ISOLATION OF MICROORGANISM FROM OIL PALM SAP

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ABSTRACT

The oil palm sap is come from the oil palm trunk. The outer of the trunk is being process to become plywood but for the most moisture part, it has been squeezed in order to get the sap. The unfermented sap is clean, sweet, colorless syrup containing about 10–12 % sugar, which is mainly. Sap also rich with other simple sugar such as glucose, fructose, raffinose, amino acid, vitamins and other essential nutrients. This research is aim to isolate bacteria using streaking plate method from palm oil sap and identified by using Bergey's manual. . A total of 15 colonies were successfully isolated from the palm oil sap. From analysis, *Lactobacillus spp.* was the dominant species isolated followed by *Pseudomonas spp., Corynebacterium spp* and *Vibro spp.* For the kinetic growth study, the kinetic values that need to determine are maximum growth rate (μ_m), decay coefficient (K_d), yield coefficient (Y) and substrate coefficient (K_s). The growth kinetic is calculated by measured the substrate concentration and biomass weight. K_d was determined to be 0.034 h^{-1} and Y as 0.124 g/L. Meanwhile, μ_m was 0.2532 h^{-1} and K_s was 536.4557 g/L. As a conclusion, it is well known that the environment condition influence the bacteria species and growth activities of bacteria.

ABSTRACT

Sap kelapa sawit adalah berasal dari batang pokok kelapa sawit yang telah diperah. Kebiasaannya, bahagian luar batang pohon kelapa sawit dijadikan papan dan bahagian yang mempunyai kandungan air yang tinggi diperah bagi mendapatkan sap kelapa sawit. Sap segar mempunyai warna yang jernih, rasa manis dan mengandungi 10 – 12% gula. Selain itu, sap kelapa sawit juga mengandungi gula ringkas lain seperti glukosa, fruktosa, raffinose, asid amino, vitamin dan nutrien-nutrien baik yang lain. Kajian ini dijalankan bagi mengasing dan mengenalpasti bakteria yang terdapat dalam sap kelapa sawit tersebut. Kaedah yang digunakan bagi mengasingkan bakteria ialah dengan menggunakna kaedah ‘ *streaking plate method’*. Bagi tujuan mengenal pasti bakteria pula, manual Bergey diaplikasikan. Sejumlah lima belas koloni berjaya diasingkan. Bakteria spesis *Lactobacillus.*dikenalpasti sebagai koloni dominan. Ini diikuti oleh bakteria spesis *Pseudomonas,* spesis *Corynebacterium* dan spesis *Vibro.* Bagi kajian menentukan pertumbuhan kinetik bakteria, nilai kinetik yang perlu dikira ialah *‘growth rate’* (μ_m), *‘decay coefficient’* (K_d), *‘yield coefficient’* (Y) dan *‘substrate coefficient’* (K_s). Berdasarkan pengiraan, nilai K_d ialah 0.034 h^{-1} dan Y ialah 0.124 g/L. Manakala, nilai bagi μ_m ialah 0.2532 h^{-1} dan K_s ialah 536.4557 g/L. Kesimpulannya, keadaan persekitaran mempengaruhi kewujudan spesis dan pertumbuhan bacteria dalam sap kelapa sawit.

TABLE OF CONTENTS

CHAPTER	TITLE	PAGE
	DECLARATION	**ii**
	DEDICATION	**iii**
	ACKNOWLEDGEMENT	**vii**
	ABSTRACT	**viii**
	ABSTRAK	**x**
	TABLE OF CONTENTS	**xi**
	LIST OF TABLES	**xiii**
	LIST OF FIGURES	**xiv**
	LIST OF APPENDICES	**xv**
	LIST OF SYMBOLS/ABBREVIATIONS	**xvi**
1	**INTRODUCTION**	
	1.1 Research Background	1
	1.2 Problems Statement	3
	1.3 Objective	4
	1.4 Research Scopes	4
	1.5 Rationale & Significance	5
2	**LITERATURE REVIEW**	
	2.1 Plant Sap	6
	2.1.1 Oil Palm (*Elaeis guineensis*) Sap	6

