ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF MICROORGANISMS FROM SOIL CONTAMINATED WITH PESTICIDE

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A thesis submitted in fulfillment
of the requirements for the award of the degree of
Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical & Natural Resources Engineering
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May, 2008

DECLARATION

I declare that this thesis entitled “Isolation, Characterization and Identification of Microorganisms from Soil Contaminated with Pesticide.” Is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.”

Signature	:…….
Name of Candidate	: Adawiah Binti Ismail
Date	: 16th May 2008

DEDICATION

Special Dedication to my family members that always support me,
My friends, my fellow colleague
and all faculty members

For all your Care, Support and Believe in me.

ACKNOWLEDGEMENTS

In the name of Allah, the Most Gracious, and the Most Merciful. Alhamdulillah, finally I finished my thesis. Here, I would like to forward my appreciation to my thesis supervisor, Mrs. Nina Suhaity Binti Azmi for her guidance and support.

My fellow colleagues should be noted for their support. Thank you for the time sacrificed to accompany me when I'm down and the time we share our University life I would like to extend my gratitude to everyone who has been helping me directly or indirectly from the beginning until the final stage of this project.

To all the staff in Faculty of Chemical & Natural Resources Engineering, very big thanks you to all because of their sincerity and patient .I'm very thankful because I have been surround with people that always give me support. Thank you very much, may Allah bless you all.

ABSTRACT

This study is investigated the type of microorganisms that have a history of been treated of pesticide. When the microorganisms can survive in pollutant environment, it has potential to degrade substance that harmful to our environment. This is because environmental pollution, caused by the leaking of chemical fertilizers and pesticides to surface and groundwater, caused serious environmental and social problems throughout the world. The soil samples had been taken from dragon fruit plantation that has history in using of pesticide. Isolation of these microorganisms until get single colony had been done by using serial dilution method and streaking method. For the identification of these microorganisms, several biochemical identification methods such as Gram stain, Starch Hydrolysis and Triple sugar–Iron Agar Test had been used. As a result, four single colonies that represent Cocci in their morphology had been isolated. Sample 1 and 2 show its characteristic as gram negative in gram staining, meanwhile for Sample 3 and 4, its represent as gram positive. There were starch hydrolysis for Sample 1 and 4, but no starch hydrolysis for Sample 2 and 3. At the end of this study, Sample 1 and Sample 2 can be classified as *Neisseria* or *Veillonella* species. Meanwhile, Sample 3 and Sample 4 can be classified as *Micrococcus sp* or *Staphylococcus sp.* For further study, all of these samples can be introduce into bioremediation technique to study its potential in degrading pesticide.

ABSTRAK

Kajian ini adalah untuk mengkaji jenis mikroorganisma yang mempunyai sejarah yang telah dirawat dengan racun serangga. Apabila mikroorganisma itu boleh bertahan di kawasan yang tercemar, ia mempunyai potensi untuk mengurai bahan yang berbahaya kepada persekitaran kita. Ini kerana pencemaran alam sekitar, yang disebabkan oleh kebocoran bahan kimia dan racun serangga kepada permukaan dan air tanah menjadi masalah persekitaran yang serius dan masalah sosial di seluruh dunia. Sampel tanah ini di ambil dari tanaman buah naga yang mana ia mempunyai sejarah penggunaan racun serangga. Pengasingan mikroorganisma ini sehingga mendapat satu koloni, telah dibuat dengan menggunakan cara pencairan berturut-turut dan kaedah mengores. Untuk pengecaman mikroorganisma ini, beberapa cara pengecaman biokimia seperti *Gram stain*, *Starch Hydrolysis* and ujian *Triple sugar–Iron Agar* telah digunakan. Keputusannya, empat koloni yang mempunyai bentuk Cocci telah diasingkan. Sampel 1dan 2 menunjukkan sifat gram negatif dalam gram staining, manakala Sampel 3 dan 4 menunjukkan gram positif. Terdapat hidrolisis kanji pada Sampel 1 dan 4, tapi tiada hidrolisis kanji pada Sampel 2 dan 3. Di akhir kajian ini, Sampel 1 and Sampel 2 boleh diklasifikasikan sebagai spesis *Neisseria* atau *Veillonella*. Manakala, Sampel 3 dan Sampel 4 boleh diklasifikasikan sebagai spesis *Micrococcus sp* atau *Staphylococcus sp.* Untuk kajian lanjutan, kesemua sampel ini boleh diperkenalkan kepada teknik bioremediasi untuk mengkaji kebolehan ia dalam mengurai racun serangga.

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%	-	Percentage
µ	-	Micro
h	-	Hours
L	-	liter
M	-	molar
min	-	minutes
ml	-	milliliters
^{o}C	-	degree Celsius
pH	-	potential hydrogen
v	-	Volume
w	-	Weight

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CHAPTER 1

INTRODUCTION

1.1 Background of study

Increasing environmental awareness has resulted in regulatory measures that aim to remedy past mistakes and protect the environment from future contamination and exploitation. These measures intend to preserve the environment and protect human health. Some of the pollutants of concern are chemical from pesticide, were banned when it was discovered that they were hazardous to human health. In our country, about 99 per cent of the pesticides are imported in bulk and in concentrated form (based on 1996 statistic). They are diluted and/or mixed with other chemicals by local manufacturers to obtain the formulation desired for local conditions. Unfortunately, in many cases, these compounds are also persistent in nature. Long after their use has been discontinued, these chemicals remain in soils and sediments where they can enter the food chain directly or percolate down to the water table. Once in the groundwater, these pollutants can enter drinking water wells and cause health problems.

Indirect accumulation in higher trophic level organisms, such as mammals, may cause health problems over time because of the increasing levels of toxic compounds within the body. A degree of persistence is often desired in chemicals such as pesticides. If microorganisms degraded them as soon as they were applied, then they would not serve their desired function. There are two main reasons that these compounds persist in nature. First, the conditions necessary for their biodegradation are not present. The microorganisms that are capable of biodegrading these toxic compounds may be absent at the contaminated site (Frazar, 2000). If the necessary microorganisms are present,

some limiting factor, such as a nutrient shortage, may create unfavorable conditions for the biodegradation of the contaminant (Frazar, 2000). The second possibility is that the compound could be recalcitrant, or resistant to biodegradation. Compounds are recalcitrant for a number of reasons. The compound may be unable to cross the cell membrane for breakdown by intracellular microbial enzymes (Frazar, 2000).

However, there were some microorganisms can survive in the place that contaminated with pesticide. So, identification and characterization of these microorganisms is important to study about its potential as a microorganism that can degrade the pesticide or use in bioremediation. Where bioremediation can be defined as a natural process which relies on bacteria, fungi, and plants to alter contaminants as these organisms carry out their normal life functions. Metabolic processes of these organisms are capable of using chemical contaminants as an energy source, rendering the contaminants harmless or less toxic products in most cases.

