ISOLATION AND IDENTIFICATION OF ACETIC ACID PRODUCER FROM SOIL USING BANANA STEM WASTE AS A SUBSTRATE

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ISOLATION AND IDENTIFICATION OF ACETIC ACID PRODUCER FROM SOIL USING BANANA STEM WASTE AS A SUBSTRATE

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Thesis submitted in fulfillment of the requirements

for the award of the degree of

Master of Engineering (Bioprocess)

Faculty of Chemical Engineering and Natural Resources

UNIVERSITI MALAYSIA PAHANG

JULY 2013

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DEDICATION

Special for my beloved mum and dad…..

My lovely fiancé….

Sisters…..

Also friends who always support me…..

(Zulsyazwan, Abu, Zul, Maho, Fatihah , Ding, Liu Meng, Li Yan, Hazimah)

Thanks for everything……

ACKNOWLEDGEMENTS

In the name of Allah, the most gracious and merciful;

I am thankful to Allah SWT, belongs all that is in the heavens and earth because with His permission I can complete this research and thesis successfully and peace be upon to our prophet Muhammad (SAW).

I would like to express my sincere gratitude to my respected supervisors, Dr. Norazwina Zainol and Prof. Ir. Dr. Jailani Salihon for their support, advice and motivation while doing this research. Their germinal ideas, invaluable guidance, continuous encouragement and constant support did help me much in making this research possible and finish on time.

I highly value the additional support from all my lab mates and members of the staff of the Faculty of Chemical Engineering and Natural Resources and Centre for Graduates Studies, UMP who help me in many ways and made my stay at UMP pleasant and unforgettable. Indeed, their invaluable assistance and efforts did much to ensure the smooth running of the project.

I would also like to extend my heartfelt thanks and sincere indebtedness to my beloved parents and family for their precious love, dream and sacrifice throughout my life. I am also grateful to my fiancé for her sacrifice, patience and understanding that was inevitable to make this work possible. I cannot find the appropriate words that could properly describe my appreciation for their devotion, support and faith in my ability to attain my goals.

Finally, again I would like to express my thanks to all who involve in completing this research. Only Allah will reciprocate your goodness.

ABSTRACT

This research dealt with the identification of acetic acid producer (AAP) from soil mixed culture (SMC), production of acetic acid from banana stem waste (BSW) and kinetic study of the acetic acid production. 57 bacterial strains were isolated from SMC. From the isolation, 15 strains produced acetid acid. Later, only five most efficient bacterial strains were identified using biochemical test and biomolecular test. Those five strains were labeled as A1, A2, A6, A7, and A8. All isolated strains were in rod shape and the colonies size ranged was from 1.0mm to 3.0mm. A1 was identified as *Bacillus cereus PG06* with 92% match, A2 as *Klebsiella pneumonia ST258* with 92% match, A6 as *Bacillus thuringiensis BMB171* 90% match, A7 as *Bacillus sp. SJ1* with 77% match and A8 as *Enterobacter cloacae Y219* with 89% match. *Bacillus thuringiensis BMB171* (A6) is the most productive bacteria that produced resulted in highest yield of acetic acid (up to 4.4 g/l) compared to other identified strains. Later, kinetic study was conducted using this strain. The experiment was conducted using BSW and glucose medium (as control substrate) in batch fermentation for 72 hours. The proposed kinetic model have 7 kinetic constants (k_1-k_7) which determined based on the sum squared error between experimental data and theoretical data by using Microsoft Excel. The initial concentrations of biomass (S_{VSS}), glucose (S_{VDS}), and acetic acid (S_{TAA}) in the calculation were: $(S_{VSS})_0$ = 0.0002 g/l; $(S_{VDS})_0$ = 2.46 g/l; $(S_{TAA})_0$ = 0 g/l. The k_1 is the kinetic constant of the reaction, k_2 is the maximum rate of glucose uptake for growth, k_4 is the maximum rate of glucose uptake for acetic acid production, k_6 maximum rate of acetic acid consumption, and k_3, k_5, k_7 is the saturation constant. The values of calculated kinetic constants were as followed: k_1 = 0.5004 h^{-1}; k_2 = 0.0576g /l h; k_3 = 0.6197g /l; k_4= 0.1294g /l h; k_5= 0.9289g /l; k_6= 0.0545g /l h; k_7= 0.0001g /l. The kinetic constant of substrate utilization and production for BSW was lower than using glucose as medium. This is due to secondary metabolism. In comparison of kinetic study from other researchers show that, the k_1 rate were higher than other research however other kinetic constant were in acceptable range. This is due to the nutrient supplied promoted the bacteria growth. The R^2 values obtained from kinetic study were in acceptable range (above 0.8). Overall, the *Bacillus thuringiensis BMB171* (A6) was the most productive acetic acid producer from soil and, the proposed kinetic model could be used to describe the process.

ABSTRAK

Penyelidikan ini berkaitan dengan pengenalpastian penghasil asetik asid (AAP) dari kultur campuran tanah (SMC), penghasilan asetik asid dari sisa batang pisang (BSW) dan kajian kinetik penghasilan asetik asid. Dari proses pemencilan, 15 strain berjaya menghasilkan asetik asid. Hanya lima strain yang terbaik dikenalpasti menggunakan ujian biokimia and biomolekular. Lima strain tersebut dilabel sebagai A1, A2, A6, A7 and A8. Kesemua strain diasingkan berbentuk rod dan koloni saiz sebesar 1.0mm hingga 3.0mm. A1 dikenalpasti sebagai *Bacillus cereus PG06* dengan 92%, A2 sebagai *Klebsiella pneumonia ST258* dengan 92%, A6 sebagai *Bacillus thuringiensis BMB171* dengan 90%, A7 sebagai *Bacillus sp. SJ1* dengan 77%, dan A8 sebagai *Enterobacter cloacae Y219* dengan 89%. *Bacillus thuringiensis BMB171* (A6) merupakan strain terbaik yang berjaya menghasilkan paling banyak asetik asid (4.4 g/l) apabila dibandingkan dengan strain yang lain. Bakteria ini dipilih untuk kajian kinetik. Uji kaji ini dijalankan dengan menggunakan BSW dan glukosa (substrat kawalan) dalam fermentasi berkelompok selama 72 jam. Model kinetik yang dipilih mempunyai 7 pemalar kinetik (k_1-k_7) yang diperolehi menggunakan perbezaan jumlah ralat kuasa dua antara data eksperimen dan data teori dengan menggunakan Microsoft Excel. Nilai kepekatan awal biomas (S_{VSS}), glukosa (S_{VSS}), dan asetik asid (S_{TAA}) yang digunakan diperolehi dari media BSW adalah seperti berikut: $(S_{VSS})_0$ = 0.0002 g/l; $(S_{VDS})_0$ = 2.46 g/l; $(S_{TAA})_0$ = 0 g/l. k_1 merupakan pemalar kinetik bagi tindakblas, k_2 merupakan pemalar bagi kadar maksima pengambilan glukosa ketika pertumbuhan, k_4 pemalar bagi kadar maksima pengambilan glukosa ketika penghasilan asetik asid, k_6 merupakan pemalar bagi kadar maksima pengunaan asetik asid, dan k_3,k_5,k_7 merupakan pemalar ketepuan. Nilai pemalar yang diperolehi adalah seperti berikut: k_1 = 0.5004 h^{-1}; k_2 = 0.0576g /l h; k_3 = 0.6197g /l; k_4= 0.1294g /l h; k_5= 0.9289g /l; k_6= 0.0545g /l h; k_7= 0.0001g /l. Pemalar kinetik penggunaan substrat dan penghasilan untuk BSW lebih rendah dari glukosa. Perbezaan ini disebabkan oleh metabolisma kedua. Hasil dari perbandingan antara kajian menunjukkan bahawa pemalar k_1 lebih tinggi dari kajian lain, manakala pemalar kinetik lain berada dalam kadar bersesuaian. Ini disebabkan oleh nutrisi yang dibekalkan menggalakkan pertumbuhan bakteria. Nilai R^2 yang diperolehi turut berada dalam kadar bersesuaian (melebihi 0.8). Kesimpulanya, *Bacillus thuringiensis BMB171* (A6) merupakan penghasil asetik asid dari tanah yang paling produktif dan model kinetik yang digunakan sesuai untuk menjelaskan proses yang terlibat.

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LIST OF NOMENCLATURE

h	Hour
min	Minutes
L	Liter
ml	Milliliters
M	Mole
℃	Degree Celsius
pH	Potential Hydrogen
µl	Microliter
%	Percentage
g	Gram
ml	Mililiter

LIST OF ABBREVIATIONS

AAP	Acetic Acid Producer
BSW	Banana Stem Waste
SMC	Soil Mixed Culture
NA	Nutrient agar
NB	Nutrient Broth
PCR	Polymerase Chain Reaction
OD	Optical Density
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
rRNA	Ribosomes Ribonucleic Acid
rpm	Revolutions per minute
HPLC	High Performance Liquid Chromatography

CHAPTER 1

INTRODUCTION

This chapter gives the fundamental information of the research. It is divided into the subtopics of background of study, problem statement, research objectives, scope of study, and thesis organization.

1.1 BACKGROUND OF STUDY

The agriculture activity is becoming one of the most important industries in Malaysia. The expansion of agricultural activity in Malaysia gives a lot of advantages by increasing the economic and development in rural area. But, there are also disadvantages where too many agricultural wastes were produced. Malaysia peninsular only, produced up to 4.2 million tons of crop residue (USM RCE, 2006). Usually, the wastes are not treated and left on the fields, discarded into the rivers or treated by open burning by the settlers and farmers. So, it is becoming increasingly difficult to ignore that the agricultural wastes are one of major organic pollutants that polluting Malaysia's rivers and air (USM RCE, 2006).

The agricultural waste or crops can be considered as renewable alternative feedstock than fossil fuel (petroleum). One of the largest agricultural wastes in Malaysia is banana stem waste.

Banana remains as the second most important fruit crop after durian in Malaysia. Banana stem waste, abundantly available in banana production fields and market, appears to be a favorable substrate for acetic acid production as it cheaply and easily to get in the tropical and subtropical countries. According to Baharudin (2009), in every 60 kg of banana grown, almost 200 kg of waste stem are thrown away. Banana plant is in Musacae family, and it is a monocotyledon. Banana stem waste is lignocelluloses, which consist of lignin, cellulose and hemicellulose. It consists of 15.42% lignin, 53.45% cellulose and 28.56% hemicellulose (Silveira et al., 2007). This data shows that high cellulose content can be recovered from banana stem. Thus, it is very suitable to be feedstock for biomass energy and other products such as for acetic acid production.

Acetic acid is one of the organic acids. This high demand material is widely used in industrial application. It is applied in production of plastic monomer, in food industry as vinegar or food additives and few in pharmaceutical industry. There are two fermentation processes for production of acetic acid which are aerobic or anaerobic process. Acetic acid is carbon sources for acetoclastic methanogen, which convert acetate from acetic acid to produce methane (Naidoo et al., 2002). Zainol and Rahman (2009) found that the reaction to produce methane occurred between mixed cultured from a soil sample with banana stem waste in anaerobic condition.

There is also a need to find a potential strain of bacteria that can directly convert the waste to acetic acid from soil. It was cost effective and highly feasible. Specific types of bacteria only involved in specific process. It is due to the product from the process may become toxic to the bacteria. In several cases, some species may involve in several processes such as *Enterobacter sp.* Common isolation method by using selective agar is not viable for this strain. Isolation of acetic acid producer (AAP) from soil and kinetic study of the most efficient AAP were conducted in this study.

1.2 PROBLEM STATEMENT

Some of the criteria to select raw material for production of acetic acid via

fermentation process are the material must contained high amount cellulose, abundantly, and not expensive (Busche, 1991; Ebner et al., 1996; Ravinder et al., 2001; Veny and Hasan, 2003). However, there has been little discussion about banana stem waste (BSW) as raw material for production of acetic acid.

Bacteria play an important role in the fermentation process. Besides, the research to date has focused more on aerobic fermentation rather than anaerobic fermentation. Aerobic fermentation of glucose to acetic acid involved two stages process, which are conversion of substrate into ethanol followed by ethanol oxidation into acetic acid. Therefore, this process needs two reactors which increased the cost (Veny et al., 2003; Veny and Hasan, 2005). Furthermore, researches nowadays are dealing with commercial bacteria. Those bacteria are expensive and hard to manage (Song et al., 2010; Veny et al., 2003).

Hence, there is a need to find anaerobic AAP that can produce higher yield, convert complex substrate directly to acetic acid, easy to obtain, easy to handle and cheap. Besides, Zainol and Rahman (2008) found that mixed culture from soil have the ability to produce acetic acid. Hence, soil is a good source to find the pure strains that can replace the aerobic AAP. The potential bacteria such as *Clostridium sp.*, *Aeromonas sp.*, *Syntrophomonas sp.*, and *Enterobacter sp.* can be obtained from soil (Hatamoto *et al.*, 2007; Manter et al., 2010; Lee et al., 2009; Song et al., 2010; Sousa *et al.*, 2007; Zhang *et al.*, 2004).

Kinetic study is needed in order to understand the performance of the system. It is necessary for balanced and rational design of a biological waste-treatment system to forecast the system stability, quality of effluent (Garcia-Ochoa et al., 1999). In some process different type of bacteria will affect the kinetic model used due to some bacteria can secrete several types of enzymes. This may affect the kinetic constant and R^2 value. Therefore, it is important to find the suitable model for specific bacteria. The most common model is Monod model, due to the flexibility of the model. The model implements the effect of a growth limiting nutrient on the specific growth rate. The relation between growth rate, substrate concentration and concentration of product is used in order to understand the process.

1.3 RESEARH OBJECTIVE

There are two main objectives in this research,:

1. To isolate and identify bacteria from soil involved in banana stem waste (BSW) fermentation
2. To determine the kinetic parameter for acetic acid production via BSW fermentation.

1.4 SCOPES OF THE STUDY

The scope of the study is based on the research objective above:

1. To isolate and identify bacteria from soil involved in banana stem waste (BSW) fermentation
    a. To isolate anaerobic bacteria from soil containing banana stem waste as substrate.
    b. To identify anaerobic bacteria that produces acetic acid using banana stem waste via fermentation.
    c. To identify the most efficient anaerobic bacteria that produces acetic acid.

2. To determine the kinetic constant for acetic acid production using BSW via fermentation.
    a. To apply the available kinetic model for acetic acid production using BSW via fermentation.
    b. To use the highest acetic acid producer in the kinetic study.

1.5 THESIS ORGANIZATIONS

This thesis consists of five main chapters. Chapter 1 is outline of the study on isolation of AAP from soil mixed culture and kinetic study in general. For chapter 2, will be describing the literature reviews of every scope covered in this study. Then, the methodology, apparatus and equipment works are discussed in Chapter 3. In addition, the

experimental results are discussed in Chapter 4. Finally, chapter 5 concludes the study and consists of suggestion for future research. This thesis is completed with references and appendices.

CHAPTER 2

LITERATURE REVIEW

2.1 ACETIC ACID

Acetic acid, also known as ethanoic acid (CH_3COOH) is one of many types of organic acids. It is the main component in vinegar and has a unique sour taste and strong smell. Industrially, acetic acid is widely used in a production of plastic monomer and reagents for pharmaceutical industries (Hosea et al., 2005).

2.1.1 Acetic acid in Food Industries

Acetic acid in food industries is commonly known as vinegar. It is commonly produced by the fermentation process and regularly used for pickling processes, vinaigrettes, salad dressings, and component in sauces such as mustard, ketchup and mayonnaise (Das and Sarin, 1938). The vinegar is typically produced from raw food such as fruit, vegetables and even fishes. Local people in Malaysia usually produce vinegar using rice, coconut water and nypa water (nira Nipah). In a western country, the vinegar is produced from wine, cider, balsamic, raisin, cane and other fruit juices (Hill et al., 2005).

2.1.2 Acetic acid in Pharmaceutical Industries

Acetic acid can also be used for medical purposes. Prophet Muhammad which lived about 1,400 years ago stated that the best condiment is vinegar (Sahih Muslim, a). He also

stated that vinegar is a comfort for man (Sahih Muslim, b), which can be said that, vinegar is good for health. Avicenna who wrote ‘The Canon of Medicine', and Ibn Qayyim Al-Jawziyya claimed that vinegar is a powerful clotting agent. It helps in healing skin burns, gastric inflammations, heartburns and also prevents the effects of toxic medications or poisonous mushrooms. Besides that, the effect of using vinegar is it relieves headaches caused by heat, and prevents tumour from occurring.

Furthermore, it can significantly lower values for serum total cholesterol and triacylglycerol, which will reduce the risk of fatal ischemic heart disease (Fushimi et al, 2006; Johnston et al, 2006). Vinegar is also used to promote lysis of red blood cells in order to do manual white blood cell counts. Moreover, it is applied for cervical cancer screening tool and antimicrobial uses due to its acidity (Szarewski, 2007; Medina et al., 2007).

2.1.3 Acetic acid in other application

Instead of vinegar, acetic acid also can be found in the form of glacial acetic acid. Glacial acetic acid is 99% pure acid which is dangerously corrosive. It is an important reagent used to produce plastic drinking bottles, cellulose acetate for photographic film and polyvinyl acetate for wood glue (Cedar Petrochemicals, 2011). Other than that, acetic acid also acts as a solvent for various applications. Acetophenone is one of the solvent produce from acetic acid and used for the making of alcohol-soluble resins via dry distillation process with calcium salts, acetic acid and other acids. Then, the resins are applied in a whole range of perfumes.

2.1.4 Acetic acid market demand

Acetic acid is a global product. China, United States of America, the rest of Asia and Western Europe are largest acetic acid consumer. The annual worldwide demand of acetic acid is around 6.5 million tonnes of which, approximately 1.5 million tonnes are produced by a fermentation process (Starcontrol, 2011). According to Malveda and Funada (2010), from the entire global acetic acid capacity (virgin acid), 65% is in the Asia where China is the major capacity, 19% in the United States of America and the rest is from other regions. These two regions make up to 84% of whole world capacity. Moreover, China is the largest consumer with 30% of total demand. The rest of Asia accounted for 27% of global demand including Malaysia, with 1 to 3% acetic acid consumption in the world, which is nearly 0.12 million tons acetic acid per annum, followed by 20% in the United States of America and 14% in Western Europe. These regions totalled over 91% of worldwide acetic acid consumption (Malveda and Funada, 2010).

In addition, acetic acid is an important feedstock for production of plastic monomer such as vinyl acetate monomer (VAM) and terephtalic acid (TPA) which holds about 80% of world consumption. In Malaysia, 92,000 tonnes acetic acid per year is needed as raw material to produce TPA, which is locally produced at Kerteh, Terengganu and generates almost 535,000 tonnes acetic acid per annum. Most of the production of acetic acid in Malaysia is exported to China. Although China is the main acetic acid consumer and also the world major plastic producer, they could not afford to fulfil their own demand due to the limited resources in China, hence the need to import them from other countries. Malaysia is known as one of the top supplier acetic acids for China's market, which contributes 10 - 20% total import of acetic acid in China (IHS chemicals, 2012).

The acetic acid market will continue to grow due to the expansions of VAM and TPA quantity in the Asia region, especially in China and Middle East (Goliath, 2006). Due to high demand but low production or supply around the world, the price for acetic acid is really expensive which cost almost RM 3500.00 per tonnes (Lee and Shen, 2011). Because of the total global acetic acid demand that is massively increasing per year, an alternative

method to produce acetic acid need to be introduced. The usage of non-renewable source can be substitute with renewable source because non-renewable source will be depleted. Biological process was introduced as an alternative method that can utilize the renewable source in acetic acid production.

2.2 RAW MATERIALS (LIGNOCELLULOSE MATERIALS)

Raw material is the unprocessed items that are broken down, undergo process or combine with some materials to create a finished product (Roland et al., 2011). In a biological process such as production of acetic acid, the raw materials used are usually natural resources or biomaterials. Few examples of biomaterials are waste from banana stem, corn plant, yam plant and renewable raw materials such as households waste, municipal waste, paper mills and many others. This diversity of biomass can be used to convert cellulose, and then into acetic acid (Okonko et al., 2009). The recycling industry has long started in producing raw materials out of other products.

2.2.1 Banana Stem Waste (BSW)

Banana is a herbaceous perennial of the genus Musa (family Musaceae) that grows in high humidity weather like tropical and subtropical climates. Malaysian tropical rain forest is well known as the centre of origin for this genus. Banana is a main crop of many regions generating a huge pile of waste after harvesting (Roux et al., 2008). Most of the fruits were consumed by local people and about 10% are exported to Singapore, Brunei, Hong Kong and Middle East (Pawiro, 2008). The banana waste, being abundantly available, easy to get and awfully inexpensive, appears to be a favourable substrate for cellulose production.

This agro waste is used as the substrate to produce cellulose in a single step fermentation process. The stem being the most valuable part in a banana tree is mainly composed of 53.45% cellulose, 28.56% hemicellulose and 15.42% lignin that come together as lignocelluloses (Silveira et al., 2008; Elanthikkal et al., 2010). Lignocelluloses

are a complex material of rigid cellulose fibers surrounded in a cross-linked medium of lignin and hemicellulose. This shows that the cellulose content that to be recovered from banana stem is high.

2.2.2 Corn Stalk Waste

Dracaena fragrans also known as Corn is a flowering plant. It is a type of food for the livings. Under the family name of *Asparagace*, it is widely grown as office plants and houseplants. Native to western Africa, *Agave* is also grown as ornamentals in tropical climates. These plants were popular because of only minimal maintenance were needed. According to Indexmundi (2012), Malaysia produced up to 100,000 MTA in 2011 and its growth rate was increased up to 5% from the previous year.

According to Chahal et al., (1990), stalks remaining after it was harvested contain 43% polysaccharides consisting mainly of hemicellulose, 29% cellulose, 7% lignin, 5% ash, and 16% others. Cellulose is the main component in plant cell walls. It gives plants structure and provides them with strength and stability. It is also the basis of grasses, woods, and plants stalks, and accounts for 44% of all plants' biomass. Corn stalks are one of them.

In order to obtain acetic acid, the cellulose needs to break down using hydrolysis process. The hydrolysis process breaks the cellulose molecules into its basic sugar form such as glucose and other pentose sugar. This sugar is utilized by microbes, which then would produce acetic acid. The acetic acid will be produced along with the other sub-products such as sulfuric acid that will reduce the acetic acid quality (Guo et al., 2011).

2.2.3 Yam Waste

Yam plant is a monocot colic root with a twining and tuberous vine. Most of the Yam species came from the same genus *Dioscorea*, where 600 wild species that grow well in damp woodlands and thickets, but not all them contain Diosgenin (Coursey, 1967;

Davidson 1999; Kiple et al., 2000). Wild yam usually has a perennial vine with pale brown, knotty, woody cylindrical rootstocks, or tubers. The roots are dry, narrow, and crooked. Its stem can grow to a length of over 30 feet. This differs with sweet potato where sweet potato is a dicot plant and has much complex structure, and also, the Yam root has unique taste (Dhir et al., 2009; Schultheis and Wilson, 1993). The wild yam plant has clusters of tiny, greenish-white and greenish-yellow flowers. The heart-shaped leaves are extended, wide and long-stemmed. The upper surface of the leaves is smooth while the underside is furry (Asiedu et al., 1997). The dietary fiber, non-cellulose polysaccharides, cellulose and lignin content in yam reported to be 13.2%, 9.7%, 2.9% and 0.1%, respectively (Akingbala, 1995; Adamson, 1985). It is reported that waste from yam peels and leaves contained high amount of cellulose which is 40.7% and 13.7% hemicelluloses (Akinfemi et al., 2009).

2.2.4 Cassava Waste

Cassava (*Manihot esculenta Cranz*) or tapioca is one of the important sources of food in tropical countries such as Asia, Africa and South America. It is considered to be originated from South America. It produces tubers and made up from two parts, which are aboveground and underground. The above ground part can be as high as 2-4 m with a trunk and branches on it. The underground part consists of two types of roots which one is responsible for the plant nutrition, while others with axial outlook surrounding the trunk. These parts are called tubers and are the parts that commonly used for food. Each plant may have 5-20 tubers, which may reach a length of 20-80 cm and 5-10 cm diameter (Pandey et al., 2000).

According to Howeler (2002), Malaysia produces 430,000 tonnes from 41,000 hectare around Malaysia. Almost 1.16 tonnes peels, 42 tonnes cassava bagasse from 300 tonnes cassava root used were produced. These wastes are usually left as it is in the environment without any treatment (Pandey et al., 2000). The cassava peels and bagasse contained of 33.2% cellulose and 21% hemicelluloses (Akinfemi et al., 2009).

2.2.5 Rice (Paddy) Waste

Rice is the seed of the monocot plants where *Oryza sativa* (Asian rice) or *Oryza glaberrima* (African rice) is widely known and commonly used for agricultural production. As a cereal grain, it is the most important food source and consumed in a large quantity widely around the world, especially in Asia and the West Indies. According to FAOSTAT (2006), paddy is the third-highest worldwide agricultural production, after maize (corn) and wheat. Paddy is produced mainly on the northern part of west peninsular Malaysia, and some part in Borneo part of Malaysia. The total land used for paddy plantation in Malaysia, is around 673,745 hectares (FAOSTAT, 2010).

Usually, after the rice is harvested, the straws and hulls or husks are left on the field and burned to dispose the waste. Rice husks are the layer that protects the grains of rice. Nowadays, consumers have shown efforts in reducing greenhouse gas emissions by contributing new alternative methods to reduce air pollution. In California, rice straw was considered as renewable sources alongside softwood as the base substance for building materials (Jenkins et al., 2000). It contained 33.9% cellulose, 25.6% hemicellulose and 10.2% lignin (Jin and Chen, 2006; Jenkins et al., 2000). Other than that, Abdel Wahab et al. (2005) reported that the rice hulls contained of 32.24% cellulose, 21.34% hemicellulose, 21.44% lignin. Due to high lignin contained in the rice hulls, it is normally used as additives in composite production (Chandra, 1997; Klyosov and Klesov, 2007). Furthermore, according to Karagoz et al. (2005), rice hulls can also be used as an acetic acid substrate.

2.2.6 Oil Palm Waste

The oil palms consist of two species of the *Arecaceae*, or palm family. Both of the species are used in commercial agriculture in the production of palm oil. The African Oil Palm *Elaeis guineensis* is native to West Africa, come from Angola and Gambia, while the American Oil Palm *Elaeis oleifera* is from tropical Central America and South America (DOA, 2009; Lötschert and Beese, 1983). According to Lam (2009), almost 4.3 million

hectares land was used for plantation of oil palms in Malaysia. This plantation includes the Federal Land Development Authority (Felda) and Sime Darby Group, which combines to form the world biggest oil palm planter.

Usually, the oil palm trees need to be replanted after 10 years of harvesting. The oil palm trunk (OPT) fiber wastes were obtained after the Oil Palm Trees were cut down. These fiber wastes contained 39.9% if cellulose, 28.9% hemicelluloses, 20.3% lignin and 3.6 % ash content (Chow et al., 1993). The cellulose content in the OPT fiber makes the fiber as one of the favourable substrate for acetic acid production. The empty fruit bunch (EFB) from Oil Palm Tree can also become substrate. According to Norhayati, (2006), 230 kg of EFB could be obtained from one tonnes fresh fruit bunch (FFB) processed. The EFB contained high amount of cellulose varied from 45% to 50%. However, the lignin content (25% - 35%) in the EFB was high. High lignin content would reduce bacteria efficiency to access cellulose (Jamaluddin et al., 2012).

2.2.7 Selection of Raw Material

A good raw material must have suitable properties with high excellencies to achieve a quality product that serves its needs and purposes. For example, in the acetic acid production, it requires an anaerobic process where the raw material to be used must contain high amount of cellulose, should be inexpensive, easily accessible and always available all year round.

One of the profoundly potential and profitable raw materials is banana stem waste since it is a very good source of cellulose and it's also an abundant natural source in the tropical and subtropical regions (Li et al., 2010). After harvesting banana fruits from the trees, each of the stem will be cut down and left in the soil to degrade itself naturally. This leaves a huge pile of abandoned biomass organic waste, ready to be exploited and used as a renewable source.

It has been estimated that for every 60kg of full-grown banana, 200kg of stem are

thrown away. Abdul Khalil et al. (2006) stated that in Malaysia, the area of banana plantation is estimated to be 34,000 hectares. Structural materials in banana stem are composed mainly of three bio-based chemicals called cellulose, hemicelluloses and lignin. Typical biomass contains 40% to 60% of cellulose, 20% to 40% of hemicelluloses and 10% to 15% of lignin. Only less than 10% of the dry biomass weight is account for other minerals such as phosphorus, nitrogen, potassium, manganese and others (Abdul Rahman, 2005). Whereas, banana stem waste consists of 15.42% lignin, 53.45% cellulose and 28.56% hemicelluloses (Silveira et al., 2007). Zainol and Abdul-Rahman (2008) stated that in their findings, the banana stem waste contains of 15% lignin and 55% cellulose.

Banana stem will be used as a raw material due to the high amount of cellulose. Its low lignin content will also benefit bacteria that involved in production of acetic acid. Low lignin content will increase bacteria efficiency to access cellulose. Table 2.1 presents the scientific classification of banana plant.

Table 2.1: Scientific Classification of Musa sp.

Class	Monocotyledonae
Order	Scitamineae
Family	Musaceae
Genus	*Musa*
Section	Eumusa
Species	*M. acuminate* (AA) *M. balbisiana* (BB)

2.3 PROCESS AND CHEMICAL REACTION

Process is a definition of subject to a treatment, with the intention of readying for some functions or purposes, improving, or remedying a condition to convert inputs into outputs. It is combined principles, which work in many diverse systemic contexts. In this study, biological process is required in order to produce acetic acid. In the biological process, there are series of chemical reactions catalyzed by enzymes form metabolic pathways, where the syntheses and decompositions can be performed within a cell or

extracellular process (Karlson et al., 2005; Nic et al., 2006).

2.3.1 Biological Process in Production of Acetic Acid

A biological process is a process of living organisms that are fabricated from chemical reactions or other events that result in transformation. An example of biological process is the biodegradation of the lignocelluloses waste, where method to produce acetic acid. The methods can be divided into two, which are aerobic fermentation and anaerobic fermentation. Commercial companies that are producing acetic acid are generally using basic raw material such as pure glucose, pure ethanol and expensive pure strains.

