Developing a unique microbial hydrolysis process for converting lignocellulosic waste into reducing sugars

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**DEVELOPING A UNIQUE MICROBIAL HYDROLYSIS
PROCESS FOR CONVERTING LIGNOCELLULOSIC
WASTE INTO REDUCING SUGARS**

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SCHOOL OF CIVIL AND ENVIRONMENTAL ENGINEERING

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WASTE INTO REDUCING SUGARS**

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School of Civil and Environmental Engineering

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Summary

Lignocellulosic waste, e.g., leaves, uprooted weeds, twigs, grass clippings and small branches, with high cellulose and hemicellulose content ($54.6 \pm 2.5\%$ of dry matter, by weight), is a cheap and plentiful feedstock for the production of reducing sugars that could be converted to fuels or useful chemicals, e.g., ethanol, lactic acid and xylitol. Hydrolysis of lignocellulosic waste to reducing sugars is one of the key steps for further treatment. The traditional hydrolysis methods, e.g., acid hydrolysis and enzymatic hydrolysis, have some critical drawbacks which are still being optimized and developed. Microbial hydrolysis uses reducing sugars-producing microorganisms directly to convert lignocellulosic waste to reducing sugars. Few studies about this have been drawn to date. This thesis attempted to develop a unique microbial hydrolysis process for converting lignocellulosic waste into reducing sugars for biofuels or other useful chemicals production.

In this thesis, 1-year compost, four-month compost, aerated activated sludge, garden soil and guts of mealworm in tropical area were selected as inocula. After 1 year of sequential cultivation, four microbial communities were built up with high lignocellulose degradation efficiency from 1-year compost (C1), 4-month compost (C4), aerated activated sludge (AAS) and garden soil (GS). The optimal polysaccharides degradation efficiency was 85.4-88.5%. The maximum reducing sugars yield, 12 mg/g lignocellulosic waste, was recorded in the microbial community from AAS. Another six microbial communities were isolated from the guts of *Tenebrio Molitor Linnaeus* (yellow mealworm) by using three specifically selected carbon sources, e.g., Carboxymethyl cellulose (CMC), Filter paper (FP) and lignocellulosic waste (LIG) in two basic nutrients (M1 and M2). The lignocellulose degradation efficiency was 59-78%. The maximum reducing sugars yield, 15.2 mg/g substrate, was obtained in the microbial community from M2-CMC.

A molecular profiling method, 16S/18S rRNA gene-based Polymerase Chain Reaction (PCR) followed by denaturing gradient gel electrophoresis (DGGE),

was used to study microbial diversity during conversion of lignocellulosic waste to reducing sugars in cultivation. Nineteen dominant microbes were successfully isolated and characterized. *Cellulomonas* and *Pseudomonas* were the most abundant phylum in all the isolates. The genera of *Bacilli* were ubiquitous among the mixture of microbial community. Three fungal isolates from the microbial community in this study were assigned to *Pseudallescheria* sp. which is known as potential animal pathogen and can degrade a wide range of organic waste.

To obtain more reducing sugars, seven pure strains of microorganisms were selected and randomly combined to investigate the best microbial combination. These pure strains consisted of *Microbacterium* sp. F28 (M1), *Cellulosimicrobium* sp. C10 (M2), *Tsukamurella* sp. C35 (M3), *Pseudallescheria* sp. D42 (M4), *Bacillus* sp. F4 (M5), *Strenotrophomonas* sp. B15b (M6) and *Pseudomonas* sp. E8 (M7). The best defined microbial cocktail for reducing sugars production was the combination of *Microbacterium* sp. F28, *Tsukamurella* sp. C35, *Pseudallescheria* sp. D42, and *Bacillus* sp. F4. The maximum reducing sugars yield was 165.3 mg/g lignocellulosic waste, which was more than ten times higher than the original consortia. During combination tests, the roles played by each member of these pure cultures and its impact on the other members were investigated.

To minimize the negative effects of environmental conditions on microbial hydrolysis and maximize reducing sugars yield, the individual effects and interactive effects of pH, temperature and initial lignocellulosic waste concentration on the maximum reducing sugars production yield from lignocellulosic waste by the defined microbial cocktail were also investigated in this study. A central composite design (CCD) and response surface methodology (RSM) were employed in designing the experiments. In order to determine the optimum conditions for reducing sugars production, three types of pretreatment methods including organosolv pretreatment, hot water pretreatment and diluted sulfuric acid pretreatment were explored to enhance reducing sugars yield. A maximum reducing sugars yield of 173.1 mg/g lignocellulosic waste was obtained

under pH 5.8, temperature 56.9 °C and 28.9 g/L of initial lignocellulosic waste concentration after organosolv pretreatment experiment.

The maximum reducing sugars yield after optimization in this study was higher than some of enzymatic hydrolysis and meanwhile faster and cost effective. Microbial hydrolysis process could therefore be an alternative method for lignocellulose hydrolysis.

Publications

- 1. Qi Wei**, Niu Dongjie, Wang Jing-Yuan (2011), “Characterization of Microbial Communities during Hydrolysis of Lignocellulosic Waste to Reducing Sugars,” *Journal of Biobased Materials and Bioenergy* 5(1): 75-84.
- 2. Qi Wei**, Chen Chia-Lung, Wang Jing-Yuan (2011), “Reducing Sugars-Producing Bacteria from Guts of *Tenebrio Molitor Linnaeus* (Yellow mealworm) for Lignocellulosic Waste Minimization,” *Microbes and Environments* 26 (4): 354-359.
- 3. Qi Wei**, Chen Chia-Lung, Wang Jing-Yuan (2011), “Enhanced Microbial Hydrolysis of Green Waste to Reducing Sugars by a Microbial Cocktail,” *Biobiomass and Bioenergy* (Revised and Resubmitted).
- 4. Qi Wei**, Chen Chia-Lung, Wang Jing-Yuan (2011), “Further Optimization of Microbial Hydrolysis of Lignocellulosic Waste to Reducing Sugars by Different Pretreatment Methods,” *Resources Conservation and Recycling* (In preparation).
- 5. Qi Wei**, Wang Jing-Yuan (2010), “Characterization of Microbial Community from Mealworm Guts during Conversion of Lignocellulose to Reducing Sugars for Bioethanol Production,” *Renewable Energy Conference (RE2010)*. 27 June-2 July 2010, Yokohama, Japan (Oral Presentation).
- 6. Wei Qi**, Jing-Yuan Wang, Rainer Stegmann (2010), “Optimization of Microbial Conversion of Lignocellulosic Waste to Reducing Sugars,” *Third International Symposium on Energy from Biomass and Waste (VENICE 2010)*. 8-11 November 2010, Venice, Italy (Oral Presentation).
- 7. Qi Wei**, Wang Jing-Yuan, Chen Chia-Lung (2010), “Enhancement of Microbial Hydrolysis of Lignocellulosic Waste to Reducing Sugars by Different Pretreatment Methods,” *International Conference on Bioenergy from Wastes: Green Chemistry*

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Acronyms

C1	1-year compost
C4	4-month compost
AAS	Aerated activated sludge
GS	Garden soil
CMC	Carboxymethyl cellulose
FP	Filter paper
LIG	Lignocellulose waste
B1	Basic nutrients I
B2	Basic nutrients II
PCR	Polymerase Chain Reaction
DGGE	Denaturing gradient gel electrophoresis
M1	<i>Microbacterium</i> sp. F28
M2	<i>Cellulosimicrobium</i> sp. C10
M3	<i>Tsukamurella</i> sp. C35
M4	<i>Pseudallescheria</i> sp. D42
M5	<i>Bacillus</i> sp. F4
M6	<i>Stenotrophomonas</i> sp. B15b
M7	<i>Pseudomonas</i> sp. E8
CCD	Central Composite Design
RSM	Response surface methodology
CO ₂	Carbon dioxide
CGW	Cotton gin waste
SHF	Separate hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation
AFEX	Ammonia fiber explosion
NA	Nutrient Agar
SDA	Sabouraud Dextrose Agar
RDP	Ribosomal Database Project
SEM	Scanning Electron Microscopy
DNS	3, 5-Dinitrosalicylic acid
OS	Organosolv pretreatment
LHW	Liquid hot water pretreatment
DSA	Diluted sulfuric acid pretreatment
X ₁	pH
X ₂	Temperature
X ₃	Substrate concentration
R ²	Regression coefficient
RSY	The maximum reducing sugars yield
OLR	Organic loading rate

Chapter 1 Introduction

1.1 Background

Lignocellulosic waste represents one of the foreseeable sustainable sources in nature because of its relatively low cost and plentiful supply (Fang et al., 2010; Gnansounou, 2010; Goh et al., 2010; Mabee and Saddler, 2010). In Singapore, the amount of lignocellulosic waste generated in 2008 was 229,300 tonnes; only 42% of the lignocellulosic waste was recycled, mainly through composting (NEA Web site, <http://www.nea.gov.sg>). The rest of lignocellulosic waste was directly incinerated without utilization. Current treatment approaches, either composting or landfill, however, required a great amount of land, which posed a very serious problem for this land-scarce country.

Lignocellulosic waste consists of cellulose, hemicellulose and lignin (Van and Jacobus, 2001). The use of lignocellulosic waste as a raw material can broaden the options of the chemical industry giving it more flexibility and a broader application range because it would rely on more-biodegradable products and processes that create less pollution and generally have fewer harmful environmental impacts, develop less expensive products and reduce the dependence on fossil fuel. To develop the carbohydrate potential of bio waste materials, its cellulose content has to be converted into reducing sugars such as glucose that can be used as basic compounds in the biosynthesis of many bioproducts. Hemicelluloses are branched polymers of xylose, arabinose, galactose, mannose and glucose (Van and Jacobus, 2001). These biopolymers bind bundles of cellulose fibrils to form microfibrils, which enhance the stability of the cell wall. They also cross-link with lignin, creating a complex web of bonds that provide structural strength but also challenge microbial degradation. Lignin is a complex polymer of phenylpropane units, which are cross-linked to each other with a variety of different chemical bonds. This complexity has far proven to be as

resistant to detailed biochemical characterization as it is to microbial degradation (Del Río et al., 2007).

The hydrolysates from lignocellulosic waste offer an attractive possibility to be used as substrates for the production of useful chemicals and fuels, e.g., xylitol, butanol, lactic acid and ethanol (Parajó et al., 1997; Garde et al., 2002; Lee et al., 2008). The detail information of conversion of lignocellulosic waste to those useful materials is briefing as below.

Xylitol

Xylitol is a pentitol with an increasing interest owing to its dietetic and clinical properties. This low caloric sweetener has negative heat of solution, anticariogenic properties, and is suitable as a sugar substitute for diabetics (Parajó et al., 1997). Extraction of xylitol from natural sources where it is found in small amount is not economical. Alternatively, xylitol can be produced by fermentation since this compound is an intermediate in the D-xylose metabolism of yeasts and bacteria. Bioconversion of xylose from lignocelluloses into xylitol is an alternative to the traditional chemical process (Gurgel et al., 1995).

Butanol

Butanol is a chemical and also an alternative liquid fuel (Lee et al., 2008). *Clostridium acetobutylicum* has been documented for its ability to produce acetone and n-butanol from hexoses (Compere and Griffith, 1979) and pentoses (Langlykke et al., 1948), particularly xylose and arabinose of lignocellulose. The prospect for using fermentations to replace the production of acetone and butanol from chemical processes depends largely on the availability of inexpensive and abundant raw materials and efficient conversion of these materials to solvents (Lee et al., 1985). Hemicellulose has great potential as a fermentation substrate because it is least expensive and it is often underutilized or even an undesirable byproduct in certain industries and agricultural processes (Sun and Liu, 2010).

Lactic acid

Many organic acids, such as lactic acid, fumaric acid, acetic acid and malic acid, can be produced by fungi through the aerobic fermentation of reducing sugars (Soccol et al., 1994). The worldwide production of L-lactic acid through fermentation was 60,000 tons in 1998. Lactic acid is an important chemical used in a wide variety of applications, being used primarily in food industry as an acidulent, preservative, and for production of emulsifying agents. One of the biggest potentials is as a precursor for biodegradable polylactic acid (PLA) production. By co-polymerization with other functional monomers, specific properties can be obtained, making it possible to substitute many existing petroleum-derived polymer products (Garde et al., 2002).

Ethanol

The most popular application of lignocellulosic waste currently should be used to produce ethanol as fuel. In the early 1970s, Brazil started a program to substitute gasoline by ethanol which was produced from sugarcane. Studies have shown that in the coming years, ethanol yield per hectare of sugarcane could be increased from 6000 L/ha to 10,000 L/ha, if 50% of the produced bagasse could be converted to ethanol (Speck, 1943). In January 2008, 136 ethanol plants in the US produced 7.5 billion gallons of ethanol annually. That number is expected to increase by 13.3 billion gallons per year after construction of 62 additional plants (Sivakumar et al., 2010). Canadian bioethanol production is currently based on corn and wheat grain, some reports show that there were 8.7 metric tons per hectare (t ha^{-1}) harvested for bioethanol production between 2007 and 2009 (Weon et al., 2010). However, the current food-based feedstock for bioethanol production may not be practical since it will cause the competition between food and energy crops for land use.

1.2 Key issues and challenges for bioethanol production from lignocellulosic waste

Bioethanol production utilizing lignocellulosic waste takes place in three phases. First, lignocellulosic matter can be delignified to liberate cellulose and

hemicellulose from lignin. Second, complex carbohydrate polymers (cellulose and hemicellulose) are depolymerized into reducing sugars (pentose and hexose). Finally, conversion of reducing sugars to ethanol fuel through further fermentation. Although bioethanol production from lignocellulosic waste has been greatly improved by new technologies, there are still challenges that need further investigations. These challenges include developing more efficient pretreatment technologies for lignocellulose, investigating more feasible hydrolysis method, maintaining a stable performance in commercial scale fermentation operation and integrating the optimal components into economic ethanol production systems. Among these, delignification of lignocellulosic matter to reducing sugars (pretreatment and saccharification) seems to be the most crucial one.

Dilute acid hydrolysis is the oldest pretreatment technology of lignocellulosic matter delignification. However, this method has some important limitations: expensive materials were required for the construction of reactor due to the acid corrosion, and the added acid has to be neutralized before fermentation. Concentrated acid hydrolysis was an alternative technique of acid hydrolysis, which was generally followed by a dilution with water to dissolve, hydrolyze and convert the substrates into sugars. This process has such advantages as the high sugar recovery (totally 90% of both hemicellulose and cellulose sugars), low temperature and low pressure employed, which allowed the use of relatively low cost materials such as fiberglass tanks and piping. However, this method has some inherent disadvantages too. Most importantly, concentrated acid hydrolysis formed some byproducts, acid must be recycled according to economic reasons, separation and concentration of the sulfuric acid added more complexity to the process. In addition, sulfuric acid was highly corrosive and difficult to handle. The high operating temperature (100-200 °C) can degrade the sugars, so that reduce the carbon source and ultimately lower the ethanol yield.

Compared to chemical hydrolysis methods, enzymatic hydrolysis has the potential for high efficiency conversion of cellulose with less production cost. To increase the efficiency of enzymatic hydrolysis and reduce the use of expensive

enzymes, lignocellulosic material should be pretreated to separate cellulose and hemicellulose from lignin. However, the main drawback of enzymatic hydrolysis was the high cost of enzyme because free cellulases were of low specific activity, susceptible to inactivation and difficult to recycle. It was stressed that immobilization of cellulases on the soluble or reversible soluble polymers was under development.

Many microorganisms like bacteria and fungi secrete enzymes that can break down large molecules such as cellulose, hemicellulose and lignin (Eriksson et al., 1990; Beguin and Aubert, 1994; Atkinson et al., 1997; Das et al., 2007). During microbial degradation of lignocellulose, the polymer is partially hydrolyzed and solubilized through the activity of exoenzymes, reduced to a molecular weight appropriate for uptake by microbial cells, and converted into biomass and other end products of microbial metabolism (Moran and Hodson, 1989). Therefore, a microbial route could be a promising and competitive way to convert lignocellulose into reducing sugars and subsequently to ethanol fuel. However, current research only focuses on lignocellulose degradation by microorganisms (Mullings and Parish, 1984; Moran and Hodson, 1989; Kim, 1999; Landy et al., 2008). Little is known about microbial conversion of lignocellulose to reducing sugars for ethanol production in tropical region, including Singapore, since most studies have been conducted acid hydrolysis and enzymatic hydrolysis.

1.3 Objectives and scopes

This thesis attempted to develop a unique microbial hydrolysis process for converting lignocellulosic waste into reducing sugars for subsequent biofuel or biochemicals production. To achieve this goal, the reducing sugars-producing microorganisms in tropical area were planned to be isolated and finally optimized to achieve maximum reducing sugars yield. Eventually, the ultimate goal was to build up a microbial cocktail to make the microbial hydrolysis process

inexpensive and environmentally friendly. The scopes of this study are as follows:

1. To isolate reducing sugars-producing microorganisms from natural sources, e.g., 1-year compost, 4-month compost, aerated activated sludge and garden soil, monitor the polysaccharides degradation ability, reducing sugars production yield and identify the dominant strains of microbial communities;
2. To isolate reducing sugars-producing microorganisms from guts of mealworm, a tropical animal in two basic nutrients with three different carbon sources, e.g. lignocellulosic waste, filter paper and Carboxymethyl cellulose (CMC), monitor the polysaccharides degradation ability, reducing sugars production yield and identify the dominant strains of microbial communities;
3. To make a microbial “cocktail” by using the isolated pure cultures to select the best combination according to the reducing sugars yield;
4. To optimize environmental conditions, e.g., pH, temperature and initial lignocellulose concentration by three types of pretreatment methods to achieve much more reducing sugars yield.

