SOLID-STATE XANTHAN FERMENTATION BY USING SUGARCANE BAGASSE AS CARBON SOURCE

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ABSTRACT

Xanthan gum is a highly demand polysaccharides in many industries especially in food and pharmaceutical products. Additionally, xanthan is produced by liquid fermentation in various parts of the world. However, the major bottleneck for a broader industrial application is their high cost with glucose as the main carbon source being used in the production process. Recently, solid-state fermentation (SSF) has gained renowned attention because it offers many economical advantages for production of added-value bio-products. In addition, cheaper and yet renewable indigenous feedstock could be employed in order to make the overall xanthan production process more competitive. In the present study, sugarcane bagasse was used as an alternative carbon substrate in producing xanthan gum. The project was initiated by comparing effects of chemicals pretreatment on sugarcane bagasse (SB). The aim of pretreatment process is to breakdown the complex structure of SB for easy access of subsequent enzymatic hydrolysis. From the study, the highest level of reducing sugar yield (0.153 g sugar per g of dry bagasse) was achieved by pretreat the SB with 3% sodium hydroxide for 30 min of treatment period. Scanning electron microscope (SEM) imaging proved the significant rupture of SB structure after alkaline treatment. Upon identifying the putative treatment method, xanthan production by *Xanthomonas* sp. in batch mode of SSF was performed. In this study, Plackett Burman Design (PBD) followed by Response Surface Methodology (RSM) was used to optimize the production of xanthan. Through screening analysis with PBD, out of nine variables, four

variables (substrate loading, NH_4NO_3, yeast extract, moisture content) with significant percentage of contribution (more than 5% of contribution) and *p*-value less than 0.05 on the yield of xanthan were further optimised by using CCD. The ANOVA of CCD indicated that the RSM model was significant with coefficient of determination (R^2) of 0.9964. The results also illustrated that xanthan yield increased with increasing concentration of NH_4NO_3, while low level of substrate loading, moisture content and yeast extract enhanced the productivity of xanthan gum. The results of the validation experiments proved that by using 2.72 g of pretreated SB impregnated with 3.14 g/L of NH_4NO_3 and 7.1 g/L of yeast extract at 81.8% of moisture level, the mean of xanthan yield recorded was 0.1221 ± 0.0059 g/g, which was near to the predicted value of 0.1304 g/g. The percentage error was 6.4% which explained the RSM experiments based on CCD were found practical to derive a statistical model in optimizing the xanthan production. Through rheological and chemical composition analysis, xanthan produced via SSF in the present study exhibited pseudoplastic and shear thinning behaviors, with acetyl content of 10.61%, uronic acid content of 2.2% and pyruvate content of 5.87%. Fourier transform infrared spectroscopy analysis further confirmed that the characteristics of xanthan from the current study is similar and comparable with those commercial xanthan,

In conclusion, results of the present study suggested that sugarcane bagasse is an appealing and promising choice for the production of xanthan as this raw material is with low starting value and yet ubiquitous as well as abundant with sustainable production in Malaysia.

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APPROVAL SHEET

This thesis/dissertation entitled "**SOLID-STATE XANTHAN FERMENTATION BY USING SUGARCANE BAGASSEAS CARBON SOURCE**" was prepared by SIEW SEOK PEAK and submitted as partial fulfillment of the requirements for the degree of Master of Science at Universiti Tunku Abdul Rahman.

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SUBMISSION OF THESIS

It is hereby certified that (**Siew Seok Peak**) (ID No: **11UEM06159**) has completed this thesis entitled "**SOLID-STATE XANTHAN FERMENTATION BY USING SUGARCANE BAGASSE AS CARBON SOURCE**" under the supervision of Dr. Hii Siew Ling from the Department of Chemical Engineering, Faculty of Engineering and Science, and Dr. Chee Swee Yong from the Department of Chemical Science, Faculty of Science.

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DECLARATION

I Siew Seok Peak hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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(SIEW SEOK PEAK)

Date _____

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ABBREVIATIONS

ADF	Acidic Detergent Fibre
ANOVA	Analysis of Variance
CCD	Central Composite Design
CFU	Colony forming units
ºC	Temperature in degree Celsius
g	Gram
g/L	Gram per litter
g/g	Gram per gram
h	Hour
H_2SO_4	Sulphuric acid
M	Mole
min	Minute
mL	Milliliter
mm	Milli meter
NaOH	Sodium hydroxide
PBD	Plackett-Burman Design
RSM	Response Surface Methodology
rpm	Revolution per minute
µL	Micro litter
µm	Micro meter
v/v	Volume per volume
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

Xanthan gum is a biodegradable polymer that was discovered by the US Scientists at the Northern Regional Research Laboratories (NRRL) during the late 1950s. Xanthan gum is the first bio-polysaccharide produced commercially from the industry. It was manufactured via fermentation process by using carbon source, mainly from glucose or sucrose, with the presence of *Xanthomonas campestris* , which is a pathogenic microorganism form plants (Tait et al., 1986).

Xanthan gum commonly has been applied in a wide range of industries, such as cosmetics, foods, water based-paints and pharmaceutical products due to its rheological properties. It's unique rheological properties such as high viscosity and pseudoplasticity, high solubility and stability, which causes it to be commonly applied in different industries. Xanthan gum acts as viscosifying and thickening agent in the food industry. Furthermore, it improves the sensory properties (flavor release, mouth feel/texture and appearance) on food products (Conttrell and Kang, 1978). As in oil recovery industry, xanthan gum should be

used as an emulsifer, lubricant and thickening agent (Margaritis and Pace, 1985).

As the demand of xanthan gum keep increasing every year, it is estimated that production of xanthan gum continuously to grow at an annual rate of 5–10% (Glazer and Nikaido, 1994). At present, about 23 million kg/year of xanthan gum were consumed, where nearly 5 million kg/year are used as a drilling fluid viscosifier in the oil industry (Yang and Silva, 1995).

For a particular fermentation process, strain improvement and medium formulation are two of the important variables that need to be taken into consideration. Glucose and sucrose plays an important role during the fermentation of xanthan (Garcia-Ochoa et al., 2000). According to Leela and Sharma (2000), glucose-based medium gave the highest yield of xanthan (14.7 g/L), followed by sucrose (13.2 g/L), maltose (12.3 g/L), and soluble starch with the lowest yield, (12.1 g/ L). Nevertheless, production of xanthan gum by using glucose or sucrose as main substrate is costly. Therefore, the growth medium should be as economical as possible and agriculture waste which is more cost-effective needs to be studied. In addition, the optimum conditions for the production of high yield and good quality of xanthan need to be identified and determined.

Recently, solid-state fermentation (SSF) is popular in biotechnology industries due to its ability in the production of biologically active metabolites. Solid-state fermentation (SSF) is the development of microbial culture under

controlled condition, and performed in the absence or near absence of free water; where the solid inert matrix must contains sufficient moisture for the production of end product (Reeta et al., 2009). The solid substrate may act as the source of nutrients or as a support impregnated together with nutrients that develops the growth of microorganisms.

The benefits of applying SSF instead of submerged liquid fermentation, as it has higher yield of the desired end product and requires low sterility due to the low water activity used in SSF (Hölker et al., 2004). In the present study, the feasibility of using SSF as the major mode of xanthan fermentation was investigated. The objectives of this research are as:

a) To assess the potential of using sugarcane bagasse as potential substrate for production of xanthan gum by *Xanthomonas campestris*.
b) To evaluate the feasibility of using solid state cultivation process for production of xanthan gum.
c) To investigate and optimise the important operating conditions of solid state fermentation (SSF) for production of xanthan gum.
d) To study the rheological properties and chemical composition of the xantham gum produced via SSF.

CHAPTER 2

LITERATURE REVIEW

2.1 History of Xanthan Gum

Xanthan gum is a microbial polysaccharide that is produced by *Xanthomonas campestris*. Xanthan gum is unique and it can produce highly viscous solution at a low concentration (Cottrell and Kang, 1978). It was discovered in the 1950s by the US Department of the Agriculture and was commercialized by industrial in 1960s. Xanthan was approved by the US Food and Drug Administration (FDA) in 1969 for use as a stabilizer and thickener in food products and approved for food usage in Europe in 1982. In 1988, the World Health Organization of the United Nations (WHO) has issued an acceptable daily intake (ADI) for xanthan, which confirmed as a safe food stabilizer. Hence, xanthan was widely used in many other countries for various food uses.

At present, xanthan gum is produced globally, especially in China recently. The major producer in China is Saidy Chemical. In Austria and US, the main manufacturer of xanthan is Jungbunzlauer and Kelco, respectively (Faria et al., 2011). The production of xanthan is about 30,000 tons per year in the year of

2003 (Kalogiannis et al., 2003) and increased to 86,000 tons per year (Vorholter et al., 2008). The demand of xanthan gum is expected to increase steadily with about 5% to 10% annually. Export price for xanthan range from RM9.33 to RM15 ($3.11 to $5.06) per kilograms. Prices of xanthan depends on the grading, where the grades of xanthan gum varies in terms of viscosity at 1 wt% concentration, moisture content of packed xanthan gum and particle size which range from 75 μm to 180μm (Jess, 2010).

2.1.1 Structure of Xanthan Gum

Chemically, xanthan gum (Figure 2.1) is a heteropolysaccharide. The primary structure of xanthan gum is composed of D-glucose (Glc), D-mannose (Man), and D-glucuronic acid (GlcA) in the ratio of 2:2:1 (Jansson et al., 1975). Xanthan polymer consists of repeating petasaccharide unit. Its main chain consists of β-D-glucose units linked at 1 and 4 positions. As for the cellulosic back bone, it composed of β-D-mannose (1-4) - β-D-glucuronic acid (1-2)- α-D-mannose which are glycosidically linked at the O-3 position of every alternate glucose in the main chain. The α-D-mannose which is linked to the main chain glucose has an acetyl group which is attached as an ester at the O-6 position. As for the β-D-mannose, its pyruvic acid is condensed as ketal with terminal mannose units (Jansson et al., 1975). The fermentation conditions also vary out the pyruvic acid content. The presence of acetic and pyruvic acid in

xanthan causes the polysaccharide turn into anionic (Standford and Baird, 1983).

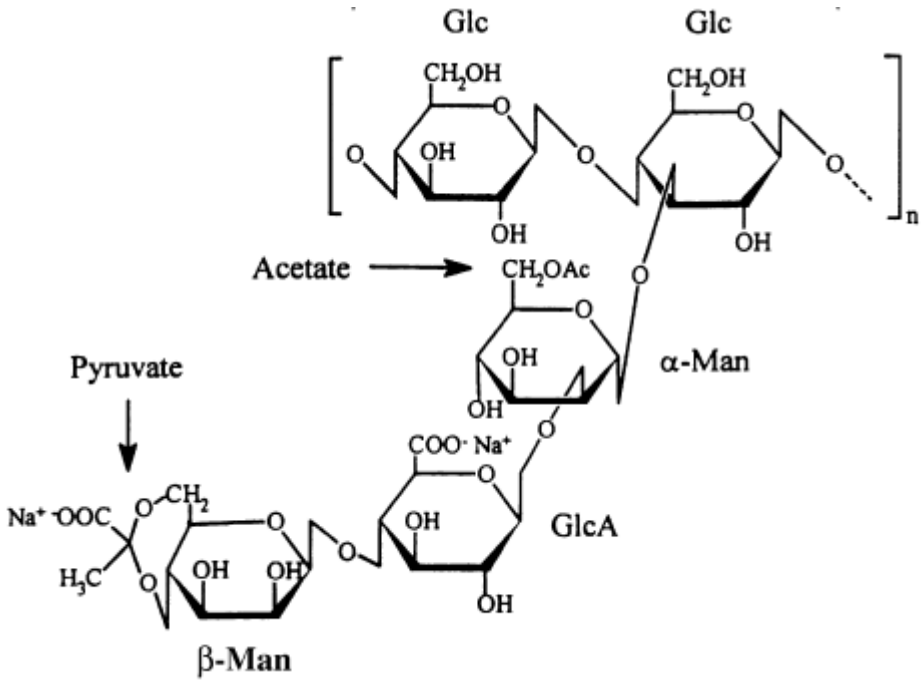

Figure 2.1: Structure of extracellular xanthan polysaccharide of *X. campestris.* (Source: Jansson et al., 1975)

2.1.2 Properties and Applications of Xanthan Gum

Xanthan gum is pseudoplastic and has a wide insensitivity of temperature, pH and electrolyte variations. Due to the marvellous properties of xanthan, hence it is widely used in cosmetic products, food, pharmaceutical products, paint, adhesives and etc.

Highly Viscous

One of the most outstanding properties of xanthan gum is its ability to produce high viscosity of a solution by adding a small amount of xanthan in water. Even at low polymer concentration, xanthan gum creates a high viscosity solution compared to other polysaccharide solution. The viscosity of xanthan solution becomes thicker when the concentration of xanthan increases. This is due to intermolecular interaction which increases the macromolecule dimension and molecular weight (Smith and Pace, 1982).

Effects on addition of salt on xanthan solutions influence xanthan viscosity. Nevertheless, this depends on gum concentration. Addition of small amount of salt in a low polymer concentration does affect the viscosity of xanthan solution. This is due to decreasing of molecular electrostatic forces, causing the reduction of the entire molecular dimension (Smith and Pace, 1982). According to Kang and Pettit (1993), the addition of 0.1% of sodium chloride increased the viscosity of xanthan, as xanthan structure was stabilized in an ordered form, giving rise to intermolecular association. Beyond this level, the addition of salt has negligible effect on viscosity.

High Temperature Resistance

Other than concentration of xanthan and addition of salt, the viscosity of xanthan gum solution also depends on temperature and pH of solution.

Increasing temperature will decrease the viscosity of xanthan gum solution. However, this behaviour is reversible between 10 to 80ºC (Garcia-Ochoa et al., 2000). Since xanthan is nearly unaffected by a wide range of temperature, thus, the viscosity of xanthan solution can be kept in fridge or stored at room temperature after heated.

Pseudoplastic Behaviour

Xanthan exhibits high pseudoplasticity in aqueous solutions. As the shear rates increase, the viscosity of xanthan solution decreases. In applications where pourability and flow are needed from a viscous solution, xanthan gum solutions will provide the pseudoplastic flow characteristics. By pouring, mixing, or pumping, the rigid structure of xanthan molecules will immediately dissociate and show a dramatic fall in viscosity. When the shear force is removed, it then re-associates to the original structure (Sharma et al., 2006).

Resistance in Degradation

Xanthan gum is extremely resistant to enzymatic degradation as the sugar side chain of the substituents is strongly bound to the polysaccharide backbone. However, xanthan can be subjected to depolymerisation by xanthanases when the molecules of xanthan are in disordered form. Besides, xanthan also can be

degraded with strong oxidizing agent such as peroxides and persulfates (Sharma et al., 2006).

2.2 *Xanthomonas campestris* as Xanthan Producing Strain

Xanthan is secreted by *Xanthomonas campestris*, a pathogenic bacterium that is from the family of *Brassicacea*e. The Gram-negative *Xanthomonas* cell occurs as a single straight rod, 0.4 to 0.7 μm wide, 0.7 to 1.8 μm long and owns a single polar flagellum with 1.7 to 3.0 μm (Bradbury and Genus, 1984). It causes a serious disease on the leaves, stems, or fruits on certain plants, such as 'black rot' on crucifers such as cabbage and cauliflower (Leach et al., 1957). Figure 2.2 shows the structure of *Xanthomonas campestris* under transmission electron micrograph.

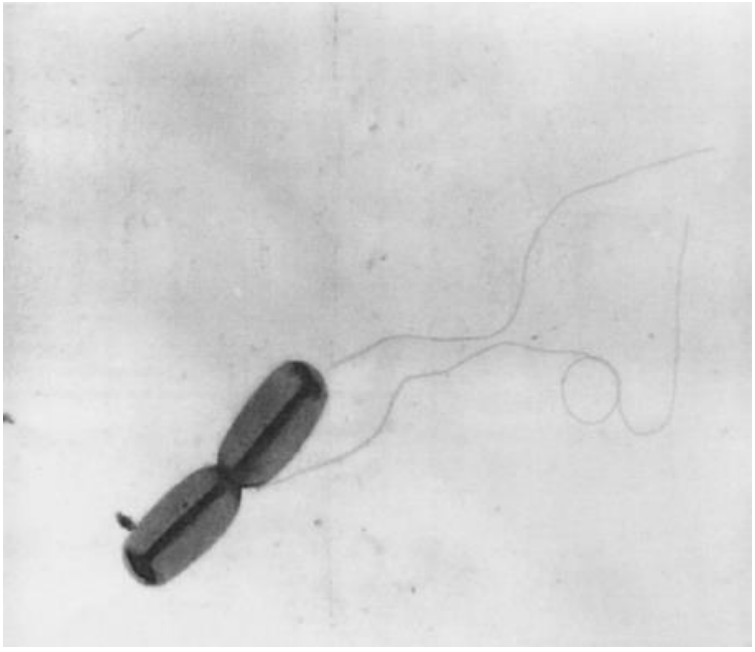

Figure 2.2: Transmission electron micrograph of *Xanthomonas campestris* (Magnification: ×12000) (Source: Garcia-Ochoa et al., 2000).

Xanthomonas is very resistant to drought under long periods. The resistance is due to the protective layer of the polysaccharide excreted from the bacteria. Xanthan gum also protects *Xanthomonas* from direct sunlight, and causes wilting of the leaves by blocking water movements. Although it can survive in harsh condition, however the temperature of the surrounding might affect the growth of *Xanthomonas*. The optimal growth temperature for *Xanthomonas* is approximately 28ºC (Leach et al., 1957).

According to Tuncay et al. (2010), the xanthan gum could be produced from various *Xanthomonas* strains such as *Xanthomonas arbicola pv. juglandis*, *Xanthomonas axonopodis pv. vesicatoria*, *X. axonopodis pv. begonia*, and *X. axonopodis pv. dieffenbachia*. The viscosity of the polysaccharide were tested at range of temperature (25 to 80ºC), at different pH values and compared to commercial xanthan. As a result, *Xanthomonas arbicola pv. juglandis* produced the highest xanthan productivity (8.22±1.52 g/L gum) followed by *X. axonopodis pv. begonia* with 7.74±1.30 g/L gum, while *Xanthomonas axonopodis pv. vesicatoria* showed the lowest productivity (6.40±0.55 g/L gum). In addition, xanthan gum produced from the strain of *Xanthomonas axonopodis pv. vesicatoria* showed the maximum viscosity value (428 mPa·sec at 1% solution) as compared to others *Xanthomonas* strains isolated from plants.

2.3 Biosynthesis of Xanthan Gum

The xanthan gum produced by *Xanthomonas* is catalyzed by multienzyme system. Repeating unit of xanthan gum is formed from addition of monosaccharide involving acetyl-CoA and phosphoenolpyruvate. The biosynthesis of xanthan gum involved (i) the uptake of substrate to necleotidal derivatives, (ii) attachment between pentasaccharide subunits and isopentyl pyrophosphate and (iii) polymerisation of pentasaccharide repeats units (Ielpi et al., 1983; Ielpi et al., 1993).

According to Sutherland (1977), firstly, the substrate enters the cell of *Xanthomonas sp.* usually through active transport and group translocation which involved substrate phosphorylation to form glucose-6-phosphate (Glc-6-P). A part of Glc-6-P transformed to glucose-1-phosphate (Glc-1-P) and fructose-1-phosphate (Fru-1-P) and lastly transformed to xanthan polymers. At the same time, some Glc-6-P undergoes Entner–Doudoroff pathway to form phosphoenopyruvate (PEP), which further dephosporylated to pyruvate. The residues of acetyl and pyruvyl are linked at the pentasaccharide. The biopolymer is released from the cytoplasmic membrane of the cell across the outer layer of membrane. This process requires energy and via a specific transport system. Figure 2.3 shows a biosynthetic pathway for xanthan gum as reported by Hsu and Lo (2003).

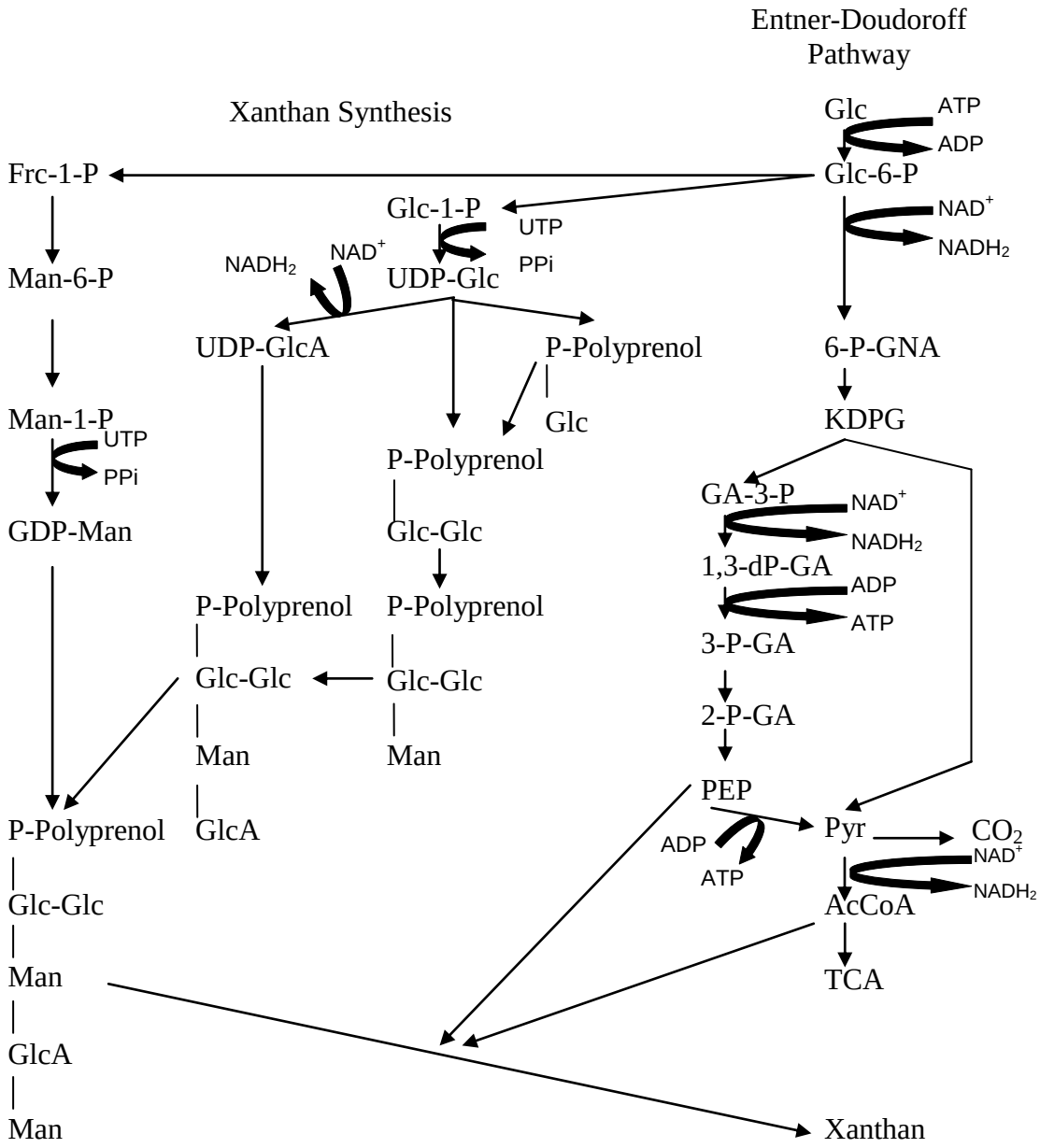

Figure 2.3: Biosynthesis of Xanthan Biopolymer. Symbols: Glucose, (Glc); Glcuose-1-phosphate, (Glc-1-P); Glcuose-6-phosphate, (Glc-6-P); 6-phosphogluconic acid, (6-P-GNA); 2-keto-3-deoxy-6-phophogluconic acid, (KDPG); glyceraldehyde-3-phosphotate, (GA-3P); 1,3-diphosphoglycerate, (1,3-dP-GA); 3-phospoglycerate, (3-P-GA); 2-phospoglycerate, (2-P-GA); phosphoenopyruvate, (PEP); pyruvate, (Pyr); acetyl-CoA, (AcCoA); tricarboxylic acid cycle, (TCA); fructose-1-phosphate, (Fru-1-P); mannose, (Man); mannose-6-phosphate, (Man-6-P); (mannose-1-phosphate, (Man-1-P). (Source: Hsu and Lo, 2003).