1.2 Objectives of the study

The objective of the study is:

i. To study about microorganisms that can live in a place that contaminated with pesticide usage.

1.3 Scope of the study

The scopes of this study are as follows:

i. To isolate the microorganisms.
ii. To identify and characterize the microorganisms.

1.4 Problem statement

Malaysia agriculture sector has been growing rapidly over the years. In order to obstruct the insect from attacking their crops, farmers use pesticide. Pesticide encompasses a variety of different types of chemicals including herbicides, insecticides, fungicides, and rodenticides, among others (Frazar, 2000). For chlorinated agrochemicals they are characterized by their long persistence, toxicity, and teratogenicity. They are widely used for pest control worldwide, especially in the developing countries. Their residues result in serious problems not only in the cultivated soils where they are applied, but also in the crops that systemically retain part of these residues, in non-target organisms, and finally in surface and underground water, through runoff an seepage of agricultural drainage. However, due to its high persistence and illegal applications, residues still contaminate some environments that serve as the last destination for agricultural drainage. But certain microorganisms, can survive in this contaminated place. In order to find local sources of microorganisms that have a potential as pesticide degrading microorganism, Dragon Fruit Plantation is the target place to make a research about isolation of these microorganisms.

CHAPTER 2

LITERATURE REVIEW

2.1 Environmental fate

Pesticides are often applied directly to soil. They may also reach the soil through application to foliage via spray drift, run-off, or wash-off vectors (Racke *et al.*, 1990). Pesticides in soil partition between at least three phases: soi1 air, soil solution, and soil sorbed (Glotfelty *et al.*, 1989). Pesticides have therefore become integrated into transport and degradation processes that characterize soi1 ecosystems (Sawhney *et al.*, 1986). This review examines the major environmental fates of pesticide compounds including volatilization, leaching, adsorption, photodecomposition, degradation by other non-biological processes, and biodegradation. Emphasis is placed on the physical and chemical properties of pesticides and soil with respect to their influence on environmental fate. The major fates for these pesticides are summarized in Table 2.1.

Major factors influencing the fate of pesticides in the environment are their volatility, sorption ion mineral or organic matter, solubility, and biological and non-biological degradation. Pesticide biodegradation involves a wide variety of microorganisms including bacteria and fungi operating under dynamic anaerobic and aerobic conditions. It is suggested that biodegradation of pesticides in soil ecosystems can only take place through the synergistic interactions of a microbial consortium, the activity of which is affected by many soil physical and chemical properties, as well as the nature and extent of the pesticide contamination. Many pesticides have proven resistant to microbial biodegradation and therefore persist in the environments in which they are found. Enhance biodegradation of pesticides in agricultural soils and the ability

of microorganisms to adapt and rapidly catabolic some pesticides have resulted in economically significant pest control failures (Racke *et al.*, 1990). This recognition of microbial degradation as a primary means of degrading many pesticides in soil ecosystems prompted the development of biodegradable herbicides, insecticides, and Fungicides in the mid 1970's (Racke *et al.*, 1990). Ideally, these pesticides would persist only long enough to complete their intended mission or benefit and then degrade to harmless products. The fate and effects of pesticides in mil, however, is extremely complicated. Numerous interactions between the solid, liquid and gaseous phases of soil and between living and biotic components of soil significantly influence the environmental fate of pesticides in soil.

An understanding of the soil processes affecting pesticides is essential if methods for controlling pesticide persistence and minimizing undesirable environmental effects are to be found (Kaufman *et al.*, 1974). This statement is true with respect to the use of soil microorganisms to remediate soils contaminated with organic pollutants. Conditions must be favorable for growth and survival of pesticide-degrading microorganisms. In addition, contaminants must be accessible to microorganisms that degrade them. The biological availability of soil contaminants, such as pesticides, is determined by their fate in the environment, which is directly influenced by soil processes. The soil processes themselves are greatly affected by physical and chemical properties of mils and soil contaminants.

Table 2.1: Major Fates for Pesticides (Fisher *et al.*, 1999)

Pesticide	Volatilization Potential	Leaching Potential	Potential for Adsorption to Soil Colloids	Potential for Adsorption to Organic Matter	Non-Bio Degradation Potential	Biotransformation (Mineralization) Potential	Factors Reported to Enhance Biodegradation
DDT	Low	Low	Low	High	Low	Moderate (low)	Anaerobic condition; high moisture content; low redox potential; nonionic surfactants; fungal enzymes; metal porphyrins; finely ground solid organic matter; alternating anaerobic and aerobic conditions; fungal applications.
DDD	Low	Low	Low	High	Low	Low to moderate	
DDE	Low	Low	Low	High	Low	Low to moderate	
2,4-D	Low	High	Low	Low to moderate	Low	High (moderate to high)	Anaerobic conditions; high moisture content; fungal applications.
2,4,5-T	Low	High	Low	Low to moderate	Low	Low to moderate (low)	Anaerobic conditions; high moisture content; fungal applications;

2.2 Environmental Factors Affecting Biodegradation of Pesticide

2.2.1 Bioavailability

The persistence of organic contaminants is often mediated, in part, by the extent of partitioning between liquid and solid phases of the soil. Compounds with high solubility are more mobile and susceptible to leaching, but are also in closer proximity to microorganisms in the liquid phase. Sorption to the surface of soil particles reduces mobility but increases the proximity of contaminants to surface-bound microorganisms. Thus, the same factors that affect solubility and sorption of pesticide influence their movement within the soil matrix, thereby affecting their bioavailability and biodegradation. Factors that affect the volatility of pesticide (temperature, humidity, vapor pressure, soil organic matter and moisture) can influence biodegradation rates, in that the extent to which they volatilize through air pockets of the soil, or escape from the surface, affect their concentrations in the solid and liquid phases of the soil and, as a consequence, their bioavailability (El Beit *et al.*, 1981). It is generally assumed, for organic contaminants, that increased soil organic matter results in increased adsorption (El Beit *et al.*, 1981), thus reducing bioavailability.

However, a higher organic matter content might also increase microbial activity thus enhancing biodegradation rates. In addition to affecting sorption and soil pH, soil composition can also affect bulk density and water retention, all of which, in turn, affect aeration, nutrient availability, bioavailability and biodegradability.