1.4 Thesis outline

This thesis is organized into 7 different chapters. Chapter 2 is a comprehensive literature review of the current technologies, issues and challenges about fuel ethanol production. Chapter 3 describes the results and detailed discussion on the detection and characterization of microbial communities from natural sources, i.e., 1-year compost (C1), 4-month compost (C4), aerated activated sludge (AAS) and garden soil (GS) during conversion of lignocellulosic waste to reducing sugars. Chapter 4 provides the process of isolation and characterization of microbial community in six different media from the guts of *Tenebrio Molitor*

Linnaeus (yellow mealworm) during conversion of lignocellulose to reducing sugars. Chapter 5 focuses on construction of a microbial cocktail to effectively convert lignocellulosic waste to reducing sugars by using the seven pure strains isolated in chapters 3 and 4. Chapter 6 optimizes the environmental conditions, e.g. pH, temperature, initial lignocellulose concentration and pretreatment methods to improve reducing sugars production yield from lignocellulosic waste by defined microbial cocktail using response surface methodology. Chapter 7 summarizes important conclusions and outlines some recommendations for future study.

Chapter 2 Literature Review

2.1 Overview

In order to obtain a clear understanding of the status and challenges of conversion of lignocelluloses to useful fuels, e.g., bioethanol, a literature review was carried out and presented in this chapter. This part reviewed first-generation food-based ethanol production and its major issues, second-generation lignocellulose-based ethanol production and its advantages compared to the first-generation one and overall processes of fuel ethanol production. The key steps including hydrolysis and pretreatment will be specifically highlighted. Finally, research focuses will be proposed in section summary.

2.2 First-generation food-based ethanol production

Sugarcane

The production of ethanol from sugarcane was an extremely feasible option. Bronzeoak Limited, a UK based company, saw an opportunity in the Philippines, a 100% fuel importer, for integrated and dedicated sugarcane to ethanol plant, which has been designed for minimum carbon footprint. The plant produces ethanol and commercially exploitable carbon dioxide as well as co-generation of power. The use for all byproducts, including effluent, results in zero discharge (Marwaha, 2007). Bioethanol production as an integrated part of sugarcane processing can be based on raw juice, molasses or a mixture of both. On average, a yield of 85 L of ethanol per ton of sugarcane was calculated. Intelligent fermentation concepts such as "high density processes" through yeast treatment and recycling were generating fast batch cycles of less than 12 h and high conversion yields of up to 92% (based on the theoretical equation from sugars to ethanol) (Dorfler and Amorim, 2007).

Corn

In the United States, the major raw material for ethanol was corn grain (starch). The US has the capacity to produce 13 billion gallons per year from corn alone and will probably reach the 7.5 billion gal per year goal much sooner than expected (Gray et al., 2006). The majority of the bioethanol currently available in Canada was also produced from corn, where the starch in the corn was chemically or enzymatically hydrolyzed to glucose, which in turn was fermented to alcohol using *Saccharomyces. cerevisiae* and was carried out at 30-32 °C with the addition of ammonium sulfate or urea as nitrogen sources (Sanchez and Cardona, 2008). *Zymomonas. mobilis* also has been researched for ethanol production from dry-milled corn starch (Krishnan et al., 1999). Currently, an estimated 1000 retail locations were selling ethanol-blended gasoline in all provinces west of New Brunswick, where approximately 7% of the gasoline sold in Canada was blended with ethanol at a proportion of 5-10% (Champagne, 2007).

Wheat

In France, ethanol was also produced from wheat by a process similar to that of corn (Sanchez and Cardona, 2008). Some efforts have been done for optimizing fermentation conditions. It follows that higher ethanol concentration were obtained, lower amounts of process water were required and energy costs were decreased. To accelerate high gravity fermentations, the controlled addition of small amounts of acetaldehyde during the fermentation has allowed the reduction in cultivation time from 790 h to 585 h without effect on the ethanol yield (Barber et al., 2002). A novel design of a wheat-based biorefinery for bioethanol production system was proposed by Arifeen et al. (2007), the system could result in a production cost within the range of \$0.96-0.50 gal⁻¹ ethanol (\$0.25-0.13 L⁻¹ ethanol) when the production capacity of the plant was within the range of 10-33.5 million gal y⁻¹.

Sweet sorghum

Sweet sorghum is rich in biomass and sugar. It contains approximately equal quantities of soluble (glucose and sucrose) and insoluble carbohydrates (cellulose and hemicellulose) and has been considered as an important source for the production of fuel ethanol (Sanchez and Cardona, 2008). Sweet sorghum

polysaccharides have recently been simultaneously hydrolyzed and fermented to ethanol by *Fusarium. oxysporum* alone or in mixed cultures with other microorganisms with promising results. The high yield of sorghum crop in some countries, such as Greece, make this process worthy of further investigation for the production of fuel bioethanol (Mamma et al., 1995). Moreover, from some varieties of sweet sorghum, the following production rate can be obtained: 5 ton/Ha grains, 8 ton/Ha sugar and 17 ton dry matter/Ha lignocelluloses. In 1999, the estimated price for fuel ethanol production from this feedstock was 200-300 US\$/m³, whereas the corresponding one was 260 US\$/m³ for sugar cane ethanol, 300–420 US\$/m³ for corn ethanol and 450 US\$/m³ for lignocellulosic ethanol (Grassi, 1999).

Molasses

The most widely used sugar for ethanol fermentation was molasses that contains about 50 (w/w) of sugar and about 50 (w/w) of organic and inorganic compounds, including water. Since molasses contained microorganisms which can affect the fermentation, the molasses was first sterilized before fermentation. In addition, a very high concentration of sugar can give too much ethanol and results in a prolonged fermentation time and an incomplete sugar conversion (Lin and Tanaka, 2006). Sugarcane molasses was the main feedstock for ethanol production in India (Ghosh and Ghose, 2003); Beet molasses were other source of fermentable sugars for ethanologenic fermentation (Cardona and Sanchez, 2007). *Saccharomyces cerevisiae* was the most employed microorganisms, which can hydrolyze cane sucrose into glucose and fructose, two easily assimilable hexoses. Other yeasts, such as *Schizosaccharomyces pombe*, present the additional advantage of tolerating high amounts of salts and high solids content (Bollok and Reczey, 2000). *Zymomonas mobilis* was the most promising bacteria, which has a low energy efficiency resulting in a higher ethanol yield, which was up to 97% of theoretical maximum (Carrascosa, 2006).

2.3 Major issues concerning food-based ethanol production

Sugar (Sucrose), which was produced from sugarcane, was one of the major basic food items for people worldwide. Corn, produced from maize plant was classified under the grains category that makes up 80% world's food supply. Since the production of ethanol was from agriculture feedstock such as corn and sugarcane, it has caused several issues because of the fact that these are human food resources. One of the major issues involved was the competition for the feedstock in the food market, which raised the international prices due to insufficient supply. The increasing shortage of food supply around the world has further worsened this issue. Another typical problem arising was the reduction of other crops grown. For example in U.S. and Brazil, lands were being converted for corn and sugarcane plantations, causing other crops such as fruits crop to be reduced (Pimentel and Patzek, 2005).

The second major issue was the environmental cost generated by producing ethanol from corn and sugarcane. For example, corn production in the U.S. caused more total soil erosion than any other U.S. crop. It used more herbicides, insecticides and fertilizers than any other crop produced in the U.S. thus causing more water pollution (Pimentel and Patzek, 2005). All these factors suggested that the environmental system in which U.S. corn was produced was being rapidly degraded. As for sugarcane production in Brazil, the same environmental problems were faced namely soil degradation, emission to water during the production of sugar syrup and emission into atmosphere due to burning before manual harvesting (Rodrigues et al., 2006). Apparently, a lot of improvement needed to be made in terms of crop cultivation so as to make both corn and sugarcane environmentally sustainable.

2.4 Second-generation lignocelluloses-based ethanol production

Lignocellulosic biomass mainly consists of celluloses (35-50%), hemicelluloses (20-35%) and lignin (15-25%), which has been recognized as a major world renewable energy source to supplement declining fossil fuel resources for three main reasons. First, lignocellulosic biomass is a renewable resource that could be sustainably developed in the future. Second, it appears to have formidably positive environmental properties resulting in no net releases of carbon dioxide (CO₂) and very low sulfur content. Third, it appears to have significant economic potential provided that fossil fuel prices increase in the future (Demirbas, 2007).

In general, prospective lignocellulosic materials for fuel ethanol production can be divided into horticultural waste (aspen, poplar, pine, spruce, newsprint, waste office paper, recycled paper sludge, alfalfa hay, switchgrass, reed canary grass, coastal bermudagrass and thimothy grass) and crop residues (cane bagasse, corn stover, wheat straw, rice straw, rice hulls, barley straw, sweet sorghum bagasse, olive stones and pulp) (Sun and Cheng, 2002; Cardona and Sanchez, 2007).

Horticultural waste

Horticultural waste consists of everyday items such as product packaging, grass clippings, hardwood, softwood and all kind of paper, which represents one of the foreseeable sustainable sources in nature because of its relatively low cost and plentiful supply (Fang et al., 2010; Gnansounou, 2010; Goh et al., 2010; Mabee and Saddler, 2010). In Singapore, the amount of horticultural waste generated in 2008 was 229,300 tons, of which only 42% of the respective waste was recycled (NEA Web site, <http://www.nea.gov.sg>).

Currently, horticultural waste is dealt with in different ways including landfilling, composting, combustion, recycling and illegal dumping. Landfills are engineered areas in which waste is buried in the land with various systems and safeguards to prevent ground water contamination, but the waste still poses health risks because it generates certain greenhouse gases, such as methane, carbon dioxide, saturated hydrocarbons, acidic hydrocarbons, aromatic hydrocarbons and sulfur compounds (Brosseau and Heitz, 1994). Another method of dealing with lignocellulose waste is composting, the decomposition of such matter by

microorganisms (mainly bacteria and fungi) to produce a humus-like substance that can be used as fertilizer. A problem that faces composting is that as a consequence of modernization, the composition of waste is changing and thus pesticides and polychlorinated bi-phenyls, as well as heavy metals, might be present in such high concentrations that they could make compost undesirable for land application (Van and Jacobus, 2001). Incineration is also one method of managing lignocellulosic waste and is popular in countries with a severe shortage of landfill sites (Yu et al., 1996). It is described as the controlled combustion of waste with the primary purpose of reducing the combustible portion of it and of recovering the available energy from the process. This method is considered as the worst way of dealing with lignocellulosic waste because it destroys resources for bio renewal. Recycling of waste proves to be an effective management option because it does not involve the emission of many greenhouse gases and water pollutants. It also saves energy, supplies valuable raw materials to industry, stimulates the development of green technologies, conserves natural resources and reduce the need for new landfill sites and incinerators.

Sugarcane bagasse

The sugarcane plant (*Saccharum officinarum*) can provide food, energy and feedstock for industry. Since sugar prices on the world market have been very low for a long time, the diversification of the sugar industry has been an urgent requirement in sugar-exporting countries (Gálvez, 2000). Bagasse, as a process waste, has traditionally been burnt in low efficiency boilers to produce modest amounts of energy and to limit the disposal problem. Because of its high carbohydrate content, relatively low lignin content and its availability as an industrial waste product, bagasse was a particularly appropriate substrate for bioconversion to ethanol (Martin et al., 2002). Brazil is the largest producer of sugarcane with 495 billion tons, the Brazilian annual production of sugarcane bagasse is currently estimated at 186 tons of bagasse (Soccol et al., 2010). The development of the sugarcane bagasse ethanol technology in Brazil is favored because the production process can be annexed to the sugar/ethanol units already in place, requiring lower investments, infrastructure, logistics and energy supply. This is a promising scenario because from each 10 million tons of dry biomass,

600 million gallons of ethanol could be produced, considering the use of its cellulosic part only.

Agricultural residue

Lignocellulosic residues used by the Canadian bio fuel industry are corn cobs, corn stover or wheat straw. Iogen currently operates a demonstration plant in Ottawa using wheat straw, while Greenfield Ethanol has developed a pilot plant in Chatham, Ontario that uses corn residues including cobs and stover (Mabee and Saddler, 2010). One study on wheat straw residues shows that variation in amounts between 1.37 and 8.46 t ha⁻¹ may be expected (Bowyer and Stockmann, 2001). The amount of straw that can be removed and utilized in an industrial process is likely significantly lower than the total residues remaining on the field. It is because that soil conservation requirements will account for 50% or more of the total residues in many areas. Furthermore, a proportion of cereal straw will generally be utilized by farmers for livestock feed. So that Canadian agricultural residues from cereal crop production has been estimated at between 2.7 and 18 million dry tones per annum (Mabee et al., 2006). Cotton gin waste (CGW) consists of sticks, leaves, burs, soil particles, mote, cotton lint and other plant materials (Schacht and Lepori, 1978). In the United States, the disposal of cotton gin waste (CGW) was a significant problem in the cotton ginning industry, but CGW could be potentially used as feedstock for bioethanol. Freshly discharged CGW and stored CGW were characterized for storage stability and potential for ethanol production by determining their summative compositions (Agblevor et al., 2006).

Woody waste

In addition to agricultural residues, wood residues from forest harvest practices or forest product processing is also a suitable feedstock for bioethanol production. Currently, Lignol is operating a pilot bioconversion facility in Burnaby, British Columbia, Canada, which uses softwood or hardwood chips as a feedstock (Mabee et al., 2006). Estimated dry residues that may be sustainably removed from forest harvest operations ranges between 9.8 and 46 million dry tones per annum. In addition, residues from forest products manufacture have been conservatively estimated to range between 2 and 5.4 million dt a⁻¹ (Mabee and Saddler, 2010).

The residues of pine and spruce contain around 43–45% cellulose, 20–23% hemicellulose and 28% lignin. Hemicellulose is mainly made up of mannose, which was a hexose that can be fermented by normal baker's yeast, and the content of pentose was only around 6-7% of the total wood. Theoretically, around 410 L of ethanol can be produced per metric ton dry raw material using only the hexose fraction and 455 L if all carbohydrates are considered (Galbe and Zacchi, 2002). Canadian researchers have worked to capture the opportunity that beetle-killed wood presents for the bio fuel industry (Ewanick et al., 2007).

2.5 Advantages of lignocellulosic waste as feedstock

The following advantages of lignocellulosic waste as feedstock must be outlined in comparison with established sugar- or starch-based ethanol production.

Economical Advantages

- Lignocellulosic biomass sources are geographically more evenly distributed than the fossil fuels; thus the sources of energy will, to a larger extent, be domestic and provide security of supply.
- Lignocellulosic materials minimize the potential conflict between land use for food (and feed) production and energy feedstock production. The raw material is less expensive than conventional agricultural feedstock and can be produced with lower input of fertilizers, pesticides and energy.
- Lignocellulosics-based bioethanol production might also provide employment in rural areas (Hahn-Hagerdal et al., 2006).

Environmental Advantages

- Bioethanol from lignocellulosic biomass generates low net greenhouse gas emissions, reducing environmental impacts, particularly climate change.
- Bioethanol from lignocellulosic biomass was considered as an alternative solution of green waste treatment in the world, which can reduce the

second pollution of traditional treatment methods, e.g. landfill and compost.

- Bioethanol from lignocellulosic biomass can avoid some environmental problems, e.g. soil degradation and water pollution, because more herbicides, insecticides and fertilizers were used for corn or sugarcane production than any other crop production in the U.S.

2.6 Technologies involved in lignocellulosic ethanol production

Based on literature, the composition of cellulose, hemicellulose and lignin in Lignocellulosic waste was summarized in Table 2-1 (Galbe and Zacchi, 2002).

Table 2-1 The contents of cellulose, hemicellulose and lignin in common lignocellulosic residue and waste^a

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood stems	40-45	24-40	18-25
Softwood stems	45-50	25-35	25-35
Nut shells	25-30	25-30	30-40
Corn cobs	45	35	15
Grasses	25-40	35-50	10-30
Paper	85-99	0	0-15
Wheat straw	30	50	15
Sorted refuse	60	20	20
Swine waste	6	28	0
Coastal Bermuda grass	25	35.7	6.4
Switch grass	45	31.4	12
Waste papers from chemical pulps	60-70	10-20	5-10
Leaves	15-20	80-85	0
Cotton seed hairs	80-95	5-20	0
Newspaper	40-45	25-40	18-30

^aSource: (Sun and Cheng, 2002)

Bioethanol production utilizing lignocellulosic feedstock materials such as residual and waste biomass, generally takes place in four major units operation (Fig. 2-1): pretreatment, hydrolysis, fermentation and product separation/purification. Pretreatment is required to alter the biomass macroscopic and microscopic size and structure as well as its submicroscopic chemical composition and structure, liberates cellulose and hemicellulose from lignin. Hydrolysis includes the processing steps that convert the carbohydrate polymers into fermentable reducing sugars (hexose and pentose) to produce ethanol (Shahbazi and Li, 2006; Champagne, 2007). Reducing sugars are readily fermented to ethanol by many naturally occurring organisms, but the pentose, xylose and arabinose are fermented to ethanol by fewer native strains, and usually at relatively low yields. While pentose is not readily fermented, the ketose of xylose is converted to ethanol by *S. pombe*, *S. cerevisiae*, *S. amucae* and *Kluveromyces fragilis* (Mosier et al., 2005). Xylose and arabinose generally are significant fractions of hardwoods, agricultural residues and grasses and must be utilized to make the economics of biomass processing feasible (Lynd et al., 1999). Genetic modification of bacteria and yeast has produced recombinant strains capable of co-fermenting both pentoses and hexoses to ethanol and other valuable products at high yields.