	2.1.2	Microorganism from Oil Palm Tree	7
2.2	Cultivation of Bacteria		10
	2.2.1	Nutritional Needs	10
		2.2.1.1 Carbon	10
		2.2.1.2 Nitrogen	10
		2.2.1.3 Vitamin	11
2.3	Techniques for Isolation of Pure Culture		11
	2.3.1	Streak-Plate Method	11
	2.3.2	Spread-Plate Technique	12
	2.3.3	Role Tube Method	13
	2.3.4	Drop Plate Method	13
	2.3.5	Membrane Filtrations	14
2.4	Characterization of Microorganism Isolates		15
	2.4.1	Gram Staining Method	15
2.5	Growth Kinetics		16
	2.5.1	Monod Kinetic Equation	16
		2.5.1.1 Rate of Change For Substrate Concentration	17
	2.5.2	Contois Kinetic Equation	18
		2.5.2.1 Rate of Change of Effluent Biomass Concentration	18
2.6	Determination of the Kinetic Parameters		19
	2.6.1	Determination of Y and K_d	19
	2.6.2	Determination of μ_m and K_S	20

3 MATERIALS AND METHODS

3.1	Introduction	22
3.2	Chemical and Biological Materials	24
3.3	Source of Inoculum	24
3.4	Autoclaving	24

3.5 Culture Medium 24
3.6 Isolation and Characterization of Bacteria. 25
3.7 Bacteria Identification 26
3.7.1 Preparation of Bacteria Smears 29
3.7.2 Gram Stain 29
3.7.3 Spore stain (Schaeffer-Fulton Method) 29
3.7.4 Acid – Fast stain (Ziehl – Neelsen Method) 29
3.7.5 Glucose Fermentation Activity 30
3.8 Determination of growth kinetic
3.8.1 Microorganism 30
3.8.2 Medium and Culture Condition 30
3.8.3 Measurement of Cell Growth 31
3.8.4 Substrate analysis using Dinitrosalicyclic Colorimetric Method (DNS Assay) 31

4 RESULTS AND DISCUSSIONS

4.1 Identification of Bacteria from Palm Oil Sap 32
4.2 Kinetic Coefficient 35
4.2.1 Determination of Y, K_d, μ_m & K_S 36

5 CONCLUSION AND RECOMMENDATION

5.1 Conclusion 40
5.2 Recommendation 41

REFERENCES 42
APPENDIX A 45

LIST OF TABLES

TABLE NO.	TITLE	PAGE
4.1	Biochemical Characteristic of the Gram-positive Bacteria Strain	33
4.2	Biochemical Characteristic of the Gram-negative Bacteria Strain	33
4.3	Kinetic coefficients of bacteria from various kind of palm sap.	38

LIST OF FIGURES

FIGURE NO.	TITLE	PAGE
1.1	Images of felled oil palm trunks and diagrams of sample preparation for analyses.	3
2.1	Four-way streak-plate inoculation	12
2.2	Petri dish turntable	12
2.3	Generalized substrate consumption and biomass growth with time.	19
3.1	Research design for bacteria isolation and growth profile study of palm oil sap.	25
3.2	Medium preparation (Nutrient Agar and Nutrient Broth)	25
3.3	Streaking plate method procedure was done under sterile condition in laminar flow.	25
3.4	Colonies appear on culture medium after 24 hours incubation	26
3.5	Bergey's Manual of Determative Bacteriology for Gram Positive Rods	27
3.6	Bergey's Manual of Determative Bacteriology for Gram Negative Rods	28
3.7	Flood smear with malachite green and steam over a beaker of water placed on a hot plate.	29
3.8	Substrate analysis using DNS assay	31
4.1	Graph of specific growth rate, μ versus substrate utilization rate, U	36

4.2 Graph of BRTG/ (1+BRTG.Kd) vs 1/S 37

LIST OF APPENDICES

APPENDIX	TITLE	PAGE
A1	Experiment Data for Kinetic Growth Study	45
A2	Calculation on determination of Y, K_d, μ_m & K_S	49

