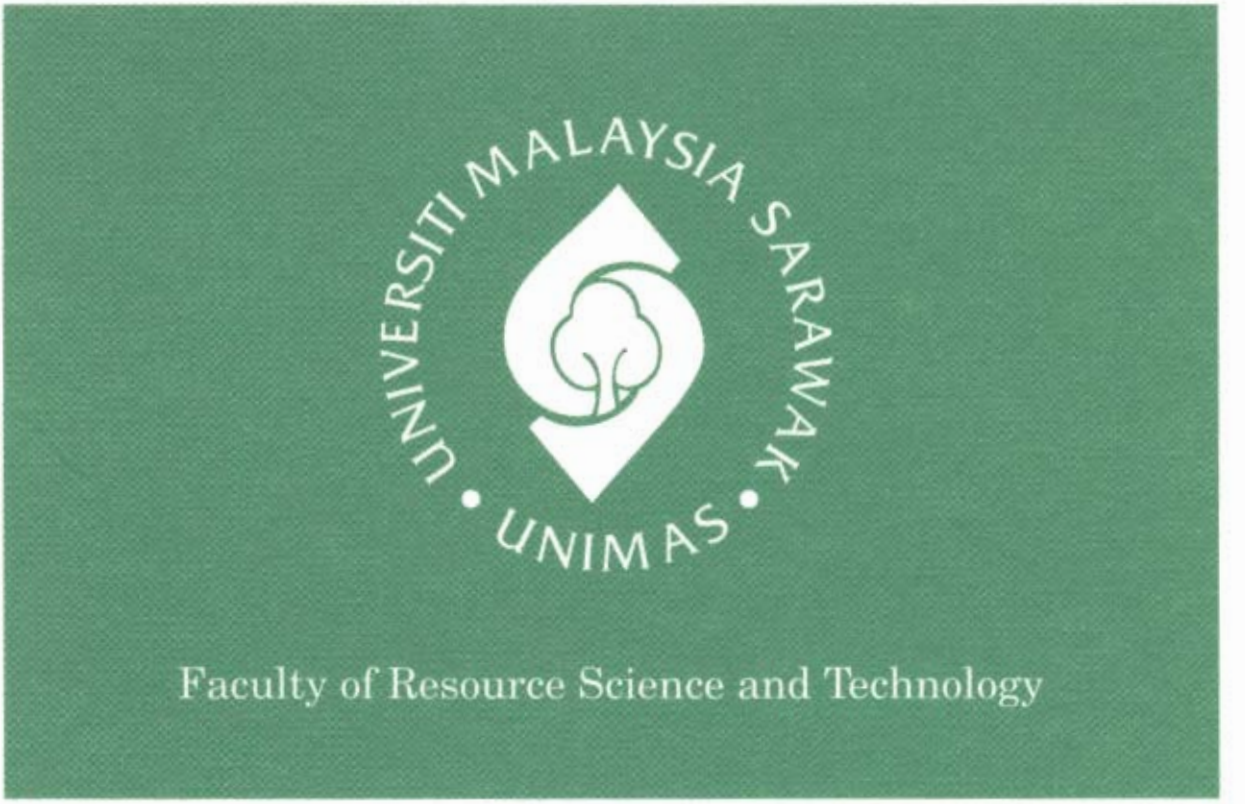

SCREENING AND MOLECULAR ANALYSIS OF BACTERIAL RESISTANCE TO LEAD AND SILVER FROM MATANG LANDFILL DISPOSAL SITE AND A WASTE DISCHARGE DOWNSTREAM OF A PAPER RECYCLING MILL

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Bachelor of Science with Honours
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Screening and Molecular Analysis of Bacterial resistant to Lead and Silver from Matang Landfill disposal site and a Waste Discharge downstream of a Paper Recycling Mill

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ABSTRACT

Twenty-four heavy metal resistant bacterial isolated from soil samples taken from Sungai Kuap and Matang Landfill disposal site. The resistant properties screened were Pb^R (1000 µl/ml) and Ag^R (30 µl/ml). Among the resistant strains that have been identified are *Enterobacter cloacae, Escherichia coli, Klebsiella pneumoniea, Proteus mirabilis, and Providencia alcalifaciens.* Simultaneous resistance towards both heavy metals have been observed in 58.33% of the isolates. Plasmid isolation was attempted but was unsuccessful. Further studies need to be carried out to characterize the bacterial strains isolated.

