streptomycete while a strain that has *meso*-DAP spot is a non-streptomycete such as mycobacterium or *Rhodococcus*.

3.4.6 Dereplication of strains

Crude genome DNA extraction

A loopful of pure actinobacterial culture was suspended into 150µl of Tris-EDTA (TE) buffer that containing glass beads. Lysozyme (5µl; 50mg/ml) was added into the suspension. Proteinase K (25µl; 20mg/ml) was added into the suspension. The suspension was mix by vortexing. After that, the suspension was incubated at 37^{o}C for 2 hours and then 56^{o}C for 3 hours or longer. Then the suspension was centrifuged at 13,500rpm for 10 minutes. The supernatant was transferred into new eppendorf tube. This supernatant was incubated at 75^{o}C for 20 minutes. Gel electrophoresis was performed to confirm the presence and quality of DNA in the supernatant.

The extracted DNA was kept at -20^{o}C. Extracted DNA was used for dereplication of strains by using BOXA1R primer.

PCR by using BOXA1R primer (Marques *et al.*, 2008)

A 25µl master mix with reagents as listed in Table 3.1 was prepared for BOX fingerprinting PCR.

Table 3.1: Recipe for master mix of BOX fingerprinting PCR.

	Final concentration	**1X reaction (µl)**
5X buffer	1X	5
25mM $MgCl_2$	1.5Mm	1.5
10mM dNTPs	0.2mM	0.5
20µM BOXAIR primer	2µM	2.5
5U/µl*Taq*	1.5U	0.075
Sterile distilled water		14.425
DNA	1	1

BOX fingerprinting PCR was carried out by using the BOXA1R primer. The condition for amplification consisted of an initial denaturation step of 7 minutes of 95^{o}C, followed by 30 cycles of denaturation at 94^{o}C for 1 minute, annealing at 53^{o}C for 1 minute, elongation at 65^{o}C for 8 minutes, and a final extension step at 65^{o}C for 15 minutes.

Gel electrophoresis

Agarose gel (2%, w/v) was prepared by mixing 0.8g of agarose into 40ml of Sodium Borate (SB) buffer. 100bp ladder and DNA samples were loaded into wells on the gel. Then gel electrophoresis was run with 100V for 120 minutes. Gels were stained in ethidium bromide (0.04%, v/v) before being visualised in a UV transilluminator.

Analysis of molecular fingerprinting using GelCompar II (Applied Maths, Belgium)

Molecular fingerprints, which were generated using BOXA1R primer, were analysed by using GelCompar II (Applied Maths, Belgium). Dendogram was generated by using the UPGMA method with the Jaccard model, 0.1% optimization and 0.7% tolerance.

3.4.7 Identification of strains

Genome DNA extraction using NucleoSpin® Tissue kit (Macherey-Nagel, Germany)

Genome DNA of presumptive actinobacteria strain was extracted by using the DNA extracton kit (MN, Germany) according to manufacturer's instructions as below.

Up to 1ml of bacterial culture can be used for the preparation depending on, for example, density of culture, culture medium, and bacterial strain. The bacterial culture was centrifuged up to 1ml for 5 minutes at 8000g. Supernatant was removed. The pellet was resuspended in 180µl Buffer T1 by pipetting up and down. Lysozyme (5µl; 20mg/ml) was

added into the sample and incubated for 1 hour. Then, Proteinase K (25µl) was added. Sample was vortexed vigorously and incubated at 56^{o}C until complete lysis is obtained (at least 1-3 hours). Vortex the sample occasionally during incubation, or use a shaking incubator. Samples can be incubated overnight as well. After that, the sample was vortexed . Buffer B3 (200µl) was added, vortexed vigorously and incubated at 70^{o}C for 10 minutes. Sample was vortexed briefly again. If insoluble particles are visible, sample was centrifuged for 5 minutes at high speed (i.e., 11000g) and supernatant was transferred to a new microcentrifuged tube. Ethanol (210µl; 96-100%) was added to the sample and vortex vigorously. For each sample, one NucleoSpin® Tissue Column was placed into a Collective Tube. The sample was applied to the column and centrifuged for 1 minute at 11000g. The flow-through was discarded and the column was placed back into the Collection Tube. If the sample is not drawn completely through the matrix, repeat the centrifugation step and discard the flow-through. Buffer BW (500µl) was added and centrifuged for 1 minute at 11000g. The flow-through was discarded and the column was placed back into the Collection Tube. Buffer B5 (600µl) was added to the column and centrifuged for 1 minute at 11000g. The flow-through was discarded and the column was placed back into the Collection Tube. The column was centrifuged for 1 minute at 11000g. The NucleoSpin® Tissue Column was placed into a 1.5ml microcentrifuge tube and prewarmed Buffer BE (100µl; 70^{o}C) was added. The column was incubated at room temperature for 1 minute and centrifuged for 1 minute at 11000g.

The extracted DNA was kept at -20^{o}C. Extracted DNA was used as template for the amplification of 16S rRNA gene.

Polymerase chain reaction (PCR) for 16S rRNA gene amplification (Tan *et al.*, 2006)

A 50µl master mix with reagents as listed in Table 3.2 was prepared for 16S rRNA gene amplification.

Table 3.2: Recipe for master mix by using PROMEGA reagent.

	Final concentration	**1X reaction (µl)**
5X buffer	1X	10
25mM $MgCl_2$	1.5mM	3
10mM dNTPs	0.2mM	1
Primer 27F	100mM	1
Primer 1492R	100mM	1
5U/µl *Taq*	1.25U	0.25
Sterile distilled water		32.75
DNA	1	1

16S rRNA genes was amplified with primer 27F and 1492R using the condition: hot start of 5 minutes at 95^{o}C followed by 35 cycles of denaturation at 94^{o}C for 45 seconds, annealing at 57^{o}C for 45 seconds, elongation at 72^{o}C for 1 minute, and a final extension step at 72^{o}C for 10 minutes.

Gel electrophoresis

Agarose gel (1%, w/v) was prepared by mixing 0.4g of agarose into 40ml of Sodium Borate (SB) buffer. 100bp ladder and nucleic acid sample were loaded into wells on the gel. Then gel electrophoresis was run with 100V for 45 minutes. Gels were stained in ethidium bromide (0.04%, v/v) before being visualised in a UV transilluminator.

16S rRNA gene sequencing

The amplified 16S rRNA gene product was sent to 1st BASE, which is a commercial servicing company, for sequencing. BigDye® Terminator v3.1 cycle sequencing kit chemistry (Applied Biosystems) is used by 1st BASE as their sequencing analyser.

Strains identification

Gene sequence of 16S rRNA of a strain was analysed by using EzTaxon (Chun *et al.*, 2007). From the results, strains with similarity values less 97% were treated as novel strains. Phylogenetic tree was generated by MEGA4 software (Tamura *et al.,* 2007) using Neighbor-Joining method, Jukes-Cantor model with 1000 bootstrap replicates, and Maximum Parsimony method with 1000 bootstrap replicates.

3.4.8 Relative enzyme activity (Bradner *et al.*, 1999)

Isolate was streaked in circular shape onto the PLA-emulsified plate (Figure 1) and incubated at 28^{o}C for 7 days. After the incubation period, the diameter of the colony and clear zone were measured and recorded. Relative enzyme activity of the particular isolate was calculated by using the formula as below.

$$\text{Relative enzyme activity} = \frac{\text{diameter of clear zone} - \text{diameter of colony}}{\text{diameter of colony}}$$

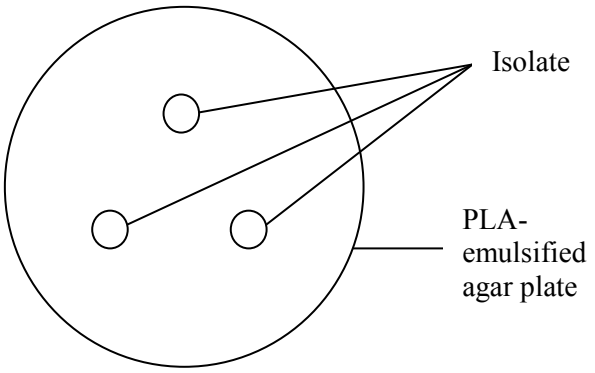


Figure 3.1: Streaking of isolate onto the PLA-emulsified plate for clear zone formation test.

3.4.9 Crude extraction of PLA-degrading enzyme (Nakamura *et al.*, 2001)

Strains P253 and P256 were grown in basal medium for 7 days. This seed culture was inoculated into PLA-emulsified broth and incubated for 7 days. After incubated for 7 days, broth culture was filtered using filter paper (F2042, CHM, Spain) and then 0.2μm filter to filter out the cell biomass. The remaining broth was treated as crude PLA-degrading enzyme.

3.4.10 Concentration of crude PLA-degrading enzyme (Wenk & Fernandis, 2007)

Saturated ammonium sulphate solution was prepared by dissolving 750g of ammonium sulphate into 1000ml of water. The solution was stirred at room temperature with magnetic stirrer for 15 minutes or until saturation. The clear supernatant solution was poured out after the undissolved solids settle on the bottom of the flask. Total volume of crude enzyme was measured and recorded. Saturated ammonium sulphate was added to the crude enzyme solution up to 20% concentration and the solution was stirred for 30 minutes. If any precipitate formed, the mixture was centrifuged at 2,000g for 20 minutes. The precipitate

was collected by carefully remove the supernatant. The precipitate was suspended in distilled water until desirable volume. Same steps were applied to the remaining supernatant with increasing concentration of saturated ammonium sulphate, that is, 40%, 60%, and 80%. The concentration of saturated ammonium sulphate for each precipitate formed was recorded. All the steps that involve enzyme solution must be done in 0-4^{o}C. The suspension was dialysed against several hundred volumes of water for 16 hours at 4^{o}C.

3.4.11 SDS PAGE (Laemmli, 1970)

The glass plate sandwich of the electrophoresis apparatus (BioRad) was assembled according to the manufacturer's instruction using two clean glass plates and two 0.75mm spacers. The sandwich was locked to the casting stand. The separating gel solution was prepared as Table 3.3 and degassed using a rubber-stopper 25ml Erlenmeyer side arm flask connected with vacuum tubing to a vacuum pump with a cold trap. After adding a specific amount of 10% ammonium persulfate and tetramethylethylenediamine (TEMED) to the degassed solution, it was stirred gently to mix. Using a Pasteur pipette, the separating gel solution was applied to the sandwich along an edge of one of the spacers until the height of the solution between the glass plates was around 11cm. Using another Pasteur pipette, the top of the gel was slowly covered with a layer (around 1cm thick) of H_2O-saturated isobutyl alcohol by gently layering the isobutyl alcohol against the edge of one and then the other of the spacers. The gel was allowed to be polymerized for 30 to 60 minutes at room temperature. The layer of H_2O-saturated isobutyl alcohol was poured off and rinsed with 1X Tris.Cl/SDS, pH8.8. The stacking gel solution was prepared according to Table 3.3. Using a Pasteur pipette, the stacking gel was allowed to trickle slowly into the center of the sandwich along an edge of one of the spacers until the height of the solution in the

sandwich was around 1cm from the top of the plate. A 0.75mm Teflon comb was inserted into the layer of the stacking gel solution. If necessary, additional stacking gel was added to fill the spaces in the comb completely. The stacking gel solution was allowed to polymerize for 30 to 45 minutes at room temperature. A portion of the protein sample was diluted 1:1 (v/v) with 2X SDS sample buffer and heated 3 to 5 minutes at 100^{o}C in a sealed screw-cap microcentrifuge tube. If the sample is a precipitated protein pellet, the protein was dissolved in 50 to 100µl of 1X SDS sample buffer and boiled 3 to 5 minutes at 100^{o}C. The Teflon comb was removed carefully without tearing the edge of the polyacrylamide wells. After the comb was removed, the wells were rinsed with 1X SDS electrophoresis buffer. Using a Pasteur pipette, the wells were filled with 1X SDS electrophoresis buffer. The gel sandwich was attached to upper buffer chamber using manufacturer's instructions. The lower buffer chamber was filled with the recommended amount of 1X SDS electrophoresis buffer. Sandwich that attached to upper buffer chamber was placed into lower buffer chamber. Using a 25 to 100µl syringe with a flat-tipped needle, the protein sample was loaded into one or more wells by carefully applying the sample as a thin layer at the bottom of the wells. Control well was loaded with molecular weight standards. An equal volume of 1X SDS sample buffer was added to any empty wells to prevent spreading of adjoining lanes. The remainder of the upper buffer chamber was filled with additional 1X SDS electrophoresis buffer so that the upper platinum electrode is completely covered. This step was done slowly so that samples were not swept into adjacent wells. The power supply was connected to the cell and run at 10mA of constant current for a slap gel 0.75mm thick, until the bromophenol blue tracking dye enters the separating gel. Then the current was increased to 15mA. After the bromophenol blue tracking dye has reached the bottom of the separating gel, the power supply was disconnected. The electrode buffer was discarded and the upper buffer chamber with the attached gel sandwich was removed. The gel was

oriented so that the order of the sample wells was known. The sandwich was removed from the upper buffer chamber and layered on a sheet of absorbent paper or paper towels. One of the spacers was slided halfway from the edge of the sandwich along its entire length. The exposed spacer was used as a lever to pry opens the glass plate, exposing the gel. The gel was removed carefully from the lower plate. A small triangle from one corner of the gel was cut off so that the lane orientation was not lost during staining and drying.

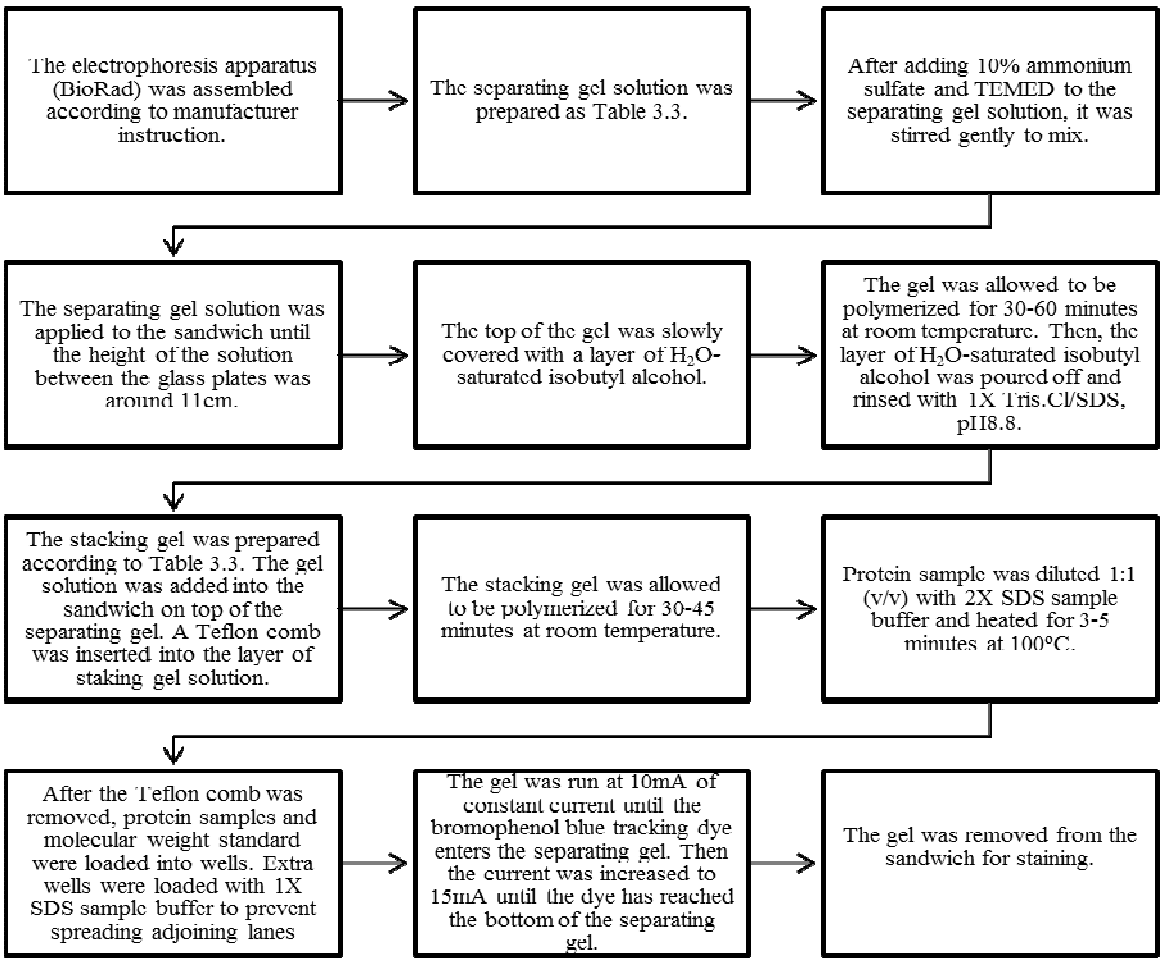


Figure 3.2: Flow chart for SDS PAGE procedure.

Table 3.3: Recipe for polyacrylamide separating and stacking gel[a].

Preparation of separating gel

Stock solution[b]	**Final acrylamide concentration in separaring gel (%)**[c]	
	10	15
30% acrylamide/0.8% bisacrylamide	5.00	7.50
4X Tris.Cl/SDS, pH8.8	3.75	3.75
H_2O	6.25	3.75
10% (w/v) ammonium persulphate[d]	0.05	0.05
TEMED	0.01	0.01

Preparation of separating gel

In a 25ml side-arm flask, 30% acrylamide/0.8% bis acrylamide solution, 4X Tris.Cl/SDS, pH8.8, and H_2O were mixed and degassed under vacuum about 5 minutes. 10% ammonium persulfate and TEMED were added. The solution was swirled gently to mix.

Preparation of stacking gel

In a 25ml side-arm flask, 0.65ml of 30% acrylamide/0.8% bis acrylamide solution, 1,25ml of 4X Tris.Cl/SDS, pH6.8, and 3.05ml of H_2O were mixed and degassed under vacuum for 10 to 15 minutes. 25µl of 10% ammonium persulfate and 5µl TEMED were added. The solution was swirled gently to mix.

[a]The recipes produce 15ml of separating gel and 5ml of stacking gel, which are adequate for a gel of dimensions 0.75mm × 14cm × 14cm. The recipes are based on the SDS (denaturing) discontinuous buffer system of Laemmli (1970).