Fig. 2-1 Possible pathways for bioethanol fermentation from lignocellulosic feedstock (Rubin, 2008)

The fermentation of biomass to alcohols has been a well-established technology. It was the primary step in the production of ethanol fuel. Hydrolysis performed separately from fermentation step is known as separate hydrolysis and fermentation (SHF). Hydrolysis carried out in the presence of the fermentative microorganism is named as simultaneous saccharification and fermentation (SSF). Simultaneous saccharification of both cellulose (to glucose) and hemicellulose (to xylose and arabinose) and co-fermentation of both glucose and xylose would be carried out by genetically engineered microbes that ferment xylose and glucose in the same broth as the hydrolysis of cellulose and hemicellulose, this process is Simultaneous saccharification and co-fermentation (SSCF). SSF and SSCF are commonly applied since both unit operations can be accomplished in the same tank, resulting in lower costs (Wright, 1998). Ethanol is recovered from the fermentation broth by distillation or distillation combined with adsorption (Cysewski and Wilke, 1977; Ishida and Shimizu, 1996; Da Silva et al., 1999; Ikegami et al., 2004). Currently, the distillation methods including vacuum distillation (Da Silva et al., 1999), gas stripping distillation (Taylor et al., 1998),

and membrane distillation (Ohashi et al., 1998). The residual of distillation may be concentrated and burned as fuel to power the process, or converted to various co products (Wooley et al., 1999; Wyman, 1999).

In this literature review, two very important processing steps, pretreatment and hydrolysis have been targeted to investigate.

2.6.1 Pretreatment

The purpose of the pretreatment was to remove lignin and reduce cellulose crystalline, alter the biomass macroscopic and microscopic sizes and structures as well as its submicroscopic chemical composition and structure so that hydrolysis of the carbohydrate fraction to reducing sugars can be achieved more rapidly and with greater yields (Mosier et al., 2005). Pretreatment must meet the following requirements: (1) improve the formation of sugars or the ability to subsequently form sugars by hydrolysis; (2) avoid the degradation or loss of carbohydrate; (3) avoid the formation of byproducts inhibitory to the subsequent hydrolysis and fermentation processes; and (4) be cost-effective. Physical, physical-chemical, chemical and biological processes have been used for pretreatment of lignocellulosic materials (Fig. 2-2).

Fig. 2-2 Schematic of goals of pretreatment on lignocellulosic feedstock (Sun and Cheng, 2002)

Mechanical comminution

Lignocellulosic waste can be comminuted by a combination of chipping, grinding and milling to reduce cellulose crystallinity. The size of the materials was usually 10–30 mm after chipping and 0.2–2 mm after milling or grinding (Sun and Cheng, 2002). Vibratory ball milling has been found to be more effective in breaking down the cellulose crystallinity of spruce and aspen chips and improving the digestibility of the biomass than ordinary ball milling (Millett, 1976). The power requirement of mechanical comminution of agricultural materials depends on the final particle size and the waste biomass characteristics (Cadoche and Lopez, 1989).

Pyrolysis

Pyrolysis has also been used for pretreatment of lignocellulosic materials. When the materials were treated at temperatures greater than 300 °C, cellulose was rapidly decomposed to produce gaseous products and residual char (Shafizadeh and Lai, 1975). The decomposition was much slower and less volatile products were formed at lower temperatures. Mild acid hydrolysis (1 N H₂SO₄, 97 °C, 2.5 h) of the residues from pyrolysis pretreatment has resulted in 80–85% conversion of cellulose to reducing sugars with more than 50% glucose (Fan et al., 1987). The process can be enhanced with the presence of oxygen. When zinc chloride or sodium carbonate was added as a catalyst, the decomposition of pure cellulose can occur at a lower temperature (Shafizadeh and Lai, 1975).

Steam explosion (Autohydrolysis)

Steam explosion was recognized as one of the most cost effective pretreatment processes for hardwoods and agricultural residues, but it was less effective for softwoods (Clark and Mackie, 1987). In this method, chipped biomass was treated with high-pressure saturated steam and then the pressure was swiftly reduced, which makes the materials undergo an explosive decompression. Steam explosion was typically initiated at a temperature of 160–260 °C (corresponding pressure 0.69–4.83 MPa) for several seconds to a

few minutes before the material was exposed to atmospheric pressure. Ninety percent efficiency of enzymatic hydrolysis has been achieved in 24 h for poplar chips pretreated by steam explosion, compared to only 15% hydrolysis of untreated chips (Grous et al., 1986). The factors that affected steam explosion pretreatment were residence time, temperature, chip size and moisture content (Duff and Murray, 1996). Optimal hemicellulose solubilization and hydrolysis could be achieved by either high temperature and short residence time (270 °C, 1 min) or lower temperature and longer residence time (190 °C, 10 min) (Duff and Murray, 1996). Recent studies indicated that lower temperature and longer residence time were more favorable (Wright, 1998). The optimal conditions of steam explosion pretreatment of sugarcane bagasse have been found to be as following: 220 °C; 30 s residence time; water-to-solids ratio, 2; and 1% H₂SO₄ (Morjanoff and Gray, 1987). Sugar production was 65.1 g sugar/100 g starting bagasse after steam explosion pretreatment.

The advantages of steam explosion pretreatment included the low energy requirement compared to mechanical comminution and no recycling or environmental costs. The conventional mechanical methods required 70% more energy than steam explosion to achieve the same size reduction (Holtzapple et al., 1989). Limitations of steam explosion included destruction of a portion of the xylan fraction, incomplete disruption of the lignin–carbohydrate matrix, and generation of compounds that might be inhibitory to microorganisms used in downstream processes (Mackie et al., 1985). Because of the formation of degradation products that were inhibitory to microbial growth, enzymatic hydrolysis and fermentation, pretreated biomass need to be washed by water to remove the inhibitory materials along with water-soluble hemicellulose (McMillan, 1994). The water wash decreased the overall saccharification yields due to the removal of soluble sugars, such as those generated by hydrolysis of hemicellulose. Typically, 20–25% of the initial dry matter was removed by water wash (Mes-Hartree et al., 1988).

Ammonia fiber explosion (AFEX)

AFEX is another type of physical-chemical pretreatment in which lignocellulosic materials were exposed to liquid ammonia at high temperature and pressure for a period, and then the pressure was swiftly reduced. The concept of AFEX was similar to steam explosion. In a typical AFEX process, the dosage of liquid ammonia was 1-2 kg ammonia/kg dry biomass, temperature 90 °C, and residence time 30 min. AFEX pretreatment can significantly improve the saccharification rates of various herbaceous crops and grasses. It can be used for the pretreatment of many lignocellulosic materials including alfalfa, wheat straw, wheat chaff (Mes-Hartree et al., 1988), barley straw, corn stover, rice straw (Vlasenko et al., 1997), municipal solid waste, softwood, newspaper, coastal bermuda grass, switchgrass (Holtzapple et al., 1992a), aspen chips and bagasse (Holtzapple et al., 1991).

The AFEX pretreatment did not significantly solubilize hemicellulose compared to acid pretreatment (to be discussed in the following section) and acid-catalyzed steam explosion (Mes-Hartree et al., 1988). Over 90% hydrolysis of cellulose and hemicellulose has been obtained after AFEX pretreatment of Bermuda grass (approximately 5% lignin) and bagasse (15% lignin) (Holtzapple et al., 1991). However, the AFEX process was not very effective for the biomass with high lignin content such as newspaper (18–30% lignin) and aspen chips (25% lignin). Hydrolysis yield of AFEX-pretreated newspaper and aspen chips were reported as only 40% and below 50%, respectively (McMillan, 1994). To reduce the cost and protect the environment, ammonia must be recycled after the pretreatment. In an ammonia recovery process, a superheated ammonia vapor with a temperature up to 200 °C was used to vaporize and strip the residual ammonia in the pretreated biomass and the evaporated ammonia was then withdrawn from the system by a pressure controller for recovery (Holtzapple et al., 1992b). The ammonia pretreatment does not produce inhibitors for the downstream biological processes, so water wash was not necessary (Mes-Hartree et al., 1988).

CO₂ explosion

Similar to steam and ammonia explosion pretreatment, CO₂ explosion was also used for pretreatment of lignocellulosic materials. It was hypothesized that CO₂ would form carbonic acid and increase the hydrolysis rate. Dale and Moreira (1982) used this method to pretreated alfalfa (4 kg CO₂:1 kg fiber at the pressure of 5.62 MPa) and obtained 75% of the theoretical glucose released during 24 h of the enzymatic hydrolysis. The yields were relatively low compared to steam or ammonia explosion pretreatment, but high compared to the enzymatic hydrolysis without pretreatment. Zheng et al. (1998) compared CO₂ explosion with steam and ammonia explosion for pretreatment of recycled paper mix, sugarcane bagasse and repulping waste of recycled paper, they found that CO₂ explosion was more cost-effective than ammonia explosion and did not cause the formation of inhibitory compounds that could occur in steam explosion.

Ozonolysis

Ozone can be used to degrade lignin and hemicellulose in many lignocellulosic materials such as wheat straw, bagasse, green hay, peanut, pine, cotton straw and poplar sawdust (Sun and Cheng, 2002). The degradation was essentially limited to lignin and hemicellulose was slightly attacked, but cellulose was hardly affected. The rate of enzymatic hydrolysis increased by a factor of 5 following 60% removal of the lignin from wheat straw in ozone pretreatment (Vidal and Molinier, 1988). Enzymatic hydrolysis yield increased from 0% to 57% as the percentage of lignin decreased from 29% to 8% after ozonolysis pretreatment of poplar sawdust. Ozonolysis pretreatment had the following advantages: (1) it effectively removed lignin; (2) it did not produce toxic residues for the downstream processes; and (3) the reactions had been carried out at room temperature and pressure (Vidal and Molinier, 1988). However, a large amount of ozone was required, making the process expensive (Sun and Cheng, 2002).

Organosolv process

In the organosolv process, an organic or aqueous organic solvent mixture with inorganic acid catalysts (HCl or H₂SO₄) was used to break the internal lignin and hemicellulose bonds. The organic solvents used in the process include methanol, ethanol, acetone, ethylene glycol, triethylene glycol and tetrahydrofurfuryl alcohol (Chum et al., 1988; Thring et al., 1990). Organic acids such as oxalic, acetylsalicylic and salicylic acid could also be used as catalysts in the organosolv process (Sarkanen, 1980). At high temperatures (above 185 °C), the addition of catalyst was unnecessary for satisfactory delignification (Sarkanen, 1980). Usually, a high yield of xylose could be obtained with the addition of acid. Solvents used in the process need to be drained from the reactor, evaporated, condensed and recycled to reduce the cost. Removal of solvents from the system was necessary because the solvents may be inhibitory to the growth of organisms, enzymatic hydrolysis and fermentation (Aziz and Sarkanen, 1989).

Liquid hot water (hydrothermal) pretreatment

Water pretreatments use pressure to maintain the water in the liquid state at elevated temperatures. Flow-through processes pass water maintained in the liquid state at elevated temperatures through cellulosics. This type of pretreatment has been termed hydrothermolysis, aqueous or steam/aqueous fractionation, uncatalyzed solvolysis and aquasolv (Mosier et al., 2005). Solvolysis by hot compressed liquid water contacts water with biomass for up to 15 min at temperature of 200-230 °C. Between 40% and 60% of the total biomass is dissolved in the process, with 4-22% of the cellulose, 35-60% of the lignin and all of the hemicellulose being removed. Over 90% of the hemicellulose is recovered as reducing sugars when acid was used to hydrolyze the resulting liquid. The pretreatment results were found to be virtually independent of temperature and time. Variability in results was related to the biomass type with lignin solubilization impeding recovery of hemicellulose sugars (Mok and Antal, 2002).

Lime pretreatment

Alkali pretreatment processes utilize lower temperatures and pressures compared to other pretreatment technologies. Alkali pretreatment may be carried out at ambient conditions, but pretreatment time is measured in terms of hours or days rather than minutes or seconds. Unlike acid-catalyzed pretreatments, a limitation occurs because some of the alkali is converted to irrecoverable salts or incorporated as salts into the biomass by the pretreatment reactions. Lime has been used to pretreat wheat straw (85 °C for 3 h), poplar wood (150 °C for 6 h with 14-atm oxygen), switchgrass (100 °C for 2 h) and corn stover (100 °C for 13 h). Playne treated sugarcane bagasse with lime at ambient conditions for up to 192 h to improve the enzyme digestibility of the cellulose from 20% before pretreatment to 72% after pretreatment (Playne, 1984). Chang et al. (1998) obtained similar digestibility results by pretreatment of bagasse with lime at 120 °C for 1 h. the addition of air/oxygen to the reaction mixture greatly improves the delignification of the biomass, especially highly lignified materials such as poplar oxidative lime pretreatment of poplar at 150 °C for 6 h removed 77.5% of the lignin from the wood chips and improved the yield of glucose from enzymatic hydrolysis from 7% (untreated) to 77% (treated) compared to the untreated and pretreated poplar wood (Chang et al., 2001).

2.6.2 Hydrolysis

Hydrolysis is the other critical step for converting lignocellulosic waste to bio ethanol. The efficiency of hydrolysis decides the subsequently bioethanol production yield from lignocellulosic waste. Although currently the technology of hydrolysis is mature and the hydrolysis yield is over 90%, the disadvantages of current methods are high cost or causing second pollution. Therefore, an alternative hydrolysis method is urgently needed for obtaining much reducing sugars for bioethanol production; this method must be low cost, fast and environmental friendly.

Acid hydrolysis

Acid hydrolysis could be performed with several types of acids, including sulphurous, sulphuric, hydrochloric, hydrofluoric, phosphoric, nitric and formic acid. These acids might be either concentrated or diluted (Galbe and Zacchi, 2002). Processes involving concentrated acid had been operated at low temperature and give high yields (e.g. 90% of theoretical glucose yield), but the large amount of acid used causes problems associated with equipment corrosion and energy-demanding acid recovery (Jones and Semrau, 1984). Furthermore, when sulphuric acid is used, the neutralization process produces large amounts of gypsum (Keller, 1996). However, the process has attracted some new interests due to novel economic methods for acid recovery proposed by the companies Masada Resource Group (Birmingham, Ala), Arkenol (Mission Viejo, Calif.) and APACE (New South Wales, Australia) (Katzen, 1997). To our knowledge, there is no on-going development of concentrated acid hydrolysis of softwood.

The main advantage of dilute acid hydrolysis was the relatively low acid consumption. However, high temperature was required to achieve acceptable rates of conversion of cellulose to glucose, and high temperature also increased the rates of hemicellulose sugar decomposition and equipment corrosion (Jones and Semrau, 1984). Sugar degradation products could also cause inhibition in the subsequent fermentation stage (Larsson et al., 1999a). The maximum yield of glucose was obtained at high temperature and short residence time, but even under these conditions the glucose yield was only between 50% and 60% of the theoretical value (Wyman, 1996). A two-stage process has been developed to decrease sugar degradation. In the first hydrolysis stage, the relatively easily hydrolysed hemicellulose was released under rather mild conditions. This enabled the second acid hydrolysis step to proceed under harsher conditions without degrading the hemicellulose sugars to furfural, hydroxymethylfurfural and other degradation products. Using a two-stage dilute acid hydrolysis process, recovery yields of as much as 70–98% of the xylose, galactose, mannose and arabinose from softwood have been reported (Nguyen et al., 1999). However, the yield of glucose was still low at 50%. In the United States, BCI

was commercializing the technology based on two-step dilute acid hydrolysis (Wyman, 1999).

Alkaline hydrolysis

Some bases can also be used for hydrolysis of lignocellulosic waste and the effect of alkaline hydrolysis depends on the lignin content of the materials (Fan et al., 1987). The mechanism of alkaline hydrolysis was believed to be saponification of intermolecular ester bonds cross linking xylan hemicelluloses and other components, for example, lignin and other hemicellulose (Tarkow and Feist, 1969). Dilute NaOH treatment of lignocellulosic materials caused swelling, leading to an increase in internal surface area, a decrease in the degree of polymerization, a decrease in crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure (Fan et al., 1987). The digestibility of NaOH-treated hardwood increased from 14% to 55% with the decrease of lignin content from 24–55% to 20%. However, no effect of dilute NaOH pretreatment was observed for softwoods with lignin content greater than 26% (Millet et al., 1976; Bjerre et al., 1996). Dilute NaOH pretreatment was also effective for the hydrolysis of straws with relatively low lignin content of 10–18% (Bjerre et al., 1996) used the combination of irradiation and 2% NaOH for pretreatment of corn stalk, cassava bark and peanut husk. The glucose yield of corn stalk was 20% in untreated samples compared to 43% after treatment with electron beam irradiation at the dose of 500 Kg/y and 2% NaOH, but the glucose yields of cassava bark and peanut husk were only 3.5% and 2.5%, respectively. Ammonia was also used for the pretreatment to remove lignin. Iyer et al. (1996) described an ammonia recycled percolation process (temperature, 170 °C; ammonia concentration, 2.5–20%; reaction time, 1 h) for the pretreatment of corn cobs/stover mixture and switch grass. The efficiency of delignification was 60–80% for corncobs and 65–85% for switch grass.