Keywords: Heavy metal resistance, bacterial strains

ABSTRAK

Sejumlah dua puluh empat bakteria yang rintang terhadap logam berat telah dipencilkan daripada sampel tanah yang diambil dari tempat pembuangan efluen dari sebuah kilang pengitaran semula kertas di Samarahan, Sarawak dan tempat pembuangan sampah sarap di Matang, Sarawak. Ciri-ciri kerintangan yang dikenalpasti termasuk terhadap Pb (2400 µl/ml) dan Ag (30 µl/ml). Di antara strain-strain bakteria yang telah dipencilkan ialah Enterobacter cloacae, Escherichia coli, Klebsiella pneumoniea, Proteus mirabilis, and Providencia alcalifaciens. Kerintangan terhadap kedua-dua logam berat serentak telah dikesan iaitu sebanyak 58.33% peratus daripada bakteria-bakteria yang dipencilkan. Percubaan untuk mengekstrak plasmid bakteria telah dilakukan beberapa kali tetapi gagal mendapat keputusan. Kajian selanjutnya perlu dilakukan bagi mencirikan starin-strain bacteria yang telah dipencilkan.

Kata Kunci: Rintangan terhadap logam berat, strain-strain bakteria

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Sites Review

Landfill disposal refers to the placement of waste on or beneath the surface of the ground with the intention of isolating the wastes from the environment. Lead and Silver were considered hazardous or potentially hazardous by 3 or more countries (Dawson & Mercer, 1986). Heavy metal cations (Ag^+, Cd^+, $Pb^+$and Hg^+) are non-essential and toxic (Gupta *et al.*, 2001).

TABLE 1: Sources of Hazardous Waste by Type.

Waste Disposal Category	Estimated Dry Weight in Tons	Industry	Percent of Total For Category	Types of Wastes
Metals/ metal finishing	3.5	Batteries	< 1%	Acid Solution
		Electroplating	80-90%	Metal –bearing sludges
		Primary metals, smelting, refining	10%	
		Special Machinery		
		Electronic	3-6%	
			2%	
Paints/ solvent/ coatings Organic	0.1	Paint and allied products	100%	Organic solvent
	2.7	Organic chemicals	80-90%	Pesticides
		Pharmaceuticals	3-5%	Biological
		Rubbers and Plastics	8-12%	Plastics, rubber
		Textiles, dyeing, finishing	5%	
Petroleum	0.8	Petroleum Refining	< 87%	Oily waste
		Waste oil-refining	> 13%	
Inorganic	0.8	Inorganic chemicals	80-90%	Aqueous solutions of salts, metals, etc.
		Leather tanning	10-20%	

Adapted from Foster Smell. (1976)

Bacteria are the most adaptive organisms as they can readily develop resistance to a broad spectrum of bactericidal agents including antibiotics and heavy metals (Meyer, 1999).

Studies conducted on an old landfill in the town of São Carlos (SP, Brazil) revealed the presence of a pollution plume (Ellert *et al.*, 1990) as well as the presence of an abundant microflora dominated by bacterial populations (Fusconi *et al.*, 1999). Sewage sludge often contains undesirable chemical contents, specifically heavy metals (Alloway, 1990). Bacteria are common inhabitants of metal-contaminated sites, where they accumulate and immobilize heavy metals (Yoshida *et al.*, 1998). The cell walls of gram-positive bacteria have strong metal-binding properties (Silver, 1993). Gram-negative bacteria are known to be more widespread in metal-contaminated soil than Gram-positive bacteria (Dietrich, 2000). Selective pressures from contaminated environments have led to the emergence of microorganisms that are resistant to virtually all toxic metals (Rouch *et al.*, 1995). Metal-resistant bacteria may have developed shortly after prokaryotic life started and are present in nearly all genera (Silver *et al.*, 1996). According to Bruins *et al.*, 2000, this occurred because bacteria normally exist in environment that has always been contaminated with heavy metals.