In this subtopic, the fermentation using lignocelluloses waste in the production of acetic acid is deliberately discussed, to ensure that this process is worth to be implemented in industry or for scale up purposes. Although it is believed that using lignocelluloses waste as raw material will results more highly cost-effective production, there is still a need to find the most efficient and feasible selection of processes. In a same purpose using aerobic process, Gaensakoo et al. (2005) stated in his findings that the process need an extra reactor which in the end will increase the total cost. In contrast, for anaerobic fermentation, limited oxygen supplies are required in the whole time which could lead to a low cost production as well. Hence, the advantages and disadvantages of both processes should be compared.

The aerobic fermentation of lignocelluloses waste to acetic acid required two stages of process. First is to convert waste into ethanol, followed by oxidation of the ethanol into acetic acid. So, it requires two separate fermentors (Veny et al., 2003). The problem with the conventional fermentation process is that it could only give acetic acid yield around 0.67g acetic acid/g glucose (Veny et al., 2003). There is also a need to purge gas in order to eliminate oxygen for ethanol production in the first stage, and the yield of acetic acid depends on how much ethanol is produced. It has been proven by Kondo and Kondo (1996) in their findings to produce ethanol that, when less oxygen exists in the reactor, the amount of ethanol is increased. Advantages of using this type of fermentation are, it could minimize the production cost since it uses a renewable resource as raw material and energy utilization

will be reduced (Veny et al., 2003).

Meanwhile, the anaerobic fermentation of lignocelluloses waste to acetic acid only needs one reactor (Veny and Hasan, 2005). The advantages of using this fermentation are, minimize the production cost significantly since it uses only one reactor, uses renewable resource as raw material, and low energy utilization. Only one type of anaerobic strain can be used in acetic acid production which is either strictly anaerobe or facultative anaerobe. None oxygen environment is vital for strictly anaerobic strain, while the facultative anaerobe can be used in low oxygen environment. Furthermore, facultative anaerobe usually requires less supervision and tougher compare to strictly anaerobe. In order to create the anaerobic environment, gas purging into the reactor need to be done. The reactor also needs to be seal properly. Even though, this method will increase the cost, it was proved to be less expensive than having to operate two reactors (Veny and Hasan, 2005).

2.3.2 Selection of Process

The biological process chosen for this research is anaerobic fermentation. Lignocelluloses source is used as raw material for the fermentation. The acetic acid yield is depending on the cellulose amount in lignocelluloses source.

Acetic acid could be produced by anaerobic fermentation. Direct conversion of cellulosic biomass to acetic acid could eliminate separate fermentation reactors. It is economical and time-efficient (Veny et al., 2003). Single step anaerobic fermentation process requires an absence of oxygen. Hence, the condition during all fermentations should be strictly anaerobic by supplying nitrogen (N_2) gas (Veny et al., 2003). However, there is a need to find the suitable bacteria that is efficient, inexpensive and easily available.

2.4 MICROOORGANISM IN ACETIC ACID PRODUCTION

Microorganism plays an important role in a fermentation process. Without microorganism, no product can be produced in the fermentation process. In this sub-

chapter, the potential aerobic and anaerobic bacteria will both be discussed. The sources for inoculums also play an important role in the process. There are several types of sources which are domestic sewage, activated sludge, brewery waste, farm waste, and soil. The most feasible source is from the plantation soil. The isolation of microorganism from soil and their cultivation in a pure culture are important requirements before accurate identification, and characterization can be accomplished. Several different processes are used for this purpose, including the classic plate casting process invented by Robert Koch (De Lucas et al., 2001; Lopez et al., 2001), as well as dilution methods or selective enrichment (Navarette-Bolanos et al., 2007)

2.4.1 Soil Bacteria

Almost 30 percent of the earth's surface consists of soil, of variety of sorts. Microorganisms or microbes had existed in the soil of the earth for billions of years before plants, and animals appeared. Therefore, their evolutionary diversity has far outpaced that of higher organisms. This huge diversity accounts for some of the stunning properties of microbes. Microbes can be defined as a small living thing or organism. They are of microscopic size and need the aid of a microscope to view them. Microbes can be divided into seven groups, which are bacteria, archaea, fungi, protists, microscopic animals, microscopic plants and viruses.

The aerobic bacteria require oxygen to live while anaerobic bacteria do not, where some of the anaerobic bacteria may not survive in oxygen because it will become toxic for them. There are a variety of types or species of these two common bacteria which live in the soil. Anaerobic bacteria grow well in a completely drained soil. But, aerobic bacteria prefer moist and damp soil. The anaerobic bacteria may produce toxic compounds, which can cause root disease to plants (Reid and Wong, 2005). However, some of the anaerobic bacteria live symbiosis with the plant beneath the soil in a nitrogen fixation process. The nitrogen fixation is indispensable as the nitrogen is important to all life forms. The protein basic building block (amino acids), cell information and regeneration (DNA and RNA) for plants, animals and other life forms require nitrogen in order to complete the biosynthesis

process. Good amount of nitrogen may enhance the plant growth rate. Thus, nitrogen fixation can improve the agricultural activity, and fertilizer development (Reid and Wong, 2005).

There are two types of bacteria able to produce acetic acid, one is aerobe bacteria and the other is anaerobe bacteria. The most commonly used is the aerobic bacteria such as *Acetobacter sp.* and *Gluconobacter sp.* These bacteria are well-known but mostly unwanted in wine and beer industry because they successively act as a parasite that corrupts the quality and yield of alcohol production. Some of *Acetobacter* species can be found in garden soil and canal water, and some of the species may live in a semi anaerobic environment where only small amount of oxygen may exist. *Gluconobacter* species is commonly found in food waste, and some can be found in soil (Swingley et al., 2007).

Clostridium sp. is a well known anaerobic bacteria that produce acetate, found in woodland soil (Küsel et al., 2001; Küsel et al., 2002; Li et al., 2007). *Clostridium tyrobutyricum* grouped is a mesophilic bacteria found in soil and it is one of many *Clostridium sp.* used to produce acetic acid. Others such as, *Desulfovibrio sp.* can be found in water logged-soil (Madigan and Martinko, 2006), *Syntrophomonas sp.* is found in mesophilic conditioned soil (Wu et al., 2006), and *Aeromonas sp.* can be found in anaerobic conditioned soil (Lee et al., 2008; Lee et al., 2009). Besides that, lactic acid bacteria commonly found from spoiled wine and beer that is also can be found in soil, can produce acetic acid (Magnusson et al., 2006). However, only heterofermentative lactic acid bacteria is able to produce acetic acid, while others can only produce lactic acid (De Vuyst et al., 2009; Vrancken et al., 2008).

2.4.2 Aerobic Bacteria

The aerobic type bacteria to produce acetic acid belong to the family group of *Pseudomoadaceae*. Its cells are normally rod-shaped, but it may be formed elongated, swollen or branched shapes. The cells can be motile and do not have endospores properties (Niir, 2003). Gullo and Giudici (2008) found that, the *Acetobacter sp., Gluconobacter sp.,*

and *Gluconoacetobacter sp.*, can be found in Malaysian soil.

Acetobacter sp.

Acetobacter sp. is commonly known as the acetic acid bacteria because it has the ability to convert ethanol to acetic acid in aerobic environment. Almost all the species in this genus can form acetic acid under various conditions (Cleenwerck et al., 2002). The most common properties between these genuses are the ability to convert ethanol to acetic acid (Madigan and Martinko, 2005). It is a nitrogen-fixing bacteria, obligatory aerobic, gram-negative, motile, and able to oxidize lactate and acetate to CO_2 and H_2O where it can produce approximately 0.40 to 0.88 g acetic acid/ g glucose (Horiuchi et al., 2004; Gullo and Godiaci, 2006; Veny and Hasan, 2005). However, this species involved two stages of fermentation. One, to convert glucose to ethanol, then followed by ethanol to acetic acid. Thus, it needs an extra reactor due to the additional process.

Gluconobacter sp.

Gluconobacter sp. can be found in soil (Gupta et al., 2001). It is a gram-negative microbe and has membrane with outer cell wall. No flagella exist within the species, which make it non-motile. Since it is aerobes, this *Gluconobacter sp*. must first oxidize ethanol or glucose to acetic acid for energy production. The process involves oxidation of sugars, aliphatic and cyclic alcohols, and steroids to oxidation product (Macauley et al., 2001). One of the species such as *Gluconobacter oxydans* has unique feature where it can synthesis glutamate, aspartate, and succinate. This feature is due to membrane-bound dehydrogenases in *Gluconobacter oxydans* as their oxidative capability is used to oxidize polyols into ketones and sugars into acids (Macauley et al., 2001). However, this species is not able to oxidize acetic acid to carbon dioxide and H_2O unlike *Acetobacter sp*. (Kadere et al., 2008). This characteristic is a major difference between the two species, *Gluconobacter sp*. and *Acetobacter sp*.

Gluconacetobacter sp.

Another species is the *Gluconacetobacter sp*. where most of these bacteria are gram-negative, acid tolerant and obligate aerobe, and the cells structures are straight rods with rounded ends. These bacteria can fix nitrogen under microaerophilic, a condition where oxygen concentration is low. The *Gluconacetobacter sp.* are also able to produce acetic acid. However, the species also has the ability to convert reducing sugar to other product. One example is *Gluconacetobater diazotrophicus*, which can hydrolyse sucrose into fructose and glucose using levansucrase, an enzyme (Muthukumarasamy et al., 2002).

2.4.3 Anaerobic Bacteria

The anaerobic bacteria usually produced volatile fatty acids from carbohydrate in anaerobic environment. Those bacteria are commonly known as acid-former. There are two types of anaerobic bacteria that produce acids from cellulose, which are cellulolytic and non-cellulolytic bacteria. These two bacteria may co-operate with each other to produce acids. This is because the non-cellulolytic could not utilize cellulose but will release and provide nutrition to be used by cellulolytic species. This could enhance their growth and at the same time, utilizing a lot of cellulose to reducing sugar and acids. This type of bacteria will produce several types of acids such as formic, acetic, butyric, propionic, lactic, succinic and isobutyric acids from cellulose. Then, the non-cellulolytic species will also utilize the reducing sugar to produce acids (Niir, 2003). Still, the celluloytic bacteria are not the main part of acid formers as it needs suitable type of waste. Common cellulolytic bacteria are mostly gram negative in form of cocci or short rods.

Clostridium sp.

Clostridium sp. is generally known as spore forming bacteria, gram-positive, can grow without oxygen and some are obligate anaerobe. *Clostridium sp*. is usually in the rod form but when producing spores, the bacteria appear more like drumsticks with a bulge at one end (Collins et al., 1994; Ou et al., 2003). According to Song et al. (2008) the

Clostridium sp. is able to produce volatile fatty acids including acetic acid. Their research was conducted using *Clostridium tyrobutyricum* in fed-batch fermenter. This species is able to produce acetic acids up to 5 g/l. *Clostridium acetobutylicum* is another potential bacterium that can also produced the same acid (Zhang et al., 2006).

Clostridium sp. can be considered as the most efficient bacteria in the production of acetic acid due to the high yield of acid and cost effective. It needs only one reactor while *Acetobacter sp.* as mentioned earlier, needs two reactors in order to complete the process. This fact is confirmed by Veny et al., (2005), where they used *Clostridium thermoaceticum* and reported that this species is able to produce up to 0.88 g acetic acid/ g glucose. Also, a work done by Song et al. (2010) found that the *Clostridium tyrobutyricum* can produce acetic acid up to 0.99 g acetic acid/ g glucose. Furthermore, *Clostridium sp*. is also well-known in biohydrogen production due to the ability to convert many types of carbon sources to hydrogen (Zhang et al., 2006; Chong et al., 2009).

However, all the *Clostridium sp.* need to produce acetic acid as an intermediate product before the hydrogen is obtained. In Malaysia, *Clostridium sp.* was used as a hydrogen producer in palm oil mill effluent (POME) anaerobic degradation (Kamal et al., 2011; Chong et al., 2009). The species used is *Clostridium butyricum* where it can be obtained from mixed culture of soil and POME (Kamal et al., 2011).

Aeromonas sp.

Aeromonas sp. is a species of gram-negative bacteria. These organisms are oxidase-positive and anaerobic. *Aeromonas sp.* is a group in the *Aeromonadaceae* family. *Aeromonas sp. is* rod-shaped and has polar flagella (Hugh and Ryschenkow, 1961; Pidiyar et al., 2002). One of the species, *Aeromonas caviae*, is able to produce acetic acid and can be found in soil. It can produce a variety of volatile fatty acids and can also produce acetic acid up to 0.2 g acetic acid/ g glucose (Lee et al., 2009). Yusuf et al. (2009) stated that *Aeromonas* species can be obtained from paddy soils in peninsular Malaysia. Those species are used as natural nitrogen-fixing agents in Malaysia's paddy soils.

Syntrophomonas sp.

Syntrophomonas sp. has a unique multilayered cell wall and lack of internal membrane-bound organelles thus it is a gram-negative prokaryote. Only some strains are found to have sporulating-specific genes. Sousa and co-workers reported that when penicillin inhibits growth, the sensitivity to lysis is increased when treated with lysozyme, and peptidoglycan in the gram negative multi layer will appeared (Sousa *et al.*, 2007). The species, *Syntrophomonas wolfei* is the one able to produce acetic acid. It converts volatile fatty acids such as butyric acid or other long chain fatty acids to acetic acids in anaerobic condition.

It is also reported by Tabassum and Rajoka (2000) *Syntrophomonas wolfeii* could produced 0.92 mmol acetic acid/M glucose. This species can be found in Malaysian soil and commonly used as inoculums in anaerobic treatment of agricultural residues and wastewater (Parawira, 2004). Furthermore, *Syntrophomonas sp.* is also used as an organic acid producer in fermentation using palm oil mill effluent (Hatamoto *et al.*, 2007). *Syntrophomonas curvata*, *Syntrophomonas zehnderi*, and *Syntophomonas wolfeii* are the species that able to produce acetic acid from POME (Zhang *et al.*, 2004; Sousa *et al.*, 2007; Hatamoto *et al.*, 2007). It is also stated that *Syntrophomonas curvata* and *Syntrophomonas zehnderi* can degrade long fatty acids in co-culture with metahnobacterium and produce acetic acid (Zhang *et al.*, 2004; Sousa *et al.*, 2007).

Enterobacter sp.

Enterobacter sp. is a gram negative, facultative anaerobic, rod-shaped bacteria in the family of *Enterobacteriaceae.* Most of the *Enterobacter sp.* can utilize many types of carbon sources such as glucose, saccharose, mannitol, xylose, and sorbitol to produce hydrogen as the end product. In 2006, Mandal et al. have done a research on fermentative hydrogen production by a microbial consortium. From the research, it was indicated that the bacteria used was *Enterobacter cloacae.* The research was carried out in batch reactors

under anaerobic conditions in order to prove the efficiencies of sucrose conversion to hydrogen by *Enterobacter cloacae*. Their result showed that the intermediary products were acetic acid, butyric acid, methanol and ethanol.

Enterobacter aerogenes which can produce hydrogen from reducing sugar are also producing acetic acid. This acetic acid is altering the hydrogen production. The *Enterobacter sp.* is very sensitive to pH where it needs pH range at around 6-7 in order to produce hydrogen (Yokoi *et al.*, 1995; Yokoi *et al.*, 2001; Oh *et al.*, 2003; Rittmann and Herwig, 2012). Moreover, there is a new species known as *Enterobacter soli* found in soil by Manter *et al.* (2010), where this species can produce hydrogen and is also able to produce organic acids as intermediate product. *Enterobacter sp.* has been reported can be found in Malaysian soil (Yusuf *et al.*, 2009; Chong *et al.*, 2009).

2.5 ISOLATION OF MICROBE

Method used for isolating bacteria from its sources requires some kind of medium that allow cells to grow and produce visible colonies. These mediums contain sources of nutrients, energy and minerals essential for the growth of the various microorganisms, such as simple sugar like glucose, amino acids and nitrogen. Ingredients and incubation condition usually can inhibit other unnecessary organisms that may be present such as cycloheximide, which will inhibit the yeast growth. It can also promote the growth of potential isolates such as oxygen, which then will encourage *Acetobacter sp*. and *Gluconobacter sp*. growth. Most of these methods can be used for clinical and environmental application (Table 2.2).

Table 2.2: Some of microbiological media used to isolate and enumerate bacterial for acetic acid producer

Author	Medium	Description
Kim et al., 2002	Raka Ray No.3 Agar	• For the isolation of lactic acid bacteria. • Contained glucose, mannose and fructose for optimum recovery. • Can be made selective by the addition of cycloheximide and 2-phenylkethanol to inhibit yeast and Gram-negative bacteria respectively.
Barraquio et al., 2008	Modified MRS Agar	• MRS with added maltose to encourage growth of lactic acid bacteria unable to ferment glucose
Maal and Shafiee, 2010	Carrs's medium	• Contain bromocresol green for the selective for lactic acid bacteria. • Distinguishes between *Acetobacter* and *Gluconobacter* species by ability to oxidize acetate
Greenberg *et al.*, 2006	Dextrose sorbitol mannitol agar	• Contains cycloheximide to inhibit yeast and sodium desoxycholate to inhibit the Gram-positive bacteria. • For the selective isolation of acetic acid bacteria
Holmes *et al.*, 2007	MacConkey agar	• Contains bile salts and neutral red indicator. Selective for enterobacteria
Killinger *et al.*, 2010	Violet red bile	• For selective isolation of enteric bacteria. Similar to MacConkey agar

2.6 IDENTIFICATION OF MICROBE

The conventional biochemical tests were used to identify the unknown microorganism at a species level. This method usually takes days to weeks and can be very tedious (Guarin et al., 2010; Yakrus et al., 2001). Throughout this method, a single colony is used by streak on a nutrient agar plate to determine if their colony form shape size and also for gram determination. After the bacteria, gram stains are known, the biochemical test using API kit or other type of kit such as BIOLOG kit was followed. Sometimes, the result from the biochemical test is not sufficient. Therefore, the test was followed by the biomolecular method where a gen 16S rRNA which contained of rRNA information sequencing method was used (Heller et al., 2008). Table 2.3 explained the common method used for bacteria identification.

The gen 16S rRNA sequencing involved several steps, including DNA extraction, gel electrophoresis, polimerase chain reaction and DNA sequencing. In DNA extraction step, the DNA of sample was extracted from cells in order to purify it with minimal mechanical disruption to the DNA (Herrick et al., 1993; Lee et al., 2010). The DNA was extracted using cell lysis solution and purified and stored in the appropriate buffer. Then, the size of DNA and RNA fragment in the purified DNA extracted was determined using gel electrophoresis. This step was important before the purified DNA to be used in Polimerase Chain Reaction (PCR). This is because, the smaller size of purified DNA could easily be generating copies of a particular DNA (Herrick et al., 1993; Lee et al., 2010). Then, the PCR product which contains of DNA fragments of known size was compared with DNA ladder (molecular weight maker) using gel electrophoresis. DNA sequencing was used for determining the order of the nucleotide bases- adenine, guanine, cytosine, and thymine in a molecule of DNA . This technique was time-consuming but highly significant as it allows scientists and researchers to read the nucleic acid make-up of individual genes (Herrick et al., 1993; Lee et al., 2010).

Table 2.3: Common method used in identification process of bacteria

Method	Description
Gram Staining	• Simplest method to identify bacteria into two large groups which are Gram positive and Gram negative based on the physical properties of their cell walls which may contained peptidoglycan or not.
API kit (Biochemical Test)	• API kit consists of many tubes. • These tubes were developed for clinical use to identify bacteria. • Each tube has different type of media which give the results if there are presences of a different enzyme or set of several enzymes in the unknown bacteria.
BIOLOG kit (Biochemical Test)	• Phenotypic Fingerprint. Have 1000-5000 identity of bacteria • Develop for clinical and environmental use to identify bacteria. • Each tube contained of many types of carbon sources and chemical sensitivity assay.
Polymerase Chain Reaction (PCR)	• PCR is highly sensitive and allows quantitation of bacteria at species level.
Microarray	• Microarray based bacterial identification relies on the hybridization of pre-amplified bacterial DNA sequences to arrayed species-specific oligonucleotides. • Each probe is tagged with a different colored dye which fluoresces upon hybridization.

2.7 KINETIC STUDY

The kinetic study is a way to understand the performance of the system. Besides that, it is necessary to ensure a balanced and rational design or to determine a standard operation of the biological waste-treatment system for estimation of the system stability, quality of effluent and stabilization of waste (Garcia-Ochoa et al., 1999). The kinetic studies of anaerobic degradation using organic substrates can contribute to the basic process analysis, optimal design and operation, and the maximum substrate utilization rates in anaerobic degradation (Pavlostathis & Giraldo-Gomez, 1991). Modeling of biological processes in anaerobic digestion is a good tool to describe and convert complex biological problems and extensive experimental data into a simple formula (McCarty and Mosey 1991).

2.7.1 Fundamental of Kinetic Study

Kinetic modeling of anaerobic degradation is needed in order to build better understanding of the performance of the system. It is design to predict the system stability and effluent quality. In describing the microbial growth rate, this equation is introduced:

$$\frac{dx}{dt} = \mu x \qquad (2.1)$$

In which $\frac{dx}{dt}$ is a microbial growth rate, X is biomass concentration and μ is specific growth rate. However, the relationship between microbial growth rate and substrate growth rate is coupled as follows:

$$\frac{ds}{dt} = -\frac{1}{Y}\frac{dx}{dt} \qquad (2.2)$$

In which $\frac{ds}{dt}$ is substrate utilization rate, Y is a microbial growth yield coefficient. Later, the correlation between the substrate concentration and microbial growth rate

produced the equation:

$$\mu = \bar{\mu} \frac{S}{Ks+S} \quad (2.3)$$

From equation 2.3, the $\bar{\mu}$ is maximum growth rate, and Ks is substrate concentration at one- half the maximum rate. The equation 2.3 merge with equation 2.2 to form equation 2.4, which result:

$$\frac{ds}{dt} = -\frac{\bar{\mu}x}{Y}\frac{S}{Ks+S} \quad (2.4)$$

2.7.2 Michaelis-Menten Model

One of the popular kinetic models is Michaelis-Mentel Model. This model is a fundamental in enzymology studies (Dowd and Riggs, 1965). It explains about the relationship between the rates of substrate conversion by an enzyme to the concentration of substrate. This relationship is shown by following equation:

$$V = \frac{V_{max}[S]}{K_m + [S]} \quad (2.5)$$

Where:

v = reaction velocity

V_{max} = Maximum velocity

K_m = Michaelis Menten constant

[S] = Concentration of substrate

Generally, half of V_{max} will gives K_m value. The substrate with a small value of K_m will approach V_{max} more quickly. Very high [S] values are required to approach V_{max}, which is reached only when [S] is high enough to saturate the enzyme. The parameters V and K_m

(the 'Michaelis constant') of the equation can be evaluated from the slope and intercept of a linear plot of v^{-1} vs. $[S]^{-1}$ (a 'Lineweaver–Burk plot') or from slope and intercept of a linear plot of v vs. v/[S] ('Eadie–Hofstee plot'). One of the researches that used Michaelis Menten Model is a theory in abiotic catalysis: catechol oxidation by δ-MnO_2 (Naidja *et al.*, 2002).

2.7.3 Monod Model

In 1942, Jaques Monod proposed that the following mathematical relationship could be used to describe the effect of a growth limiting nutrient on specific growth rate. The relation between the specific growth rate and substrate concentration play an important role in biotechnology, where μ is specific growth rate of a population of microorganisms and *S* is a subtarate concentration. This relationship is represented by a set of empirically derived rate laws referred to as theoretical models below:

$$\mu = \mu_{max} \frac{S}{K_s + S} \tag{2.6}$$

Where:

μ = specific growth rate

μ_{max} = maximum specific growth rate,

K_s = the saturation or Monod constant

S = concentration of substrate.

Monod model is often used in bacterial growth on acetate for description of acetoclastic methanogenesis (Vavilin *et al.*, 2001). According to Harris *et al*. 1985, the way that growth rate is measured may become guidance on how the unit for biomass and substrate can be determined. Thus, this model shows that the biomass and substrate could affect the growth rate. The growth rate will be zero when there is no substrate and have upper limitation when the substrate is too much in the system. These two conditions are described in a rectangular hyperbola figure in the graph (Lobry et al. 1992). This phenomenon is also known as substrate inhibition effect.

2.7.4 Haldane Model

Haldane's growth kinetics model is commonly used for degradation model. It is widely used in mathematical simplicity (Shen *et al.*, 2009). It predicts that final biomass representing the growth kinetics data of an inhibitory concentration became doubled. The following equation shows Haldane's inhibitory growth kinetics:

$$\mu = \frac{\mu_{max} s}{k_2 + S + \frac{S^2}{k_i}} \quad (2.7)$$

Where:

μ = specific growth rate

K = Haldane's growth kinetics inhibition coefficient (mg/l)

In the exponential phase is calculated using equation:

$$\mu = \frac{dX}{X.dt} \quad (2.8)$$

Where, X is indicated as microorganism concentration (mg/l). There were two researches used Haldane model for kinetic study. The research on biodegradation kinetics of phenol and catechol using *Pseudomonas putida* MTCC 1194 used Haldane Model to determine the growth kinetics of inhibitory substrates (Kumar *et al.*, 2005). Other research was on kinetics of high strength phenol degradation using *Bacillus brevis*. This research used Haldane Model to determine the kinetic of phenol biodegradation (Arutchelvan *et al.*, 2006).

2.7.5 Other model for acetic acid production

In 2007, Song *et al.* has done a research on modeling of batch fermentation kinetics for succinic acid production by *Mannheimia succiniciproducens*. They preferred this kinetic model to determine the growth of *M.succiniciproducens* together with inhibitions of glucose and organic acid build up in the culture broth. In this research, the Michaelis Menten model is not selected because it is suitable for determination of the enzyme kinetic.

Their model was based solely on the production of the acetic acid and did not consider any consumption of the acetic acid. This model was based on model proposed by Song *et al.* (2008). The kinetic equations are as followed

$$\frac{dX}{dt} = k_1 S_{vss} \tag{2.9}$$

In equation 2.9, (dX/dt) is the increase rate of non-soluble organic matter (g/l h); k_1 is the kinetic constant of the reaction (l/h); S_{vss} is effluent concentration of biodegradable non-soluble organic matter (g/l) or biomass.

$$-\frac{dY}{dt} = \frac{(k_1 Svss)}{k_2} + \frac{k_4(k_1 Svss) + k_5 Svss}{k_3} \tag{2.10}$$

In equation 2.10, (dY/dt) is the removal rate of soluble organic matter (g/l h); k_2 is the stoichiometric yield coefficient for biomass on glucose (g/g), k_3 is the stoichiometric yield coefficient for acetic acid on glucose (g/g), k_4 is the kinetic coefficient constant of (g/l), and k_5 is the kinetic coefficient (g/l).

$$\frac{dP}{dt} = k_4 (k_1 S_{vss} + k_5 S_{vss}) \tag{2.11}$$

In equation 2.11 as will be shown later, (dP/dt) is the rate of acetic acid production (g/l h); k4 is the growth associated acetic acid formation parameter (g/g), and k_5 is the non-

growth associated acetic acid formation parameter (g/g h).

Other kinetic model such as model proposed by Borja et al., (2005) and used by Zainol et al., (2012), which consider the characteristic of lignocelluloses from banana stem waste (BSW), and from the collected experiment data, the following hypotheses below were assumed:

1. The first transformation occurred in the first kinetic order when the insoluble organic matter or volatile suspended solids were transformed to soluble organic matter (VDS).
2. The volatile fatty acids was produced by the dissolved organic matter (VDS) resulting from the decomposition process of the insoluble organic matter which initially present in raw BSW. This process is following a Michaelis-Menten kinetic model.
3. Then, the acetic acid produce from decomposition of VDS which initially present in the raw BSW were converted to other product which is also following a Michaelis-Menten kinetic model.
4. The reactor was assumed to be completely mixed reactor.
5. All the concentrations of the organic matter were expressed as g/l. With these considerations, the following kinetic model can be established, using the following differential equations:

$$-\frac{dSvss}{dt} = k_1 S_{vss} \qquad (2.12)$$

In equation 2.12, (dS_{vss}/dt) is the removal rate of non-soluble organic matter (g/l h); k_1 is the kinetic constant of the reaction (l/h); Svss is effluent concentration of biodegradable non-soluble organic matter (g/l).

$$\frac{dSvds}{dt} = k_1 Svss - \left(\frac{k_2 Svds}{k_3 + Svds}\right) \qquad (2.13)$$

In equation 2.13, (dS_{vds}/dt) is the production rate of soluble organic matter (g/l h); k_2 is the maximum removal rate of soluble organic matter (g/l h); Svds is effluent concentration of soluble biodegradable organic matter (g/l) and k_3 is the saturation constant (g/ l)

$$\frac{dStaa}{dt} = \left(\frac{k_4 Svds}{k_5 + Svds}\right) - \left(\frac{k_6 Staa}{k_7 + Staa}\right) \quad (2.14)$$

In equation 2.14, (dS_{taa}/dt) is the rate of acetic acid production (g/l h); Staa is effluent concentration of acetic acid concentration (g/l); k_4 is the maximum rate of VDS uptake (g/l h), k_5 is the saturation constant (g/ l), k_6 maximum rate of TAA (acetic acid concentration) consumption (g/l day), and k_7 is the saturation constant (g TAA/l).

2.7.6 Selection of Kinetic Model

While selecting the reaction mechanism for mathematical model, the degradation complex materials such as lignocelluloses to acetic acid, the concept of enzymatic hydrolysis, acidogenic and acetogenic process needs to be considered (Angelidaki et al., 2000). This is important since almost all kinetic models are used for specific reaction involved in the system. Some of the model may only for one reaction and could not regard other reactions in the system. Therefore, there is probability where one system needs to implement all or several theoretical models to explain the system for the whole process. Sometimes, the models need some alteration to be applied in the system. Moreover, the system that involved with variety of reactions, which includes different type of substrate, organism or catalyst may need a little modification. As an example, if the process is involving catalyst and organism (enzyme and microbe), it is better to use Monod and Michaelis-Menten model as one hybrid model. By this, the hybrid model can represent on how the system operates precisely, without neglecting important factors involved.