2.4 Factors Affecting Production of Xanthan

Oxygen, minerals, carbon and nitrogen sources, control of pH, and temperature are the essential needs to favour the growth of xanthan-producing organism in production of xanthan gum.

2.4.1 Nutrients

Glucose and sucrose are the most commonly used carbon sources to produce xanthan gum. The quantity of carbon sources influences the xanthan yield. The concentration in the range of 2 to 4% was reported to be optimal for xanthan production (Souw and Demain, 1980). However, the conversion of glucose at higher concentration gave poor yield of xanthan (Leach et al., 1957).

Nitrogen source in the medium also affect the xanthan production. Nitrogen which is an essential nutrient can be categorized into organic compound or inorganic compound. Stredansky and Conti (1999) reported that medium with Na-glutamate produced the highest yields of xanthan (2 to 5 g/L) followed by diammonium citrate and soybean peptone which also gave satisfactory results.

By applying limiting nutrient technique, Souw and Demain (1979) reported that the best carbon and nitrogen source for high xanthan yield were glucose and glutamate, respectively. Low carbon/nitrogen ratio enhanced bacteria growth and xanthan production. In addition, carbohydrate to protein ratio is

essential for the growth of bacterium and xanthan production (Lindblom et al., 1967). Biopolymer production increased when carbon and phosphorous used as limiting nutrients (Souw and Demain, 1979). Addition of small amount of organic acids such as succinic acid and citric acid also enhanced the production of polysaccharide (Souw and Demain, 1979).

A nutritional study proved that nitrogen, phosphorus and magnesium possess significant effect on growth of *Xanthomonas* while nitrogen, phosphorus and sulfur have effects on the synthesis of xanthan (Garcia-Ochoa et al., 1992). Based on the research, lower concentration of nitrogen, phosphorus, and sulfur with higher level of magnesium in the medium favoured the biomass growth and produced high yield of xanthan. Sulfur was preferred at lower level because it brought negative effect on xanthan production. However, magnesium in a higher level brought positive effect on the bacterium growth.

2.4.2 pH

The xanthan fermentation overall is slightly acidic. During the initial exponential growth phase, pH slightly rises near neutrality, followed by a rapid drop in pH during xanthan production due to rapid metabolism of nitrogen production in the production medium (Chandel et al., 2007). Furthermore, due to the free carboxylic groups and other acidic by-products of sugar metabolism caused a drop in pH.

2.4.3 Incubation Temperature

Different species of *Xanthomonas* has its own optimum growth temperature for growth and for the biopolymer production (Starr, 1946). The influence of temperature on production of xanthan has been widely investigated.

The incubation temperature during the fermentation of biopolymer not only affects the percentage of yield, but also the structure and properties of xanthan gum. Higher culture temperature increased the yield of xanthan but decreased the pyruvate content of polymer (Cadmus et al., 1978). Shu and Yang (1990) summarised that temperature between 31 to 33ºC was suitable for high xanthan yield; while high pyruvate content of the polymer could be achieved with incubation temperature between 27 to 31ºC.

2.5 Sugarcane

Sugarcane is established as an agricultural crop during 2500BC (Daniels and Roach, 1987). It is belong to the grass family, under the genus *Saccharum, tribe Andropogoneae.* It can be widely found worldwide and it is originally from tropical country such as South and South Asia (Peter, 1998). This plant was planted as crop in New Guinea firstly for the purpose of high demand of sugar worldwide (Kew, 2004). There are various species of sugarcane where the major species and popular nowadays are *S. barberi* from India, *S. edule* and *S. officinarum* from New Guinea (Peter, 1998).

Currently, most sugarcane are hybrids (*Saccharum spp.*), selected from the crosses between the species of *S. officinarum* and *S. spontaneum* (Cox et al., 2000). *S. officinarum* or so called "noble canes" are popular due to its high amount of sugar in the stem compared to others species. However, this species is poor in resisting diseases. As a result, Bull and Glasziou (1979) cultivated sugarcane which has high sucrose content with strong disease resistance by combining *S. officinarum* and *S. spontaneum* through back crossing. This newly commercial hybrid species were derived 80% from *S. officinarum* and 10% from *S. spontaneum*, with the remainder by the natural process of synapsis during meiosis (D'Hont et al., 1996).

Sugarcane is averagely grows three to four meters in height and about five centimeter in diameter. The growth of sugarcane requires moderate rainfall and a dry tropical climate with an average temperature of 20ºC to 30ºC. It is a tropical plant that can be grown organically. However, it hardly can tolerate hot climate such as warm ocean currents that sweep down the coast. As a result, sugarcane can be found at countries near the equator such as Indonesia, Brazil, Malaysia and Peru.

Commercial cultivation of the sugarcane has been carried out in Brazil, Vietnam, India, China and Thailand. In 2010, FAO estimated about 23.8 million hectares of sugarcane were planted worldwide, with a production of 1.68 billion tons. Brazil was known to be the world largest producer of sugarcane, followed by countries such as India, China and Thailand.

Sugarcane was firstly grown as crop and used as a source of sucrose for sugar production. Harvested sugarcane usually were shredded and crushed in where sugarcane juice was produced. The juice was further processed by heating with lime and phosphorus to eliminate impurities through sedimentation of calcium phosphate. Concentrated juice, "syrup" was obtained by heating and evaporating the processed juice. Then, syrup was undergone several times of crystallization to extract sucrose (Mackintosh, 2000).

2.6 Sugarcane Bagasse: A Lignocellulosic Material

The remaining residue after processing of sugarcane is known as sugarcane bagasse. Sugarcane bagasse is normally burnt into ash where the energy generated was used to produce electric power (Aigbodion et al., 2010). However, the emission of carbon dioxide and methane gas during the burning process caused global warming and degradation of ozone layer (Sirirat et al., 2010). An alternative method to generate electricity from bagasse is through biogas.

Sugarcane bagasse has been used as feed after enriched with protein through the process of solid state fermentation (SSF). Zadrazil and Puniya (1995) tested on the nutritive value of sugarcane bagasse for the purpose of animal feed.

In addition, bagasse is now well known in the production of biofuel or bioethanol, especially in Brazil. However, this reduces the fuel supply from the

sugar mills as sugarcane bagasse mostly is primary fuel source for the boilers. Production of bioethanol also requires large amount of enzyme (cellulase) for saccharification process.

As a result, some of current research focused on production of variety of enzymes, such as cellulase, xylanase, amylase and lipase by using sugarcane bagasse as inert support during solid state fermentation. Other valued end-product such as glutamic acid and citric acid were also been studied. Nampoothiri and Pandey (1996) studied the production of glutamic acid with sugarcane bagasse as inert support, with the addition of nutrients such as mineral salts and vitamins. Manonmani and Sreekantiah (1987) reported the production of citric acid by using alkaline treated sugarcane bagasse under solid state fermentation.

Lignocellulosic material, such as sugarcane bagasse, is renewable inexpensive energy supplies. Sugarcane bagasse, citrus peels and red dates wastes are very popular and usually are used as carbon source during fermentation for the production of valuable end product (Parameswaran et al., 2012; Stredansky and Conti, 1999; Ben Salah et al., 2010). The major compositions of these lignocellulosic wastes are cellulose, hemicellulose, and lignin. These components are closely bound together (Agbor et al., 2011). Hence, treatment is needed for these wastes to separate main fractions of lignocellulosic material and increase surface area for the subsequent fermentation process for by microorganism.

2.6.1 Cellulose

Cellulose (Figure 2.4) plays an important role by giving structural support to plant cell wall. It is the main component of the plant cell wall, and is formed by repeating units of cellobiose and linked each other by β-1,4 glycosidic linkage and form polymer chains (Klemm et al., 1998). The cellulose chain connected into an ultrastructure via hydrogen bond and van der Walls bonds, and packed the cellulose into microfibrils (Ha et al., 1998). The chain of polymers are position either parallel (crystalline structure) or in a disordered amorphous region. Strength of the forces determines the crystalinity structure of cellulose (Laureano-Perez et al., 2005).

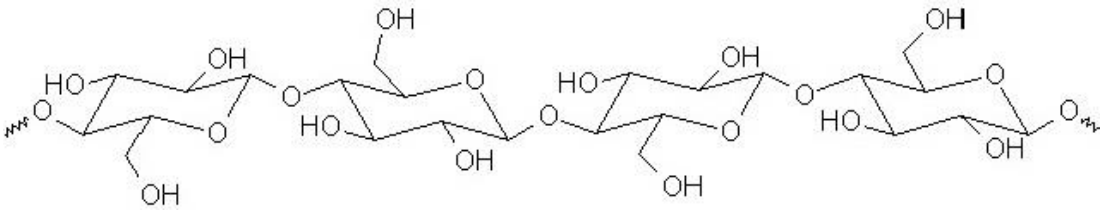

Figure 2.4: Structure of cellulose (Source: Gardner and Blackwel, 1974)

2.6.2 Hemicellulose

By comparing with cellulose, hemicellulose (Figure 2.5) has a lower molecular weight than cellulose. In addition, hemicellulose consist a variety of sugar units, with shorter chains, and it is in the amorphous region of a plant (Fengel and Wegener, 1989). The sugar units of hemicellulose are pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose) (Figure 2.5) and acetylated

sugars. As hemicellulose has lower molecular weight, shorter chains and is in the armophous region, hence it it is more easily hydrolysed compared to cellulose (Saha, 2003). It is connected to lignin and provides boundary between the lignin and cellulose. The interaction between the lignin and the cellulose fibres are hence more rigid (Laureano-Perez et al., 2005).

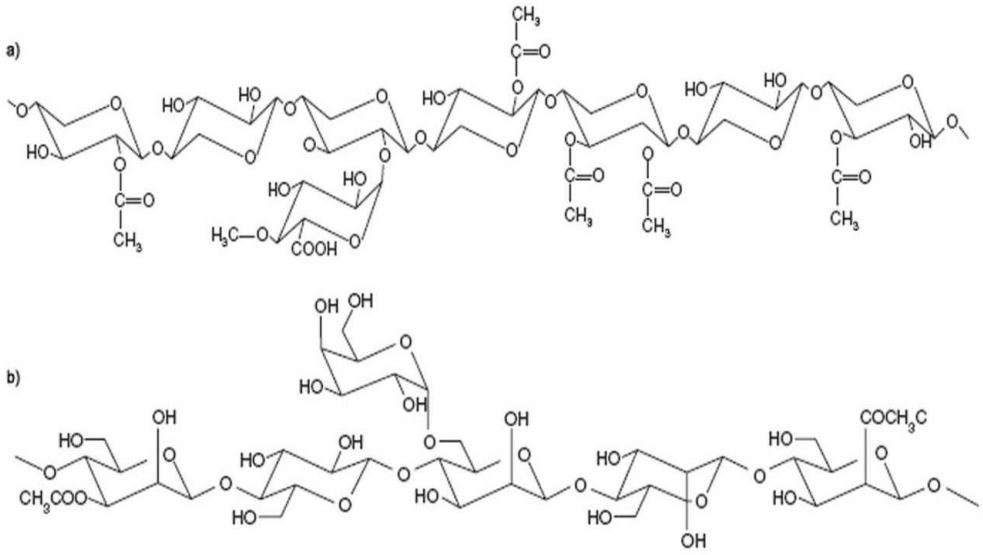

Figure 2.5: Structure of hemicellulose. Symbols: a is structure of *O*-acetyl-(4-OMe-glucurono)xylan; b is structure of structure of *O*-acetyl-galactoglucomannan (Source: Spiridon and Popa, 2008)

2.6.3 Lignin

Lignin (Figure 2.6) is an abundant polymer that can be found in the in the cellular wall of plant. It is responsible for the rigidity of the plant cell wall. Lignin is from the amorphous layer and consists of three different types phenylpropane units, which are p-coumaryl, coniferyl and sinapyl alcohol (Hendriks and Zeeman, 2009). Lignin has a strong connection with cellulose microfibrils, hence, lignin is impermeable and resistance of microbial attack

and oxidative stress to the plant (Hedriks and Zeeman, 2009). Indirectly, this causes cellulose hardly to be hydrolyzed by enzymes (Hedricks and Zeeman, 2009).

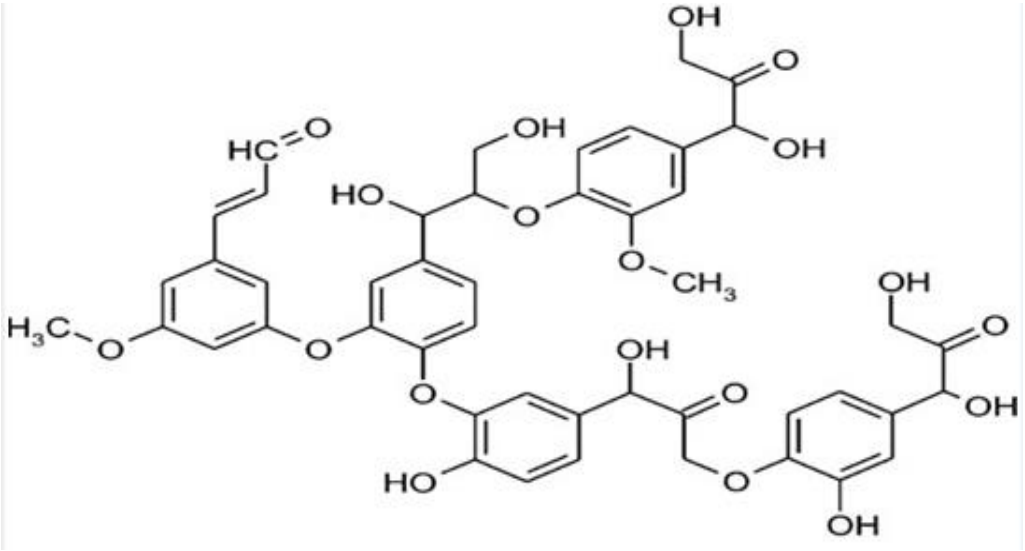

Figure 2.6: Structure of lignin. (Source: Namrata, 2012)

As a result, delignification process is needed to improve the extent of enzymatic hydrolysis. Lignin melts during pre-treatment process and precipitated out. Delignification process caused biomass swelling and alternation of lignin structure (Lynd et al., 2002). Indirectly, this increases the internal surface area of substrate which enhanced enzymes accessibility and microbial attack to cellulose fibers. Studies by Chang and Holtzapple (2000) proved that biomass digestibility is improved with increasing lignin removal.

2.7 Pretreatment of Lignocellulosic Materials

The strongly bond complex structure of lignocelllosic materials and presence of lignin caused cellulose hardly can be hydrolyzed by enzymes and microorganisms. Thus, pretreatment of lignocellulosic biomass is needed in order to ease the hydrolysis of cellulose into fermentable sugar (Gutierrez et al., 2008).

The pretreatment processes including physical, chemical and physiochemical techniques. The main purpose of pretreatment is to remove lignin content of lignocellulosic feedstock and release cellulose from lignin (Champagne, 2006). Alteration of the structure of lignocellusic material after pre-treatment improves the rate of saccharification and hence enhances the yield of fermentable sugar (Mosier et al., 2005).

2.7.1 Physical Pretreatment Techniques

Physical pre-treatment including comminution, iradation and microwave. The main goal of physical pre-treatment is to reduce the particle size of lignocellulosic substrate before the process of saccharification and fermentation. The reduction in particle size causes the surface to of substrate increase (Palmowski and Muller, 2000).

Comminution and Milling

Comminution is a common treatment technique used to improve the digestibility of lignocellulosic biomass (Palmowski and Muller, 2000), which including chipping, grinding, milling and shredding. The purpose of this treatment is to increase surface area, decrease the degree of polymerization (DP) as well as to decrease the cellulose crystallinity (Sun and Cheng, 2002). Usually, the size of lignocellulosic material after chipping is 10 to 30 mm and 0.2 to 2 mm after milling or grinding (Sun and Cheng, 2002). The final particle size depends on the energy input of the mechanical comminution of agricultural materials (Cadoche and López, 1989).

Autogenous mill, rod mill and pebble mill are used for dry materials, while colloid mill, fibrillator and dissolver are suitable only for moisten materials. The most popular milling is the ventory ball milling due to its high effectiveness in decreasing cellulose crystalinity compared to others common ball milling (Millett et al., 1976). In addition, ventory ball milling is suitable for wet and dry materials.

The efficiency of milling process was studied in the combination with enzymatic saccharification and crystalinity in order to improve hydrolysis process. Fan et al. (1980) proved that the crystalinity index reduced from 74.2% to 4.9% after treatment with ball milling. According to Ayla et al. (2010), ball milling enhanced enzymatic saccharification by decreasing the crystallinity. From their research, the optimal sugar yields were 78.7% glucose

(338.6 mg/g bagasse) and 72.1% xylose (193 mg/g bagasse) from bagasse, and 77.6% glucose (289 mg/g straw) and 56.8% xylose (164.6 mg/g straw) from straw.

Irradiation Technique

Irradiation is categorized under physical pretreatment which inclusive of gamma-rays, electron bean and microwave. The purpose of irradiation on lignocellulosic materials is to degrade cellulose component into fragile fiber and to produce low molecular weight oligosaccharides cellobiose. Bagasse treated with irradiation has a higher sugar yield compared to the untreated bagasse during enzymatic hydrolysis (Kumakura and Kietsu, 1983). However, the irradaion treatment is very costly in a large scale process with environment safety concern and has difficulties in industrial application.

Microwave Pretreatment

Microwave treatment is an alternative way for conventional heating due to its high heating efficiency and easy operation (Hu and Wen, 2008). Microwave pretreatment is another division of physical pretreatment which shows feasible effectiveness in enzymatic hydrolysis. This pretreatment method vibrates the polar bonds of the lignocellulosic material and its surrounding liquid, causing an internal heating to biomass. Besides, microwave irradiation also generates

magnetic field and cause polar bonds vibrate and to be align with the magnetic field. In this circumstance, the polar bonds of lignocellulosic materials were disrupted (Sridar, 1998). In addition, the thermal generated in the biomass will release acetic acid, causing auto-hydrolysis of the biomas (Lora and Wayman, 1978). When autohydrolysis occurred, the acetyl groups of lignocellulosic materials were cleaved from hemicelluloses (Ooshima et al., 1984).

According to Keshwani et al. (2007), there was a slight increase of reducing sugar from 4.87 mg/mL to 5.18 mg/mL when switchgrass was immersed in water and treated with microwave at 1250W for 1 min. Nikolic et al. (2008) reported that an increase of sugar with 6.4% over untreated sample, by immersing corn meal in water, with microwave at 80W for 5 min.

The reported findings indicated that low sugar yield when biomass was immersed in water and treated with microwave irradiation. Hence, microwave irradiation usually is assisted with chemical reaction, such as acid or alkaline. This can accelerate the chemical reaction rate (Caddick, 1995). Besides, the benefits of microwave-based irritation include minimizing the process energy usage, and it is able to start and stop the process instantaneously (Datta, 2001).

2.7.2 Chemical Pretreatment Techniques

Acid (sulphuric acid, hydrochloric acid) and alkaline (sodium hydroxide, ammonia) solutions are commonly used method to pre-treat lignocellulosic material (Mosier et al., 2005). The purpose of chemical pretreatment is to alter the structure of lignin with cellulose, as well as to dissolve hemicelluloses (Mosier et al., 2005).

Acid Treatment

Acid pre-treatment on lignocellulosic biomass had been widely used nowadays. Sulphuric acid, hydrochloric acid and nitric acid are commonly used to treat agricultural residues (Taherzadeh and Karimi, 2007). The aim of this treatment is to solubilize hemicellulose (Ramos, 2003), for achieving structure that are appropriate for fermentation and to enhance cellulose for microbial and enzyme attack (Yang and Wyman, 2004). The removal of hemicellulose increases the porosity of biomass and enhances enzymatic digestibility (Chen et al., 2007). Solubilised hemicelluloses easily subjected to hydrolysis process, generating monomers such as furfural and hydroxymetylfurfural (HMF) which will inhibit the fermentation process (Ramos, 2003).

Acid pretreatment can be done by using either strong or dilute acid. Strong acid can be done at lower temperature and shorter time. In contrast, dilute acid

pretreatment usually done at high temperature with a longer treatment time. Najafpour et al. (2007) mentioned that the sugar yield was found to be dependent on acid concentration and the incubation temperature employed. They reported that with 40 min treatment time, empty fruit bunches (EFB) waste exhibited conversion of sugar up to 80% when 5% EFB was treated with 30% (w/w) HCl. However, there are a few drawbacks with the strong acid treatment. High concentration of acid used during strong acid treatment is tremendously corrosive and hazardous. As a result, strong acid treatment needs neither particular non-metallic nor expensive reactors that are resistant to corrosion. Besides, acid recovery or neutralization on the waste of concentrated acid is needed. The high cost and expensive maintenance on the constructions caused less commercial interest in this kind of treatment (Sun et al., 2004).