[b]All reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.

[c]Units of numbers in table body are milliliters. The desired percentage of acrylamide in the separating gel depends on the molecular size of the protein being separated. See annotation to step 3, Basic Protocol 1.

[d]Best to prepare fresh.

3.4.12 Determination of PLA-degrading enzyme's molecular weight by zymography

The crude enzyme was run with SDS-PAGE. The gel was soaked in 2.5% (v/v) Triton X-100 for 1 hour at room temperature. A PLA agar indicator gel plate (Appendix A, page 58) was prepared to detect the molecular weight of PLA-degrading enzyme which responsible to the PLA-degrading activity. After 1 hour, the SDS-PAGE gel was washed with distilled water and laid on the PLA agar indicator gel plate. The indicator gel plate was incubated at 30^{o}C overnight in humid condition to ensure the gel wasn't dried up. The position of clearance zone formed on the indicator gel plate was compared with the protein molecular weight ladder.

CHAPTER 4

4.0 Results

4.1 Pretreatment of soil sample

PLA degradation in the PLA-emulsified plate was indicated by the formation of yellow zone on PLA-emulsified medium. Shorter incubation period was needed for microorganisms in the pretreated soil to show PLA degradation activity compared to those in non-pretreated soil. However, the colony forming unit per milliliter (cfu/ml) and also the number of presumptive actinobacteria isolated from the pretreated soil was lower than the non-pretreated soil (Table 4.1). Most of the isolates isolated from pretreated DS2D soil sample were Gram negative bacteria.

Table 4.1: Comparison of the effects of soil pretreatment by using PLA film.

	cfu/ml	**Incubation period**	**Number of isolated strain (presumptive actinobacteria)**
Pretreated DS2D	3.00×10^5	2 weeks	15
Non-pretreated DS2D	1.51×10^6	5 weeks	35

4.2 Isolation of PLA-degrading actinobacteria

Bromocresol purple, a pH indicator, was added into the PLA-emulsified medium to act as an indicator for the PLA degradation activity. The colour of the medium (at pH7) with bromocresol purple supposed to be in purple colour. However, after the agar medium was poured into the Petri dish, the colour of the medium was diluted into green in colour (Figure 4.1). After incubation period, agar plates without PLA degradation activity were

remained green in colour while those with degradation activity were changed into yellow in colour. Strains were only picked and purified from those agar plates which turn yellow. A total of 251 strains were isolated in this study (Table 4.2).

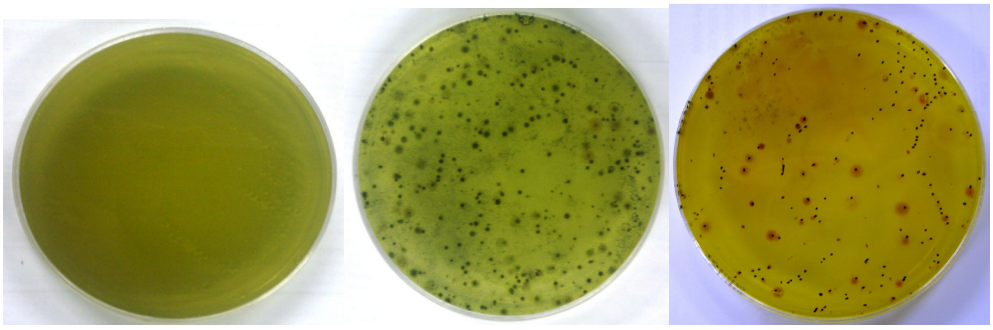


Figure 4.1: PLA-emulsified plate with and without PLA degradation activity. From left to right: fresh PLA-emulsified agar plate; PLA-emulsified agar plate without PLA degradation activity; PLA-emulsified agar plate with PLA degradation

Table 4.2: Number of isolated strain from each soil sample. There was no presumptive actinobacterial strain isolated from the landfill soil samples DS1C, DS1D, DS2C, DS2E, DS3A, DS3B, DS3D, DS3E, DS4A, DS4B, DS4C, DS4D, DS5A, DS5C, DS5D, DS5E, and the non-contaminated soils SP1, SP2, SP4 and SP5.

Soil sample	Source of soil sample	Number of strain
DS1A	Landfill site	2
DS1B	Landfill site	5
DS1E	Landfill site	19
DS2A	Landfill site	94
DS2B	Landfill site	36
DS2D	Landfill site	50
DS3C	Landfill site	17
DS4E	Landfill site	1
DS5B	Landfill site	11
SP3	Non-contaminated camp site	16

4.3 Characterization of isolated strains

4.3.1 Grouping of isolated strains according to phenotypic characteristics

4.3.1.1 Grouping based on cell morphology

Generally, there were several types of cell morphology observed through light microscope, which are rod, coccus, oval, filamentous and fragmented filament. For filamentous strains, they may or may not have spores, if they have, the spores can be either rod-shaped or coccus. Figure 4.2 are the photos of each type of cell morphology observed under light microscope after gram staining procedure.

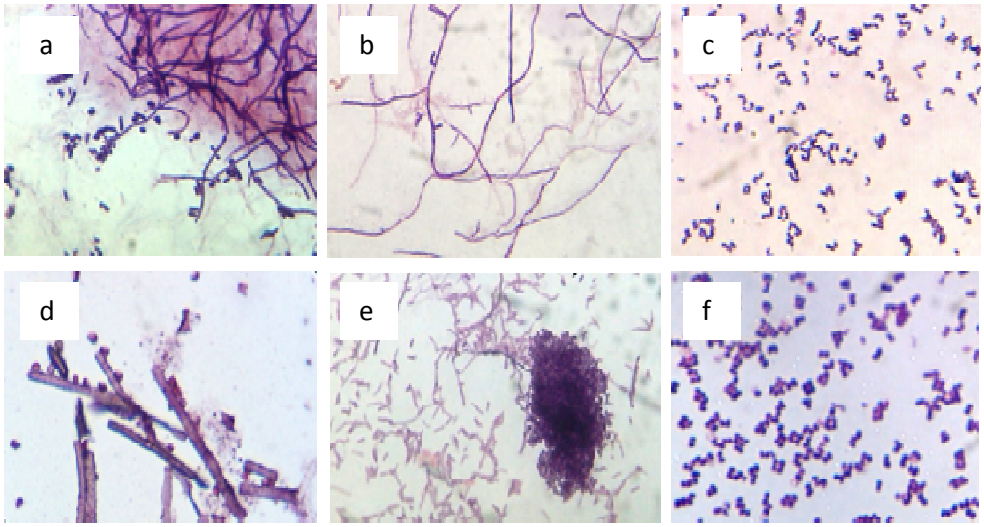


Figure 4.2: Different types of cell morphology of isolated strains. (a) strain P6 which is filamentous with rod-shape spores; (b) strain P27 which is filamentous in cell morphology; (c) strain P251 which is rod-shape; (d) strain P279 which has thick filament with spores on it; (e) strain P141 which is filamentous with coccus spores; (f) strain P252 which is coccus in shape.

Three main types of cell morphology which were observed through light microscope are rod-shaped, filamentous and also fragmented filament (Figure 4.3). Cell morphology grouping with strain's details can be found in Table B.1 in Appendix B (page 59).

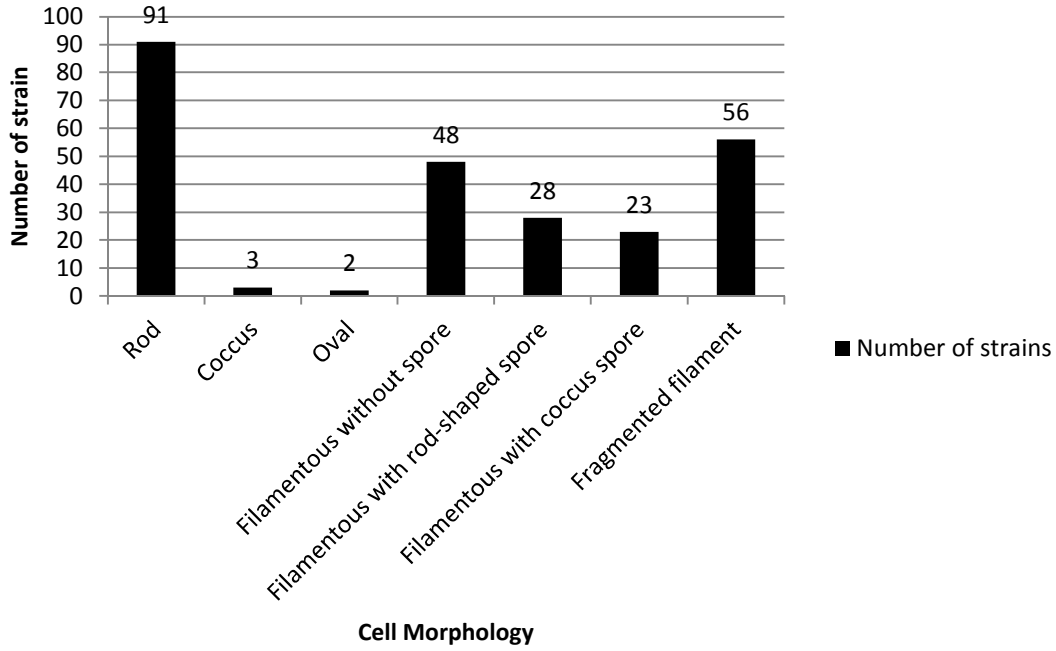


Figure 4.3: Number of strains for each type of cell morphology.

4.3.1.2 Colour grouping

All the 251 strains were grouped into a total of 50 colour groups based on the colour of their aerial mycelia, substrate mycelia and pigmentation. All the strains were grown for 7 days at 28^{o}C and compared for their colour (Table 4.3).

Table 4.3: Colour grouping of isolated strains based on colour of their aerial mycelia, substrate mycelia and pigmentation. Colour was determined by using National Bureau of Standards Colour Name Charts.*

No.	Colour group			No. of strains
	Aerial mycelia	Substrate mycelia	Pigmentation	
1	Vivid yellow	Vivid yellow	No	18
2	Vivid orange	Vivid orange	No	42
3	Vivid orange yellow	Deep yellow	No	9
4	Vivid orange yellow	Vivid orange yellow	No	4
5	Strong yellow	Strong yellow	No	2
6	Vivid greenish yellow	Vivid greenish yellow	No	6
7	Deep orange yellow	Deep orange yellow	No	1
8	Brilliant greenish yellow	Strong greenish yellow	No	3
9	Brilliant yellow green	Brilliant yellow green	No	42
10	Light greenish yellow	Brilliant greenish yellow	No	50
11	Pale orange yellow	Brilliant greenish yellow	No	2
12	White	Strong greenish yellow	No	6
13	Light gray	Vivid yellow	Deep yellow	2
14	Light gray	Vivid yellow	No	3
15	White	Vivid yellow	No	1
16	Light grayish olive	Dark brown	No	1
17	Light gray	Deep yellow	Vivid greenish yellow	1
18	Grayish olive green	Deep orange yellow	Strong greenish yellow	2
19	Light grayish brown	Strong greenish yellow	No	1
20	Light gray	Strong greenish yellow	No	1
21	White	Deep orange yellow	Deep yellow	1
22	White	Deep orange yellow	Vivid greenish yellow	1
23	White	Deep orange yellow	Dark orange yellow	1
24	White	Deep orange yellow	No	1
25	White	Deep yellowish pink	Strong greenish yellow	1
26	Light grayish brown	Deep orange yellow	No	1
27	White	Moderate olive green	No	1
28	Light gray	Deep yellow	No	1
29	Grayish olive green	Deep yellow	Moderate olive	1
30	Reddish black	Strong greenish yellow	No	1
31	Light gray	Reddish black	Moderate olive brown	1
32	Vivid yellow green	Deep greenish yellow	No	1
33	Vivid reddish orange	Vivid reddish orange	No	1
34	Deep reddish orange	Deep reddish orange	No	3
35	Black	Deep orange	Moderate yellow green	4
36	Black	Deep orange	No	6
37	Black	Black	No	2
38	Black	Black	Dark olive green	1

'Table 4.3, continue'

39	Reddish black	Brownish black	Moderate olive brown	9
40	Reddish black	Brownish black	No	3
41	Reddish black	Brownish black	Deep yellow	1
42	Reddish black	Brownish black	Light olive	1
43	White	Brownish black	Moderate olive brown	1
44	Light yellow green	Light yellow green	No	1
45	Brilliant greenish yellow	Brilliant greenish yellow	No	2
46	Olive black	Bluish black	Bluish white	3
47	Light orange	Deep yellowish pink	Strong yellow	1
48	Deep yellowish pink	Deep yellowish pink	Brilliant yellow green	1
49	Dark olive green	Dark olive green	Dark olive green	1
50	Deep orange	Deep orange	Moderate yellow green	1

*Colour grouping with strain's details can be found in Table B.2 in Appendix B (Page 60).

4.3.2 Oxidase test and catalase test

All isolated strains showed negative oxidase reaction except for strains P208, P226, P230, P202, P147, P148, P149, P146, P145, P69, P204, P56, P166, P236, P84, P85, P89, P109, P120, P121, P123, P122 and P125.

All isolated strains showed positive catalase reaction except for strains P141, P200, P227, P229, P217, P41, P79, P80, P71, P36, P104, and P131.

4.3.3 Novel strains identification

Two of the isolated strains, P86 and P224, were identified as novel strains through their 16S rRNA gene sequences. Strain P86 has 97.77% similarity with *Agromyces humatus* CD5^{T} with 30 nucleotides differences over 1344 nucleotides while strain P224 has 98.19% similarity with *Mycobacterium tokaiense* ATCC 27282^{T} with 24 nucleotides differences over 1328 nucleotides. Phylogenetic trees for strains P86, P224 and their closely related

strains were generated by using MEGA 4 software (Figures 4.4, 4.5, 4.6 and 4.7) (Tamura *et al.*, 2007).

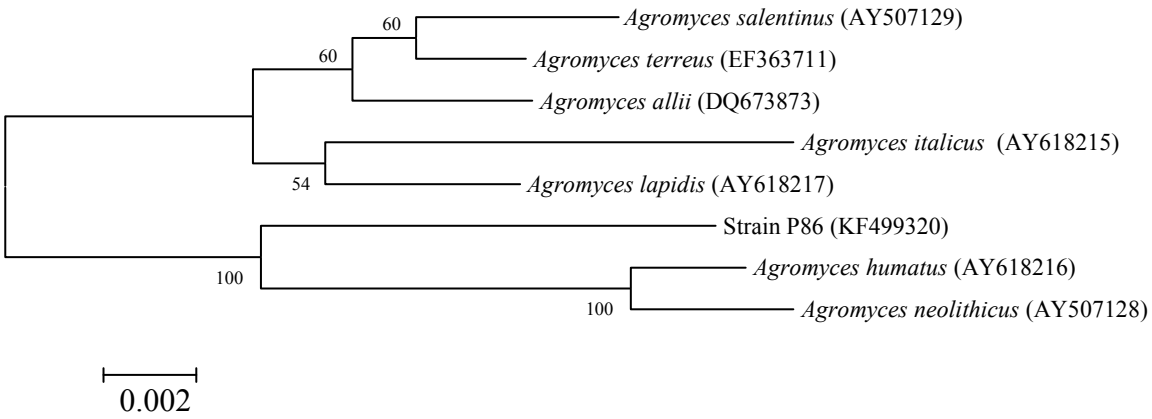


Figure 4.4: Phylogenetic tree of strain P86 and its closely related strains. This phylogenetic tree was generated by Mega4 software using Neighbor-Joining method, Jukes-Cantor model, and with 1000 bootstrap replicates.

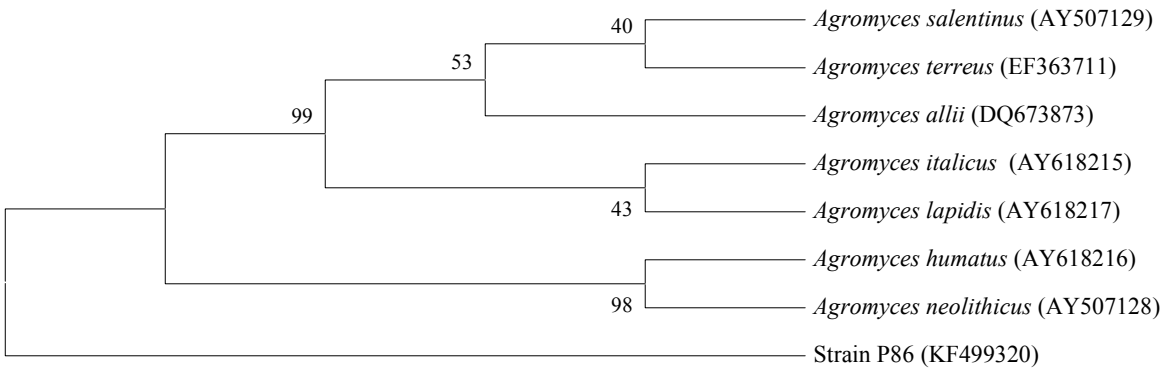


Figure 4.5: Phylogenetic tree of strain P86 and its closely related strains. This phylogenetic tree was generated by Mega4 software using Maximum Parsimony method with 1000 bootstrap replicates.

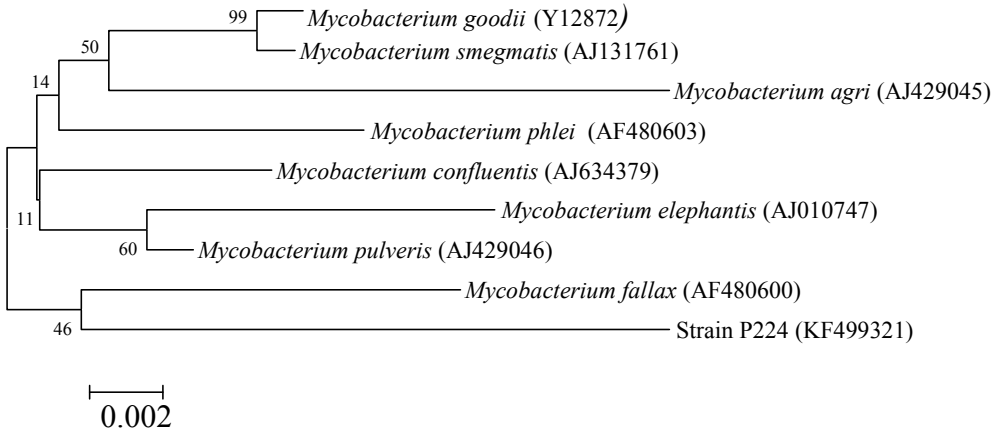


Figure 4.6: Phylogenetic tree of strain P224 and its closely related strains. This phylogenetic tree was generated by Mega4 software using Neighbor-Joining method, Jukes-Cantor model, and with 1000 bootstrap replicates.