LIST OF SYMBOLS/ABBREVIATIONS

DNS	-	Dinitrosacyclic
g	-	Gram
g/l	-	Gram per litre
hr	-	Hour
L	-	Litre
mg/ml		Miligram per mililitre
OD	-	Optical density
rpm	-	Round per minute
Uv-Vis	-	Ultraviolet Visible Spectroscopy
w/v	-	Weight per volume
˚C	-	Degree Celsius
%	-	Percentage

CHAPTER 1

INTRODUCTION

1.1 Research Background

Palms are believed to be among the oldest flowering plants in the world (Redhead, 1989) There are various species of palm trees among which are *Elaeis guineensis, Raphia regalis, R.sudanica, R.vinifera and R.hookeeri* (Obire, 2005). The oil palm is a tropical plant that grows in warm climates at altitudes below 500 meters above sea level. Its introduction to tropical America is attributed to Portuguese colonizers and slave traders, who used it as part of the diet for their slaves in Brazil.

Oil palm *(Elaeis guineensis)* for palm oil production needs to be replanted at an interval of 20 to 25 years in order to maintain oil productivity. The plantation area in Malaysia and Indonesia in 2007 was 4,304,913 ha (Al Widyan, 2002) and nearly 7 million ha respectively. Considering the replanting interval, 450,000 ha to 560,000 ha of the oil palm plantation area is expected to be replanted annually during the next 25 years. This means on average 64 million to 80 million old palm trees will be felled every year in the two countries, as approximately 142 oil palms are usually planted in one hectare (Pramila and Subhash, 2007).

For centuries, many palm species have been tapped throughout the tropical world in order to produce fresh juice (sweet toddy), fermented drinks (toddy, wine, arak), syrup ("honey"), brown sugar (jaggery) or refined sugar. One of mankind's first sources

of sugar was probably *Arenga pinnata* (Redhead 1989). Evidence of the use of *Borassus flabellifer* sugar in India has been reported by the Greek historian Megasthenes, ambassador to the court of Chandragupta, in the 4th century BC. Hindus knew how to extract it about 4,000 years ago (Ferguson, 1888; Fox, 1977). Jaggery and treacle extracted from *Caryota urens* sap in Sri Lanka has been an important source of sugar from antiquity (Dissanayake, 1977). In Africa, the main traditional use of palm sap is for wine production. It has been reported in Egypt (date palm) long before the birth of Christ (Barreveld, 1993) and on the Guinea coast by early navigators in the 15th century (Sodah Ayernor and Matthews, 1971).

The oil palm sap is come from the oil palm trunk. The outer of the trunk is being process to become plywood but for the most moisture part, it has been squeezed in order to get the sap (Yutaka et al., 2007). The unfermented sap is clean, sweet, colorless syrup containing about 10–12 % sugar, which is mainly sucrose (Bassir, 1962 : Okafar, 1975). Sap also rich with other simple sugar such as glucose, fructose, raffinose , amino acid , vitamins and other essential nutrients (Okafar, 1978).

This pure sap has a short life of stability because the condition of the sap is very suitable for microorganisms to growth (fermentation). The presence of various microorganism especially bacteria and yeast responsible for the fermentation of palm-wine (Bassir, 1962 ; Faparusi, 1966 ; Okafar, 1977). So, the sugar level of the sap will decrease rapidly due to the fermentation by the natural microbial flora and converted to alcohol and other products. The types of bacteria present appear are depends on the stage of fermentation and the composition of the sap (Bassir, 1962 ; Okafar, 1977).

1.2 Problem Statement

Palm oil production has been documented as a cause of substantial and often irreversible damage to the natural environment. Most researcher nowadays were focusing on the study of empty fruit bunch or POME, however less research were done to investigate other part from the palm oil tree itself. In major practices, palm sap usually were fermented to be used as traditional drink which known as palm wine. In African society, palm wine has a significant role in customary practices. Generally, both brands of palm wine have several nutritional, medical, religious and social uses which have been reported else where (Faparunsi, 1966; Odeyemi, 1977; Ikenebomeh and Omayuli, 1988; Uzogara *et al.,*1990; Iheonu, 2000), to have increasingly enhanced the demand for this natural product. Sugars in the sap of the felled trunk and observed a large quantity of high glucose content sap in the trunk. Studies over the years have been deboted to the isolation and identification of the microorganism responsible for various fermentation processes.