In contrast, dilute acid pre-treatment usually done at high temperature and for a longer period of treatment time. Most of the xylan in biomass was converted to xylose when biomass was treated with dilute acid (Taherzadeh and Karimi, 2008). This kind of treatment is more popular in commercial compared to strong acid treatment, as xylan takes up to a third of the total carbohydrate in many lignocellulosic materials (Hinman et al., 1992). Usually, sulfuric acid or hydrochloric acid is diluted in 2% to 5% (w/w) and the process takes place either at higher temperature (above 160^{o}C) (Converse et al., 1989), with short treatment time (1 to 5 min) or at lower temperature (below 160^{o}C) (Esteghlalian et al., 1997), with a longer treatment time (30 to 90 min) (Hsu, 1996). The *Eucalyptus grandis* pretreated with dilute acid at 200^{o}C with the

yield of cellulose conversion (90%) (Emmel et al., 2003). Cara et al. (2007) also reported that the highest enzymatic saccharification yield (76.5%) was achieved when olive tree biomass was treated by sulphuric acid with the concentration of 1.4% (w/w) at 210°C.

Acid pre-treatment might have a few drawbacks, such as formation of inhibitors, i.e., carboxylic acids, furans and phenolic compounds (Taherzadeh and Karimi, 2008). During acid pretreatment, xylose from hemicellulose might degrade to form 2-furfuraldehyde while glucose might degrade to form hydroxymethylfurfural (HMF), as shown in Figure 2.7 (Meyer and Perdesen, 2010). Both of these inhibitors are known to hamper glycolytic enzymes. When the process proceed, it caused the formation of formic acid and levulinic acid, which are considered as weak acid and has the potential inhibit saccharification or fermentation process (Thomsen et al., 2009). The formation of high concentrations of these inhibitors can affect the fermentation organism by changing the pH of fermenting medium (Almeida et al., 2007). As a result, the condition of acid pretreatment should be chosen appropriately in order to reduce the formation of these inhibitors.

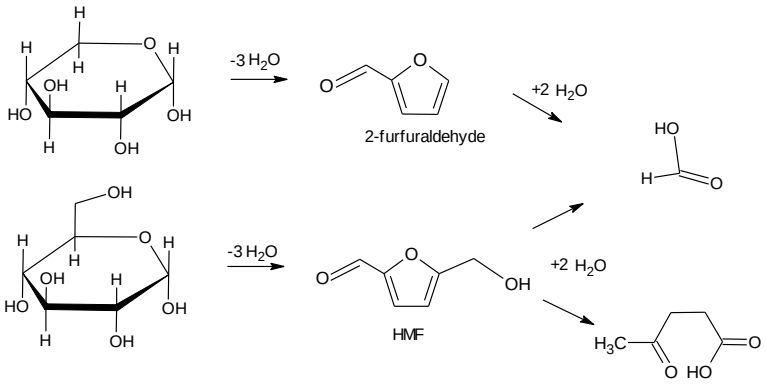

Figure 2.7: Degradation pathway of xylose. (Source: Pedersen et al., 2010)

Alkaline Treatment

Sodium hydroxide, calcium hydroxide, lime and ammonia are commonly used in alkaline treatment. Alkaline treatment caused saponification of intermolecular ester bonds cross-linking lignin. The diminution of this cross-linking causes porosity of lignocellulosic material increase (Tarkow and Feist, 1969). Mostly, alkaline pretreatment removes amorphous phase (lignin and hemicellulose) of lignocellulosic material, hence crystalinity of biomass usually increased after alkaline ptretreatment (Kim and Holtzapple, 2006). Lignin removal increases the accessibility of enzyme and microorganism towards treated biomass during fermentation. According to Parameswaran et al. (2012), crystalinity index increased from 53.44% to 65.29% when sugarcane bagasse was treated with alkaline-assited microwave treatment, with significant increment on reducing sugar. The study proved that enzyme effectiveness was boost up when lignin was removed and the accessibility of cellulose and hemicellulose were increased. Xu et al. (2007) also reported that when ammonia solution (10%) was used to treat soybean straw for 24 h at room temperature, the percentage of hemicellulose and lignin decreased by 41.45% and 30.16%, respectively.

In addition, alkaline treatment is also used to remove acetyl group and uronic acid substitutions (Chang and Holtzapple, 2000). Acetyl group content of substrate influence directly on yield of sugar from enzymatic hydrolysis. According to Kong et al. (1992), when acetyl group in biomass was removed, reduction of the steric hindrance of hydrolytic enzymes occurred, which

significantly enhance carbohydrate digestibility of microorganism during fermentation.

Sodium hydroxide is the most widely used alkaline pretreament agents while calcium hydroxide can be considered as the most commercially available alkaline compared to other alkaline treatment agents (Soto et al., 1994). By comparing with acid treatment, alkaline treatment has less problem with sugar degradation. The NaOH-pretreated biomass enzymatic conversion ratio of cellulose is more effective as compared with H_2SO_4 pretreated biomass (Zhao et al., 2007). Silverstein et al. (2007) also compared the efficiency of sulfuric acid, sodium hydroxide, hydrogen peroxide, and ozone pretreatments for enzymatic conversion of cotton stalks. Their result proved that sodium hydroxide pretreatment reported the maximum point of cellulose conversion (60.8%).

2.8 Solid State Fermentation

Solid state fermentation (SSF) is defined as the development of microbial culture on solid materials in the absence or near absence of free water (Cannel and Moo-Young, 1980). The moisture content in the inert solid support however must be suitable to support growth of microorganism (Pandey, 1992). The major difference between submerged liquid fermentation (SLF) and SSF is the amount of water content in the substrate. As for SSF, the solid substrates were involved during fermentation apart from free water around. This

technology has been developed as it is more attractive compared to liquid fermentation for a number of valued productions. Unlike liquid fermentation, SSF requires smaller fermenters, smaller volumes of inputs, less energy is required and produce lesser waste water.

There are several factors that need to be considered before commencing a SSF process, which includes the selection of microorganism and types of inert support. Fungi and yeast are both can be well adapt due to their physiological, enzymological and biochemical properties (Singhania et al., 2009). The hyphal mode of fungi can easily penetrate into solid substrates and directly uptake carbon source or nutrients from solid substrates (Pérez-Guerra et al., 2003). However, bacteria also found to be used is SSF process (Mitchell and Lonsane, 1992). Besides, Pandey (1992) also stated that bacteria can grow well and manipulated in SSF. Currently, bacteria have been used for enzymes production and on food processes such as fermented soybean paste and Chinese vinegar (Ramesh and Lonsane, 1990).

The solid inert support used in SSF must be insoluble in water. It acts as physical support and source of nutrients for the growth of microorganism. Substrates that had been used most are substrates from agricultural or waste from food industry, such as cassava, potato, beans and sugar beet pulp. Solid substrate used will act as physical support and also nutrient source for the microorganism (Pérez-Guerra et al., 2003). However, the cost for these materials is expensive. Hence, researcher nowadays seek for agricultural waste such as sugarcane bagasse, citrus peels, rice husk and corn peels which are

cheap and easily available. Usually, these inert solids are impregnates with nutrients in order to provide a suitable environment for the growth of micoorganism (Weber et al., 1999). Treatment process of these lignocellulosic waste is needed to make cellulose more accessible and their physical structure more susceptible for the growth of microorganism (Manpreet et al., 2005).

The environmental factor such as moisture content might affect the growth of microorganism and also the yield of end product. Moisture content plays an important role in SSF. Low moisture content can cause low solubility of nutrients in the substrate. Nevertheless, high moisture content can cause reduction in porosity of the solid substrates and directly interfere oxygen transfer (Lonsane et al., 1985). The most favorable condition of moisture content depends on both microorganism and types of solid substrates used.

2.9 Xanthan from Agricultural Waste

Production of xanthan gum is costly as the main carbon source (glucose or sucrose) and the very strict purity of standards of food grade stipulated by Food and Drug Administration. Reduction of cost can be achieved by using less expensive substrates, such as agricultural waste products.

Xanthan gum can be produced by variety of cheap substrate including agicultural industrial products, by-products and wastes. The application by using agriculture waste for the bioprocess not only provides alternative

substrates, but reduces pollution. Hence, biotechnological fermentation, especially solid-state fermentation (SSF), has contributed extremely for such utilization.

According to Stredansky and Conti (1999), dried apple pomace and grape pomace were used as agriculture waste to produce xanthan by solid state fermentation. Dried apple pomace was choosen as substrate because its high concentration of sugar. The apple was treated and dry malt grains were added into the fermentation medium. The research group proved that the addition of malt grain improved xanthan yield. The porous characteristic of dry malt grains increased oxygen availability in malt grains. The overall result showed that apple pomance substrate produced high yield of xanthan with 52.1 g/L, while grape pomance substrate gave poor yield of 10 g/L. Low yield from grape substrate might be due to low absorption capacity of the substrate and low sugar content.

Sugarcane molasses was used as solid substrate and it yield as high as 70.5 g/L of xanthan under certain defined condition (Abd El-Salam et. al., 1994). The optimum medium were adding 25% sugar in medium, and impregnated with 2.4 g/L of nitrogen content from ammonium chloride, with the air/medium ratio of 3:2, and incubated for 3 days at 30ºC. Sugarcane molasses have high potential on yielding high percentage of xanthan because it has about 50% of cellulose and 25% each of hemicellulose and lignin. As it has low ash content, it is suitable for micro-organisms including bacteria, yeasts and fungi.

2.10 Concluding Remarks

At present, xanthan gum has been recognized as an important thickening and stabilizing agent in a wide range of industry such as food industry, personal care products and pharmaceutical products. There are strong demands on this gum worldwide due to its rheological properties, as described earlier in Section 2.1.2.

Due to the high demand and the increasing of price on xanthan annually, further investigation is required in order to search for the most economic and viable technique for the production of xanthan. In the present study, sugarcane bagasse was used as carbon to replace the usage of pure glucose which are expensive and has been used commercially. Nevertheless, treatment on sugarcane bagasse is necessary in order to breakdown the complex structure of lignocellulosic compound, causing cellulose more accessible for enzymatic attack.

Solid state fermentation (SSF) has been applied in the present study. Less energy input is required for SSF and hence is an alternative to submerged liquid fermentation. However, information related to the production of xanthan via SSF is very limited in the literature. Optimization of the medium condition and culture condition not only results in improvement on xanthan production, but also reduces the cost of production.

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 Collection, Processing and Treatment of Sugarcane Bagasse

Sugarcane bagasse was collected from a wholesaler of sugarcane juice, located at Jalan 8/23a Danau Kota, 53300 Kuala Lumpur, Malaysia. The bagasse was washed with de-ionized water to remove dirt and debris. Then, it was dried in oven at 50ºC until constant weight. The dried bagasse was grinded into small particles with size 600 to 850 µm for further usage. The dried sugarcane bagasse was subjected to several pretreatment conditions, as described in Chapter 4 (Section 4.2.1)

3.2 Preparation of Inoculum

Strain of *Xanthomonas campestris* NRRL 1459 was used throughout the study. The microorganism were cultured on yeast malt (YM) agar plate (yeast extract, 5 g/L; malt extract, 3 g/L; dextrose, 10 g/L; agar powder, 20 g/L) and incubated for 48 h at 30ºC. Single colonies of *Xanthamonas campestris* from

YM agar plate were cultured into yeast malt broth (yeast extract, 5 g/L; malt extract, 3 g/L; and dextrose 10 g/L) for 24 h in a rotary shaking incubator with 200 rpm at 30ºC, and used as inoculum for the subsequent solid-state fermentation process.

3.3 Solid-State Fermentation

In this present study, solid-state fermentation (SSF) technique was applied in producing the xanthan from pretreated sugarcane bagasse. The SSF process was performed in conical flask under static condition and the flasks containing pretreated sugarcane bagasse and impregnated with required nutrients. Sterilization of contents was conducted at 121ºC for 15 min prior to addition of inoculum and enzyme cellulase. Incubation process started thereafter and sampling was done on the daily basis until the system achieved equilibrium stage for growth of culture and xanthan formation. The SSF process was initiated by employing Plackett Burman Design (PBD) to indentify and select those significant and affecting variables. Following this, the optimization of SSF was continued with Response Surface Methodology (RSM) with Central Composite Design (CCD). Detail information on PBD and RSM with CCD are explained in Chapter 5 (Section 5.2.1 and 5.2.2).

3.4 Analytical Methods

3.4.1 Determination of Xanthan

Determination of xanthan gum is based on methods as proposed by Stredansky and Conti (1999). Xanthan gum was extracted from fermented mass by adding 200 mL of distilled water into flask and by shaking at 250 rpm for 30 min. Coarse solid particles were removed by filtration. Following this, finer particles such as microorganism were centrifuged at 11,000 rpm for 20 min at 4ºC (SIGMA 3-18K centrifuge machine, Germany). The cold cell free xanthan gum was extracted with three volumes of cold acetone. The mixture was then centrifuged again to separate xanthan gum from the aqueous phase. The aqueous phase was discarded and the precipitated xanthan gum was re-dissolved in distilled water, centrifuged and precipitated again with acetone for purification of xanthan. Xanthan gum was then dried at 50^{o} C until constant weight.

3.4.2 Determination of Reducing Sugar

Reducing sugar content of crude supernatant was analyzed by using 3,5 dinitrosalicylic acid method (Miller, 1959). The DNS solution consist of dinitrosalycylic acid, 10g/L; phenol, 2 g/L; sodium sulfide, 0.5 g/L; sodium hydroxide, 10 g/L; sodium potassium tartarate, 182 g/L. One mL (1 mL) of sample was added with 1 mL of DNS solution and 2 drops of 1 M NaOH in a

test tube. The solution was mixed well and boiled in a water bath at 100^{o} C for exact 5 min. Then, the mixture was cooled under running tap water instantly and 10 mL of distilled water was added into the solution. The solution was left for 20 min for colour development, and the absorbance of the suspension was recorded at 540 nm by using single beam spectrophotometer (JENWAY 6320D). The standard curve of reducing sugar content was conducted following the standard procedure using series of glucose with known concentration (Figure 3.1).

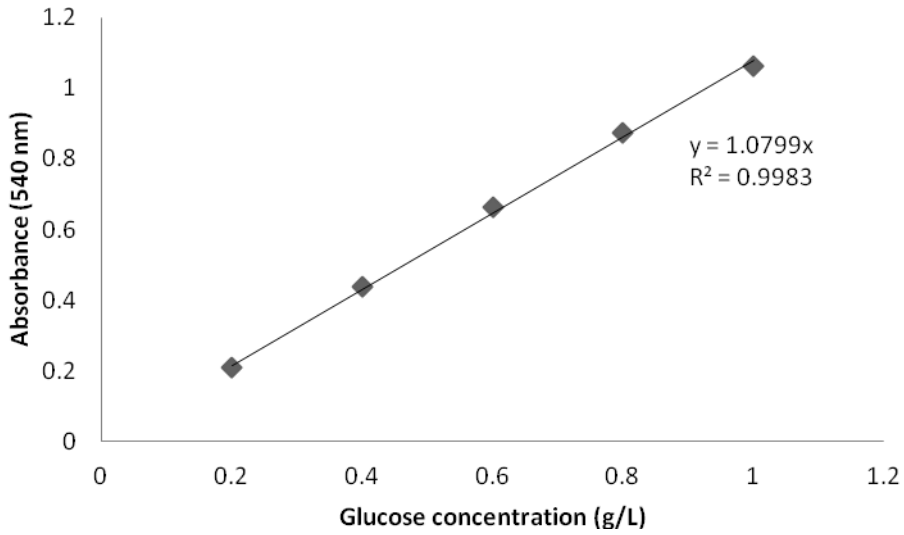

Figure 3.1: Standard calibration curve for reducing sugar

3.4.3 Viable Cell Counts

The number of viable cells was determined in terms of colony forming units (CFU). Serial dilution of each sample were plated in duplicate onto yeast malt (YM) agar plates. Agar plates were incubated at 30^{o} C for 48 h. Counting of the bacteria growth were done in terms of colony forming units per mL (CFU/mL).

3.5 General Plan of Experimental Work

Figure 3.2 represents the flow of current experimental works.

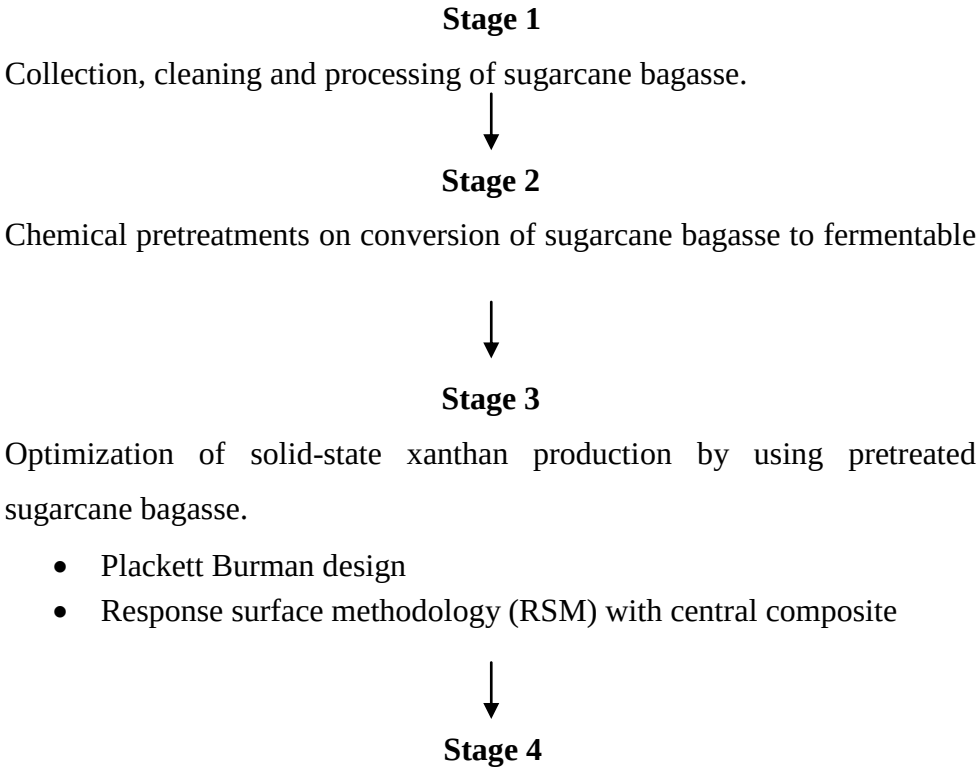

Figure 3.2: Flow chart of experimental work

CHAPTER 4

INFLUENCE OF CHEMICAL PRETREATMENTS ON CONVERSION OF SUGARCANE BAGASSE BIOMASS TO FERMENTABLE SUGAR

4.1 Introduction

Pretreatment on sugarcane bagasse was conducted to breakdown the complex structure of biomass for the ease of subsequent fermentation process. Through pretreatment process, the porosity of lignocellulosic material increases (Sun and Cheng, 2002).

In the present study, sulphuric acid (H_2SO_4) and sodium hydroxide (NaOH) were used during acid and alkaline treatment, respectively. Different concentrations of solution ranging from 1% (w/v) to 5% (w/v) were used. The chemical pretreatment processes were conducted at different treatment duration, ranging from 30 to 90 min.

4.2 Materials and Methods

4.2.1 Chemical Pretreatment

Sulphuric acid (Fisher Scientific Brand, USA) and sodium hydroxide (Unichem Brand, India) are two chemicals used during chemical pretreatment process. Grinded sugarcane bagasse were immersed in dilute sodium hydroxide and sulpuric acid, respectively, at concentration ranging from 1% (w/v) to 5% (w/v). The mixtures were then incubated in the shaker at 250 rpm. The treatment time range from 30 to 90 min. After pretreatment process, the treated biomass was filtered and washed with de-ionized water until the pH of mixture became neutral (pH 7). Then, the treated sugarcane bagasse was dried in oven at 50ºC until constant weight and kept in a sealed plastic bag with silica gel for further usage.

4.2.2 Enzymatic Hydrolysis of Pretreated Sugarcane Bagasse

Enzymatic hydrolysis was carried out in conical flask where pretreated sugarcane bagasse was moistened with appropriate amount of distilled water to achieve the desired moisture content. Then, flask containing moistened pretreated sugarcane bagasse was autoclaved at 121ºC for 15 min. Commercial cellulase, Cellulase ® 1.5L (Novozymes, Denmark) was filtered, sterilized and added into flask. The saccharification process was started thereafter. The enzymatic hydrolysis was conducted in solid state, at a static condition for

seven consecutive days. Samples were withdrawn every 24 h. Distilled water was added to the flask and the mixture was shaken for 20 min at 250 rpm. Then, the mixture was centrifuged at 11,000 rpm for 20 min. The clear supernatant was used for determination of reducing sugar by using 3,5 dinitrosalicylic acid as describe in Section 3.4.2. In addition, cellulose, hemicellulose and lignin content of untreated and treated sugarcane bagasse were also analyzed. Morphological changes and crystalinity of sugarcane bagasse before and after pretreatment process were determined by using scanning electron microscopy (SEM) and X-ray diffraction (XRD), respectively.

4.2.3 Analytical Procedures

4.2.3.1 Determination of Cellulose, Hemicellulose and Lignin Contents

The lignocellulosic content (hemicellulose, cellulose and lignin) of untreated and treated sugarcane baggasse were determined by neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin test (Goering and Van Soest, 1970). Equations 4.1 to 4.3 are used to determine the amount of hemicellulose, cellulose and lignin of untreated and treated sugarcane bagasse.

Determination of Neutral Detergent Fibre (NDF)

The NDF reagent was prepared by diluting 30 g sodium laurel sulphate, 18.61 g disodium dihydrogen ethylenediamine tetracetate, 6.81 g disodium tetraborate, 10 mL of ethylene glycol and 4.56 g disodium hydrogen phosphate in 1 L of distilled water. One hundred milliliter (100 mL) of NDF reagent was mixed with 1 g of sample, 2 mL of decalin and 0.5 g of sodium sulphite. The mixture was heated in a water bath at 100ºC for 1 h. Then, the residues were filtered with a dry and pre-weighed filter paper. Residues left on top of filter paper were washed with hot distilled water followed by acetone. Next, it was dried in oven at 105ºC for 24 h. The net weight of dry residue was the net weight of NDF.

Determination Acidic Detergent Fibre (ADF)

The ADF reagent was prepared by diluting 20 g of cetyl trimethylammmonium bromide in 1 L of 0.5 M H_2SO_4. One hundred mL (100 mL) of ADF reagent was mixed with 1 g of sample and boiled for 1 h at water bath. Then, the residues were filtered with a dry and pre-weighed filter paper. Residues left on the filter paper were washed with hot distilled water followed by acetone. Next, it is dried in oven at 105ºC for 24 h. The net weight of the dry residue is the net weight of ADF.