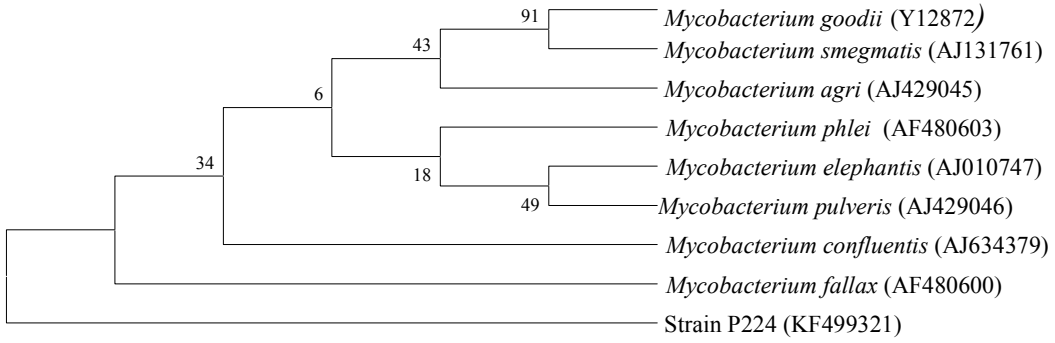


Figure 4.7: Phylogenetic tree of strain P224 and its closely related strains. This phylogenetic tree was generated by Mega4 software using Maximum Parsimony method with 1000 bootstrap replicates.

4.3.4 PLA degradation by strains

Clear zone formation test was performed onto all the 251 strains to confirm on their PLA-degrading ability. There were only 235 strains confirmed to have the ability to degrade PLA. The remaining 16 strains were not able to form a clear zone on the PLA-emulsified agar plate, which indicate that they were not capable in degrading PLA.

4.3.4.1 Relative enzyme activity of PLA-degrading strains

Relative enzyme activity of the 235 PLA-degrading strains on both low and high molecular weight PLA was determined. Strains were grouped according to their relative enzyme activity and a chart was generated according to that (Figure 4.8). The relative enzyme activity of all the strains was following a normal distribution pattern, that is, there are more strains with average relative activity.

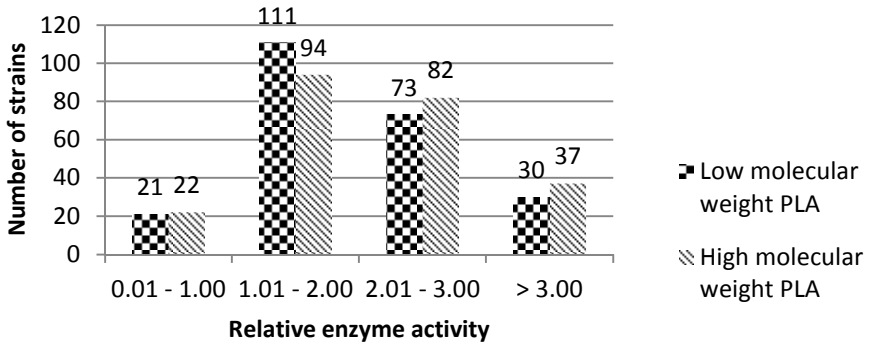


Figure 4.8: Number of strains in each group of relative enzyme activity.

4.4 Crude enzyme extraction

Strains P253 and P256 were chosen for crude enzyme extraction because these two strains had relative enzyme activity higher than 3.00. After the cell biomass was filtered out, the broth was clear in colour (Figure 4.9) instead of the milky white colour of the PLA-emulsified broth. The clear colour of the broths indicates that the PLA present in the broth was either degraded or assimilated by strains P253 and P256. The broth without cell biomass was treated as crude enzyme and was used for further tests.

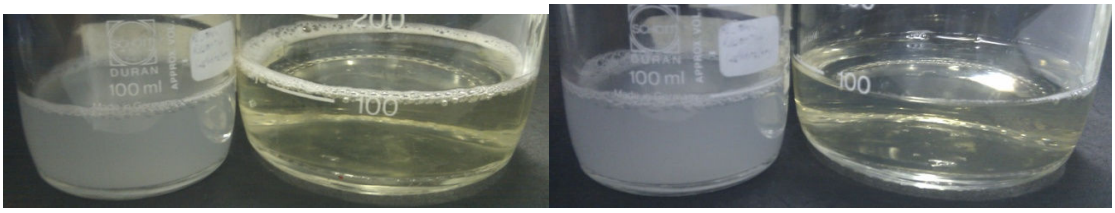


Figure 4.9: PLA-emulsified broth and filtered broth of strain P253 (left); PLA-emulsified broth and filtered broth of strain P256 (right).

4.5 Determination of PLA-degrading enzyme's molecular weight

Crude enzymes were run by using SDS-PAGE to visualize the extracted enzymes (Figure 4.10a). After that, the denatured enzymes in the PAGE were renatured by washing with Triton-X 100 before the PAGE gel was overlaid onto the PLA indicator gel plate for PLA-degrading enzyme(s) detection (Figure 4.10b).

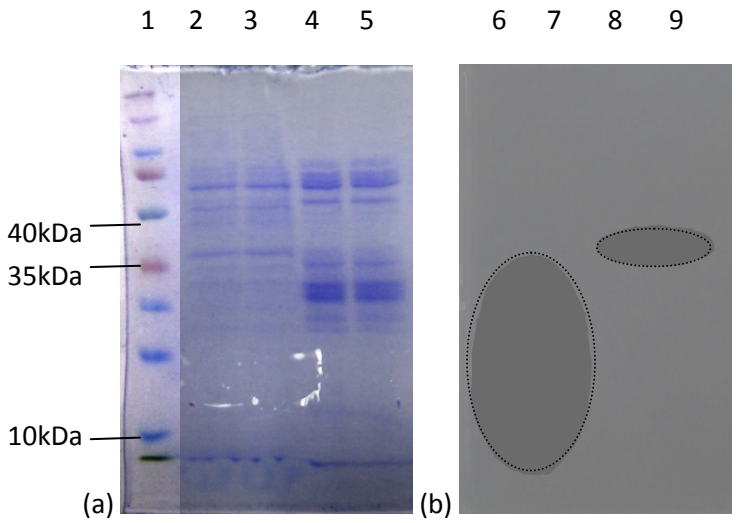


Figure 4.10: SDS PAGE and PLA gel indicator plate of zymography. (a) Crude enzymes of strain P253 (lane 2 and 3) and strain P256 (lane 4 and 5) on Commasie Blue stained SDS-PAGE. (b) PLA indicator gel with clearing zones (dash line indicating the clearing zone).

CHAPTER 5

5.0 Discussions

5.1 Isolation of PLA-degrading actinobacteria

In this study, there were two groups of soil samples, which were used to isolated PLA-degrading actinobacteria. First group of soil samples (labeled as "DS") were collected from landfill site in Perak, Malaysia. This is because we believe that in a landfill site, which has large number of buried plastics, will have higher number of microorganisms that could degrade plastic and utilize the degradation products as carbon source compared to site which is not contaminated with plastic materials. Another group of soil samples (labeled as "SP") were obtained from a camp site on Gunung Jerai, Gurun, where it is free from plastic wastes contamination. This site was chosen randomly to check whether PLA-degrading actinobacteria are commonly present in any conditions or places. From the results, a total of 16 strains were isolated from soil sample SP3 while there were 235 strains isolated from landfill site. This shows that PLA-degrading actinobacteria are also present in non-polluted places. However, their number is much lower than those present in plastic wastes polluted places. This may indicate that the carbon metabolism mechanism for those actinobacteria present in plastic wastes contaminated sites is shifting from normal organic carbon sources to plastic wastes over long period of time after the organic carbon sources become depleted. Surface soils were collected and used for isolation of PLA-degrading actinobacteria because actinobacteria is aerobic bacteria.

Soil sample DS2D was randomly chosen and used to check the effects of pretreatment on the isolation, especially the effect on the number of PLA degrader isolated and the incubation period. Isolation was performed using both pretreated and non-pretreated DS2D

soil sample. During the pretreatment step, PLA degraders, including both Gram positive and Gram negative bacteria, increased in numbers. Therefore, shorter incubation period was needed for the PLA degraders in the pretreated DS2D soil sample to grow, degrade the PLA in the isolation medium and change the medium into yellow colour compared to non-pretreated DS2D soil sample (Table 4.1, page 31). However, at the second week of incubation where the isolation medium started to turn yellow, colony of most of the slow growers like actinobacteria were not formed yet or they were probably too small and were overlooked when counted by naked eye. This is the reason why the cfu/ml count of pretreated DS2D soil sample was lower than non-pretreated DS2D soil sample. On the other hand, non-pretreated DS2D soil sample required longer incubation period for the yellow zone to be observed and has higher cfu/ml count because the number of PLA degrader in the non-pretreated DS2D soil sample was lower and most of the slow growers start to form colony after a longer incubation period. Therefore, there was no pretreatment step carried out for other soil samples since our aim is to isolate more actinobacteria so that more PLA-degrading actinobacteria can be discovered.

In this study, a total of 251 presumptive actinobacteria strains were isolated from 10 different soil samples. Throughout the whole isolation process, modified PLA-emulsified medium was used as selective isolation medium (Sukkhum *et al.*, 2009). This medium consists of mineral salts, yeast extract, PLA-powder, polysorbate-80 and bromocresol purple. There are only minimum amount of mineral salts and yeast extract present in this medium to fulfill the basic requirement of microorganisms' growth. Hence, PLA will be the sole carbon source in this medium, which means, only microorganism that can degrade and utilize PLA as their carbon source can grow on this medium. Besides, 1% of polysorbate 80 was added into the medium as emulsifier so that the PLA can disperse

evenly in the medium. Moreover, polysorbate 80 also has the ability to inhibit the biofilm formation by both Gram positive and Gram negative bacteria, therefore, has a positive effect in the isolation of slow grower, actinobacteria (Toutain-Kidd *et al.*, 2009). Other than this, 0.01% of bromocresol purple, which is a pH indicator, was added into the medium to detect the degradation of the PLA. Bromocresol purple will change to yellow colour when the pH drops to below 5.2. If the PLA in the PLA-emulsified agar plate is degraded, that particular agar plate will change to yellow colour (Figure 4.1, page 32). This is due to the fact that PLA-oligomer and lactic acid will be produced as PLA degradation product (Tokiwa & Jarerat, 2004). All the strains were picked and purified from the isolation plate once the plate change to yellow colour. After they were purified on the ISP2 medium, Gram reaction of all isolated strains were determined. Only strains that gave Gram positive reaction were kept for further tests since actinobacteria are Gram positive bacteria. Number of presumptive actinobacteria strains isolated from each soil sample was shown in Table 4.2 (page 32). However, non-PLA-degrader may also grow in the yellow zone coincidently especially actinobacteria, which has the ability to survive in the environment with low nutrient content. Therefore, isolated strains were subject to clear zone formation test to confirm their ability to degrade PLA. In this clear zone formation test, clear zone will be formed around the strain's colony if the strain has the ability to degrade PLA. Two types of antifungals, which are nystatin and cyclohexamide, were added into the isolation medium to exclude or reduce the number of fungus in the isolation.

5.2 Characterization and grouping of isolated strains

All isolated strains were grouped based on their phenotypic and physiological characteristics, which are, colour of their aerial mycelia, substrate mycelia and pigmentation, gram reaction, cell morphology, and their ability to produce enzyme cytochrome oxidases and catalase. A dendrogram (Figure 5.1) was generated based on these characteristics by using PAST software (Hammer *et al.*, 2001). Isolated strains were clustered together based on similarities in their characteristics, which means, strains with similar characteristics were joined into clusters and most similar clusters were joined into superclusters and the process go on until all the clusters are joined.

From Figure 5.1, the dendrogram can be separated into 4 main clusters, where the strains were listed in Table 5.1. For cluster 1, it consists of strains that are having characteristics similar to micromonospora, which is, their colonies were completely covered by mucoid, black mass of spores. Strains in cluster 2 were having characteristics resemble to mycobacterium, which are, they all have whitish or cream-coloured colonies and their cells are fragmented or rod-shaped. While cluster 3 consists of strains with bright yellow, orange or red coloured colonies, and their cells were in rod, fragmented filament or filamentous shape. These characteristics were resembled to those agromyces, rhodococcus, and also mycobacterium. The last cluster, cluster 4, consists of strains varies in the colour of their aerial mycelia and substrate mycelia, some of them produce diffusible pigment, and they sporulate. These characteristics suggest that they are streptomycetes.

Then, a few strains from each cluster were selected for identification through their partial 16S rRNA gene sequence to confirm the clustering result generated using phenotypic and physiological characteristics. Identification results by using partial 16S rRNA gene sequence were consistent with the clustering result (Table 5.1).

After that, strains from cluster 3 were further grouped based on their molecular characteristics that is, their molecular fingerprint, in order to have a finer grouping of the strains (Figure 5.6). This is because cluster 3 consists of genera *Agromyces, Rhodococcus* and *Mycobacterium* which cannot be separate into distinct groups based on limited phenotypic and physiological characteristics. Strains from same genus were grouped together based on their molecular fingerprints.

Previous studies showed that almost all PLA degraders are members of *Amycolatopsis* (Pranamuda & Tokiwa, 1999). However based on the grouping results in this study, members from other genera, which are *Agromyces, Rhodococcus, Micromonospora, Mycobacteria* and *Streptomyces*, were shown to have the ability to degrade PLA too.

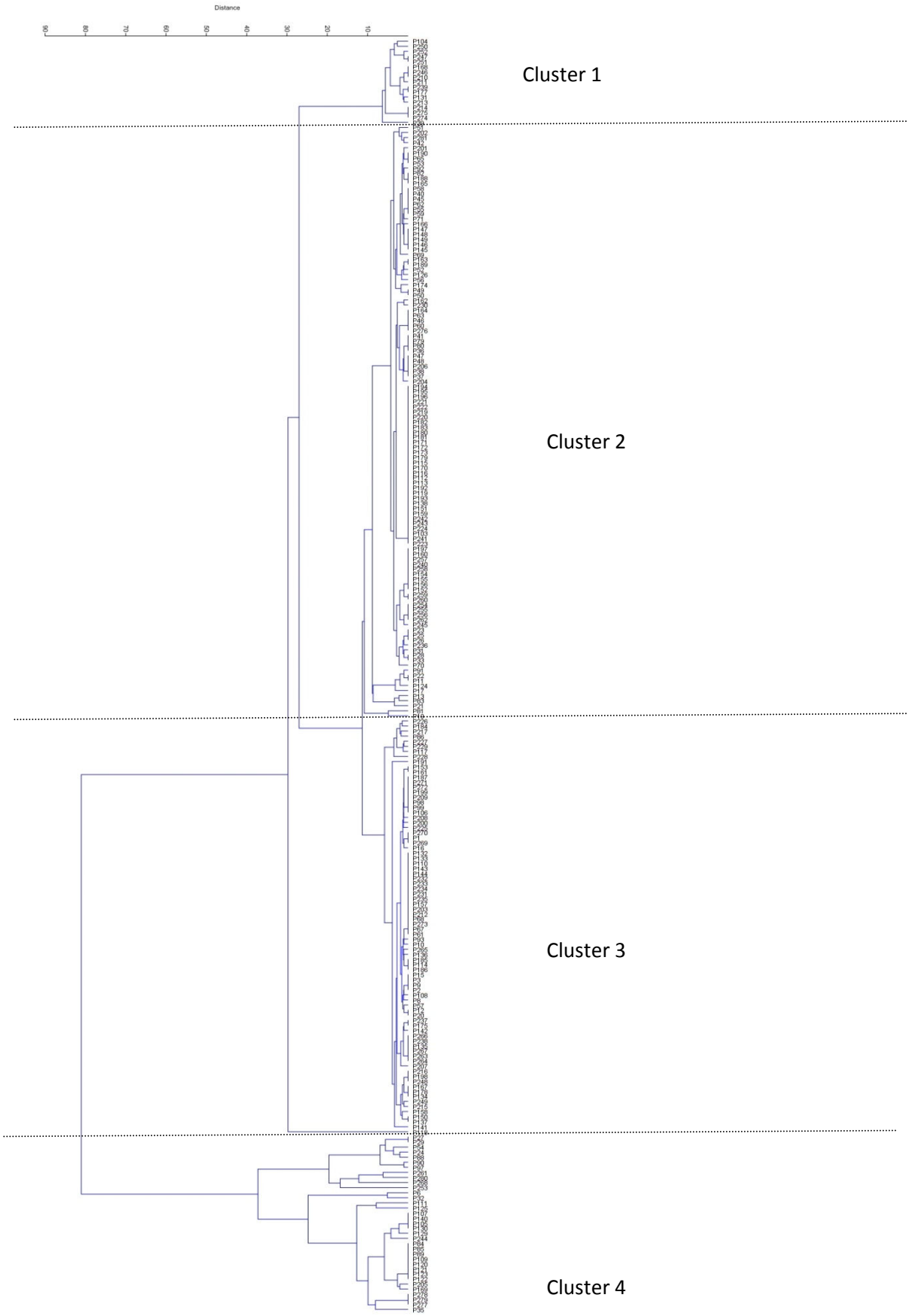


Figure 5.1: Dendogram generated by PAST software according to phenotypic and physiological characteristics.