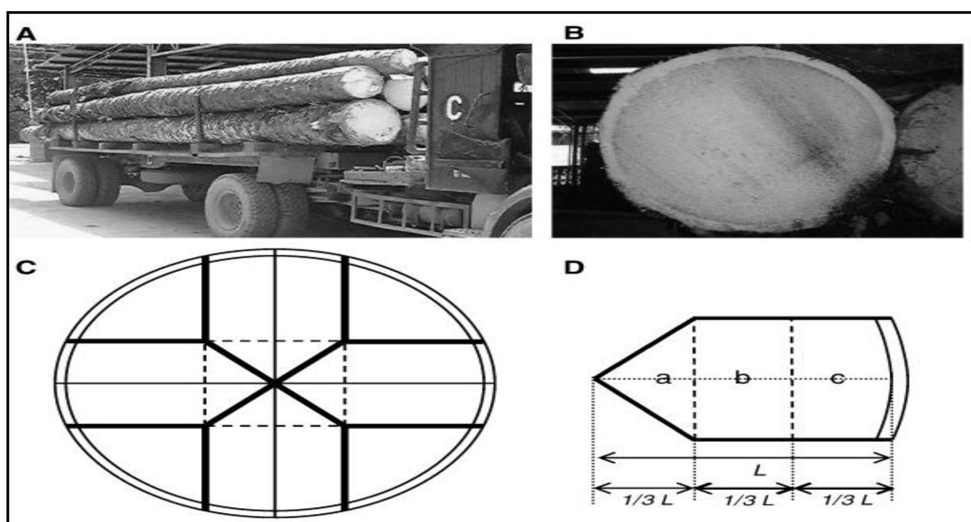

Figure 1.1: Images of felled oil palm trunks and diagrams of sample preparation for analyses. (A) Felled trunks carried to a plywood factory. (B) A disk taken from a trunk. (C,D) Disks were cut into inner (a), middle (b), and outer (c) parts.

1.3 Objective

i. This research is aim to isolate bacteria using streaking plate method from palm oil sap.

ii. Determine the kinetic models values of bacteria mix culture from palm oil sap.

1.4 Research Scope

There are research scopes that have been identified for this study to achieve the objective:

i. To isolate the bacteria from the oil palm sap using the streaking plate method.

ii. To identify bacteria colonies by using Bergey's manual.

iii. To study the growth kinetics of bacteria mix culture from oil palm sap by using Monod Kinetic Model. The kinetic values that need to determine are maximum growth rate, decay coefficient, yield coefficient and substrate coefficient. The growth kinetic is calculated by measured the substrate concentration and biomass weight.

1.5 Rational And Significant

Palm sap is the tropical plants of the palmae family. It is produced and consumed in very large quantities in the southeastern Nigeria. It contains nutritionally important components including amino acids, proteins, vitamins and sugars (Okafor, 1987). These make this sap a veritable medium for the growth of a consortium of microorganisms, whose growth in turn, change the physicochemical conditions of the wine, giving rise to competition and successions of organisms. Many workers have indeed carried out studies aimed at isolating and exploiting palm wine yeasts for industrial processes. These include for baking, portable ethanol production and single cell protein production.

CHAPTER 2

LITERATURE REVIEW

2.1 Plant Sap

Plant sap is distinct from nectar and latex as well as from the gum and resin that sap carry (David, 1989). Sap is aqueous, tends to have a high pH, and is carried in plant xylem or phloem. Xylem sap tends to have lower sugar concentration than that of phloem (Darkee, 1983). The sugar content of phloem primarily sucrose and from a sample of 500 trees species, ranged from 1%-30% in concentration (Zimmerman and Ziegler, 1975). In addition to carbohydrate, phloem sap contain some amino acid, proteins, vitamins, salts and elemental metals (Chino *et al*., 1982 ; Richardson, Bakerand Ho , 1982 ; Stemmer *et al*., 1982 ; Simpson and Neff, 1983). Notably, the sap in the inner part of the trunk accounted for more than 80% of the whole trunk weight.