Determination of Lignin

Dried residues from ADF test were kept and used for lignin test. The leftover residues were immersed in 72% of H_2SO_4 and stirred for 3 hours. Then, the residues were filtered with dry and pre-weighed filter paper, followed by washing with hot distilled water and acetone. The filter paper was dried in oven at 105ºC for 24 h. The net weight of dry residues was the mass for lignin and ash. The leftover residues were sent to furnace and heated at 550ºC for 3 h. The residue left in the furnace was the net weight of lignin.

$$\text{Hemicellulose} = \frac{(\text{Weight of NDF - Weight of ADF})}{1 \text{ g of sample}} \times 100\% \quad \text{Eq. 4.1}$$

$$\text{Cellulose} = \frac{(\text{Weight of ADF - Weight of lignin and ash})}{1 \text{ g of sample}} \times 100\% \quad \text{Eq. 4.2}$$

$$\text{Lignin} = \frac{(\text{Weight of lignin})}{1 \text{ g of sample}} \times 100\% \quad \text{Eq. 4.3}$$

4.2.3.2 X-ray Diffraction (XRD) Analysis of Sugarcane Bagasse

The crystalinity of untreated and treated sugarcane bagasse was measured by using X-ray diffraction (XRD) (SHIMADZU XRD-6000, Japan). All samples were scanned at 40Kv, 40mA, between the range of 2º to 30º. Crystalinity index (CrI) was calculated as CrI (%) = $[(I_{002}-I_{am})/ I_{002}] \times 100$, where I_{002} is the maximum intensity at between 21º -22º and I_{am} is the minimum intensity between 18º and 19º (Segal et al., 1959).

4.2.3.3 Scanning Electron Microscopy (SEM) Observation

Changes of physical structure on untreated and treated sugarcane bagasse were observed by using HITACHI S-3400N Scanning Electron Microscopy (SEM). The magnification used was 750X and 1500X at the voltage of 15kV.

4.3 Results and Discussion

4.3.1 Reducing Sugar Yield from Pretreated Sugarcane Bagasse (SB)

As observed from the Figure 4.1, by using 1% (w/v) NaOH, the reducing sugar yield was only 0.071 g/g after 30 min treatment. The yield of reducing sugar nearly double up to 0.153 g/g when the concentration of NaOH was increased from 1% to 3% (w/v). No significant improvement of reducing sugar yield could be observed when the sugarcane bagasse was pretreated for prolonged treatment durations (60 and 90 min) by using 1% to 3% (w/v) of NaOH.

As for acid treatment, significantly low level of reducing sugar was recorded, regardless the treatment duration or the concentration of H_2SO_4 used. By applying 5% (w/v) H_2SO_4 for 30 min treatment, the highest reducing sugar yield, 0.056 g/g was achieved and it is 38% lower as compared with alkaline treatment under similar conditions.

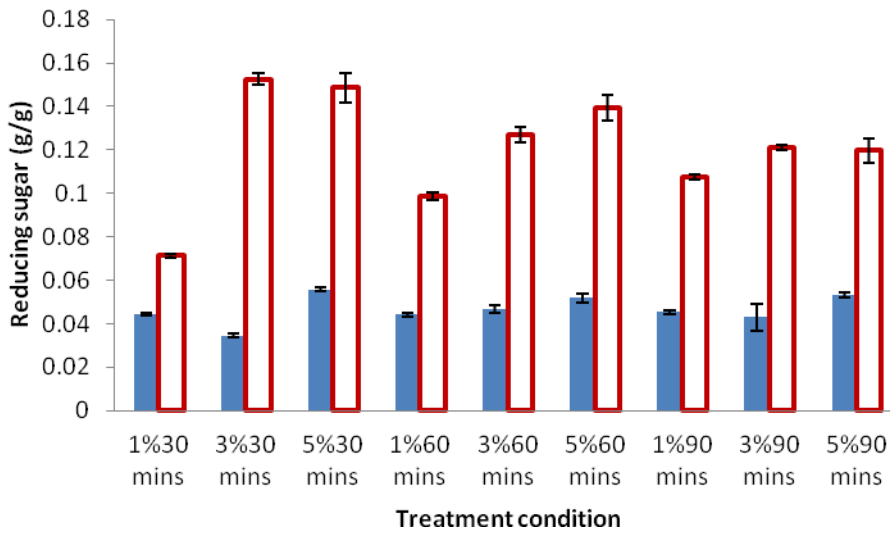

Figure 4.1: Yield of reducing sugar under different pretreatment conditions. Symbol: ( ), H_2SO_4 treatment; ( ), NaOH treatment.

Generally, the effects of acid and alkaline treatment on SB were noticeable. Alkaline-treated SB exhibited a better yield of reducing sugar as compared with acid-treated SB. The purpose of alkaline treatment is to remove lignin from lignocellulosic material while acid pre-treatment help to solubilize mostly the hemicellulose (Ramos, 2003). The reason of lower yield on acid-treated bagasse roughly correlates with reported values of low lignin removal during acid treatment, as discussed in Section 4.3.2. The low removal of lignin content caused low yield of reducing sugar during subsequet saccharification process. Lignin present in the biomass and gives rigidity to the plant cell wall. Hence, the higher content of lignin in acid-treated bagasse caused it resistance to enzymatic attack. Chang and Holtzapple (2000) proved that biomass digestibility is enhanced with lignin removal. The increase of reducing sugar is due to the removal of lignin content during pre-treatment process.

With alkaline pretreatment, the lignin content of lignocellulosic biomass is altered, and hence increasing the digestibility of cellulose and hemicelluloses (Pedersen et al., 2010). When most of the lignin in alkaline-treated lignocellulosic biomass was removed, it is no longer acting as a protective layer, hence making cellulose more susceptible to enzymatic attack (Yang and Wyman, 2004). By referring to Table 4.1, lignin content of NaOH-treated SB is considerably lower as compared to H_2SO_4-treated SB, and hence leading to higher yield of reducing sugar during enzymatic saccharification process. The breakdown of cellulose structure by cellulase enzyme is illustrated in Figure 4.2. Cellulose from treated lignocellulosic biomass is hydrolyzed by enzyme cellulase, creating free chain ends in cellulose. Cellulose is further degraded and breakdown to cellulobiose units from the chain ends. The cellulobiose units breakdown by enzymes, producing glucose (Cao and Tan, 2002; Prasad et al., 2007).

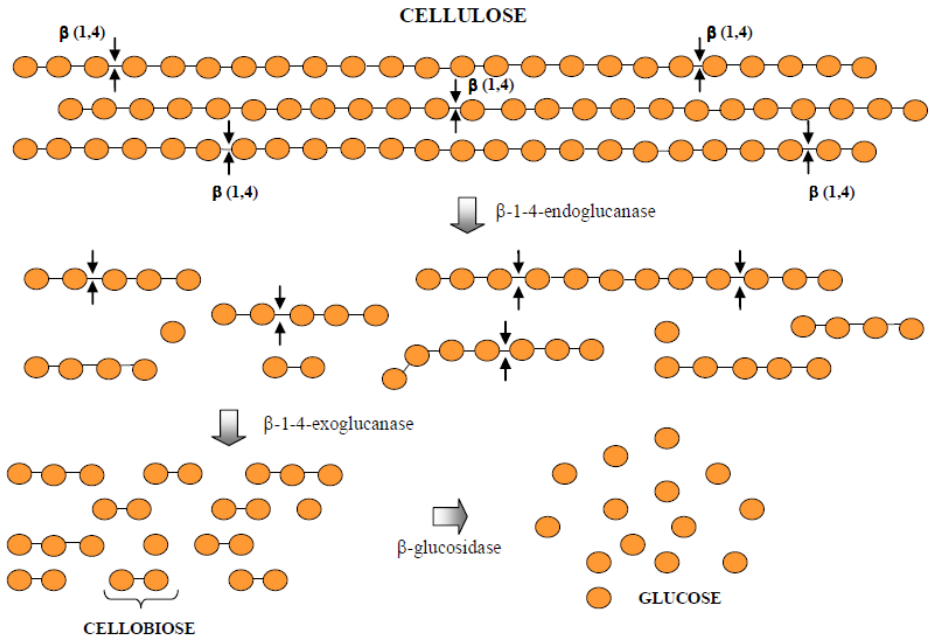

Figure 4.2: Breakdown of cellulose structure by celluase enzyme (Source: Mussatto and Teixeira, 2010)

Concentration of chemicals used and treatment time are main factors affecting the enzymatic saccharification and hydrolysis process. According to Shah et al. (2011), empty fruit bunches (EFB) treated by using 3% NaOH at 50^{o}C gave the highest yield of reducing sugar (175.03 mg/g). Xu et al. (2011) also reported better performance of enzymatic hydrolysis on mild NaOH pre-treated switchgrass, with the reducing sugar yield of 0.4314 g/g, while mild H_2SO_4 pre-treatment gave significant lower sugar yield of 0.1994 g/g. Parameswaran et al. (2012) found that when sugarcane bagasse was treated with 1% NaOH by using microwave for 4 min, followed by enzymatic saccharification, the yield of reducing sugar (0.665 g/g) is higher as compared to 1% H_2SO_4, under the same condition with the yield was only 0.091 g/g. A combination of treatment increases not only improve the rate of enzymatic hydrolysis, but also reduce the time of treatment and the usage of chemical during pre-treatment.

Main findings of the previously reported researches shared the same idea of pre-treatment by using low concentration of chemical to extract fermentable sugar from lignocellulosic waste. In terms of economical feasibility, high concentration usage of chemicals involves expensive cost in increasing the accessibility of cellulose content by enzymatic attack. In addition, neutralization of the waste generated is required after the strong acid or alkaline pretreatment. Hence, the high cost and expensive treatment process causes less commercial interest in this kind of treatment (Sun et al., 2004). In the present study, mild NaOH treatment on SB is sufficient to examine the bioconversion feasibility of SB into fermentable sugar. Current results proved

that alkaline-treated SB has the potential to produce high level of fermentable sugar for subsequent fermentation process.

4.3.2 Characterization of Pretreated Sugarcane Bagasse

Analysis of Lignocellulosic Compounds

Table 4.1 summarizes the compositions of cellulose, hemicellulose and lignin of native SB, acid-treated and alkaline-treated SB. From the results, treatment time and treatment concentration possess significant effect on the content of cellulose and lignin. Lignin content was significantly removed during NaOH treatment as compare with native SB and acid-treated SB. The maximum removal of lignin was 51.07% by using 5% (w/v) NaOH and treatment duration of 90 min with the highest level of cellulose (63.24%) was achieved. As for H_2SO_4 treatment, the highest removal of lignin content was only 27.22% by using 5% (w/v) H_2SO_4 for 30 min indicated that only small amount of lignin was removed from sugarcane bagasse.

Cellulose content of SB treated by using 5% (w/v) of NaOH for 90 min was about three times higher (63.24%) as compared to the cellulose content of the native SB (27.54%). The cellulose content increased while the hemicellulose and lignin content decreased during the entire NaOH treatment process, with increasing of NaOH concentration from 1% to 5% (w/v) and also with increasing of treatment time (Table 4.1).

Table 4.1: Compositions of cellulose, hemicellulose and lignin of native SB, acid-treated and alkaline-treated SB

Treatment Conditions	Hemicellulose (%)	Cellulose (%)	Lignin (%)
Native SB	28.82	27.54	15.10
H_2SO_4-treated			
1% 30min	24.87	56.72	11.29
1% 60min	26.03	58.37	11.94
1% 90min	25.39	57.44	11.37
3% 30min	25.61	54.97	11.95
3% 60min	21.55	60.83	12.71
3% 90min	21.70	60.61	13.35
5% 30min	26.73	55.18	10.99
5% 60min	25.43	53.98	11.27
5% 90min	22.56	58.88	12.57
NaOH-treated			
1% 30min	28.63	55.05	10.83
1% 60min	27.23	57.35	11.08
1% 90min	28.39	56.85	8.85
3% 30min	26.46	58.98	9.25
3% 60min	23.65	58.75	8.96
3% 90min	23.53	60.17	8.90
5% 30min	23.57	60.64	8.07
5% 60min	20.28	61.77	8.53
5% 90min	19.93	63.24	7.39

Hemicellulose acts as a physical barrier and avoids cellulose from enzymatic attack (Taherzadeh and Karimi, 2007). Hence, removal of hemicellulose is necessary to enhance enzymatic attack on cellulose in order to increase fermentable sugar content during fermentation process. From Table 4.1, the hemicellulose content decreased from 28.82% to 19.93% by using 5% (w/v) of NaOH with the treatment time of 90 min. From the current study, it is suggested that removal of hemicellulose was successful by using alkaline solution (NaOH) as treatment agent to pretreat the sugarcane bagasse.

According to Fan et al. (1980), NaOH treatment causes swelling of lignocellulosic materials, leading to an increase in the internal surface area of the lignocellulosic biomass. As a result, the structure of lignin is disrupted and separated from cellulose and hemicellulose. Lignin is removed in the form of phenolic compound through the washing process after pre-treatment (Mussatto et al., 2007). However, lignin content can be easily re-condensed and precipitate during acid pre-treatment (Hendriks and Zeeman, 2009). In the present study, percentage removal of lignin by H_2SO_4 pretreatment is two times lower compared to those sugarcane bagasse treated by NaOH (Table 4.1), and thus explain the merits of using NaOH pretreatment on lignocellulosic materials. The removal of lignin content disrupted the structure of lignocellulosic materials causing cellulose in sugarcane bagasse no longer being shielded by lignin and hence the higher cellulose content was found in NaOH treated sugarcane bagasse.

In the present study, an increase of cellulose content increase the fermentable sugar availability for enzymatic attack, resulting in a high yield of reducing sugar with 0.153 g/g. At the same time, a decrease of hemicellulose and lignin content were observed. Selig et al. (2009) performed the treatment of corn stover at pH 11.5, 50^{o} C, with the treatment time of 3 hours. They found that the removal of lignin and hemicellulose reached up to 56.3% and 14.0%, respectively. In addition, Sun et al. (2000) conducted alkaline treatment and found that 83.3% and 70.0% of lignin and hemicellulose was removed from the native rye straw, with the alkaline treatment at pH 11.5, 50^{o} C, for 12 hours.

X-ray Diffraction Analysis

Crystalinity of biomass plays an important role in affecting the enzymatic hydrolysis process (Sun and Cheng, 2002). In order to further examine the condition of treated and native bagasse, crystalinity index (CrI) of the bagasse was measured. From Table 4.2, the native sugarcane bagasse has the lowest crystalinity index of 73.55%. After pre-treatment with H_2SO_4 for 30 min, the crystalinity index of SB slightly increased to about 78%. As the treatment time prolonged to 60 min, there was a minor increase of CrI to 82.35% for the treatment by using 5% (w/v) H_2SO_4. The crystalinity index of H_2SO_4 pre-treatment reached a plateu (CrI of about 80%) when the treatment time extended to 90 min. In contrast, a noticeable increase of crystalinity index was observed in NaOH-treated SB with increase dosage of NaOH and prolong of treatment time. The highest CrI recorded for NaOH-treated SB is 85.30%, by treatment using 5% (w/v) of NaOH for 60 min of treatment time.

According to Parameswaran et al. (2012), the removal of lignin content at the armophous region of biomass caused increasing of the crystalinity index. Findings from Kim and Holtzapple (2006) also proved an increase of CrI from 43% to 69% when lignin content of corn stover was removed by calcium hydroxide. As discussed earlier, higher percentage of lignin removal was achieved when NaOH treatment was applied (Table 4.1). Hence, when percentage of lignin decreased, the CrI increased.

Table 4.2: Cyrstalinity index (CrI) of native, H_2SO_4 and NaOH treated sugarcane bagasse with different concentration and treatment time

Treatment Condition	Crystalinity Index (%) of Sugarcane Bagasse		
	Native	**H_2SO_4-treated**	**NaOH-treated**
Nil	73.6		
1% 30min		78.0	80.2
3% 30min		78.0	81.7
5% 30min		78.7	82.3
1% 60min		81.0	81.1
3% 60min		81.9	81.9
5% 60min		82.4	85.3
1% 90min		79.1	80.9
3% 90min		80.9	82.0
5% 90min		80.6	84.6

Scanning Electron Microscopy (SEM)

SEM image of the native sugarcane bagasse clearly shown that the native sugarcane bagasse has a smooth and tearless surface (Figure 4.3) as compared to NaOH and H_2SO_4 treated bagasse which both have rough surface with apertures (Figure 4.4 and Figure 4.5). The appearance of NaOH-treated bagasse (Figure 4.4) has more holes compared to H_2SO_4-treated biomass (Figure 4.5), indicating NaOH treatment increased surface area of the substrate effectively. Hence, the cellulose of NaOH-treated SB is now more accessible to enzymatic hydrolysis, which was proven by higher yield of reducing sugar from this bagasse.

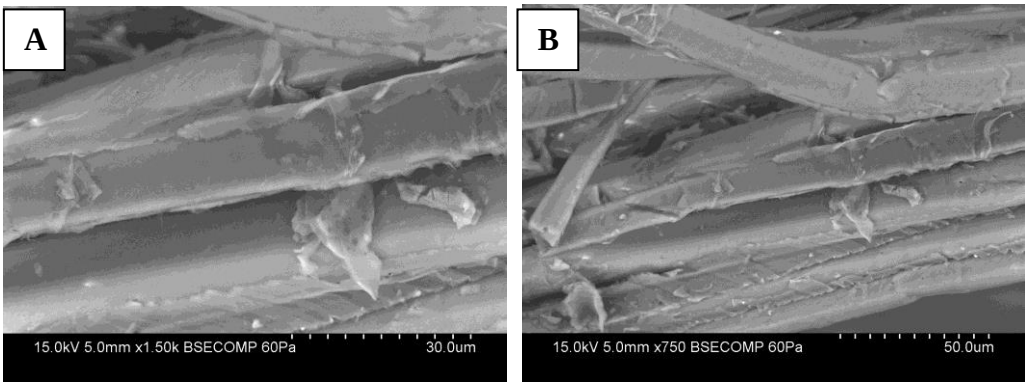

Figure 4.3: SEM images of native sugarcane bagasse. Symbols: (A), x1500 magnification; (B), x750 magnification

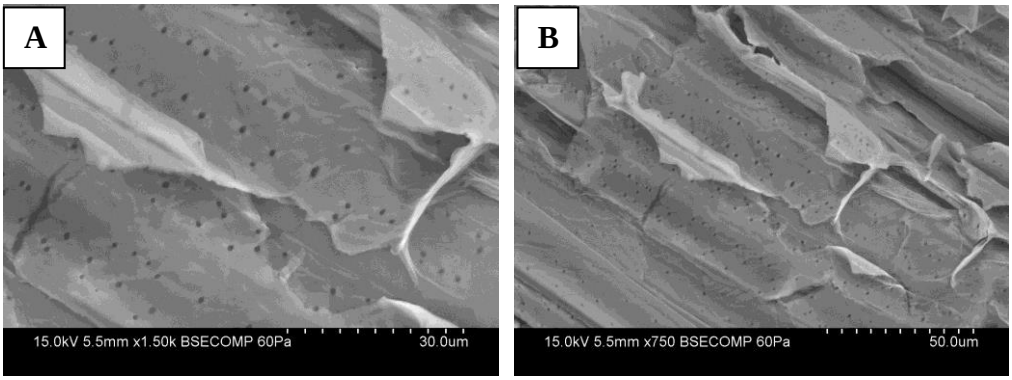

Figure 4.4: SEM images for alkaline-treated sugarcane bagasse. Symbols: (A), x1500 magnification; (B), x750 magnification

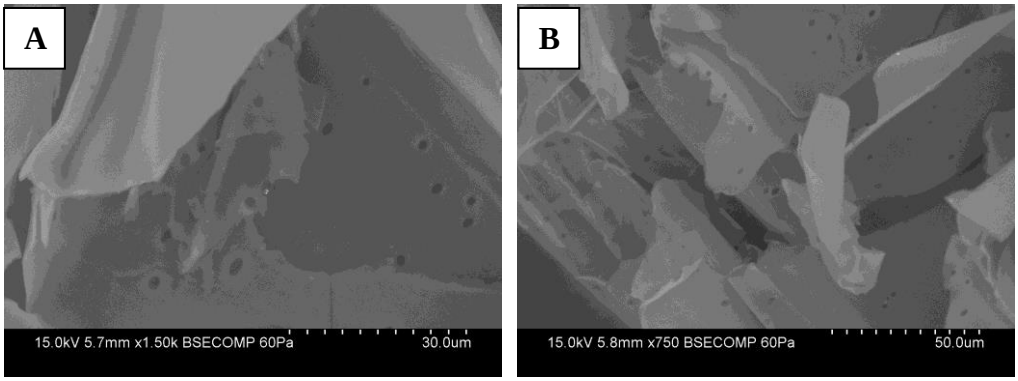

Figure 4.5: SEM images for acid-treated sugarcane bagasse. Symbols: (A), x1500 magnification; (B), x750 magnification

4.4 Concluding Remarks

In order to select the most promising treatment method on conversion of SB to fermentable sugars for subsequent xanthan gum production, NaOH and H_2SO_4 were two chemicals used to pretreat SB prior to enzymatic hydrolysis. According to the analysis of lignocellulosic compounds, NaOH-treated SB has higher cellulose content compared to H_2SO_4-treated SB.

The impact and changes of structure of NaOH- and H_2SO_4-treated SB were studied by using X-ray diffraction (XRD) analysis. The crystalinity index (CrI) of SB increased after treating either by using NaOH or H_2SO_4 solution, due to removal of lignin content after pretreatment. Moreover, the structure of NaOH-treated bagasse had seriously altered compared to the structure of H_2SO_4-treated SB through SEM analysis.

Sugarcane bagasse treated with 3% (w/v) NaOH for 30 min of treatment time gave the highest yield of reducing sugar (0.1526 g/g). The reducing sugar produced from sugarcane bagasse after enzymatic hydrolysis is an important factor that must be taken into consideration for maximum production of xanthan gum. Hence, the pretreatment conditions by using 3% (w/v) NaOH and 30 min duration was selected and used to pretreat the SB throughout the subsequent projects.

CHAPTER 5

STATISTICAL OPTIMIZATION OF SOLID-STATE XANTHAN FERMENTATION PROCESS

5.1 Introduction

The cost on production in xanthan is high and been the major concern of the industry. Hence, most of the recent researches have been focused on using agricultural waste producing xanthan (Stredansky and Conti, 1999; Kianoush et al., 2011). In this present study, sugarcane bagasse, a locally and abundantly available agricultural waste was used as substrate for producing xanthan under solid-state fermentation process.