In the kinetic model of acetic acid production from banana stem waste, there are four differential equations that described the overall process, which consists of several

kinetic constants. For the step in anaerobic fermentation, first step is hydrolysis and solubilization of organic matter. Next step is acidogenesis, follow by the methanogenesis. The kinetic constant obtained and purposed equations were used to simulate the step in the anaerobic digestion process of banana stem waste (Zainol et al., 2012). There is also kinetic model known as Methane model had been constructed (Noike et al., 1985). After 3 years published, the model improved, with the modification to include a sum of three types of VFAs (acetic, propionic and butyric acids) and the inhibition factors (Vavilin and Lokshina, 1996).

As a conclusion, the kinetic model that will be chosen in this present study is the model by Borja *et al.*, (2005). This is because it offers a promising approach to a rational mathematical model for the anaerobic process involved, and clearly reflects the important effect of biomass in the reactor. This model was used to describe the process involved during degradation of banana stem waste using soil mixed culture (Zainol et al., 2009). Thus, this model is much convincing to be used for kinetic study.

2.7.7 Kinetic evaluation on the most efficient acetic acid producer

After the model is identified based on its suitability for the process involved in the system, it is then verified by minimizing sum squared error of theoretical value and experimental value. The theoretical value is obtained using engineering mathematical equation available on the kinetic model. Most of the engineering problem is actually solved using a variety of numerical equations, but ordinary differential equation (ODE) is very common and widely used in bioprocess (Harvey and Douglas, 1996; Katoh and Yoshida, 2009).

2.8 ORDINARY DIFFERENTIAL EQUATION (ODE)

Differential equations are of basic equation in mathematics, and it is important in engineering because the physical laws and relations between it appears in form of mathematical differential equation. Ordinary Differential Equation (ODE) can be used in

various physical and geometric problems that can be solved with the differential equations, but emphasis more on modeling, which transforms it from the physical situation into a “mathematical model." In ODE, there will be 3 parts of methods involved in engineering, which are Runge-Kutta Method, Stiffness/ Mutistep Methods and Boundary Value / Eigenvalue Problem (Chapra & Canale, 2010).

Most kinetic studies in bioprocess are using the ordinary differential equations to solve the kinetic parameter and to find the theoretical value. The research done by Lee et al. (2000) used the ODE to find the theoretical value of acetic acid and succinic acid formations in their study on continuous cultures of *Anaerobiospirillum succiniciproducens* grown on glycerol. The results is convincing because the error obtained from the experimental data is small. The ODE is also used in modeling of batch fermentation kinetic for succinic acid production by *Mannheimia succiniciproducens* (Song et al., 2008). This is proving that ODE can be used to find the kinetic value from the model that suitable for organic acids.

In other works, Garcia-Ochoa et al. (1999) used the ODE in two unstructured segregated kinetic models to describe the anaerobic digestion of livestock manure in batch fermentation and to find the values of kinetic parameters. It is also applied by Borja et al. (2005) to find the value of kinetic parameter and theoretical data in their simplified kinetic model for studying the hydrolysis, acidogenic and methanogenic steps of the anaerobic digestion process of two-phase olive pomace (TPOP). Also, Vrancken et al., (2008) used ODE to analyze the growth and sugar consumption of *Lactobacillus fermentum*. These researches proven that the ODE can be used in different types of model that utilized complex substrate in fermentation.

It can be concluded that the ODE can be used in various types of kinetic model. But the method to be used is most importantly needed to be chosen first, in order to find the accurate model. Runge-Kutta (RK) method is the widely used method by scientists, researchers and engineers to find the value of the kinetic parameters.

2.8.1 Runge-Kutta Method (RK)

In RK method, there are several methods can be applied, which are Eular Method, Heun Midpoint Method, Runge-Kutta methods or System of ODE. Runge-Kutta fourth order is the most popular RK method of all. There are an infinite number of versions as the second-order approaches. The following equation is commonly called as the classical fourth order RK method (Chapra & Canale, 2010):

$$y_{i+1} = y_i + \frac{1}{6}(k_1 + 2k_2 + 2k_3 + k_4)\,h \qquad (2.15)$$

where,

$$k_1 = f(x_i, y_i) \qquad (2.15.1)$$

$$k_2 = f\left(xi + \frac{1}{2}h,\ y_i + \frac{1}{2}k_1 h\right) \qquad (2.15.2)$$

$$k_3 = f\left(xi + \frac{1}{2}h,\ y_i + \frac{1}{2}k_2 h\right) \qquad (2.15.3)$$

$$k_4 = f(xi + h,\ y_i + k_3 h) \qquad (2.15.4)$$

The derivation of fourth order Runge-Kutta method involves tedious computation of many unknowns, and the detailed step by step derivation and analysis can hardly be found in the literatures. It played vital role in the field of computation and applied science/engineering (Musa et al., 2010). The Runge-Kutta was used since it gives high efficiency and stability of the data obtained. But, the stability is much important than efficiency. However, in this research, the steps in the Runge-Kutta are chosen to yield accuracy rather than stability similar with Bogacki and Shampine, (1996).

A non-linear multiple-response regression technique with a fourth-order Runge-Kutta algorithm is employed in a study on anaerobic digestion of livestock manure to obtain the values of the parameters involved in the kinetic model (Garcia-Ochoa et al.,

1999). They found that this method can be used to determine the kinetic parameter. Besides, the method also able replicate almost same experimental data obtained. Song et al., (2010) also used this method to find the value of kinetic parameters for two models that they proposed. The first model was described by Monod model with terms describing substrate inhibition and product inhibition. The second model used was Luedeking-Piret model with terms describing the growth and non-growth associated with acetic acid formation. The theoretical values and kinetic parameters fit satisfactorily with all the models they proposed. Zainol et al., (2012) also used the Runge-Kutta method to find the theoretical value and kinetic parameters in anaerobic digestion of banana stem waste using soil mixed culture. The kinetic parameters obtained were used to describe the production of biogas from banana stem waste fermentation using soil.

2.9 KINETIC CONSTANT CALCULATION USING PACKAGES

In order to calculate the kinetic parameter and sum squared error, the most effective method is by using software program such as Excel Solver, Mathcad, Sigma Plot or Matlab. These softwares could increase the efficiency in calculating the kinetic parameter and values because it helps in saving laborious time for manual calculation. Besides, the results are more precise as the iteration of the value is much higher than manual calculation. The higher the iterations, the less the error will be. The less error means high-quality results can be obtained.

2.9.1 Microsoft Excel (Solver)

Solvers or optimizers in Microsoft Excel are software tools that help users find the best way to assign and distribute scarce resources. The resources can be almost everything from raw materials, process time, cost, and to anything else that suffer limited supply. The optimal solution usually means maximizing profits or products, minimizing costs or time, or achieving the best possible quality product. Vrancken et al. (2008) used Excel solver to analyze the growth and sugar consumption of *Lactobacillus fermentum*. All the model equations in their study were integrated using Euler's integration technique with a time step

of 0.25 h. The software could minimize the sum of residual squares between modelled and measured valued. Furthermore, this software can be access easily using Microsoft Excel in data analysis. The cost for this software is also inexpensive compared to the other engineering calculation software. It is also user-friendly and easy to learn for a beginner.

2.9.2 Mathcad

Mathcad is the industry-standard software for engineering calculations. It is released by Parametric Technology Corporation. Mathcad is dedicated to engineering calculation application where it combines the ease and familiarity of an engineering equation. It is user friendly as it has an easy interface. Besides, it can solve, share, re-use and analyze calculations without need to learn or rewrite a new programming language. Borja et al. (2005) used Mathcad software to find the kinetic parameter from *k1* – *k7* based on experimental results by mathematical adjustment to the condition of obtaining a minimum value of the sum of the squares of the differences between the experimental and theoretical values.

2.9.3 Matlab

Matlab is a programming environment for algorithm development, data analysis, visualization, and numerical computation. It is released by The MathWorks Incorporation. Matlab can solve various technical computing problems and also faster than traditional programming languages, such as C, C++, and FORTRAN. Most of the time, it is used in signal and image, data processing, communications, control design, test and measurement, financial modeling and analysis, and also computational biology. The software is widely used by professional engineers and researchers to solve their mathematical problems. Most of the researchers used Matlab to determine the kinetic parameter and theoretical values as well as minimizing the sum squares error of the differences between the experimental and theoretical values (Song et al., 2008; Lee et al., 2009; Song et al., 2010)

2.10 CONCLUSION

Banana stem waste can be used as a substrate for production of acetic acid. It contained high amount of cellulose, making it beneficial for the acetic acid production. Soil is a good microbe source.

There are no other research able to isolate specific anaerobic strain for production of acetic acid. Therefore, the isolation can only be done using basic techniques such as aseptical technique using the glucose medium and anaerobic incubation. Furthermore, the hybrid kinetic model can be used to explain the process involved namely hydrolysis, acidogenesis and acetogenesis in fermentation of complex substrate.

The hybrid kinetic model used is based on the basic model such as Monod, Michaelis Menten, or Haldane Model. The ordinary differential equation is a mathematical equation that can be used to solve the kinetic model. The Runge-Kutta method is the best method to find the value of theoretical model and kinetic parameters. Microsoft Excel is an attractive option to be used in determining the kinetic parameter in the kinetic model since it is inexpensive, easily available, user-friendly and great for beginners. Furthermore, the software can also be used to find the value of theoretical value and sum squares error between experimental and theoretical values.

CHAPTER 3

METHODOLOGY

3.1 METHODOLOGY

The research methodology for this research was separated into two main parts based on the research objective; the isolation and identification of the efficient anaerobic acetic acid producer and the kinetic study of the selected acetic acid producer. The brief method in this research was shown in the Figure 3.1. Further information on the main parts was discussed in the next subchapter.

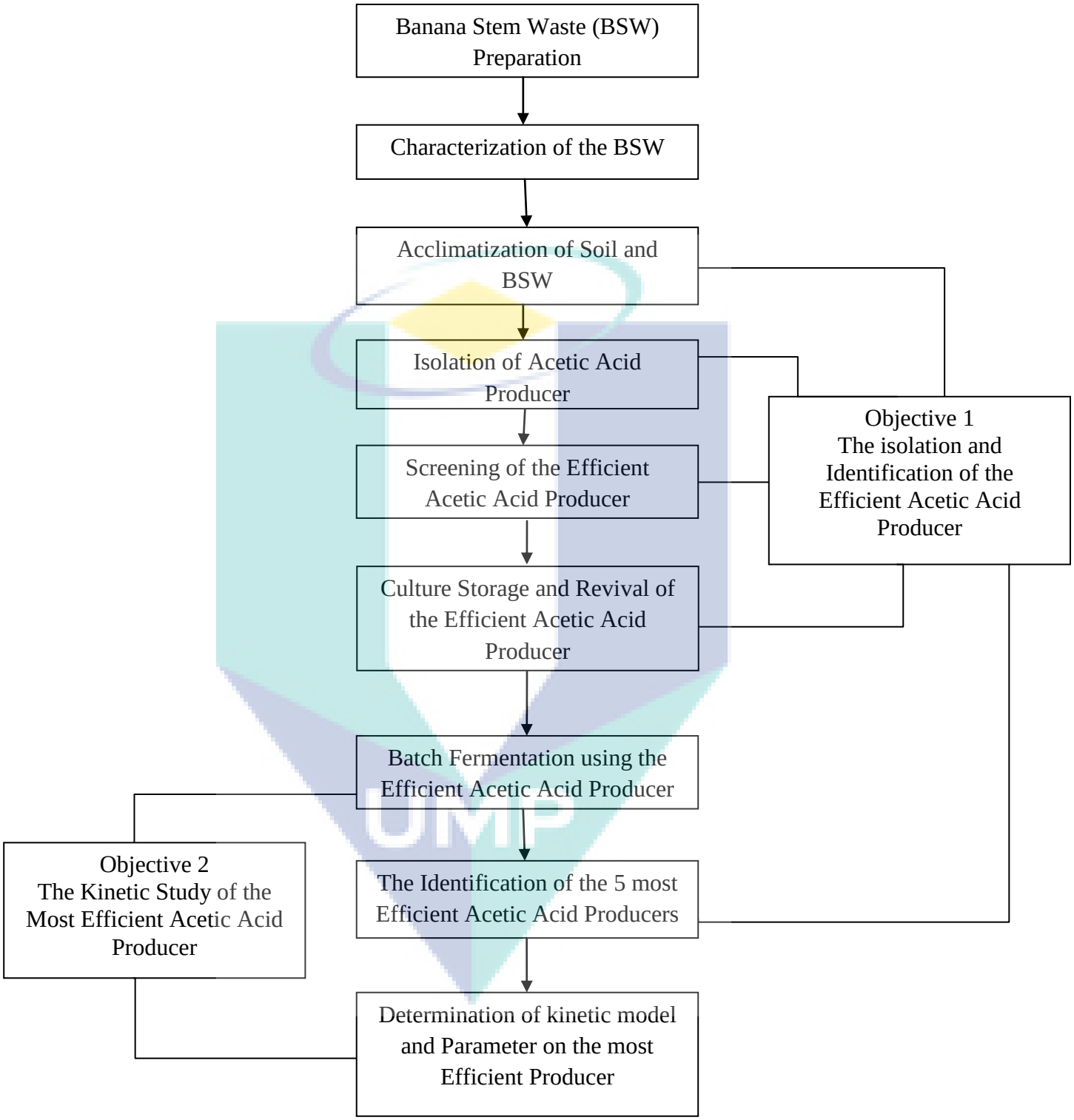

Figure 3.1: The brief process of the isolation and identification of acetic acid producer microbe from soil

3.2 THE ISOLATION AND IDENTIFICATION OF THE EFFICIENT ACETIC ACID PRODUCER (AAP)

The acclimatization of soil and banana stem waste were conducted to enhance the growth of microbes in the mixed culture before the isolation of AAP. Then, the isolation was conducted using serial aseptically diluted on the agar plate before incubation in anaerobic condition. This method would ensure that the isolated anaerobic species could be obtained. Then, the batch fermentation was conducted to obtain the potential AAP from isolated species. Later, five most efficient AAP were identified using biochemical test and molecular method.

3.2.1 Banana Stem Waste (BSW) Preparation

The banana stem waste (BSW) and soil was obtained from local banana plantation located near Kuantan, Pahang. BSW was used as feedstock in the experiments. Before it could be used, BSW was washed to remove the soil and dried until constant weight was achieved before used in the experiment. This BSW was cut into small pieces around ~5cm. Sharma et al., (1988) found that the small size of BSW will give better results on product yields.

3.1.2 Characterization of the Banana Stem Waste

BSW is non-wood plant's waste, which contained cellulose, lignin, and hemicellulose (Mohapatra et al., 2010). Each tree of the BSW contained different chemical content making it difficult to characterize the BSW. Chemical analysis was carried out according to the standard method used to characterize material approximately.

Holocellulose Analysis

Holocellulose is compound that is soluble in alkali and water. This type of compound consists of hemicellulose and α-cellulose. This compound was known as one of

the major compounds available in banana stem waste. As for this analysis, the extracted sample from ethanol-toluene solubility was used. The details procedure was available in Appendix A.

α-cellulose (TAPPI T 203) Analysis

The α-cellulose also known as cellulose is a refined and insoluble fibrous residue. It is usually obtained from the removal of other soluble materials. As a part of the chemical characterization analysis, the α-cellulose content was determined based on standard TAPPI method T 203. The analysis was carried out in water bath. 17.5% NaOH was added to the holocellulose. NaOH was added again gradually and the solution was stirred to well mix. The details procedure was explained in Appendix A.

Acid insoluble lignin

Acid-insoluble lignin analysis is a method used in order to determine the lignin content of BSW. This method emphasize on the acid hydrolysis of BSW that will degrade almost every other compound except lignin. The residue left at the end of this process is the lignin content of the BSW. The standard method of Klason-lignin determination method is used in this part. The extracted sample from ethanol-toluene solubility was used for this analysis. A 72% sulfuric acid was added to the extracted sample. The acid was added gradually in small increments while stirring and macerating the material with a glass rod while the beaker was kept in an ice bath. A certain amount of distilled water was added to the sample. A 3% of sulfuric acid was added to the material. The solution was boiled and the constant volume was maintained by frequent addition of hot water. The insoluble material (lignin) was allowed to settle and the flask was kept in an inclined position. The lignin was then transferred to the filtering crucible. The lignin was rinsed using hot water to free of acid. The lignin with crucible was dried in oven. The full procedure on this method was explained in Appendix A.

3.2.3 Acclimatization of Soil and BSW

The soil mixed culture (SMC) was obtained from agricultural soil. The sample was collected using the plastic pipes (polyvinyl chloride) under 10cm depth from the surface. The end of the pipe was then fitted with stopper and be processed within a period of hours. This step was important to ensure that anaerobic mixed culture would be collected.

The SMC and water were put into 35 ml's serum bottle, which contained of BSW. The serum bottle was flushed with nitrogen for five minutes before incubation in anaerobic jar for a month (Benner et al., 1984; Zainol 2009). After that, all the ingredients in the serum bottles were put into the container volume of one liter of anaerobic (Benner et al., 1984; Zainol 2009). This step could increase biomass amount for later application. The anaerobic container was flushed with nitrogen before incubation for two months (Benner et al., 1984; Zainol, 2009). The incubation was conducted in ambient temperature.

3.3 THE ISOLATION OF ACETIC ACID PRODUCER (AAP)

Isolation was done by aseptically serial diluted, and each dilution was cultured on Petri's dish. The colony was determined and differentiated based on their morphology, size and color. All the isolated pure cultures were used for screening purposes in the glucose medium.

3.3.1 Media Preparation and Initial Isolation

The media preparation from commercial products was simple and straightforward. A specific amount of agar and broth were weighed by using a balance. It was then suspended in distilled water. The suspension was mixed thoroughly in an Erlenmeyer flask. Next, it was dispensed and autoclaved. The agar was later poured into a Petri dish. The same method and procedure were used in broth preparation, but it was poured into the bottle.

After two months of acclimatization, the samples in the one liter anaerobic container were diluted (Zainol, 2009). The samples were prepared at 10% dilution. The sample was then stirred and further diluted into several dilution concentrations (1%, 0.1%, 0.01%, and etc). Then, small amount from each dilution tube was poured into prepared agar plates. The plates were inverted and placed in incubator for few days.

3.3.2 Screening of Acetic Acid Producer (AAP)

Only several pure cultures obtained were acetic acid producer (AAP). Therefore, the pure cultures were screened in batch fermentation to obtain AAP. The medium for batch fermentation contained of glucose, peptone, meat extracts, KH_2PO_4, $MgCl_2$, and $NH_4Cl_2H_2O$. The materials used in the medium have specific function to enhance bacteria growth. Peptone and meat extract were for protein supply. KH_2PO_4 was used for fungicide, while $MgCl_2$ to catalyze the metabolism in the bacteria cell. $NH_4Cl_2H_2O$ was used for nitrogen sources.

The batch fermentation was carried out in anaerobic condition for 24 hours at 30^{o}C and 150 rpm. Then, the results were collected and tested for acetic acid concentration. The acetic acid concentration was analyzed using High-Performance Liquid Chromatography. The system was equipped with diode array detector and with Agilent Zorbaq Sb-Aq C-18 column. Only five most efficient AAP were chosen for identification process. The AAP that produced highest amount of acetic acid was chosen to be used in the kinetic study.

3.3.3 Morphology Observation and Gram-staining

The morphology observation and gram-staining results were analyzed on five most efficient AAP. The colony morphology of these AAP were described in terms of size, shape, margin, elevation, consistency, color and transparency to give an accurate description of the colonies.

In the gram-stain, the cells were heat-fixed and then stained with basic dye, crystal violet, I_2-KI mixture (mordant) to fix the stain, washed briefly with 95% alcohol (destained), and finally counter-stained with safranin. Gram-positive organisms retained the crystal violet stain, while gram-negative organisms were decolorized by the organic solvent and hence showed the pink counter-stain. The difference between gram-positive and gram-negative bacteria was the ability of the cell wall to retain the crystal violet dye.

3.3.4 Culture Storage and Revival of the Efficient Acetic Acid Producer

The pure culture was cultured in a slant agar and nutrient broth before being kept under 4°C as culture stock. This pure culture was pre-cultured twice a week on the new agar plate. Meanwhile, the pure culture was stored in sub-cultured (glycerol stock) in -80^{o}C. If the pure culture in the nutrient agar was contaminated, the pure culture in glycerol stock was used as source for sub culture. The technique to shift the pure culture from glycerol stock (in ice form) to become active in agar plate was known as revival technique. One loop of pure culture from glycerol stock (when it still in ice form) was put into broth and left overnight at 30^{o}C in incubator. The broth would be cloudy on the next day. One loop of pure culture from the broth was put onto agar plate and incubated at 30^{o}C.

3.3.5 Materials and Equipment

The acetic acid producers (AAP) were isolated from soil mixed culture with several chemicals and equipments. The chemicals and equipments involved were different from identification part. The chemicals and equipments were listed in Table 3.1.

Table 3.1: The chemical and equipment list for isolation part.

Material		Instrumentation	
Company	**Chemicals**	**Company**	**Equipment**
Merck (Malaysia)	Microbiological agar, Broth media, Peptone and Meat extract	Hirama (Toyono, Japan)	50L Autoclave
		Heraeus (Hanau, Germany)	Microbiological incubator
Chemar (Poland)	Potassium dihydrogen Phosphate (KH_2PO_4), Sodium Nitrate ($NaNO_3$), Magnesium Chloride ($MgCl_2$), Magnesium Sulphate ($MgSO_4$)	Esco (Hatboro, USA)	Laminar Air Flown Chamber
		Carl Zeiss (New York, USA)	Microscope
QReC (New Zealand)	Potassium Chloride (KCl)	Mikro Makmur Enterprise	Sterile Petri Plate

3.4 THE IDENTIFICATION OF THE ACETIC ACID PRODUCERS (AAP)

Five most efficient acetic acid producers (AAP) were identified based on biochemical test and biomolecular method. The biochemical test was conducted using BIOLOG kit. Then, these AAP were identified using polymerase chain reaction (PCR) where proteins sequences from AAP were used and compared with the large database from BLAST.

3.4.1 Biochemical Test (BIOLOG Microplate)

The BIOLOG Microplate used was Gen (III) microplate with IFB (Inoculating Fluid B) protocol. The IFB was chosen because the acetic acid producers (AAP) obtained from soil and these species were strong reducing bacteria. Several steps were briefly listed here; the actual procedure can be acquired in Appendix B.

All five AAP were cultured on BIOLOG recommended agar media. Then, the AAP were prepared at desired cell density. If the density was within range, the inoculation of microplate carried out. Then, the microplate was incubated in the OmniLog incubator/reader for 48 hours. Finally, the results were obtained from Biolog's Microbial Identification System software. It detected color densities in wells of the carbon sources

and chemical sensitivity assays. The fingerprint was compared with the reference in the software library.

3.4.2 Molecular Biology Method

Molecular biology has modernized as it is now possible to extract the proteins from diverse microorganism, create huge database of the sequences, and use the database as great identification tools. DNA extraction kit was used to extracting the proteins from acetic acid producers (AAP). Agarose gel electrophoresis was used for estimation the size and of DNA and RNA fragments by charge (Kryndushkin, 2003). The polymerase chain reaction (PCR) was used to identify AAP. It was fast, has high sensitivity, much accurate and specificity on the diagnostic methods.

3.4.3 DNA Extraction and Agarose Gel Electrophoresis

The DNA of five most efficient AAP was extracted using commercial kit, the brief steps as following: the cells were harvested before the pre-buffer and lysozyme solution were added to the cells. The binding buffer was added after the G-buffer solution inserted into the cells lysates. Next, the column containing the cells lysates was washed with buffer A. Then, the column was put in a clean 1.5ml micro centrifuge tube, and elution buffer were added directly onto the membrane in the column. Each interval in those steps was conducted with centrifuging process for several minutes and incubation at certain temperatures. Finally, the samples were centrifuged before kept at -20℃ freezer.

A common method to separate and analyze DNA was by using agarose gel. It was used to quantify AAP DNA and to isolate a particular band in the DNA. The AAP DNA was interpreted using ethidium bromide, which would visualize the DNA band. The ethidium bromide was bonded with the DNA by intercalating between the bases and fluorescent. It would make it absorbed invisible UV light, then the energy transmitted back as visible orange light.

The agarose powder was suspended in 1X TAE buffer, and thoroughly dissolved in a microwave oven. Ethidium bromide was added before it was allowed to cool with special comb. The gel was placed in the gel rig with the wells closest to the cathode (black) end. It was covered with 1X TAE running buffer. Then, few drops of glycerol loading dye were placed onto the waxy side for each sample. The loading dye was added to the sample. The sample and 1 Kb ladder was analyzed at 90V for 40 min. Later, the completed gel was visualized with U.V. light and photograph with a Bio-imaging machine. The gel must be disposed properly because it contained carcinogenic materials.

3.4.4 PCR Amplification with Universal Primers

DNA concentrations in diluted samples (by mixing distilled water) were checked using UV-Vis machine under 260nm wavelength. Two oligos universal primers were designed to be used in PCR. The first forward primer (F: 5' AGA GTT TGA TCC TGG CTC AG3') and one reverse primer (R: TAC GGY TAC CTT GTT ACG ACT T3') for initial PCR amplification. A typical reaction mixture in each PCR tube consisted of dNTPs, 10ul buffer, $MgCl_2$, two primers, distilled water and sample template. Amplification conditions were run for 30 cycles. PCR involved initial denaturation at 95°C, followed by 30 cycles of denaturation at 94°C and annealing at 55°C and a final extension at 72°C. The PCR products were then separated on 1% agarose gel containing ethidium bromide for visualization on a Bio-imaging machine.

3.4.5 Materials and Equipment

The bacteria are identified using biochemical test and molecular biology. The chemicals and equipments are listed in Table 3.2.

Table 3.2: The chemical and equipment list for identification part.

Material		Instrumentation	
Company	**Chemicals**	**Company**	**Equipment**
Merck (Malaysia)	Broth media	Alpha Innotech (USA)	Bio-imaging system
Focus Biotech (M)	Gen (III) BIOLOG Kit	Eppendorf, (New York, USA)	PCR machine
Intron Biotechnology	MEGAquick-spin PCR, Agarose Gel DNA Extraction, System	Heraeus (Hanau Germany)	Micro centrifuge, Chamber
		Sharp (Tokyo Japan)	Microwave Oven
Biosyntech Sdn. Bhd.	SYBR safe DNA gel stain	Mikro Makmur Enterprise	Sterile Petri Plate
Invitrogen (USA)	1 Kb plus DNA ladder, *Taq* DNA polymerase, $MgCl_2$, 10x Buffer	Major Science (Saratoga, USA)	Electrophoresis Apparatus
		Science Valley Sdn. Bhd.	Clear tips, Clear tubes
DKSH Technology Sdn. Bhd.	TAE-buffer		

3.5 KINETIC STUDY OF THE MOST EFFICIENT ACETIC ACID PRODUCER (AAP)

The kinetic study is important to understand the performance of the system. Not all kinetic models could be used to represent the system for production of acetic acid from BSW. The model needs to represent important components such as biomass growth, glucose utilization and acetic acid production. Therefore, the kinetic model proposed in this study represented the concentration of each component at each interval of time. The data would be used in kinetic constants determination. The batch fermentation is conducted by using most efficient AAP in glucose and BSW medium for 72 hours.

3.5.1 Batch Fermentation for Kinetic Study

The most efficient AAP was inoculated in the conical flask. The anaerobic condition was provided by purging the flask with nitrogen gas before incubation in incubator shaker. The inoculation in glucose medium and banana stem waste medium were

prepared in 13 set triplicate. The incubation was performed for 72 hours. The samples were collected at 6 hours time interval.

3.5.2 Data Analysis from Batch Fermentation

The acetic acid concentration was determined by using High-Performance Liquid Chromatography (HPLC) at each time interval. Meanwhile, glucose content in the samples was quantified using Glucose Analyzer. The biomass concentration was determined via UV-vis Spectrometer and Analytical Balance. Based from the data collected, the graph will be plotted between concentrations of acetic acid, glucose or biomass vs. time.

The phosphate buffer (20mm) and acetonitrile were used for determination of acetic acid by using column Zorbax-Sb Aq C18. The acetic acid concentrations in each sample at each interval of time were promptly determined after the sample was collected. The glucose concentrations at each time intervals were determined using glucose analyzer. The biomass concentration was determined based on the dry cell weight in specific volume of sample and the absorbance value at 550nm using UV-vis. The theoretical value for growth, substrate utilization and production were determined by using proposed model. The model was fitted to the experimental data by using Microsoft Excel.

3.5.3 Determination of Kinetic Constants of the Most Efficient Producer

The mathematical method used to find the value of kinetic constants was done by using Fourth Order Runge-Kutta (RK 4TH Order). It was solved in Microsoft Excel software by minimized the sum square error between experimental data and theoretical data.

3.6 CONCLUSION

The isolation of acetic acid producer (AAP) was completed using aseptical serial dilution and incubation in anaerobic condition. The characterization of isolated strains was

conducted using morphological observation and gram staining method. The colony and cell morphology of these isolated strains was observed in terms of size, shape, margin, elevation, consistency, color and transparency. The batch fermentation on glucose medium was conducted for 24 hours. It was used to determine the AAP strain. Five most efficient AAP were then identified using BIOLOG microplate and polymerase chain reaction (PCR).The IFB protocol was used in biochemical test because the AAP obtained from soil and these species were strong reducing bacteria. The DNA from the AAP was extracted using G-spin Genomic DNA extraction kit. The gel electrophoresis method was used to determine the AAP DNA size and length. Two oligos universal primers were used to determine the AAP in PCR sequencing. The first forward primer (F: 5' AGA GTT TGA TCC TGG CTC AG3') and one reverse primer (R: TAC GGY TAC CTT GTT ACG ACT T3') for initial PCR amplification. The sequencing results were compared with BLAST library.

The kinetic model proposed in this study represented the concentration of biomass, glucose, and acetic acid at each interval of time. The batch fermentation on glucose and BSW medium was conducted using the most efficient AAP. The concentration for these three components in glucose and BSW medium were collected at each 6 hours. Microsoft Excel was used as a tool to find the constant and theoretical data. The Runge-Kutta method was used in determination of kinetic constants by minimizing the sum squared error between experimental and theoretical data. All the results obtained are discussed in Chapter 4.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 PROXIMATE CHEMICAL ANALYSIS

Some types of wood or non-wood species are characterized by using proximate chemical analysis. These analyses are to determine quantitatively the major components of the pulp under normal circumstances (Rahim, 1996). From the previous chapter, three types of analysis were applied to the banana stem waste (BSW), which was the holocellulose analysis, α-cellulose analysis (TAPPI T 203) and acid-insoluble lignin analysis.