Enzymatic hydrolysis

Enzymatic hydrolysis of cellulose has been carried out by cellulase enzymes which were highly specific. The products of the hydrolysis were usually reducing sugars including glucose. Utility cost of enzymatic hydrolysis was low compared to acid or alkaline hydrolysis because enzyme hydrolysis was usually conducted at mild conditions (pH 4.8 and temperature 45–50 °C) and did not have a corrosion problem (Duff and Murray, 1996). Both bacteria and fungi could produce cellulases for the hydrolysis of lignocellulosic materials (Sun and Cheng, 2002). There were known cellulase and hemicellulase enzymes from both bacterial and fungal sources. The most commonly used enzymes in lab and pilot-scale bioethanol production were cellulases from the aerobic fungi *Trichoderma reesei* (Gray et al., 2006). Cellulase of the thermophilic, ethanol-producing anaerobic bacterium *Clostridium thermocellum* could be also used for the pretreatment of substrate in ethanol production (Lynd and Grethlein, 1987). Cellulases were used in different quantities in the range from 8 to 40 FPU/g of substrate, depending on the type and concentration of substrate. For example, application of cellulase in quantity 8 FPU/g (Lark et al., 1997), 15 FPU/g (Ballesteros et al., 2004) and 40 FPU/g substrate (Krishna and Chowdary, 2000) were reported.

Enzymatic hydrolysis has several advantages. First, the specificity of enzymes allows the production of sugar syrups with well defined physical and chemical properties. Second, the milder enzymatic hydrolysis results in few side reactions and less “browning.” Indeed, for the production of glucose syrups from starch, enzymatic hydrolysis is essential (Lin and Tanaka, 2006). However, the main drawback for enzymatic hydrolysis was the high cost of enzyme because free cellulase was of low specific activity, susceptible to inactivation and difficult to recycle. Technology of immobilization of cellulase in the soluble or reversible soluble polymers was under development. A hydrolysis time of several days is necessary for enzymatic hydrolysis. The sugars released inhibit the hydrolysis reaction, in order to overcome this problem, SSF process was developed, in which the sugar released from the hydrolysis are directly consumed by the present fermentation microorganisms. Direct microbial conversion seems to be a feasible alternative of converting cellulosic biomass to reducing sugars.

2.6.3 The advantages of microbial hydrolysis process

Since loss of enzyme is the big challenge of enzymatic hydrolysis which will increase the cost of application, directly use microorganisms to hydrolyze lignocellulosic waste might be an alternative solution. The advantages of microbial hydrolysis process include the following aspects: (a) Low cultivation cost; (b) Hydrolysis time is shorter than enzymatic hydrolysis (24-36h); (c) Working conditions are similar to the fermentation step; (d) Low corrosion; (e) Low utility consumption; (f) Low toxicity of the hydrolysates.

A number of microorganisms including fungi, bacteria and actinomycetes had the ability to produce cellulose-degrading enzymes (Bisaria et al., 1989). *Poria placenta*, *Lanzites trabeaum*, *Tyromyces palustris* and *Coniophora puteana* were brown-rot fungi among the most studied for their cellulolytic activities (Eriksson et al., 1990). White-rot group of fungi have the ability to degrade lignin as well as other lignocellulosic components (Eriksson, 1981). The most studied white-rot fungi were *Phanerochaete chrysosporium* that was first isolated from wood chip piles. Other important white-rot fungi are *Sporotrichum thermophile* and *Coriolus versicolor*. Rumen fungi often described as a complex of enzymes that could solubilize cellulose efficiently (Wood, 1991; Wood and Ingram, 1992). Cellulolytic enzyme systems of bacteria were not directly comparable to those of fungi. Bacteria often produced cellulases in small amounts and degradation of cellulose seemed to take place by a cluster of multi-enzyme complexes, which were difficult to disrupt without loss of total activity as well as of the individual components (Rabinovich, 2002a).

Hemicellulose-degrading system is widespread as it is produced by fungi and bacteria from terrestrial environments, rumen microorganism, yeasts and marine algae. Fungi, yeasts and some bacteria secreted hemicellulolytic enzymes extracellularly, although cell wall bound and intracellular hemicellulolytic enzymes had also been reported (Kuhad et al., 1997a). Only a few yeast species

such as some species of *Aureobasidium*, *Cryptococcus* and *Trichosporon* were known to produce xylan-degrading enzymes (Eriksson et al., 1990).

Lignin is degraded to different extents by different microorganisms, of which wood-rotting fungi were the most effective, white rot fungi in particular (Eriksson et al., 1990). Brown-rot fungi prefer coniferous wood. These fungi caused extensive degradation of the polysaccharides and only a limited degradation of lignin. These were some brown-rot fungi, which had the capability of degradation of lignin *Fomitopsis pinicola*, *Gleophyllum trabeum*, *Poria placenta*, *Lentinus lepideus*, *Pholiota adiposa* and *Tyromyces palustris* (Kuhad et al., 1997a). White-rot fungi were the most effective wood-rotting fungi. These predominantly degrade wood from deciduous trees but coniferous wood trees were also degraded (Eriksson et al., 1990). Most white-rot fungi degraded wood by a simultaneous attack on the lignin, cellulose and hemicelluloses, but a few were rather specific lignin degraders (Blanchette, 1991). Bacteria generally caused a low percentage of mineralization of lignin in lignocellulosic materials (Zimmermann, 1990). Actinomycetes had been reported to mineralize lignin successfully, although not as fast or comprehensive as white-rot fungi (Eriksson et al., 1990).

2.6.4 Factors affecting microbial hydrolysis process

Temperature

Of the physical factors affecting microbial growth in any environment, one of the most influential in the selection of species is temperature (Brock and Darland, 1970). Microorganisms possess no means of controlling internal temperature, and the temperature within the cell is therefore determined by the external temperature. Each microorganism is able to grow only within a specific range of temperatures. Most microorganisms are capable of growing over a range of 30 to 40 °C. Three temperature values, often called the cardinal temperatures, are commonly used to characterize the effect of temperature on the growth of a microbial species or strain (Aksu and Eren, 2005). The

maximum and minimum temperatures define the limits of the temperature range within which growth is possible; at lower or higher temperatures than these, no growth occurs. The optimum temperature is that at which growth is most rapid, i.e., at which the growth rate reaches its maximum value (Tempest and Hunter, 1965). The optimum temperature for most microorganisms is much closer to the maximum than to the minimum temperature. The cardinal temperatures are considered to be characteristic of the organism, although they may be altered to some extent by other environmental factors such as pH, the concentration of salts, or the available nutrients (Margesin et al., 2000).

Growth at the minimum temperature is typically quite slow; the rate increases exponentially with increasing temperature, reaching a maximum at the optimum temperature and falling abruptly to zero at a few degrees above the optimum. For most microorganisms, the growth rate increases two- to threefold for each 10 °C rise in temperature between the minimum and the optimum (Tempest and Hunter, 1965).

Culture pH

A second environmental factor that, like temperature, influences the growth rate and limits growth is pH of the aqueous environment (Brock and Darland, 1970). Each microbial species is characterized by minimum and maximum pH values defining the limits of the range of pH values within which growth is possible (Aksu and Eren, 2005). The optimum pH value for any species is that at which growth rate is most rapid. The minimum and maximum values that limit growth usually differ by only three or four pH units. Most bacteria have pH optima near neutrality and minimum and maximum pH values for growth near 5 and 9, respectively. Most fungi prefer acidic environment and have minimum pH values between 1 and 3 with an optimum pH near 5. Most protozoa are able to grow in the pH rang 5 to 8, with an optimum pH near 7 (Margesin et al., 2000).

Substrate concentration

The carbon source is the third important factor (Brock, 1967). The ability of any species to use any specific carbon source depends on its genetic information. Some prototrophs such as some species of the genus *Pseudomonas*, are capable of utilizing any one of approximately 100 different types of organic molecules as sole carbon source, whereas others are much more restricted in their carbon source utilization (Robert et al., 1989; Durner et al., 2001). For most microorganisms, a single carbon source can supply almost all of the cellular carbon if one or a few auxotrophic requirements are available. Since carbon is the essential element in all organic compounds and comprises more than 50 percent of the dry weight of the cell, the presence of an utilizable carbon source is a major determining factor in the ability of any microbial species to flourish in a given habitat (Hempfling and Mainzer, 1975).

2.7 Summary

Lignocellulosic waste, e.g., agricultural residues, herbaceous residues and horticultural waste, is recognized as a major world renewable energy source to supplement declining fossil fuel resources. However, high production cost is one of the most critical issues currently. Although bioethanol production from lignocellulosic waste has been greatly improved by new technologies, there are still challenges that need further investigations. These challenges include developing more efficient pretreatment technologies for lignocellulose, investigating more feasible hydrolysis method, maintaining a stable performance in commercial scale fermentation operation and integrating the optimal components into economic ethanol production systems.

Pretreatment is necessary because it is an important tool to alter the structure of lignocellulosic waste to make cellulose and hemicellulose more accessible to the hydrolysis step. Pretreatment has been viewed as one of the most expensive processing steps in lignocellulosic waste-to-fermentable reducing sugars conversion. Pretreatment also has great potential for improvement of efficiency and lowering of cost through research and development. Acid hydrolysis of plant lignocellulosic biomass has been successfully commercialized and widely used

over the world. However, more energy input was required by the process of acid hydrolysis as the reactions generally occur either at high temperatures or at high pressures. In addition, massive use of acid catalyst would cause environmental pollution. Although enzymatic hydrolysis was a promising alternative, high costs but low specific activities of commercial enzymes had limited its application in the pilot-scale ethanol production.

In tropical area, microbial hydrolysis seems to be a unique process for converting lignocellulosic waste into reducing sugars due to its climatic advantages. A further knowledge of the microbial hydrolysis of lignocellulosic waste, however, needs to be understood as low hydrolysis rates were indicated in literatures without exception. It is believed that microbial conversion of lignocellulosic waste to reducing sugars is low cost and environmental friendly.

Chapter 3 Isolation and Characterization of Microbial Communities from Natural Sources during Conversion of Lignocellulosic Waste to Reducing Sugars

3.1 Introduction

Lignocellulosic waste, including leaves, uprooted weeds, twigs, grass clippings and small branches, represents one of the foreseeable sustainable sources in nature because of its relatively low cost and plentiful supply. Lignocellulose is the main component of lignocellulosic waste, and it is an alternative to food-based feedstock (i.e., corn and sugarcane) for fuel ethanol production.

Lignocellulose consists of three types of polymers including cellulose, hemicellulose and lignin that are strongly intermeshed and chemically bonded by non-covalent forces and covalent cross-linkages. A variety of chemical, mechanical and enzymatic approaches have been commonly employed in hydrolysis to release primarily hexose and pentose sugars for subsequent fermentation to ethanol, lactic acid and xylitol (Katrib et al., 1992; Anne Belinda Bjerre, 1996; Iranmahboob et al., 2002). However, there are some drawbacks using these conventional approaches for hydrolysis. For example, acid is harmful to the environment and may inhibit the subsequent fermentation process. The cost of specific functional enzyme is high (Jimenez and Bonilla, 1993; Nguyen et al., 1999; Saha et al., 2005; Sun and Cheng, 2005). Recently, studies on biodegradation of large molecules such as cellulose, hemicellulose and lignin have been suggested that bioconversion is a potential alternative for treatment of lignocellulosic waste (Kim, 1999; Das et al., 2007; Landy et al., 2008). However, the efficient microbial strains for lignocellulosic waste degradation and its production of reducing sugars which can be subsequently used for production of fuel ethanol remain to be further explored.

In this chapter, microbial hydrolysis of lignocellulosic waste to reducing sugars was investigated. Two composts, one sludge and one garden soil samples in tropical area were selected as inocula suggested by previous reports (containing more lignocellulose-degraders) (Mullings and Parish, 1984; George et al., 1993) for studying efficiency of lignocellulose degradation and reducing sugars yield. Both culture-independent and culture-dependent approaches were used to characterize the lignocellulose-degrading microbial isolates. DGGE was performed on PCR products representing the 18S rRNA genes of fungi and the 16S rRNA genes of bacteria to reflect community structure. Finding in this section was expected to extend our understanding of lignocellulose-degrading microorganisms, and to provide a basis for developing optimal control and management strategies of converting lignocellulosic waste to reducing sugars for fuel ethanol production in the future.

3.2 Materials and methods

3.2.1 Preparation of lignocellulose

Lignocellulosic waste was obtained from a local landscape and construction firm in Singapore. The raw waste including leaves, uprooted weeds, twigs, grass clippings and small branches was washed thoroughly with water to remove the soil and clay. Subsequently, it was milled to powder with a size of less than 1 mm, followed by the removal of extractives (e.g., waxes, fats, some resins, and portions of wood gums) and liberating cellulose and hemicellulose from lignin with toluene-ethanol (2:1, v/v) in a Soxhlet apparatus (Exelfit, Duran[®]) for 6 hours and drying at 60 °C until completely dry (Sun et al., 2004).

3.2.2 Sources of microorganisms

Four natural samples, including 1-year compost (C1), 4-month compost (C4), aerated activated sludge (AAS) and garden soil (GS) were collected as inoculums.

The compost samples were collected from a local composting plant in Singapore, the sample ages were 1 year and 4 month, respectively. Both of compost samples were taken from 10cm from the composting pile surface downward. Aerated activated sludge was collected from primary setting tank of local wastewater treatment plant. Garden soil was obtained from Yunnan garden at Nanyang Technological University. The sample-collected tools were sterilized and samples were stored in sterilized sampling bags and bottles. All the samples were kept cold (4 °C) and in the dark until cultivation of microorganisms was performed. Cultivation processes were conducted on the same day.

3.2.3 Nutrients for enrichment

The inoculated flasks were fed with lignocellulose as the sole carbon source with the following basic nutrients (g·L⁻¹): (NH₄)₂SO₄ 2, CaCl₂ 0.1, KH₂PO₄ 0.5, K₂HPO₄ 2.0, MgSO₄·7H₂O 0.1, NaCl 6.0, Yeast extract 1.0, and lignocelluloses waste powder 10.

3.2.4 Batch enrichment

10 g/L of green waste was used as the substrate for microorganisms cultivation. 10 g of each of 1-year compost, 4-month compost, garden soil, and 50 mL of aerated activated sludge with a MLSS concentration of 2.6 g/L) were suspended in 200 mL of sterilized aerobic cultivation medium. The medium included the following basic nutrients (g/L): (NH₄)₂SO₄ (2.0), CaCl₂ (0.1), KH₂PO₄ (0.5), K₂HPO₄ (2.0), MgSO₄·7H₂O (0.1), NaCl (6.0) and yeast extract (1.0). The cultivation was conducted at (30 ± 2) °C and pH 7 ± 0.2. The flasks were placed in a reciprocal incubation shaker (OLS 200, Grant Instruments, UK) with a horizontal rotational speed of 150 rpm.

For the starting of enrichment, 50% (v/v) of enrichment (including the used medium, cultures and lignocellulose waste) was removed after 15 days of incubation of the first batch, and replaced with 100 mL fresh sterilized medium and 10 g/L of sterilized lignocellulose waste powder. From the third batch

onwards, 75% (v/v) of the enrichment was removed and supplemented with 150 mL of the fresh sterilized medium and 10 g/L of sterilized lignocellulose waste powder. The temperature, initial pH and rotational speed of the tests were kept constant as described above. Soluble polysaccharides degradation and reducing sugars yield were monitored 1-2 times a week. This enrichment process was conducted for up to 12 months (after 24 successive transfers). Afterward, the effective lignocelluloses-degrading microbial consortia were used for batch degradation assays and microbial community analyses. For batch degradation tests, soluble polysaccharides depletion and reducing sugars production were measured during incubation and continuously monitoring upon 8 days.

3.2.5 Analytical methods

The amounts of hemicellulose, cellulose and lignin in collected lignocellulosic waste were characterized. To determine the amount of extractives in the waste solvent extraction, toluene-ethanol (2:1, v/v) for 1 g of dried waste sample was used, and refluxed in a Soxhlet apparatus for 6 hours. Subsequently, the sample was dried in a 60 °C oven until a constant weight was obtained. The difference in mass before and after the extraction is the amount of the extractives (Sun et al., 2004). The lignin compositions were determined according to NREL Ethanol Project Laboratory Analytical Procedure #003 for acid-insoluble lignin (David and Tina, 1995) and Procedure #004 for acid-soluble lignin (Erhman, 1996). Content of polysaccharides (soluble and insoluble) including cellulose and hemicellulose were calculated by difference, assuming that extractives, hemicellulose, lignin, and cellulose are the only components of the entire lignocellulosic waste. A phenol-sulfuric acid method was used to quantify polysaccharides (DuBois et al., 1956), with glucose as the standard. Monosaccharides were measured by DNS method with glucose as the standard (Miller, 1959). Each sample was measured in triplicates. pH was measured with a pH meter (CyberScan PCD 6500 pH meter, Germany).