In general, the optimum temperature for pesticide biodegradation ranges from 25 to 30°C in soil, soil slurry, and bacterial cultures (Siddique *et al.,* 2002).The optimum temperature for degradation may vary in field environments depending on the bacterial population. An increase in temperature might enhance pesticide removal simply by increasing biological activity (within limits), or might also affect biodegradation by enhancing bioavailability through reduced sorption (El Beit *et al.*, 1981). Bacteria capable of degrading pesticide at extreme temperatures (<5°C or >40°C) have not been

reported. Some studies suggest that the influence of temperature is not as significant as that of other soil conditions and characteristics (i.e., redox conditions, moisture content) that affect volatility, sorption and bioavailability. Increased soil water may also enhance bioavailability if the degradation rate is limited by dissolved pesticide concentrations or desorption velocities (Rijnaarts *et al.,* 1990), unless the pesticide is so hydrophobic that dissolution depends on biosurfactants, in which case, lower moisture contents may be more desirable.

2.2.2 Volatilization

The aerial transport of pesticides from soil to the atmosphere (volatilization) has received considerable attention. The volatilization of pesticide can be influenced by soil moisture content and may be facilitated by a proposed wicking or capillary effect, through which more compounds that are soluble are brought to the soil surface more quickly and compounds that are more volatile disappear from the surface more rapidly (Spencer *et al.,* 1973). Although soil moisture content is critical (Glotfelty *et al.*, 1989), volatilization of pesticides in soil is also dependent on temperature, soil organic matter (influencing sorption), ambient relative humidity and other interacting factors. For example, it has been observed that adsorption of pesticide decreases as temperature increases (El Beit *et al.,* 1981). Soil moisture content has been identified as a key factor influencing pesticide transport within soil and, along with temperature, was incorporated into a model for volatilization from surface soil (Cohen *et al.,* 1989). Paraiba *et al.,* 2002 reported a relationship between soil temperature and the retardation factor, a calculated number that represents the delay of pesticide leaching in soil as it is affected by sorption, volatilization and solubility. Oxic bioremediation practices for contaminated soil normally involve tillage to promote aeration. It is important to consider that tillage not only promotes aeration and aerobic biodegradation processes, but affects the exposed surface area and soil moisture content, with consequences with respect to volatilization (Glotfelty *et al.,* 1989).

2.2.3 Physical and chemical properties of pesticides

The volatility of a particular pesticide in soil is dependent on a variety of physical and chemical properties of that pesticide. Vapor pressure, solubility in water, nucleus type and number, kind(s), and position(s) of functional groups all play important roles and can greatly modify the kinetics of volatilization (Racke, 1990) and enable volatility of a compound to be predicted. Generally, volatilization from soil is only considered a factor in the fate of pesticides if the vapor pressure of the compound is greater than 10^4 mmHg (Racke, 1990)

2.2.4 Concentration of pesticide

A significant influence on biodegradation of pesticide could be the concentration at which they are present in contaminated soils. There is evidence that pesticide biodegradation rates in soil follow first-order kinetics and are concentration dependent. Pesticide may affect soil microbial populations, stimulating growth of certain microorganisms and exerting toxic effects and inhibiting growth of others. In soil and culture media containing higher pesticide concentrations, that are more representative of what might be found at post-production sites and waste disposal sites, the numbers of some microbial species or their activities may be affected. Bioremediation of pesticide contaminated soil is usually only necessary on industrial post-production, or waste dumping sites, as opposed to crop fields where application rates were lower and the pesticide are expected to be degraded by naturally occurring microorganisms.

2.3 Hazardous characteristics of pesticide

Majority of pesticides are liquid and have different vapor pressures at room temperature. The compounds used for agricultural purposes are available mainly as

emulsifiable concentrates or wet table powder formulations for reconstitution as liquid sprays, but also as granules for soil applications. A limited number are also available as fogging formulations, smokes, impregnated resin strips for use indoors, and as animal or human pharmaceutical preparations.

Dispersion of spray droplets by wind is possible, but in general, only small amounts are likely to be dispersed in this way. All pesticides are subject to degradation by hydrolysis, yielding water-soluble products that are believed to be non-toxic at all practical concentrations. The toxic hazard is therefore essentially short-term in contrast to that of the persistent organochlorine pesticides, although the half-life at neutral pH may vary from a few hours for dichlorvos to several weeks for parathion. At the pH of slightly acidic soils (pH 4 to 5), these half-lives will be extended many times. However, constituents of soil and of river water may themselves catalyse degradation.

2.4 Bioremediation of pesticide

Bioremediation as a technology for reclaiming chemically contaminated land has been steadily growing in acceptance since the 1980's, but detailed strategies for optimizing treatments on sites containing pesticide remain to be discovered. Bioremediation strategies typically involve enhanced natural attenuation, or optimization of the environmental conditions discussed previously, to stimulate growth and biodegradation of pesticide by indigenous microorganisms. Supplemental nutrients and organic amendments may be added to enrich the habitat for degrading organisms. Bioaugmentation (inoculation with previously acclimated pesticide -degrading microorganisms that are not necessarily indigenous to the site) is a less popular approach, albeit a potentially effective one. Each of these approaches can enhance bioremediation, either by increasing the population of microorganisms in soil capable of degrading the target contaminant, or by rendering the contaminant more bioavailable. Combinations of these strategies might be used to further enhance the effectiveness of a treatment protocol.

2.4.1 Inorganic and organic amendments

Amendments for bioremediation of pesticide -contaminated soil can be divided into two general classes. The inorganic amendments include nutrients in the form of fertilizers, salts or metals, and might include emulsifiers/surfactants to improve bioavailability of the contaminant, and lime or other amendments for adjusting pH and improving soil drainage properties. The organic amendments are carbon-based nutrient sources. Often, for economic reasons, organic amendments used in field-scale bioremediation protocols are derived from plant or other natural sources although commercially manufactured organic fertilizers also fall under this category. Before amendments are utilized in situ at field-scale, they are typically tested in bench-scale microcosms of soil or water. Inorganic macronutrient sources of nitrogen (N), phosphorus (P), magnesium (Mg) or potassium (K) might be added in pure form or as constituents of organic fertilizers in a scaled-up bioremediation study. Nitrate, sulfate and ferric iron are commonly used as alternative electron acceptors, in the absence of oxygen (i.e. under anoxic conditions) and might be used to enhance anaerobic degradation processes. Other auxiliary carbon sources, added to enrichment cultures or soils where microorganisms are capable of utilizing pesticide for growth, might either enhance or retard pesticide degradation.

A number of studies have compared multiple sources of carbon for their effect on pesticide removal. Sodium acetate inhibited γ-HCH removal in cultures of Pseudomonas sp. that used γ-HCH as a carbon source to promote aerobic growth in minimal salts media (Sahu *et al*, 1993). With the exception of fertilizers and other compounds made for agricultural use (e.g., urea), nutrient amendments consisting of manufactured chemicals are not cost-effective enough for full-scale bioremediation projects. Plant-derived organic amendments are often used in bioremediation because of the availability of large quantities of raw material at low cost.