Table 4.1: Chemical composition in BSW determined using proximate chemical analysis

Proximate analysis	%
Hemicellulose	35.33
α-cellulose	55.15
Lignin	9.52

The results from characterization of BSW show that, the cellulose was 55.15%, hemicellulose was 35.33% and the lignin content was only 9.52 %. These amounts were

higher than findings from Li et al., (2010) found. They obtained 39.12% cellulose, 52% hemicelulose, and 8.88% lignin (Li et al., 2010). However, Silveira et al., 2007 had higher amount of lignin which were 15.22%, 53.45% cellulose and 31.33% hemicellulose (Silveira et al, 2007). The amount of cellulose in banana stem waste was different because of several factors. The main factor was due to the selection of different banana plant species for each research. The climate and environment conditions of the banana plantation were also different from one another (Li et al., 2010; Silveira et al., 2007). Therefore, it was important to analyze the BSW sample in order to determine the chemical composition.

4.2 ISOLATION AND SCREENING OF THE BACTERIA

Bacteria isolation and identification are important for acclimatization process. In this study, several types of anaerobic acetic acid producer (AAP) were isolated and identified from the soil mixed culture. The performance of acetic acid production from BSW was also studied. The soil micro flora consists of lignin degrading bacteria can release many types of enzyme. This lignin degrading bacteria able to produce various useful compounds including metabolites intermediate such as sugar and acetic acid (Raj et al., 2007).

The methods for production of acetic acid using soil anaerobic bacteria were limited compared to aerobic acetic acid bacteria (Morinaga and Kawada 1990; Ravinder et al., 2001; Talabardon et al., 2000). However, the acetic acid production from each isolated strain could be determined from the fermentation process. In this study, glucose fermentation was done to find the potential AAP. The process was conducted in anaerobic condition for 57 isolated strains. Only 15 strains out of 57 strains were able to produce acetic acid from glucose fermentation.

4.2.1 Isolation of Acetic Acid Producer

A pure culture of anaerobic acetic acid producer theoretically contains a single bacterial species. There are number of procedures available for the isolation of pure

cultures from soil mixed culture. A pure culture can be isolated by using special media with specific chemical or physical agents. It will allow the enrichment and selection of one organism over another (Bernalier et al., 1996; Fuentes-Ramírez et al., 2001). The DNA extraction is often an early step in diagnostic processes used to detect bacteria in the environment. The extracted DNA is used in either Fluorescence in Site Hybridization (FISH), Terminal Restriction Fragment Length Polymorphism (T-RFLP) or Polymerase Chain Reaction (PCR) sequencing. A pure culture used during diagnostic processes will avoid contamination and doubt on the sequencing results (Dale et al., 2006; Schneegurt et al., 2003).

There are few methods available to isolate mesophilic anaerobic AAP from soil (Bernalier et al., 1996; Braus-Stromeyer et al., 1993; Fuentes-Ramírez et al., 2001; Küsel et al., 2000). Only aerobic AAP can be isolated from soil using selective medium consist of alcohol (Lisdiyanti et al., 2002; Lisdiyanti et al., 2003). In this study, the isolation of anaerobic AAP was done by using glucose media at mesophilic temperature. It has been reported that only few bacteria such as *Clostridium sp.*, *Ruminococcus sp.*, and *Bacteroides xylanolyticus* could produce acetic acid from glucose in anaerobic condition (Bernalier et al., 1996; Hunger et al., 2011; Küsel et al., 2000; Ravinder et al., 2001; Sim et al., 2008). The morphology of the isolated colony in nutrient agar (NA) plate was observed after incubation in anaerobic condition at 30^{o}C for 48 hours. 57 strains were found and fermentation was conducted using these strains.

4.2.2 Screening of Acetic Acid Producer

From 57 strains, only 15 strains were able to produced acetic acid from glucose fermentation. The most efficient strain was selected based on acetic acid yield. The most efficient bacteria would be the highest acetic acid producer. The concentration of acetic acid for each strain was determined using High-Performance Liquid Chromatography (HPLC). A diode array detector and Agilent Zorbaq Sb-Aq C18 analytical column were equipped with a corresponding guard column. The condition of the column was controlled at 35°C. Phosphate buffer was used as mobile phase at a flow rate of 0.8 ml/min and

volume of injection for every vial was 5µl. Table 4.2 showed the acetic acid concentration and pH according to their strains.

Table 4.2: Result of acetic acid concentration and pH for 15 strains

Strain	Amount of Acetic Acid (g/l)	pH
A1	1.98	5.55
A2	1.83	5.55
A3	1.57	5.57
A4	0.32	4.75
A5	1.55	5.21
A6	2.90	3.63
A7	1.83	5.68
A8	1.73	5.09
A9	0.51	4.79
A10	0.55	4.70
A11	0.28	5.34
A12	0.26	4.95
A13	1.16	4.87
A14	1.49	4.87
A15	0.81	5.14

From Table 4.2, strain A1, strain A3, strain A6, strain A7, strain A8, strain A5, strain A11, strain A13 and strain A14 produced more than 1.0g/l acetic acid. From these strains, only five strains that produced the highest acetic acid were chosen for further analysis. They were strains A1, strain A6, strain A8, strain A2 and strain A7 with concentration range from 1.7 – 3.0 g/l. Later, only strain A6 was used to be inoculum for kinetic study.

4.3 IDENTIFICATION OF ACETIC ACID PRODUCERS (AAP)

There are three common methods available for identification of bacteria. The conventional methods for bacteria identification are by morphological observation and biochemical test. The latest method for identification process is bio molecular method. The anaerobic AAP was identified by using these methods. It was characterized by morphological observation, phenotypic test in BIOLOG kit and analysis using 16S rRNA sequences.

The conventional method was used to identify the physical and chemicals properties of the bacteria. However, it had some significant weaknesses (Janda and Abbott, 2002; Woo et al., 2008 and Cloud et al., 2010). This method was reported to be insufficient since the information was very limited and high probability of inaccuracies (Woo et al., 2008). It could be influenced by changes in environmental conditions such as the growth substrate, temperature and pH levels (Janda and Abbott, 2002). Besides, these tests were also time-consuming (Kotay and Das, 2007).

Molecular biological methods were used to overcome the drawbacks of the conventional methods. Technique chosen was the 16S rRNA gene sequencing which had been used in the study of phylogeny and taxonomy (Janda and Abbott, 2002). Although it could not guarantee 100% accuracy, all types of bacteria have the Housekeeping gene known as 16S rRNA gene sequence (16S rDNA). The function has not ever changed, and the size is 1500 bp (Janda and Abbott, 2007). Based on the sequence analysis, species or genus of bacteria was determined based on the correspondence of the sequence obtained with the sequences available in GeneBank database

4.3.1 Identification Based on Morphological

The isolated colonies were observed after incubation for 48 hours (Figure 4.1) in anaerobic condition at 30^{o}C. The biggest colony size was A8, while the smallest size was A2. The different colony size and shape proved that all the strains were different.

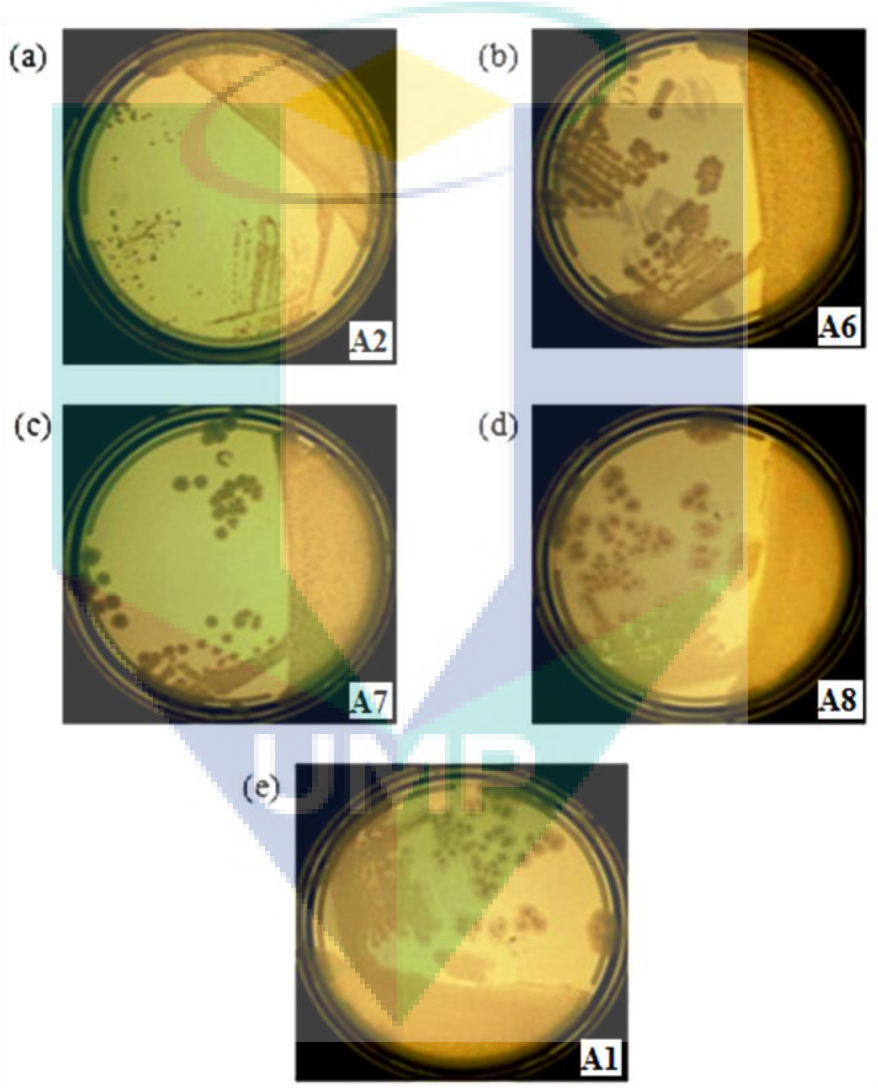

Figure 4.1: Isolated colonies for all efficient strains. (a) A2, (b) A6, (c) A7, (d) A8, (e) A1

Table 4.3 show the gram staining, shape and cell size, and colony size. From Table 4.3, all strains were in rod shape but in different sizes. Two strains were gram negative

while the rest were gram positive. The biggest cell size was the A6 strain, and it was gram positive. All strains were in the same family but not the same species. The results also show that the cell size is not directly proportional with colony size. A6 strain has the biggest cell size but the A6 colony size was smaller compared to A8. It has been reported by Young (2006) and that different cell shape, size and staining show that the bacteria were not same but it may be from the same family. According to Scheffers and Pinho (2005), gram staining was not sufficient to be used as the only method to identify bacteria at the species level. Certain bacteria are able to retain the crystal violet and remove the crystal violet in the same conditions. The cell wall of those bacteria may synthesis various structure of peptidoglycan (Madison, 2001; Ryan and Ray 2004).

Table 4.3: Gram staining results for all 5 strains

Strains	Gram Stains	Shape and Size	Colonies size (mm)
A2	-	Rod (0.15-0.25µm)	1.0
A6	+	Rod (0.15-0.45µm)	2.0
A7	-	Rod (0.15-0.25µm)	2.0
A8	-	Rod (0.10-0.20µm)	3.0
A1	+	Rod (0.10-0.15µm)	2.5

In this study, gram negative rod shape showed that the strains were from *Proteobacteria* or *Enterobacteriaceae* or *Bacilli* family (Drucker and Whittaker, 1971; Zhang et al., 2012). However, several species from *Bacilli* family can also be gram positive such as *Bacillus thuringiensis* and *Bacillus cereus* (Ammouneh et al., 2010; Tallent et al., 2012). Therefore, the approach to find the exact species using the morphological properties was not effective and accurate. The results cannot be presented accurately due to many uncertainties. Identification based on the biochemical and the bio molecular tests were necessary to provide more precise result.

4.3.2 Identification Based on Biochemical Test

Biochemical test was conducted using Biolog Microplate. It contained 71 carbon source utilization assays and 23 chemical sensitivity assays in 96 wells. Phenotypic fingerprint provided in the test panel could identify bacteria at the species level. After 48 hours incubation, the optical density (OD) readings in each well were taken. Table 4.4 showed the species ID obtained from the Biolog Microplate. The percentage showed that the pure strains obtained were considerably matched (more than 50%) with the species id in the Biolog database. The species was differentiated according to their reaction to carbon and chemical sensitivity assays test. All species in the same family and genus reacted with the same carbon sources. But, some species has special ability to react with extra carbon sources. This characteristic can be used to differentiate species in the same family and genus.

Table 4.4: The results from biochemical test by using Biolog Microplate.

Parameter	Results (ID)	Probability (%)
A2	*Arthrobacter xylinum*	58.9
A1	*Bacillus cereus/thuringiensis*	80.1
A7	*Bacillus cereus/thuringiensis*	58.9
A6	*Bacillus cereus/thuringiensis*	60.3
A8	*Enterobacter cowanii*	62.4

Strain A8 was taken as an example to clarify the above statement. The common carbon sources that can be utilized by genus *Enterobacter* is glucose, mannitol, inositol, sorbitol, rhamnose, saccharose, melibiose, amygdalin and arabinose (Inoue et al., 2000; Manter et al., 2008). From the Biolog Microplate test, A8 was identified as *Enterobacter cowanii* and has 62.4% matches. Some of the carbon source that cannot be utilized by *Enterobacter cowanii* has been utilized by A8. A8 has the ability to utilized arginine, ornithine, lysine and tryptophan. The Biolog library did not have the exact fingerprint for A8. Manter et al., 2008 reported that new species, *Enterobacter soli* have same characteristic with *Enterobacter cowanii,* except that *Enterobacter soli* can utilize same

carbon sources as A8. It showed that the Biolog database was not up to date. Because of this, further test was carried out to clarify the species ID. The identification based on gen 16S rRNA sequencing was used and discussed in the next subchapter.

4.3.3 Identification Based on Gen 16S rRNA Sequencing

The analysis of nucleic acids in gen 16S rRNA sequencing play an important role in species identification, studies of biodiversity, phylogeny, species definition, interactions study, detection of metabolite producers, degradation processes study, gene expression and gene cloning. The gen 16S rRNA sequencing has several steps, including DNA extraction, gel electrophoresis, polymerase chain reaction and DNA sequencing.

Figure 4.2 below showed the image taken from gel electrophoresis for DNA extraction and PCR product. For the DNA extraction, all the samples were compared with 1kb DNA ladder. In Figure 4.2(a), the DNA from all strains was successfully extracted. The white markers for all strains were stretched below than the DNA ladder marker. Results for PCR products were also compared with 1kb DNA ladder. The marker showed that the PCR product for strain A7 and A8 were quite low because there was no bright or clear mark obtained in the columns.

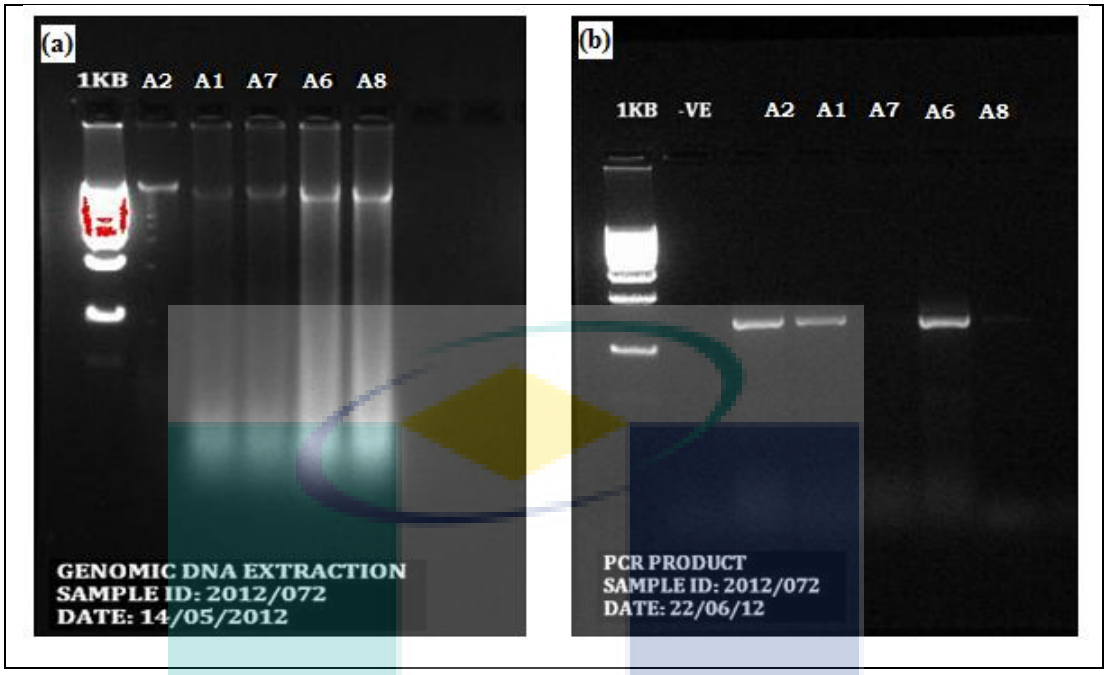

Figure 4.2: Gel electrophoresis images of selected five strains for; (a) Genomic DNA extraction and (b) PCR product.

The sequencing results obtained are shown in Table 4.5. All the results obtained from gel electrophoresis for DNA extraction and PCR products were all positives. It meant that the DNA extraction and PCR were successful and applicable for sequencing (Herrick et al., 1993). Two strains were in the same genus of *Enterobacteriaceae*, which were A2 (*Klebsiella pneumonia ST258*) and A8 (*Enterobacter cloacae Y219*) with probability of 92% and 89%. Both have less than 20% error. A1 (*Bacillus cereus PG06*), A7 (*Bacillus sp. SJ1*), and A6 (*Bacillus thuringiensis BMB171*) with probability of 92%, 77%, and 90% respectively, were from *Bacilli* family. A7 has the lowest percentage compared to the other three strains. It was due to the low PCR product obtained by A7 (Herrick et al., 1993: Lee et al., 2010). Besides, the signal intensity for nucleotide base of adenine, guanine, cytosine, and thymine for A7 was low compared to the original *Bacillus sp. SJ1*.

Table 4.5: The sequencing, gel electrophoresis results from PCR product and DNA extraction.

Sample	Gel Electrophoresis		ID	Probability
	DNA extraction	PCR product		
A2	+	+	*Klebsiella pneumonia ST258*	92%
A1	+	+	*Bacillus cereus PG06*	92%
A7	+	+	*Bacillus sp. SJ1*	77%
A6	+	+	*Bacillus thuringiensis BMB171*	90%
A8	+	+	*Enterobacter cloacae Y219*	89%

Enterobacteraceae genus (A2 and A8) was a facultative anaerobe, rod shape, gram negative and could be found in soil. The *Klebsiella pneumonia*, A2, have some similarities in DNA homology with other six strains of *Klebsiella sp.* According to Yu and Saddler (1982), this species grown in high sugar concentration with the presence of acetic acid but high amount of acetic acid might inhibit its growth. Furthermore, the acetic acid would act as a catalyst to enhance substrate utilization of glucose or xylose for the production of 2,3-butanediol. Biebl et al., (1999) reported that the *Klebsiella pneumonia* can produce acetic acid with 1,3-propanediol in a ratio of 1 to 2. However, this species can shift from acetic acid to 2,3-butanediol formation under conditions of decreased pH (Biebl et al., 1998). This species are naturally produce indole acetic acid (IAA) from rhizosphere plant (Acuna et al., 2011; El-Khawas and Adachi, 1999; Sachdev et al., 2009). This IAA would actually promote the plant's growth by increasing the root length and it is very good for agriculture.

Bacillus cereus PG06, A1, was a facultative anaerobe, gram positive, rod shape, could be found in soil, similar to findings from Stenfors et al. (2008). This species was a well known bacteria that spoiled food and harmful to human and animal. *Bacillus thuringiensis BMB171*, A6, and Bacillus *cereus PG06,* A1, were able to produce indole acetic and bacteriocin (Gray et al., 2006; Raddadi et al., 2008). Finally, *Bacillus sp. SJ1*, A7, was a gram negative, rod shape, facultative anaerobe and could also be found in soil. These three species were in the same *Bacilli* genus.

It can be concluded that all the species ID from BLAST report was accepted with probability more than 70%. The A6 strain which was the most efficient bacteria that able to produce acetic acid from banana stem waste was identified as *Bacillus thuringiensis BMB171*.

4.4 ACETIC ACID PRODUCTION FROM BANANA STEM WASTE AND *BACILLUS THURINGIENSIS* (A6)

The most efficient acetic acid producer (AAP) was A6. It produced 2.90 g/l acetic acid from 2.0 g/l glucose with pH around 3.63. The comparison with other research on acetic acid production has been tabulated as in Table 4.6.

Table 4.6: Comparison of acetic acid production from different type of waste

Researcher (Year)	Research Title	Amount of acetic acid produced, g/l
This research, 2011	Production of acetic acid from banana stem waste (BSW) using A6 (*Bacillus thuringiensis BMB171*)	1.1 – 4.4
Kim *et. al*., 2002	Acetic acid production using xylose and corn steep liquour(CSL) by *thermoaceticum* strain	15.2
Surawin et al., 2006	Acetic acid production from molasses by *Clostridium thermoaceticum*	3.06 – 6.29
Jin et al., 2008	Production of Acetic Acid by Hydrothermal Two-step Process of Vegetable Wastes	16.5-17.5

In this research, the amount of acetic acid produced in 72 hours by *Bacillus thuringiensis* (A6) was 1.1 – 4.4 g/l. The yield was affected by quantity of glucose in the process. When banana stem waste (BSW) was used as the material, the acetic acid produced was up to 1.1 g/g BSW. But, if pure glucose was used, the yield was up to 4.4 g/g glucose. The acetic acid yield from BSW is much lower due to the lignin content in BSW. The species need to change the cell metabolism to access glucose in the substrate. Thus, it required more time to produce high amount of acetic acid.

Kim et al., (2002) used xylose and corn step liquor (CSL) with *Clostridium thermoaceticum* for acetic acid production. The yield was 15.2 g/l acetic acid from 20 g/l substrate. Even though the yield was high, the substrate was not abundantly available in Malaysia. Furthermore, the substrate becomes limited because corn plantation was decreasing in Asia. From the website of index Mundi (2011), the corn harvested area in Korea for year 2002 until 2011 was surprisingly decreased which was from 21.43% to 0.05%. In Malaysia too, the growth of corn plantation decreased from 4.55% to 0.05%. The production of corn steep liquor became unfeasible since the raw material was hardly accessible.

The acetic acid production from molasses by using *Clostridium thermoaceticum* was performed by Surawin et al, (2006). It was reported that the yield increased from 3.06 g/l to 6.29 g/l. This happened when acclimatized culture was mixed with diluted 1:50 molasses. Even though the substrate was agricultural waste, it seemed difficult to obtain molasses nowadays. Molasses is a by-product of white sugar production from sugarcane. Sugarcane plantation is not abundantly available compared to BSW in Malaysia. According to FAO Corporate Document Repository (2012), the cultivation of sugarcane in Malaysia was small compared to banana plantation.

Lots of investments were needed to obtain molasses. Currently, the sugarcane harvested area has averaged between 20,000 and 24,000 hectares (Index Mundi, 2011). The limitation of producing the white sugar might cause the unavailability of the molasses which in turn could suspend the acetic acid production. In this research, BSW was used as substrate to produce acetic acid. This waste was plenty in Malaysia, where at least 34,000 hectares of banana plantation area in Malaysia (Abdul Khalil et al., 2006). In fact, the cost of raw materials could significantly reduced when BSW were used compared to molasses. It is due to the inexpensive price and its accessibility.

Another research of acetic acid production was done by Jin et al. (2008). The production was conducted by using hydrothermal two-step process with vegetable wastes. The vegetables used were Chinese cabbage, cabbage, potatoes, carrots and white radish. It

was completed under same conditions (Jin et al., 2008). The yields obtained from those five vegetables were almost same. It was around 16.5 – 17.5 g/l from 20 kg of waste. Jin et al., 2008 also compared their findings with starch where 17.5 g/l of acetic acids was produced. There were no significant differences observed.

The two-step processes used in the research were consisted of hydrothermal reaction without oxygen supply and oxidation reaction. First they prepared the feed slurry which was a mixture of water and vegetables then, the system was heated. The system mainly consists of a slurry pump, plunger pump, preheater and cooler for the whole process that also involving reaction media, reactor, solid-liquid separator, filter, back-pressure regulator and a gas-liquid separator (Jin et.al, 2008). Even though the used of vegetable wastes was not expensive, the technology applied demand high pressure and power consumption. Compared to this research, the yield was much lower than the research done by Jin et al., (2008). However, the energy consumption by Jin et al., (2008) was higher. The temperature needed was up to 300^{o}C to initiate the reaction. The higher the energy needed, the higher the cost will be. Thus, it can be concluded that this process is much feasible compared to hydrothermal process.

Moreover, high amount of glucose could produce high yield of acetic acid (Amin et al., 2008). The production of acetic acid using this species was limited. It was due to the insecticide produced was much valuable compared to acetic acid (Amin et al., 2008; Kannan et al., 2012; Vassilev et al., 2008).

4.5 COMPARISON OF ACETIC ACID PRODUCTION WITH OTHER RESEARCHES.

Table 4.7 showed several types of soil acetic acid producer (AAP) used in other researches. Factors affecting growth and acetic acid yield were also discussed. All types of soil AAP required high amount of substrate for higher yield. Cellulose, ethanol and glucose

were common substrates for acetic acid production. From Table 4.7, the highest yield was obtained by *Bacillus thuringiensis*. Theoretically in anaerobic fermentation, one mole of glucose ($C_6H_{12}O_6$) can produce up to three mole acetic acid (CH_3CO_2H) (Niir, 2003). However, the production from this strain was up to 1.8 g acetic acid per g glucose. It was 60% conversion of glucose to acetic acid.

The *Bacillus thuringiensis* is a facultative anaerobe. Meanwhile, other types of AAP were obligate anaerobe and aerobe. This strain was much durable compared to other AAP because it can survive in either oxygen or non-oxygen environment. The system must be implemented with strict supervision on oxygen level for obligate anaerobe AAP.

Table 4.7: Factor affecting yield of acetic acid and growth

Researches	Factor Affecting Yield and Growth	Type of Bacteria	Yield of Acetic Acid g acetic acid / g glucose
This research (2012)	Yield: i. Substrate –Cellulose and Glucose ii. Biomass – High amount increase yield Growth: i. Temperature – 27 - 40^{o}C ii. pH – 3.0 – 7.0	Strain *A6 @ Bacillus thuringiensis (facultative anaerobic)*	1.8
Gaensakoe et al., (2005) Brener et al., (2005)	Yield: i. Substrate – Ethanol and Glucose ii. Biomass – High amount increase yield Growth: i. Temperature – 30^{o}C ii. pH – 5.0	*Acetobacter aceti (aerobic)*	0.40
Horiachi et al.,, (2004)	Yield: i. Substrate – Ethanol and Glucose ii. Biomass – High amount increase yield iii. Aeration rate- High aeration rate increase O_2 in the process Growth: i. Temperature – 30^{o}C ii. pH – 5.0	*Acetobacter pasteurianus (aerobic)*	0.88

Researches	Factor Affecting Yield and Growth	Type of Bacteria	Yield of Acetic Acid g acetic acid / g glu
Veny and Hasan (2005)	Yield: i. Substrate –Ethanol and Glucose ii. Biomass – High amount increase yield Growth: i. Temperature – 30°C ii. pH – 5.0	*Acetobacter aceti (aerobic)*	0.67
Song et al., (2010)	Yield: i. Substrate – Ethanol and Glucose ii. Biomass – High amount increase yield Growth: i. Temperature – 37°C ii. pH – 7.0	*Clostridium tyrobutyricum (obligate anaerobic)*	0.99
Lee et al., (2009)	Yield: i. Substrate – Ethanol and Glucose ii. Biomass – High amount increase yield Growth: i. Temperature – 35°C ii. pH – 7.0	*Aeromonas caviae (anaerobic)*	0.22

Bacillus thuringiensis has higher resistant on temperature and pH compared to other strains. It grew at temperature from 27^{o}C to 40^{o}C and pH as low as 3.0 to 7.0. The soil temperature is changing constantly during daytime until night. It also changes pH at different weather throughout the year (Donahue et al, 1977). Therefore, the soil microorganisms should have the capability or certain unique characteristics in order to survive in that kind of environment (Pettersen and Baath, 2006).

From Table 4.7, *Clostridium tyrobutyricum* was the second highest AAP. It produced 0.99g acetic acid from one gram glucose. *Clostridium tyrobutyricum* was obligate anaerobe and grew at 37^{o}C and pH 7.0 (Song et al., 2010). However, this strain could only reach 33% of total glucose conversion to acetic acid. The conversion was low because the main product produced was not acetic acid. This reason was supported by Song et al., (2010) and Jo et al., (2008); they reported that this strain was actually a butyric acid producer. It could produce a fraction amount of acetic acid while producing butyric acid. Other than that, *Aeromonas caviae* was one of anaerobic AAP. The highest yield was at 0.22g acetic acid/ g glucose. The suitable growth temperature and pH for this species were at 35^{o}C and 7.0 respectively (Lee et al., 2008).

The most common species used for acetic acid production was *Acetobacter sp.* In the Table 4.7, two species from *Acetobacter* strain have been used for production of acetic acid. These species were *Acetobacter aceti* (Gaensakoe et al., 2005; Brener et al., 2005; Veny and Hasan, 2005) and *Acetobacter pasteurianus* (Horiuchi et al., 2004). These species were frequently used in acetic acid production (Brener et al., 2005; Gaensakoe et al., 2005; Horiachi et al., 2004; Veny and Hasan, 2005). These AAP could only produce acetic acid in aerobic condition. The suitable temperature and pH for *Acetobacter* growth was at 30^{o}C and 5.0, respectively. However, this species could only convert ethanol to acetic acid. It might be a problem for a system that does not have ethanol as raw material. Extra reactor will be needed to convert this material to ethanol (Veny et al., 2003).

The comparison in the Table 4.7 showed that the *Bacillus thuringiensis* was able to utilized glucose to produce high amount of acetic acid. It could produce up to 1.8g acetic

acid/g glucose. Furthermore, this AAP was more resilient compared to others. It has wide range of growth temperature and pH.