2.1.1 Oil Palm (*Elaeis guineensis*) Sap

Oil palm (*Elaeis guineensis*) is widely planted for its edible oil in tropical countries such as Malaysia and Indonesia. The production of palm oil is 39 Mt per year in 2007, which is the most produced plant oil in the world (H.Yamada *et al.,* 2010). The oil is mainly used for food and related industries, and is also used as a raw material for various products such as detergents and cosmetics. Moreover, a number of research studies have been carried out for biodiesels and bio-plastic materials from the oil in

recent years (Chongkhong *et al.,* 2007; Tanaka *et al.,* 2008; Kalam *et al.,* 2002; Al-Widyan *et al.,* 2002; Pramila.T *et al.,* 2007).

It has been traditionally practiced to produce palm sugar and palm wine using sap obtained by tapping the inflorescence of various species of palms including Arenga pinnata, Borassus flabellifer, Cocos nucifera, Nypa fruticans and oil palm (Dalibard C, 1999). Among these palm species, oil palm is considered to produce much smaller amount of tapped sap, or low sugar yield (Niger J, 1987). Oil palm sap was reported to contain approximately 11% sugars with sucrose as a major component accounting for approximately 90% of total sugar (Eze MO and Ogan AU, 1988). Oil palm sap was found to be rich in various kinds of amino acids, organic acids, minerals and vitamins.

2.1.2 Microorganism from Oil Palm Tree

Previous studies on the microbiology of oil palm tree (*E. guineensis)* and *R. hookeri* have incriminated several bacterial and yeast flora to be involved in the fermentation process (Faparunsi and Bassir, 1972a; Okafor, 1972ab; Okafor, 1975b; Eze and Ogan, 1987; Amanchukuru *et al.,* 1989; Ejiofor, 1994; Orimaiye, 1997; Nester *et al.,* 2004). These organisms have also been reported to originate from several sources, which include tapping equipment, containers and the environment (Faparunsi and Bassir, 1972a; Eapen, 1979).

During fermentation, the sugars in the palm-sap are metabolized to alcohol and organic acids with the result that the sap loses its sweetness (Okafor, 1975). The types of bacteria present appear to depend on the stage of fermentation and the composition of the sap (Bassir, 1962; Okafor, 1977). Although alcohol production is common among yeasts, it is rare among bacteria (Ingraham and Ingraham, 2004). Yeasts are used to make most alcohol beverages. But *pulque* is an exception. Pulque is an alcoholic beverage from the juice of the agave plant fermented by *Zymomonas mobilis* (Talaro and Talaro, 1999; Ingraham and Ingraham, 2004).

Acetobacter species were earlier isolated from oil palm wine (Faparusi and Basir, 1972; Faparusi, 1973; Okafar, 1975) and from immature spadix of palm tree (Faparusi, 1973). *A. pasteurianus* was isolated from palm wine (Simmonart and Laudelout, 1951), and *A. aceti subp. Xylinium* from the leaflets of the palm tree and the surrounding air (Faparusi, 1973). The strains of acetic acid bacteria are useful for vinegar production; however, lack of defined pure starter cultures is due to problems in strain isolation, cultivation and preservation of vinegar bacteria (Kittleman *et al.,* 1989; Sievers *et al*., 1992; Sokollek and Hammes, 1997). Acetic acid bacteria are able to produce high amounts of acetic acid from alcohol. Furthermore, these bacteria can produce other compounds, apart from acetic acid, that can influence wine quality (Drysdale and Fleet, 1989a). Earlier research has also shown that acetic acid bacteria (genera Acetobacter and Gluconobacter) were able to produce some polysaccharides such as cellulose, levan and dextran (Hibbert and Barsha, 1931; Loitsyanskaya, 1965; Hehre and Hammilton, 1953).