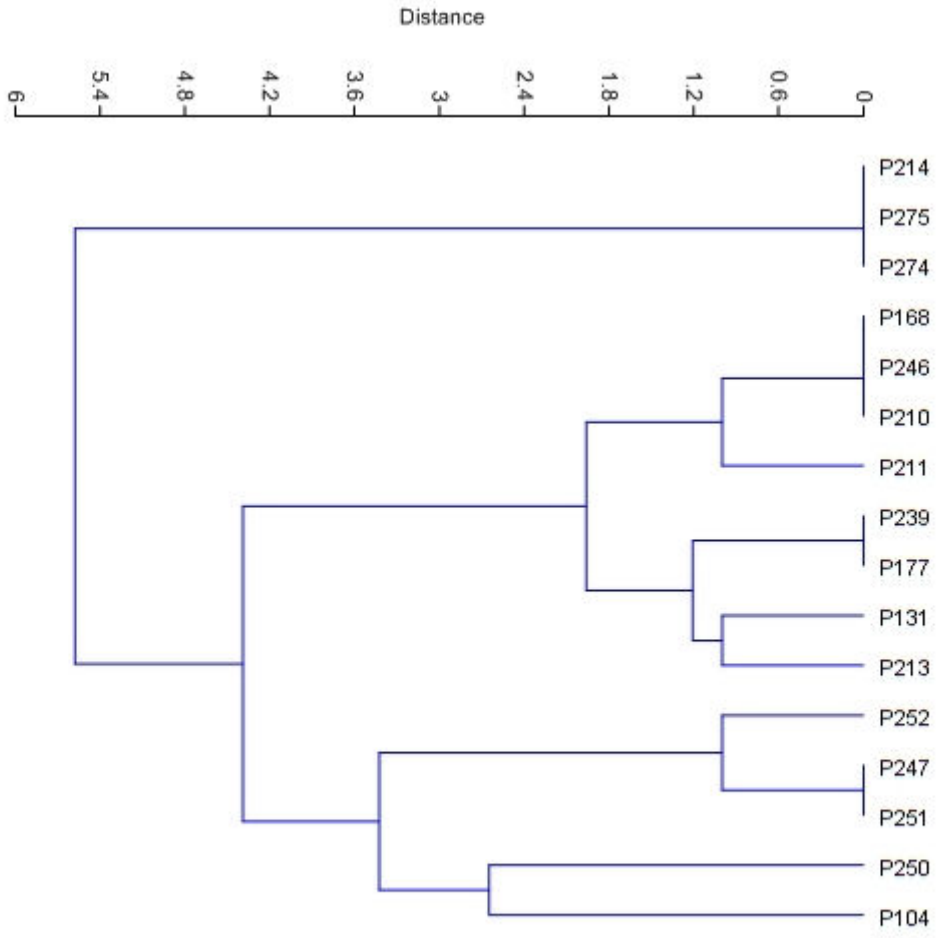


Figure 5.2: Cluster 1 which consists of strains from genus *Micromonospora*.

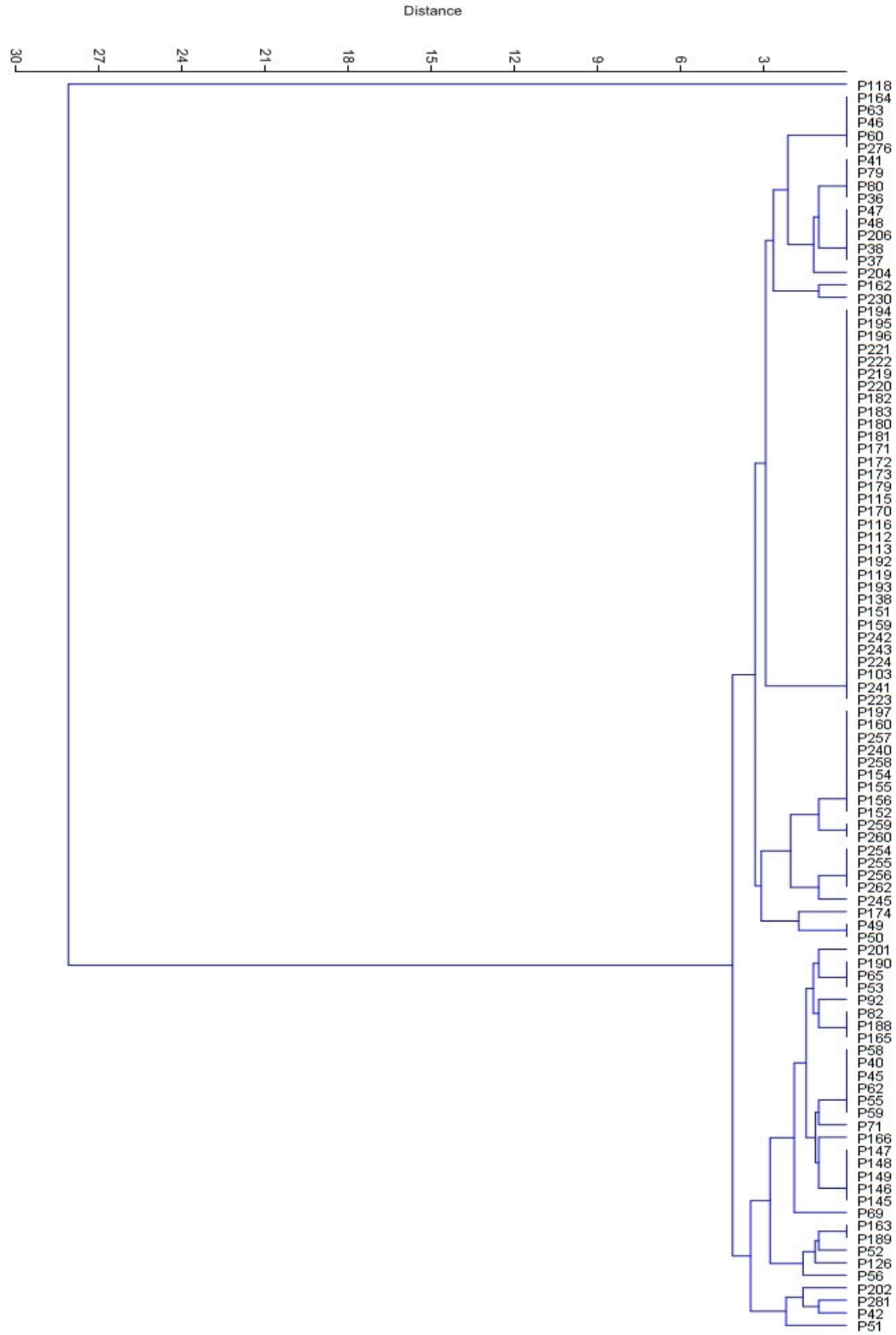


Figure 5.3: Cluster 2 which consists of strains from genus *Mycobacterium*.

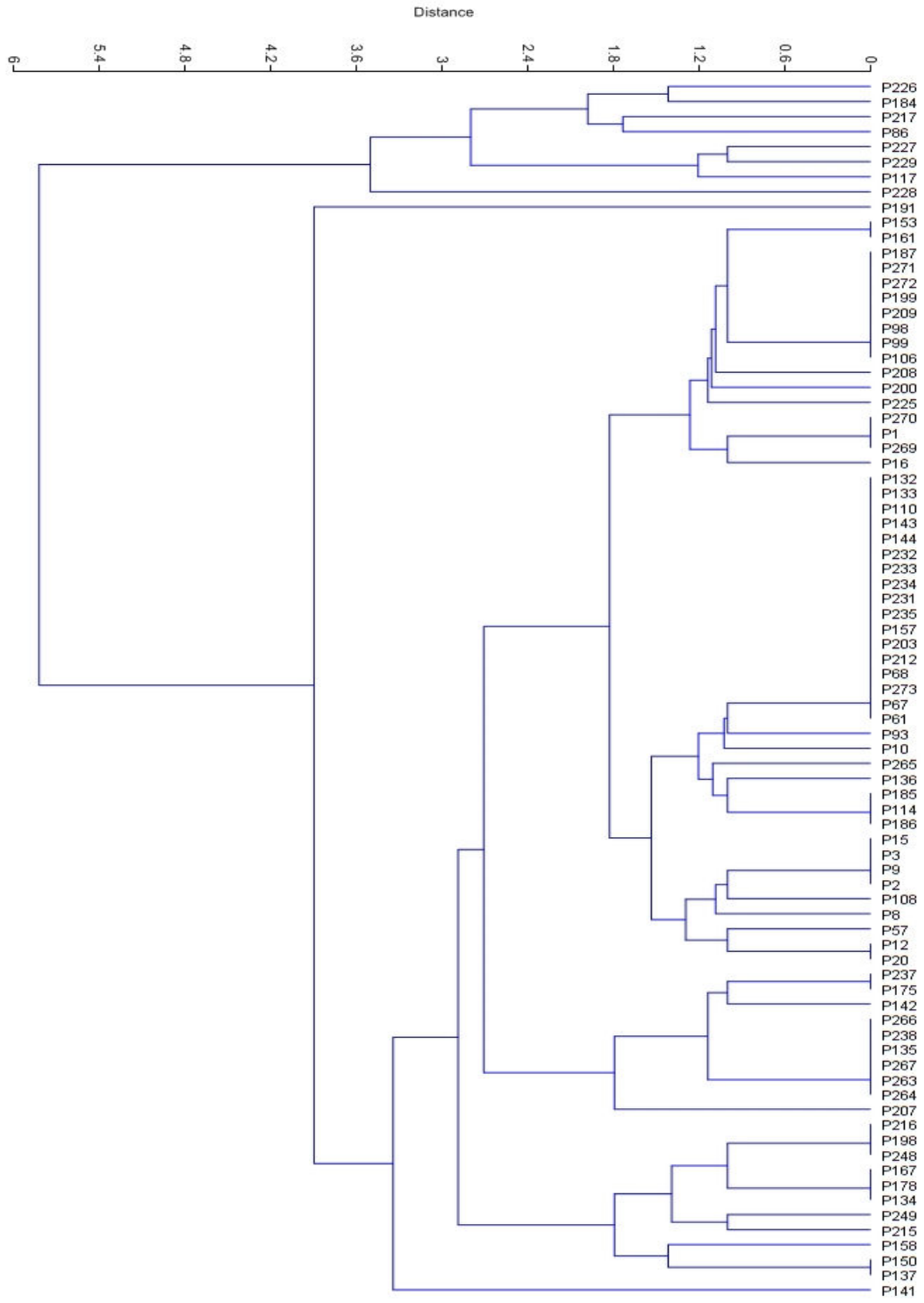


Figure 5.4: Cluster 3 which consists of strains from genera *Agromyces, Rhodococcus* and *Mycobacterium*.

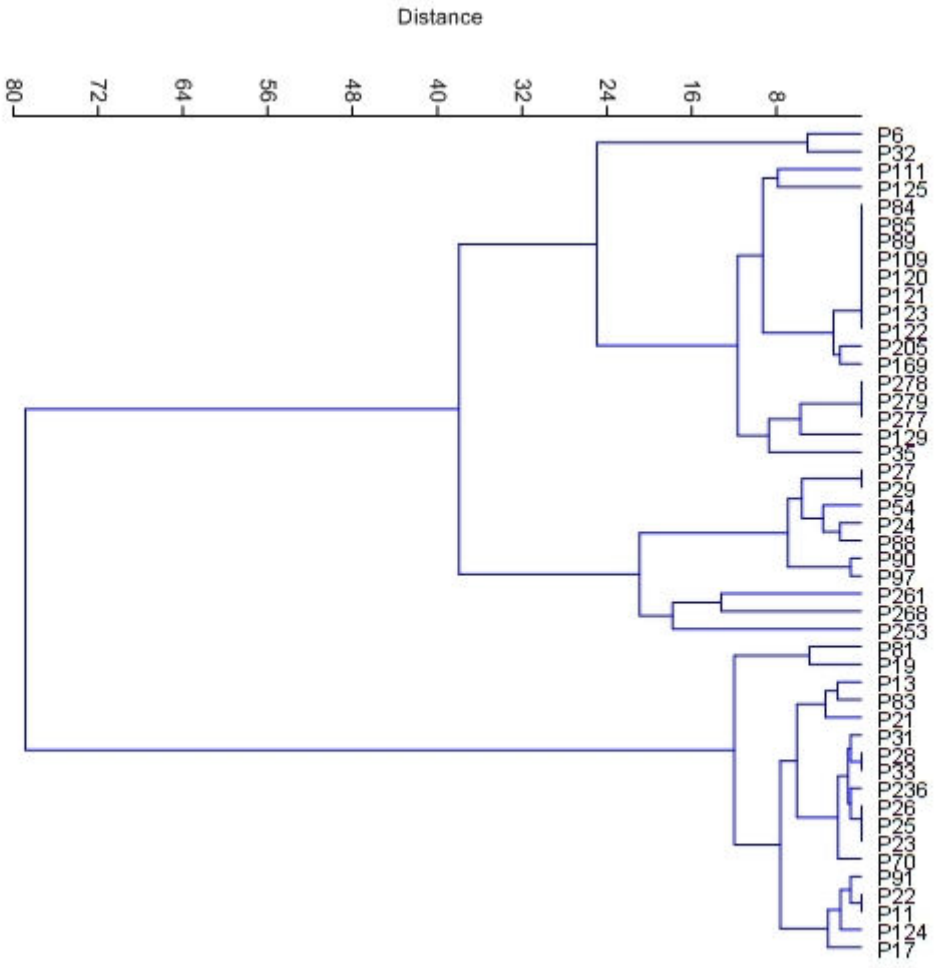


Figure 5.5: Cluster 4 consists of strains from genus *Streptomyces*.

Table 5.1: Strains of each cluster, which was generated by using PAST, their cell wall type, and their closest match in BLAST results.

Strain label	PAST cluster	Cell wall analysis	Strain (*Closest match in BLAST results*)*
P214, P275, P274, P168, P246, P210, P211, P239, P177, P131, P213, P252, P247, P251, P250, P104	1	*meso*-DAP	P168, P131 (*Micromonospora chersina*, DSM 44151^T)
P118, P164, P63, P46, P60, P276, P41, P79, P80, P36, P47, P48, P206, P38, P37, P204, P162, P230, P194, P195,			P36 (*Mycobacterium fortuitum subsp. Fortuitum*, ATCC 6841^T)

‘Table 5.1, continue’

P196, P221, P222, P219, P220, P182, P183, P180, P181, P171, P172, P173, P179, P115, P170, P116, P112, P113, P192, P119, P193, P138, P151, P159,			P103, P197 (*Mycobacterium moriokaense*, DSM 44221^T)
P242, P243, P224, P103, P241, P223, P197, P160, P257, P240, P258, P154, P155, P156, P152, P259, P260, P254, P255, **P256**, P262, P245, P174, P49, P50, P201, P190, P65, P53, P92, P82, P188, P165, P58, P40, P45, P62, P55, P59, P71, P166, P147, P148, P149, P146, P145, P69, **P253**, P163, P189, P52, P126, P56, P202, P281, P42, P51	2	*meso*-DAP	P40 (*Mycobacterium mageritense*, CIP 104973^T)
P226, P184, P217, P86, P227, P229, P117, P228, P191, P153, P161, P187, P271, P272, P199, P209, P98, P99, P106, P208, P200, P225, P270, P1, P269, P16, P132, P133, P110, P143, P144, P232, P233, P234, P231, P235, P157, P203, P212, P68, P273, P67, P61, P93, P10, P265, P136, P185, P114, P186, P15, P3, P9, P2, P108, P8, P57, P12, P20, P237, P175, P142, P266, P238, P135, P267, P263, P264, P207, P216, P198, P248, P167, P178, P134, P249, P215, P158, P150, P137, P141	3	*meso*-DAP, DAB	P86 (*Agromyces humatus*, CD5^T) P16, P9 (*Agromyces aurantiacus*, YIM 21741^T) P67 (*Mycobacterium neoaurum*, ATCC 25795^T) P114 (*Mycobacterium phlei*, ATCC 11758^T) P10 (*Rhodococcus ruber*, ATCC 27863^T)

‘Table 5.1, continue’

P6, P32, P111, P125, P84, P85, P89, P109, P120, P121, P123, P122, P205, P169, P278, P279, P277, P129, P35, P27, P29, P54, P24, P88, P90, P97, P261, P268, P81, P19, P13, P83, P21, P31, P28, P33, P236, P26, P25, P23, P70, P91, P22, P11, P124, P17, P30, P34	4	LL-DAP	P6 (*Streptomyces cavourensis*, NBRC 13026^{T}) P109 (*Streptomyces purpeofuscus*, NBRC 12905^{T}) P88 (*Streptomyces roseoviridis*, NBRC 12911^{T}) P83 (*Streptomyces laurentii*, LMG 19959^{T}) P23 (*Streptomyces chrysomallus subsp. Fumigatus*, NBRC 15394^{T}) P17 (*Streptomyces phaeoluteichromatogenes*, NRRL 5799^{T})

* 16S rRNA gene sequence were blast by using EzTaxon (Chun *et al.*, 2007).