On solid surfaces exposed to aqueous environments bacterial growth form biofilms in which the microbial cells are associated with large amounts of exopolysaccharides (EPS) (Sutherland, 1990). Exopolysaccharides are essential to the biological success of most bacteria living within the biofilm in varied natural environments in which they are observed since they can concentrate nutrients from water flow and protect the bacteria from antibacterial agents and from predators (Costerton, 1985). A number of reports have described the resistance mechanism of bacteria to either the ion or soluble form of heavy metals. But the interaction between bacteria with insoluble forms of heavy metals has not been fully understood (Yoshida *et al.*, 1998).

High levels of heavy metal contamination have been detected in the wastewater discharged from a local paper-recycling mill in Sarawak (Lim, 1997). The contaminants found in excessive amounts include Pb, Cu, Ca, Cd, Fe, Mn and Zn (Lim, 1997).

1.2 Objectives

1. To isolate and identify bacterial strains that are resistant to lead and silver.
2. To determine whether the resistant genes are carried by plasmid or the chromosome.
3. To isolate plasmids that encode resistance towards lead and silver.
4. To clone the genes that encode resistance towards lead and silver.

1.3 Resistance to Lead

Microorganisms have developed mechanisms of coping with a variety of toxic metals. A few studies have explored microbial resistance to lead. Lead is not very toxic to microorganisms, but its excessive use by man makes lead a problem (Roane, 1996). When lead is released into the environment it has long residence time compared with most other pollutants. Lead in the form of PbO_2 is used to fabricate the lead-acid accumulator battery (Alloway, 1990). Lead is used in automotive electric batteries, solder for electronic devices and pigments for house paints until the 1950s (Wilner, 1997). All of these materials can be seen in Table 1 as sources of hazardous wastes.

Resistance of soil bacteria towards lead has been studied in soil contaminated by smelting wastes in Derbyshire, England, although the percentage of resistance is less in lead-contaminated soil than in those polluted with zinc or cadmium (Alloway, 1990). A few studies have investigated microbial resistance to lead. Roane (1996) found that in *Pseudomonas marginalis*, the lead resistance level was of 2.5 mM of total lead, involving an extracellular lead exclusion mechanism. The only detailed study on the molecular basis of lead resistance is carried out on *Alcaligens eutrophus* CH:34. Other bacterial species studied include *Thibacillus intermedius, Escherichia coli,* and *Alcaligens radiobacter* strain which were found to be extremely sensitive to lead under high-level, short-term exposure (Yoshida *et al.*, 1998). Dietrich (2000) reported that Pb-resistance isolates they found were all gram-negative bacteria such as *Pseudomonas* spp. or *Actinomycetes*.

1.4 Resistance to Silver

Silver has been used in a variety of application since ancient times to control infections and spoilage. Its compounds and products are increasingly utilized as antimicrobicidal agents in hygiene, agricultural and industry, in addition to clinical uses (Silver *et al.*, 1996). The toxicity of silver makes it unattractive as trace element, but it has been used for a long time for medical purposes (Slawson *et al.*, 1992). For example, diluted silver nitrate is used as an antiseptic (Wilner, 1997). Major contemporary uses of silver include the production of very thin films for electroplating; manufacture of reflecting mirrors, electrical contacts, alloys, coinage and jewelry; and various applications in dentistry, optics, and photography (Alloway, 1990). These materials also can be seen in Table 1 as sources of hazardous wastes. Silver compounds are used as antimicrobial agents in medicine and bacteria that develop resistance to silver cation (Ag^+) pose problems similar to those antibiotic-resistant bacteria (Gupta *et al.*, 2001).