In Mexico, the distilled spirit tequila is traditionally made from the fermentation of juices from the agave plant using the bacterium *Z. mobilis* (Ingraham and Ingraham, 2004; Nester *et al*., 2004). *Zymomonas* species are perhaps the most important alcoholic fermenters of the bacterial group in plant saps and juices. *Zymomonas* are facultative aerobes with both respiratory and fermenting capabilities. As in yeast, *Zymomonas* decarboxylates pyruvate non oxidatively with the formation of acetaldehyde; which is subsequently reduced to ethanol by the Entner-Doudoroff’s pathway (Swings and De Ley, 1977; Talaro and Talaro, 1999; Ingraham and Ingraham, 2004). The production of acetaldehyde and the characteristic fruity odour of *Zymomonas* also contribute to the odour and taste of wines (Swings and De Ley, 1977; Ingraham and Ingraham, 2004).

Lactic acid bacteria (LAB) are recognized as having important roles in the fermentation and preservation of a great variety of food and feed, while the roles of acetic acid bacteria (AAB) in the development of the vinegary taste in palmwine have also been investigated (Amoa-Awua *et al.,* 2006). LAB and AAB have been found at high levels, while the yeast species *S. cerevisiae* and *Schizosaccharomyces pombe* have

been reported to be the dominant yeast species (Odunfa and Oyewole, 1998). However, other yeast species have also been found, such as *Kloeckera apiculata, Candida krusei,* and other Candida *sp*., Pichia *sp.* (Atacador-Ramos, 1996; Amoa-Awua *et al*., 2006).

The difficulty of storing palm-wine to retain its normal characteristics due to the fermentative ability of probably *Zymomonas* species and other microorganisms present in the wine has been a major problem in the bottling of palm-wine in Nigeria and consequently its distribution for consumption (Obire O, 2005). In addition to their fermentative ability, *Zymomonas* species are known to be harmless to man and cattle. Some of its fermentation products are found useful in the treatment of various diseases ranging from chronic enteric metabolic disorders to gynecological infections (Swings and De Ley, 1977). The presence of *Zymomonas* species in palm-wine may thus be beneficial to man. Other components in the squeezed sap that may affect fermentation, namely, amino acids, organic acids, minerals and vitamins.

In recent years, culture-independent methods based on molecular biology techniques have been developed to study microbial population dynamics. Today, culture-independent methods are particularly attractive, as they provide a good and rapid strategy for yeast detection, and they represent a valid alternative to classical microbiological analyses. In addition, culture-independent analysis offers the possibility of detecting species that may be present in the habitat at viable, but non-culturable, levels (Head *et al.,* 1997; Rappe and Giovannini, 2003; Ercolini, 2004). Indeed, classical microbiological methods based on plate counts, and isolation and biochemical identification have been criticized, since only easily culturable microorganisms can be detected, and members of microbial communities that need elective enrichment are not identified.

2.2 Cultivation of Bacteria

As do all other living organisms, microorganism require certain basic nutrients and physical factors for the sustenance of life. However, their particular requirements vary greatly.

2.2.1 Nutritional Needs

Nutritional needs of microbial are supplied in the laboratory through a variety of media.

2.2.1.1 Carbon

Carbon is the most essential and central atom common to all cellular structures and functions. Among microbial cells, two carbon-dependent types are noted, autotrophs and heterotrophs.

2.2.1.2 Nitrogen

Nitrogen also an essential atom in many cellular macromolecules, particularly proteins and nucleic acids. Proteins serve as the structural molecules forming the so-called fabric of the cell and as functional molecules, enzymes, that are responsible for the metabolic activities of the cell. Nucleic acid include DNA, the genetic basis of cell life, and RNA , which plays an active role in protein synthesis within the cell.

2.2.1.3 Vitamin

Besides, vitamin, organic substances contribute to cellular growth and are essential in minute concentrations for cell activities. some microbes requires vitamin for normal metabolic activities. Some possess extensive vitamin-synthesizing only a limited number from other compounds present in the medium.