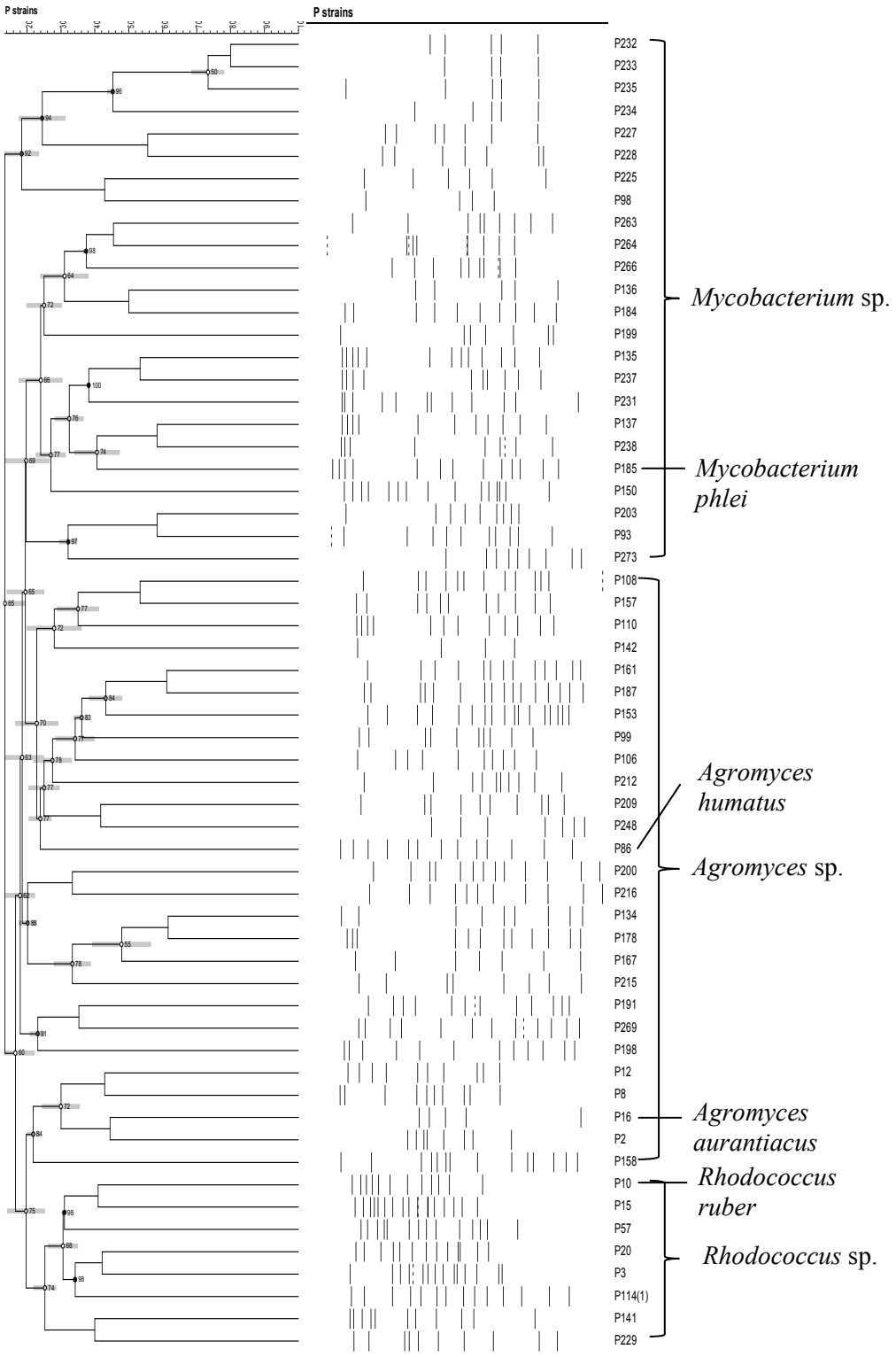


Figure 5.6: Dendrogram generated based on molecular fingerprints of strains from Cluster 3 using GelCompar II. This dendogram was generated by using UPGMA method, Jaccard model, 1.0% optimization, and 0.7% tolerance.

5.3 PLA degradation

All isolated strains were streaked onto the PLA-emulsified agar plate following the method in 3.4.8 (page 24) to confirm their capability to degrade PLA and their relative enzyme activity. This is because some of the isolated strains just coincidently grew inside the yellow zone instead of really having the ability to degrade PLA. Result shows that 235 strains from the total of 251 isolated strains were confirmed to have the ability to degrade PLA. Among these 235 strains, most of them have medium relative enzyme activity, that is, between 1.00 and 3.00. Strains P253 and P256, which were members of genus *Mycobacterium* (Table 5.1, page 51), with higher relative enzyme activity were selected for crude enzyme extraction. Both strains P253 and P256 have relative enzyme activity that were higher than 3.00 for both low and high molecular weight PLA. Their higher relative enzyme activity indicates that either they can produce higher volume of enzyme or their enzymes have stronger enzyme activity than other strains. Choosing these strains will ease the downstream processes because loss of enzyme quantity in these steps is expected.

The cell biomass of each strain was removed from the PLA-emulsified broth by filtration. The remaining broth was treated as crude enzyme and concentrated by using ammonium salt precipitation method. Enzyme present in the remaining broth was concentrated and check for its activity by using the method stated in section 3.4.10 (page 25). These results also indicate that the enzyme produced was released into and remained in the broth since the extraction method did not involve any step that destructs the cells. Hence, the enzyme produced is an extracellular enzyme (Nakamura *et al.*, 2001).

Crude enzyme of strains P253 and P256 were separated based on their molecular weight using SDS PAGE. Then, the separated enzymes in the PAGE gel were soaked the gel in

2.5% (v/v) Triton-X 100 for one hour. This step was to renature the denatured enzymes in the PAGE gel by washing away the SDS using Triton-X100. After the renaturation step, the PAGE gel was laid on the PLA indicator gel plate to allow the enzyme to diffuse from the PAGE gel into the PLA indicator gel plate. By doing this, the particular band that is responsible to the PLA degradation activity and its molecular weight can be determined simultaneously. From the result, clearing zone was observed in the area with molecular weight of 10-35kDa and 35-40kDa for strains P253 and P256, respectively. This indicates that the molecular weight of PLA-degrading enzymes from strains P253 and P256 are in that particular range. However, there were more than one protein band within the clearing zone. Therefore, further steps such as protein purification have to be done in order to determine the exact molecular weight of the PLA-degrading enzyme and identify them. Enzymes from strain P253 with molecular weight range of 10-35kDa was similar to those found by Li and his group (Li *et al.*, 2008), and Nakamura (Nakamura *et al.*, 2001). While enzyme extracted from strain P256 may be a novel enzyme because no PLA-degrading enzyme with this range of molecular weight was reported up to date.

CHAPTER 6

6.0 Conclusion

A total of 251 strains of PLA degrading actinobacteria were isolated from collected soil samples. They were members of genera *Agromyces, Rhodococcus, Micromonospora, Mycobacterium* and *Streptomyces*. These results showed that PLA-degrading activities were also present in genera other than *Amycolatopsis* (Pranamuda *et al.*, 1997) *Kibdelosporangium* (Jarerat *et al.*, 2003), *Pseudonocardia* (Konkit *et al.*, 2012) and *Saccharothrix* (Jarerat & Tokiwa, 2003). Among these strains, two novel strains, strains P86 and P224, were discovered and they are members of *Agromyces* and *Mycobacterium*, respectively. Full characterization of these two strains will be carried out in future for species description. Crude enzyme was extracted from strains with the highest relative enzyme activity, strains P253 and P256, which were members of genus *Mycobacterium*. PLA-degrading enzymes extracted from strain P253 were found to have molecular weight between 10 to 35kDa, which was similar to Li's and Nakamura's finding, while the molecular weight of PLA-degrading enzyme from strain P256 was between 35 to 40kDa indicating it may be a novel enzyme. In order to have a clearer insight on the extracted enzymes and their mechanisms, further studies have to be carried out to identify the PLA-degrading enzyme and the genes that code for the enzymes.

APPENDICES

Appendix A

Preparation of reagents.

¼ strength Ringer's solution

A Ringer's pellet (Oxoid) was dissolved in 500ml of distilled water.

6N HCl

One liter of 6N HCl was prepared by mixing 617ml of HCl (35%) to 382ml of distilled water.

PLA agar indicator gel plate

PLA (0.1%; w/v) with 5000Mw was dissolved by using dichloromethane. The dissolved PLA together with 1% (v/v) Tween 80 were added into 50ml distilled water. The solution was sonicated for 5 minutes. Then, dichloromethane was evaporated by using a hot plate stirrer. After that, 2.5% (w/v) agarose powder was mixed into the solution. The solution was heated until the agarose powder dissolves completely. The solution was poured into a cast and allows to be solidified at room temperature.

Appendix B

Raw data for characterization of PLA-degrading actinobacteria.

Table B.1: Grouping of isolated strains based on their cell morphology.

Cell morphology	Present of spores	Strains	Number of strain
Rod	No	P1, P153, P161, P187, P208, P271, P272, P200, P199, P209, P98, P99, P106, P270, P269, P132, P133, P110, P143, P144, P232, P233, P234, P231, P235, P157, P203, P212, P68, P273, P67, P185, P114, P186, P12, P20, P10, P136, P61, P57, P216, P198, P248, P167, P178, P134, P150, P137, P226, P184, P86, P51, P162, P230, P202, P82, P92, P41, P79, P80, P147, P148, P149, P146, P145, P58, P71, P40, P45, P62, P55, P59, P47, P48, P204, P206, P38, P37, P166, P188, P165, P36, P174, P49, P50, P252, P247, P251, P39, P281, P42	91
Coccus	No	P225, P69, P93	3
Oval	No	P52, P56	2
Filamentous	No	P16, P108, P15, P158, P190, P53, P197, P160, P257, P240, P258, P236, P27, P22, P280, P35, P3, P9, P2, P265, P215, P249, P217, P65, P154, P155, P156, P152, P23, P25, P26, P29, P11, P13, P261, P19, P168, P246, P210, P84, P85, P89, P109, P120, P121, P123, P122, P125	48
	Rod	P201, P254, P255, P256, P259, P260, P91, P124, P83, P253, P8, P262, P28, P33, P81, P97, P54, P31, P24, P88, P6, P70, P17, P32, P21, P104, P211, P111	28
	Coccus	P141, P250, P107, P213, P105, P130, P177, P205, P275, P268, P279, P277, P129, P140, P131, P239, P244, P214, P169, P274, P278, P245, P90	23
Fragmented filament	No	P237, P175, P142, P266, P238, P135, P267, P263, P264, P191, P207, P227, P228, P229, P117, P163, P126, P164, P63, P46, P60, P276, P189, P194, P195, P196, P221, P222, P219, P220, P182, P183, P180, P181, P171, P172, P173, P179, P115, P170, P116, P112, P113, P192, P119, P193, P138, P151, P118, P159, P242, P243, P224, P103, P241, P223	56

Table B.2: Colour groups and strains belong to each group.

No.	Colour group			Strains	No. of strains
	Aerial mycelia	**Substrate mycelia**	**Pigmentation**		
1	Vivid yellow	Vivid yellow	No	P1, P16, P141, P153, P161, P187, P208, P271, P272, P200, P199, P209, P98, P99, P106, P270, P269, P225	18
2	Vivid orange	Vivid orange	No	P132, P133, P110, P143, P144, P232, P233, P234, P231, P235, P157, P203, P212, P68, P273, P67, P185, P114, P186, P237, P175, P108, P93, P142, P15, P8, P3, P9, P12, P2, P20, P10, P136, P266, P238, P135, P267, P263, P264, P265, P61, P57	42
3	Vivid orange yellow	Deep yellow	No	P215, P216, P198, P248, P167, P178, P134, P191, P249	9
4	Vivid orange yellow	Vivid orange yellow	No	P150, P137, P207, P158	4
5	Strong yellow	Strong yellow	No	P226, P184	2
6	Vivid greenish yellow	Vivid greenish yellow	No	P227, P228, P229, P117, P217, P86	6
7	Deep orange yellow	Deep orange yellow	No	P51	1
8	Brilliant greenish yellow	Strong greenish yellow	No	P162, P230, P202	3

‘Table B.2, continue’

9	Brilliant yellow green	Brilliant yellow green	No	P201, P82, P163, P190, P65, P53, P92, P52, P126, P164, P63, P46, P41, P79, P80, P147, P148, P149, P146, P145, P58, P71, P40, P45, P62, P55, P59, P69, P47, P48, P204, P206, P38, P37, P60, P276, P56, P166, P188, P189, P165, P36	42
10	Light greenish yellow	Brilliant greenish yellow	No	P197, P194, P195, P196, P221, P222, P219, P220, P182, P183, P180, P181, P171, P172, P173, P179, P115, P170, P116, P112, P113, P192, P119, P193, P138, P151, P118, P159, P160, P254, P255, P256, P257, P245, P240, P242, P243, P259, P260, P224, P258, P154, P155, P156, P152, P103, P241, P223, P262, P174	50
11	Pale orange yellow	Brilliant greenish yellow	No	P49, P50	2
12	White	Strong greenish yellow	No	P23, P25, P26, P28, P33, P236	6
13	Light gray	Vivid yellow	Deep yellow	P27, P29	2
14	Light gray	Vivid yellow	No	P22, P11, P91	3
15	White	Vivid yellow	No	P124	1
16	Light grayish olive	Dark brown	No	P81	1
17	Light gray	Deep yellow	Vivid greenish yellow	P54	1

‘Table B.2, continue’

18	Grayish olive green	Deep orange yellow	Strong greenish yellow	P90, P97	2
19	Light grayish brown	Strong greenish yellow	No	P13	1
20	Light gray	Strong greenish yellow	No	P31	1
21	White	Deep orange yellow	Deep yellow	P24	1
22	White	Deep orange yellow	Vivid greenish yellow	P88	1
23	White	Deep orange yellow	Dark orange yellow	P6	1
24	White	Deep orange yellow	No	P70	1
25	White	Deep yellowish pink	Strong greenish yellow	P261	1
26	Light grayish brown	Deep orange yellow	No	P83	1
27	White	Moderate olive green	No	P19	1
28	Light gray	Deep yellow	No	P17	1
29	Grayish olive green	Deep yellow	Moderate olive	P32	1
30	Reddish black	Strong greenish yellow	No	P21	1
31	Light gray	Reddish black	Moderate olive brown	P111	1
32	Vivid yellow green	Deep greenish yellow	No	P104	1
33	Vivid reddish orange	Vivid reddish orange	No	P250	1

‘Table B.2, continue’

34	Deep reddish orange	Deep reddish orange	No	P252, P247, P251	3
35	Black	Deep orange	Moderate yellow green	P107, P140, P105, P130	4
36	Black	Deep orange	No	P168, P246, P210, P211, P131, P213	6
37	Black	Black	No	P239, P177	2
38	Black	Black	Dark olive green	P244	1
39	Reddish black	Brownish black	Moderate olive brown	P84, P85, P89, P109, P120, P121, P123, P122, P205	9
40	Reddish black	Brownish black	No	P214, P275, P274	3
41	Reddish black	Brownish black	Deep yellow	P268	1
42	Reddish black	Brownish black	Light olive	P169	1
43	White	Brownish black	Moderate olive brown	P125	1
44	Light yellow green	Light yellow green	No	P39	1
45	Brilliant greenish yellow	Brilliant greenish yellow	No	P281, P42	2
46	Olive black	Bluish black	Bluish white	P278, P279, P277	3
47	Light orange	Deep yellowish pink	Strong yellow	P253	1
48	Deep yellowish pink	Deep yellowish pink	Brilliant yellow green	P280	1
49	Dark olive green	Dark olive green	Dark olive green	P35	1
50	Deep orange	Deep orange	Moderate yellow green	P129	1

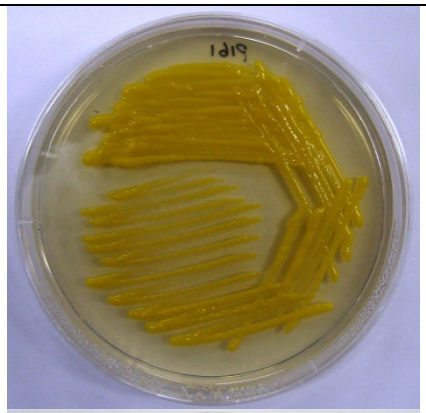
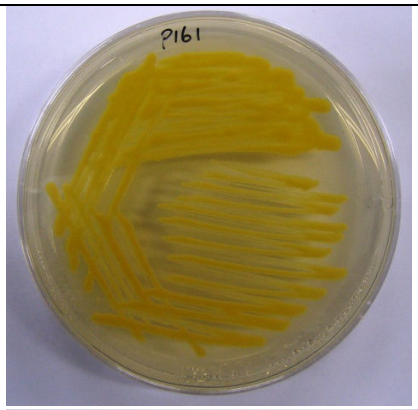
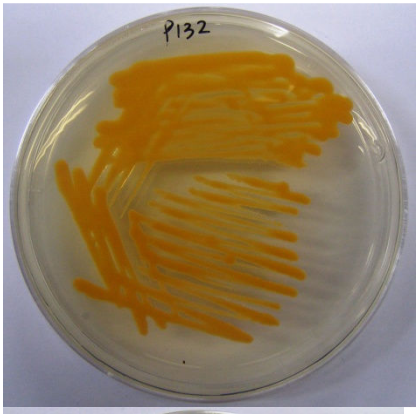
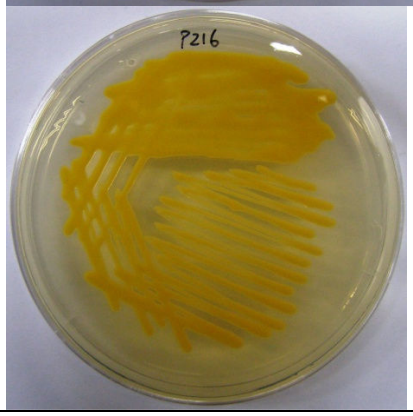
Table B.3: Characteristics that differentiate P86 and their closest relatives within the genus *Agromyces.* 1: strain P86; 2: *A. Humatus*; 3: *A. neolithicus*; +, positive; -, negative; V,variable (data in parentheses are for the type strain); W, weakly positive; ND, not determined or not described; Glc, glucose; Gal, galactose; Man, mannose; Rha, rhamnose; Rib, ribose. All strains produced acid from L-arabinose, D-fructose, galactose, glucose, glycerol, maltose, mannitol, D-mannose, but not from B-gentiobiose, lactose or D-turanose. All strains do not decompose adenine, xanthine or urea. All strains can grow in the presence of 2% NaCl but not at 10% NaCl. All strains can grow at 28^{o}C and 37^{o}C. All strains showed positive reaction in catalase test.