In this study, solid-state fermentation was proposed as an alternative way for the production of xanthan gum by using sugarcane bagasse as carbon source. Plackett Burman design (PBD) was first used to screen out variables which possess significant effect which contribute the most on the yield of xanthan gum. Several affecting variables such as moisture content, substrate loading, organic nitrogen sources (yeast extract and tryptone), trace elements (NH_4NO_3, KH_2PO_4 and $Mg.Cl_2.6H_2O$) and inoculum size on xanthan production were

studied. Following this, the significant variables were then further optimized by using Response Surface Methodology (RSM), based on central composite design (CCD).

5.2 Materials and Methods

5.2.1 Plackett Burman Design (PBD)

Plackett Burman design (PBD) was applied to find and screen the significant variables on the production of xanthan gum. This design is able to screen and eliminate variables which are important in the model (Ooijkaas et al., 2000).

This design is based on first order model:

$$Y = \beta_0 + \Sigma \beta_i X_i \qquad \text{Eq. 5.1}$$

where Y is the response, β_0 is the model intercept, β_i is the linear coefficient, and X_i is the level of independent variable (Plackett and Burman, 1946). A PBD with nine variables, resulting in 12 runs with 3 runs of center point were used to determine the most significant parameters on the yield of xanthan gum. Each parameter was designated into high level (+) and low level (-) as described in Table 5.1. The choices and selection of nutrients (NH_4NO_3, yeast extract, tryptone, KH_2PO_4, $MgCl_2.6H_2O$, and $NaSO_4$) and moisture content were based on the literature survey of related researchers on xanthan production (Abd El-Salam et al., 1994; Stredansky and Conti, 1999; Lopez et al., 2004; Marceli et al., 2009; Marzieh et al., 2010). In this design, all

variables were assumed have no interactions and a first order multiple regression model was applied. Two dummy variables (K and L) were also studied in order to calculate the standard error (Reddy et al., 2012).

Table 5.1: Plackett Burman design and statistical analysis for xanthan gum production.

Code	Independent Variable	Unit	Low Level (-1)	High Level (+1)
A	Substrate Loading	g	5	15
B	Moisture Content	%	70	90
C	NH_4NO_3	g/L	0	10
D	Yeast Extract	g/L	0	10
E	Tryptone	g/L	0	10
F	$KH_2\ PO_4$	g/L	2	9
G	Na_2SO_4	g/L	0	3
H	$MgCl_2.6H_2O$	g/L	0.1	0.6
J	Inoculum Size	% v/w	3	15

5.2.2 Response Surface Methodology (RSM) with Central Composite Design (CCD)

Based on the results from PBD, the variables with high percentage of contribution (> 5%) on the yield of xanthan was selected and further optimized for xanthan production. Four important variables substrate loading, moisture content, NH_4NO_3, and yeast extract were selected as response variable (Table 5.2). Details on the selection of variables are explained in Section 5.3.1.

Table 5.2: Actual factor levels and coded factor levels

Code	Independent Variable	Unit	Actual and Coded Factor Level				
			-α	**-1**	**0**	**1**	**α**
A	Substrate Loading	g	2	4	6	8	10
B	Moisture Content	%	80	82.5	85	87.5	90
C	NH4NO3	g/L	0	1.25	2.5	3.75	5
D	Yeast Extract	g/L	5	7.5	10	12.5	15

Each variable in the design was varied over 5 levels, plus and minus alpha (axial points) with 6 runs, plus and minus 1 (factorial points) with 18 runs and center point with 6 runs, leading to a total of 30 runs were carried out and studied. The actual and coded factor level is shown in Table 5.2.

All of the variables were coded according to the following Equation 5.2 (Chen et al., 2012):

$$x_i = (X_i - X) / \Delta X_i \qquad \text{Eq. 5.2}$$

where x_i and X_i are coded and actual values respectively of independent variable $_i$, X is the actual value of the independent variable at the central point and ΔX_i is the step change value.

The experimental data were fitted into polynomial model to find the best model. The model expected to be a quadratic (Equation 5.3) or cubic (Equation 5.4) equation, where optimum xanthan production can be found.

$$Y = \beta_0 + \Sigma \beta_i x_i + \Sigma \beta_i x_i^2 + \Sigma \beta_{ij} x_i x_j \quad \text{Eq. 5.3}$$

or

$$Y = \beta_0 + \Sigma \beta_i x_j + \Sigma \beta_{ij} x_i x_j + \Sigma \beta_{ijk} x_i x_j x_k \quad \text{Eq. 5.4}$$

where Y is the predicted response, β_0, β_i, and β_{ij} are the regression coefficient for intercept, linear and interaction terms respectively, while x_i and x_j are both independent variables. Analysis of variance (ANOVA) is performed and three dimensionless response surface graphs were plotted to understand the interaction of variables on xanthan production. Validation of the model was conducted in order to check the adequacy of the developed model.

5.3 Results and Discussion

5.3.1 Plackett Burman Design (PBD) Analysis

In the present study, the Plackett Burman design (PBD) was used to search for significant variables which affect the yield of xanthan gum. An experiment design matrix with the application of two-level factorial model was used in order to screen significant variables on the production of xanthan. The design matrix with 9 factors, resulting in 12 run, with 3 center points, each with different combination of high, medium and low level of the parameters were

tested. Table 5.3 illustrates overall design setup of the experiment and experimental results of maximum xanthan yield.

Table 5.3: PBD variables (in coded levels) with xanthan yield as response

Run	A	B	C	D	E	F	G	H	J	Xanthan yield (g/g)
1	-1	1	1	-1	1	1	1	-1	-1	0.0512
2	-1	1	1	1	-1	-1	-1	1	-1	0.0952
3	1	-1	1	1	-1	1	1	1	-1	0.0275
4	1	-1	1	1	1	-1	-1	-1	1	0.0277
5	1	1	-1	1	1	1	-1	-1	-1	0.1000
6	1	1	1	-1	-1	-1	1	-1	1	0.0445
7*	0	0	0	0	0	0	0	0	0	0.0588
8*	0	0	0	0	0	0	0	0	0	0.0584
9	-1	-1	-1	1	-1	1	1	-1	1	0.0616
10	-1	-1	1	-1	1	1	-1	1	1	0.0280
11	1	1	-1	-1	-1	1	-1	1	1	0.0464
12	1	-1	-1	-1	1	-1	1	1	-1	0.0485
13	-1	1	-1	1	1	-1	1	1	1	0.1048
14*	0	0	0	0	0	0	0	0	0	0.0588
15	-1	-1	-1	-1	-1	-1	-1	-1	-1	0.0416

* Center point

Analysis of variance (ANOVA) of Plackett Burman Design (PBD)

The adequacy of the model was calculated, and the variables with statistically significant effects were monitored by using ANOVA analysis. As observed in Table 5.4, substrate loading (A), moisture content (B), NH_4NO_3 (C), yeast extract (D), tryptone (E), KH_2PO_4 (F), $MgCl_2.6H_2O$ (H), and inoculum size (J) are significant variables with confidence level above 95%. In addition, the low

p-value of all variables (*p*-value < 0.0001) indicates that these variables had a direct relationship on yield of xanthan. Out of eight significant variables, only moisture content, yeast extract, tryptone, and $MgCl_2.6H_2O$ exerted positive effect while substrate loading, NH_4NO_3, KH_2PO_4, and inoculum size possess negative effect towards yield of xanthan (Table 5.4). The effect of Na_2SO_4 was found insignificant and hence was excluded from further studies.

Table 5.4: Analysis of variance of PBD model

Code	Independent Variable	Coefficient Estimate	Percentage of Contribution (%)	*p*-value Prob > F
A	Substrate loading	-0.0073	7.3	< 0.0001
B	Moisture content	0.0173	40.6	< 0.0001
C	NH_4NO_3	-0.0107	15.7	< 0.0001
D	Yeast extract	0.0130	23.2	< 0.0001
E	Tryptone	0.0036	1.8	< 0.0001
F	$KH_2 PO_4$	-0.0040	2.2	< 0.0001
H	$MgCl_2.6H_2O$	0.0020	0.5	< 0.0001
J	Inoculum size	-0.0042	2.5	< 0.0001

R^2: 0.99998; Adjusted- R^2: 0.99994; Curvature: *p*-value = 0.0006; Lack of fit: *p*-value = 0.4226

Plackett Burman design has shown a good fit with the experimental data, with correlation coefficient, R^2 and Adjusted-R^2 are 0.99998 and 0.99994, respectively. Value of R^2 of the model near to 1.0 would explain better variability of experimental values to predicted response (Sayyad et al., 2006). A significant *p*-value of model (*p*-value< 0.0001), non-significant value lack of fit (*p*-value = 0.4226) indicates that the model is significant (Box et al., 1978).

Nonetheless, significant value of curvature (*p*-value = 0.0006) explained that the predicted value at the centre point is significantly different from the value obtained when conducting the centre point conditions. Highly significance of curvature in other words shows that at least one variable is involved in an order higher than one (Tasharrofi et al., 2011), and hence a linear model will not be suitable in identifying the maximum concentrations of significant variables. Hence, a higher order model such as response surface methodology can be employed for further studies.

Selecting Significant Operating Variables by Plackett Burman Design (PBD)

Half-normal plot and Pareto chart provide a convenient way to understand results of Plackett Burman design, as well as to indentify the most crucial factors affecting the production of desired product (Haaland, 1989; Strobel and Sullivan, 1999). In addition, half-normal plot and Pareto chart allows classification of parameters that possess positive or negative effect. Variable possessed positive effect on xanthan yield is indicated in orange color; while variable possessed negative effect on xanthan yield is indicated in blue color (Figure 5.1).

Half-normal probability plot is a graphical method to test if a data set is approximately normally distributed. Experimental data were plotted against the theoretical normal distribution (red line). Important variables are well dislodge in the plot and appear at the upper-right side of the graph, showing a significant

effect on xanthan yield; while the unimportant variables which have minimal effect on xanthan yield appear at the lower-left part of the graph. Figure 5.1 shows eight variables are significant and contributed on the production of xanthan. Replicates which are the center points (green triangle box) that had a negligible effect on xanthan yield were within the normal distribution.

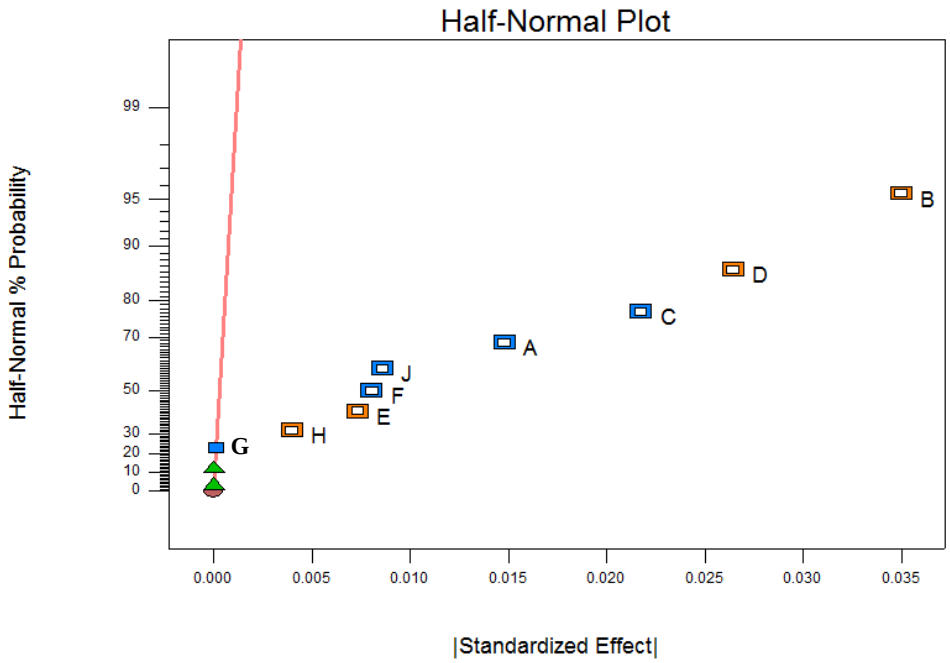

Figure 5.1: Half-normal probability plot of the standardized effect for xanthan yield. Symbols: error from replicates, (▲); positive effect, (▭) and negative effect, (▭)

Pareto chart (Figure 5.2) illustrates the maximal effect was shown on the left side and then shifted towards right side for the minimal effect. In addition, two horizontal lines in red and blue can be observed across the chart. The red line represents the Bonferroni limit, while the blue line represents the *t*-value limit. The significance of each variable was determined by the *t*-value limit, where variables above the *t*-value were significant. Parameters above the Bonferroni limit were relatively more significant than *t*-value limit. This is due to

Bonferroni limit is more conservative compared to *t*-value limit (Anderson and Whitcomb, 2000), as the Bonferroni controls making false discovery and the probability of eliminating correct null hypothesis (Abdi, 2007); while the *t*-value limit had a 95% significant. Hence, from Figure 5.2, substrate loading (A), moisture content (B), NH_4NO_3 (C), yeast extract (D), tryptone (E), KH_2PO_4 (F), $MgCl_2.6H_2O$ (H), and inoculum size (J) are significant variables with confidence level above 95%.

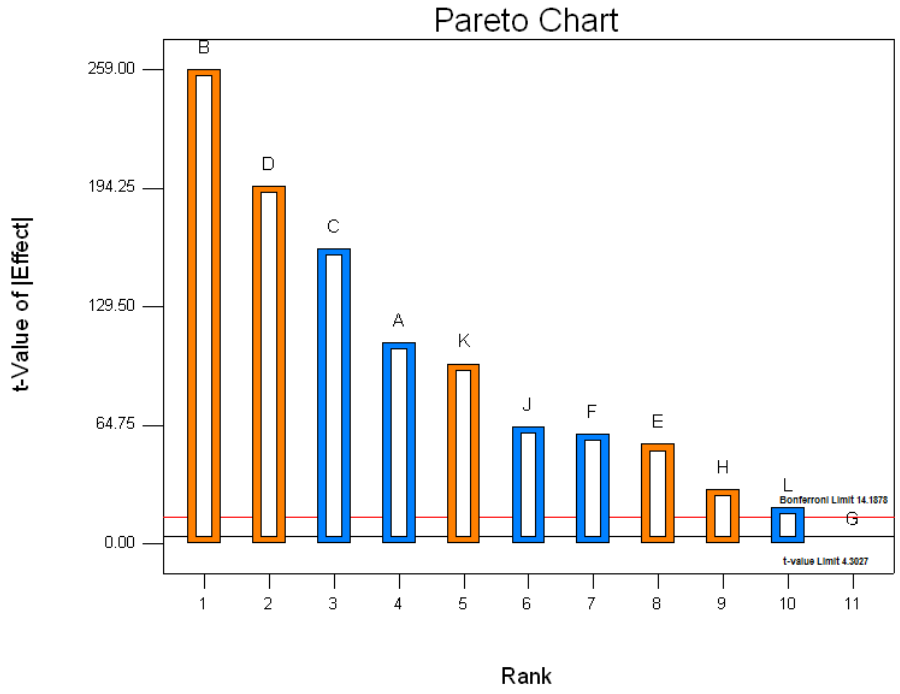

Figure 5.2: Pareto chart from Plackett Burman design (PBD). Symbols: positive effect (▭) and negative effect (▭)

Table 5.4 also explains the size of coefficient estimate and the percentage of contribution of all the parameters on xanthan yield. Size of coefficient estimate and percentage of contribution gives the direct measurement of the importance of each variable (Psomas et al., 2007). From Table 5.4, the maximal effect on the production of xanthan gum was moisture content with the percentage of

contribution 40.64%, followed by yeast extract (23.2%), NH_4NO_3 (15.71%) and substrate loading (7.29%).

On the other hand, tryptone, KH_2PO_4, $MgCl_2.6H_2O$ and inoculum size possess lesser effect on production of xanthan gum with percentage of contribution (%) lower than 5%. For the ease of subsequent optimization study, these four variables were fixed at either low or high level (Table 5.1), depends on the negative or positive effects, as observed from PBD analysis, respectively. For example, tryptone and $MgCl_2.6H_2O$ are with positive values of coefficient estimate, hence, their concentrations were fixed at 10 g/L and 0.6 g/L for subsequent optimization process, respectively. For variables KH_2PO_4 and inoculum size with negative values of estimate, the concentration used were fixed at 2 g/L and 3% (v/w), respectively.

In the present study, moisture content is the most influencing effect on the production of xanthan. Water content on the inert support of solid substrate is one of the major factors which influence the microbial growth and productivity of xanthan. According to Stredansky and Conti (1999), the highest productivity of xanthan was observed with 75% of moisture content while no significant improvement was observed when the moisture content further increased to 83%.

As observed from Table 5.4, nutrient source such as KH_2PO_4, $MgCl_2.6H_2O$, tryptone and inoculum size had less effect on the yield of xanthan. The mineral salts and trace elements such as phosphorus and magnesium are mainly

required for the growth of microorganism (Garcia et al., 1992). In addition, Davidson (1978) found an increase of xanthan yield when phosphorus was the limiting nutrient, which further explained by the negative effect of KH_2PO_4 on the production of xanthan in the present study (Figure 5.1 and 5.2).

According to Cadmus and Knutson (1983), an inoculum size between 5% (v/v) to 15% (v/v) is suitable for the production of xanthan. Lower percentage of inoculum size causes prolonged lag initial growth of the microorganism, resulting in longer fermentation time to achieve maximum xanthan yield. Their earlier research also stated that the usage of organic nitrogen sources hardly enhances the production of xanthan in comparison with inorganic nitrogen sources. There were controversies between researchers about the usage of organic and inorganic nitrogen source for microorganism growth and xanthan production. Detail information on the effect of nitrogen sources are explained in Section 5.3.2, under the subtopic of nitrogen sources.

5.3.2 Optimization Process

Based on results from Plackett Burman design (PBD), only four variables (substrate loading, moisture content, NH_4NO_3 and yeast extract) were selected and further optimized by using Response Surface Methodology (RSM) with a full factorial design of Central Composite Design (CCD) in optimizing the xanthan yield. Tryptone, KH_2PO_4, $MgCl_2.6H_2O$, and incoulum size are four significant variables with low percentage of contribution, ranging from 0.53%

to 2.46% (Table 5.4). Hence, these variables were not included in CCD studies but were fixed according to the negative or positive effects of the PBD results.

As illustrated in Table 5.5, the model of RSM on the production of xanthan gum was highly significant, with *p*-value <0.0001, with an acceptable R^2 value and insignificant lack-of-fit (*p*-value >0.05).

Table 5.5: Regression analysis (ANOVA) of RSM for production of xanthan gum

Source	Sum of squares	Mean square	F-value	*p*-value Prob>F
Block	6.27×10^{-5}	6.27×10^{-5}		
Model	1.57×10^{-2}	7.46×10^{-4}	92.31	< 0.0001
Residual	5.65×10^{-5}	8.08×10^{-6}		
Lack of Fit	3.43×10^{-5}	1.14×10^{-5}	2.06	0.2482
Pure Error	2.22×10^{-5}	5.56×10^{-6}		
Correlation Total	1.58×10^{-2}			

R^2: 0.99640; Adjusted- R^2: 0.98561; Lack of fit: *p*-value = 0.2482

The ANOVA analysis shows that the model was significant with high coefficient of determination (R^2), 0.9964. This measure the significance of the model which explained that 99.64% of the variability in the response could be explained by the model; while less than 1% of the total variations were due to noise. Besides, the R^2 value showed that only 0.36% of the total variation cannot be explained by the model. Hence, the R^2 value reflected an excellent fit between the observed and predicted values. The value of lack-of-fit was not significant (*p*-value = 0.2482) indicates that the model was suitable in predicting xanthan yield under permutation of the values of the parameters involved (Zhang, 2012).

The observed response yield of each run was shown in Table 5.6 and the significance of each variable was verified by *p*-values (Table 5.7). The smaller the *p*-value, the higher the significance of the variable (Khuri and Cornell, 1987). The *p*-value which more than 0.05 indicates the corresponding variable is not significant. As observed from ANOVA analysis in Table 5.7, all linear parameters were statistically significant (*p*-value < 0.05), except NH_4NO_3 with *p*-value 0.8313 (Table 5.7). Nevertheless, this variable was included in the cubic model to make sure a hierarchical model, as the variable NH_4NO_3 was involved in the interaction at second and third order of the model.

As for higher order, interaction of substrate loading and moisture content (AB), moisture content and NH_4NO_3 (BC), and interaction between moisture content and yeast extract (BD) were significant with *p*-value less than 0.05. The significant interaction indicates a direct relationship on the yield of xanthan gum by combining the two variables during cultivation process.

The optimal conditions for xanthan production were determined by constructing countor plots in order to study and understand the interaction of each variable on xanthan yield. By using the three-dimensional plots, the optimal levels can be easily seen and understood. From the response surface plots (Figure 5.3 to 5.5), the maximum yield of xanthan achieved was 0.13 g/g.

The yield of xanthan in this study was affected by:

(a) Substrate loading and moisture content;

(b) Nitrogen sources (NH_4NO_3 and yeast extract)

Table 5.6: Actual and predicted yield of xanthan from CCD of RSM

Run	Substrate Loading	Moisture Content	NH_4NO_3	Yeast extract	Actual yield (g/g)	Predicted xanthan (g/g)
1	6	85	2.5	10	0.049	0.050
2	6	85	2.5	10	0.049	0.050
3	6	85	2.5	10	0.051	0.050
4	8	87.5	1.25	7.5	0.057	0.061
5	8	82.5	3.75	12.5	0.040	0.042
6	8	87.5	3.75	7.5	0.049	0.050
7	4	82.5	1.25	12.5	0.076	0.076
8	4	87.5	3.75	7.5	0.085	0.087
9	4	82.5	1.25	7.5	0.088	0.092
10	8	82.5	3.75	7.5	0.075	0.077
11	4	82.5	3.75	7.5	0.112	0.114
12	4	87.5	1.25	12.5	0.085	0.089
13	8	87.5	3.75	12.5	0.054	0.055
14	4	87.5	3.75	12.5	0.077	0.079
15	4	82.5	3.75	12.5	0.064	0.066
16	6	85	2.5	10	0.047	0.050
17	8	82.5	1.25	7.5	0.072	0.071
18	8	87.5	1.25	12.5	0.046	0.045
19	8	82.5	1.25	12.5	0.043	0.046
20	4	87.5	1.25	7.5	0.096	0.096
21	6	85	5	10	0.050	0.048
22	6	90	2.5	10	0.047	0.045
23	6	85	2.5	10	0.054	0.050
24	6	85	0	10	0.059	0.057
25	6	85	2.5	15	0.053	0.052
26	2	85	2.5	10	0.130	0.128
27	6	85	2.5	10	0.049	0.050
28	10	85	2.5	10	0.049	0.048
29	6	85	2.5	5	0.106	0.104
30	6	80	2.5	10	0.090	0.088

Table 5.7: Regression coefficient and *p*-value of ANOVA analysis

Factor	Coefficient Estimate	Standard Error	F value	*p*-value Prob > F
Intercept	0.050067	0.001175	92.31	< 0.0001
A-Substrate Loading	-0.020200	0.001005	404.07	< 0.0001
B-Moisture Content	-0.010833	0.001005	116.22	< 0.0001
C- NH_4NO_3	0.000222	0.001005	0.05	0.8313
D-Yeast Extract	-0.013175	0.001005	171.89	< 0.0001
AB	-0.0016875	0.000711	5.64	0.0493
AC	0.000500	0.000711	0.50	0.5044
AD	0.000563	0.000711	0.63	0.4546
BC	-0.002000	0.000711	7.92	0.0260
BD	0.006188	0.000711	75.83	< 0.0001
CD	-0.001375	0.000711	3.74	0.0942
A^2	0.009483	0.000543	305.34	< 0.0001
B^2	0.004167	0.000543	58.94	0.0001
C^2	0.000667	0.000543	1.51	0.2590
D^2	0.006996	0.000543	166.16	< 0.0001
ABC	0.001875	0.000711	6.96	0.0335
ACD	0.002750	0.000711	14.98	0.0061
BCD	0.003875	0.000711	29.74	0.0010
A^2B	0.009521	0.001231	59.84	0.0001
A^2D	0.003863	0.001231	9.85	0.0164
AB^2	0.004638	0.001231	14.20	0.0070
C^3	-0.000597	0.000410	2.12	0.1888

Substrate loading and moisture content

Figure 5.3 depicts the interaction between substrate loading and moisture content on the yield of xanthan gum, while the other variables were kept at optimal level. Yield of xanthan gum significantly increases with a decrease of substrate loading and moisture content. When the substrate loading was at high level, xanthan yield was low. Xanthan yield rose slightly with a decline in moisture content. However, when substrate loading was at low level, there was a significant enhancement on xanthan yield by decreasing the moisture content.