2.3 Techniques for Isolation of Pure Culture

In nature, microbial population do not segregate themselves by species but exist with a mixture of many other cell types. These population can be separated into pure culture. These culture contains only one type of organism and are suitable for the study of their cultural, morphological, and biochemical properties. The techniques commonly used for isolation of discrete colonies initially required that the number of organisms in the inoculum be reduced (James and Natalie, 7th ed., microbiology laboratory manual)

2.3.1 Streak-Plate Method

The streak-plate is a rapid qualitative isolation method. It is essentially a dilution technique that involves spreading a loopful of cultures over the surface of an agar plate. The four-way or quadrant streak is preferred.

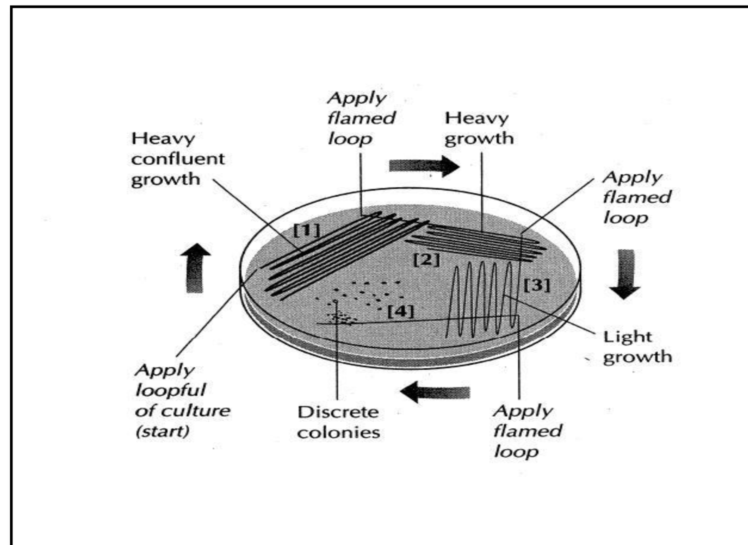

Figure 2.1: Four-way streak-plate inoculation

2.3.2 Spread-Plate Technique

The spread plate techniques required that a previous diluted mixture of microorganism be used. During inoculation, the cells are spread over the surface of a solid agar medium with a sterile, L-shaped bent rod while Petri dish is spun on a "lazy-Susan"turntable.

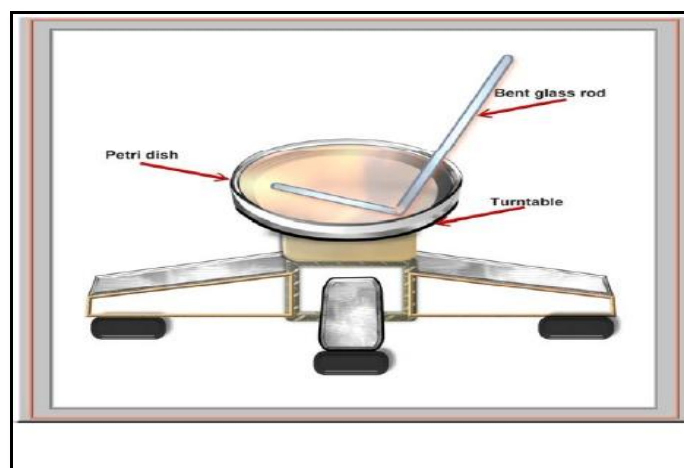

Figure 2.2: Petri dish turntable

2.3.3 Role Tube Method

Roll tubes are test tubes with a nutrient medium solidified in a thin layer around the inner surface of the tubes. The roll tube, as originated by Esmarch (E.1886), has not been a popular system for the isolation and enumeration of bacteria since the availability of petri dishes. However, a rolltube method for counting bacteria in milk is still used (American Public Health Association. 12th ed., 1967).

Esmarch used roll tubes for anaerobic culturing by filling the hollow core with a solid medium, and Frankel (Frankel, 1888) and Ewell (Ewell, 1897.) used hydrogen gas methods to reduce the medium after sterilization. These methods were replaced principally by petri plates used with anaerobic jars.