4.6 Batch Fermentation Profile

After identification process, growth profile for *Bacillus thuringiensis* (A6) in BSW and glucose mediums was performed. The fermentation was conducted at 30°C and 150 rpm for 72 hours. The result was taken every 6 hours by observing the absorbance via UV-Vis Spectrophometer at 550nm wavelength. The growth profile was presented in Figure 4.3 and Figure 4.4.

From Figure 4.3 and 4.4, the growth could be divided into number of stages. In early stage, there was no growth appeared for a certain period. This period was referred as lag phase and considered as a time of adaptation. Then, there was log phase where cells growth rate gradually increased at a constant rate. After that, growth ceased where cells entered the stationary phase. The cell number declined as the culture entered the death phase. The profile could describe the growth and culture behavior. The behaviour could be monitored by examining products generated during different stages of the growth curve. The products during the log phase, (e.g.; nucleic acids, carbohydrates, proteins, lipids) were essential to cell growth (Stanbury et al., 2003). These products known as primary metabolites and were produced during primary metabolism (Bu'Lock et al., 1965). Trophophase is a phase where primary metabolites are formed (Liao et al, 1995; Stanbury et al., 2003). It usually occurred during log phase. Primary metabolites generated during trophophase were sometimes beneficial and economically worth. Therefore, it was important to modify the cultural conditions to improve bacteria productivity on wanted products (Liao et al, 1995; Stanbury et al., 2003).

After trophophase, the growth entered the idiophase. In this phase, secondary metabolites will be produced (Bu'Lock et al., 1965). The secondary metabolites did not have any obvious function in cell metabolism. The cells growth during idiophase was slow or not growth at all. Thus, it could be the reason this phase normally occurred during

deceleration and stationary phases (Liao et al., 1995; Pirt, 1975; Standbury et al., 2003). Stanbury et al., (2003), also reported that the secondary metabolism could occur at low growth rates in continuous cultures. The fact was confirmed by Liao et al., (1995), where they found that the secondary metabolites could be obtained during biomass accumulation after assimilation of either carbon or nitrogen source. Besides, the idiophase state usually prevails in natural environment rather than the trophophase. Microorganism usually has low growth rates in their natural environments. Meanwhile, the trophophase normally prevails when the microorganisms is in media culture (Stanbury et al., 2003).

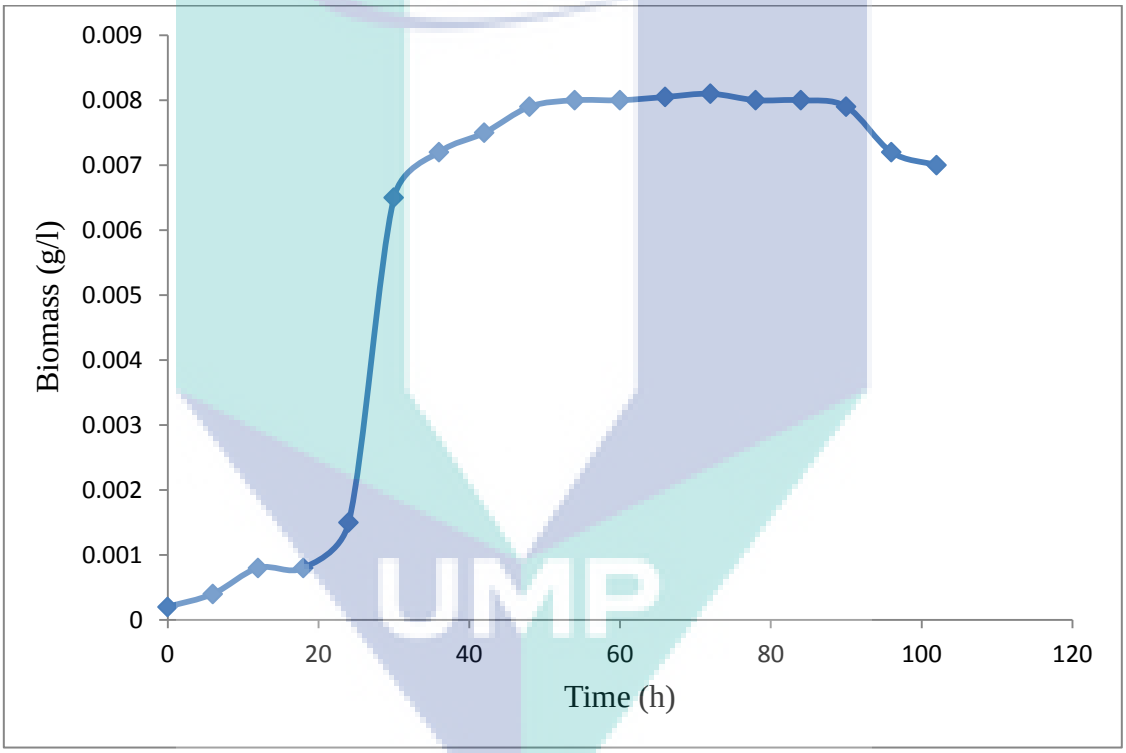

Figure 4.3: Growth profile of *Bacillus thuringiensis* using BSW as growth medium

Figure 4.3 showed the growth profile of *Bacillus thuringiensis* in BSW medium. The profile was fit with the standard growth profile. In Figure 4.3, the growth was slowly developed during early stages. After the inoculation process with BSW medium, there was a slow growth period. *Bacillus thuringiensis* was actually adapting with new environment during this period. Subsequently, the growth rate gradually increased and it entered the log

phase in 24 to 36 hour. Eventually, growth ceased and the cells entered the stationary phase in 48 to 84 hour. After 84 hours, the growth rate was declined where it entered the death phase.

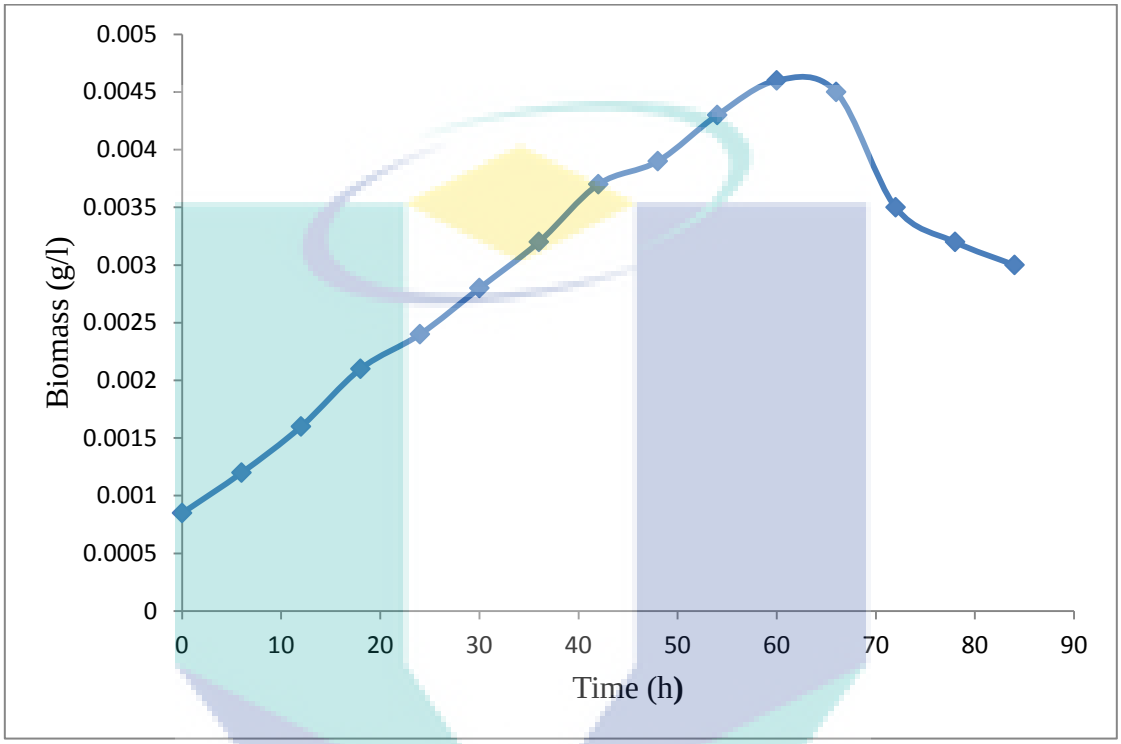

Figure 4.4: Growth profile of *Bacillus thuringiensis* using glucose as growth medium

Meanwhile, Figure 4.4 showed the growth profile of *Bacillus thuringiensis* in glucose medium. The profile was not fit with the standard growth profile. No lag phase could be observed and only log and death phase were appeared. At the early stage, growth rate appeared to increase at constant rate. This event took place until 60 hours. After that, the growth rate declined and it entered death phase. No stationary phase and lag phase involved in this growth profile. It showed the cell growth was dominated by primary metabolism until 60 hours. The cell growth rate declined when the primary metabolism completely shifted to secondary metabolism.

Stanbury et al., (2003), reported that the microorganisms could have primary metabolism whenever it is in culture medium. The culture medium contained important nutrients that can enhance the production of primary metabolism and cell growth (Liao et al., 1995; Hugh and Leifson, 1953; Stanbury et al., 2003). In this research, glucose was supplied in media culture to enhance *Bacillus thuringiensis* growth.

4.7 DETERMINATION OF THE KINETIC CONSTANTS

Only one kinetic model was used for this study based on the evaluation of kinetic model. The evaluation of the kinetic model was based on the sum squared error between experimental data and theoretical data. The model that used by Borja et al., (2005) and Zainol et al., (2012) was selected to explain the kinetic of the process. The model equations are presented below:

$$-\frac{dSvss}{dt} = k_1 Svss \tag{2.12}$$

In equation 2.12, (dS_{vss}/dt) is the removal rate of non-soluble organic matter (g/l h); k_1 is the kinetic constant of the reaction (h^{-1}); Svss is effluent concentration of biodegradable non-soluble organic matter (g/l)

$$\frac{dSvds}{dt} = k_1 Svss - \left(\frac{k_2 Svds}{k_3 + Svds}\right) \tag{2.13}$$

In equation 2.13, (dS_{vds}/dt) is the production rate of soluble organic matter (g/l h); k_2 is the maximum removal rate of soluble organic matter (maximum glucose uptake during growth)(g/l h); Svds is effluent concentration of soluble biodegradable organic matter (glucose concentration)(g/l) and k_3 is the saturation constant (g/ l)

$$\frac{dStaa}{dt} = \left(\frac{k_4 Svds}{k_5 + Svds}\right) - \left(\frac{k_6 Staa}{k_7 + Staa}\right) \tag{2.14}$$

In equation 2.14, (dS_{taa}/dt) is the rate of acetic acid production (g/l h); Staa is effluent concentration of acetic acid concentration (g/l); k_4 is the maximum rate of VDS uptake (maximum rate of glucose uptake during acetic acid production)(g/l h), k_5 is the saturation constant (g/ l), k_6 maximum rate of TAA (acetic acid concentration) consumption (g/l day), and k_7 is the saturation constant (g /l).

There were two parts involved in this kinetic study. These two parts were the collection of experimental data and the simulation by using Microsoft Excel. The experimental data was divided into three components. The components were microbe cell concentration, glucose concentration and acetic acid concentration. These data were collected from batch fermentation (Song et al., 2008; Zainol et al., 2012). In the simulation, the proposed model was used to determine the theoretical value for growth, substrate utilization and production. The model was fitted to the experimental data by using Microsoft Excel. Runge-Kutta 4th Order was implemented for determination of kinetic constants (k_1-k_7) value by minimizing sum squared error between theoretical and experimental data (Song et al., 2008; Lee et al., 2009; Zainol et al., 2012).

The kinetic model involved the biomass accumulation, biomass growth and loss together with substrate inhibition effect or liquid flow effect (Borja et al., 2005). However, parts of the biomass may attach onto the reactor walls making it almost impossible to quantify. Therefore, it was assumed that the biomass remains constant as the biomass accumulation was restrained by its adhesion or effluent loss (Borja et al., 2005; Zainol et al., 2012). The proposed equations were validated by comparing the theoretical curves obtained with the corresponding experimental data.

4.8 KINETIC CONSTANTS FOR BOTH MEDIUMS

By using Microsoft Excel, the kinetic constants (k_1-k_7) of the model for banana stem waste medium were calculated according to the following initial condition: $(S_{VSS})_0$ = 0.0002 g/l; $(S_{VDS})_0$ = 2.46 g/l; $(S_{TAA})_0$ = 0 g/l. The kinetic constants for glucose medium

were based on the following initial condition: $(S_{VSS})_0$ = 0.00085 g/l; $(S_{VDS})_0$ = 4.38 g/l; $(S_{TAA})_0$ = 0 g/l. The following values for the kinetic constants were tabulated in Table 4.8.

Table 4.8: Kinetic constant obtained from this study

Kinetic Constant	Banana Stem Waste (BSW)	Glucose
k_1	0.5004 h^{-1}	0.4667 h^{-1}
k_2	0.0576g /l h	0.0671 g /l h
k_3	0.6197g /l	0.0229 g /l
k_4	0.1294g /l h	0.2382 g /l h
k_5	0.9289g /l	0.5382 g /l
k_6	0.0545g /l h	0.1138g /l h
k_7	0.0001g /l	0.001 g /l

4.8.1 Biomass

In Figure 4.5(a), the biomass concentration slowly increased until 48 hours. The growth increased rapidly after 48 hours until 72 hours. No stationary and death phase existed in the Figure 4.5(a). The *Bacillus Thuringiensis* has the ability to utilized acetic acid in secondary metabolism. It only entered stationary and death phase after the products became inhibitor to the cells. The stationary and death phase might be in the curve if the growth was prolonged more than 72 hours. Liao et al., (1995) reported all the microorganism has their own specific time for stationary and death phase. It was depending on the substrate used in fermentation. The curve also supported the theory proposed by Stanbury et al., (2003). They stated that microorganism growth became slow or in deceleration state when the amount of secondary metabolites increased. The growth also became slow when the primary metabolites low in concentration or depleted (Standbury et al., 2003). R^2 value for biomass concentration obtained from this study was closed to 1.0. It indicated that a regression data fits the proposed model. The R^2 values for Figure 4.5(a) and Figure 4.5(b) were 0.98 and 0.91, respectively. These values were satisfactory, which means this model could be used for data prediction in future work.

The growth rate in Figure 4.5 (b) was gradually increased constantly until 72 hours. Both Figure 4.5(a) and Figure 4.5(b) only had log phase but different type of growth. The growth rate increased slowly in glucose medium compared to BSW medium. According to Liao et al., (1995), microorganism growth could be induced by using substrates that enhanced growth. Glucose was supposedly became substrate that enhanced *Bacillus Thuringiensis* growth. However, Magasanik (1961) appointed that excess glucose could inhibit growth for nitrogen fixation bacteria by catabolic repression. Therefore, the growth rate in glucose medium was low compared to BSW medium. *Bacillus Thuringiensis* was also one of several types of nitrogen fixation bacteria (Gray et al., 2006; Raddadi et al., 2008).

In Monod model, the saturation constants were known as the substrate utilization constants. Saturation constant was determined at half of the value of maximum specific growth rate. This was to measure the affinity of the organism for its substrate. The saturation constants for glucose utilization for BSW and glucose medium were at k_3 = 0.6197g /l and k_3 = 0.0229g /l respectively. The values showed that the organism has high affinity towards reducing sugars in BSW medium (Ahmad and Holland 1995; Monod 1942; Stanbury et al., 2003). Jamaluddin et al, (2012) reported that BSW contained C5-C6 sugar. BSW contained of xylose, galactose, arabinose and mannose (Mohaptara et al., 2010). As a result, the growth rate in BSW medium was higher than glucose medium.

The k_1 value obtained for BSW= 0.5004 h^{-1} was higher than glucose = 0.4667 h^{-1}. This species has the ability of secondary metabolism, which might affect the substrate utilization and the cell growth rate. If the species has high affinity towards the limiting substrate, the growth rate will get affected when limiting substrate concentration decreased to a very low level (Ahmad and Holland 1995; Stanbury et al., 2003). According to Bushell (1988), *Bacillus Thuringiensis* has secondary metabolism because the strain is sporing bacteria. However, it is hard to tell either the cell metabolism has already shifted to secondary metabolism or primary metabolism by only looking at the bacteria growth (Stanbury et al., 2003). The substrate and product during metabolism could indicate the exact metabolism occurred.

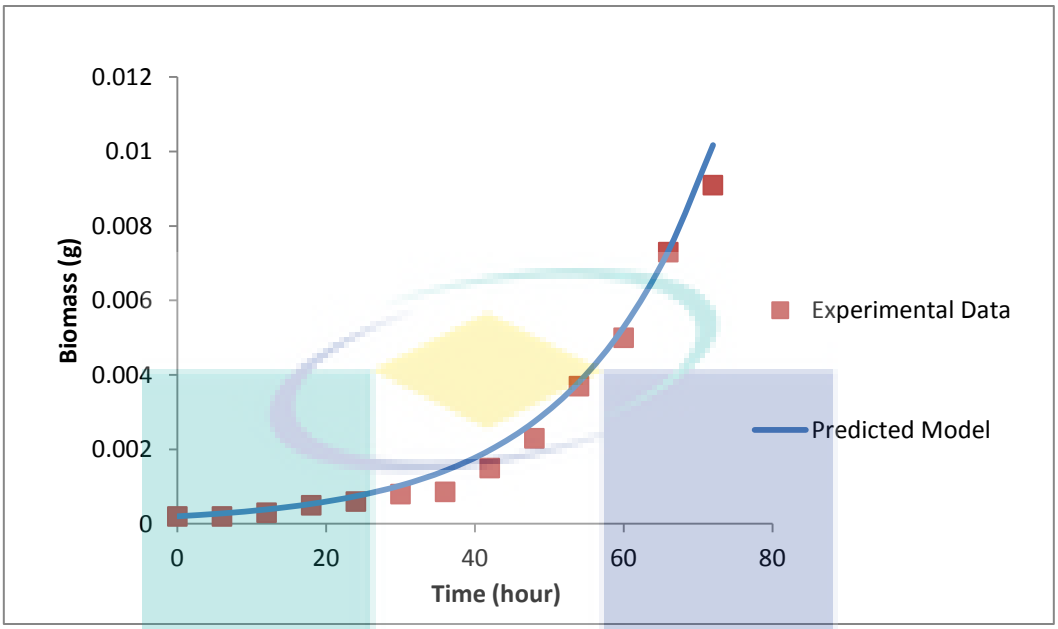

Figure 4.5(a): Graph of biomass (banana stem waste substrate) from experimental and predicted data. R^2=0.98

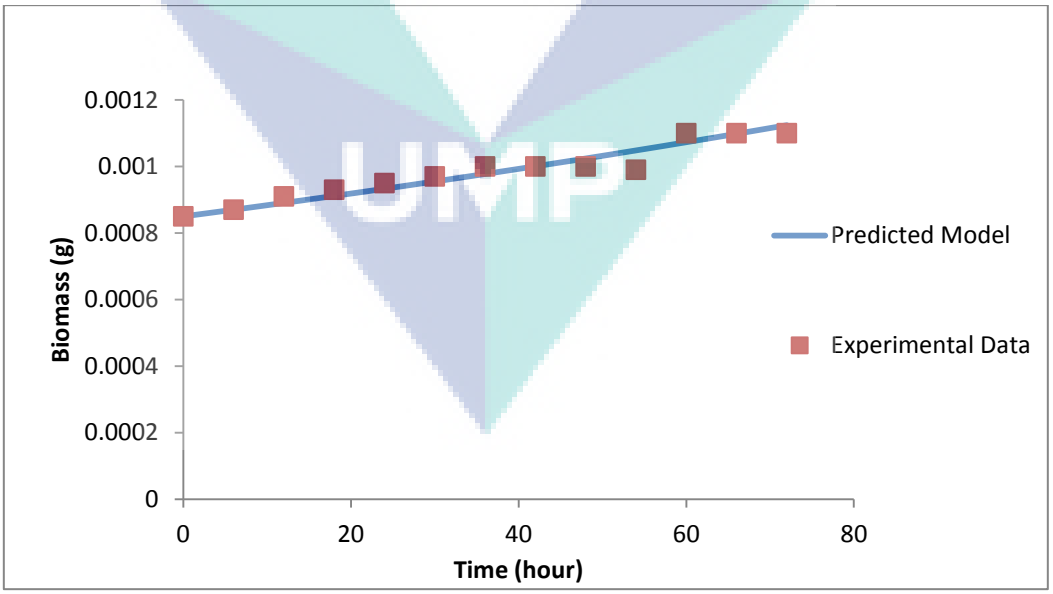

Figure 4.5(b): Graph of biomass (glucose substrate) from experimental and predicted data. R^2= 0.91

4.8.2 Substrate Utilization (Glucose consumption)

The glucose concentration decreased rapidly and became constant after 40 hours until 72 hours in Figure 4.6(a). The glucose concentration also become constant after it was below than 0.5 g/l. This was due to either the exhaustion of substrate or the accumulation of toxic products (Stanbury et al., 2003). The *Bacillus Thuringiensis* metabolism shifted from 42 to 72 hours. This was when glucose concentration lower than 0.5 g/l (in Figure 4.6(a)). For glucose medium (Figure 4.6(b)) the glucose concentration was decreasing in constant rate. The situation was distinctly different from the curve in Figure 4.6(a). The *Bacillus Thuringiensis* metabolism shifted from primary to secondary after 60 hours (Figure 4.6(b)). The primary metabolism took longer time to shift in glucose medium due to high glucose concentration compared to BSW medium (Figure 4.6(b)). The initial concentration of glucose in nutrient medium increased the glucose utilization (Cohen, 1951; Wright and Lockhart 1965). The initial glucose concentration in BSW (2.5 g/l) was lower than in glucose medium (4.5 g/l). The R^2 values obtained for Figure 4.6(a) and Figure 4.6(b) were 0.97 and 0.99, respectively.

The kinetic constants of k_2 and k_4 showed the *Bacillus Thuringiensis* dependency on glucose. The k_2 is maximum removal rate of glucose during growth and k_4 is maximum rate of glucose uptake during acetic acid production. The k_2 values for glucose medium and BSW medium were 0.0671g/ l h and 0.0576 g/ l h, respectively. The k_4 values for glucose medium and BSW medium were 0.2382g/ l h and 0.1294 g/ l h, respectively. The kinetic values (k_2 and k_4) showed that glucose utilization for acetic acid production was higher than for growth. The k_4 value was higher than k_2 because this species utilized only glucose to produce acetic acid (Amin et al., 2008; Kannan et al., 2012; Vassilev et al., 2008;Yousten and Rogoff, 1969). This species utilized other types of reducing sugar for growth. Thus, the rate of glucose utilization during growth was lower compared to acetic acid production (Amin et al., 2008; Kannan et al., 2012; Vassilev et al., 2008; Yousten and Rogoff, 1969).

Bacillus Thuringiensis had very high affinity for the limiting substrate (glucose) since the rate would not be affected until the substrate concentration declined to very low

level. This explained the missing lag phase (deceleration phase) and stationary phase in biomass concentration (Figure 4.5(a) and 4.5(b)). Besides, the bacteria growth was also affected by secondary metabolism. High initial amount glucose also increased the glucose utilization rate.

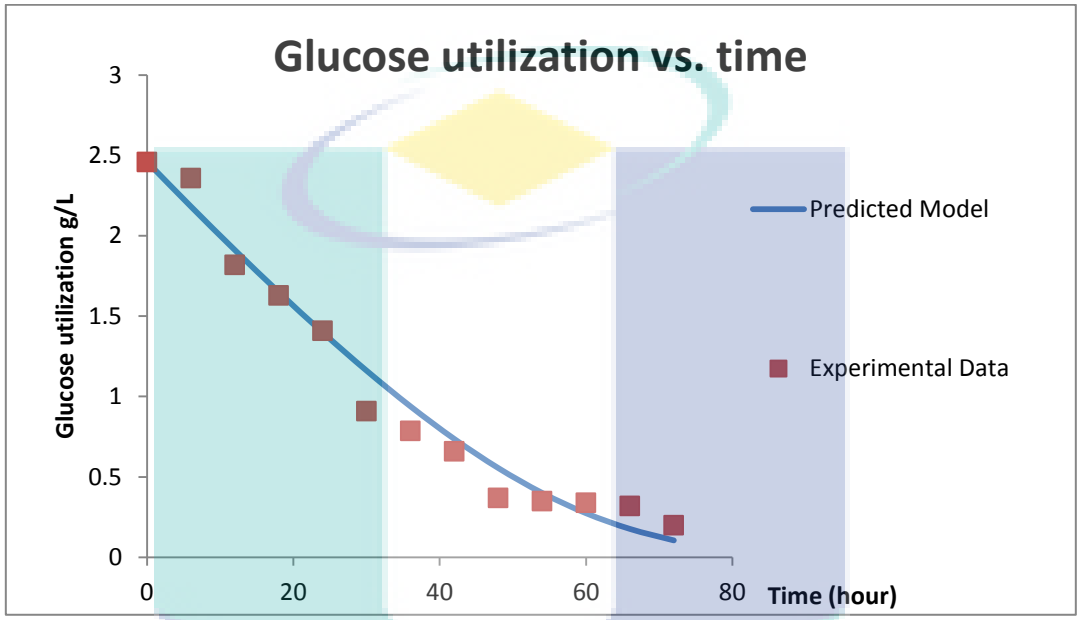

Figure 4.6(a): Graph of glucose utilization (banana stem waste substrate) from experimental and predicted data. R^2= 0.97

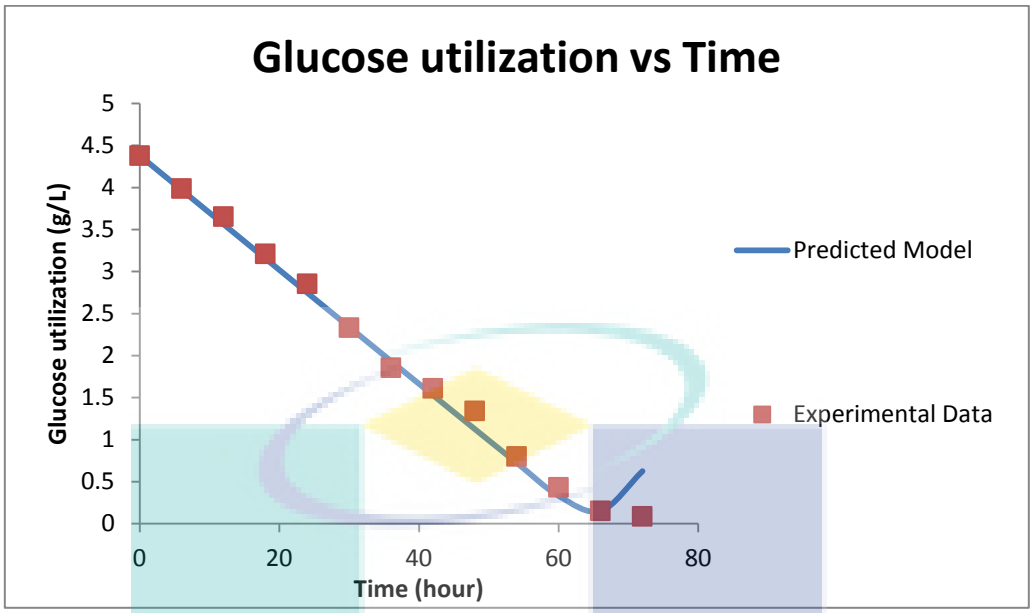

Figure 4.6(b): Graph of glucose utilization (glucose substrate) from experimental and predicted data. R^2= 0.99

4.8.3 Product Formation (Acetic Acid Production)

Figure 4.7(a) showed that the acetic acid concentration increased with time, but the concentration remained constant after 36 hours and started decreasing after 54 hours. Stanbury et al., (2003) stated that the secondary metabolism occurred when limiting substrate was in low concentration. The low glucose concentration shifted *Bacillus Thuringiensis* metabolism from primary to secondary metabolism at 42 hours. *Bacillus Thuringiensis* consumed acetic acid after 36 hours of fermentation (in Figure 4.7(a)). Other types of reducing sugars such as xylose, galactose, arabinose and mannose in BSW were used by *Bacillus Thuringiensis* for acetic acid production in primary metabolism (Jamaluddin et al., 2012; Mohaptara et al., 2010; Stanbury et al., 2003; Wright and Lockhart 1965). In glucose medium, the acetic acid concentration increased until 60 hours and later decreased (in Figure 4.7(b)). The curve did not have stationary phase as in Figure 4.7(a) because only one carbon source available in the glucose medium. *Bacillus*

Thuringiensis consumed acetic acid when the concentration of glucose was below than 0.5 g/l.

Normally, final product concentration will increase with time and become constant at the end of fermentation. This study proved that the *Bacillus thuringiensis* has secondary metabolism and acetic acid was not the final product. This study agreed with the research done by with Yousten and Rogoff, (1969) where *Bacillus thuringiensis* produced acetic acid from glucose. The acetic acid was an intermediate product from primary metabolism that would be utilized in secondary metabolism (Stanbury et al., 2003). By referring to the kinetic model, the phenomenon could be explained using the saturation constants (k_5 and k_7). The k_5 was referred as glucose saturation constant, and k_7 was referred as acetic acid saturation constant. The saturation constants (k_5) in BSW and glucose medium were 0.9289g/l and 0.5382g /l, respectively. Meanwhile, the saturation constants (k_7) in BSW and glucose medium were both 0.0001g/l These showed that the saturation constant for acetic acid was lower than glucose. The values showed that the *Bacillus thuringiensis* has higher affinity towards glucose than acetic. It also showed that the *Bacillus thuringiensis* preferred to utilize glucose than acetic acid. When the amount of glucose became critically low *Bacillus thuringiensis* shifted to secondary metabolism and utilized acetic acid..

As mentioned in subchapter 4.8.1 and 4.8.2, the value of kinetic constant would determine the *Bacillus thuringiensis* affinity towards substrate. The *Bacillus thuringiensis* affinity towards substrate from both metabolisms is explained using the saturation constants (k_5 and k_7). The k_5 was referred as glucose saturation constant in acetic acid production, and k_7 was referred as acetic acid saturation constant. The saturation constants (k_5) in BSW and glucose medium were 0.9289g/l and 0.5382g /l, respectively. Meanwhile, the saturation constants (k_7) in BSW and glucose medium were both 0.0001g/l . The values obtained showed that the *Bacillus thuringiensis* preferred glucose more than acetic acid. This finding agrees with other few researches. The primary metabolism has higher rate of substrate utilization than secondary metabolism (Ahmad and Holland 1995; Bu'Lock et al., 1965; Liao et al., 1995; Pirt, 1975; Stanbury et al., 2003). Glucose was needed in primary

metabolism while acetic acid in secondary metabolism. Therefore, the value of k_5 should be higher than k_7.