The amendments are selected on the basis of desirable C:N:P ratios and not only serve as a nutrient source, but improve aeration or water retention in the soil where required, reduce toxicity and create habitats for indigenous microorganisms. In addition,

because of the high carbon content in plant matter, organic amendments tend to create a respiratory burst, thereby contributing to the generation of anoxic conditions in the soil, especially at high moisture content.

2.5 Soil microbiology

The soil is a complex environment colonized by an immense diversity of microorganisms. Soil microbiology focuses on the soil viruses, bacteria, actinomycetes, fungi, and protozoa, but it has traditionally also included investigations of the soil animals such as the nematodes, mites, and other microarthropods. These organisms, collectively referred to as the soil biota, function in a belowground ecosystem based on plant roots and litter as food sources. Modern soil microbiology represents an integration of microbiology with the concepts of soil science, chemistry, and ecology to understand the functions of microorganisms in the soil environment.

Microorganisms have a key role in the processing of materials that maintain life on the Earth. The transformations of elements between forms are described conceptually as the elemental cycles. Soil microorganisms play key roles in the nitrogen cycle. The atmosphere is approximately 80% nitrogen gas (N_2), a form of nitrogen that is available to plants only when it is transformed to ammonia (NH_3) either by soil bacteria (N_2 fixation) or by humans (manufacture of fertilizers). Soil bacteria also mediate denitrification, which returns nitrogen to the atmosphere by transforming NO_3^- to N_2 or nitrous oxide (N_2O) gas. Microorganisms are crucial to the cycling of sulfur, phosphorus, iron, and many micronutrient trace elements.

The numerous natural substances that are used by microorganisms indicate that soil microorganisms have diverse mechanisms for degrading a variety of compounds. Human activity has polluted the environment with a wide variety of synthetic or processed compounds. Many of these hazardous or toxic substances can be degraded by soil microorganisms. This is the basis for the treatment of contaminated soils by

bioremediation, the use of microorganisms or microbial processes to detoxify and degrade environmental contaminants.

2.6 Bergey's manual

Bergey's manual is a manual that use to identify microbe as to their genus and species. Below are some examples of the identification flow chart of Bergey's Manual.

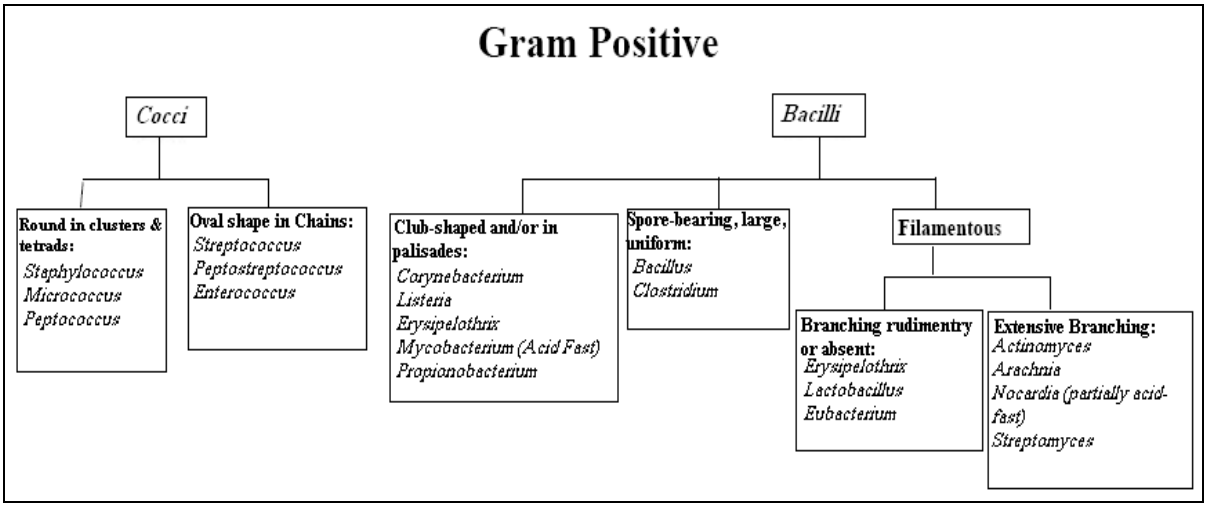

Figure 2.1: Differentiation via Gram Stains and Cell Morphology (Gram Positive)

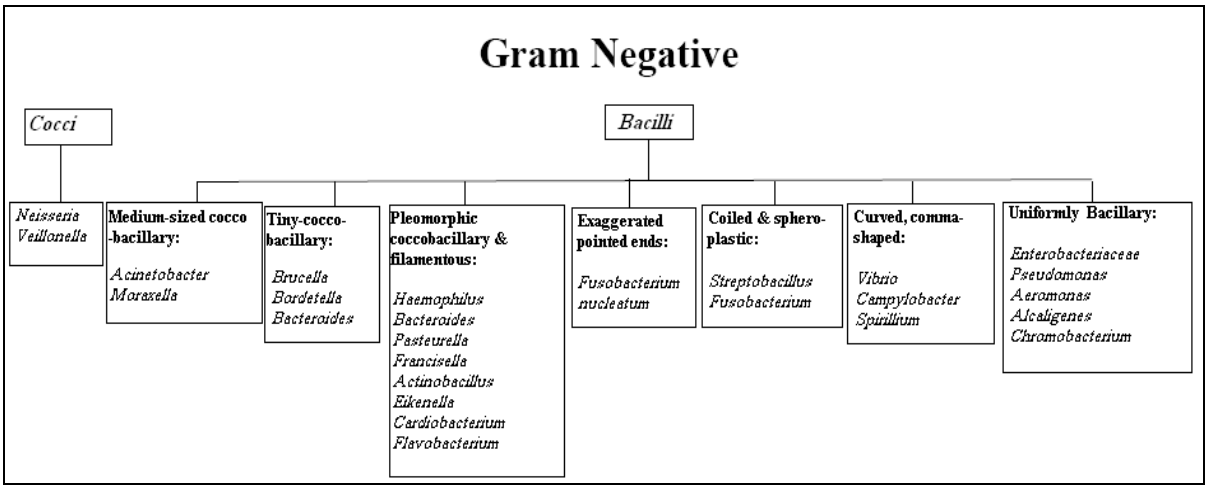

Figure 2.2: Differentiation via Gram Stains and Cell Morphology (Gram Negative)