Characteristic	**1**	**2**	**3**
Acid production:			
Arbutin	-	+	+
D-Arabinose	-	-	+
Cellobiose	+	V	+
L-Fucose	-	-	+
Melibiose	+	-	+
Inulin	-	+	-
N-acetylglucosamine	+	V	(+)
Rhamnose	+	+	-
Ribose	+	-	-
Salicin	+	-	-
Trehalose	+	-	-
D-xylose	+	-	-
Decomposition of:			
Tyrosine	-	+	+
Tween 80	+	-	-
Casein	-	+	+
Starch	-	+	+
Aesculin	-	+	+
Utilization of:			
Malate	-	+	-
Succinate	-	+	-
Growth in the presence of:			
4% NaCl	-	-	-
Growth at:			
10^{o}C	+	-	-
Microaerophilic growth	+	-	+
Oxidase test	-	-	+/-
Enzyme activities			
a-Chymotrypsin	+	-	+
a-Galactosidase	+	-	+
b-Galactosidase	+	+	-
b-Glucuronidase	-	+	+
N-Acetyl-b-glucosaminidase	+	-	-
a-Mannosidase	+	+	+

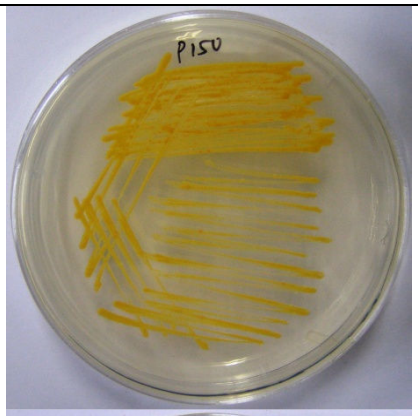
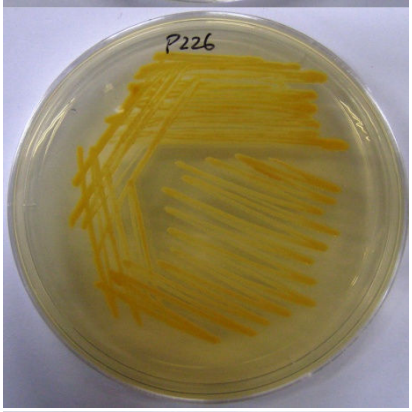
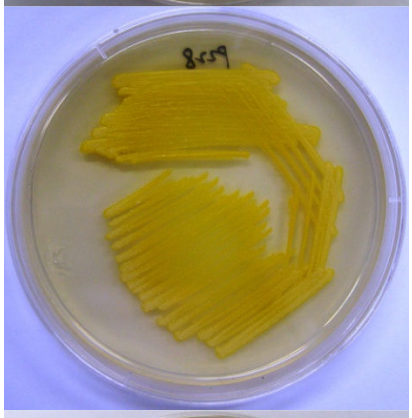
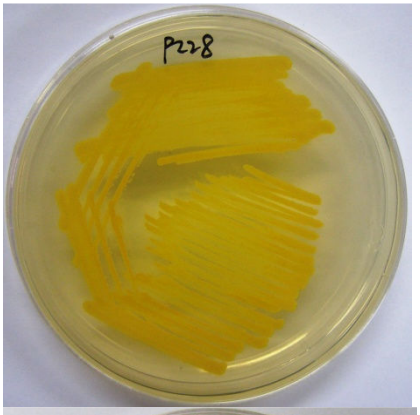
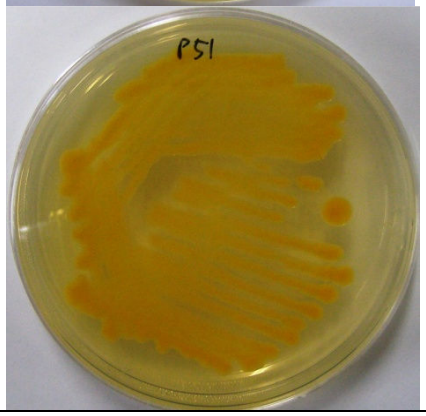
Appendix C

Pictures of representative strain from each colour group

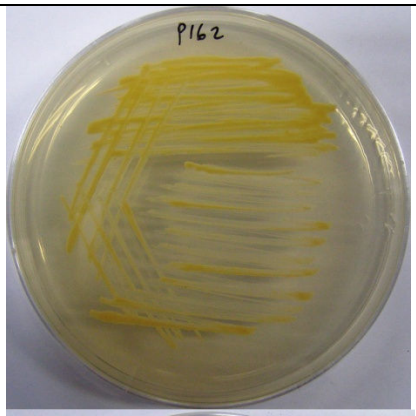
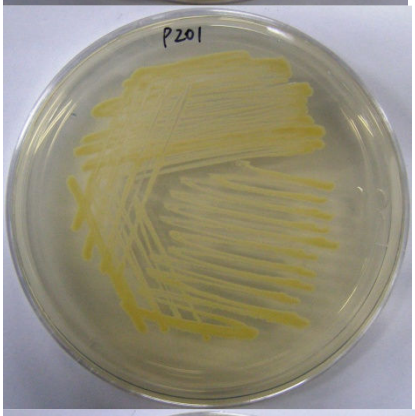
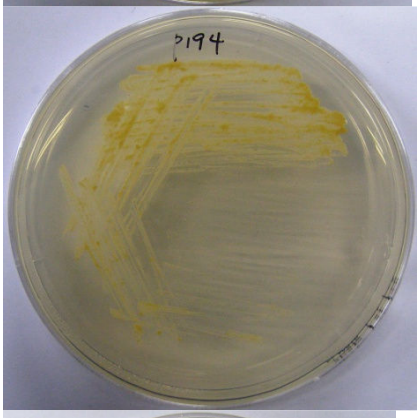
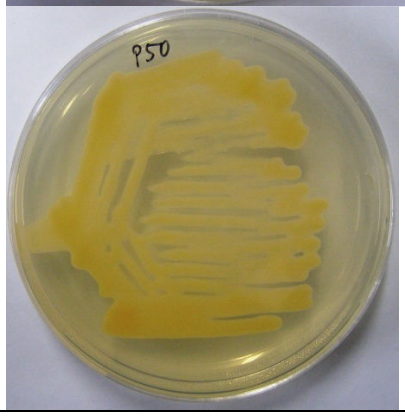
Table C.1: Pictures of representative strain from each colour group

Colour group (Representative strain)	Aerial mycelia	Substrate mycelia
Group 1(P161)		
Group 2 (P132)		
Group 3 (P216)		

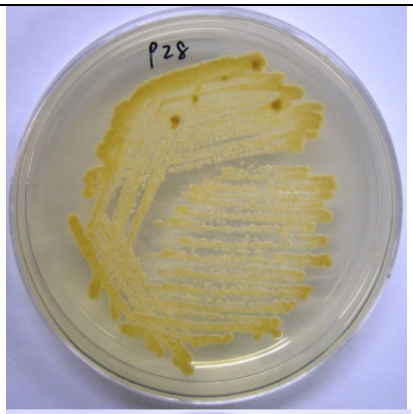
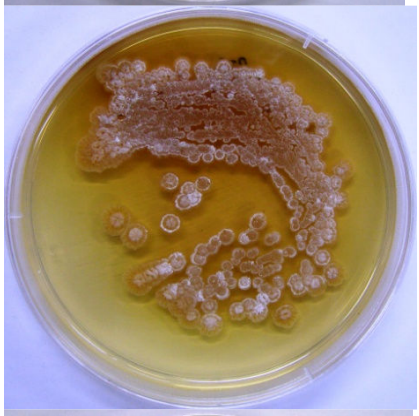
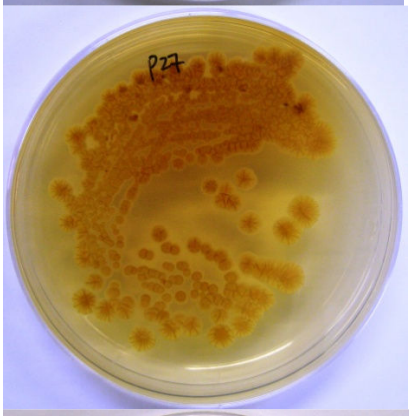
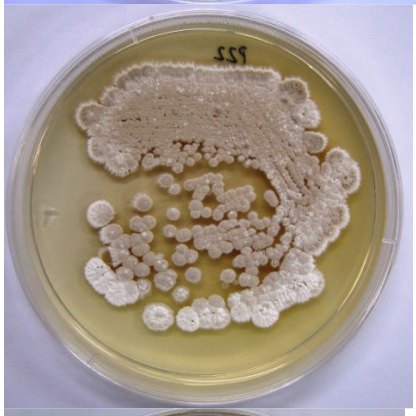
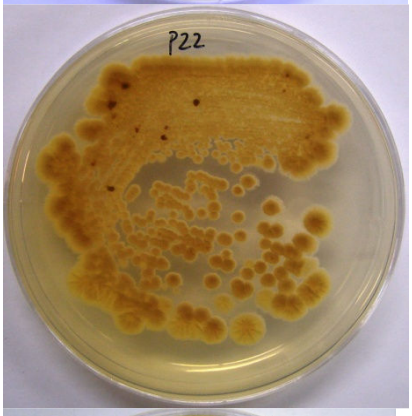
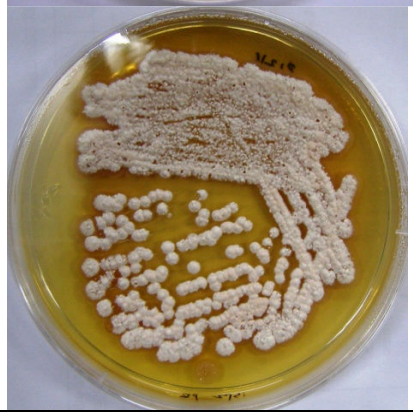
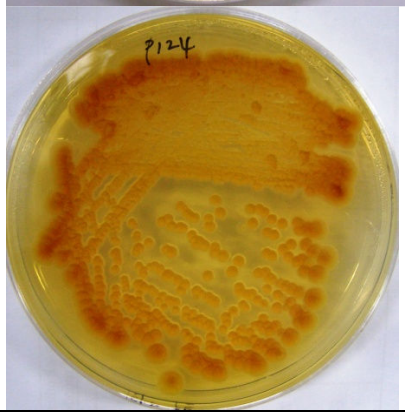
‘Table C.1, continue’

Group 4 (P150)		
Group 5 (P226)		
Group 6 (P228)		
Group 7 (P51)		

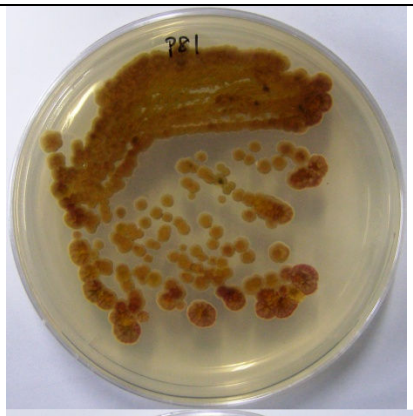
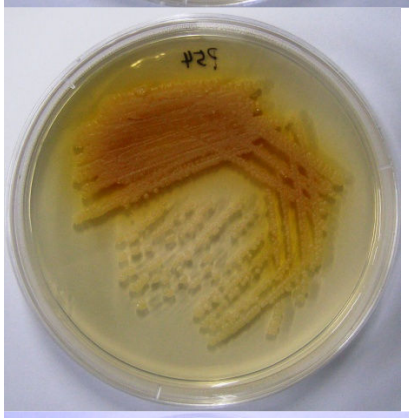
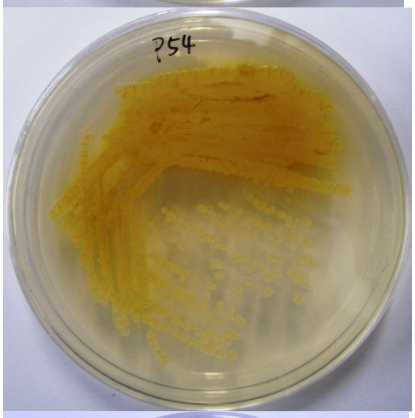
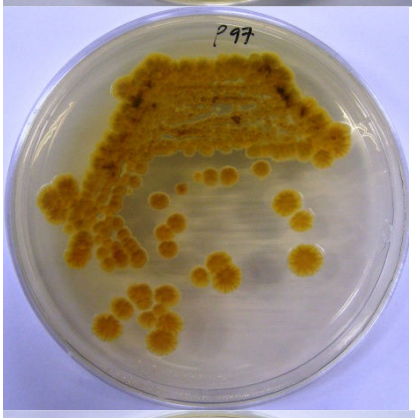
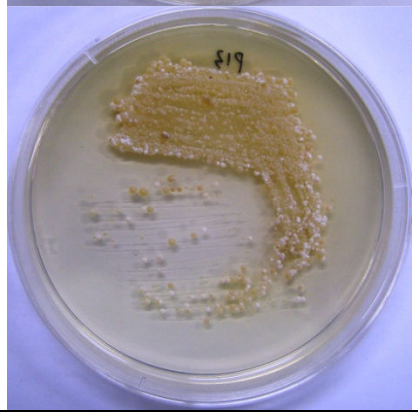
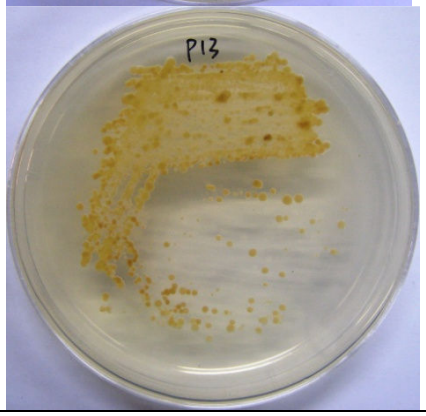
‘Table C.1, continue’

Group 8 (P162)		
Group 9 (P201)		
Group 10 (P194)		
Group 11 (P50)		

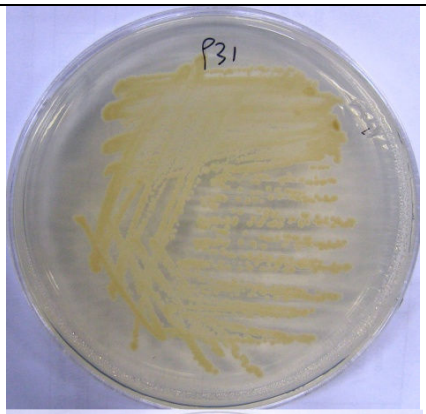
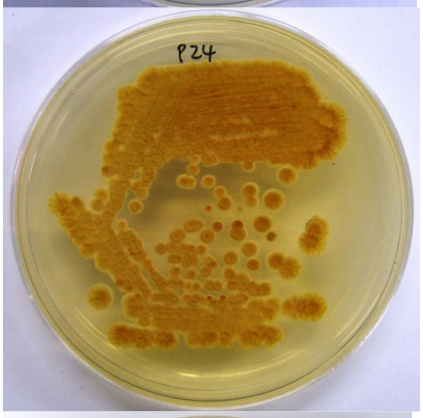
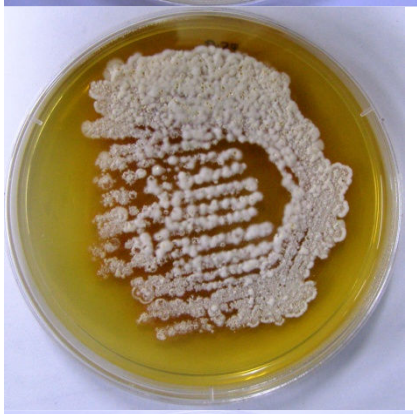
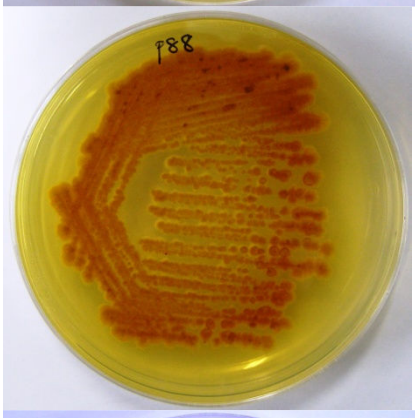
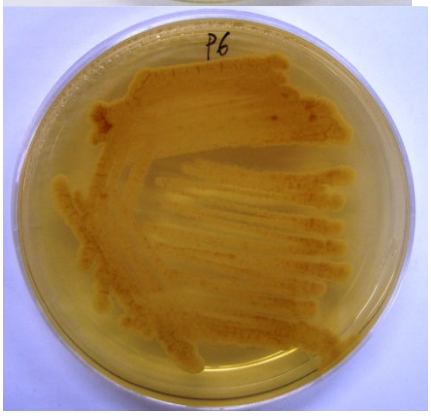
‘Table C.1, continue’

Group 12 (P28)		
Group 13 (P27)		
Group 14 (P22)		
Group 15 (P124)		

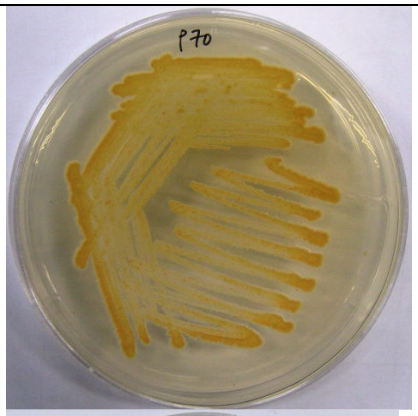
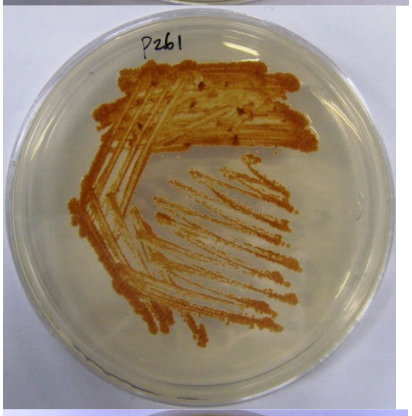
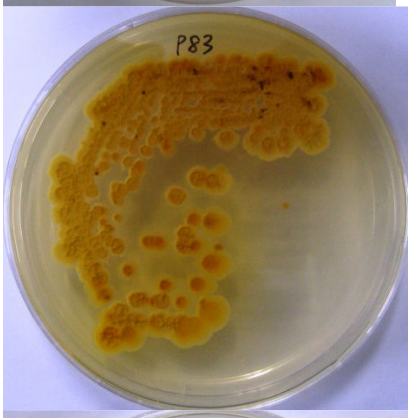
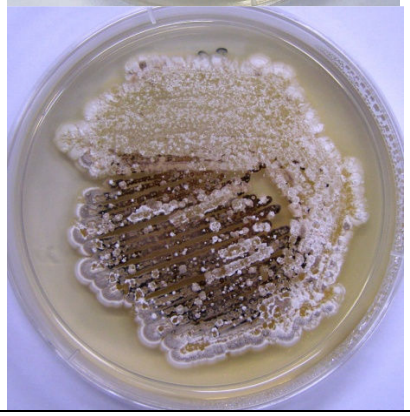
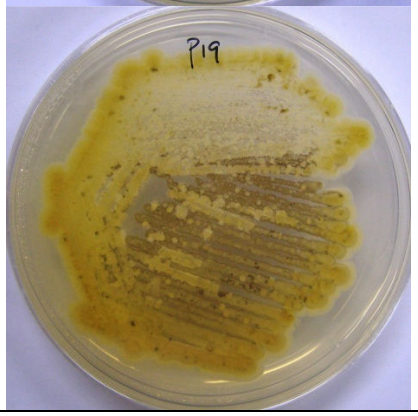
‘Table C.1, continue’