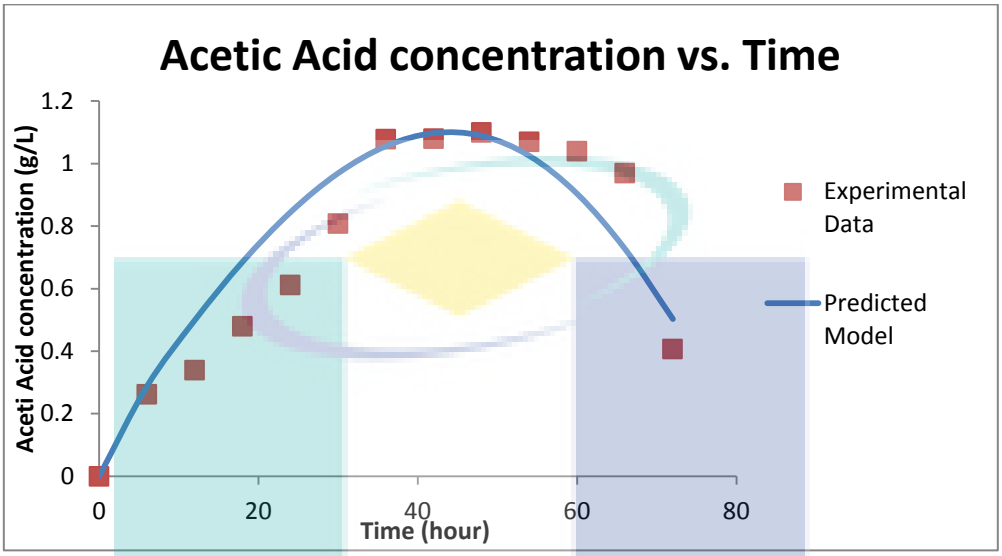

Figure 4.7(a): Graph of acetic acid concentration (banana stem waste substrate) from experimental and predicted data. R^2= 0.86

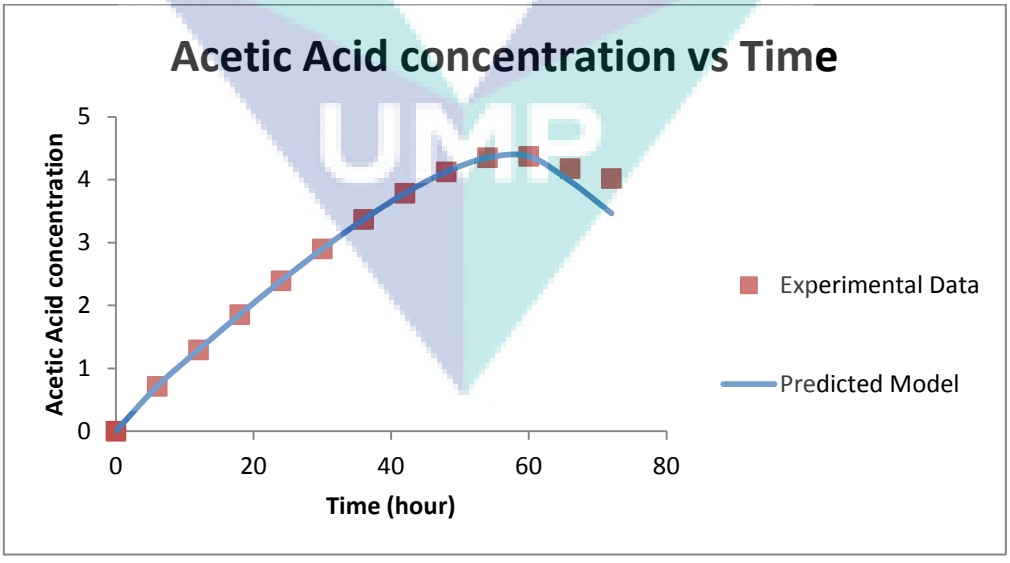

Figure 4.7(b): Graph of acetic acid concentration (glucose substrate) from experimental and predicted data. R^2= 0.98

4.8.4 Comparison of Kinetic Constants with Other Researches

Table 4.9 showed the comparison of k_1 value with other researches. The k_1 interpreted as reaction rate of bacteria in the hydrolysis process (Borja et al., 2005; Song et al., 2008; Lee et al., 2009; Zainol et al., 2012). High value of k_1 indicated high rate of hydrolysis. The highest value of k_1= 0.5004 h^{-1} was obtained from this research when compared to other researches in the Table 4.9.

Pelillo et al., (2006) obtained k_1= 0.00500 h^{-1} from aerobic degradation of two phase olive mill effluents. The research was conducted in stirred batch reactor at 25^{o}C. The inoculum was obtained from olive mill wastewater. The waste was acclimatized with the inoculum in the reactor for 20 day periods to enhance the bacteria growth. Borja et al., (2005) also conducted a kinetic study on anaerobic degradation of two phase olive mill effluents. The research used completely stirred batch reactor at mesophilic temperature 35^{o} andobtained k_1= 0.00238 h^{-1}. However, the waste was not acclimatized with the bacteria. The acclimatization will increase the biomass and production (Jamaluddin et al., 2011; Jamaluddin et al., 2012; Pellilo et al., 2006)

Siles et al., (2008) obtained k_1= 0.00085 h^{-1} from the pressing orange rind. The study was conducted in batch at mesophilic temperature (35^{o}C). Brewery wastewater was used as bacteria source. The orange rind wastewater used was supplied with nitrogen and phosphorus with the aim to improve the microorganism metabolism in the process. In this study, the *Bacillus Thuringiensis* metabolism was improved with nitrogen and phosphorus supply. Zainol et al., (2012) used same substrate with this research but different types of bacteria. The k_1 value from this research was higher than Zainol et al., (2012). They used soil mixed culture and no extra nutrients were supplied for their soil mixed culture.

In Table 4.10, k_2 (maximum rate of glucose uptake during growth (g/l h)) was compared with other researches. The rate of glucose removal was proportional to glucose utilization by bacteria d. The k_2 value obtained in this research was 0.0576g /l h. The

highest value k_2= 0.1760g /l h was obtained by Borja et al., (2005) was followed by Blonskaja et al., (2003) and Pelillo et al., (2006) at k_2= 0.1590g /l h and k_2= 0.1340g /l h, respectively. Only certain type of bacteria prefer to utilize glucose as main carbon sources for growth (Stanbury et al., 2003). Therefore, the rate of glucose uptake during growth would be slow if there were other carbon sources available in the substrate. Most of the substrate used (in Table 4.9) contained many types of carbon sources that can be utilized by the bacteria. Siles et al., (2008) has the lowest value for k_2 at 0.0009g /l h. Orange rind wastewater contained other types of reducing sugar such as sucrose and fructose (Sánchez et al., 2012). These were pentose sugar and simple than glucose, thus bacteria tend to utilized it first (Price et al., 2013).

Besides, high k_2 values when olive mill effluents used as substrate indicated high glucose concentration in olive mill effluents (Anastasiou et al., 2011; Borja et al., 2005; Pellilo et al.,2006). BSW contained high amount of glucose compared to orange rind wastewater (Mohapatra et al., 2010; Price et al., 2013). However, the olive mill effluents wastewater was easy to degrade compared to BSW due to low lignin content. Thus, high amount of glucose can be utilized when this waste was used.

The acetic acid consumption rate showed the level of acetic acid utilization by the bacteria. In Table 4.11, the rate of acetic acid consumption (k_6) for this research was compared with other researches. The k_6 value obtained was around 0.0545 g/ l h. The highest rate was obtained by Blonskaja et al., (2003) with the value of 0.4330 g/ l h. The study was done using upflow anaerobic sludge blanket (UASB) reactor with distillery waste at mesophilic temperature for 39 days. The k_6 value was high due to acetic acid consumption by acetoclastic methanogens for biogas production. Acetoclastic methanogen is a bacteria that consumed acetate or acetic acid in order to produce methane (Blonskaja et al., 2003). The kinetic value from this research was low compared to other studies. The *Bacillus Thuringiensis* species consumed acetic acid in secondary metabolism. This species is not an acetoclastic methanogens because it does not produce methane. Thus, the rate of acetic acid utilization in this study lower compared to Blonskaja et al., 2003.

According to Pellilo et al., (2006), the phenolic compound available in olive mill effluents was difficult to degrade by aerobic microorganism. The value obtained by Pellilo et al., (2006), was k_6 = 0.0133g/ l h. It was lower than value obtained by Borja et al., (2005), k_6 = 0.1800 g/ l h. Both studies using olive mill effluents but the with different type of processes Borja et al., (2005) used anaerobic process and required more time compared to Pellilo et al.,(2006). The lowest value of k_6 was obtained by Siles et al., (2008). Low acetic acid utilization was due to low acetic acid concentration in orange rind wastewater (Siles et al., 2008).

Table 4.9: The comparison of k_1 value with other researches

Researcher (Year)	Research Title	Kinetic Parameter Results, k_1 (h^{-1})
This Research, (2012)	Production of acetic acid from soil microbe	0.5004
Zainol et al., (2012)	Biogas generation from banana stem waste	0.00015
Siles et al., (2008)	Production of biogas from orange rind wastewater	0.00085
Pelillo et al., (2006)	Aerobic degradation of two phases olive mill effluents	0.00500
Borja et al., (2005)	Study of anaerobic digestibility of two phases olive mill solid waste	0.00238
Keshtkar et al., (2003)	Mathematical modeling of anaerobic digestion of cattle manure	0.00010-0.00238
Cuevas-Rodríguez et al., (1998)	Fermentation of wastewater in sequencing batch reactors	0.00011-0.00238
McCarty and Mosey (1991)	Modeling of anaerobic digestion process	0.00014-0.00238

Table 4.10: The comparison of k_2 value with other researches

Researcher (Year)	Research Title	Kinetic Parameter Results, k_2 (g/ l h)
This Research, (2012)	Production of acetic acid from soil microbe	0.0545
Zainol et al., (2012)	Biogas generation from banana stem waste	0.0010
Siles et al., (2008)	Production of biogas from orange rind wastewater	0.0009
Pelillo et al., (2006)	Aerobic degradation of two phases olive mill effluents	0.1340
Borja et al., (2005)	Study of anaerobic digestibility of two phases olive mill solid waste	0.1760
Blonskaja et al., (2003)	Use of two stage anaerobic treatment for distilling waste	0.1590

Table 4.11: The comparison of k_6 value with other research

Researcher (Year)	Research Title	Kinetic Parameter Results, k_6 (g/ l h)
This Research, (2012)	Production of acetic acid from soil microbe	0.0545
Zainol et al., (2012)	Biogas generation from banana stem waste	0.0003
Siles et al., (2008)	Production of biogas from orange rind wastewater	0.0005
Pelillo et al., (2006)	Aerobic degradation of two phases olive mill effluents	0.0133
Borja et al., (2005)	Study of anaerobic digestibility of two phases olive mill solid waste	0.1800
Blonskaja et al., (2003)	Use of two stage anaerobic treatment for distilling waste	0.4330

4.9 CONCLUSION

Only 5 strains from 52 strains were identified as the most efficient acetic acid producer. Those five strains were labeled as A1, A2, A6, A7, and A8. All strains were in rod shape with colonies size ranged from 1.0mm to 3.0mm. From both biochemical and biomolecular tests, A1 was identified as *Bacillus cereus PG06* with 92% probability, A2 as *Klebsiella pneumonia ST258* with 92% probability, A6 as *Bacillus thuringiensis BMB171* 90% probability, A7 as *Bacillus sp. SJ1* with 77% probability and A8 as *Enterobacter cloacae Y219* with 89% probability. It was found that strain A6 (*Bacillus thuringiensis BMB171*) was the most effective acetic acid producer from soil. A6 (*Bacillus thuringiensis BMB171*) was able to produce 1.1- 4.4 g/l acetic acid.

The kinetic model by Borja et al., (2005) and Zainol et al., (2012) were used in this study. The results showed that the proposed model fit the *Bacillus thuringiensis* species. The R^2 values obtained from kinetic study were satisfactory (above 0.8). The values of kinetic constants were as followed for BSW: k_1 = 0.5004 h^{-1}; k_2 = 0.0576g /l h; k_3 = 0.6197g /l; k_4= 0.1294g /l h; k_5= 0.9289g /l; k_6= 0.0545g /l h; k_7= 0.0001g /l. Meanwhile, the values for glucose medium: k_1 = 0.4667 h^{-1}; k_2 = 0.0671 g /l h; k_3 = 0.0229 g /l; k_4= 0.2382 g /l h; k_5= 0.5382 g /l; k_6= 0.1138g /l h; k_7=0.001 g /l. The kinetic constants represented the microbial activity in the process. The k_3 = 0.6197g /l (BSW medium) was higher than k_3 = 0.0229 g /l (glucose medium). The values showed that the organism has high affinity towards reducing sugars in BSW medium. The glucose was important substrate for acetic acid production. The k_4 value was higher than k_2 because this species utilized only glucose to produce acetic acid. This species utilized other types of reducing sugar for growth. Thus, the rate of glucose utilization during growth was lower compared to acetic acid production.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

The banana stem waste (BSW) was normally discarded during the harvesting of the banana fruit. This waste could be used as favorable substrate for acetic acid production since it contained a lot of cellulose which can be degraded to glucose. The analysis on BSW composition showed that cellulose was the dominant component, followed by the hemicelluloses and lignin. The soil bacteria could be used in acetic acid production from BSW. The acetic acid producer (AAP) was isolated from soil. The screening to find the most efficient AAP was conducted in batch fermentation. The A6 was found to be the most efficient AAP. This strain was able to produce up to 1.8 g acetic acid per g glucose. It was 60% conversion of glucose to acetic acid. The result was higher than common acetic acid bacteria such as *Acetobacter sp.*, and *Gluconobacter sp.* These two strains were widely used commercially and in industry for vinegar production. Besides, these two strains need oxygen, ethanol as substrate and two reactors to complete the production of acetic acid.

5.1.1 Isolation and Identification of Microbes Involved in Biodegradation of Banana Stem Waste and Soil Mixed Culture.

The isolation and identification of microbes involved in biodegradation of banana stem waste and soil mixed culture (SMC) was successfully done. 57 strains were isolated

from SMC. 5 most efficient acetic acid producers (AAP) were identified using biochemical test and bio molecular method. Those five strains were labeled as A1, A2, A6, A7, and A8. All strains were in rod shape with colonies size ranged from 1.0mm to 3.0mm. From both biochemical and biomolecular tests, A1 was identified as *Bacillus cereus PG06* with 92% probability, A2 as *Klebsiella pneumonia ST258* with 92% match, A6 as *Bacillus thuringiensis BMB171* 90% match, A7 as *Bacillus sp. SJ1* with 77% match and A8 as *Enterobacter cloacae Y219* with 89% match. All the results showed error less than 10% except for A7. The A7 might be new wild strain because the sequencing data obtained was not accessible within BLAST library.

The SMC contained many types of AAP that can be used in production of acetic acid. A6 (*Bacillus thuringiensis BMB171*) was the most effective acetic acid producer obtained from SMC. The A6 (*Bacillus thuringiensis BMB171*) was able to produce acetic acid up to 4.4 g/l.

5.1.2 Determination of Kinetic Constants for Acetic Acid Production from BSW Fermentation

The *Bacillus thuringiensis BMB171* was used in kinetic study of acetic acid production from banana stem waste. The results showed that the proposed model was suitable for *Bacillus thuringiensis* species. The R^2 values obtained from kinetic study were satisfactory (above 0.8). The initial concentrations (BSW) of biomass (S_{VSS}), glucose (S_{VDS}), and acetic acid (S_{TAA}) in the calculation were: $(S_{VSS})_0$ = 0.0002 g/l; $(S_{VDS})_0$ = 2.46 g/l; $(S_{TAA})_0$ = 0 g/l. The initial concentrations for glucose medium were: $(S_{VSS})_0$ = 0.00085 g/l; $(S_{VDS})_0$ = 4.38 g/l; $(S_{TAA})_0$ = 0 g/l. The k_1 is the kinetic constant of the reaction, k_2 is the maximum rate of glucose uptake for growth, k_4 is the maximum rate of glucose uptake for acetic acid production, k_6 maximum rate of acetic acid consumption, and k_3, k_5, k_7 is the saturation constant.

The values of kinetic constants were as followed for BSW: k_1 = 0.5004 h^{-1}; k_2 = 0.0576g /l h; k_3 = 0.6197g /l; k_4= 0.1294g /l h; k_5= 0.9289g /l; k_6= 0.0545g /l h; k_7= 0.0001g /l. Meanwhile, the values for glucose medium: k_1 = 0.4667 h^{-1}; k_2 = 0.0671 g /l h; k_3 = 0.0229 g /l; k_4= 0.2382 g /l h; k_5= 0.5382 g /l; k_6= 0.1138g /l h; k_7=0.001 g /l. The kinetic constants represented the microbial activity in the process. The k_3 = 0.6197g /l (BSW medium) was higher than k_3 = 0.0229 g /l (glucose medium). The values showed that the organism has high affinity towards reducing sugars in BSW medium. The glucose was important substrate for acetic acid production. The k_4 value was higher than k_2 because this species utilized only glucose to produce acetic acid. This species utilized other types of reducing sugar for growth. Thus, the rate of glucose utilization during growth was lower compared to acetic acid production.

5.2 IMPROVEMENT AND RECOMMENDATION

As this study achieved its objectives, several recommendations were proposed to improve the work quality and to give better results. The recommendations are listed below.

5.2.1 The Study on Acetic Acid Producer Properties

These findings provide the following insights for future research where there is a potential the acetic acid producer (AAP) found is a new strain. It will become great discovery and novelty. There is new field where properties of AAP can be determined by studies on metabolite producers, studies on biodiversity, and studies of interaction. All of these studies are available within the analysis and characterization of nucleic acids of the DNA of the AAP. The gene cloning, screening of gene expression and phylogeny may also become interesting field for future study. All of these studies can show whether the wild strain AAP DNA is mutated to improve the ability of the same species by bringing different properties and extra specialty. These studies can fill the gap of knowledge to find the suitable range of temperature, condition, suitable materials for growth and etc.

5.2.1 The Production Scale Up and Separation of Acetic Acid.

Further research in the reactor field using the pure strain would also be a great help by comparing the production yield of using mixed culture and pure strain. It is recommended that the production is re-run on other types of fermentation such as continuous fermentation or in immobilized system. It is also suggested that the experiment is continued with the scale up bioreactor using the same species obtained from this study before continued with pilot scale bioreactor. Future research in the acetic acid extraction and purification from the study can be a good option. Membrane and chemical separation can be employed to develop a system that contains production and separation as one unit.

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APPENDIX A

GRAM – STAINING REAGENTS AND ANALYSIS METHOD

GRAM -STAINING REAGENTS

A.1: Crystal violet

Crystal violet 85% dye 2 g

95% ethyl alcohol 20 ml

Mix and dissolve.

Ammonium oxalate 0.8 g

Distilled water 80.0 ml

A.2: Gram's iodine solution

Iodine crystals 1 g

Potassium iodide 2 g

Distilled water 300 ml

Store in an amber bottle; discard when the color begins to fade.

A.3: Safranin solution

Safranin 2.5 g

95% ethyl alcohol 100.0 ml

For a working solution, dilute stock solution 1/10 (10 ml of stock safranin to 90 ml of distilled water).

A.4 Moisture content (oven method)

Overview of the oven-drying method: The primary oven-drying method (this is "Method A – Oven-Drying Primary" as detailed in ASTM D4442-07) is intended as the sole primary method and is structured for research purposes where the highest accuracy or degree of precision is needed and requires a specific oven type (i.e. a vented forced convection oven), closed weighing jars, and the performance of additional special procedures. The oven-drying method most practically suited for use in determining the moisture content of wood biomass that is typically or most commonly in the forest industry is often simply referred to by most people in forest industry as "the oven-drying method" (this is actually "Method B – Oven-Drying Secondary" and is also detailed in ASTM D4442-07 which provides detail regarding calibration and standardization details for both methods). This oven-drying method is appropriate for use with woody biomass samples regardless of moisture content, and although it takes some time to complete the test (usually about 24 hours, and possibly more), the time spent in dealing with each of the individual samples is minimal, and given a large drying oven, a fairly large number of samples can be handled at one time. For these reasons, the ovendrying method is the method that is most commonly used, and other methods (while also suitable) are typically compared to the results of the oven-drying method to ensure their accuracy.

The oven-drying method procedures: The oven being used must be capable of maintaining temperatures of 103°C +/- 2°C (or holding between 101°C to 105°C) near the drying endpoint (this is the same as holding between about 214°F and 221°F). The sensitivity of the balance (scale) that is being used to weigh the samples must be to within a minimum of 0.1% of the weight of the sample being tested (for example, if samples being tested are expected to be about 100 grams in weight when dry, the scale should be able to read to at least 0.1 gram (or a tenth of a gram) – this would be the minimum sensitivity allowable, but a somewhat greater sensitivity, such as a sensitivity of 0.01 gram (or a hundredth of a gram) would be preferred in this situation. Samples collected for moisture content determination are to be kept in individual vapor-tight containers if there is any delay between the collection of the sample and the initial weighing which will determine

the "green weight" that is the weight of wood and water combined. (Note: If the sample is placed, kept and weighed in a container, such as a small aluminum pan typically used to hold chips or sawdust in drying ovens, the weight of the empty pan needs to be known and subtracted from all numbers in the calculation, or else the balance needs to be tare weighted to eliminate the pan's weight from what is recorded with each weighing.) The following equation takes place:-

$$Moisture\ Content\ \%\ (Green\ basis) = \left(\frac{weight\ of\ water}{weight\ of\ water + dry\ weight\ of\ wood}\right) x\ 100$$

After initial weighing, the sample is placed in the oven and kept there until the endpoint has been reached and is then removed and reweighed as soon as possible. It is known that the endpoint is reached when there is no appreciable change in the final weight at approximately 4 hour intervals. The weight of the sample at endpoint is a direct measurement of the dry weight of wood in the sample, which is subtracted from the green weight of the sample (from the initial weighing) to calculate the weight of water. The weight of water is then divided by the green weight of the sample (from the initial weighing) to calculate the Green basis moisture content of the sample (round to the nearest 1%). In most cases the oven-drying method can be completed in about 24 hours of testing, assuming the materials contained in the samples being tested are not very large in size (e.g. in testing samples of chips and smaller material, these samples can typically be processed within a 24 hour period in a good forced convection oven that is properly vented and is not overloaded). If relatively large sizes of material are included as part of the samples being tested, or if a very large volume of high-moisture material is placed in the oven at one time, this can result in the samples requiring a longer time in the oven to reach endpoint. In using an oven in testing various types of samples over time, an operator who has appropriately tested for endpoints when running samples of various types and moistures (and with various loads in the oven and during various climatic conditions) could typically be expected to reasonably estimate the time to endpoint for the samples being tested relative to the conditions at hand. It should be obvious that it is important to get the samples

to endpoint in using the oven-drying method of moisture calculation or the recorded dry weight for the sample will be overstated (i.e. what is recorded as dry weight of wood will be too high as it will have a residual water fraction) and consequently the resulting moisture content calculation will then understate the moisture content (i.e. the calculated moisture content from the test will be less than the actual moisture content of the sample). In the circumstance where woody biomass is purchased on a dry ton basis, this failure to reach endpoint in drying of samples and resulting understatement of moisture content will overstate the dry tons of material actually received by the purchaser and will result in an overpayment to the supplier – consequently it is not an error a purchaser can afford to make in a significant way on a regular basis. For this reason it is important for the purchaser to use good procedures and to ensure the samples being tested reach endpoint.

A.5 Holocellulose analysis

The extracted sample from ethanol-toluene solubility was used for this analysis. The sample quantitatively was transferred to 250 ml beaker. 100 ml of water, 1.5 g of $NaClO_2$ and 5 ml of 10% acetic acid was added to the beaker. The beaker was then heated on a boiling water bath. 5 ml of acetic acid was added after 30 minutes reaction. After further 30 minutes (1 h after started), 1.5 g of $NaClO_2$ was added. The reaction was continued with alternate acetic acid and $NaClO_2$ additions at 30 minutes and intervals until 6 g $NaClO_2$ has been added. The solution was heated for 30 minutes after last addition of $NaClO_2$. The residue should be white in color and retain woody structure. The residue was cooled in an ice bath until it became crystallize. The entire residue was filtered using fritted glass crucible (porosity 1) and weighted. Then rinsed the residue with cool distilled water and finally with acetone. Let the residue dried by air.

Standard method of holocellulose analysis:-

1) Weigh 3 g of an extractive-free sample and place in a 300-mL flask.
2) Soak the flask in a 70°C water bath, and add 180 mL of 0.2% sodium acetate (pH 3.5).
3) Add 1.2 g of sodium chlorite and stir.

4) After 30 min, repeat Step 3.
5) After 45 min, filter with a preweighed glass filtering crucible
6) (30-mL, medium porosity), and wash with 600 mL of cold distilled water followed by 600 mL of acetone.
7) Dry under a high vacuum and weigh the residue.

Small-scale method of holocellulose analysis:-

1) Withdraw an extractive-free sample to the point where about 90 mg are left in the same glass filtering crucible as that used for extraction.
2) Wash with acetone and dry under a high vacuum to weigh.
3) Plug the crucible leg with a septum and wrap the joint with labosealing tape (3M, St. Paul, MN).
4) Soak the crucible in a 70°C water bath, and add 1.4 mL of 0.2M sodium acetate (pH 3.2).
5) Add 0.1–0.2 mL of 20% sodium chlorite and stir with a small glass rod.
6) After 30 min, repeat Step 5.
7) After 45 min, remove the septum in ice-cold distilled water, and soak the glass crucible for 5 min.
8) Squeeze and wash with ice-cold 1% acetic acid three times and with ice-cold acetone twice.
9) Dry under a high vacuum and weigh the residue.

1.1. TAPPI T203

Alpha-, beta- and gamma-cellulose in pulp (Reaffirmation of T 203 cm-99)

1. Scope

This method for determination of alpha-, beta- and gamma-cellulose can be applied to bleached or delignified pulps only. Unbleached and semi-bleached pulps must be delignified before testing.

2. Summary

Pulp is extracted consecutively with 17.5% and 9.45% sodium hydroxide solutions at 25°C. The soluble fraction, consisting of beta- and gamma-celluloses, is determined volumetrically by oxidation with potassium dichromate, and the alpha-cellulose, as an insoluble fraction, is derived by difference

3. Significance

3.1 Separation of the cellulose in pulp into alpha-, beta- and gamma-cellulose fractions is an empirical procedure, originally devised by Cross and Bevan (1) around 1900, and has been widely used to evaluate pulps for various purposes, such as aging characteristics and response to refining operations. In a modified form, the method was adopted first as a TAPPI tentative standard in 1931 (2).

3.2 In general, the alpha-cellulose indicates undegraded, higher-molecular-weight cellulose content in pulp; the beta-cellulose indicates that of a degraded cellulose, and the gamma-cellulose consists mainly of hemicellulose (3, 4).

4. Definitions

Alpha-cellulose is the pulp fraction resistant to 17.5% and 9.45% sodium hydroxide solution under conditions of the test. Beta-cellulose is the soluble fraction which is reprecipitated on acidification of the solution; gamma-cellulose is that fraction remaining in the solution.

5. Apparatus

5.1 Pulp dispersion apparatus consisting of a variable speed motor and a stainless steel stirrer with a shell. The speed of the motor and the angle of the blades should be adjusted so that no air is drawn into the pulp suspension during stirring.

5.2 Constant temperature bath, to maintain a temperature of 25°C ± 0.2°C.

5.3 Timer, stop watch or electric timer.

5.4 Filtering funnel or crucible, 50 or 100 mL, with a fritted glass disk of coarse (40 to 60 mm) porosity.

5.5 Other glassware: beakers, tall-form, 300-mL; pipets, 10, 25, 50, and 75 mL; buret 50-mL; flasks, 250- and 300-mL; filtering flasks, 250-mL; graduated cylinders, 25-, 50-, and 100-mL; glass stirring rods.

6. Reagents

6.1 Sodium hydroxide solution, 17.5% NaOH by weight, 5.21 ± 0.005N, carbonate free. Prepare a concentrated (about 50%) NaOH solution and let stand until the suspended carbonates have settled. Then decant the clear solution, dilute with CO2-free (recently boiled) distilled water, and adjust the normality as specified to 5.21 ± 0.005N.

6.2 Potassium dichromate solution, 0.5N. Dissolve 24.52 g of K2Cr2O7 in water and dilute to 1000 mL.

6.3 Ferrous ammonium sulfate solution, 0.1N. Dissolve 40.5 g of Fe(NH4)2 (SO4)2 • 6H2O in water, add 10 mL of concentrated H2SO4, and dilute to 1000 mL. The solution is not stable and the exact normality should be determined daily by titration with 0.100N potassium dichromate standard solution.

6.4 Phenanthroline - ferrous sulfate. Dissolve 1.5 g of 1, 10-phenanthroline monohydrate, C12H8N2 • H2O and 0.7 g of FeSO4 • 7H2O in 100 mL of water. The indicator solution is also available commercially as "Ferroin."

6.5 Sulfuric acid, concentrated H2SO4, 96 to 98%, sp gr 1.84.

6.6 Sulfuric acid, 3N. Add 83.5 mL of concentrated H2SO4 to an excess of water and dilute to 1000 mL.

7. Sampling

7.1 Obtain a representative sample of bleached pulp equivalent to about 5 g oven-dry in accordance with the sampling section of TAPPI T 210 "Weighing, Sampling, and Testing Pulps for Moisture" or another appropriate sampling procedure.

7.1.1 If the sample is a pulp sheet, split it in layers and tear by hand in small pieces about 10 mm across. Do not cut or shred the pulp by mechanical devices.

7.1.2 If the sample is a slush pulp, remove water by filtering and by pressing between blotters. Tear the pulp in small pieces and dry thoroughly in air or in an oven at a temperature not higher than 60°C.