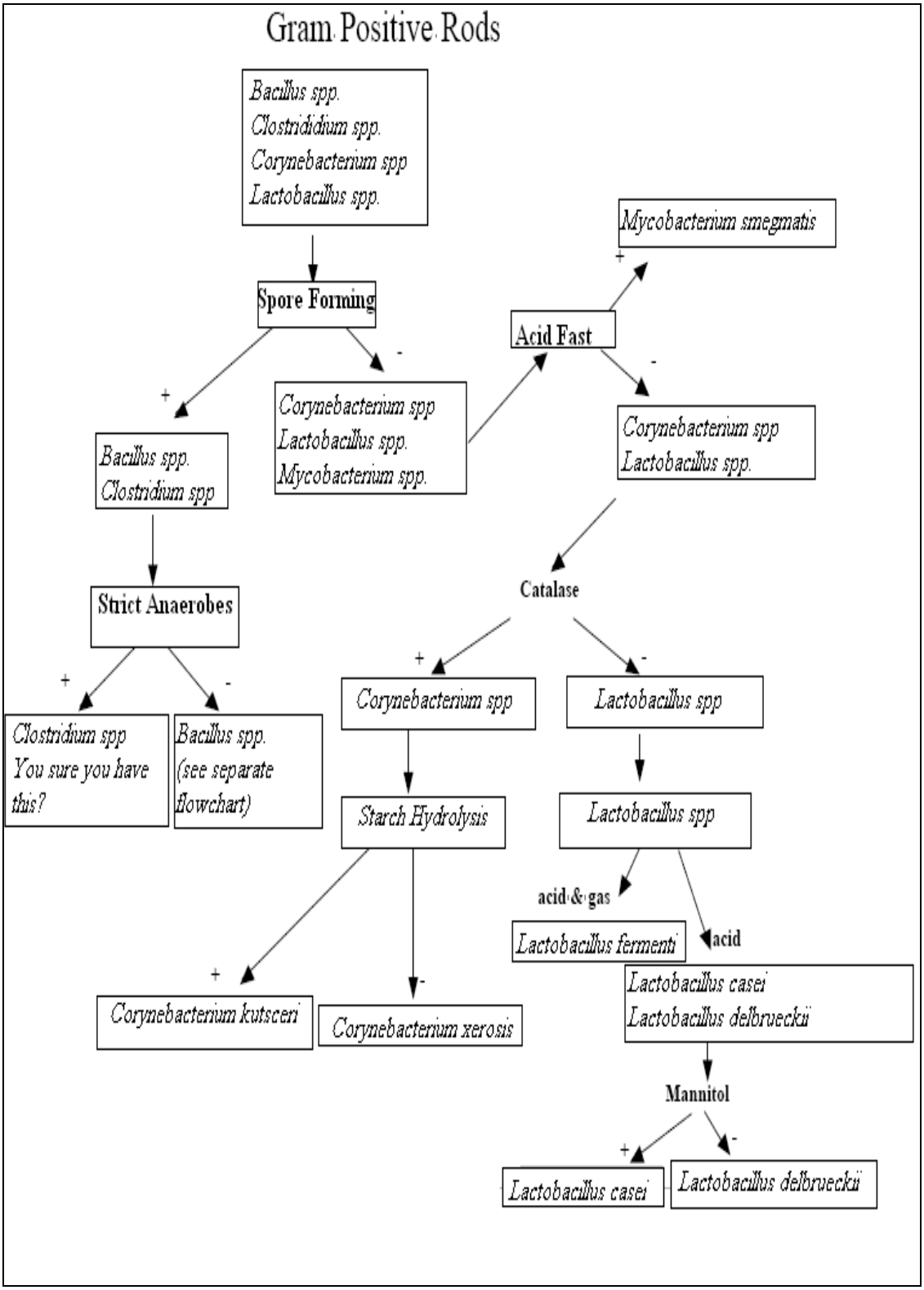

Figure 2.3: Gram Positive Rods ID Flow Chart

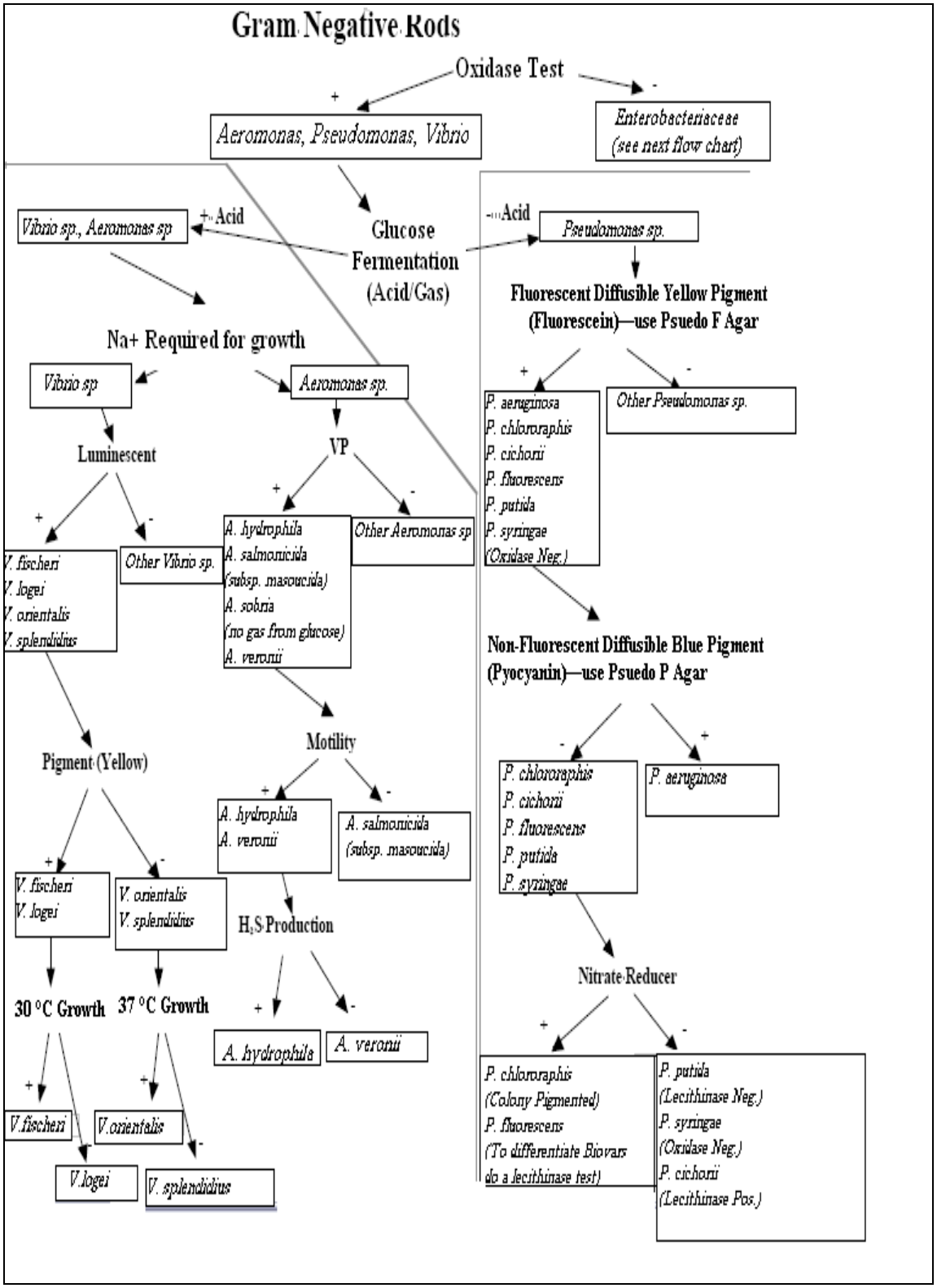

Figure 2.4: Gram Negative Rods ID Flow Chart

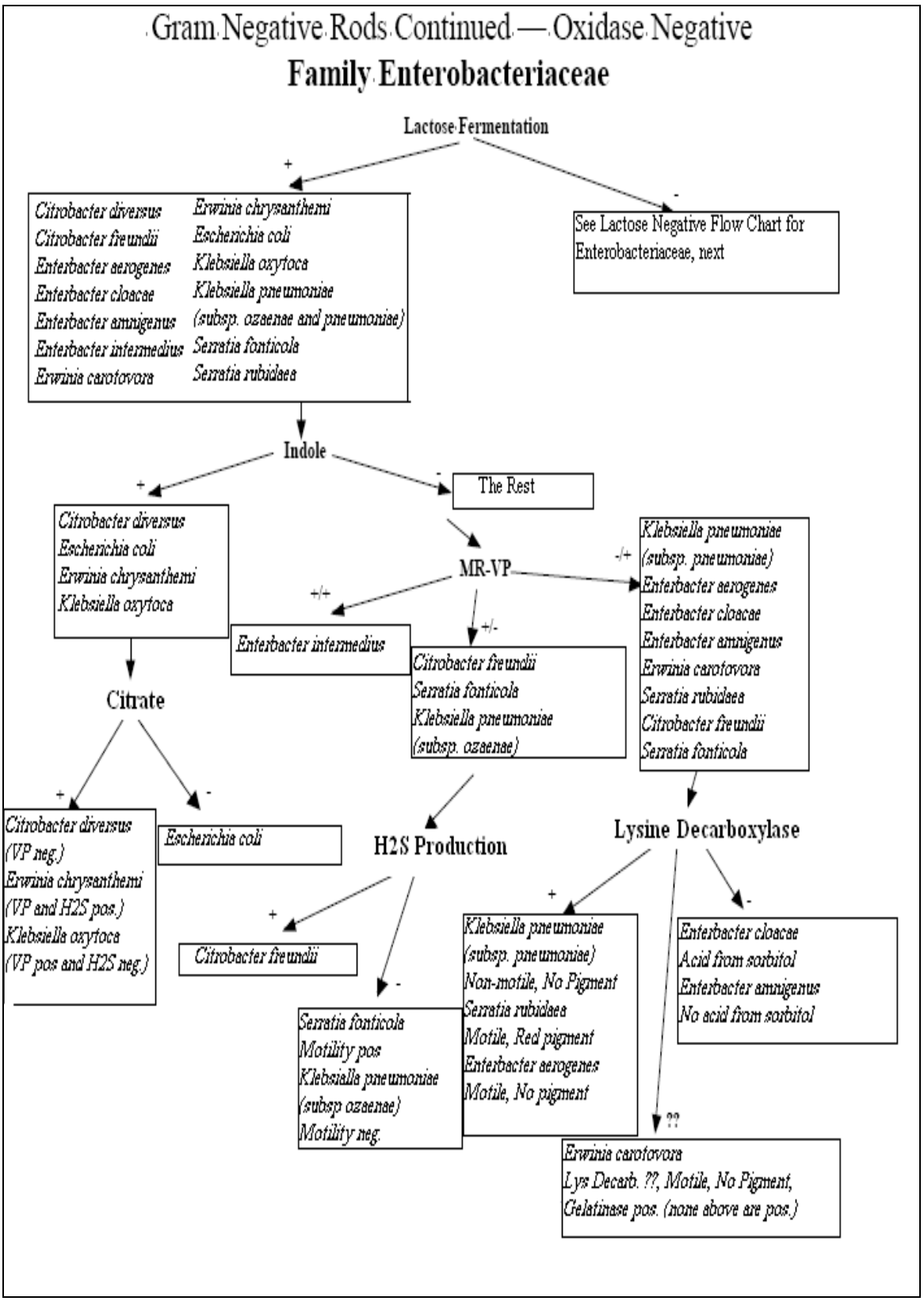

Figure 2.5: Family Enterobacteriaceae Lactose-Positive ID Flowchart

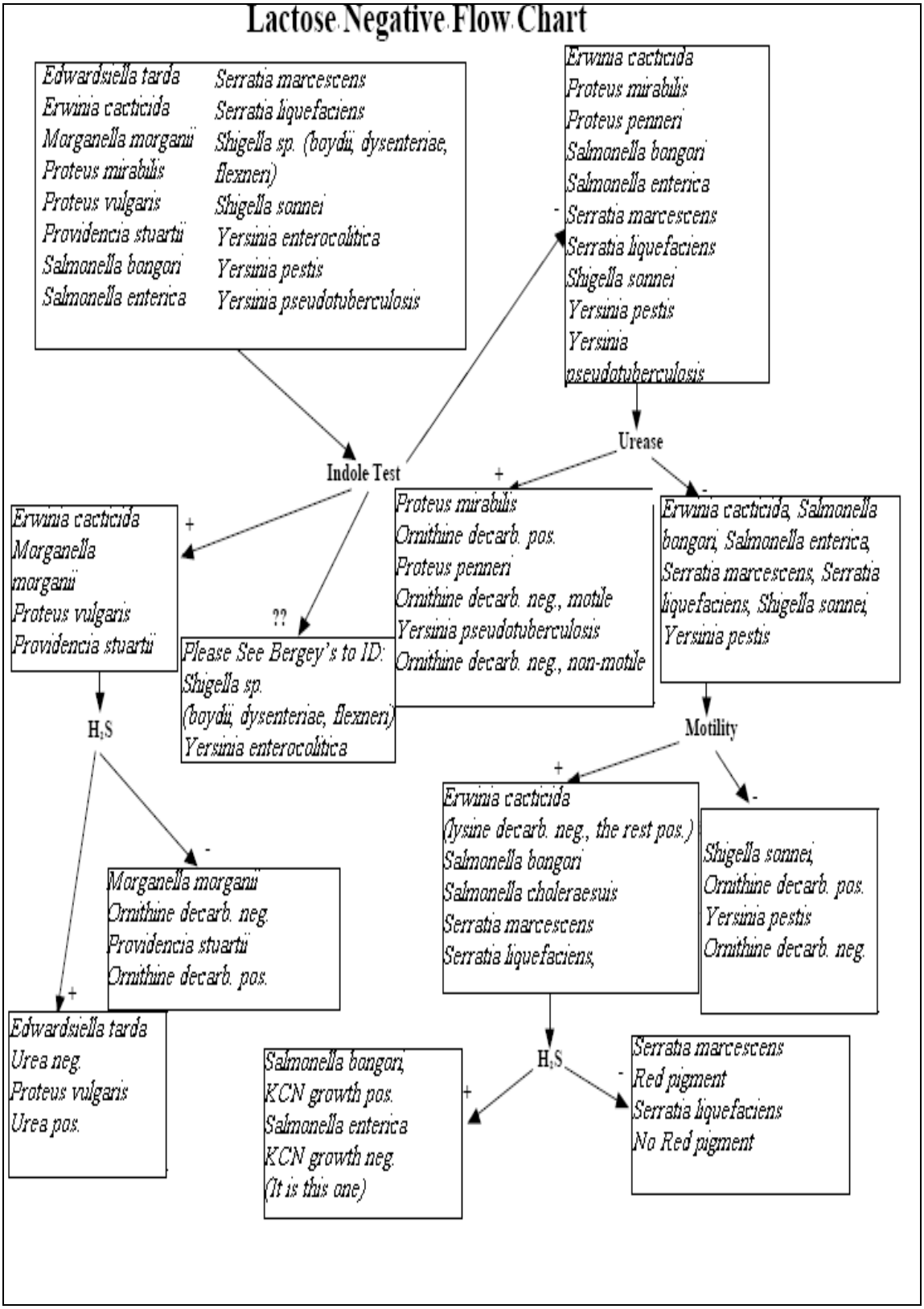

Figure 2.6: Family Enterobacteriaceae Lactose-Negative ID Flowchart

CHAPTER 3

METHODOLOGY

3.1 Collecting Sample

Soil's sample is taken from Dragon fruit plantation at Gambang Kuantan. Aseptic technique is applied to prevent contamination during taking the sample.

3.2 Isolation of microbes

The sample is mixed with 1L pure water to make solution sample. This solution sample is diluted up to 10^{-5} serial dilution. Serial dilution is a process of diluting a sample several times. The sterile Petri dish is label. Than 90ml of phosphate buffer is transferred into each of 5 tubes using sterile pipettes (10ml) with aseptic technique. By using sterile pipette (1ml), 1ml of water sample is transferred into 10^{-1} tube. Mix the test tube properly. Continue dilution with aseptic technique for test 10^{-2} until 10^{-5}.

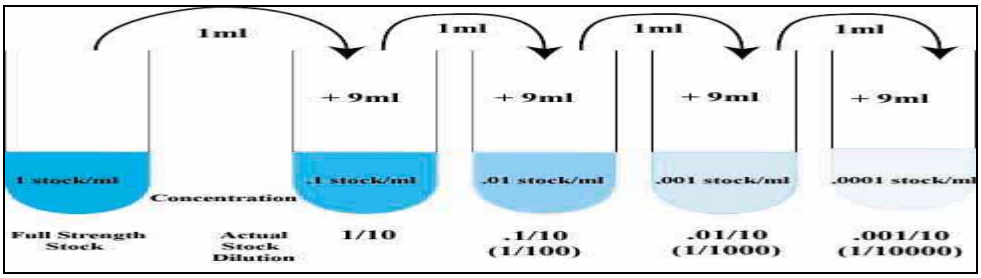

Figure 3.1: Serial Dilution Method