Both of these variables possess an important role on diffusion of oxygen into the solid matrix during solid state fermentation porcess.

Solid state fermentation (SSF) is defined as microorganism that grows on the surface in the absence of free water (Reeta et al., 2009). The advantages of using SSF including it has higher productivity and higher concentration of end-products, as nutrients which support growth of microorganism are not diluted as compared to submerged liquid fermentation. Furthermore, SSF requires lower energy input and sterility due to the low moisture availability for growth of fungi during SSF (Hölker et al., 2004).

From Figure 5.3, xanthan yield increased with decrease of moisture content from 87.5% to 82.5%. It is believed that the over moisture substrate causes solid clumped together and limits oxygen transfer to the bacteria growth (Lonsane et al., 1985), hence, affecting the yield xanthan gum. In addition, the porosity of solid matrix was reduced with increase of moisture content. On the other hand, too low of moisture content might cause reduction of nutrients solubility from the substrate to the microorganism, causing low production of xanthan gum (Lonsane et al., 1985).

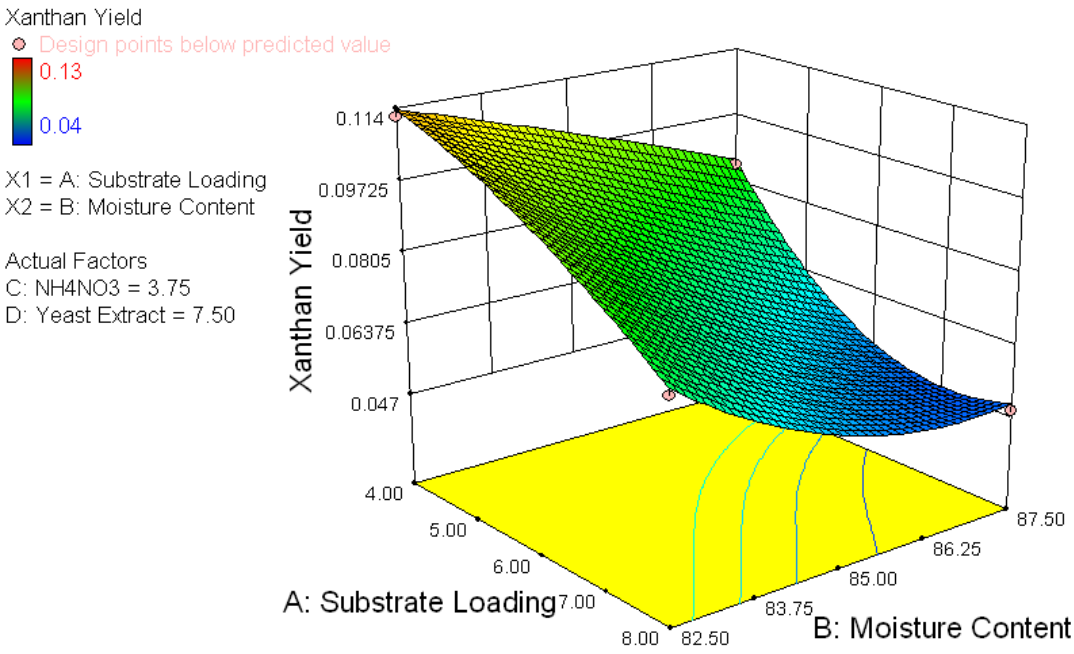

Figure 5.3: Contour plot for interaction of substrate loading and moisture content on the production of xanthan

In the present study, substrate loading is the most significant variable affecting the yield of xanthan gum (Table 5.7). Production of xanthan gum decreased with increasing of solid matrix weight from 4.0 g to 8.0 g. The increase of substrate loading might decrease the contact surface area of air to the microorganism. The gases diffusion might be limited with the reduction of diameter in conical flask as the substrate was packed compactly in the flask (Cannel et al., 1980).

Works by Stredansky and Conti (1999) proved that huge amount of substrate loading decreased the yield of xanthan gum. Besides compact packaging of substrate at high level of loading, the presence of inhibitors after the pre-treatment process may affect the growth of *Xanthomonas campestris* as well as the production of xanthan. Kianoush et al. (2011) pointed out that an increase

in concentration of date extract from 40 g/L to 60 g/L reduced the xanthan yield and cell growth ceased. The reasons might be due to the inhibitors produced from high level of substrate loading after pretreatment process, causing difficulties of cell growth. Inhibitors such as furan, phenolic and acetic acid can be found easily from pretreated lignocellulosic waste (Chandel et al., 2007).

Nitrogen sources

Figure 5.4 illustrates interaction effect between moisture content (B) and yeast extract (D) on the production of xanthan, with the other factors were kept at their optimal level. As can be observed, the increase of xanthan yield was noticeable at low level of moisture content when the concentration of yeast extract decreased. Conversely, at high level of moisture content, decreasing of yeast extract caused an increment on xanthan yield. This phenomenon explained the negative effect of yeast extract on the production of xanthan.

Figure 5.5 shows the interaction between moisture content (B) and ammonium nitrate (NH_4NO_3) (C). Yield of xanthan was greatly enhanced with the presence of NH_4NO_3 when the moisture content was kept at its optimal level (82.5%). By comparing the usage of organic nitrogen source (yeast extract) and with inorganic nitrogen (NH_4NO_3), the addition of yeast extract did not improve the production of xanthan at 82.5% of moisture content.

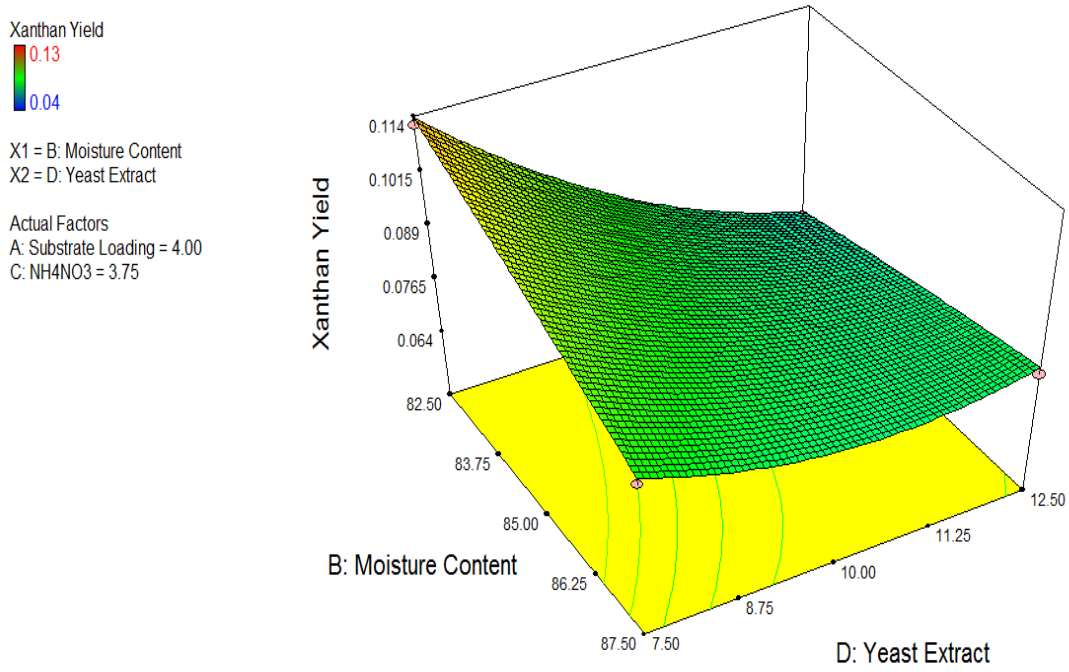

Figure 5.4: Contour plot for interaction of moisture content and yeast extract on the production of xanthan

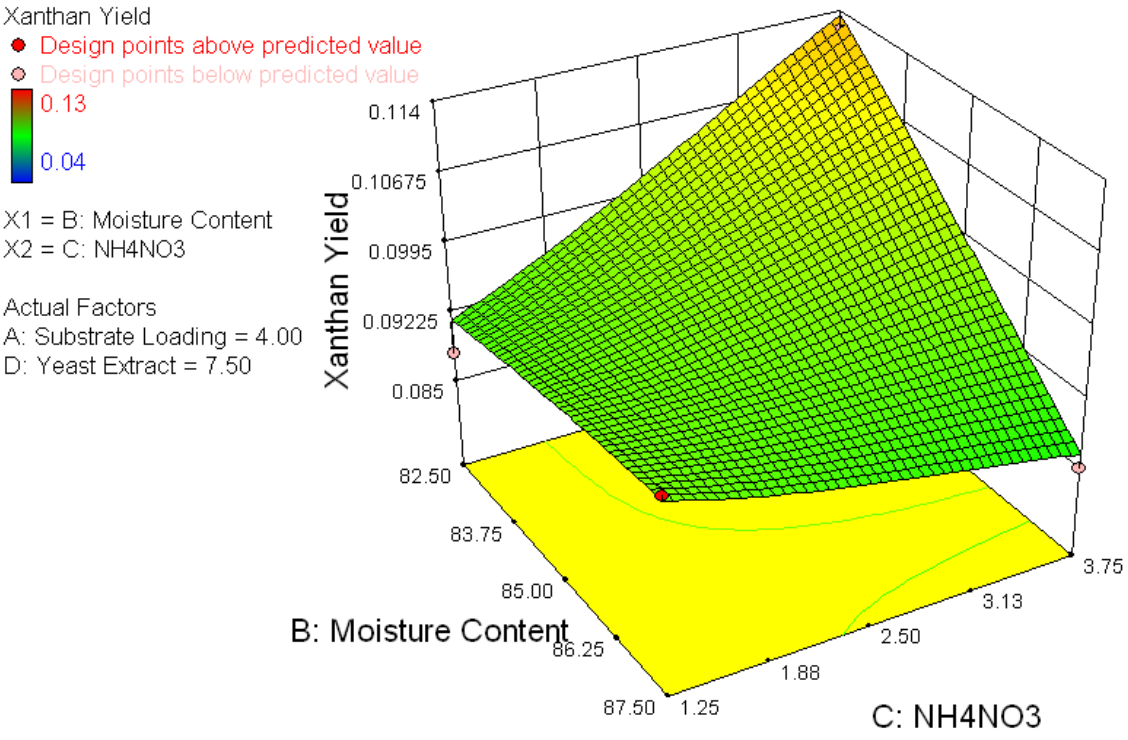

Figure 5.5: Contour plot for interaction of moisture content and NH_4NO_3 on the production xanthan

With reference to Table 5.8, by selecting substrate loading (A) and moisture content (B) at their optimal level, it was shown that Run 11 with the total nitrogen content 151.05 mM had the highest yield of xanthan (0.112 g/g) as compared to Run 9 and Run 15 with the total nitrogen content of 88.55 mM and 189.25 mM, respectively. Nitrogen source is needed for the growth of *Xanthomonas campestris* during microbial fermentation; however, excess addition of nitrogen source decreased xanthan production (Cadmus and Knutson 1983). Besides, Letisse et al. (2001) reported that by increasing the concentration of nitrogen source, xanthan yield decreased though growth of *Xanthomonas campestris* increased. A report from Casas et al. (2000) revealed that initial nitrogen concentration affect xanthan production. Results from their report showed that growth of *Xanthomonas* increased as the initial nitrogen source (NH_4NO_3) increased from 0.57 g/L to 1.14 g/L. Nonetheless, the growth of the microorganism decreased as the concentration of initial nitrogen source increased to 2.29 g/L, leading to a decrease on the production of xanthan gum. Furthermore, Kianoush et al. (2011) explained that limited nitrogen source is needed during the production phase (as it does not prevent cell growth), however, in growth phase, high level of nitrogen source could function as cell growth inhibitor. As a result, a small quantity of total nitrogen source would be sufficient for cell growth while excess nitrogen source might inhibit the formation and production of xanthan.

Table 5.8: Comparison of results for Run 9, 11 and 15 to depict effects of total amount of nitrogen on xanthan yield

Run	A (g)	B (%)	C (g/L)	C (mM)	D (g/L)	D (mM)	Total nitrogen (mM)	Actual yield (g/g)
9	4	83	1.25	31.25	7.5	57.3	**88.55**	0.088
11	4	83	3.75	93.75	7.5	57.3	**151.05**	0.112
15	4	83	3.75	93.75	12.5	95.5	**189.25**	0.064

In addition, as illustrated in Figure 5.4 and Figure 5.5, yeast extract (organic nitrogen source) is with negative effect while NH_4NO_3 (inorganic nitrogen source) possess positive effect on the production of xanthan gum. According to Letisse et al. (2001), inorganic nitrogen sources, such as ammonium nitrate enhanced maximum yield of xanthan as compared with other nitrogen sources. In the present study, similar phenomena was recorded when NH_4NO_3 was used and exerted positive effect on xanthan yield.

Cadmus and Knutson (1983) mentioned that the nitrogen content in the culture medium not only affects the productivity of xanthan, it also influences the quality of xanthan. Their earlier studies reported that the usage of organic materials fail to stimulate high-pyruvate xanthan gum and causes the formation of dark coloration in xanthan gum, which indirectly reduced the gum quality. According to their findings, the usage of inorganic nitrogen source (ammonium nitrate) in fermentation medium produces xanthan with better quality.

5.3.3 Validation Experiments

A validation experiment on xanthan gum production under optimized condition (substrate loading, 2.72 g; moisture content, 81.77%; NH_4NO_3, 3.14g/L and yeast extract, 7.1 g/L), as suggested by RSM software was conducted in order to verify the validity of the statistical experimental strategies.

The changes of pH were measured throughout the fermentation process. The initial pH of cultivation medium was pH 7 (Figure 5.6). In the early stage of the fermentation process, the pH of fermentation medium was relatively steady. After third day of fermentation, the pH of the medium decreased sharply to pH 6.69. As the fermentation process proceeds, the pH tends to drop to pH 5.27 at the seventh day. According to Gilani et al. (2011), the decrease of pH value (acidic condition) was due to the formation of organic acids (acetate and pyruvate) during fermentation process. The acidity of culture medium is one of the factors causing the reduction of xanthan production after fourth day of incubation.

As shown in Figure 5.7, the maximum yield of xanthan (0.1221 g/g) and bacteria growth $2.86x10^{50}$ cfu/mL were achieved at 4th day of incubation. As observed from Figure 5.7, sugar was released from the fermentation process, causing an increase of the bacteria growth, hence, yielding the maximum xanthan at the fourth day during the fermentation process. The reducing sugar from the saccharified sugarcane bagasse was used as the main carbon source for growth of microorganism and xanthan production.

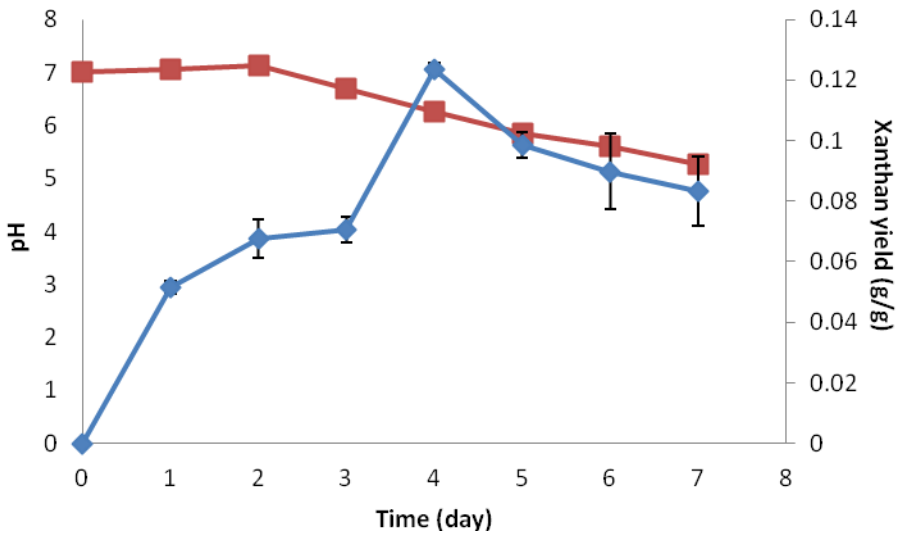

Figure 5.6: **Time course data of pH and xanthan yield under optimized condition. Symbols: pH (■) and xanthan yield (◆)**

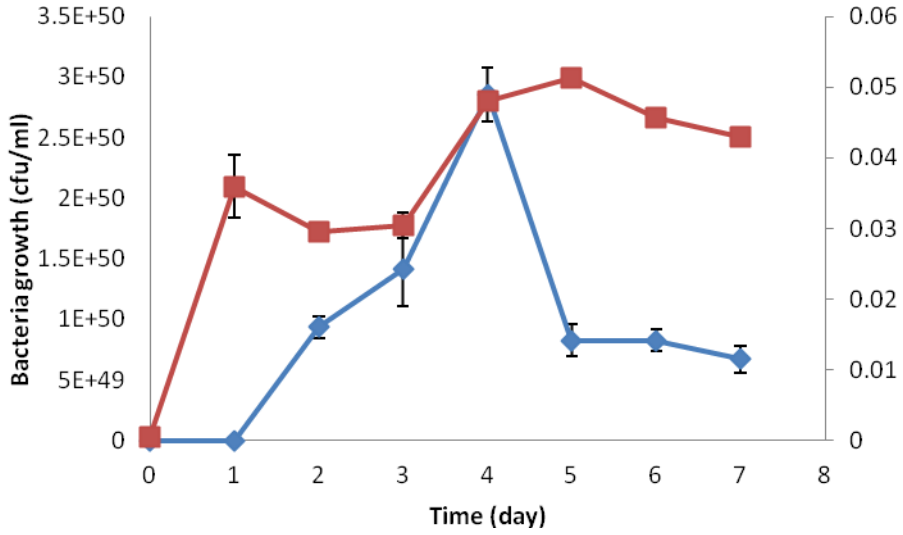

Figure 5.7: **Time course data on bacteria growth (*Xanthomonas campestris*) and reducing sugar under optimized condition. Symbols: Bacteria growth (◆) and reducing sugar (■)**

The validation results proved that by using 2.72 g of pretreated sugarcane bagasse, 3.14 g/L of NH_4NO_3 and 7.1 g/L yeast extract in solid medium with 81.77% of moisture content, the mean of xanthan yield achieved was 0.1221 $\pm$ 0.0059 g/g, and the value is near to the predicted value of 0.1304 g/g. The percentage error was 6.4% which explained the RSM experiments based on

CCD were found practical to derive a statistical model in optimizing the xanthan production.

5.4 Concluding Remarks

This present work reported the feasibility of using sugarcane bagasse as solid substrate for xanthan production via solid state fermentation. The statistical experiment design (PBD and RSM) offer an efficient and time saving method to search for significant variables and to optimize the production of xanthan with minimal experimental run. For PBD, eight variables, including substrate loading (A), moisture content (B), NH_4NO_3 (C), yeast extract (D), tryptone (E), KH_2PO_4 (F), $MgCl_2.6H_2O$ (H), and inoculum size (J) were found significant on the yield of xanthan gum. Parameters with percentage contribution higher than 5% on xanthan yield were selected for further study by using RSM.

From the RSM analysis, a maximum xanthan yield was achieved with the condition of substrate loading, 2.72 g; moisture content, 81.77%; NH_4NO_3, 3.14g/L and yeast extract, 7.1 g/L; whereas tryptone, KH_2PO_4, $MgCl_2.6H_2O$ and inoculum size were kept at their optimum level (results from PBD). Validation experiment was carried out in order to confirm the adequacy of the model and the result was found in a good agreement with a small percentage error (6.4%). Generally, the findings in this present study suggested that sugarcane bagasse has the potential to be used as cost effective substrate in the production of xanthan gum.

CHAPTER 6

RHEOLOGICAL ANALYSIS AND CHEMICAL CHARACTERIZATION OF XANTHAN GUM

6.1 Introduction

A crucial characteristic of xanthan gum is their ability to cause thickening at a low concentration, promoting high viscosity at high strain rate, thermal and pH stable (Kang and Pettit, 1993). Due to the marvelous properties of xanthan gum, its application covers a wide range of industry such as food, pharmaceutical, cosmetic. paper mill and textile industry. Furthermore, it has been effectively applied in drilling fluids and oil recovery processes (Gallino et al., 2001).

In order to understand further on the rheological behaviour of xanthan gum, analysis was conducted by using purified xanthan from the present study, in comparison with commercial xanthan. The levels of chemical composition (acetyl, uronic acid and pyruvic acid) were also determined, which allows us to relate the chemical composition of xanthan gum to the rheological behavior of the colloid. For the ease of explanation, xanthan gum from the current study is designated as XCS while commercial xanthan is designated as CX.