3.2.7 Scanning electron microscopy (SEM) observation

After 12 months enriching processes, all enriched microorganisms were gently washed with a phosphate buffer solution and fixed with 4% paraformaldehyde for 4 hours. The fixed samples were dehydrated by successive passages through 40, 60, 80 and 100% ethanol and dried by a Freeze Dryer (Edwards, Crawley, West Sussex, UK) or Critical Point 6 Dryer (E3000) (VG Microtech, East Grinstead, West Sussex, UK) and subsequently observed by a scanning electron microscope (JSM-6400; JEOL, Tokyo) at an accelerating voltage of 12 or 15 kV.

3.2.8 Isolation procedure

Nutrient Agar (Difco™) and Sabouraud Dextrose Agar (Difco™) were used for bacterial and fungal isolation, respectively. The aliquot from each of the four enrichments (after 12 months enriched) was serially diluted with sterilized water (10^1 to 10^6 - fold dilutions), and 0.1 mL of each dilution was spread onto agar plates. The plates were incubated in an incubator at 30 °C until obvious colonies show up. After three successive transfers, the distinct colonies had been further transferred to lignocellulose agar plates (with lignocellulose waste powder as sole carbon source, 10 g/L) to confirm the isolated strains were able to utilize lignocellulose. Redundancy of obtained microbial strains was eliminated based on colony morphology, color and size.

3.2.9 DNA extraction, PCR and DGGE

After one year of cultivation, genomic DNA of lignocellulose-decomposing cultures was extracted by a ZYMO research soil microbe DNA extraction kit (ZYR - Zymo Research, USA).

Genomic DNA of the four enrichments (i.e., after 1, 6, 9, 10, 11 and 12 months) was extracted by a ZYMO research soil microbe DNA extraction kit (ZYR - Zymo Research, USA). PCR primers P2 and P3-GC (i.e., 40-bp GC clamps at 5'-end) were used to amplify the V3 region of the bacterial 16S rRNA gene corresponding to positions 341 and 534 in the *Escherichia coli* sequence (Muyzer et al., 1993). For the detection of Fungi, nested-PCR strategy with primers NS1 and NS8 (Kowalchuk et al., 1997) and with touch-down thermocycling program (Muyzer et al., 1993) were used.

The PCR-amplified fragments were separated by a DCode universal mutation detection system (Bio-Rad Laboratories) as described by Muyzer et al (1993). Briefly, the gel was placed in 1× TAE buffer and run at 85 V at 60 °C for 15 hours. After electrophoresis, the gel was stained with 2% ethidium bromide for 15 min and destained with 1× TAE buffer for 30 min. Result was viewed and photographed with an EDAS 290 gel imaging system (Kodak).

3.2.10 16S/18S rRNA gene sequencing and phylogenetic analyses

The whole-cell direct-lysis PCR amplification method was used to amplify the 16S rRNA gene of microbial isolates. The 16S rRNA gene and 18S rRNA gene for bacterial and fungal isolates were amplified with primer sets 8F/1490R (Weisburg et al., 1991) and NS1/NS8 (Kowalchuk et al., 1997), respectively. The PCR products were purified with a WIZARD SV GEL AND PCR CLEAN UP SYSTEM (Promega, USA) according to manufacturer's instructions and stored at -20°C before sequencing. The 16S rRNA gene sequence of the isolates was obtained from a local sequencing service company (1st BASE Pte Ltd, Singapore).

For phylogenetic analyses, the obtained sequences were compared to the sequences available from the Ribosomal Database Project (RDP) and the EMBL/GenBank databases. All relevant sequences were aligned using the Clustal W algorithm (program version Clustal ×1.83), and a phylogenetic tree was

constructed using the neighbor-joining algorithms (PHYLIP program package 3.6). Tree topologies were evaluated by bootstrap analysis of the neighbor-joining tree using the original dataset and 100 bootstrap datasets. *Cyanobacterium stanierii* (GQ350222) was used as an outgroup. The 16S/18S rRNA gene sequences obtained in this study were deposited in the EMBL nucleotide sequence database under accession GU183385 to GU183397.

Table 3-1 Taxonomic and phylogenetic characterization of isolates

Isolate	Gram staining	Shape	Closest relative	16S/18S rRNA gene sequence identify (%)	Accession number
F4	Half stain	Oval undulate	<i>Bacillus coagulans</i> (AY138271)	99	GU183397
G10	Half stain	Oval undulate	<i>Bacillus</i> sp. <i>KHc1</i> (FJ379319)	98	GU183387
G35	No stain	Filamentous	<i>Psedallescheria</i> <i>ellipsoidea</i> (PEU43911)	99	GU183396
D42	No stain	filamentous	<i>Psedallescheria</i> <i>ellipsoidea</i> (PEU43911)	99	GU183392
E41	No stain	Filamentous	<i>Pseudallescheria</i> <i>boydii</i> (PBU43915)	99	GU183395
C10	+	Coccid	<i>Cellulosimicrobium</i> sp. HY-13 (FJ375341)	100	GU183386
F28	-	Coccid	<i>Microbacterium</i> sp. DCY37 (EU873314)	98	GU183390
B15b	-	Coccid	<i>Strenotrophomonas</i> <i>acidaminiphila</i> (EU352763)	99	GU183393
B2a	-	Coccid	<i>Xenophilus azovorans</i> (NR_025114)	99	GU183388
G7	-	rod	<i>Pseudomonas</i> sp. ZZ5 (DQ113452)	100	GU183389
E8	-	rod	<i>Pseudomonas</i> sp. MT-13 (EU727192)	100	GU183385
C35	+	rod	<i>Tsukamurella</i> <i>tyrosinosolvans</i> (AB478956)	100	GU183394

3.3 Results and discussions

3.3.1 The composition of lignocellulosic waste

Lignocellulosic waste is often incinerated or dumped in the vicinity of greenhouses, causing several undesirable agronomic and environmental effects, such as unpleasant smell or appearance of plant diseases, particularly if the pathogens involved can infect subsequent crops. Lignocellulose, the main component of lignocellulosic waste, can be degraded to reducing sugars for fuel ethanol production by certain fungi and bacteria but is indigestible by most animals (Benner and Hodson, 1985). In this study, the composition of lignocellulosic waste included soft wood, leaves, tree trunk, tree branch, grass and tree bark, which were collected in Singapore. The effective content (% dry weight) of lignocellulose in lignocellulosic waste was found to be considerably high ($87.0 \pm 2.6\%$), in which lignin accounted for $32.4 \pm 1.1\%$ and polysaccharides in terms of cellulose and hemicellulose accounted for $54.6 \pm 2.5\%$ (Table 3-2). The results were well in agreement with the reported constituents of lignocellulosic materials in literature (Galbe and Zacchi, 2002; Hahn-Hägerdal et al., 2007). Champagne (2007) reported that the major constituents of lignocellulose were cellulose and hemicelluloses (48-80%) and lignin (18-30%). This indicated that the lignocellulosic waste can be a potential feedstock for reducing sugars production.

Table 3-2 Polysaccharides components in lignocellulosic waste

Component	Test 1 (%)	Test 2 (%)	Test 3 (%)	Average (%)	Total (%)
Extractives	12.3	10.8	15.8	13.0	13.0 ± 2.6
Acid insoluble lignin	29.4	31.6	30.5	30.5	30.5 ± 1.1
Acid soluble lignin	1.9	1.9	1.9	1.9	1.9 ± 0.0
Polysaccharides	56.4	55.6	51.8	54.6	54.6 ± 2.5
Lignocellulose	87.7	89.2	84.2	87.0	87.0 ± 2.6

3.3.2 Degradation properties of the microbial community

The ability of any microorganism to utilize polysaccharide depends on its ability to produce the specific enzyme or enzymes required for hydrolysis of the polymer, its ability to transport the products of hydrolysis into the cell and its ability to convert them to intermediates that can enter the central pathway (Gaudy and Gaudy, 1980). Hydrolysis may convert the polysaccharides to reducing sugars, which are small enough to enter the cell and may be taken up and then hydrolyzed by specific intracellular enzymes. In this study, after one year of sequential cultivation and selection, the reducing sugars-producing microbial communities were successfully selected from the four sources of inoculums, i.e., C1, C4, AAS and GS. All the four microbial communities reached a steady state, as evidenced by very high polysaccharides reduction efficiency and reducing sugars production yield (Figs. 3-1 and 3-2).

Table 3-3 shows the microbial communities performance from the four sources. The initial soluble polysaccharides concentration was 276.2, 266.8, 253.9 and 267.3 mg/g lignocelulosic waste in C1, C4, AAS and GS, respectively. The soluble polysaccharides degradation efficiency increased to their peaks at 144 h at 88.5%, 86.2%, 86.5% and 85.4% in each of the four microbial communities respectively, and declined afterwards (Fig. 3-1). The highest reducing sugars yield was obtained in the microbial community from AAS, 12 mg/g lignocelulosic waste on day 3. The maximum reducing sugars yield produced by microbial communities from C1, C4, AAS and GS were obtained on day 1, 7, 3 and 3, respectively. These results indicated that the lignocellulose was degraded very quickly within 72 hours by the four selected microbial communities, while reducing sugars were produced mainly in this period. The results are consistent with Haruta et al (2004) which showed that the efficiency and microbial community are stable by using a mixed culture of aerobic and anaerobic bacteria. However, the degradation rate was lower than that of this study. It found that aerobic lignocelluloses degradation rates were typically much higher than anaerobic degradation rates; 15 to 30% of the lignocellulose were mineralized aerobically during 25 d of incubations, whereas 250 d of

incubation was required to mineralize a similar percentage of the lignocelluloses under anaerobic conditions (Benner and Hodson, 1985; Yang et al., 2009).

When polysaccharides are degraded to monosaccharides or to small oligosaccharides or disaccharides, the ability of a microorganism to utilize the products depends on the specific metabolic enzymes required (Anderson and Wood, 2003). Lignocellulose is readily metabolized to reducing sugars after hydrolysis (Fraenkel and Vinopal, 2003). Some degradation products were taken up immediately by the microorganisms associated with the degrading particles, in which case the soluble products were never detected (Wells et al., 1995). This is why the reducing sugars yield was lower compared to the high polysaccharides degradation efficiency in present study.

Table 3-3 The performance of microbial communities during lignocellulose degradation

Source of microorganisms	Initial soluble polysaccharides concentration (mg/g lignocellulosic waste)	Maximum reducing sugar concentration produced, as glucose (mg/g lignocellulosic waste)	Maximum polysaccharides degradation efficiency (%)
1-year compost (C1)	276.2	11.2 ± 1.3	88.5
4-month compost (C4)	266.8	11.1 ± 0.7	86.2
Garden Soil (GS)	253.9	10.9 ± 1.5	86.5
Aerated Activated Sludge (AAS)	267.3	12.0 ± 0.9	85.4

Fig. 3-1 Polysaccharides degradation efficiency by four microbial communities

Fig. 3-2 Reducing sugars concentration by the four microbial communities

The products of degradation are then transported into the cell and used. The metabolism of polysaccharides is initiated by the hydrolytic degradation of the polymer catalyzed by enzymes excreted by the cell, i.e., extracellular enzymes. These may be released into the environment or in some cases, remain bound to the cell surface (Moran and Hodson, 1989). The hexuronic acids, e.g., glucuronic, galacturonic and mannuronic acids occur as components of microbial capsules

during lignocellulose degradation. They are generally metabolized through the Entner-Doudoroff pathway after conversion to 2-keto-3-deoxy-6-phosphogluconate (Fraenkel and Vinopal, 2003). These acids cause the pH of all the reactors to decrease slightly, with the exception of the C1 reactor, whose pH increased steadily during the first 96 hours and finally returned to its initial value (pH7) (Fig.3-3).

Fig. 3-3 pH variation during reducing sugars production by the four microbial communities

In the latter case, the cell must make contact with the polysaccharide. Since many polysaccharides are insoluble, this is facilitated by growth of the microorganisms on the surface of polysaccharide materials (Fig. 3-4). Fig. 3-4 shows the microbial morphotypes present in the four different microbial consortia after 12 months enriching processes for lignocelluloses degradation and reducing sugars production. These SEM pictures show that the dominant microorganisms enriched from C1 were small, wide and curved rod-shaped bacteria embedded in lignocelluloses (Fig. 3-4 (A)). Few filamentous and some coccoid microorganisms were observed in the microbial community from C4 (Fig. 3-4 (B)). In AAS and GS, different morphotypes were found (Fig. 3-4 (C) and (D)), and it seemed like fungal hyphae was confined in the intercellular spaces in plant cell walls which are in close proximity to substrate even if they are not strictly “adhering to” (Fig. 3-4 (D)).

Fig. 3-4 Images of lignocellulose-degrading microorganisms by SEM from sources (A) 1 year compost (C1), (B) 4 months compost (C4), (C) aerated activated sludge (AAS), and (D) garden soil (GS). Scale bar indicates 5 μ m.

3.4 Community diversity determined by DGGE

Since the hydrolysis of soluble polysaccharides occurs extracellularly, the products of hydrolysis may be available to organisms other than the ones that produce the hydrolytic enzyme. Complete degradation of polysaccharides may require the action of more than one microorganism, since several different enzymes may be required to break the different bonds and no one organism may be able to elaborate all of the enzymes required (Anderson and Wood, 2003). The dynamic changing of the population of the four reducing sugars-producing microbial communities at different stages of cultivation and shifts in the community composition were monitored by DGGE in the PCR-amplified V3 region of the 16S rRNA gene, since microbial communities from natural sources have extremely phenotypic and genotypic diversity (Dilly et al., 2004). As shown

in Fig. 3-5 (A-D) and Table 4, each band on the DGGE profile corresponded to a gene fragment of unique 16S rDNA sequences and represented a specific species in the microbial community accordingly; the intensity of a band represented the relative abundance of the corresponding microbial species. Bacterial DGGE revealed that the lowest number of DNA bands was in C1 and the highest number was obtained after six months of degradation in C4. The dominating bands were hardly seen during the first month in C1, AAS and GS in contrast to the later stages of cultivation in month 6, 9 and 10 when the bands were more diverse. After 11 months, the dominant bands decreased to 8-10 in each source. Approximately 12-21 dominant strains were present initially in all the inoculated sources after two months. However, the dominant DGGE bands associated with each source did not migrate along with sequential cultivation, except for a few discernible bands that migrated further after 12 months of cultivation.

The bacterial community in C1 is shown in Fig. 3-5 (A); most of the bands in the 1-month lane and the 2-month lane did not co-migrate with cultivation and selection. Fig. 3-5 (B) shows the microbial population selected from C4 during one year of cultivation. The microbial species from C4 is more diverse than C1 in the beginning; however, some dominant bands only migrated during the first 8 months of cultivation and then disappeared after 9 months. Bacterial population in AAS was not diverse and discernible initially (see Fig. 3-5 (C) 1-month lane), but after six months of cultivation, 14 clear bands showed up. Only one of three bands in the 1-month lane remained after 12 months of cultivation. Some bands co-migrated from the 6-month lane to the 12-month lane. In Fig. 3-5 (D), the microbial community cultivated from GS shows 10 distinguished bands after one month, but some of the bands disappeared in the 11-month lane. However, the microbial community from GS is not stable, especially for band H, which might be a high G+C species; it showed up in the 1-month lane, but vanished from the 6-month lane to the 11-month lane and finally became one of the dominant bands in the 12-month lane.

Fig. 3-5 An ethidium bromide-stained 10% polyacrylamide denaturing gradient gel (30 to 70%) with DGGE profiles of 16S rRNA gene fragments after PCR amplification of nucleic acids from (A) 1 year compost (C1), (B) 4 months compost (C4), (C) aerated activated sludge (AAS), and (D) garden soil (GS). The source of each fingerprint is indicated above each lane.

These findings indicated that the major microbial species were dramatically changed in the cultivation process (Table 3-4). Microbial community adaptation

towards lignocellulose degradation occurred as the sole carbon source in inoculated flasks forced the weak microorganisms to die and the robust microorganisms to survive. For fungal communities, after one year cultivation, only one band survived in each source, although there are 3 bands found in the 10-month lane of C1 (data not shown).

Table 3-4 Bacterial diversity as indicated by the number of DNA bands during lignocellulose degradation by the microbial communities cultivated from different sources

Sources	Cultivation time (month)	No. of DNA bands
1 year compost (C1)	1	14
	2	16
	6	11
	9	11
	10	11
	11	11
	12	8
4 months compost (C4)	1	15
	6	21
	8	21
	9	11
	10	11
	11	11
	12	9
Aerated Activated Sludge (AAS)	1	10
	6	14
	8	11
	9	16
	10	15
	11	16
	12	9
Garden Soil (GS)	1	10
	6	12
	8	10
	9	14
	10	14
	11	11
	12	10

The number of microbial DNA bands decreased as lignocellulose degradation proceeded, whereas the microbial biomass and performance increased. This indicated that only a few robust bacteria can adapt to the complex composition of

lignocellulose and consumed lignocellulose as the only carbon source. The decrease in diversity with increase in degradation activity reflected the conversion of lignocellulose to organic matter, i.e., reducing sugars and the concomitant development of diverse microbial communities adapted to low availability of nutrients (Pielou, 1974). The initial consortia in the four sources were remarkably different from that after one year of cultivation. Those quite stable bands during the cultivation period could have contributed to a higher polysaccharide degradation as observed in regulating microbial communities for utilizing the diverse and complex substrates (Haruta et al., 2002). Therefore, the lignocellulose degradation and reducing sugars production ability could be considered as a criteria to select the reducing sugars-producing microbial communities in this study.