In recent years, new scientific evidence suggests that certain microorganisms can overcome silver lethal effect. Some bacterial communities have been isolated from soils which are able to tolerate high concentrations of Silver (100 mM Ag^+) (Dietrich, 2000). These soil bacteria, particularly *Pseudomanas* species and *Thiobacillus*, have the ability to accumulate silver and become tolerant to the metal (Alloway, 1990). In *Pseudomonas* species, silver is reduced to the metallic form and complexes with cellular components (Trevors, 1994). Resistances to silver compounds by bacteria have been shown to be due to plasmid-borne resistance genes (Silver, 1998).

Most of the studies of silver disinfection reported either single exposures to bacteria under laboratory conditions and results monitored for a season or less. If these experiments were continued for several years, development of resistant strains would be expected (Meyer, 1999). A search of literature reveals that many microorganisms including bacteria such as from *Salmonella* species are not effectively killed by exposure to silver (Meyer, 1999). Ag+ resistance is an example of such efflux pumping and is the first co-transcribed resistance system that has both classes of efflux pumps (Gupta *et al.*, 1999). Silver-resistant bacteria have frequently been reported (McHugh, 1975), but these initial reports have generally not been followed by further work (Silver, 2003). Silver-resistant bacteria have been found repeatedly in environments where silver toxicity might be expected to select for resistance, in particular from burn wards of hospitals where silver salts (silver nitrate but especially silver sulfadiazine) are used as antiseptics to treat burns Ag(I) binds tightly to the bacterial cell surface, inhibiting respiration and having other toxic effects (Bragg *et al.*, 1974, Gandour *et al.*, 1988, Schreurs *et al.*, 1982). The Ag(I)-resistant Salmonella strain from which pMG101 was isolated resulted in the death of several patients and required closing of the burn ward at the Massachusetts General Hospital (McHugh, 1975). Now that the means for identifying silver resistance determinants in Enterobacteriaceae is available, similar efforts are needed with other common pathogens on large burns (specifically *Pseudomonads* and *Staphylococci*) (Silver, 2003).

1.5 Involvement of Bacterial Plasmids

In addition to the chromosome, many bacteria posses large numbers of tiny circular DNA molecules that may contain only several thousand nucleotide (Watson, 1992). These minichromosomes called plasmid were first discovered as genetic elements that were not linked to the main chromosome and carried genes that conveyed resistance to antibiotics such as tetracyclines or kanamycin (Watson, 1992). Certain plasmids called episomes, has the ability to move in and out of the main chromosomal element. This capacity often reflects the possession of mobile genetic elements whose movements are accomplished through the fusion of two independently replicating DNA units (replicon) (Watson, 1992). Bacterial plasmids contain genes that provide extra functions to the cells, among which resistance to toxic metals.

Plasmids are small circular DNA molecular that can move from one cell to another (Silver, 1998). Plasmids that can integrate into bacterial chromosome can be transferred from one bacterium to another when the cells mate and a copy of the 'male' chromosome is transferred to the 'female' cell (Watson, 1992). Thus, the transfer of toxic metal resistance from one cell to another will be facilitated in bacteria. It is suggested that most of the time, resistance genes are found on these plasmids, but some systems may be determined by chromosomal genes in other organisms. Several studies have focused on the association between plasmids and heavy metal resistance in enteric bacteria isolated from the environment (Baya *et al.*, 1986). It is known that bacteria can transfer resistance plasmids *in situ* to indigenous microflora. Interspecies and intergeneric transfer of R plasmids has also been shown to occur (Baya *et al.*, 1986). Baya *et al.* (1986) reported that bacterial isolates derived from toxic chemical wastes more frequently contain plasmid DNA and also demonstrate antimicrobial resistance.