8. Test specimen

Allow the sample to come to moisture equilibrium in the atmosphere near the balance, and weigh out two test specimens of 1.5 ± 0.1 g to the nearest 0.1 mg. At the same time weigh out another specimen for moisture determination. See TAPPI T 550 "Determination of Equilibrium Moisture in Paper and Paperboard for Chemical Analysis."

9. Procedure

9.1 Place the test specimen in a 300-mL tall-form beaker and add 75.0 mL of 17.5% NaOH reagent, adjusted previously to 25° ± 0.2°C. Note the time at which the reagent is added.

9.2 Stir the pulp with the apparatus until it is completely dispersed. Avoid drawing air into the pulp suspension during stirring.

NOTE 1: Some pulps can be dispersed readily by stirring and macerating with a glass rod; however, complete dispersion is essential and too high an alpha-cellulose value will be obtained if pulp dispersion is not complete.

9.3 When the pulp is dispersed, raise the stirrer and remove the adhered pulp fibers with a pointed glass rod. Rinse the stirrer with 25.0 mL of 17.5% NaOH reagent, adding it to the beaker, so that exactly 100.0 mL of the reagent have been added to the pulp. Stir the pulp suspension with a rod and place in a bath at 25° ± 0.2°C.

9.4 After a period of 30 min from the first addition of the NaOH reagent, add 100.0 mL of distilled water at 25° ± 0.2°C to the pulp suspension and stir thoroughly with a rod.

9.5 Leave the beaker in the bath for another period of 30 min so that the total extraction time is 60 ± 5 min.

9.6 At the end of the 60-min period, stir the pulp suspension with a rod and transfer to a filtering funnel. Discard the first 10 to 20 mL of the filtrate, then collect about 100 mL of the filtrate in a clean and dry filtration flask.

CAUTION: Do not rinse or wash the pulp with water and do not draw air through the pulp on the filter.

9.7 Alpha-cellulose determination

9.7.1 Pipet 25.0 mL of the filtrate and 10.0 mL of 0.5N potassium dichromate solution into a 250-mL flask. Add cautiously, while swirling the flask, 50 mL of concentrated H_2SO_4 (see Note 3).

9.7.2 Allow the solution to remain hot for 15 min, then add 50 mL of water and cool to room temperature. Add 2 to 4 drops of Ferroin indicator and titrate with 0.1N ferrous ammonium sulfate solution to a purple color.

NOTE 2: If available, an electrometric apparatus such as an automatic titrator may be used instead of the indicator solution to determine the endpoint of the titration, applying standard techniques used with such equipment.

NOTE 3: If the solubility of a pulp is high (alpha-cellulose content low) and the back-titration of dichromate takes less than 10 mL, reduce the volume of the pulp filtrate to 10 mL and that of the sulfuric acid to 30 mL.

9.7.3 Make a blank titration substituting the pulp filtrate with 12.5 mL of 17.5% NaOH and 12.5 mL of water.

9.8 Beta- and gamma-cellulose determination.

9.8.1 Pipet 50.0 mL of the pulp filtrate into a 100-mL graduated cylinder having a ground glass stopper. Add 50.0 mL of 3N H_2SO_4 and mix thoroughly by inverting.

9.8.2 Heat the cylinder submerged in a hot water bath at about 70°-90°C for a few minutes to coagulate the beta-cellulose. Allow the precipitate to settle for several hours, preferably overnight, then decant or filter, if necessary, to obtain a clear solution.

NOTE 4: A centrifuge may be used for a rapid separation of the beta-cellulose after precipitation.

9.8.3 Pipet 50.0 mL of the clear solution and 10.0 mL of 0.5N $K_2Cr_2O_7$ into a 300-mL flask and add cautiously 90 mL of concentrated H_2SO_4. Allow the solution to remain hot for 15 min, then proceed with titration as outlined in 9.7.2.

9.8.4 Make a blank titration substituting the solution with 12.5 mL of 17.5% NaOH, 12.5 mL of water and 25 mL of 3N H2SO4.

10. Calculations

10.1 Calculate the alpha-cellulose content in pulp:

$$Alpha - cellulose, \% = 100 - \frac{6.85\,(V2 - V1) \times N \times 20}{A\,x\,W}$$

where:

V1 = titration of the pulp filtrate, mL

V2 = blank titration, mL

N = exact normality of the ferrous ammonium sulfate solution

A = volume of the pulp filtrate used in the oxidation, mL

W = oven-dry weight of pulp specimen, g

NOTE 5: Theoretically, 1 milliequivalent of K2Cr2O7 corresponds to 6.75 mg of cellulose and other hexosans, and 6.60 mg of pentosans. Under conditions of the test procedure, less oxidant is consumed, and 1 milliequivalent has been found to correspond to 6.85 mg of cellulose and other dissolved carbohydrates.

10.2 Calculate the gamma-cellulose content in pulp:

$$Gamma\ cellulose, \% = \frac{[6.85\,(V4 - V3) \times N \times 20]}{[25 \times W]}$$

where:

V3 = titration of the solution after precipitation of beta-cellulose, mL

V4 = blank titration, mL

10.3 Calculate the beta-cellulose content in pulp:

$$Beta - cellulose, \% = 100\ B\ (alpha - cellulose\ \% + gamma - cellulose\ \%)$$

NOTE 6: In the first oxidation, the total dissolved fraction (beta-cellulose plus gamma-cellulose) is determined, and the alpha-cellulose is calculated as an undissolved fraction by difference between the total pulp specimen (100%) and the dissolved fraction in per cent. In the second oxidation, the gamma-cellulose only is determined, and the beta-cellulose is found by difference between the first and the second oxidations.

11. Report

Report the percentages of alpha-, beta, and gamma-cellulose content, as an average of two determinations, to the
nearest 0.1%.

12. Precision

12.1 Repeatability of the alpha-cellulose = 0.4%; reproducibility of the alpha-cellulose = 1.3%; comparability = not known; in accordance with the definition of these terms in T 1200 "Interlaboratory evaluation of test methods to determine TAPPI repeatability and reproducibility." These values are based on an interlaboratory study conducted by five laboratories on four bleached pulp samples with alpha-cellulose content from 83.8% to 96.8%.

12.2 Repeatability of the beta-cellulose = 9.0%; repeatability of the gamma-cellulose = 8.5%, as found in one laboratory; reproducibility and comparability = not known.

13. Keywords

Pulp, Cellulose, Alpha cellulose, Beta cellulose, Gamma cellulose, Bleached pulps, Hemicellulose

14. Additional information

14.1 Effective date of issue: to be assigned.

14.2 Related method: CPPA G.29; TAPPI Useful Method 249 “Delignification of Unbleached Pulp (Chlorine Gas Method)” can also be used for delignification of unbleached pulps.

14.3 This method was reclassified as Classical by committee action in 1999.

Literature cited

1. Cross, C.F., and Bevan, E.J., “Researches on Cellulose” Vol. III, Longmans, Green and Co., London, 1912.

2. Willets, W. R., Determination of Alpha Cellulose,≅ Tech. Assoc. Papers XIV: 121 (1931).

3. Ranby, B. G., The Physical Characteristics of Alpha-, Beta- and Gamma-Cellulose,≅ Svensk Papperstid. 55(4):115 (1952).

4. Wilson, K., Ringstrom, E., and Hedlund, I., The Alkali Solubility of Pulp,≅ Svensk Papperstid. 55(2):31 (1955).

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Bray, M.W., and Andrews, T.M., An Improved Method for the Determination of Alpha-, Beta and Gamma-Cellulose,≅ Ind. and Eng. Chemistry 15(4):377 (1923).

Launer, H.F., Simplified Volumetric Determination of Alpha, Beta and Gamma Cellulose in Pulps and Papers,≅ J. of Res. N.B.S. 18:333 (1937).

Tyden, H., The Determination of Alpha-, Beta- and Gamma-Cellulose≅ (Swedish), Svensk Papperstid. 43(12):221 (1940); B.I.P.C. 11(2):45 (1940).

A.6 Klason-lignin determination

Determination of Acid-Insoluble Lignin in Biomass

Laboratory Analytical Procedure #003

1. Introduction

1.1 Biomass is composed largely of cellulose, a polymer of glucose; hemicellulose, a complex polymer of which the main chain consists primarily of xylans or glucomannans; and lignin, a complex phenolic polymer. Unlike the other cell wall components of biomass, the lignin is mostly insoluble in mineral acids. For this reason, lignin can be analyzed gravimetrically after hydrolyzing the cellulose and hemicellulose fractions with sulfuric acid.

1.2 This method contains two different procedures for determining acid-insoluble lignin. Both approaches have been shown to give equivalent results. Procedure A presents an approach where the acid-insoluble lignin procedure also generates the solutions required for total carbohydrate and acid-soluble lignin determinations, thereby making possible the "summative" analysis of the same sample. Procedure B is a modification of the classic "Klason lignin" determination. Although the filtrate generated from this procedure can be used to determine acid-soluble lignin, total carbohydrates should be determined on a completely separate sample.

1.3 This procedure has been adopted by ASTM as an ASTM Standard Test Method.

2. Scope

2.1 This test method covers the determination of acid-insoluble lignin of hard and softwoods, herbaceous materials (such as switchgrass and sericea), agricultural residues (such as corn stover, wheat straw, and bagasse), wastepaper (such as office waste, boxboard, and newsprint), acid and alkaline pretreated biomass, and the solid fraction of fermentation residues. All results are reported relative to the 105°C oven-dried weight of the sample. In the case of extracted materials, the results may also be reported on an extractives-free basis.

2.2 The residue collected contains the acid-insoluble lignin and any condensed proteins from the original sample. An independent nitrogen analysis would be required to determine the acid-insoluble lignin content separate from the condensed protein fraction and is outside the scope of this procedure.

2.3 All analyses shall be performed according to the guidelines established in the Ethanol Project Quality Assurance Plan (QAP).

3. References

3.1 Ehrman, C.I., and M.E. Himmel. 1994. "Simultaneous Saccharification and Fermentation of Pretreated Biomass: Improving Mass Balance Closure." Biotechnology Techniques, 8(2):99-104.

3.2 Moore, W.E., and D.B. Johnson. 1967. Procedures for the Chemical Analysis of Wood and Wood Products. Madison, WI: U.S. Forest Products Laboratory, U.S. Department of Agriculture.

3.3 NREL CAT Task Laboratory Analytical Procedure #001, "Standard Method for the Determination of Total Solids in Biomass".

3.4 NREL CAT Task Laboratory Analytical Procedure #002, "Two Stage Sulfuric Acid Hydrolysis for Determination of Carbohydrates".

3.5 NREL CAT Task Laboratory Analytical Procedure #004, "Determination of AcidSoluble Lignin in Biomass".

3.6 NREL CAT Task Laboratory Analytical Procedure #010, "Standard Method for the Determination of Extractives in Biomass".

3.7 TAPPI Test Method T222 om-88, "Acid-Insoluble Lignin in Wood and Pulp." In Tappi Test Methods. Atlanta, GA: Technical Association of the Pulp and Paper Industry.

3.8 TAPPI Test Method T264 om-88, "Preparation of Wood For Chemical Analysis." In Tappi Test Methods. Atlanta, GA: Technical Association of the Pulp and Paper Industry.

3.9 Vinzant, T.B., L. Ponfick, N.J. Nagle, C.I. Ehrman, J.B. Reynolds, and M.E. Himmel. 1994. "SSF Comparison of Selected Woods From Southern Sawmills." Appl. Biochem. Biotechnol. 45/46:611-626.

4. Terminology

Acid-insoluble lignin is defined to be the residue, corrected for acid-insoluble ash, retained on a medium porosity filter crucible after the primary 72% and secondary 4% H_2SO_4 hydrolysis steps described in this procedure.

5. Significance and Use

The acid-insoluble lignin content is used in conjunction with other assays to determine the total composition of biomass samples.

6. Interferences

6.1 The results of acid-insoluble lignin analysis are affected by incomplete hydrolysis of biomass. Unless the sample is hydrolyzed completely, the results will be biased high. Take care to mix the acid/biomass slurry thoroughly at the beginning and periodically throughout the concentrated acid hydrolysis.

6.2 The results of acid-insoluble lignin analysis are affected by the timing of the acid digestion steps. The insoluble lignin will slowly dissolve into solution in an irreproducible fashion. The timing within this procedure must be followed closely.

6.3 Some proteinaceous materials can also form acid-insoluble substances that are collected with acid-insoluble lignin.

7. Apparatus

7.1 Analytical balance readable to 0.1 mg.

7.2 Convection oven with temperature control of 105 ± 3°C.

7.3 Muffle furnace: an electric furnace is recommended for igniting the sample. The furnace should be fitted with an indicating pyrometer or thermocouple, so that the required temperature of 575 ± 25°C can be maintained.

7.4 Autoclave capable of maintaining 121 ± 3°C (Procedure A) or heating manifold equipped with reflux condensers with 24/40 ground glass joints (Procedure B).

7.5 Water bath set at 30 ± 1°C (Procedure A).

7.6 Filtration set-up including vacuum source and vacuum adapters for crucibles.

7.7 Desiccator containing anhydrous calcium sulfate.

8. Reagents and Materials

8.1 Reagents

8.1.1 72% w/w H_2SO_4 (12.00 ± 0.02 M or specific gravity 1.6389 at 15.6°C/15.6°C).

8.1.2 Water, 18 megohm deionized.

8.2 Materials

8.2.1 Glass test tubes, 16x100 mm (Procedure A) or 20x150 mm (ProcedureB).

8.2.2 125 mL glass serum bottles, crimp top style, with rubber stoppers and aluminum seals to fit (Procedure A).

8.2.3 Erlenmeyer flask, 1000 mL, with 24/40 ground glass joint (Procedure B).

8.2.4 Filtration flask, 250 mL (Procedure A) or 1000 mL (Procedure B).

8.2.5 30 mL (Procedure A) or 50 mL (Procedure B) glass filtering crucible, medium porosity, nominal maximum pore size of 10 mm.

9. ES&H Considerations and Hazards

9.1 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

9.2 72% H2SO4 is very corrosive and must be handled carefully.

9.3 Use caution when handling glass bottles after the autoclave step in Procedure A, as they may have become pressurized.

10. Sampling, Test Specimens, and Test Units

10.1 Test specimens suitable for analysis by this procedure are as follows:

- biomass feedstocks, dried and reduced in particle size if necessary,
- pretreated biomass, washed free of any residual acid or alkali,
- the solids fraction of fermentation residues.

10.2 The sample must not contain particles larger than 1 mm in diameter. If milling is required to reduce the particle size of the test specimen, a laboratory mill equipped with a 40 mesh (or smaller) screen should be used.

10.3 The total solids content of the "as received" test specimen (prior to any drying or extraction steps) must be determined by Laboratory Analytical Procedure #001, "Determination of Total Solids in Biomass", in parallel with the lignin analysis. Record this value as Tas received.

10.4 Material with a total solids content less than 85%, on a 105°C dry weight basis, will require drying by lyophilization, oven drying, or air drying prior to milling or analysis. The amount of moisture lost as a result of the preparation procedure must be determined. This moisture content is used to calculate the total solids content of the sample based on its preparation and is recorded as T_{prep}. This value is used to correct the weight of the prepped material used in the lignin analysis, as described in the calculations section. The prepared

sample should be stored in a manner to ensure its moisture content does not change prior to analysis.

Note: Preparing samples for analysis by oven drying can produce hard chunks of material. This material must then be milled to reduce the size of the large pieces to less then 1 mm in diameter. The sample is then redried prior to testing.

10.4 Some samples may require extraction prior to analysis, to remove components that may interfere with the analysis. Laboratory Analytical Procedure #010, "Standard Method for the Determination of Extractives in Biomass", is used to prepare extractives-free sample with a moisture content suitable for lignin analysis. As part of this procedure, the percent extractives in the prepared sample, on a 105°C dry weight basis, is determined. This value, recorded as % extractives, is used to convert the % lignin reported on a extractives-free basis to an as received (whole sample) basis.

10.5 The test specimen shall consist of approximately 0.3 g of sample for Procedure A or approximately 1.0 g of sample for Procedure B. The test specimen shall be obtained in such a manner to ensure that it is representative of the entire lot of material being tested.

11. Procedure A - Summative Analysis

11.1 This procedure is suitable for air-dried, lyophilized, and extracted biomass samples, as well as for samples that have been oven dried at a temperature of 45°C or less. It is not suitable for samples that have been dried at a temperature exceeding 45°C.

Note: The total solids content of the original sample, Tas received, as well as the total solids content determined as the sample is prepared, Tprep, must be known.

11.2 Individually label the crucibles needed for analysis, and ignite them at 575 " 25°C to achieve a constant weight of " 0.3 mg. Store the ignited crucibles in a desiccator until needed.

Note: In order to determine the absolute amounts of acid-insoluble residue and acid-insoluble ash, for quality control purposes, it is useful to weigh and record the ignited crucible to the nearest 0.1 mg.

11.3 Weigh 0.3 ± 0.01 g prepared sample to the nearest 0.1 mg and place in a 16x100 mm test tube. Record as W1, the initial sample weight. Each sample must be run in duplicate, at minimum.

11.4 Samples for total solids determination (LAP-001) must be weighed out at the same time as the samples for the acid-insoluble lignin determination. If this is done later, it can introduce an error in the calculation because ground biomass can rapidly gain or lose moisture when exposed to the atmosphere. Record the average total solids value as T_{final}.

11.5 Add 3.00 ± 0.01 mL (4.92 ± 0.01 g) of 72% H2SO4 and use a glass stirring rod to mix for 1 minute, or until the sample is thoroughly wetted.

11.6 Place the test tube in the water bath controlled to 30 ± 1°C and hydrolyze for 2 hours.

11.7 Stir the sample every 15 minutes to assure complete mixing and wetting.

11.8 Transfer the hydrolyzate to a glass bottle and dilute to a 4% acid concentration by adding 84.00 ± 0.04 mL water, or by bringing the combined weight of sample, acid, and water up to 89.22 ± 0.04 g. Be careful to transfer all the residual solids along with the hydrolysis liquor.

11.9 Stopper each of the bottles and crimp aluminum seals into place.

11.10 Set the autoclave to a liquid vent cycle to prevent loss of sample from the bottle in the event of a loose crimp seal. Autoclave the samples in their sealed bottles for 1 hour at 121 ± 3°C.

11.11 After completion of the autoclave cycle, allow the samples to cool for about 20 minutes at room temperature before removing the seals and stoppers.

11.12 Vacuum filter the hydrolysis solution through one of the previously ignited filtering crucibles.

11.13 If a carbohydrate analysis (LAP-002) and/or an acid-soluble lignin analysis (LAP-004) is desired, decant 15-25 mL of filtrate into a resealable container. If this aliquot is not used immediately for further analysis, store in refrigerator at 4°C.

Note: Acid-soluble lignin should be analyzed within 24 hours, preferably within 6 hours of hydrolysis.

11.14 Use hot deionized water to wash any particles clinging to the glass bottle into the crucible and to wash the filtered residue free of acid using vacuum filtration.
11.15 Dry the crucible and contents at 105 ± 3°C for 2 hours or until constant weight is achieved ("0.3 mg upon reheating).
11.16 Cool in desiccator and record the weight, W2, the weight of the crucible, acid-insoluble
lignin, and acid-insoluble ash to the nearest 0.1 mg.
11.17 Place the crucible and contents in the muffle furnace and ignite at 575 ± 25°C for a minimum of 3 hours, or until all the carbon is eliminated. Heat at a rate of 10°C/min to avoid flaming. If the sample tends to flare up, the container should be partially covered during this step. Avoid heating above the maximum stated temperature. Protect the test container from strong drafts at all times to avoid mechanical loss of the test specimen.
11.18 Cool in desiccator and record the weight, W3, the weight of the crucible and acidinsoluble ash, to the nearest 0.1 mg.

Note: The amount of acid-insoluble ash remaining in the crucible is not equal to the total ash content of the sample. Refer to Laboratory Analytical Procedure #005 if total ash is to be determined.

12. Procedure B - Klason Lignin Determination

12.1 This procedure is suitable for oven-dried samples (including those dried at temperatures between 45°C and 105°C) as well as air-dried, lyophilized, and extracted biomass samples.

Note: The total solids content of the original sample, T_{as} received, as well as the total solids content determined as the sample is prepared, T_{prep}, must be known.

12.2 Individually label the crucibles needed for analysis, and ignite them at 575 ± 25°C to achieve a constant weight of ± 0.3 mg. Store the ignited crucibles in a desiccator until needed.

Note: In order to determine the absolute amounts of acid-insoluble residue and acid-insoluble ash, for quality control purposes, it is useful to weigh and record the ignited crucible to the nearest 0.1 mg.

12.3 Weigh 1.0 ± 0.05 g prepared sample to the nearest 0.1 mg and place in a 20x150 mm test tube. Record as W_1, the initial sample weight. Each sample must be run in duplicate, at minimum.

12.4 Samples for total solids determination must be weighed out at the same time as the samples for the acid-insoluble lignin determination. If this is done later, it can introduce an error in the calculation because ground biomass can rapidly gain or lose moisture when exposed to the atmosphere. Record the average total solids value as T_{final}.

12.5 Add 15.00 ± 0.02 mL of 72% H2SO4, chilled to 4°C in the refrigerator. Use a glass stirring rod to mix for 1 minute, or until the sample is thoroughly wetted.

12.6 Hydrolyze the sample for 2 hours at room temperature (approximately 20°C), stirring every 15 minutes to assure complete mixing and wetting.

12.7 Transfer the hydrolyzate to a 1000 mL Erlenmeyer flask and dilute to a 3% acid concentration with 560 mL of deionized water. Be careful to transfer all the residual solids along with the hydrolysis liquid.

12.8 Place the flask on the heating manifold and attach to the reflux condenser. Heat the liquid to a gentle boil. Start timing at the onset of boiling, and reflux for 4 hours ± 5 minutes.

12.9 At the end of 4 hours, rinse the condenser with a small amount of deionized water before disassembling reflux apparatus.

12.10 Vacuum filter the hydrolysis solution through one of the previously ignited filtering crucibles.

12.11 If an acid-soluble lignin determination (LAP-004) is to be run, record the weight of the collected filtrate. Decant 15-25 mL of filtrate into a resealable container. If this aliquot is not used immediately for further analysis, store in refrigerator at 4°C.

Note: Acid-soluble lignin should be analyzed within 24 hours, preferably within 6 hours of hydrolysis.

12.12 Use hot deionized water to wash any particles clinging to the glass bottle into the crucible and to wash the filtered residue free of acid using vacuum filtration.
12.13 Dry the crucible and contents at 105 ± 3°C for 2 hours or until constant weight is achieved (±0.3 mg upon reheating).
12.14 Cool in desiccator and record the weight, W2, the weight of the crucible, acid-insoluble lignin, and acid-insoluble ash to the nearest 0.1 mg.
12.15 Place the crucible and contents in the muffle furnace and ignite at 575 ± 25°C for a minimum of 3 hours, or until all the carbon is eliminated. Heat at a rate of 10°C/min to avoid flaming. If the sample tends to flare up, the container should be partially covered during this step. Avoid heating above the maximum stated temperature. Protect the test container from strong drafts at all times to avoid mechanical loss of the test specimen.
12.16 Cool in desiccator and record the weight, W3, the weight of the crucible and acidinsoluble ash, to the nearest 0.1 mg.

Note: The amount of acid-insoluble ash remaining in the crucible is not equal to the total ash content of the sample. Refer to Laboratory Analytical Procedure #005 if total ash is to be determined.

13. Calculations

13.1 For lyophilized, air dried, or oven dried samples, or samples requiring no preparation, calculate % acid-insoluble lignin on an as received 105°C dry weight basis as follows:

$$\%\ acid\ -\ insoluble\ lignin = \frac{W_2 - W_3}{W_1 x \frac{T_{asreceived}}{T_{prep}}} x\ 100\% = \frac{W_2 - W_3}{W_1 x \frac{T_{final}}{100}\%} x\ 100\%$$

Where:

W1 = initial sample weight.

W2 = weight of crucible, acid-insoluble lignin, and acid-insoluble ash.

W3 = weight of crucible and acid-insoluble ash.

T_{as} received = % total solids content of the original sample (prior to any preparation steps) on a 105°C dry weight basis, as determined by the LAP-001.

T_{prep} = % total solids content of the sample as determined during the preparation of the sample for analysis (by lyophilization, oven-drying, or air drying).

T_{final} = % total solids content of the prepared sample used in this lignin analysis, on a 105°C dry weight basis, as determined by the LAP-001.

Note: If the sample used in the acid-insoluble lignin analysis required no preparation (analyzed as received), then T_{prep} = 100% and T_{final} = T_{as} received. If a sample was prepared by drying at 105°C prior to analysis, then T_{prep} = T_{as} received and T_{final} = 100%.

13.2 For an extracted sample, it may be necessary to report the results on an extractives-free 105°C dry weight basis or on an as received (whole sample) 105°C dry weight basis, or both.

13.2.1 Calculate % acid-insoluble residue on an extractives-free basis as follows:

$$\%\ acid\ -\ insoluble\ residue_{extractives-free} = \frac{W_2 - W_3}{W_1 x \frac{\%T_{final}}{100\%}} x\ 100\%$$

Where:

W1 = initial weight of extracted sample

W2 = weight of crucible, acid-insoluble residue, acid-insoluble ash

W3 = weight of crucible and acid-insoluble ash%

T_{final} = % total solids of the extracted sample determined at 105°C as described by the Standard Method for the Determination of Total Solids in Biomass.

13.2.2 Correct the acid-insoluble residue value calculated above on an extractivesfree basis, to an as received (whole sample) 105°C dry weight basis:

$$\%\ acid\text{-}insoluble\ residue_{whole\ sample} = \%\ AIR_{extractives\text{-}free}\ x\ \frac{(100\% - \%\ extractives)}{100\%}$$

Where:

$\%\ AI_{Rextractives\text{-}free}$ = % acid-insoluble residue on an extractives-free 105°C dry weight basis, as determined in the previous step

% extractives = % extractives in the sample extracted as described in the Standard Method for the Determination of Extractives in Biomass.

14. Report

14.1 Report the percent acid-insoluble lignin, to two decimal places, on a 105°C dry weight basis, and cite the reporting basis.

14.2 For replicate analyses of the same sample, report the average, standard deviation, and relative percent difference (RPD).

15. Precision and Bias

15.1 Data obtained by replicate testing of a hybrid poplar in one laboratory gave a standard deviation in Klason lignin content of 0.32% and a CV% of 1.26%.

15.2 Data obtained by replicate testing of a hybrid poplar sample in six different laboratories gave a standard deviation of 2.37% and a CV% of 9.92%.

16. Quality Control

16.1 Reported significant figures: The acid-insoluble lignin results will be reported with two decimal places, on a 105°C dry weight basis.

16.2 Replicates: All samples and all method verification standards are to be analyzed in duplicate, at minimum.

16.3 Blank: A blank crucible is to be run through the analysis. The dish is to be weighed empty, ashed and reweighed. The difference in weight must be less than the equivalent of a 0.5% error.

16.4 Relative percent difference criteria: The RPD must be less than 3.4%. If the RPD is too large, the sample will be rerun.

16.5 Method verification standard: A method verification standard must be run in duplicate with every batch.

16.6 Calibration verification standard: Not applicable.

16.7 Sample size: Approximately 0.6 grams of sample is required for conducting duplicate analyses by Procedure A. Two grams will be required for Procedure B. If there is insufficient sample, the result will be flagged and the lack of precision will be noted.

16.8 Sample storage: Wet samples, prior to preparation, must be stored in the refrigerator. Samples that have been prepped by extraction, lyophilization, or oven drying must be stored in tightly sealed containers or in a desiccator.

16.9 Standard storage: Not applicable.

16.10 Standard preparation: Not applicable.

16.11 Definition of a batch: Any number of samples which are analyzed together and recorded together. Samples within a batch must be of the same matrix. The maximum size of a batch would be limited by the equipment constraints. A batch cannot be larger than what is practical with the equipment.

16.12 Control charts: The result of each replicate analysis of the method verification standard is recorded along with the average, RPD, and a laboratory book/page reference. The average value obtained for each analysis of the method verification standard is to be control charted.