3.5 Isolation and phylogenetic analysis of pure isolates from mixed microbial communities after one year cultivation

Although many bacteria have been identified being able to survive in the environment of wood and timbers, very few studies reported that microorganisms or mixed cultures could degrade the feed stocks, e.g., rice straw, filter papers and not easily degraded lignocellulosic waste (Haruta et al., 2004; Haruta et al., 2005). More than 160 agar plates with pure cultures were isolated from 4 sources. Two criteria were used for identification of repeats, the characteristic of the colonies in the agar plates and the cell shape and gram staining result under a light microscope. Based on colony morphology, color and size, all colonies from the reducing sugars-producing aerobic microbial community were obtained by a direct isolation technique and gram staining, a total of 12 isolates with 3 suspected fungi and 9 suspected bacteria were initially screened (Table 3-1). The final 12 isolates were then subjected to partial 16S rRNA gene sequencing for bacteria and 18S rRNA gene sequencing for fungi to identify identical strains. The putative division and nearest relatives of the 12 sequences were investigated by BLAST, the phylogenetic relationship between isolates and reference strains is demonstrated in Fig. 3-6.

From the phylogenetic view, the isolated group of aerobic reducing sugars-producing microorganisms is rather heterogeneous and may consist of microorganisms belonging to various phyla including *Firmicutes*, *Actinobacteria*, *Bacteroidetes* and *Proteobacteria*. The commonly known genera are *Bacillus*, *Cellulomonas*, *Streptomyces*, *Cytophaga*, *Cellvibrio*, and *Pseudomonas* (Ulrich et al., 2008). The SEM pictures (Fig. 3-4) and gram staining results (Table 3-1) were in line with the identification results and *Bergey's Manual of Systematic Bacteriology* (Garrrity, 2001). The fungi isolates, i.e. F41, D42 and G35, from the microbial community in this study are assigned to *Pseudallescheria*; this strain was recorded as a filamentous fungus found in soil and plant material, which is a potential pathogen and can degrade a wide range of organic waste (Anastasi et al., 2005). This fungi species can produce hyphae, conidiophores, synnemata, conidia and rhizoid-like structure. Members of the genus are familiar to plant microbiologists because they are one of the few true pathogens of plants.

Gammaproteobacterium and *Pseudoxanthomonas* has been reported with high lignocellulose degradability, which have high similarity with the isolates of B15b (Souichiro et al., 2004). Organisms F28 and C10 of the *Cellulomonas* group, characterized as cellulose-degrading *Actinobacteria*, are small and thin rod-shaped bacteria, which are common in composting (Michael et al., 2008). The genera *Cellulomonas* are coryneform bacteria that produce at least six endoglucanases and one exoglucanase (Cex) (Chaudhary et al., 1997). The individual cellulases of *Cellulomonas* resemble the cellulose systems of aerobic fungi and contain CBMs; however, cellulosome-like protuberant structures have been noted on *Cellulomonas* cells grown with cellulose and cellobiose as carbon sources (Lamed et al., 1987). Various strains of *Cellulomonas* have been reported to be able to produce high yields of cellulose on cellulosic substrates and lower yields on xylan, galactomannan, starch, and sugars (Poulsen and Petersen, 1988). E8 and G7, members of the *Pseudomonas* group, were reported as strict aerobes and are known for the presence of a variety of metabolic pathways that are capable of degrading complex organic compounds including xenobiotics (Landy et al., 2008). *Pseudomonas* sp. is a rod and motile by means of a single polar flagellum. The

isolate F4 was assigned to *Bacillus* sp., which are large and wide rods occurring singly or in pairs. In *Bacillus* sp., xylanase is induced by xylose, but is repressed in the presence of glucose.

Fig. 3-6 Relationship of the cellulolytic isolates and selected reference strains based on the 16S/18S rDNA sequences. The tree is based on the alignment of 1,326 bp and was constructed using the neighbor joining method. Dots indicate the branches of the tree that were also formed using the maximum-likelihood method. Values are the relative proportions that a branch appeared in 100 bootstrap replications. Bar, 10 relative sequence divergences

Several reports have indicated xylanase can be induced by lignocelluloses such as wheat bran, rice straw, corncobs and sugarcane bagasse (Beg et al., 2001). *Xenophilus* and *Strenotrophomonas*, which are cells that are straight to curved rods, do not belong to the cellulolytic species. However, in nature, cellulose utilization is carried out not by pure cultures of microorganisms but by multiple cellulolytic species coexisting with each other and with many noncellulolytic species (Chen and Weimer, 2001). While cellulolytic species may compete directly for cellulose, both cellulolytic and noncellulolytic species can compete for cellodextrin products of cellulose hydrolysis, in cross-feeding of nutrients and in the production of inhibitory compounds (Shi et al., 1997).

3.6 Conclusions

In conclusion, the present work demonstrated that the isolated microbial communities from the four natural sources under aerobic condition can degrade above 85% of soluble polysaccharides (cellulose and hemicellulose) in lignocellulosic waste. The isolates identified in this study belong to bacterial groups that exist in soil or compost environments and utilize cellulose as a carbon source, e.g., *Betaproteobacteria*, *Gammaproteobacteria*, and *Cellulomonas*. *Pseudomonas* sp., existed in the mixed microbial community, has been commonly reported to be able to degrade cellulose and hemicellulose of plant cell wall. Some isolates, e.g., *Xenophilus* and *Strenotrophomonas*, though have not been reported for lignocellulose conversion in literatures, they were found in this study to have high degrading efficiency. They played a key role in high lignocellulose conversion for reducing sugars production.

Chapter 4. Isolation and Characterization of Microbial Community from Mealworm Guts during Microbial Hydrolysis of Lignocellulosic Waste to Reducing Sugars

4.1 Introduction

In Chapter 3, some high lignocelluloses-degrading microorganisms were isolated from natural sources. Ruminant animals also have been reported to be able to utilize lignocellulosic waste as an energy source (Miura et al., 1983; Koike et al., 2007). Termite, a typical ruminant animal, has been widely studied as an important player in the turnover and mineralization of complex biopolymers such as wood and other lignocellulosic materials (Eutick et al., 1978; Yamin, 1980; Odelson and Breznak, 1985; Wenzel et al., 2002; Graber et al., 2004; Hongoh et al., 2005; Hongoh et al., 2006). However, in nature, termites are very difficult to catch in quantity. Most studies focus on anaerobic or facultative anaerobic conditions, which were reported that the lignocellulose-conversion rate is lower than that in aerobic condition (Benner and Hodson, 1985).

Mealworms are primary consumers which can feed on organic residues, the ingestion and digestion of food by mealworms trigger a series of physiological and biochemical events that result in the conversion of protein, carbohydrate and lipid in food into utilizable nutrients (Terra and Ferreira, 1994). Such primary digestion is the dispersion and reduction in molecular size of food from polymers to oligomers (Terra and Ferreira, 2005). In the present study, the guts of *Tenebrio Molitor Linnaeus* (yellow mealworm) were selected as the microbial inocula due to two major reasons. Firstly, it is found that this species was maintained well on a diet of lignocellulosic biomass powder for at least 15 days. The second is that the realization that some protozoa may be involved in the digestion of wood components other than cellulose, and the gut of mealworm is the major interface between the insect and its environment (Inoue et al., 1997). Hence, isolation and selection of efficient reducing sugars-producing bacteria from guts of *Tenebrio Molitor Linnaeus* under aerobic condition are promising and significant.

In this chapter, six different medium including two basic nutrients with three different sole-carbon sources (i.e. lignocellulose powder (LIG), filter paper (FP) and Carboxymethyl cellulose (CMC)) were used for isolation and selection of microorganisms from mealworm guts. During the selection, the sole-carbon-source utilization tests of the culturable populations were explored to functional measure the metabolic potential. Polymerase Chain Reaction (PCR) followed by denaturing gradient gel electrophoresis (DGGE) in conjunction with cultivation-based method was applied to analyze the diversity of the bacterial community in the guts of mealworm during lignocellulose conversion and reducing sugars production.

4.2 Materials and methods

4.2.1 Sample collection and nutrients for enrichment

Tenebrio Molitor Linnaeus (yellow mealworm) was provided by a Final year project student in our laboratory. Before using, these mealworms were fed with decaying and decomposing lignocellulosic waste powder for 2 weeks.

The surface of mealworms were sterilized in 70% ethanol and then washed in sterilized water. Every 8 mealworms were disrupted by passing through a syringe needle for each medium. The disrupted gut debris were suspended in 200 ml of medium 1 ($\text{g}\cdot\text{L}^{-1}$) (yeast extract, 0.40; malt extract, 1, CaCO_3 , 2; pH 7.0 ± 0.2) (Wenzel et al., 2002). Medium 1 was supplemented with carboxymethyl cellulose (CMC; 10 g L^{-1} ; 21900; Fluka, Buchs, Switzerland; degree of substitution 0.70-0.85; medium 1a), filter paper strips (10 g L^{-1} , brand; medium 1b) and lignocellulosic waste powder (10 g L^{-1} ; with a size of less than 1 mm; medium 1c), respectively. The disrupted gut debris was also cultivated in 200 ml of medium 2 ($\text{g}\cdot\text{L}^{-1}$) ($(\text{NH}_4)_2\text{SO}_4$ 2; CaCl_2 0.1 ; KH_2PO_4 0.5; K_2HPO_4 2.0; $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ 0.1; NaCl 6.0; Yeast extract 1.0). Medium 2 was added with the same carbon sources as Medium 1, i.e., (CMC; 10 g L^{-1} ; 21900; Fluka, Buchs, Switzerland; degree of substitution 0.70-0.85; medium 2a), filter paper strips ($10 \text{ g}\cdot\text{L}^{-1}$, brand; medium 2b), and lignocellulosic waste powder (10

g·L⁻¹; preparation as below; medium 2c), respectively. The flasks were placed in a reciprocal incubation shaker (Grant, OLS 200, Grant Instruments, UK) at 30±2 °C and at a horizontal rotational speed of 150 rpm.

4.2.2 Preparation of lignocellulose

The lignocellulosic waste powder was provided by Meng Guan Landscape and Construction Pte Ltd in Singapore. The raw lignocellulose (the mixture of tree trunk, tree branch, tree leaves, and grass) was collected and washed thoroughly with tap water to remove the soil and clay. The lignocellulose was milled to powder with size less than 1 mm, followed by the removal of extractives and liberating cellulose and hemicellulose from lignin with toluene-ethanol (2:1, v/v) in a Soxhlet apparatus (Exelfit, Duran[®]) for 6 hours and drying in 60 °C until completely dry (Sun et al., 2004). Filter paper was cut to 2 mm width of strip. Carboxymethyl cellulose (CMC) was purchase from Fluka, Switzerland, the degree of substitution was 0.70-0.85.

4.2.3 Cultivation methods

A serial of batch tests were conducted by 250 mL Erlenmeyer flask with a working volume of 200 mL under aerobic condition. Each sample was measured in triplicates. Each flask was loaded with the disrupted gut debris of 8 mealworms; sterilized CMC, filter paper strips and lignocellulosic waste powder (10 g·L⁻¹) and 200 mL of sterilized cultivation medium, respectively. The culture pH was adjusted to 7 ± 0.2. The flasks were placed in a reciprocal incubation shaker (Grant, OLS 200, Grant Instruments, UK) at 30 ± 2 °C and at a horizontal rotational speed of 150 rpm.

The tests were operated for 15 days per batch. After the first batch, 50% (v/v) of the used medium was removed and replaced with 100 mL of the same type of sterilized medium and 10 g of respective carbon source. In the third batch, 75% (v/v) of the used medium was removed and supplemented with 150 mL of the same type of sterilized medium and 10 g of sterilized respective carbon source. The temperature, initial pH and rotational speed of the tests were kept constant. 5 mL of broth in the

cultivated flasks were analyzed every 24 hours in the cultivated flasks and the information was recorded. Each sample was measured in triplicates.

4.2.4 Analytical methods

pH was measured with a pH meter (CyberScan PCD 6500 pH meter, Germany). A phenol-sulfuric acid method was used to quantify polysaccharides (DuBois et al., 1956), with glucose as the standard. Reducing sugars were measured by DNS method with glucose as the standard (Miller, 1959). Cell concentration was measured by Spectrophotometer (Beckman and Coulter™ DU® 640B, Germany) at 600nm.

4.2.5 Scanning electron microscopy observation

All cultivated microorganisms with medium were gently washed with a phosphate buffer solution and allowed to settle naturally. The samples were then fixed with 4% paraformaldehyde and left for 4 hours. The fixed samples were dehydrated by successive passages through 40, 60, 80 and 100% ethanol and then dried either by a Freeze Dryer (Edwards, Crawley, West Sussex, UK) or Critical Point 6 Dryer (E3000) (VG Microtech, East Grinstead, West Sussex, UK) and finally observed with a scanning electron microscope (JSM-6400; JEOL, Tokyo) at an accelerating voltage of 12 or 15 kV.

4.2.6 Isolation procedure

The culture medium used for isolation was Nutrient Agar (NA) for bacteria and Sabouraud Dextrose Agar (SDA) for fungi, respectively. The supernatant from each of the four sources was serially diluted with sterilized water (10^1 - to 10^6 – fold dilutions), and 0.1 ml of each dilution was spread onto agar plates containing each of medium. The plates were inverted and incubated in a 30°C incubator until obvious colonies appeared. After three rounds of transfer, the distinct colonies had been transferred to lignocellulose agar plates two times to confirm the isolated culture could utilize lignocellulosic waste.

4.2.7 DNA extraction

Genomic DNA of isolates cultivated in lignocellulosic medium was extracted by a ZYMO research soil microbe DNA extraction kit (ZYR - Zymo Research, USA) after 4 months cultivation.

4.2.8 PCR and DGGE

PCR primers P2 and P3 with 40 bases of a GC clamp were used to amplify the V3 region of the bacterial 16S rRNA gene corresponding to positions 341 and 534 in the *Escherichia coli* sequence (Muyzer et al., 1993). Primer F243 was designed for specific amplification of actinomycete 16S rRNA templates in combination with reverse primer R513GC for DGGE analysis (Heuer et al., 1997). Nested PCR strategy and conditions for the detection of Fungi with primers NS1 and NS8 (Kowalchuk et al., 1997) and with touch-down thermocycling program (Muyzer et al., 1993). The band of the expected size from nested PCR, approximately 1.7 kb, was excised from the gel, and DNA was extracted with the aid of WIZARD SV GEL AND PCR CLEAN UP SYSTEM (Promega, USA). DNA eluted was used as the template in a second 50- μ l PCR mixture with the forward primer NS1-GC and the reverse primer NS2+10, which overlaps the site of the NS2 primer. Reaction conditions referred to the literature (Kowalchuk et al., 1997).

The PCR-amplified DNA fragments were separated by DGGE with a DCode universal mutation detection system (Bio-Rad Laboratories) as described previously (Muyzer et al., 1993). The gel was placed in 1 $\times$ TAE buffer and run at 85 V at 60 °C for 15 hours. After electrophoresis, the gel was stained with ethidium bromide for 15 min, destained with 1 $\times$ TAE buffer for 30 min, and viewed and photographed with an EDAS 290 gel imaging system (Kodak).

4.2.9 16S rRNA gene sequencing and phylogenetic analyses

A whole-cell direct lysis PCR amplification method was used to amplify the 16S rRNA gene of bacterial isolates. The nearly full-length 16S rRNA gene was amplified by PCR with forward primer 5'-AGA GTT TGA TCC TGG CTC AG-3' and reverse primer 5'-GGC TAC CTT GTT ACG ACT T-3'. The PCR products were purified with a WIZARD SV GEL AND PCR CLEAN UP SYSTEM (Promega, USA) according to manufacturer's instructions and stored at -20°C before sequencing. The 16S rRNA gene sequence of the isolates was determined by 1st BASE (Singapore).

The partial 16S rRNA sequences were compared to sequences available from the Ribosomal Database Project (RDP) and the EMBL/GenBank databases. For phylogenetic analyses, the DNA sequences were aligned using the Clustal W algorithm (program version Clustal ×1.83), the tree was constructed using the Neighbor-Joining and Maximum-Likelihood algorithms (PHYLIP program package 3.6). Tree topologies were evaluated by bootstrap analysis of the neighbor-joining tree using the original dataset and 100 bootstrap datasets. To estimate the root position of the tree, *Cyanobacterium stanierii* (GenBank accession no. [GQ350222](#)) was used as an outgroup. The 16S rRNA gene sequences obtained in this study were deposited in the EMBL nucleotide sequence database under accession GU394953 to GU394959 (Table 5).