6.2 Materials and Methods

6.2.1 Rheological Analysis of Xanthan Gum

A commercial xanthan gum (R&M Chemicals, US) was used as standard to compare with xanthan gum produced from the present study. The quality and rheological behavior of both CX and XCS were studied and compared by using a MCR 301 Rheometer (Anton Paar, GmBH) with radius 25 mm and 0.5 mm gap size. The samples were placed at the bottom plate and compressed by the upper plate. The excess sample was wiped off with a spatula. For each sample, constant shear rate test, steady shear (shear stress versus shear rate) and amplitude sweep test were studied. All measurement was performed at 25^{o} C and triplicate results were recorded.

Both CX and XCS with 1% (w/v) were prepared separately. The desired mass of xanthan gum was slowly added into distilled water in a beaker with constant stirring by using magnetic stirrer until all xanthan gum was equally dissolved.

Constant Shear Rate

The viscosity and flow properties of both CX and XCS were examined under constant shear rate. The shear rate was set to 25 s^{-1} for 120 s.

Shear Stress as Function of Shear Rate

The test was performed to measure shear stress as the function of shear rate. The shear stress was studied with increasing of shear rate, ranging from 1.0 s^{-1} to 1000 s^{-1}. The shear stress versus shear rate data were obtained as rheograms, as reported by Kim and Yoo (2006). In order to study the variation in rheological behaviour of xanthan gum, the data were fitted into power model law ($\tau = K\gamma^n$), where τ is shear stress, K is the consistency index, and γ is shear rate and n is flow behavior index.

Amplitude Sweep Test

Both CX and XCS were subjected to the amplitude sweep test by ranging the strain amplitude from 0.01 to 100% logarithmically. The samples were sheared at a fixed frequency of 1 Hz. The storage modulus G' and loss modulus G" were studied throughout the test.

6.2.2 Chemical Analysis of Xanthan Gum

The CX and XCS were compared in terms pyruvate, acetate and uronic acid content. The details on the experiment were as described below.

Pyruvic Acid Content

For sample preparation, 10 mL of 0.6% (w/v) of xanthan gum was added in 10 mL of distilled water in a stoppered conical flask. Then, 20 mL of 1 N hydrochloric acid was added and the mixture was refluxed for 3 h in a stoppered conical flask. The mixture was then cooled to room temperature. One milliliter (1 mL) of 2,4-dinitrophenyl hydrazine reagent was pipetted into a separatory funnel, followed by adding 2 mL of sample solution. The 2,4-dinitrophenyl hydrazine reagent was prepared by diluting 0.5 g of 2,4-dinitrophenyl hydrazine into 2 N of hydrochloric acid. Mixture in separatory funnel was mixed properly and was stand for 5 min. The mixture was extracted with 5 mL of ethyl acetate and the aqueous layer was discarded. Hydrazone content was extracted from ethyl acetate by using 5 mL of 10% (w/v) of sodium carbonate. The extract was collected into 50 mL volumetric flask. The absorbance of each sample was recorded by using spectophotometer at 375 nm. For standard preparation, a standard calibration of pyruvic acid (Figure 6.1) was conducted and plotted for the determination of pyruvate content in xanthan gum.

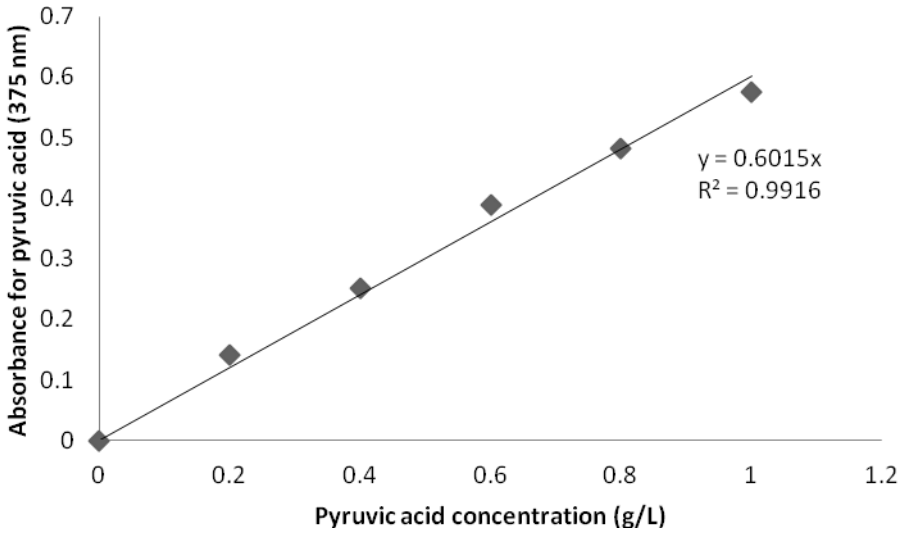

Figure 6.1: Standard calibration curve of pyruvic acid content

Acetyl Content

For sample preparation, 200 µL of 1% (w/v) xanthan gum was pipetted into a test tube, followed by 400 µL of 2 M hydroxylamine hydrochloric acid reagent (HA). The HA reagent was prepared by diluting 100 mL of 2 M hydroxylamine hydrochloric acid into 100 mL of 3.5 M sodium hydroxide. The mixture in test tube was stand for 2 min at room temperature. Then, 200 µL of 5.65 M hydrochloric acid was added into the mixture, followed by adding 200 µL of 0.37 M iron (III) chloride hexahydrate in 0.1 M hydrochloric acid. The mixture was mixed well and a brown reddish color was formed. The sample was measured by using spectophotometer at 540 nm. A standard calibration curve was prepared by diluting acetylcholine as standard into 0.001 M of sodium acetate (Figure 6.2).

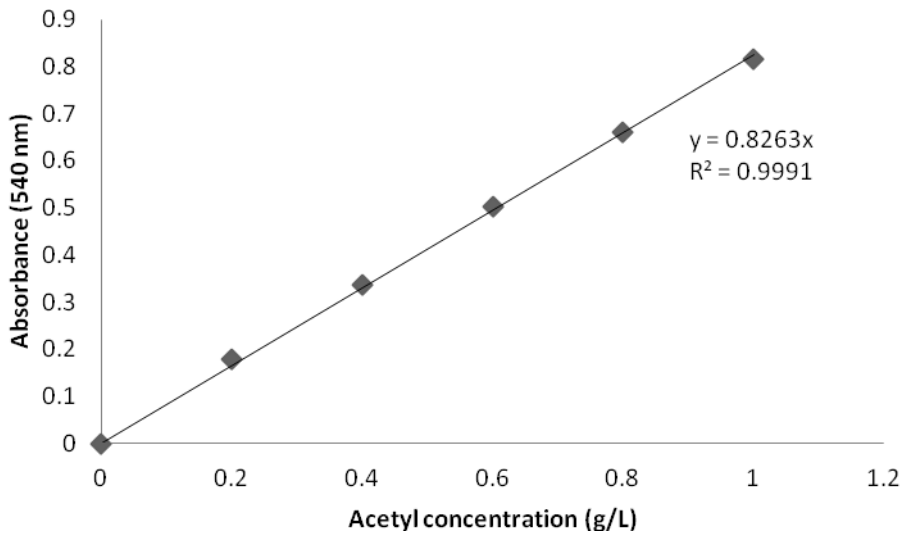

Figure 6.2: Standard calibration curve for acetyl content

Uronic Acid Content

For sample preparation, 0.2 mL of 0.1% (w/v) of xanthan gum was put into test tube, followed by adding 1.2 mL of 0.0125 M of sodium tetraborate in concentrated sulphuric acid. The mixture in test tube was chilled in ice water bath for 5 min, and then it was heated to 100℃ and chilled again in ice water bath. Then, 20 μL of 8.8 M 3-phenylphenol in 0.125M NaOH was added into the mixture. The color developed was measured at 520 nm by using spectophotometer. A standard calibration curve was prepared by diluting pure glucuronic acid as standard in distilled water.

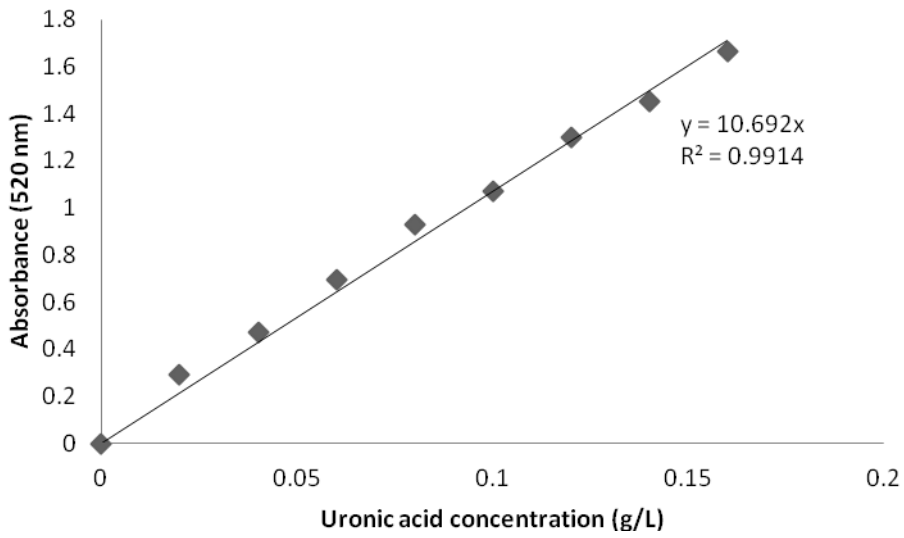

Figure 6.3: Standard calibration curve for uronic acid content

6.2.3 Fourier Transform Infrared (FTIR) Analysis

The infrared spectroscopy analysis was performed for both CX and XCS. Both samples were analyzed using Perkin Elmer Fourier Transform Infrared, with wavelength raging from 400 to 4000 waves/cm with 32 scans/sample.

6.3 Results and Discussion

6.3.1 Rheological Analysis

Constant Shear Rate Test

As shown in Figure 6.4, both CX and XCS exhibited minor decrease in viscosity with prolonged shearing time when constant shear was applied. The initial viscosity for CX at the sixth second was 0.99 Pa.s at the start of testing, which was slightly higher than XCS, 0.78 Pa.s. As the time progressed, the viscosity of both CX and XCS decreased till 0.962 Pa.s and 0.71 Pa.s, respectively. The constant shearing applied on xanthan solution disrupts and deforms the aggregated particles of xanthan, hence causing decline on the viscosity of xanthan solution.

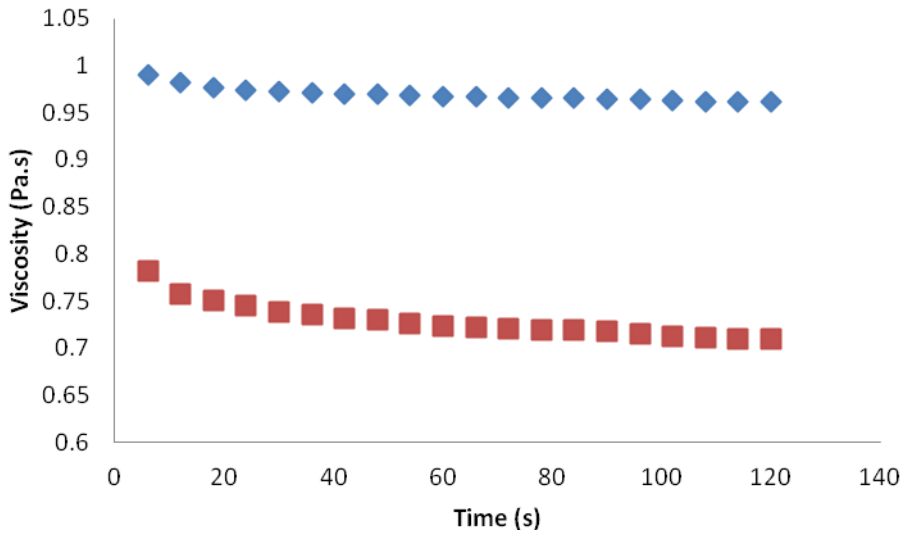

Figure 6.4: Constant shear rate tests for 1% w/v of CX(◆) and XCS (■)

During constant shear test, the viscosity reduction at a constant shear indicating that xanthan gum exhibits shear-thinning behavior, and hence known as pseudoplastic. Both CX and XCS exhibit non-Newtonian behavior when the viscosity of both solutions declined sharply with application of shear rate. The phenomenon can be explained when shear is applied, the chain orientation of xanthan is disrupted with the flow directions, thus reducing local drag (Harrison et al., 1999). The polysaccharide of xanthan gum strongly bound together via hydrogen bonding and polymer entanglement, resulting in high viscosity at rest. However, when shear was applied, the viscosity of polysaccharide declined due to disentanglement of the polymer network in the direction of shear flow, causing the decreased of viscosity (Moschakis et al., 2005).

According to Kang and Pettit (1993), xanthan gum exhibits psedoplastic property. The viscosity of xanthan gum decreases over time when shear stress was applied, causing the viscous xanthan solution to flow. However, the viscosity of xanthan solution recovered almost instantaneously once the shear stress was removed. The rheological property of xanthan gum is time dependent too, as the longer the solution of xanthan undergoes shear stress, the lower the viscosity of both CX and XCS solutions. Some fluids such as ketchup is pseudoplastic fluid and the fluid flowed out when stress was applied. However, the viscosity of the fluid remained once shear stress is removed. Due to the rheological properties of xanthan, it explained the reason that it was popular been applied in many industries, especially food industry and

pharmaceutical. The gum is usually been as thickener, emulsion and temperature stability in food industry (Kang and Petitt, 1993).

As observed from Figure 6.4, CX is with higher viscosity, as compared to XCS. According to Faria et al. (2011), the viscosity of xanthan gum obtained from medium with sugarcane broth was slightly lower than commercial xanthan. The difference might be due to the dissimilarity of the size, shape and molecular structure of both gums. In addition, the variation of bacteria strain, procedures for recovery of xanthan (such as heating, filtration and centrifugation) and solution used for extraction of xanthan are the possible reasons affecting the viscosity and rheological behavior of xanthan (Galindo et al., 1993).

Zhang and Chen (2009) found that the viscosity of xanthan produced from the mixture of xylose and glucose was much lower than the viscosity of commercial xanthan. They proposed that the dissimilarity between commercial xanthan and xanthan produced from the project might be due to the different content of pyruvate and acetate in the xanthan. Furthermore, bacteria strain used during fermentation process also affects the chemical composition of xanthan. Hence, screening of microorganism for optimization of xanthan is the interesting topic for many researchers. Rottava et al. (2009) isolated 10 different strains of *Xanthomonas campestris* and found that the best strain in terms of xanthan productivity (9.67 g/L) was *Xanthomonas campestris pv. campestris*, but with a low apparent viscosity of 440 $mPa.s^{-1}$. On the other hand,

apparent viscosity of xanthan gum produced by *Xanthomonas campestris pv. mangiferaeindicae* is 1840 mPa.s^{-1} , at a concentration of xanthan 3% (w/v).

Amplitude Sweep Test

Figure 6.5 illustrates the strain dependece of storage modulus (G') for 1% (w/v) aqueous solution of CX and XCS. The storage modulus (G') of both CX and XCS were compared. The storage modulus of CX was higher compared to XCS. A linear region was clearly observed for both xanthan gum solutions at a smaller strain amplitude (strain < 10%). Nonetheless, a nonlinear behaviour is also observed when strain aplitude was increased above 10%, with the storage modulus of both xanthan solutions declined.

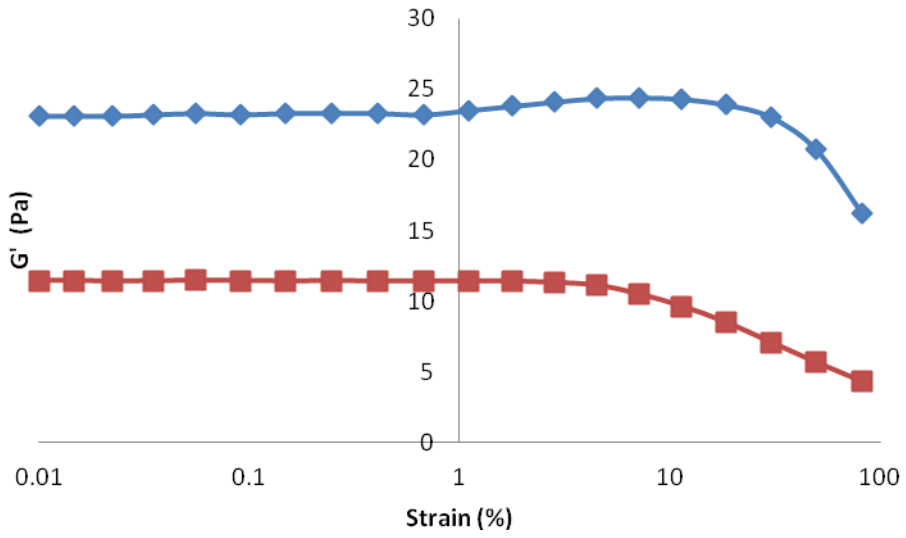

Figure 6.5: Storage modulus as a function of strain amplitude for 1% w/v aqueos xanthan solution. Symbols: CX (◆); XCS (■)

Storage modulus of both xanthan gums exhibited a constant value at small strain amplitude, followed by a decrease with increasing strain amplitude. The decrease of storage amplitude indicated strain thinning behavior of both CX and XCS. With small strain amplitude (strain < 10%), the polymer bonding and entanglement is in linear region as the balanced state sustained between the rates of structural destruction and structural reformation of xanthan (Song et al., 2006a). The elastic response of xanthan gum to the applied force caused a constant magnitude of the storage modulus, resulting in a linear behavior of xanthan. When both xanthan were subjected to larger strain amplitude, the rate of destruction increased with decreasing on the rate of structural deformation. Hence, the elastic response of xanthan gum was deformed due to the increase of applied strain, resulting in a decrease in storage modulus.

As for loss modulus (G"), a linear region is clearly observed for both of the xanthan gum solutions at a smaller strain amplitude range smaller than 2% (Figure 6.6). Both xanthan solutions showed a constant value at small range of strain amplitude. However, a nonlinear behaviour was observed at strain aplitude range larger than 2%. The loss modulus was first increased up to a certain amplitude (strain > 2%), followed by a decrease in loss modulus with further increase of strain amplitude (strain > 30%).

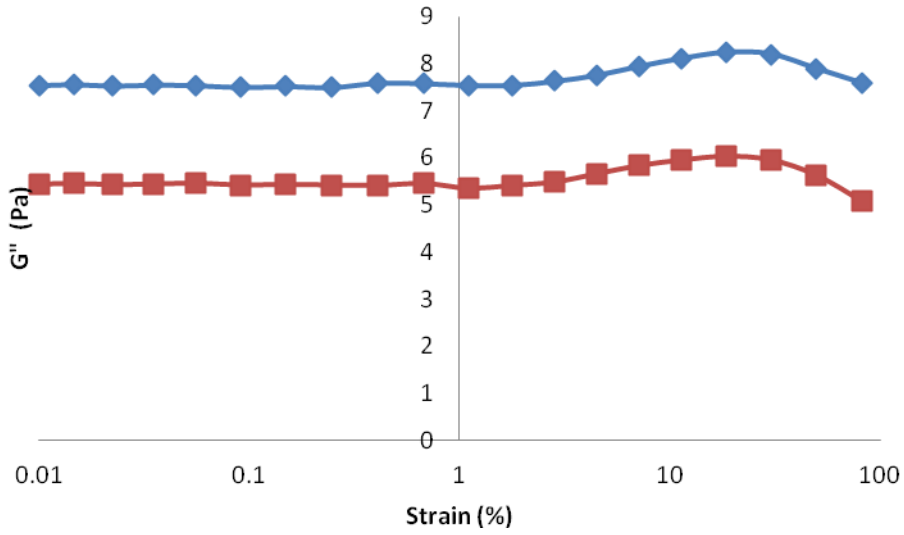

Figure 6.6: Loss modulus as a function of strain amplitude for 1% w/v aqueos xanthan solution. Symbols: CX (◆) and XCS (■)

The loss modulus (G") of both xanthan exhibited a constant value at a small range of strain amplitude (strain < 2%), which might be due to the presence of charged groups (pyruvic acid and uronic acid) on the side chain of xanthan gum (Lapasin and Pricl, 1999). The electrostatic repulsion between the charged groups caused an extend of xanthan structure, forming a weakly ordered structured material (Rochefort and Middleman, 1987), and the small strain applied hardly can breakdown the structure of xanthan, resulting a linear region at small range of strain amplitude (strain ≈ 2%). However, when both CX and XCS were subjected to larger strain, a remarkable increase in loss modulus was noticed (strain > 2%). When strain amplitude increased, the structure of xanthan resisted the imposed deformation up to certain amplitude (Song et al., 2006b), causing an increased in loss modulus (strain > 2%). When strain applied was more than 30%, the structure of xanthan deformed and broken down, leading to a decrease in loss modulus.

In addition, the storage modulus (G') of both CX and XCS were larger than the loss modulus (G") at the linear region, indicating the elastic behavior dominates the viscous behavior of xanthan gum (Song et al., 2006b). However, at the strain approximate to 50%, the loss modulus (G") of XCS intersects with the storage modulus (G') of XCS. The intersection at this point indicating that the XCS changed from solid-like to liquid-like behaviour (Song et al., 2006b). At larger strain (strain > 50%), the loss modulus of the XCS was higher than the storage modulus, indicating the viscous property of the XCS was greater than the elastic property. The CX exhibited elastic behaviour with increasing strain amplitude. As shown clearly in Figure 6.7, the storage modulus of CX was greater than loss modulus. Hence, the 1% (w/v) concentration of CX can be classified as gel system; while 1% w/v of XCS was categorized to have elastic behaviour.