Group 16 (P81)		
Group 17 (P54)		
Group 18 (P97)		
Group 19 (P13)		

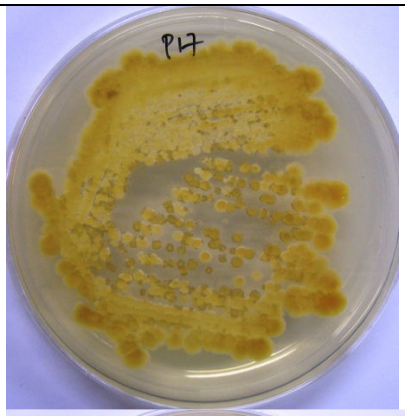
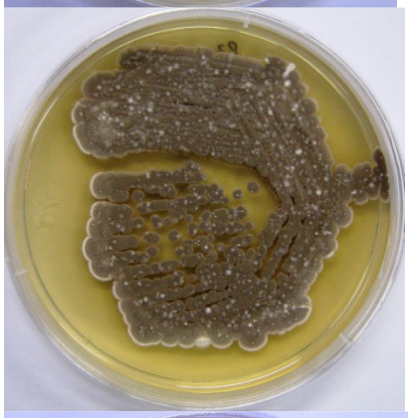
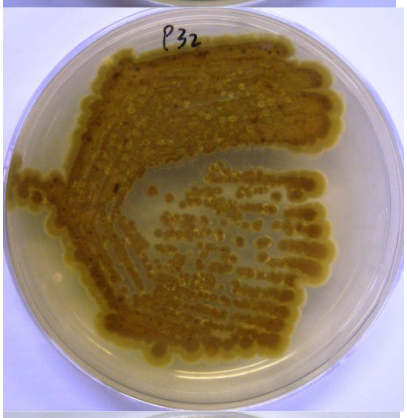
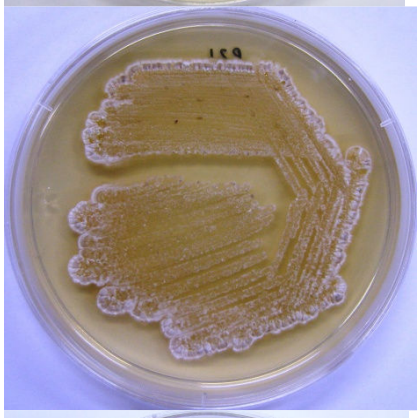
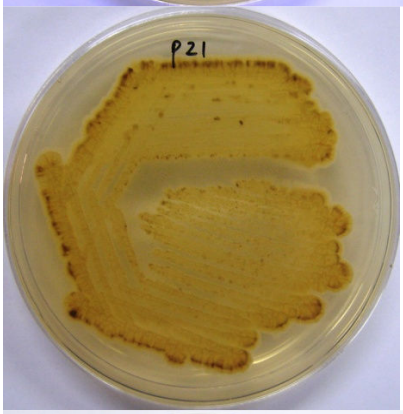
‘Table C.1, continue’

Group 20 (P31)		
Group 21 (P24)		
Group 22 (P88)		
Group 23 (P6)		

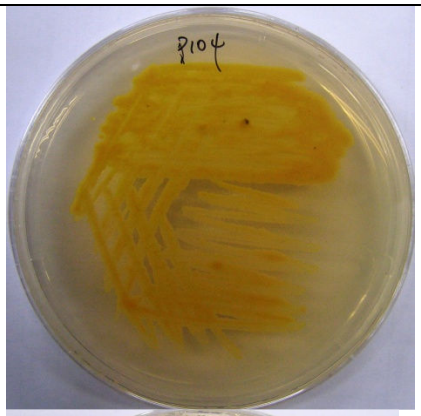
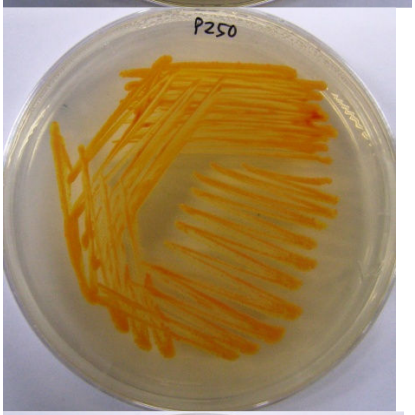
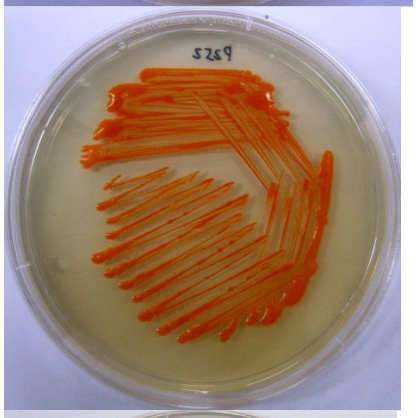
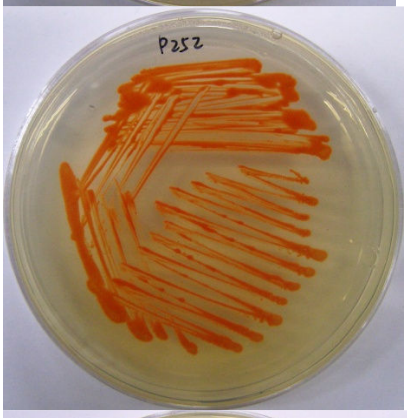
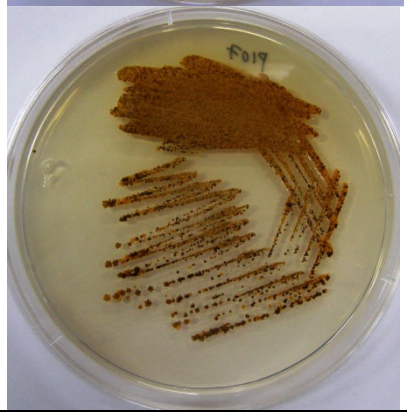
‘Table C.1, continue’

Group 24 (P70)		
Group 25 (P261)		
Group 26 (P83)		
Group 27 (P19)		

‘Table C.1, continue’

Group 28 (P17)		
Group 29 (P32)		
Group 30 (P21)		
Group 31 (P111)		

‘Table C.1, continue’

Group 32 (P104)		
Group 33 (P250)		
Group 34 (P252)		
Group 35 (P107)		

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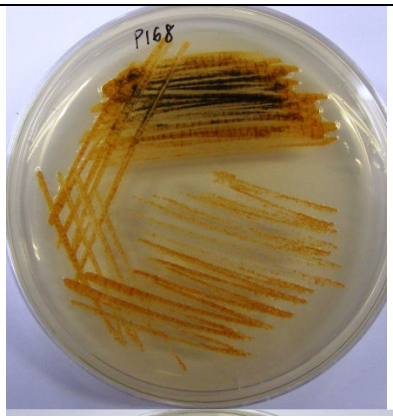
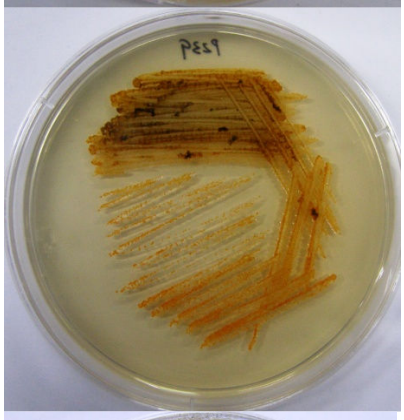
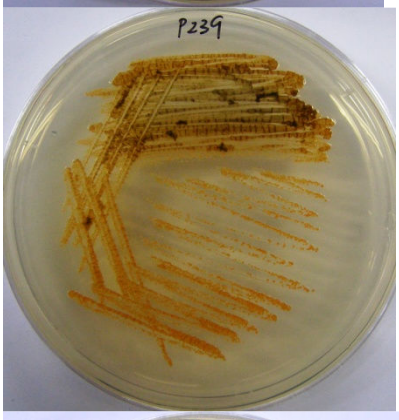
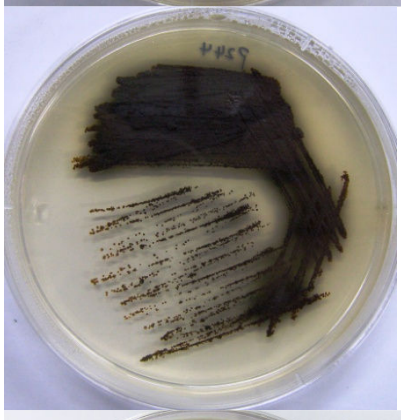
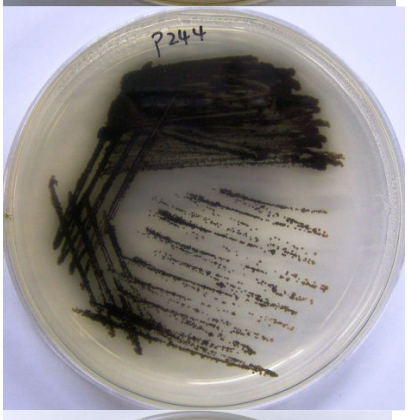
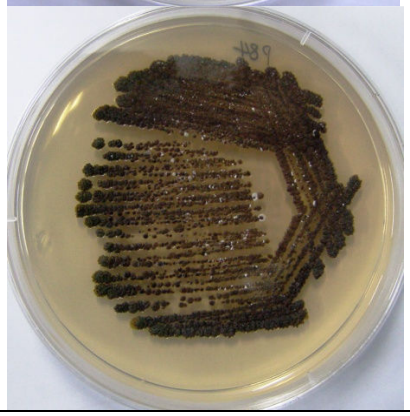
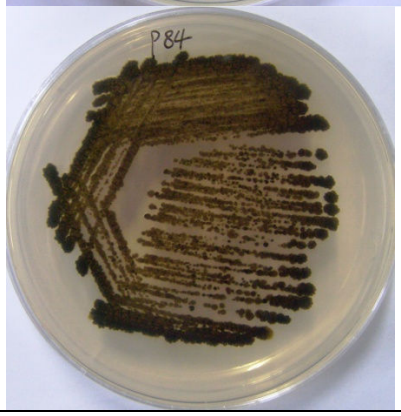
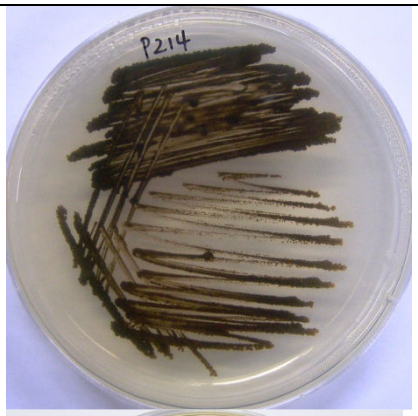
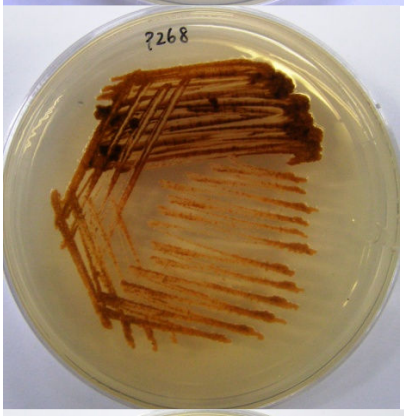
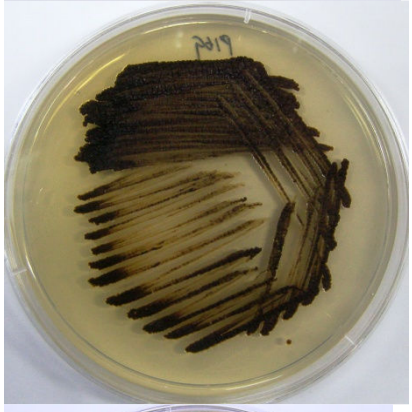
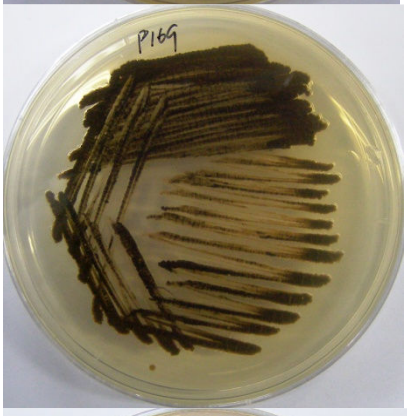
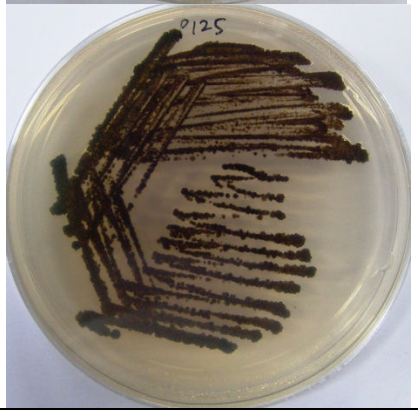
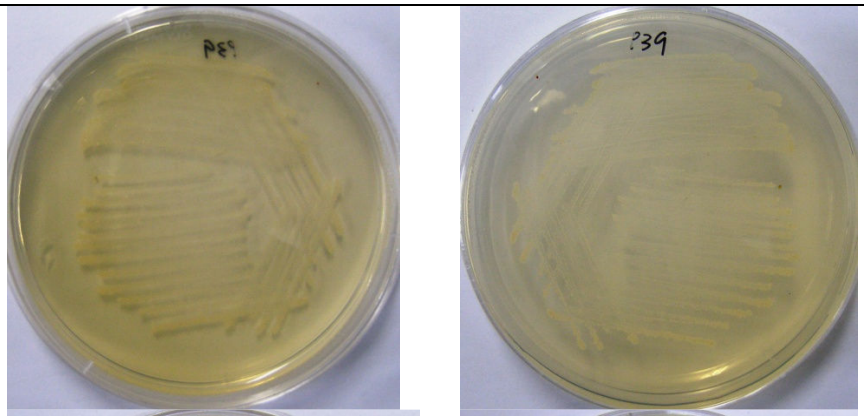
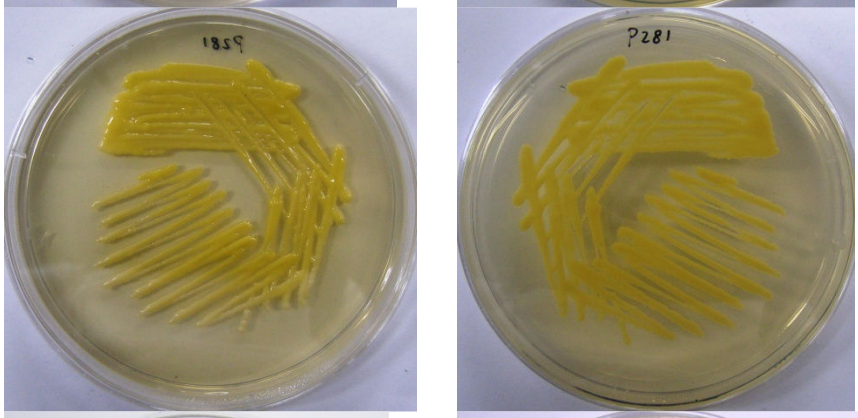
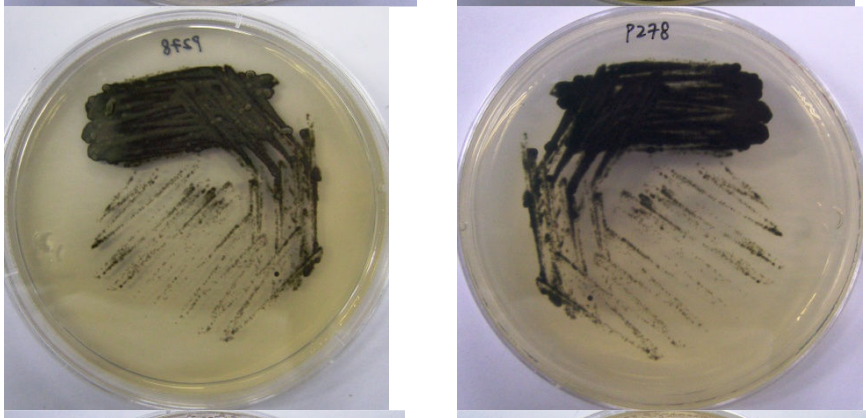
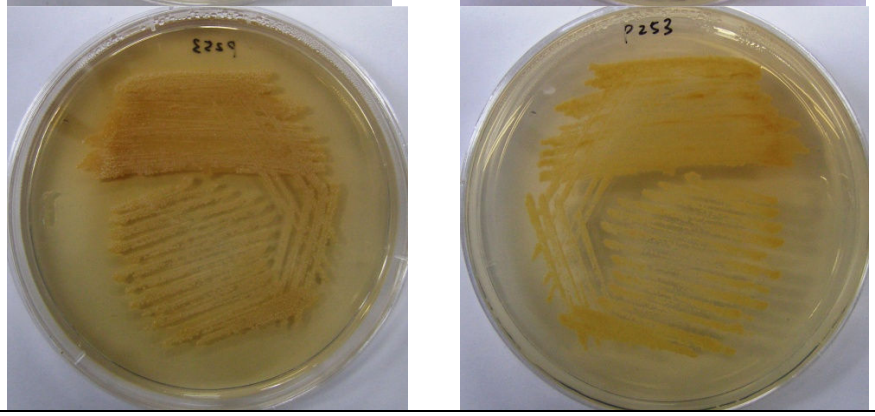
Group 36 (P168)		
Group 37 (P239)		
Group 38 (P244)		
Group 39 (P84)		