4.3 Results

4.3.1 Morphological and physiological characteristics of the isolates after 6 months of cultivation

Fig. 4-1 shows Scanning Electron Microscopy (SEM) images of reducing sugars-producing microorganisms from yellow mealworm guts after six months cultivation. A close examination of the mixed cultures revealed the presence of a large diversity of microbial morphotypes, including bacterial rods, cocci and actinomycete initially. These microorganisms were embedded in an extracellular polymeric matrix. Most of the microorganisms grown in (a) M1-CMC and (b) M2-CMC were short rod and cocci; some long shape rod microbes were also found in (b) M2-CMC, which was

different from (a) M1-CMC. In the (c) M1-LIG and (d) M2-LIG culture, cocci and filamentous microbes exit which were embedded in the lignocellulose biomass. The microorganisms isolated from (e) M1-FIL were cocci and short rod, while those from (f) M2-FIL were curved rod and cocci.

Fig. 4-1 Images of lignocellulose-degrading microorganisms by SEM from enrichments (a) M1-CMC, (b) M2-CMC, (c) M1-FP, (d) M2-FP, (e) M1-LIG and (f) M2-LIG. Scale bar indicates 5μm.

4.3.2 Lignocellulose degradation by the isolated microbial communities in batch cultures

The isolated microbial communities were grown in batch culture on six types of synthetic medium including two basic nutrients, i.e., M1 and M2 and three types of sole carbon sources, i.e., CMC, filter paper and lignocellulosic waste, respectively. The important experimental data were summarized in Table 4-1.

Table 4-1 The performance of six microbial communities from mealworm guts during reducing sugars production

Substrates	Maximum polysaccharides degradation efficiency (%)	Time achieved (hr)	Maximum reducing sugar yield (mg/g substrate)	Time achieved (hr)	Maximum cell growth yield ($\times 10^7$)	Time achieved (hr)
M1-CMC	59	192	6.9 ± 1.1	120	297	24
M1-FP	78	72	2.0 ± 0.6	120	81	120
M1-LIG	75	96	0.2 ± 1.2	48	409	24
M2-CMC	58	144	15.2 ± 0.8	168	172	192
M2-FP	76	168	4.3 ± 0.4	120	394	24
M2-LIG	72	24	7.3 ± 0.7	120	403	120

Soluble polysaccharides degradation efficiency obtained from the microorganisms in M1 was slightly higher than M2 and it was found that around 78% degradation was achieved with filter paper substrate in M1 medium, but this percentage dropped and reached 54% in 168 hour (Fig. 4-2). Such a decrease indicated that approximately the same amount of cellulose was hydrolyzed so that cellulolysis performances were close to their maximum. In this substrate, the cell growth concentration increased with reducing sugars yield, and the maximum value of both cell concentration and reducing sugars yield were reached about 81×10^7 and 2.0 mg/g substrate in 120 h, respectively (Fig. 4-4). The second highest soluble polysaccharides degradation efficiency (76%) happened in M2 medium, and also with filter paper substrate (Fig. 4-3). This indicated that the degradation efficiency was related to substrate and nothing to do with basic nutrient (M1 and M2), which is coincident with the degradation efficiency of M1 and M2 basic nutrient with lignocellulose substrate, 75% and 72%, respectively.

Fig. 4-2 Soluble polysaccharides degradation efficiency by three microbial communities isolated from mealworm guts in M1

Fig. 4-3 Polysaccharides degradation efficiency by three microbial communities isolated from mealworm guts in M2

Fig. 4-4 Cell concentration changing during reducing sugars production by three microbial communities isolated from mealworm guts in M1

Fig. 4-5 Cell concentration changing during reducing sugars production by three microbial communities isolated from mealworm guts in M2

However, the reducing sugars yield was significantly different between two basic medium with three different cellulose substrates. Compared between reducing sugars concentrations of M1 medium and those of M2 medium, the maximum reducing sugars yield in M2 medium was much higher than M1 medium (Fig. 4-6 and 4-7).

The highest reducing sugars yield, 15.2 mg/g substrate, was achieved in 144h with CMC substrate in M2 basic medium, but the yield slightly dropped afterward. At this time, the cell concentration grew very fast which indicated that microorganisms were in exponential phase. The lowest reducing sugars yield was achieved about 0.2 mg/g substrate in 96 hours with lignocellulosic waste in M1 medium, although the highest cell concentration was obtained among all substrates. It is noted that reducing sugars yield of M2-CMC shown similar pattern that of cell growth trend; the cell growth curve has three peaks, i.e., 24 h, 72 h, and 144 h, respectively, while the reducing sugars yield also reached to peak in each time. The highest soluble polysaccharides degradation efficiency, 78%, was obtained by the microorganisms from M1 with Filter paper, but the reducing sugars yield was only 2.0 mg/g substrate, although the cell growth yield was only $81 \times 10^7/L$ which is much lower than other medium (Fig. 4-4). It is noted that lignocellulose was difficult to be broken down to reducing sugars, since lowest reducing sugars yield was produced in M1 with lignocellulose as carbon source. But, CMC was easy to be converted to reducing sugars although it could only have lowest polysaccharides degradation. During cellulose degradation and reducing sugars generated, pH with all substrates in both two basic medium has not been changed significantly, which indicated that there was no acid produced during degradation process (Fig. 4-8).

Fig. 4-6 Reducing sugars concentration produced by three microbial communities isolated from mealworm guts in M1

Fig. 4-7 Reducing sugars concentration produced by three microbial communities isolated from mealworm guts in M2

Fig. 4-8 pH profile during reducing sugars production by three microbial communities isolated from mealworm guts in M2

Fig. 4-9 pH profile during reducing sugars production by three microbial communities isolated from mealworm guts in M2

4.3.3 Community diversity determined by DGGE

The diversity of bacterial communities at different stages of cultivation and shifts in the community composition were monitored by DGGE in the PCR-amplified V3 region of the 16S rRNA gene. As shown in Figs. 4-10-4-12, each band on the DGGE profile corresponded to a gene fragment of unique 16S rDNA sequences and accordingly represented a specific species in the microbial community, the intensity of a band represented the relative abundance of the corresponding microbial species. Bacterial DGGE revealed that, the population and diversity of bacterial community has changed during cellulose degradation in all substrates. For the substrate of CMC (Fig. 4-10), 11 of dominant bands were isolated from batch 1 in both M1 and M2 medium, the bands number has been increased to 17 in the second batch cultivation in M2 medium, and dropped to 12 in third batch, finally obtained 11 bands in the fourth batch. Compared with M1 medium, 15 of bands were found in the second batch, then decreased to 9 and finally dropped to 8 in the fourth batch. The band numbers in lignocellulose substrate were much lower than CMC (Figs. 4-10 and 4-11). There were 9 dominant bands presented after the first batch cultivation in M1 medium, but dropped during the following three batches and finally only 6 dominant bands shown in the fourth batch. Compared with M2 medium, the final dominant bands were also

6, although the bands increased from 7 initially to 8 in second and third batch. The dominant bands in filter paper substrate were much less than CMC and slightly less than Lignocellulose (Figs. 4-11 and 4-12). The final species numbers obtained in both M1 and M2 medium were 5 after four batches cultivation. Therefore, the lowest number of DNA bands was in filter paper substrate, and the highest number was obtained after second batches cultivation in M2 medium with CMC substrate. Fungi were not detected in the microbial community.

Fig. 4-10 An ethidium bromide-stained 10% polyacrylamide denaturing gradient gel (30 to 70%) with DGGE profiles of 16S rRNA gene fragments after PCR amplification of nucleic acids from M1-CMC and M2-CMC. The source of each fingerprint is indicated above each lane.

Fig. 4-11 An ethidium bromide-stained 10% polyacrylamide denaturing gradient gel (30 to 70%) with DGGE profiles of 16S rRNA gene fragments after PCR amplification of nucleic acids from M1-LIG and M2-LIG. The source of each fingerprint is indicated above each lane.

4.3.4 Isolation and Classification of pure isolates from microbial communities

More than 130 agar plates were isolated from all substrates. Identification of repeats was carried out in 2 steps. First were the characteristics of the colonies in the agar plates, and second was cell shape and gram staining result under a light microscope. An agar plate was placed under bright white light to observe the characteristic of the colonies. The characteristic can be classified into 5 topics – color, elevation, form, margin and texture. The color varies from an agar plate to another.

The elevation includes flat, raised, convex, pulvinate/drop-like, and umbonate, while the form of a colony can be identified as circular, filamentous, irregular and rhizoid. The margin of the colonies can be described as entire, undulate, lobate, erose, curled and filamentous/filiform. The texture of the colonies varied from dry, hard, smooth, not smooth and creamy. Based on colony morphology, color, and size, all colonies from the lignocellulose-degrading aerobic microbial community obtained by a direct isolation technique and gram staining, a total of 7 isolates without fungi were initially screened (Table 4-2).

Fig. 4-12 An ethidium bromide-stained 10% polyacrylamide denaturing gradient gel (30 to 70%) with DGGE profiles of 16S rRNA gene fragments after PCR amplification of nucleic acids from M1-FIL and M2-FIL. The source of each fingerprint is indicated above each lane.

Table 4-2 Taxonomic and phylogenetic characterization of isolates

Isolates	Shape	Putative division	Accession number	Enrichment sources
C42	Long rod	<i>Actinobacteria</i>	GU394958	M1-CMC
C43a	Short rod	<i>Actinobacteria</i>	GU394959	M2-FP
B18b	Short Rod	<i>Protreobacteria</i>	GU394953	M2-CMC
C26	Short rod	<i>Protreobacteria</i>	GU394954	M1-LIG
C12b	Oval/cocci	<i>Protreobacteria</i>	GU394956	M2-FP
A10b	Short rod	<i>Protreobacteria</i>	GU394957	M2-LIG
B3a	Cocci	<i>Protreobacteria</i>	GU394955	M1-FP

4.3.5 Phylogenetic analysis of the isolates

The finalized 7 isolates were then subjected to partial 16S rRNA gene sequencing to identify identical strains. The putative division and nearest relatives of the 7 sequences were investigated by BLAST, the phylogenetic relationship between isolates and reference strains is demonstrated in Fig. 4-13. As a result, phylogenetically diverse groups of isolates were found including *Cellulomonas* and *Pseudomonas*. The detail information of isolates was summarized in table 4-2. 2 of total isolates, which were isolated from filter paper substrate, were assigned to the genus *Cellulomonas*, 3 isolates belonged to the genus of *Pseudomonas* were isolated from CMC and filter paper substrates, and only 2 of the total isolates, which were isolated from lignocellulose and CMC substrate, were assigned to *Bacilli* branch, as supported by high bootstrap values. These results were coincident with the SEM pictures (Fig. 4-1) and gram staining results (Table 4-2).

Fig. 4-13 Phylogenetic tree of 16S rRNA gene sequences (>1,300 bp) constructed for lignocellulose-degrading isolates from guts of yellow mealworm. The tree was obtained using the neighbor-joining algorithm with bootstrapping (PHYLIP). *Aquifex pyrophilus* ([M83548](#)) was used as the outgroup. The scale bar represents the estimated number of nucleotide changes per sequence position.

4.4 Discussions

Reducing sugars-producing bacteria from rumen guts represent a group of microorganisms that are functionally related by their ability to consume polysaccharides and produce reducing sugars during saccharification. The results obtained in this study indicated that the microorganism population of the rumen is a dynamic one that responds rapidly to changes in diet. The peaks of soluble polysaccharides degradation efficiency in M1 with each source are slightly higher than those in M2 with the corresponding substrate, the maximum soluble

polysaccharides degradation (78% and 76%) happened in filter paper substrate both in M1 and M2; which were similar with the results in Haruta's study. In Haruta's study (2002), the polysaccharide degradation was obtained by a mixed culture of aerobic and anaerobic bacteria at 50 °C within 4 days. However, the maximum reducing sugars yield was only 1 mg/g lignocellulosic waste on the fifth day. In this study, the maximum reducing sugars was 15.2 mg/g substrate during CMC degradation in M2 medium. Even during filter paper degradation, the reducing sugar yield still can get 4.3 mg/g substrate.

Compared with the results of the reducing sugars yield within this study, the minimum reducing sugars yield obtained from the reducing sugars-producing microorganisms in M1 medium, which was 0.2 mg/g substrate although there were 75% of soluble polysaccharides degraded. This result indicates that lignocellulose is not readily metabolized to reducing sugars after hydrolysis. When a polysaccharide has been degraded to monosaccharides or to small oligosaccharides or disaccharides, the ability of a microorganism to utilize the products depended on the specific metabolic enzymes required (Anderson and Wood, 2003). However, hexoses, one kind of reducing sugars, are converted usually by inducible enzymes to either glucose 6-phosphate or fructose 6-phosphate, most frequently to the latter. Pentoses, another kind of reducing sugars, are also generally converted by inducible pathway to ribose 5-phosphate, ribulose 5-phosphate or xylulose 5-phosphate, intermediates in the HMP pathway. Xylose and arabinose are the most commonly occurring pentoses, since they are found in hemicelluloses and other polysaccharides produced by plants. Arabinose is readily converted to ribulose 5-phosphate, while xylose is converted to xylulose 5-phosphate (Fraenkel and Vinopal, 2003). Some degradation products were taken up immediately by the microorganisms associated with the degrading particles, in which case the soluble products were never detected (Wells et al., 1995). This is the reason why the reducing sugars yield is lower compared to the high polysaccharides degradation efficiency in present study. The products of degradation are then transported into the cell and used.

The hexuronic acids, e.g., glucuronic, galacturonic and mannuronic acids occur as components of microbial capsules during lignocellulose degradation. They are

generally metabolized through the Entner-Doudoroff pathway after conversion to 2-keto-3-deoxy-6-phosphogluconate (Fraenkel and Vinopal, 2003). These acids cause the pH of all the reactors to decrease slightly, with the exception of the C1 reactor, whose pH increased steadily during the first 96 hours and finally returned to its initial value (pH7).

It is noted that reducing sugars generated from M2 were much higher than those from M1, the possible reason was there were more nutrients in M2 than M1, such as N, P and K, and these are all basic nutrients for bacteria growth. Especially, NaCl is more important for cellulose degradation bacteria, since most of bacteria which were reported and recorded are living in salty environment (Garrity, 2001). Compared to the data of CMC degradation in both M1 and M2, the lowest soluble polysaccharides degradation efficiency was with highest reducing sugars yield, which appeared consistent with that high reducing sugars concentration can inhibit microorganism activity (Wells et al., 1995).

The metabolism of polysaccharides was initiated by the hydrolytic degradation of the polymer catalyzed by enzymes excreted by the cell, i.e., extracellular enzymes. These might be released into the environment or in some cases, remained bound to the cell surface (Moran and Hodson, 1989). In the latter case, the cell must make contact with the polysaccharide. Since many polysaccharides were insoluble, this was facilitated by growth of the microorganism on the surface of polysaccharide materials. These SEM pictures show that all kinds of microorganisms confined in the intercellular spaces in plant cell walls were in close proximity to substrate even if they were not strictly “adhering to” it. Small, wide and curved rod-shaped bacteria were embedded in filter paper and lignocellulose. Also, some coccoid bacteria existed in it. The SEM pictures changed significantly among six nutrient sources. The maximum numbers of bacteria were between 81×10^7 and 409×10^7 per ml of fluid, and the predominating morphological types were short or long rods, cocci in pairs, and filamentous.

The structure and diversity of microbial communities changed significantly during four batches of sequential cellulose degradation which was revealed by using

PCR-DGGE approach. The number of microbial DNA bands decreased as cellulose degradation proceeded, whereas the microbial biomass and performance increased. This can be explained that only a few robust bacteria can adapt to the complex composition of lignocellulose and consumed it as the only carbon source. Microbial communities from mealworm guts have extreme phenotypic and genotypic diversity (Dilly et al., 2004). The decrease in diversity with increase in degradation activity reflects the conversion of lignocellulose to organic matter, i.e., reducing sugars and the concomitant development of diverse microbial communities adapted to low availability of nutrients (Pielou, 1974).

The initial consortia in the six sources were remarkably different from that after four batches of cultivation. Some bands were detected after two batches of cultivation, and the increase in band intensity in the later two batches of cultivation could be the result that this bacterium was released into the culture solution, along with lignocellulose degradation and reducing sugars production. Those quite stable bands during the cultivation period could have been contributed to a higher reducing sugars yield as observed in regulating microbial communities for utilizing the diverse and complex substrates (Haruta et al., 2002). As a result, the reducing sugars production ability could be considered as a function of the microbial populations in the culture. Slight change of pH indicated that there was no acid produced during the whole degradation process.

From a phylogentic point of view, the group of aerobic cellulolytic bacteria consists of bacteria belonging to various phyla including *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria*. Well-known genera are *Bacillus*, *Cellulomonas*, *Streptomyces*, *Cytophaga*, *Cellvibrio*, and *Pseudomonas* (Ulrich et al., 2008). SEM pictures and gram staining results are coincident to the identification results and *Bergey's Manual of Systematic Bacteriology* (Garrrity, 2001).