After that each of serial dilution is transferred into nutrient agar plate by using spread plate method. 0.1ml of an appropriately diluted culture is spread over the surface of agar using sterile glass spreader. The plate is then incubated until the colonies appear. It is important that the surface of the plate be fairly dry so that the spread liquid soaks in. figure 3.2 shows the step for spread plate method.

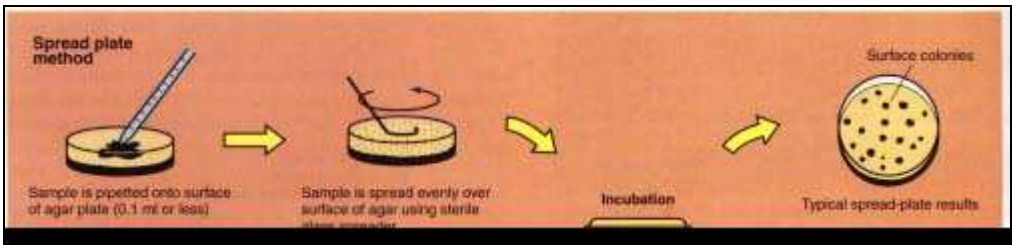

Figure 3.2: Spread Plate Method

3.3 Identification and Characterization of Microorganisms

3.3.1 Gram Staining

The smear is covered with crystal violet and stand for 20 seconds. The stain is briefly washed off using a wash bottle of distilled water. Excess water is drained off. The smear is covered with Gram's iodine solution and stand for 30 seconds. The Gram's iodine is washed off. The slide is hold 45-degree angle and allows the 95% alcohol to flow down the surface of the slide until the alcohol is colorless as it flows from the smear down the surface of the slide. Stop decolorization by washing the slide with a gentle stream of water. The smear is covered with safranin for 1 minute. The slide is washed gently for a few seconds. Blot dry with bibulous paper, air-dry. The slide is examined under oil immersion.

3.3.2 Starch Hydrolysis

Starch agar is used to demonstrate the hydrolytic activities of exoenzymes. The medium is composed of nutrient agar supplemented with starch. First, inoculate a starch plate with the organism to be tested. These microorganisms had been culture in trypticase soy broth. Then incubate at optimum temperature for at least 48 hours. After that, flood plate with iodine, allow the iodine to remain contact with medium for 30s and pour off the excess, and observe results. Blue color indicates no hydrolysis, while a clear zone indicates hydrolysis.

3.3.3 Triple Sugar-Iron Agar Test

This is a rapid screening procedure and is designed to differentiate among the different or genera of the *Enterobacteriaceae*, which are all gram negative bacilli capable of fermenting glucose with the production of acid; and to distinguish the *Enterobacteriaceace* from other gram-negative intestinal bacilli. Using sterile technique, inoculate each organism from trypticase soy broth, into the slant by means of stab and streak inoculation. Do not fully tighten screw cap. Uninoculated tube serves as a control. Table 3.1 shows the interpretation for TSI test.