Because plasmid DNA is so much smaller than even highly fragmented chromosomal DNA, it is easily separable, and highly purified plasmid DNA is readily obtained. In the laboratory, when plasmid DNA is added to plasmid-free bacteria in the presence of Ca^{2+}, the DNA is taken up to yield bacteria that will soon contain many copies of the plasmid. In general, a given bacterial cell usually harbors only one form of plasmid. The number of copies of a plasmid in a host cell depends on the genetic constitution of the plasmid and cell. The so-called relaxed-control plasmids may multiply until each cell has on the average 10-200 copies of the plasmid. In contrast, stringent-control plasmids replicate at about the same rate as the cell's main chromosome and are present in only one or few copies per cell (Watson *et al.*, 1992). Bacterial plasmids encode resistance systems for toxic metal ions, including Ag +, AsO2, AsO 3-, Cd 2+, Co 2+, CrO 2-, Cu 2+, Hg 2+, Ni 2 +, Pb 2 +, Sb 3 +, TeO32-, TI + and Zn 2 + (Silver, 1996).

CHAPTER TWO
MATERIALS AND METHODS

2.1 Sample collection

Soil samples were collected from the Matang Landfill site and the Kuap River downstream for the paper recycling plant in Kuching Division, Sarawak, Malaysia. Soil samples were collected and stored in sterile conical flasks at 4°C until needed.

2.2 Growth Medium

Nutrient agar (NA), Blood Agar Base and Mueller Hinton Agar (MHA) were purchased from Oxoid (UK), and Luria-Bertani (LB) broth was obtained from Fluka (Switzerland). All growth media were sterilized by autoclaving at 121°C for 1 and a half hour.

2.3 Heavy Metal Stock Solution

The heavy metal salts used in this study are shown in **TABLE 2**. All chemicals were of analytical reagents grade. Heavy metal stock solutions were prepared by dissolving the respective heavy metal salts in double distilled water. The concentration for the stock solutions were calculated based on the solubility of the heavy metal salts in water taking into consideration the working concentration for each heavy metal salt.

TABLE 2: Heavy Metal salts used in this study

Heavy metal salts	Heavy metal cations	Stock concentrations (mg/ml)	Working concentrations (µg/ml)
$Pb(NO_3)_2$	Lead, Pb^{2+}	400	3000-5000
$AgNO_3$	Silver, Ag^{+}	25	25-100

2.4 Isolation of bacterial strains from soil samples

Soils samples were first suspended in sterile distilled water (1 g/ml) before being serially diluted in peptone water from 10^{-1} to 10^{-4}. Aliqouts (0.1 µl) of the 10^{-1} to 10^{-4} dilution were spread on nutrient agar (NA) plates. Two replicates were prepared for each dilution and plates were incubated at 37°C overnight. Bacteria colonies were sub-cultured to obtain pure cultures before screening for heavy metal isolates.

2.5 Screening and identification of heavy metal resistant bacteria isolates

Bacterial colonies recovered on NA after overnight incubation at 37°C were picked and spotted on MHA plates supplemented with various heavy metal salts at various concentrations as shown in Table 2. The selective agar plates were incubated at 37°C overnight. Isolates that were able to grow at the highest concentrations of metal supplements were subsequently re-streaked on selective agar plates to confirm their resistance properties. Heavy metal-resistant isolates were examined for Gram stain reaction.

2.5.1 Growth of Bacterial Strain on Selective Media for Identification

For MacCONKEY agar (Merckoplate) and EMB agar (Merckoplate), the heavy metal resistances bacteria isolate were inoculated by spreading on the surface of the plates. Then they were grown for 18-24 hours at 35°C.

For Salmonella Shigella Agar (Merckoplate), heavy metal resistance strains were spread from an enrichment culture on the surface of the culture medium. They were grown for 18-24 hours at 35°C aerobically.

2.5.2 Biochemical Tests (Mesa Community College, 1998)

Methyl red and Voges-Proskauer tests

Bacteria were inoculated into MRVP broth and then incubated at 35°C for 48 hours. After incubation, 1/3 of the suspension were poured into a clean non-sterile tube and were subjected to the VP test by adding 12 drops of Barritt's A and 4 drops of Barritt's B. The other 2/3 was run with MR test by adding 6-8 drops of methyl red reagents.