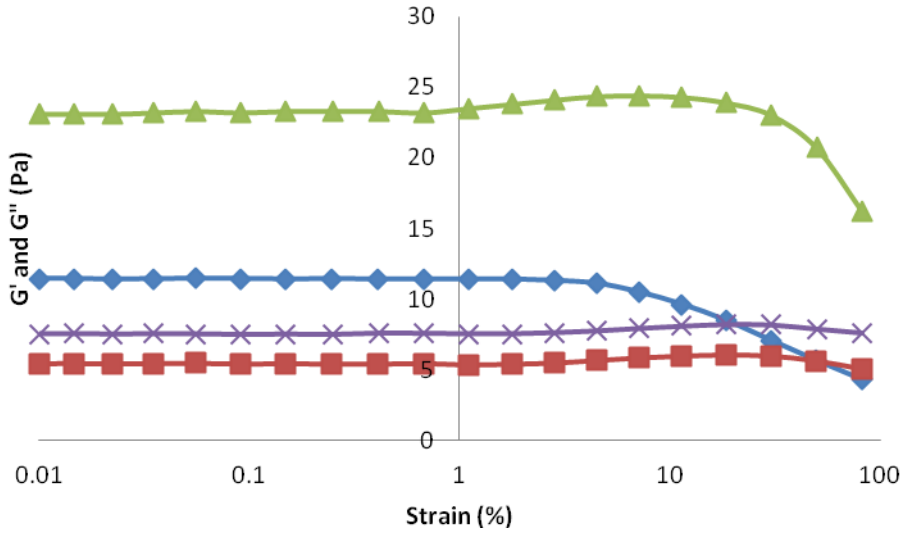

Figure 6.7: Storage modulus (G') and loss modulus (G") as a fuction of strain amplitude for 1% w/v aqueous solution. Symbols: G' of CX, (▲); G' of XCS, (◆); G" of CX, (x); G" of XCS (■)

Relation between Shear Stress and Shear Rate

The shear stress versus shear rate of both CX and XCS were fitted into Power Law Model, which can be written as bellow:

$$\tau = K\gamma^n \qquad \text{Eq. 6.1}$$

where τ is shear stress (Pa), K is concensistency index (Pa.s^n), γ is shear rate (1/s) and n is flow behaviour index. Figure 6.8 represents the rheograms of shear stress versus shear rate applied to a 1% (w/v) solution of both CX and XCS. On linearlization, a graph of $ln\,\tau$ againts $ln\,\gamma$ was plotted. A straight line was obtained with slope of n and y-intersect of *ln K*. Hence, the flow behaviour index (n) and consistency index (K) can be calculated.

$$\ln \tau = \ln K + n \ln \gamma$$

Eq. 6.2

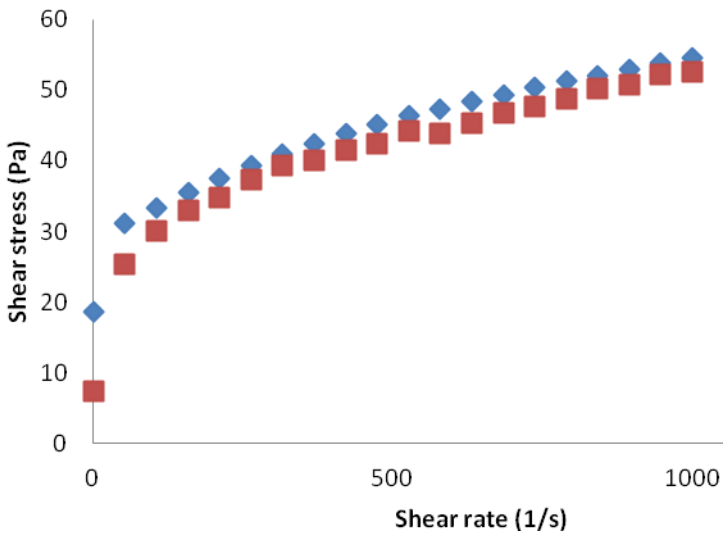

Figure 6.8: Shear stress versus shear rate of 1% (w/v) xanthan solution. Symbols: CX (◆) and XCS (■)

The data of consistency index (*K*) and flow behaviour (*n*) of both xanthan was presented on Table 6.1. The flow beaviour of both CX and XCS were fitted well to the Power Law equation, with high determination of regression coefficients (R^2), 0.9642 and 0.9949 respectively.

Table 6.1: Rheological parameters of CX and XCS

Concentration (1% w/v)	*K* (Pa.s^n)	*n*	R^2
CX	16.9302	0.1606	0.9642
XCS	7.8201	0.2764	0.9949

As observed from Table 6.1, the flow behaviour index of CX (*n* = 0.1606) was slightly lower than XCS (*n* = 0.2764). According to Lopez et al. (2004), small value of flow behaviour (*n*) indicates pseudoplastic behavior of the fluid. When the flow behavior index near to unity, the pseudoplastic behavior of the polysaccaride decrease and Newtonian behaviour is achieved when *n* equals or near to 1 (Song et al., 2006a). In addition, the flow behaviour index shows the degree of stucture properties and stability of a polysaccharide (Tipvarakarnkoon and Senge, 2008). In the present study, CX possess higher stability and showed better pseudoplastic behavior in aqueous state as compared to XCS with lower value of flow behavior index. This also further explained by the results of constant shear rate test (Figure 6.4) when the viscosity of CX is higher than XCS at a constant shear rate.

The rheological behaviour of xanthan is very much depending on the cultivation condition. Faria et al. (2011) investigated the rheological behaviour of xanthan produced by using sugarcane broth as carbon source and found the

n value of produced xanthan was 0.226. Tipvarakarnkoon and Senge (2008) compared the rheological behaviour of xanthan, guar, CMC and LBG at 1% concentration and noticed that xanthan has the lowest *n* value and hence xanthan has the highest stability among others gums.

Xanthan is an anionic polysaccharide due to the presence of uronic acid and pyruvic acid groups at the side chain of polysachharide. As a result, the structure of xanthan gum was highly extended due to electrostatic repulsion from the functional groups on the side chain (Rochefort and Middleman, 1987). The electrostatic repulsion of the charged group increase the solubility of xanthan and hence increase the viscosity of xanthan gum as compared with others polysaccharides.

Section 6.3.2 compares the chemical composition between CX with XCS, which further explained the difference of viscosity between these two gums. The rheological properties of xanthan shows the intermolecular interaction of xanthan in aqueous solutions (Pelletier et al., 2001), where the viscosity of xanthan solution is affected by the negatively charged pyruvic acid that attached at the side chain of xanthan (Kang and Pettit, 1993). According to Tako and Nakamura (1984), high level of pyruvilation increases the viscosity of xanthan as the intermolecular associations between xanthan and water are favored. In other words, the index of gum quality is usually based on the pyruvic acid content (Cadmus et al., 1978).

6.3.2 Chemical Composition Analysis of Xanthan Gum

The chemical composition of xanthan produced in the present study was compared with commercial xanthan gum. Figure 6.6 shows both CX and XCS had almost similar level of acetyl, uronic acid and pyruvate contents. The CX with 6.46% of acetyl content, which is slightly lower as compare to XCS which has higher acetyl content (10.61%). Both gums are with almost same level of uronic acid content (2.2%). As for the pyruvate content, commercial xanthan gum had a slightly higher percentage (8.14%), compared to XCS with 5.87%.

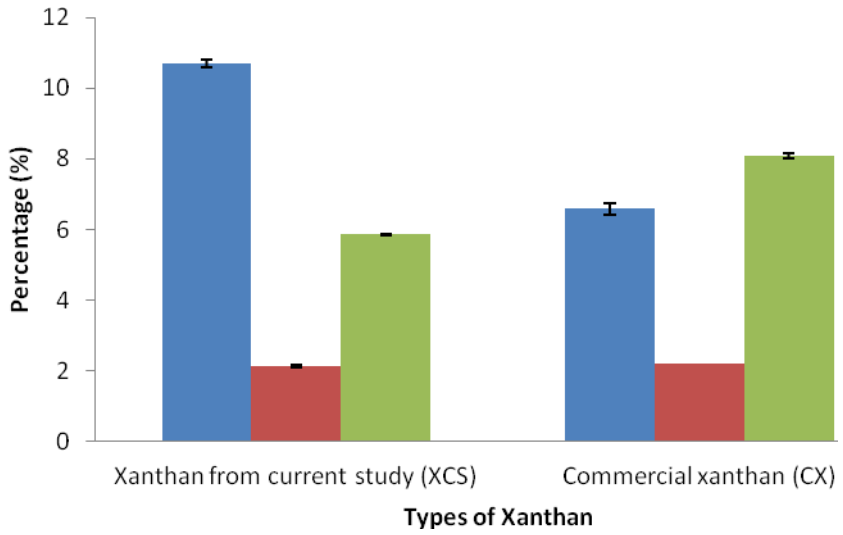

Figure 6.9: Chemical composition of xanthan from current study (XCS) and commercial xanthan (CX).Symbols: acetyl content, ( ); uronic acid content, ( ); pyruvic acid content, ( )

In the present analysis, CX is with higher pyruvate content (8.14%) as compared to XCS (5.87%). As a result, the viscosity of CX was found higher than XCS at the constant shear rate test (Figure 6.4). The negatively charged carboxylic groups (COO^-) of pyruvate repel each other, hence extend and

increase the end-end chain length of the polymer. The polysaccharide hence is more flexible in solution, they gyrate and flex, sweeping out a large space and collides each other more frequent. As a result, xanthan gum with higher pyruvate content possesses higher viscosity in water solution because intermolecular associations are favored (Tako and Nakamura, 1984). In addition, the viscosity of xanthan reduced when pyruvate content of the xanthan decreased (Tako and Nakamura, 1984).

Furthermore, the acetyl content of xanthan also affects the rheological behavior of xanthan. According to Sloneker and Jeanes (1962), the removal of acetyl groups improves the viscosity of xanthan. In aqueous solution, the acetyl group (CH_3CO) is hydrophobic and considered as non-polar and tends to aggregate. The hydrophobic character of acetate group minimizes the area of direct contact of xanthan with water; hence decrease the intermolecular association of xanthan with water. In the present study, the percentage of acetyl group of XCS (10.61%) was higher than the CX with 6.46% of acetate content, suggesting that the rheological behavior of CX was much better compared to XCS. Khouryieh et al. (2007) reported that de-acetylated xanthan gum has higher viscous property as compared to native xanthan gum.

6.3.3 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

Fourier transform infrared spectrum (FTIR) was used to identify the functional groups exists in the structure of xanthan gum. The infrared spectra of CX and XCS were compared and further analyzed (Table 6.2).

Table 6.2: FTIR spectra of CX and XCS

Wavenumber (cm^{-1})	Functional group
3400 to 3000	OH stretching of hydroxyl group
2900 to 2800	CH stretching of alkyl group
1601	C=O of carboxylate group
1406 to 1401	C-O stretching of carboxylate group
1019	C-O-C stretching of acetal

As shown in Table 6.2, the broad absorption band at 3400 cm^{-1} to 3000 cm^{-1} showing the presence of hydroxyl group (-OH) in the structure of xanthan. The -CH stretching appeared between 2900 cm^{-1} to 2800 cm^{-1}. The peak within 1601 cm^{-1} identified the presence of C=O bonding, indicating the presence of ester, acid carboxylic, aldehydes and ketones around the structure of polysaccharide. The bands in the range of 1406 cm^{-1} to 1401 cm^{-1} showing the C-O stretching of caboxylate group, followed by bands at 1019 cm^{-1}, indicating the C-O-C stretching of acetal. Both spectra show quite similar peaks with a good correlation value (R^2 = 0.9254), indicating the similarity of both spectra was high and reached a similarity of 92.54 % (Figure 6.10). Based on the FTIR spectra results, with similar spectra for both polysaccharide, suggesting that XCS possess similar characteristic with CX.

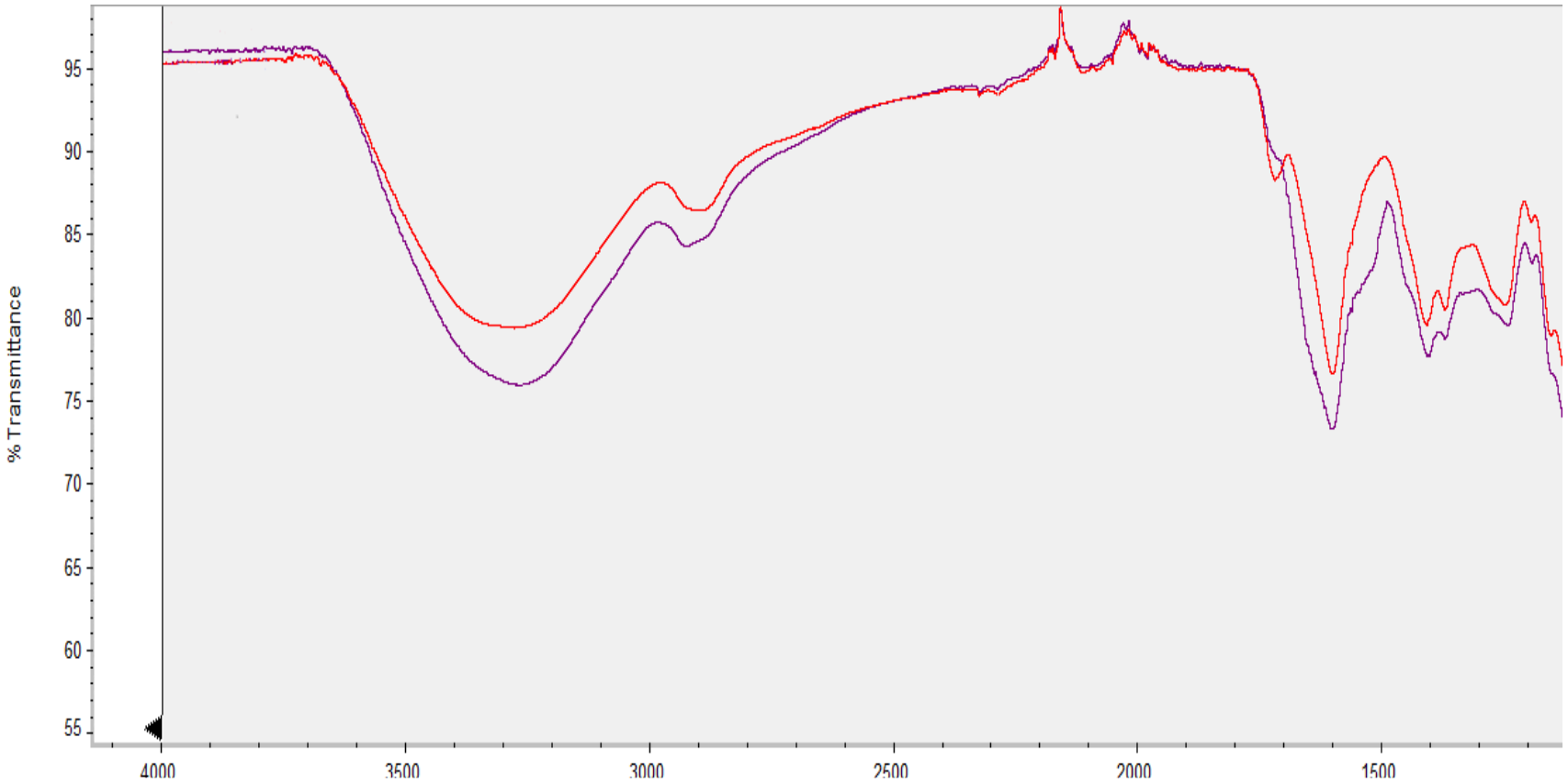

Figure 6.10: FTIR spectrum of CX (—) and XCS (—)

6.4 Concluding Remarks

In this study, the rheological behavior of xanthan produced from solid-state fermentation of sugarcane bagasse (XCS) was compared with the commercial xanthan (CX). The result shows that both CX and XCS possess pseudoplastic behavior. From the analysis, CX exhibited better rheological characteristic compared to XCS due to the low degree of pyruvate content in XCS. However, the difference on molecular structure of both CX and XCS might due to dissimilarity of strain of bacteria used and procedures applied during recovery of xanthan. Spectra from FTIR proved an excellent correlation value between CX and XCS, indicating similarity between both xanthan gums.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

Production of xanthan has recently been applied in wide range of industry, due to its outstanding rheological properties. The industrial production of xanthan usually uses pure glucose as main carbon source for the production via submerged liquid fermentation. In order to lower the cost of production, the abundantly available agricultural wastes are recently being used and studied as raw material for xanthan production. Agricultural waste represents an alternative cheap carbon source that is more beneficial for the yield of xanthan in the way of economic. In addition, solid state fermentation has also attracts attention from researchers as it reduces the cost of production. The objective of the present study was concerned with the production of xanthan via solid state fermentation, by incorporating sugarcane bagasse as solid substrate as carbon source, with the goal of maximizing the xanthan production.

The study as reported in Chapter 4 investigates the feasibility of using the sugarcane bagasse in the production of reducing sugar, as reducing sugar act as

carbon source on the production of xanthan. The alteration on the structure of sugarcane bagasse, which treated with either with NaOH or H_2SO_4 were analyzed and studied. The yield of reducing sugar was greatly affected by the concentration of chemical used and treatment time applied. From our study, sugarcane bagasse treated with 3% w/v NaOH for 30 min of treatment time gave the highest yield of reducing sugar (0.1526 g/g), and was used as solid substrate during subsequent fermentation process.

Chapter 5 summarizes the application of statistical analysis on the production of xanthan. Plackett Burman design (PBD) was first used to screen significant variables which contribute to xanthan production. Moisture content is the most significant variables on the yield of xanthan, followed by yeast extract, NH_4NO_3, substrate loading, tryptone, KH_2PO_4, $MgCl_2.6H_2O$ and inoculum size. Variables with percentage of contribution higher than 5% (moisture content, yeast extract, NH_4NO_3 and substrate loading) on the yield of xanthan were chosen and further optimized by using Response Surface Methodology (RSM) with Central Composite Design (CCD). From the analysis, the optimal condition for xanthan production was with 2.72 g of substrate loading, at a moisture content of 81.77%, with the addition of 3.14 g/L of NH_4NO_3, and 7.1 g/L of yeast extract. The maximum production of xantan under this condition was 0.1221 g/g, which has only 6.4 % of error from the predicted yield (0.1304 g/g). This indicated the effectiveness of the model for the optimization of xanthan production. From the RSM model, xanthan production was enhanced at a low substrate loading and low moisture content. A low level of total nitrogen source reduced the production of xanthan as it limited bacteria growth.

However, high concentration of nitrogen source inhibited the production of xanthan. Hence, appropriate amount of total nitrogen source (about 151.05 mM) needed for cell growth in this present study, as excess nitrogen source might inhibit the production of xanthan.

Chapter 6 presents the properties of xanthan gum produced from the current study (XCS) in comparison with the commercial xanhan gum (CX). Fourier Transform Infrared Spectroscopy (FTIR) was applied to determine the similarity between XCS and CX. Both spectra show quite similar peaks, indicating the similarity of both spectra was high with a similarity of 92.54 %. Characterization of the rheological behavior of xanthan produced from project is crucial as the quality of the gum can be further determined. Results indicated that XCS has a lower viscosity compared to CX at 1 wt% concentration. However, both gums exhibited pseudoplastic behavior.

Table 7.1 compares the xanthan production as reported in literature with xanthan produced from the current study (XCS) via SSF process. As shown in Table 7.1, sugarcane bagaasse has the potential in producing comparable or even higher amount of xanthan gum via solid state fermentation. In conclusion, results of current study confirmed that sugarcane bagasse is a potential and suitable carbon source for production of xanthan to be used in industry.

Table 7.1: Reported xanthan yield via SSF

Inert solid used	Condition	Xanthan yield (g/g)	Reference
Alkaline treated sugarcane bagasse	Carbon source: 2.72 g sugarcane bagasse in conical flask Conditions: Moisture content: 81.77% or 12.2 mL of fermentation medium/g of SB Inoculum size: 3% v/w or 24.6% v/v Nutrients: NH_4NO_3: 3.14g/L Yeast extract: 7.1g/L Tryptone: 10g/L KH_2PO_4: 2g/L $Mg.Cl_2.6H_2O$: 3g/L	0.1304g of xanthan/g of sugarcane bagasse	Present study
Trimmed and grounded potato peels (PP)	Carbon source: 50g of PP in conical flask Conditions: Moisture content: 0.4mL of water/g of PP	0.0574g of xanthan/ g of potato peels	Vidhyalakshmi et al. (2012)
Apple pomace and spent malt grains	Carbon source: 20 g of substrate (8 g apple pomace and 12 g spent malt grains) in conical flask Conditions: Moisture content: 70% Inoculum size: 5% v/v Nutrients: Sodium glutamate: 4g/L K_2HPO_4: 1g/L $MgSO_4.7H_2O$: 0.2g/L	0.1124g of xanthan/ g total substrate	Miroslav and Elena (1998)

7.2 Main Conclusion

Summary of this present study are as bellow:

a) Alkaline treated sugarcane bagasse is a potential substrate for production of xanthan gum by *Xanthomonas campestris*. The sugarcane bagasse provided sufficient carbon source during fermentation process.

b) Application of solid state fermentation (SSF) successfully produce xanthan gum in a more environment friendly and economical way.

c) Optimization of xanthan production has been successfully conducted. Plackett Burman Design aid to screen out unsignificant variable and four important variables on xanthan production was further optimized by Response Surface Methodology.

d) Rheological properties and chemical composition of the xantham gum produced from current study (XCS) was quite similar with commercial xanthan (CX). A correlation of 0.9254 between XCS and CX from FTIR spectra showed 92.54% similarity of XCS and CX.

7.3 Recommendations

The application of solid state fermentation was proved to be very encouraging on the production of xanthan. The production can be upscale by using fermenter such as tray fermenter and rotary drum fermenter instead of using conical flask. Hence, supply of oxygen can be controlled at a sufficient level for a better yield of xanthan.

The quality and quantity of chemical compositions of xanthan produced are greatly affected by the rheological behavior of xanthan gum. Operational conditions during fermentation processes could influence the appearance of xanthan gum. Further study on several affecting parameters such as stirring and aeration, could be conducted, in investigating the effect of these variables on the quality of gum.

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APPENDIX

The findings of the research has been presented

1. Poster presentation: in AKEPTs 1st Annual Young Researchers Conference and Exhibition, organized by MOHE, on 19 – 20 December 2011 at PWTC Malaysia.

UTILISATION OF AGRICULTURAL WASTE FOR PRODUCTION OF XANTHAN

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Abstract

A *Xanthomonas* species was used for production of xanthan gum using pretreated sugarcane bagasse as substrate. The bacteria was cultivated on two different moisture conditions (70% and 80%) in order to evaluate their ability to produce xanthan gum under Solid State Fermentation (SSF) process. From the present study, *Xanthomonas* produces significantly high yield of xanthan (0.141g/g) when it is incubated with 80% moisture content of sugarcane bagasse.

2. Poster presentation: in National Postgraduate Seminar 2012, organized by University of Malaya, on 11 July 2012 at University of Malaya.

SUGARCANAE BAGASSE AS POTENTIAL SUBSTRATE FOR BIOPOLYMER PRODUCTION

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Abstract

Chemically pretreated sugarcane bagasse was used as substrate for xanthan gum production with *Xanthomonas* species. Preliminary investigation on the effective variables was conducted by using Plackett-Burman design, by searching the significant ANOVA model with p-value <0.0001. The variables included substrate loading, moisture content, NH_4NO_3, yeast extract, tryptone, KH_2PO_4, Na_2SO_4, $MgCl_2.6H_2O$ and inoculum size. The results indicated that substrate loading, moisture content and yeast extract has the most significant effect on the yield of xanthan gum with p-value <0.0001.