Table 3.1: Fermentative Activities of the Organisms

Results (slant/butt)	Symbol	Interpretation
Red/yellow	Alkaline reaction/ Acid production	Glucose fermentation only; Peptone catabolized
Yellow/yellow	Acid production / Acid production	Glucose and lactose and/or sucrose fermentation
Red/red	Alkaline reaction / Alkaline reaction	No fermentation; Peptone catabolized
Red/no color change	Alkaline reaction / No Change	No fermentation; Peptone used aerobically

Yellow/yellow with bubbles	Acid production / Acid production, Gas production	Glucose and lactose and/or sucrose fermentation; Gas produced
Red/yellow with bubbles	Alkaline reaction / Acid production, Gas production	Glucose fermentation only; Gas produced
Red/yellow with bubbles and black precipitate	Alkaline reaction / Acid production, Gas production, H_2S	Glucose fermentation only; Gas produced; H_2S produced
Red/yellow with black precipitate	Alkaline reaction / Alkaline reaction, H_2S	Glucose fermentation only; H_2S produced
Yellow/yellow with black precipitate	Acid production / Acid production, H_2S	Glucose and lactose and/or sucrose fermentation; H_2S produced
No change/no change	No Change	No fermentation

3.3.4 Catalase Test

1. Principle - To detect the presence of the enzyme catalase. Catalase enzyme is found in most bacteria. It catalyses the breakdown of hydrogen peroxide (H_2O_2) with the release of free Oxygen.
2. Method
    1. Dip a capillary tube into 3%H_2O_2
    2. Touch a colony
    3. Observe the tube for bubble indicating a positive reaction
3. Result

Bubbles	positive
No bubbles	negative

4. Special Features - catalase is found in most aerobic and facultative anaerobic bacteria. The main exception is Streptococcus spp. Catalase is not found in anaerobes.
5. Precautions in interpretation
    - It is important not to contaminate the bacterial colony under test with blood agar. Red blood cells contain catalase and their presence will give a false positive result.
    - Old cultures may loose their catalase activity, possible resulting in a false negative result.

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CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation results

The isolation process is a procedure of isolation the mixture of colonies to a single colony. This process was done by using streaking method to obtain pure cultures. The soil's sample was added with 1 Liter pure water to obtain solution sample before transferred into nutrient agar plate. It is important that the numbers of colonies developing on the plates are not being too large. On crowded plates some cells may not form colonies, and some colonies may fuse, leading to erroneous measurements. So, to obtain the appropriate colony number, the samples need to be diluted. This solution samples were diluted up to 10^{-5}. By using spread plate method, the diluted samples were transferred into nutrient agar plate and the bacteria were grown on it. From the observation, these samples take about three until four days to growth on the plate. Figure 4.1 shows the growth of the bacteria on plate after 5 days.

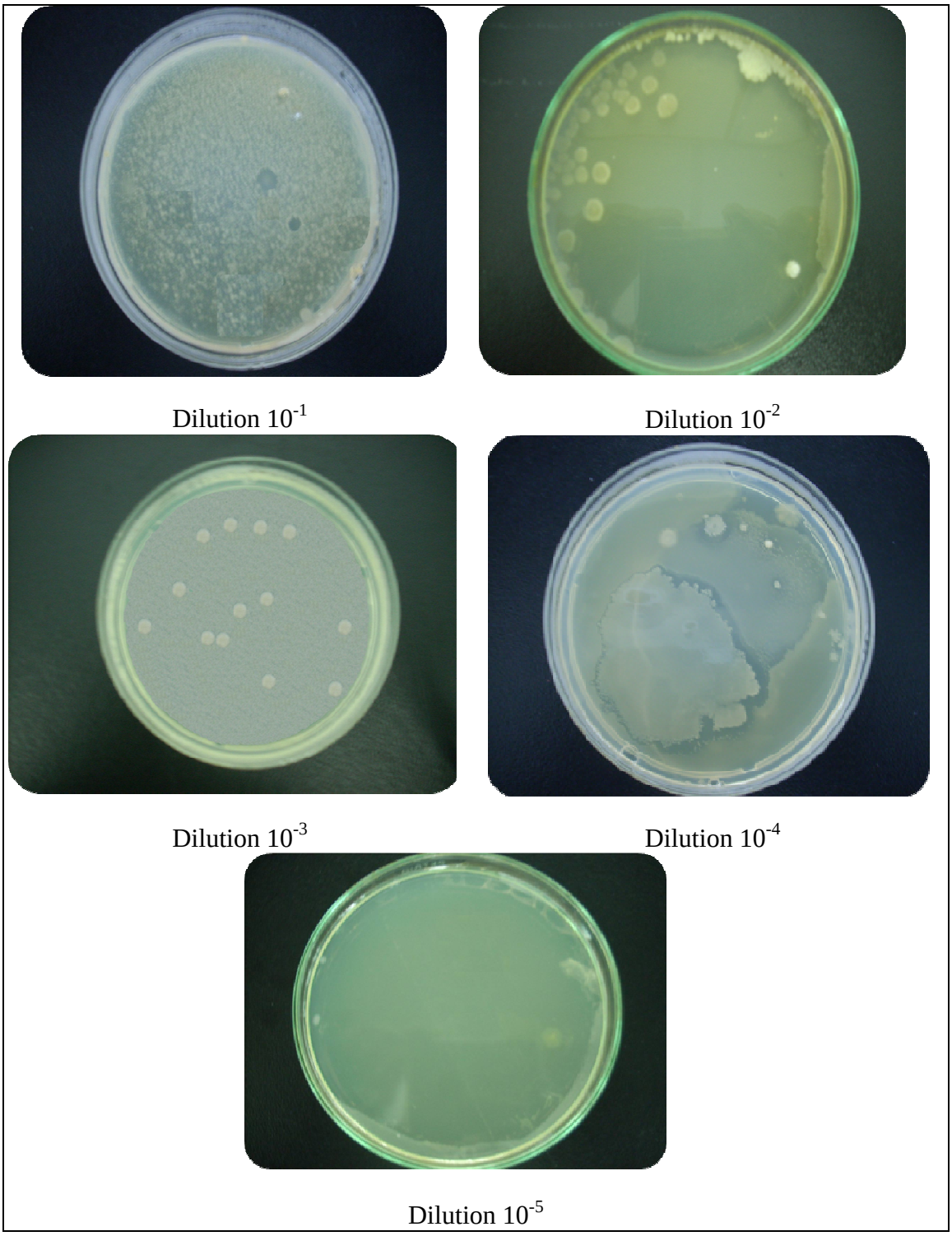

Figure 4.1: The growth of bacteria on plate for serial dilution (10^{-1} – 10^{-5})