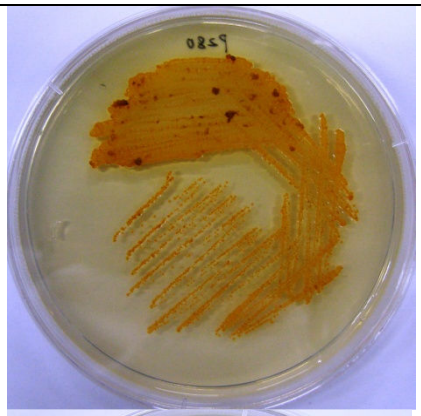
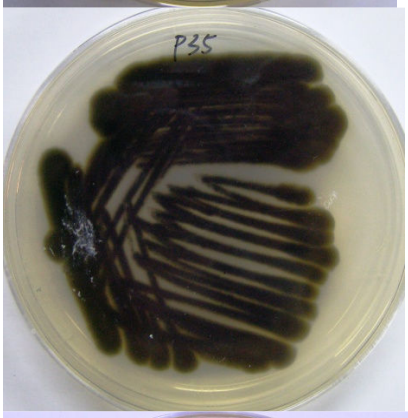
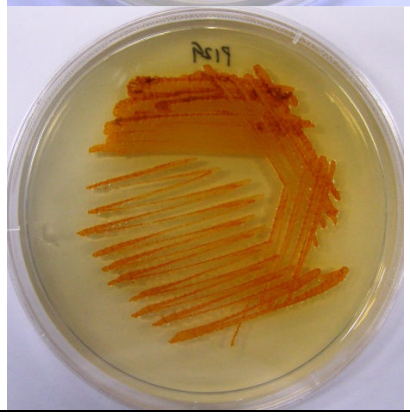
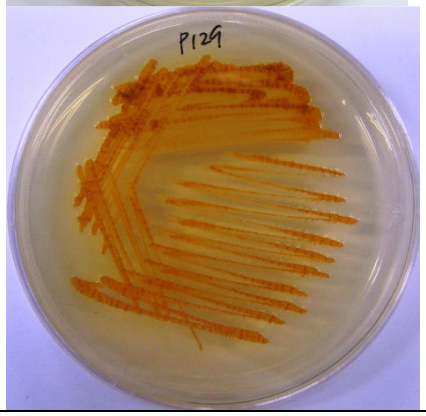
‘Table C.1, continue’

Group 40 (P214)		
Group 41 (P268)		
Group 42 (P169)		
Group 43 (P125)		

‘Table C.1, continue’

Group 44 (P39)	
Group 45 (P281)	
Group 46 (P278)	
Group 47 (P253)	

‘Table C.1, continue’

Group 48 (P280)		
Group 49 (P35)		
Group 50 (P129)		

Appendix D

16S rRNA gene sequences for novel strains

Sequence for strain P86 (KF499320)

CAAGTCGAACGATGAACTGAGAGCTTGCTCTTGGGGATTAGTGGCGAACGGGT
GAGTAACACGTGAGTAACCTGCCCTGGACTCTGGGATAACCCCGAGAAATCGG
AGCTAATACCGGATAGGACCTTGAGCGGCATCGCTTGGGGTGGAAAGTTTTTTC
GGTTTGGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCA
AGGCGTCGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGA
GACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGG
CGCAAGCCTGATGCAGCAACGCCGCGTGCGGGATGACGGCCTTCGGGTTGTAA
ACCGCTTTTAGTAAGGAAGAAGGGGAGCTTGCTCCTTGACGGTACTTGCAGAA
AAAGGACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTCCGAGC
GTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCT
GTGAAAACTAGAGGCTCAACCTCTAGCCTGCAGTGGGTACGGGCAGACTGGAG
TGCGGTAGGGGAGAATGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCA
GGAGGAACACCGATGGCGAAGGCAGTTCTCTGGGCCGTAACTGACGCTGAGGA
GCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAA
ACGTTGGGCGCTAGATGTGGGGACCTTTCCACGGTTTCCGTGTCGTAGCTAACG
CATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT
TGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCG
AAGAACCTTACCAAGGCTTGACATAACCGAGAACACCGTAGAAATACGGGACT
CTTTGGACACTCGGTTACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAG
ATGTTGGGTTAAGTCCCGCAACGAGAGCAACCCTCGTCGCATGTTGCCAGCAC
GTCATGGTGGGAACTCATGTGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGG
GATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAAT
GGCCGGTACAAAGGGCTGCGATGTCGTAAGGCGGAGCGAATCCCAAAAAGCC
GGTGTCAGTTCGGATTGAGGTCTGCAACTCGACCTCATGAAGTCGGAGTCGCTA
GTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACA
C C G C C C G T C A A G T C A T G A A A G T C G G T A A C A C C C G A A G C C G

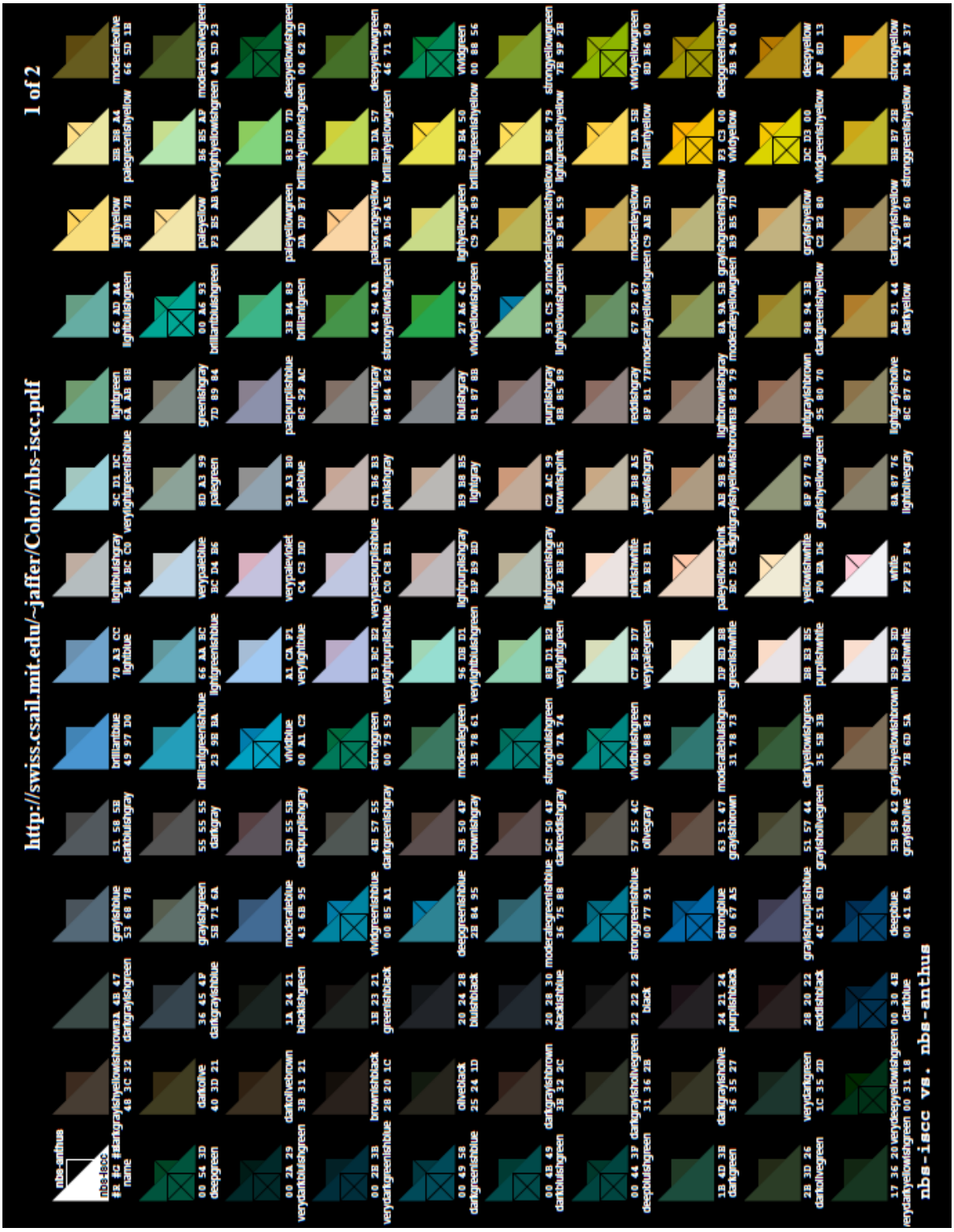
Sequence for strain P224 (KF499321)

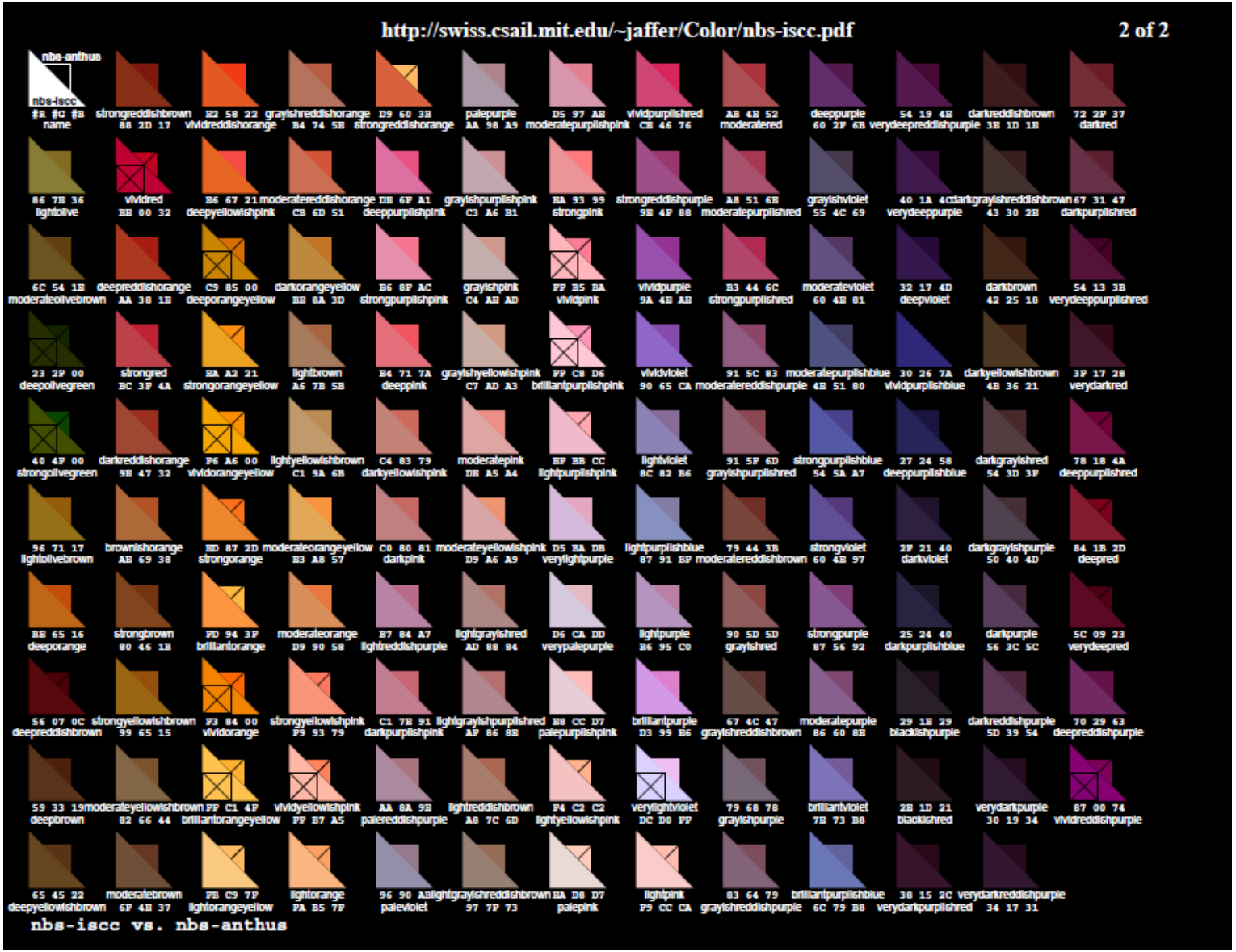
GGACTCGAGTGGCGAACGGGTGAGTAACACGTGGGTGATCTGCCCTGCAC
TTTGGGATAAGCCTGGGAAACTGGGTCTAATACCGGATAGGACTACGCAC
TGCATGGTGTGTGGTGGAAAGCTTTTGCGGTGTGGGATGGGCCCGCGGCC
TATCAGCTTGTTGGTGGGGTGATGGCCTACCAAGGCGACGACGGGTAGCC
GGCCTGAGAGGGTGTCCGGCCACACTGGGACTGAGATACGGCCCAGACTC
CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATG
CAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCA
GCATCGACGAAGCGCAAGTGACGGTAGATGCAGAAGAAGCACCGGCCAAC
TACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAAT
TACTGGGCGTAAAGAGCTCGTAGGTGGTTTGTCGCGTTGTCCGTGAAAAC
TCACAGCTTAACTGTGGGCGTGCGGGCGATACGGGCAGACTGGAGTACTG

CAGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGG
AGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGG
AGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCC
GTAAACGGTGGGTACTAGGTGTGGGTTTCCTTCCTTGGGATCCGTGCCGT
AGCTAACGCATTAAGTACCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAA
CTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGATTAA
TTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACATGCACAGGACGTG
CCTAGAGATAGGTATTCCCTTGTGGCCTGTGTGCAGGTGGTGCATGGCTG
TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTTGTCTCATGTTGCCAGCGCGTTATGGCGGGGACTCGTGAGAGACTG
CCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCC
TTATGTCCAGGGCTTCACACATGCTACAATGGCCGGTACAAAGGGCTGCG
ATGCCGTGAGGTGGAGCGAATCCTTTCAAAGCCGGTCTCAGTTCGGATCG
GGGTCTGCAACTCGACCCCGTGAAGTCGGAGTCGCTAGTAATCGCAGATC
AGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCA
C G T C A T G A A A G T C G G T A A C A C C C G A A G C

Appendix E

National Bureau of Standard Colour Name Chart




http://swiss.csail.mit.edu/~jaffer/Color/nbs-iscc.pdf
2 of 2
nbs-anthus
nbs-iscc
#R #G #B
name
strongreddishbrown 88 2D 17
vividreddishorange E2 58 22
grayishreddishorange B4 74 5B
strongreddishorange D9 60 3B
palepurple AA 98 A9
moderatepurplishpink D5 97 AB
vividpurplishred C8 46 76
moderatered AB 4B 52
deeppurple 60 2F 6B
verydeepreddishpurple 54 19 4B
darkreddishbrown 3B 1D 1B
darkred 72 2F 37
lightolive 86 7B 36
vividred BB 00 32
deepyellowishpink E6 67 21
moderatereddishorange CB 6D 51
deeppurplishpink DB 6F A1
grayishpurplishpink C3 A6 B1
strongpink EA 93 99
strongreddishpurple 9B 4F 88
moderatepurplishred A8 51 6B
grayishviolet 55 4C 69
verydeeppurple 40 1A 4C
darkgrayishreddishbrown 43 30 2B
darkpurplishred 67 31 47
moderateolivebrown 6C 54 1B
deepreddishorange AA 38 1B
deeporangeyellow C9 85 00
darkorangeyellow BB 8A 3D
strongpurplishpink B6 8F AC
grayishpink C4 AE AD
vividpink FF B5 BA
vividpurple 9A 4B AB
strongpurplishred B3 44 6C
moderatevioletish 60 4B 81
deepviolet 32 17 4D
darkbrown 42 25 18
verydeeppurplishred 54 13 3B
deepolivegreen 23 2F 00
strongred BC 3F 4A
strongorangeyellow EA A2 21
lightbrown A6 7B 5B
deeppink B4 71 7A
grayishyellowishpink C7 AD A3
brilliantpurplishpink FF C8 D6
vividviolet 90 65 CA
moderatereddishpurple 91 5C 83
moderatepurplishblue 4B 51 80
vividpurplishblue 30 26 7A
darkyellowishbrown 4B 36 21
verydarkred 3F 17 28
strongolivegreen 40 4F 00
darkreddishorange 9B 47 32
vividorangeyellow P6 A6 00
lightyellowishbrown C1 9A 6B
darkyellowishpink C4 83 79
moderatepink DB A5 A4
lightpurplishpink EF BB CC
lightviolet 8C 82 B6
grayishpurplishred 91 5F 6D
deeppurplishblue 27 24 58
strongpurplishblue 54 5A A7
darkgrayishred 54 3D 3F
deeppurplishred 78 18 4A
lightolivebrown 96 71 17
brownishorange AB 69 38
strongorange ED 87 2D
moderateorangeyellow B3 A8 57
darkpink C0 80 81
moderateyellowishpink D9 A6 A9
verylightpurple D5 BA DB
lightpurplishblue 87 91 BF
moderatereddishbrown 79 44 3B
strongviolet 60 4B 97
darkviolet 2F 21 40
darkgrayishpurple 50 40 4D
deepred 84 1B 2D
deeporange BB 65 16
strongbrown 80 46 1B
brilliantorange FD 94 3F
moderateorange D9 90 58
lightreddishpurple B7 84 A7
lightgrayishred AD 88 84
verypalepurple D6 CA DD
lightpurple B6 95 C0
grayishred 90 5D 5D
strongpurple 87 56 92
darkpurplishblue 25 24 40
darkpurple 56 3C 5C
verydeepred 5C 09 23
deepreddishbrown 56 07 0C
strongyellowishbrown 99 65 15
vividorange F3 84 00
strongyellowishpink F9 93 79
lightgrayishpurplishred AF 86 8B
palepurplishpink B8 CC D7
brilliantpurple D3 99 B6
grayishreddishbrown 67 4C 47
moderatepurple 86 60 8B
blackishpurple 29 1B 29
darkreddishpurple 5D 39 54
deepreddishpurple 70 29 63
deepbrown 59 33 1F
moderateyellowishbrown 82 66 44
brilliantorangeyellow FF C1 4F
vividyellowishpink FF B7 A5
palereddishpurple AA 8A 9B
lightreddishbrown A8 7C 6D
lightyellowishpink F4 C2 C2
verylightviolet DC D0 FF
grayishpurple 79 68 78
brilliantviolet 7E 73 B8
blackishred 2B 1D 21
verydarkpurple 30 19 34
vividreddishpurple 87 00 74
deepyellowishbrown 65 45 22
moderatebrown 6F 4B 37
lightorangeyellow FB C9 7F
lightorange FA B5 7F
palevioletish 96 90 AB
lightgrayishreddishbrown 97 7F 73
palepink EA D8 D7
lightpink F9 CC CA
grayishreddishpurple 83 64 79
brilliantpurplishblue 6C 79 B8
verydarkpurplishred 38 15 2C
verydarkreddishpurple 34 17 31
nbs-iscc vs. nbs-anthus

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