Roll-tube methods allow cultivation of fastidious anaerobes that have not been successfully cultured by petri plate and anaerobic jar methods (Hungate, R. E. 1950 ; Hungate, R. E. 1966 ; Spears, R. N., and R. Freter. 1967). Roll-tube techniques have been used to study the rumen of herbivores and, as a result, the rumen is now the best ecologically understood anaerobic microbial habitat.

Roll-tube anaerobic counts have been compared to standard anaerobic jar plate counts by using normal flora of animals and man (Spears, R. N., and R. Freter. 1967; M.Stutman and D. F. Gordon, Jr., Int. Ass. DentalRes. Abstr. 181, p. 86, 1969), and the roll-tube method has always given a significantly higher count.

2.3.4 Drop Plate Method

The drop plate (DP) method exhibits many positive characteristics. The plating and counting procedures require less labor than alternative methods. The plating and counting steps are very convenient and manageable. On appropriately dried plates, the drops will absorb quickly into the agar. By distributing the sample in drops, colony counting can be done faster and perhaps more accurately.

The drop plate method expends relatively few supplies. A bibliographic-database search and a worldwide web search showed that the drop plate method is being used in numerous laboratories across the world. In spite of its widespread use, the DP method has not been standardized. Accurate and precise measurement of the drop volume is absolutely necessary to the DP method.

Donald 1915 was the first to describe a method for the precise measurement of fluid volume by means of drops. The DP method is a mixture of microbiological components and design components.

2.3.5 Membrane Filtrations

Membrane filtration usually used for isolation of microorganism from surface water samples. In recent research, evaluation of membrane filters application were used to isolated *Leptospira* from surface water samples. The filter materials evaluated included nitrocellulose (0.22 and 0.45 μm pore diameters), polyvinylidene fluoride (Durapore 0.22 and 0.40 μm pore diameters), nylon mesh (37 μm), and glass fiber (1.0 μm). Millipore polyvinylidene fluoride filter (0.22 μm) was examined by scanning electron microscopy to verify that leptospires were present following filtration (Kaboosi H, Razavi M.R and Noohi, 2005).

2.4 Characterization of Microorganism Isolates

The isolates were grouped accorded to their colonial morphology and cell characteristics. The colonies were counted and re-isolated in pure culture using the medium on which they had grown as described by Njoku *et al*. (1990). Isolates were thereafter subjected to biochemical tests as described by Collins and Lyne (1984) and Ogbulie *et al.* (1994). The probable identities of the isolates were determined as recommended by Bergey's manual.

2.4.1 Gram Staining Method

The Gram stain was developed by Christian Gram in 1884 and modified by Hucker in 1921. The Gram stain is a differential stain that allows classifying bacteria as either gram-positive or gram-negative. Gram-positive microorganism that retain the primary dye (Crystal violet) and Gram-negative microorganism that takes the color of the counterstain (usually Safranin). These result are due to the differences in the structure of the cell wall. Crystal violet is attracted to both Gram-positive and Gram-negative microorganism. The second step (Gram's Iodine) stabilizes the Crystal violet into the peptidoglycan layer of the cell wall. The peptidoglycan layer is much thicker in Gram-positive bacteria than in Gram-negative bacteria. Hence the Crystal violet is more extensively entrapped in the peptidoglycan of Gram-positive bacteria. The third step (alcohol decolorization) dissolves lipids in the outer membrane of Gram-negative bacteria and removes the Crystal violet from the pepridoglycan layer. In contrast, the Crystal violet is relatively inaccessible in Gram-positive microorganism and cannot readily be removed by alcohol in Gram-positive microorganisms, after the alcohol step, only the colorless Gram-negative microorganism can accept the Safranin, Carbol-fuchsin, and Basic-fuchsin are sometimes employed in the counterstain to stain anaerobes and other weakly staining Gram-negative, including *Legionella spp*., *Campylobacter spp*., and *Brucella spp*. (Emanuel G and Lorrence H.G, 2009).