Oxidase Test

A piece of filter paper was place in an unused Petri dish and flooded with freshly made 1% aqueous solution of TETRAMETHYL-p-PHENYLENEDIAMINE HYDROCHLORIDE. Distilled water was added to the solid and the plate was rocked before pouring of excess fluid. Bacteria were smeared across the wet filter paper using the corner of a cover slip and color changes were observed.

Citrate Utilization Test

Simmon's Citrate Agar (Merckoplate), was streaked with the bacteria and then incubated at 37°C for 48 hours.

H_2S production and Motility Test

SIM agar was prepared in a screw-cap test tube. Then, the heavy metal resistance isolated was inoculated by stabbing the agar. The culture was then incubated for 24-48 hours at 35°C. The presence of black precipitate along the line of the stab inoculation was examined.

TSI Agar

The media were prepared by slanting screw cap test tube and kept room temperature until used. Inoculation was done by long straight wire and stabbed into the deep of the tube and also streaked the slant surface with a back and forth motion. The culture was then incubated at 35°C for 18-24 hours.

2.5.3 API 20-E System

The system has 20 miniature capsule reaction chambers which can run 23 biochemical tests following the procedure recommended by the manufacturer (Biomeriux).

2.6 Measurement of minimal inhibitory concentrations (MIC)

The level of heavy metal resistance was measured using minimal inhibitory concentrations (MIC) assays for each of the respective heavy metals. A single colony was inoculated in LB broth and grown overnight at 37°C. A 10^{-1} dilution of the culture was prepared and 100 µl of this dilution was spread on MHA plates added with different concentrations of heavy metal salt solutions. The plates were incubated at 37°C overnight and the MIC is defined as the lowest concentration that completely inhibits growth. The range of concentrations of the respective heavy metal salts are shown in **TABLE 3**.

TABLE 3: Concentrations of Heavy metals used

Heavy Metal ions	Heavy metal concentrations (µl/ml)
Pb^{2+}	50-100-200-400-800-1200-1600-2000
Ag^{+}	15-25-50

2.7 Mini Plasmid Prep (Alkaline Lysis Method) (Blake, 2004)

The resistant bacterial strains obtained from the selective media were then inoculated into nutrient broth and incubated at 30°C with vigorous shaking at 180 rpm, overnight. 1.5 ml of the culture was poured into an Eppendorf tube. The tube was then spun for 30 seconds to pellet it. The supernatant was poured off and the tube was air-dried. The pellet was resuspended by vortexing in 100µl of Solution 1 (50 mM glucose, 10 mM EDTA, 25 mM Tris <pH 8.0>). Then, 200µl of solution 2 (0.2 NaOH, 1% SDS) were added into the tube. The

Eppendorf tube was inverted several times to mix the content. The tube was left on ice for 5 minutes. 150µl of cold solution 3 was then added into the tube. The tube was then inverted for several times to mix. Then, it was centrifuged for 5 minutes. 350µl of the supernatant were transferred into a fresh tube and phenol/chloroform solution was added for extraction of plasmid DNA. 2 volumes of EtOH were added at room temperature. The tube was vortexed and centrifuged for 1 minute at full speed (14, 000) rpm. The spin column was discarded and the plasmid was contained in the phenol/chloroform solution in the collection Eppendorf tube. The plasmid identified was analyzed by Agarose Gel Electrophoresis.

2.8 Agarose Gel Electrophoresis

Agarose gel of 0.8% was prepared by melting the agarose in 1X TAE buffer. Exactly 5 µl of DNA sample was mixed with 1 µl of 6X gel loading dye before being loaded into the well. Electrophoresis was conducted at 90 V for 1-2 hours. After electrophoresis, the gel was stained in 1.0 g/µl of ethidium bromide for 20 minutes. The gel was then viewed under the UV transilluminator. Pictures of the gel was taken by using Direct Screen Polaroid Camera DS34 manufactured by Ultralum, Inc.