A.7 Acid Hydrolysis

GLUCOSE ASSAY BY DINITROSALICYLIC COLORIMETRIC METHOD

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Method

This method tests for the presence of free carbonyl group (C=O), the so-called reducing sugars. This involves the oxidation of the aldehyde functional group present in, for example, glucose and the ketone functional group in fructose. Simultaneously, 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino,5-nitrosalicylic acid under alkaline conditions:

```
                          oxidation
aldehyde group           ----------> carboxyl group

                          reduction
3,5-dinitrosalicylic acid ----------> 3-amino,5-nitrosalicylic acid
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Because dissolved oxygen can interfere with glucose oxidation, sulfite, which itself is not necessary for the color reaction, is added in the reagent to absorb the dissolved oxygen.

The above reaction scheme shows that one mole of sugar will react with one mole of 3,5-dinitrosalicylic acid. However, it is suspected that there are many side reactions, and the actual reaction stoichiometry is more complicated than that previously described. The type of side reaction depends on the exact nature of the reducing sugars. Different reducing sugars generally yield different color intensities; thus, it is necessary to calibrate for each sugar. In addition to the oxidation of the carbonyl groups in the sugar, other side reactions such as the decomposition of sugar also competes for the availability of 3,5-dinitrosalicylic acid. As a consequence, carboxymethyl cellulose can affect the calibration curve by enhancing the intensity of the developed color.

Although this is a convenient and relatively inexpensive method, due to the relatively low specificity, one must run blanks diligently if the colorimetric results are to be interpreted correctly and accurately. One can determine the background absorption on the original cellulose substrate solution by adding cellulase, immediately stopping the reaction, and

measuring the absorbance, i.e. following exactly the same procedures for the actual samples. When the effects of extraneous compounds are not known, one can effectively include a so-called internal standard by first fully developing the color for the unknown sample; then, a known amount of sugar is added to this sample. The increase in the absorbance upon the second color development is equivalent to the incremental amount of sugar added.

List of Reagents and Instruments

A. Equipment

Test tubes
Pipets
Spectrophotometer

B. Reagents

Dinitrosalicylic Acid Reagent Solution, 1%
Dinitrosalicylic acid: 10 g
Phenol: 2 g (optional, see Note 1)
Sodium sulfite: 0.5 g
Sodium hydroxide: 10 g
Add water to: 1 liter
Potassium sodium tartrate solution, 40%

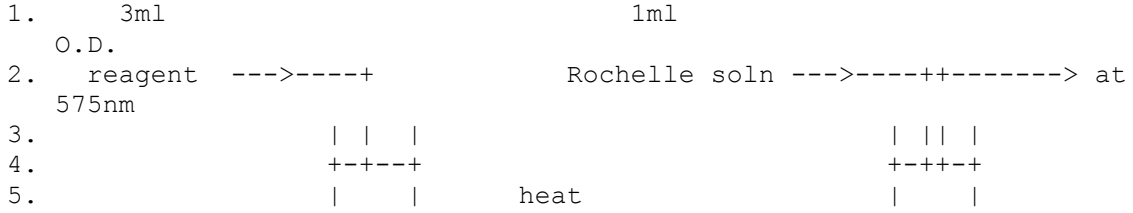
Procedures

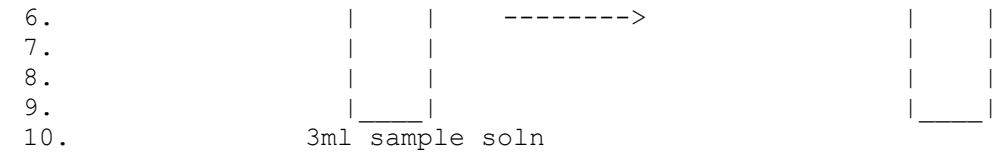
Add 3 ml of DNS reagent to 3 ml of glucose sample in a lightly capped test tube. (To avoid the loss of liquid due to evaporation, cover the test tube with a piece of paraffin film if a plain test tube is used.)

Heat the mixture at 90º C for 5-15 minutes to develop the red-brown color.

Add 1 ml of a 40% potassium sodium tartrate (Rochelle salt) solution to stabilize the color.

After cooling to room temperature in a cold water bath, record the absorbance with a spectrophotometer at 575 nm.





Notes

Phenol, up to 2g/l, intensifies the color density. It changes the slope of the calibration curve of absorbance versus glucose concentration but does not affect the linearity. The above procedure yields an absorbance of 1 for 1 g/l of glucose in the original sample in the absence of phenol in the reagent, as opposed to an absorbance of 2.5 for 1 g/l of glucose in 2 g/l of phenol. This property can be exploited to achieve the maximum sensitivity for dilute samples.

References

Miller, G.L., Use of dinitrosalicylic acid reagent for determination of reducing sugar, Anal. Chem., 31, 426, 1959.

APPENDIX B

RECIPE OF CULTURE MEDIA AND BIOLOG METHOD

APPENDIX B

Recipe of Culture Media and Biolog Method

B.1: Recipe of nutrient agar (pH 7.0)

Peptone	5.0 g
Beef extract	3.0 g
Agar	15.0 g
Distilled water	1,000.0ml

B.2: Recipe of nutrient broth (pH 7.0)

Peptone	5.0 g
Beef extract	3.0 g
Distilled water	1,000.0ml

B.3: Recipe of BSW Medium

BSW	20.0g

Water	1000 ml

B.4 Recipe of Artificial Glucose Medium

Peptone	5g
Meat Extract	3g
Artificial Glucose	5g
KH_2PO_4	2g
$MgCl_2$	0.5g
$NH_4Cl_2H_20$	0.5g
Water	1,000 ml

INSTRUCTIONS FOR USE OF THE BIOLOG GEN IIIMICROPLATE™

Intended Use

The GEN IIIMicroPlate™ test panel provides a standardized micromethod using 94 biochemical tests to

profile and identify a broad range of Gram-negative and Gram-positive bacteria

1,2

. Biolog's Microbial

Identification Systems software (e.g.OmniLog

®

Data Collection) is used to identify the bacterium from its

phenotypic pattern in the GEN IIIMicroPlate.

Description

ƒ The Biolog GEN III MicroPlate analyzes a microorganism in 94 phenotypic tests: 71 carbon source

utilization assays (Figure 1, columns 1-9) and 23 chemical sensitivity assays (Figure 1, columns

10-12). The test panel provides a "Phenotypic Fingerprint" of the microorganism that can be

used to identify it at the species level.

ƒ All necessary nutrients and biochemicals are prefilled and dried into the 96 wells of the

MicroPlate. Tetrazolium redox dyes are used to colorimetrically indicate utilization of the carbon

sources or resistance to inhibitory chemicals.

ƒ Testing is performed very simply, as shown in Figure 2. The isolate to be identified is grown on

agar medium and then suspended in a special "gelling" inoculating fluid

(IF) at the recommended

cell density. Then the cell suspension is inoculated into the GEN IIIMicroPlate, 100 µl per well, and

the MicroPlate is incubated to allow the phenotypicfingerprint to form. All of the wells start out

colorless when inoculated. During incubation there is increased respiration in the wells where cells

can utilize a carbon source and/or grow. Increased respiration causes reduction of the tetrazolium

redox dye, forming a purple color. Negative wellsremain colorless, as does the negative control

well (A-1) with no carbon source. There is also a positive control well (A-10) used as a reference for

the chemical sensitivity assays in columns 10-12. After incubation, the phenotypic fingerprint of

purple wells is compared to Biolog's extensive species library. If a match is found, a species level

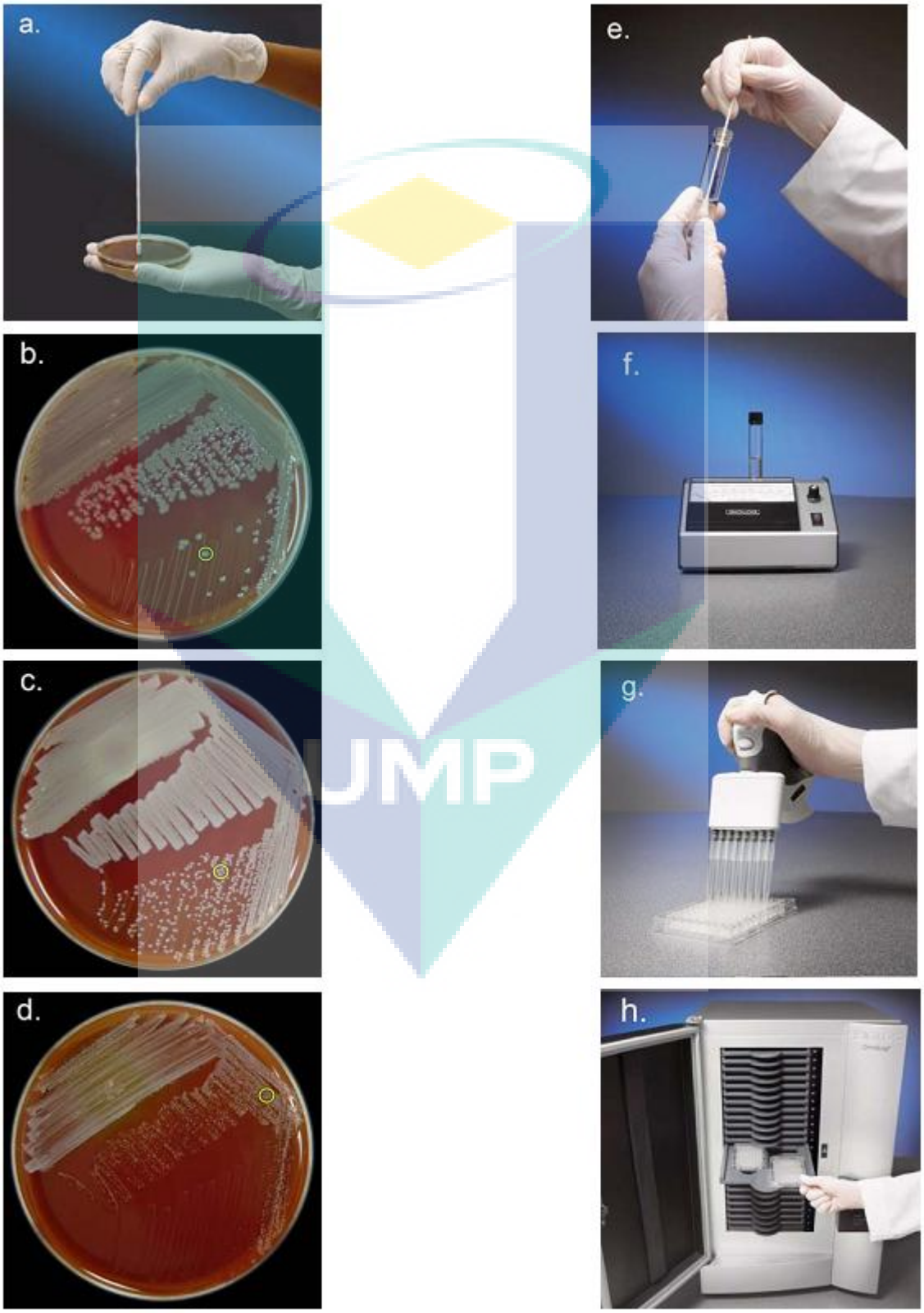
identification of the isolate is made.

Figure 1. Layout of assays in the MicroPlate

GEN III MicroPlate ™

A1 Negative Control	A2 Dextrin	A3 D-Maltose	A4 D-Trehalose	A5 D-Cellobiose	A6 Gentiobiose	A7 Sucrose	A8 D-Turanose	A9 Stachyose	A10 Positive Control	A11 pH 6	A12 pH 5
B1 D-Raffinose	B2 α-D-Lactose	B3 D-Melibiose	B4 β-Methyl-D-Glucoside	B5 D-Salicin	B6 N-Acetyl-D-Glucosamine	B7 N-Acetyl-β-D-Mannosamine	B8 N-Acetyl-D-Galactosamine	B9 N-Acetyl Neuraminic Acid	B10 1% NaCl	B11 4% NaCl	B12 8% NaCl
C1 α-D-Glucose	C2 D-Mannose	C3 D-Fructose	C4 D-Galactose	C5 3-Methyl Glucose	C6 D-Fucose	C7 L-Fucose	C8 L-Rhamnose	C9 Inosine	C10 1% Sodium Lactate	C11 Fusidic Acid	C12 D-Serine
D1 D-Sorbitol	D2 D-Mannitol	D3 D-Arabitol	D4 myo-Inositol	D5 Glycerol	D6 D-Glucose-6-PO4	D7 D-Fructose-6-PO4	D8 D-Aspartic Acid	D9 D-Serine	D10 Troleandomycin	D11 Rifamycin SV	D12 Minocycline
E1 Gelatin	E2 Glycyl-L-Proline	E3 L-Alanine	E4 L-Arginine	E5 L-Aspartic Acid	E6 L-Glutamic Acid	E7 L-Histidine	E8 L-Pyroglutamic Acid	E9 L-Serine	E10 Lincomycin	E11 Guanidine HCl	E12 Niaproof 4
F1 Pectin	F2 D-Galacturonic Acid	F3 L-Galactonic Acid Lactone	F4 D-Gluconic Acid	F5 D-Glucuronic Acid	F6 Glucuronamide	F7 Mucic Acid	F8 Quinic Acid	F9 D-Saccharic Acid	F10 Vancomycin	F11 Tetrazolium Violet	F12 Tetrazolium Blue
G1 p-Hydroxy-Phenylacetic Acid	G2 Methyl Pyruvate	G3 D-Lactic Acid Methyl Ester	G4 L-Lactic Acid	G5 Citric Acid	G6 α-Keto-Glutaric Acid	G7 D-Malic Acid	G8 L-Malic Acid	G9 Bromo-Succinic Acid	G10 Nalidixic Acid	G11 Lithium Chloride	G12 Potassium Tellurite
H1 Tween 40	H2 γ-Amino-Butyric Acid	H3 α-Hydroxy-Butyric Acid	H4 β-Hydroxy-D,L-Butyric Acid	H5 α-Keto-Butyric Acid	H6 Acetoacetic Acid	H7 Propionic Acid	H8 Acetic Acid	H9 Formic Acid	H10 Aztreonam	H11 Sodium Butyrate	H12 Sodium Bromate

Figure 2. Steps in the testing protocol



Materials Provided

ƒ MicroPlates:Catalog No.1030- Biolog GEN III MicroPlates (10/box).

ƒ On receipt, inspect foil pouches and MicroPlates for damage in shipping. To maintain the full shelf

life, the foil pouched MicroPlates must be stored at 2-8°C. The expiration dateis printed on each

pouch. Do not use the MicroPlates after the expiration date.

Materials Not Provided

ƒ Agar Culture Media: Catalog No.71102-BUG Agar with 5% sheep blood (BUG+B); Catalog No.

Bio-M1012- Chocolate Agar; Catalog No.70101- Biolog Dehydrated Growth Agar, 500 gm (BUG

Agar).

ƒ Inoculating Fluid: Catalog No.72401- IF-A, Catalog No.72402-IF-B, Catalog No. 72403- IF-C.

ƒ Inoculatorz™: Catalog No.3321- Sterile disposable inoculator swabs (20x50); Catalog No.3323

(100x1).

ƒ Streakerz

TM

: Catalog No.3025- Sterile disposable wooden agar plate streakers (10x100); Catalog

No.3026 (50x20).

ƒ Transfer Pipets: Catalog No.3019- Sterile disposable 9 inch transfer pipets.

ƒ Reservoirs: Catalog No.3102- Sterile disposable reservoirs.

ƒ Multichannel Pipettes: Catalog No.3711- 8 channel electronic pipettor.

ƒ Pipet Tips: Catalog No. 3201- Sterile racked pipet tips for Ovation multichannel pipettor; Catalog

No. 3001- Matrix multichannel pipettor tips.

ƒ Turbidimeter: Catalog No.3531- 110 volt model, Catalog No.3532 -220 volt model, Catalog

No.3585 - 240 volt model.

ƒ Turbidity Standards: Catalog No.3441 - 85% T; Catalog No.3440 - 65% T.

Determine Appropriate Protocol to Use (Inoculating Fluid and Cell Density)

ƒ All protocols are performed in the same manner, the only difference being the choice of inoculating

fluid (IF) and cell density for inoculation.

ƒ Protocol Ais used for the vast majority of species.

ƒ Protocol Bis used for a small number of stronglyreducing species and capsulated species

(primarily some strains of Aeromonas, Vibrio,and spore-forming Gram-positive rods). These

species will give a false-positive result in the A-1 well with Protocol A. If this occurs, simply repeat

the test using Protocol B.

ƒ Protocol C1is used for slow growing bacteria that typically form pinpoint-sized colonies (less than

1 mm in diameter) on BUG+B Agar in 24 hours of growth (see example in Figure 2.d.). These are

primarily microaerophilic and capnophilic Gram-positive cocci and tiny rods. See Table 1. below for

a list.

ƒ Protocol C2is used for fastidious, capnophilic, and very oxygen-sensitive bacteria that grow very

slowly or not at all on BUG+B Agar. For example, it is used for fastidious Gram-negative species

that would most likely be encountered from respiratory tract specimens after cultivation on

Chocolate Agar with 6.5% CO2. Some very oxygen-sensitive Gram-positive bacteria also require

the higher inoculation density of Protocol C2. See Table 1. below for a list.

ƒ If unsure of the appropriate test protocol, use protocol A. If the result fails to yield an identification

because of a false-positive A-1 well,then use Protocol B. If the result fails because of insufficient

positive carbon source reactions, then try, in succession, Protocols C1 and C2

Table 1. Test Protocols

Protocol	IF	Cell Density	Species
A	A	90-98% T	**Nearly all** – this is the default protocol
B	B	90-98% T	**Strongly reducing and capsule producing GN** (e.g., some *Aeromonas, Vibrio*) **and GP** (e.g., some *Bacillus, Aneurinibacillus, Brevibacillus, Lysinibacillus, Paenibacillus,* and *Virgibacillus*)
C1	C	90-98% T	**Microaerophilic, capnophilic GP** (e.g., *Dolosicoccus, Dolosigranulum, Eremococcus, Gemella, Globicatella, Helcococcus, Ignavigranum, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Weissella,* and some *Aerococcus, Arcanobacterium, Corynebacterium* and *Enterococcus sp.*)
C2	C	62-68% T	**Fastidious, capnophilic, oxygen sensitive GN** (e.g., *Actinobacillus, Aggregatibacter, Alysiella, Avibacterium, Bergeriella, Bordetella, Capnocytophaga, Cardiobacterium, CDC Group DF-3, CDC Group EF-4, Conchiformibius, Dysgonomonas, Eikenella, Francisella, Gallibacterium, Gardnerella, Haemophilus, Histophilus, Kingella, Methylobacterium, Moraxella, Neisseria, Oligella, Ornithobacterium, Pasteurella, Simonsiella, Suttonella,* and *Taylorella*) **and GP** (*Actinomyces, Aerococcus, Alloiococcus, Arcanobacterium, Camobacterium, Corynebacterium, Erysipelothrix, Granulicatella, Lactobacillus, Pediococcus,* and *Tetragenococcus*)

TEST PROCEDURE

Preparation

ƒ Before starting, prewarm MicroPlates and IF to room temperature and review the entire protocol,

including precautions.

Step 1. Culture Organism on Biolog Recommended Agar Media

ƒ Isolate a pure culture on Biolog recommended agar media (BUG+Bor Chocolate Agar)and

incubate at 33° C. Some species may require special culture conditions, for example either lower

or higher temperature (26° - 37° C.) and elevated CO2 (6.5% - 10%).

ƒ Use of alternative media should be validated. For laboratories that need to use agar media

without blood, we recommend using BUG Agar. However, some species will grow extremely

slowly or not at all if blood is omitted, for example the genera listed for Protocols C1 and C2 in

Table 1. R2A Agarand Tryptic Soy Agar without or with blood (TSA, TSA+B) can be substituted,

but they will not culture as wide a range of bacteria as BUG+B. Furthermore, their recipes and

performance characteristics from different vendors may vary.

ƒ The cells must be freshly grown since many strains lose viability and metabolic vigor in

stationary phase. The recommended incubation period for most organisms is 4-24 hours. Spore-forming gram-positive bacteria (Bacillus and related genera) should be grown for less than 16

hours to help minimize sporulation.

ƒ If insufficient growth is obtained to inoculate the panel, restreak heavily (as a lawn) onto one or

more agar plates. Incubate for 4-48 hours. This should give enough growth to inoculate the panel.

Step 2. Prepare Inoculum

ƒ Check the calibration of the turbidimeter periodically. Use an appropriate turbidity standard

(85% T or 65% T) and follow instructions in the turbidimeter manual to verify that the turbidimeter is

calibrated and operating properly.

ƒ Blank the turbidimeter with a clean tube (wiped clean of dirt and fingerprints) containing

uninoculated IF. Because the tubes used are notoptically uniform, they should be blanked

individually. Set the 100% transmittance adjustment knob so that the meter reads 100%.

ƒ Prepare the inoculum at the desired turbidity. The target cell density should be in the range

of 90-98%T for Protocols A, B, and C1. Protocol C2 requires a higher cell density of 62-68%T for species that are sensitive to oxygen. Use a cotton-tipped Inoculatorz swab to pick up

a 3 mm diameter area of cell growth from the surface of the agar plate. As shown in Figure 2.a.,

grasp the swab at its tip and, holding the swab vertically, touch it to the cell growth. Figure 2.b.,

c., and d.show examples of fast, medium, and slow growing bacteria, and the yellow circle

indicates where to touch the end of the cotton swab. For fast growing bacteria, touch a single

colony, for medium growing bacteria, touch a cluster of colonies, and for slow growing bacteria

touch the first area of confluent growth. Release the bacteria into the IF by rubbing the swab tip

against the bottom of the tubecontaining IF as shown inFigure 2.e.Crush any cell clumps

against the tube wall or remove them from the IF bycatching them on the swab. Stir the IF with the

swab to obtain a uniform cell suspension and read it in the turbidimeter, as shown in Figure 2.f.If

the cell density is too low, add more cells. If the cell density is too high, add more IF.

For extremely clumpy bacteriathat cannot be dispersed directly, use the following procedure.

First prepare a dense suspension in 2 ml of IF as follows. Use a sterile wooden Streakerz stick

to remove a clump of cell mass from the agar surface without gouging the agar. If the bacteria are

extremely dry and embedded in the agar, use the edge of a sterile glass microscope slideto

gently scrape a mass of cells onto the glass slide, again, without gouging the agar. The cells can

then be scraped off the glass slide with a sterile Streakerz stick. Then use the Streakerz stick to

deposit the cell mass onto the inner wall of a dry tube. Use the Streakerz stick to crush, break up,

and spread the clumps of cells against and along the inner wall of the tube. Then add 2 ml of IF,

and gradually slide the dispersed cells into the IF. The resulting cell suspension will be a mixture

of suspended cells and residual clumps. Stand the tube in a rack for about 5 minutes and allow

the clumps to settle to the bottom. Use a small pipet and transfer the suspended cells at the top

into a fresh tube of IF to achieve the target cell density.

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Step 3. Inoculate MicroPlate

ƒ Pour the cell suspension into the multichannel pipet reservoir.

ƒ Fasten 8 sterile tips securely onto the 8-Channel Repeating Pipettor and fill the tips by drawing up

the cell suspension from the reservoir.

ƒ Fill all wells with precisely 100 µl as shown inFigure 2.g. Be careful not to carry over chemicals

or splash from one well into another. The inoculating fluid will form a soft gel shortly after

inoculation.

ƒ Cover the MicroPlate with its lid and eject the pipettor tips.

Step 4. Incubate MicroPlate

ƒ Place the MicroPlateinto the OmniLog incubator/reader as shown in Figure 2.h., or into an

incubator, for 3 to 36 hours. Incubate at 33° C., oruse incubation conditions that were found to be

optimal for the bacterium in Step 1.

RESULTS

Reading and Interpretation of Results

ƒ Read MicroPlatesusing Biolog's Microbial Identification Systems software (e.g.OmniLog

®

Data

Collection). Refer to the User Guide for instructions.

ƒ Biolog's Microbial Identification SystemsSoftware performs all reading and interpretation of

results.

ƒ The color densities in wells of the carbon source utilization assaysin columns 1-9 are

referenced against the negative control well, A-1. All wells visually resembling the A-1 well

should be scored as "negative" (-) and all wells with a noticeable purple color (greater than well A-1) should be scored as "positive" (+). Wells with extremely faint color, or with small purple flecks or

clumps are best scored as “borderline” (\). Most species give dark, clearly discernible “positive”

reactions. However, it is normal for the “positive”reactions of certain genera to be light or faint

purple.

ƒ The color densities in wells of the chemical sensitivity assaysin columns 10-12 are referenced

against the positive control well, A-10. All wells showing significant sensitivity to the inhibitory

chemical, with less than half the color of the A-10 well are considered “negative” (-) for growth. All

other wells showing normal or near normal purple color (similar to well A-10) are considered

“positive” (+). If there is uncertainty about the interpretation, it is best to score the well as

“borderline” (\).

ƒ “False positive” coloris defined as purple color forming inthe negative control well (A-1) and in

other “negative” wells. This is seen with only a few species such as from the generaAeromonas,

Vibrio, and Bacillus. If such a result occurs, the cells are simply retested with Protocol B and IF-B.

ƒ See Biolog’s Microbial Identification Systems softwareUser Guide for further assistance

in interpreting identification results.

Precautions

To obtain accurate and reproducible results, the recommendations below must be followed.

ƒ Readthe “Instructions for Use” prior to using the GEN III MicroPlate and follow the procedures.

ƒ Pure cultures must be usedto obtain identifications. The system is not designed to identify

individual bacterial strains from within mixed cultures. The most common problem in identification

is that microbiologists are not aware that they have a mixed culture. Streaking for isolated colonies

may not be sufficient because isolated colonies can arise from a clump of cells as well as a single

cell. Bacteria have sticky surfaces and they tightly adhere to other bacteria. This is particularly a

problem with mucoid bacteria, fresh environmental isolates, and staphylococci.First,

examine cultures with care using a dissecting microscope or some colony magnifying lens, to make

sure that only one colony morphologyis present in the culture. If no species identification is

obtained, you may still have a mixed culture.Restreak the cells onto a multi-chromogenic agar

medium and let the original agar plate and the chromogenic agar plate sit at room temperature for 3

or 4 days. Examine both plates carefully, looking for the outgrowth of “bumps” or non-uniform

growth in the areas of confluent growth. On the chromogenic agar plate, look for more than one

color. If necessary, reisolate the colony types that are present and performthe identification assay

a second time.

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ƒ Culture media and repeated subculturing may affect the results. Strains may produce different

phenotypic patterns depending upon how theyare cultured prior to inoculation.

ƒ Sterilecomponents and aseptic techniques must be used in set-up procedures. Contamination will

affect results.

ƒ Disposable glasswareshould be used to handle all cell suspensions and solutions. Glassware

that has been washed may contain trace amounts of soap or detergentthat will affect results.

ƒ Prewarm the IF and the MicroPlates to room temperature before use. Some species (e.g.,

Neisseria sp.) are very sensitive to cold shocks.

ƒ Check the calibration of your turbidimeter carefully and always prepare your inoculum within

the specified density range.

ƒ Biolog's chemistrycontains components that are sensitive to temperature and light. Store the

inoculating fluids inthe dark with refrigeration. Brown or yellow wells in the GEN III MicroPlate

indicate deterioration of the chemistry.

ƒ Always keep in mindthat you are testing the metabolic properties of live cells. Some species can

lose their metabolic vigor when subjected to stresses (e.g., temperature, pH, and osmolarity) for

even a few minutes. To get the best performance possible from these MicroPlates, be aware that

the cells are alive and take care in how you handle them.

Trouble Shooting

If all wells in columns 1-9 are positive, make sure that:

ƒ You are using a microorganism thatis appropriate for the GEN III MicroPlate. If the bacterium is a

strongly reducing or capsulated species causing false positive color in the A-1 well, repeat the test

using Protocol B and IF-B.

ƒ You are not carrying over any nutrients from the agargrowth medium into the inoculating fluid.

ƒ Your inoculum is free of all clumps.

ƒ Your inoculum density is not excessive – check the calibration of your turbidimeter.

ƒ The A-1 well is not under-filled. It is used as a reference well by Biolog's MicrobialIdentification

Systems software.

If all wells in columns 1-9 are negative, make sure that:

ƒ You are using a microorganism thatis appropriate for the GEN III MicroPlate. Oligotrophic species

or extremely slow growing or oxygen sensitive bacteria, for example, may give all negative wells.

ƒ Your cells are freshly grown and you haveused the recommended agar culture medium.

ƒ Your incubation temperature and atmosphere are correct for the organism that is being tested.

ƒ The inoculating fluid was stored correctly and was prewarmed prior to use.

ƒ You are handling the cells with all disposable hardware (soap residues are toxic).

ƒ Your inoculum density is sufficient – check the calibration of your turbidimeter.

ƒ The A-1 well is not over-filled. It is used as a reference well by Biolog's Microbial Identification

Systems software.

Performance Characteristics

The GEN IIIMicroPlate performance characteristics have been determined by establishing a database using

a large collection of microorganisms from diverse sources. The database is designed to give identifications

of all species in the database, in accordance with current standards of classical identification methods and

current taxonomic nomenclature. To obtain accurate and reproducible results,all procedures and

recommendations in these Instructions for Use must be followed precisely.

Limitations

The GEN IIIMicroPlate is designed to identify pure cultures of Gram-negative and Gram-positive bacteria.

The panel will only identify members ofthe species in the current database. Other species will usually be

reported out with the message “no identification.” Atypical strains may also yield a low similarity index and

therefore will be reported out as “no identification.” This product is not for human in vitro diagnostic

use.Some bacterial species are reportable to government and public health agencies in certain

circumstances. For any isolate that is identified as Salmonella or Shigella or E. coli O157:H7, we

recommend confirmation by serology. Neisseria gonorrhoeae identifications should also be confirmed.

Appropriate caution and confirmation should be used for isolates suspected of being Dangerous

Pathogens.

8

Quality Control

Biolog MicroPlates are tested and meet internal quality control standards before being released for sale.

However, some laboratories may desire or may be required to perform independent quality control checks

on each manufacturing lot.

To test the performance of the GEN III MicroPlateuse the 2 Gram-negative and 2 Gram-positive strains

specified below using Protocol A. These are available from Biolog as a set (Biolog Catalog No.8050).

1. Escherichia coli ATCC 11775

2. Paenibacillus polymyxa ATCC 842

3. StaphylococcusepidermidisATCC 12228

4. Stenotrophomonas maltophiliaATCC 13637

Inoculate each bacterium following the TEST PROCEDURE as specified. When lyophilized or frozen

cultures are used, they should be subcultured at least twicebefore being tested.

Read the panels after appropriate incubation. The resulting identification should correctly correspond to

the identity of the quality control strain.

If the identification does not match, review the test procedures and check the purity of your culture. Repeat

the test.

Technical Assistance

For help or to report problems with this product contact Biolog Technical Service either by phone (510-785-2564) by fax (510-782-4639) or by email (tech@biolog.com) during business hours (7:30 A.M. to 5 P.M.

Pacific Standard Time), or contact the Biolog Distribution Partner in your area.

General information, Certificates ofAnalysis and MSDS can now be found at www.biolog.com.

References

1

Bochner, BR 1989. Sleuthingout Bacterial Identities. Nature 339:157-158.

2

Bochner, BR 1989. “Breathprints” at the Microbial Level. ASM News55:536-539.

3

Biolog, Inc., US Patent # 5,627,045.

APPENDIX C

LIST OF PUBLICATION

LIST OF PUBLICATION

Journal Paper

D.1: Jamaluddin Mohd Faizan, Zainol Norazwina, Salihon, Jailani. 2011. *Factorial Analysis on Acetic Acid Production Using Banana Stem Waste (BSW).* International Journal of Chemical and Environmental Engineering 2(4).

D.2: Jamaluddin Mohd Faizan, Zainol Norazwina, Norsam Nur Aidilla. 2012. *Isolation and Identification of acetic acid producer from mixed culture of soil and banana stem waste in anaerobic condition.* International Journal of Chemical and Environmental Engineering 3(2)

Conference Paper

D.1.1 Jamaluddin MF, Zainol N. *Factorial analysis on acetic acid production using banana stem waste.* International Renewable Energy and Environment Conference 2011.

D.1.2 Zainol N, Jamaluddin MF, Norsam NA. 2012. *Kinetic study of acetic acid production by facultative anaerobe isolated from soil.* International Conference of Environment 2012

D.1.3 Jamaluddin MF, Zainol N, Norsam NA. 2012. *Isolation and identification of acetic acid producer from soil mixed culture of soil and banana stem waste in anaerobic condition.*

Patent Paper

UMP