CHAPTER 3
RESULTS AND DISCUSSION

3.1 Isolation, Purification and Identification of Bacterial Strains

3.1.1 Isolation of bacterial isolates

Bacteria were isolated from Site 1 (Kuap River), downstream from the wastewater discharge pond of a paper recycling plant and Site 2, the municipal landfill site at Matang. A total of 120 bacterial strains were isolated from each site.

Morphological observation of these bacteria on Nutrient Agar showed that most of the bacterial isolated formed yellowish and circular colonies. Some of them were also creamy, sticky, beige, with rough circular forms. Orange, red and even purple pigments were also observed from some of the colonies. Morphological examination also showed that some bacteria were in filamentous and rhizoid forms. **FIGURE 1** is an example of the morphological observation. Subs-culturing were done to obtained fresh cultures for the screening of heavy metal resistance bacterial.

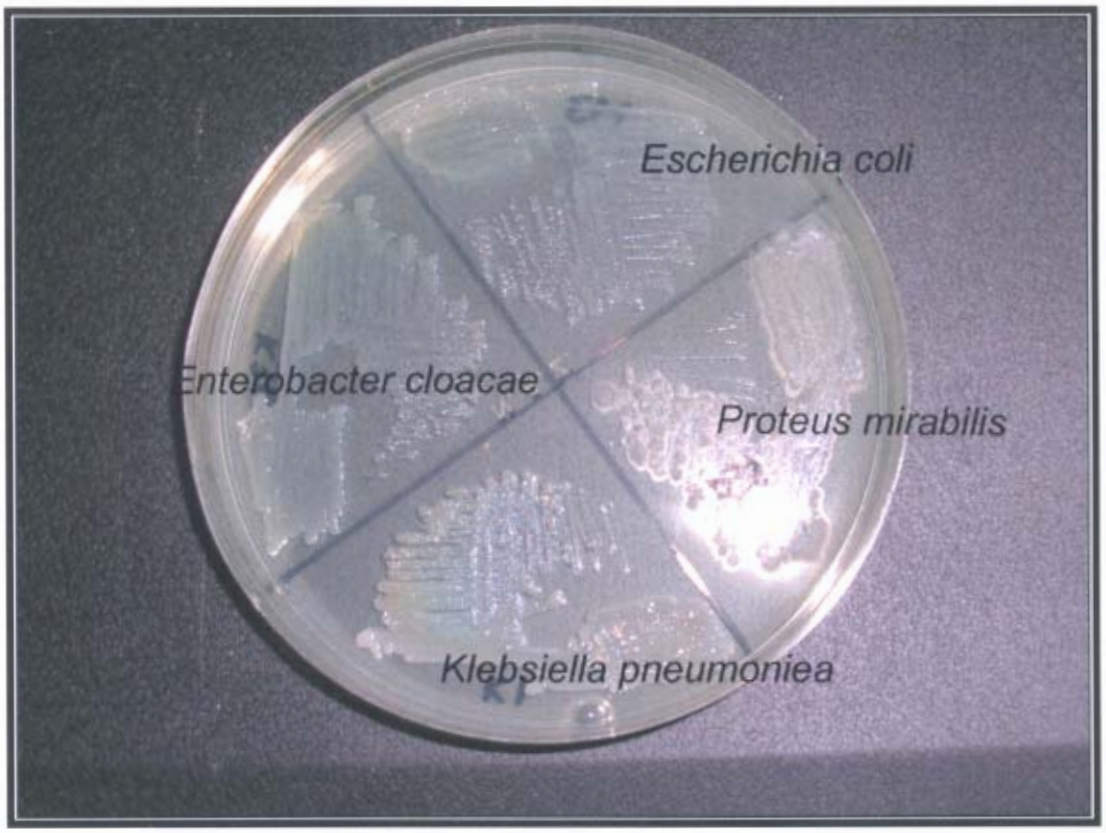

Figure 1: Example of morphological characteristics of the bacterial isolates.

3.1.2 Identification of Bacteria Isolates

Gram staining was done on the bacteria isolated and most of them were rod-shape and coccus-shape gram negative bacteria based on microscopic analyses. From the literature review, it is learnt that only gram negative bacteria shown resistance abilities towards heavy metal. Therefore, the gram positive bacteria were omitted from further identification while the gram negative strains were subjected to further analysis. Biochemical tests were done first for a presumptive test before the API 20-E kits were used to confirm the bacteria taxonomic groups. However, only 12 strains from each site were taken for the biochemical tests. The results of biochemical test on the bacterial isolated were shown in **TABLE 4.**

TABLE 4: Biochemical Test Results

Strain Designation	TSI Agar		Oxidase	Citrate	MR	VP	H_2S & Motility
	Butt	Slant					
M1	Black	Yellow	+	-	-	+	+
M3	Yellow	Yellow	-	-	+	-	-
M17	Black	Yellow	+	+	-	+	+
M19	Black	Yellow	-	-	-	+	+
M22	Black	Yellow	+	-	+	-	+
M30	Black	Yellow	+	-	-	+	+
M35	Black	Yellow	+	-	-	+	+
M39	Nil	Nil	Nil	Nil	Nil	Nil	Nil
M41	Nil	Nil	Nil	Nil	Nil	Nil	Nil
M43	Black	Yellow	+	Nil	Nil	Nil	+
M48	Black	Yellow	-	+	Nil	Nil	+
M49	Yellow	Yellow	+	+	-	+	-
K1	Yellow	Yellow	+	+	-	+	-
K3	Nil	Nil	Nil	Nil	Nil	Nil	Nil
K4	Red Black	Yellow	+	+	Nil	Nil	-
K6	Red	Yellow	-	+	+	-	Nil
K17	Nil	Nil	+	-	-	+	-
K21	Yellow	Red	+	+	-	+	-
K36	Yellow	Yellow	+	+	-	-	-
K60	Yellow	Yellow	+	-	-	-	-
K61		Nil	Nil	Nil	Nil	Nil	Nil
K71	Yellow	Yellow	+	+	-	+	-
K75	Yellow	Red	+	+	-	+	-
K76	Red	Yellow	-	-	-	+	-

Note: Strains with prefix 'M' were isolated from the Matang Landfill site soil sample. Those with the prefix 'K' were from the Kuap river soil samples.

After the various biochemical tests were done, further identification were done using selective media. The selective media used are EMB agar, MacCONKEY agar and Salmonella agar. The results are shown in **TABLE 5**.

TABLE 5: Selective Media Test

Strain Designation	MacCONKEY	EMB	Salmonella Shigella
M1	Pink	Purple	Nil
M3	Pink to colorless	Green metallic	Dark blue
M17	Pink red	Purple to black	Dark blue
M19	Red	Purple	Dark blue
M22	Colorless	Purple	Rust color
M30	Colorless	Nil	Dark blue
M35	Colorless	Purple	Dark purple
M39	Red	Transparent	Nil
M41	Red	Transparent	Nil
M43	Red	Purple	Purple
M48	Pink	Purple to black	Dark purple
M49	Red	Purple to black	Dark blue
K1	Pink	Purple to black	Blue
K3	Nil	Nil	Nil
K4	Nil	Transparent	Nil
K6	Nil	Transparent	Nil
K17	Nil	Red	Nil
K21	Nil	None	Nil
K36	Colorless	Purple	Pink
K60	Colorless	Transparent	Red
K61	None	Nil	Nil
K71	Pink	Purple to black	Rust color
K75	Colorless	Purple	Rust color
K76	Colorless	Nil	Nil

Note: Strains with prefix 'M' were isolated from the Matang Landfill site soil sample. Those with the prefix 'K' were from the Kuap river soil samples.

Various biochemical tests and later on the usage of selective media had yielded preliminary characterization of the bacterial species that were isolated from both sites. The results are shown on **TABLE 6**.