There were no fungi isolated from mealworm guts in this study. *Gammaproteobacterium* and *Pseudoxanthomonas* had been reported with high lignocellulose degradability, which have high similarity with the isolates of B15b (Souichiro et al., 2004). Organisms C42 and C43a of the *Cellulomonas* group,

characterized as cellulose-degrading *Actinobacteria*, are small and thin rod-shaped bacteria, which are common in composting (Michael et al., 2008). The genera *Cellulomonas* are coryneform bacteria that produce at least six endoglucanases and one exoglucanase (Cex) (Chaudhary et al., 1997). The individual cellulases of *Cellulomonas* resemble the cellulose systems of aerobic fungi and contain CBMs; however, cellulosome-like protuberant structures have been noted on *Cellulomonas* cells grown with cellulose and cellobiose as carbon sources (Lamed et al., 1987). Various strains of *Cellulomonas* have been reported to produce high yields of cellulose on cellulosic substrates and lower yields on xylan, galactomannan, starch, and sugars (Poulsen and Petersen, 1988). B18b, C26 and C12b, the members of *Pseudomonas* group, which are reported strict aerobes and renowned for the presence of a variety of metabolic pathways were capable of degrading complex organic compounds including xenobiotics (Landy et al., 2008). *Pseudomonas sp.* is a rod and motile by means of a single polar flagellum. The isolates of A10b and B3a were assigned to *Bacillus sp.*, which are large and wide rods occurring singly or in pairs. In *Bacillus sp.* xylanase is induced by xylose, but is repressed in the presence of glucose. Several reports have shown xylanase induction by lignocelluloses such as wheat bran, rice straw, corncobs and sugarcane bagasse (Beg et al., 2001).

4.5 Conclusions

In conclusion, the present work demonstrated that the isolated microbial communities from the mealworm guts under aerobic condition can degrade about 58% - 78% of polysaccharide and produce more than 15 mg reducing sugars/g substrate. Among three carbon sources, filter paper can be easily degraded but with low reducing sugars yield; lignocellulose can also be degraded over 72% - 75%. Although only 58% - 59% of CMC were degraded, much reducing sugars were generated. Compared the isolates obtained from the six different nutrients, bacterial phylogenetic groups changed during reducing sugars production. The isolates identified by this study belong to bacterial groups that existed in soil or rumen environments and utilize cellulose as an energy source, e.g., *Betaproteobacteria*, *Gammaproteobacteria*, and *Cellulomonas*. *Pseudomonas sp.* also existed in the mixed

microbial communities, which has been often reported to degrade cellulose and hemicellulose of plant cell wall. Although there were some isolates, i.e. *Xenophilus* and *Strenotrophomonas*, which have not been verified to relate with lignocellulose degradation in literature, their roles in this study is under developed. Furthermore, it is indicated that some novel microorganisms with high degradation efficiency were found out in the microbial community, which played a key role in high lignocellulose degradation and reducing sugars production. In the future, the construction of a high efficiency community with isolated microorganisms would be a promising way to improve lignocellulose degradation and reducing sugars yield. Further study is conducted on constructing an efficient microbial community.

Chapter 5. Construction of a Microbial Cocktail for Effective Conversion of Lignocellulosic Waste to Reducing Sugars

5.1 Introduction

In Chapters 3 and 4, nineteen pure strains of reducing sugars-producing microorganisms were successfully isolated from natural sources and mealworm guts. However, the reducing sugars yield was too low to compete with acid hydrolysis and enzymatic hydrolysis. It has been reported that a mixed culture of one cellulolytic bacterium together with another noncellulolytic bacterium is ideal for degrading lignocellulose, with this “symbiosis” apparently promoting lignocellulose degradation (Odom and Wall, 1983; Lewis et al., 1988). As such, utilization of a defined microbial community could be effective in achieving high reducing sugars yield from lignocellulosic waste.

In various natural and engineered environments, many species of microorganisms stably coexisted by interacting with each other and effectively exert various functions (Fernandez et al., 1999; Briones and Raskin, 2003). However, the mechanisms responsible for coexistence of many species of microorganisms have not yet been clarified. One factor hampering the elucidations of these mechanisms is the difficulty of defining all members included in such microplex microflora. Furthermore, it is even more difficult to clarify the roles of each member and the relationships among each of the members of such a community. Hence, we expected that it would facilitate our understanding of these mechanisms by constructing a defined mixed culture consisting of microorganisms isolated from the consortia. Such approaches have often been applied to examine various microbial communities, especially in the field of pollutant biodegradation (Carvalho et al., 2002; Dejonghe et al., 2003). In addition, some reports have described the construction of a stable coculture consisting of two bacterial strains in mutualistic or commensalistic association (Drzyzga et al., 2001; Thomas et al., 2002). However, other report have noted that the addition of a third microorganism alters the relationships between two microorganisms (Dollhopf et al., 2003).

In order to gain a better understanding of natural (or engineered) complex microbial communities, relationships among many (more than two) species of microorganisms should be investigated. If such a defined mixed culture were to be successfully constructed, it would facilitate the examination of general characteristics of all of the members in pure culture and the monitoring of the dynamics of the members in the community throughout a given cultivation period. Furthermore, by constructing a “knock-out community” in which one of the members is eliminated from the defined mixed culture, the roles played by the eliminated member in situ and its impact on the other members of the community could be evaluated. This approach is derived from the same perspective as gene disruption studies, in which the role of a gene in an organism can be evaluated by the elimination of that gene. In the field of microbial ecology, similar approaches have been applied frequently to investigate the function of a specific group of microorganisms via the inhibition of a specific type of metabolism (Glissmann and Conrad, 2000).

In this chapter, 7 aerobic lignocellulosic microorganisms have been selected for conducting microbial cocktail experiments the cocktail including one or more representatives of each of six groups, the betaproteobacteria, gamaproteobacteria, bacilli, actinomycetes and fungi. *Microbacterium* sp. F28 (M1), *Cellulosimicrobium* sp. C10 (M2) and *Tsukamurella* sp. C35 (M3) are actinomycetes, *Bacillus* sp. F4 (M5) is a bacillus, *Strenotrophomonas* sp. B15b (M6) is a gamaproteobacteria, *Pseudomonas* sp. E8 (M7) is betaproteobacteria, and *Pseudallescheria* sp. D42 (M4) is the only fungi strain.

Serial combination tests had been done from two strains to seven strains among seven selected pure strains to find out the best “cocktail” which can produce the most reducing sugars. The knockout communities were constructed in which one of the members was eliminated from the combination in order to investigate the relationships and mechanism of seven strains in terms of the respective reducing sugar production yield. Furthermore, the characteristics of each of the members

were explored in detail. The roles and relationships among the members of the community in situ were evaluated based on these results.

5.2 Materials and methods

5.2.1 Preparation of lignocellulose

The lignocellulosic waste was provided by a local landscape and construction firm in Singapore. The raw waste (leaves, uprooted weeds, twigs, grass clippings, and small branches) was collected and washed thoroughly with tap water to remove the soil and clay. Then it was milled to powder with a size of less than 1 mm, followed by the removal of extractives (e.g., waxes, fats, some resins, and portions of wood gums) and liberating cellulose and hemicellulose from lignin with toluene-ethanol (2:1, v/v) in a Soxhlet apparatus (Exelfit, Duran[®]) for 6 hours and drying at 60 °C until completely dry (Sun et al., 2004). Organic solvent must be evaporated completely; otherwise the solvent might be inhibitory to the growth of microorganisms, enzymatic hydrolysis and fermentation (Kumar et al, 2009).

5.2.2 Nutrients for enrichment

The inoculated flasks were fed with lignocellulose as the sole carbon source with the following basic nutrient: $(\text{NH}_4)_2\text{SO}_4$ 2 g L⁻¹, CaCl_2 0.1 g·L⁻¹, KH_2PO_4 0.5 g·L⁻¹, K_2HPO_4 2.0 g·L⁻¹, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1 g·L⁻¹, NaCl 6.0 g·L⁻¹, Yeast extract 1.0 g·L⁻¹, and lignocellulosic waste 10 g·L⁻¹.

5.2.3 Experimental procedure

200 ml of basic nutrient and 10 g/L of pretreated lignocellulosic waste were mixed inside a 250 mL conical flask to form a culture medium for each microbial community. Before inserting cell biomass into each culture medium, 20ml cell

solution with consistent cell concentration must be prepared. Firstly, cell biomass of each microbial strain were taken out from nutrient agar plate and deposited into sterilized de-ionized water. Secondly, the mixture was mixed to form homogenous cell solution by using vortex machine (VWR Scientific Vortex Gene-2). Then, the cell concentration was measured by using spectrophotometer (Beckman and CoulterTM DU® 640 B, Germany). The ABS reading from spectrophotometer at 600 nm was expected to be 0.017 (± 0.001) to ensure same cell concentration for cell solution of each microbial strain. This trial and error approach can be done by concentration or dilution. The prepared volume of each cell solution must be enough for each cycle. Each cycle of cultivation takes about 7 to 10 days. All culture mediums then were placed inside shaking incubators (Multitron Infros, Japan) with 30 °C and 120-150 revolutions per minute (rpm).

5.2.4 Analytical methods

Samples in the cultivated flasks were analyzed regular period in the cultivated flasks. Reducing sugars were measured by DNS method with glucose as the standard (Miller, 1959). The ABS readings of reducing sugars concentration of each sample at zero hour were taken after cell solution was inserted into culture medium. The value after conversion is taken as the initial reducing sugars concentration, which is an essential value for the calculation of relative reducing sugars yield. Each sample was measured in triplicates.

5.3 Results

5.3.1 The best defined microbial community

Preliminary experiments had been done to determine the reducing sugars production capability of each single strain. From Fig. 5-1, M1 and M2 can produce almost the same quantity of reducing sugars, which were 26.9 mg/g lignocellulosic waste and 26.8 mg/g lignocellulosic waste; the maximum reducing sugars yield,

38.6 mg/g lignocellulosic waste, was obtained by M4; M3 can produce the second higher reducing sugars yield, which was 30.2 mg/g lignocellulosic waste; while, 25.4 mg reducing sugars /g lignocellulosic waste can be produced by either M5 or M7; however, M6 can only produce 14.2 mg reducing sugars /g lignocellulosic waste, which was the least among the seven selected strains. All these pure strains showed more excellent reducing sugars production capability than the original microflora.

Fig. 5-1 Reducing sugars production by each pure strain

The results showed that the combination microorganisms could produce much more reducing sugars from lignocellulosic waste than single strain (Fig.5-2). The maximum reducing sugars yield, 165.3 mg reducing sugars /g lignocellulosic waste, was produced by the combination of M1, M3, M4 and M5. The second most reducing sugars yield can be obtained by M1, M3, M4 and M6, the yield was 122.2 mg reducing sugars /g lignocellulosic waste. These results indicated that M1, M3, M4, M5 and M6 were the best strains for reducing sugars production among the seven selected strains; meanwhile, M5 was much better than M6 based on the reducing sugars yield. When the combination lack of anyone of them, the reducing sugars yield was decreased dramatically, which was verified by the combination of M1, M3 and M5, the combination of M1, M3 and M4, the combination of M1, M3

and M6 and the combination of M1, M4 and M5, the maximum reducing sugars yield obtained by them were 56.0, 51.6, 81.0 and 84.2 mg reducing sugars /g lignocellulosic waste, respectively. This phenomenon was further confirmed by the combination of M1, M4 and M6, the combination of M3, M4 and M5 and the combination of M3, M4 and M6, as the maximum reducing sugars yields produced by them were 38.8, 44.9 and 32.3 mg reducing sugars /g lignocellulosic waste, respectively (Fig. 5-3).

Fig. 5-2 The maximum reducing sugars yield among all the combination tests

M7 showed negative effect on the combination of M1, M3, M4 and M5 and the combination of M1, M3, M4 and M6, as the maximum reducing sugars yield were dropped to 67.3 mg reducing sugars /g lignocellulosic waste and 69.0 mg reducing sugars /g lignocellulosic waste, respectively. Although the maximum reducing sugars yield from the combination of M1, M3, M4, M5 and M6 was 60.7 mg reducing sugars /g lignocellulosic waste, the yield has been decreased to 18.9 mg reducing sugars /g lignocellulosic waste when M7 was put into the combination, which further verified that M7 played negative role to produce reducing sugars (Fig. 5-4).

Fig. 5-3 Reducing sugars production by the combinations of M1, M3, M4, M5 and M6

Fig. 5-4 Negative effect of M7 on the combination of M1, M3, M4, M5 and M6

Fig. 5-5 Positive effect of M4 on other strains during combinations

Fig. 5-6 The negative role of M2 on other combinations

M4 played a necessary role as the maximum reducing sugars yield produced by the combination of M1, M3, M5 and M7 and the combination of M1, M3, M6 and M7 were decreased to 1.7 and 29.3 mg reducing sugars /g lignocellulosic waste,

respectively. The same situations had been also observed when M4 was get rid of the combination of M1, M3, M4, M5 and M6, the maximum reducing sugars yield was decreased from 60.7 to 24.2 mg reducing sugars /g lignocellulosic waste. When M4 was added into the combination of M1, M5, M6 and M7, the maximum reducing sugars yield was increased from 20.2 to 53.8 mg reducing sugars /g lignocellulosic waste (Fig. 5-5.).

It has been found that M2 also played a negative effect on the combination of M1, M3, M4 and M5 and the combination of M1, M3, M4 and M6, because that the maximum reducing sugars yields were decreased to 26.0 mg reducing sugars /g lignocellulosic waste and 28.8 mg reducing sugars /g lignocellulosic waste when M2 joined these two best combinations. The maximum reducing sugars yields produced from the combination of M1, M2, M3, M4, M5 and M7 and the combination of M1, M2, M3, M4, M6 and M7 were 6.0 mg reducing sugars /g lignocellulosic waste and 6.5 mg reducing sugars /g lignocellulosic waste, which further indicated that M2 played a negative role (Fig. 5-6.).

5.3.2 The relationship and effect of each member on reducing sugars production

As M1, M3, M4, M5 and M6 showed excellent reducing sugars production ability, necessary combination experiments were carried out to investigate the relationship between each other and the effect of each one on reducing sugars production. The results show that the combination of M1 and M3 can produce 34.1 mg reducing sugars /g lignocellulosic waste maximumly, which was higher than the single strain of M1 and M3. When M4 was put in the combination of M1 and M3, the maximum reducing sugars yield was increased to 51.6 mg /g lignocellulosic waste. When M5 was put in the combination, the yield was further increased to 56.0 mg reducing sugars /g lignocellulosic waste, which was further increased to 81.0 mg reducing sugars /g lignocellulosic waste when M6 was added in M1 and M3. However, the reducing sugars yield was decreased to 31.1 mg /g lignocellulosic waste and 300 mg /g lignocellulosic waste when M2 and M7 were

added, respectively. These results further verified that M2 and M7 played more negative effect than other strains (Fig. 5-7.).

The combination of M4 and M5 can produce 23.1 mg reducing sugars /g lignocellulosic waste at the peak time. When M1 was put in to the combination, the maximum reducing sugars yield was increased to 84.2 mg /g lignocellulosic waste. If M3 was replaced of M1, the yield was 449 mg /g lignocellulosic waste which is higher than the original combination, but much lower than the combination of M1, M4 and M5. When M6 was added in the combination of M4 and M5, the maximum reducing sugars yield was 46.4 mg/g lignocellulosic waste, which was slightly higher than the combination o f M3, M4 and M5. But the reducing sugars yield was dropped to 14.5 mg /g lignocellulosic waste by the combination of M4, M5 and M7, which was further decreased to 2.5 mg reducing sugars /g lignocellulosic waste by the combination of M2, M4 and M5 (Fig. 5-8.).

Fig. 5-7 The effect of other players on the combination of M1 and M3

Fig. 5-8 The effect of other players on the combination of M4 and M5

Fig. 5-9 The effect of other players on the combination of M3 and M4

52.0 mg reducing sugars /g lignocellulosic waste was produced by the combination of M3 and M4. The yield was decreased to 51.6 mg /g lignocellulosic waste as M1 was added in the combination, which was further decreased to 3.7 mg reducing sugars /g lignocellulosic waste when M7 was involved. The combination

of M2, M3 and M4 could only produce 23.5 mg reducing sugars /g lignocellulosic waste, which was slightly increased to 26.9 mg reducing sugars /g lignocellulosic waste when M5 was added. M7 might decrease the reducing sugars yield to 43.8 mg /g lignocellulosic waste when it was combined with M3 and M4, but the yield was increased to 54.2 mg reducing sugars /g lignocellulosic waste as M5 was added (Fig. 5-9.).

The combination of M1 and M5 can obtain 48.8 mg reducing sugars /g lignocellulosic waste during the reaction, which was increased to 50.6 mg reducing sugars /g lignocellulosic waste when M3 was put into the combination. M4 can make the reducing sugars yield higher (842 mg/L) when it was added into the combination of M1 and M5. The reducing sugars yield can be increased to 59.3 mg /g lignocellulosic waste when M6 was put into the combination, but the maximum reducing sugars was decreased to 26.1 mg /g lignocellulosic waste when M7 was added (Fig. 5-10.).

Fig. 5-10 The effect of other players on the combination of M1and M5

The maximum reducing sugars yield was increased from 39.5 to 51.6 mg/g lignocellulosic waste when M3 was added to the combination of M1 and M4. The yield was further increased to 84.2 mg reducing sugars /g lignocellulosic waste as M3 was replaced by M5. However, the combination of M1, M4 and M7 can only

produce 38.5 mg reducing sugars /g lignocellulosic waste. Almost the same quantity reducing sugars (38.9 mg /g lignocellulosic waste) was obtained by the combination of M1, M2, M4 and M7, which was further increased to 41.7 mg reducing sugars /g lignocellulosic waste and 48.0 mg reducing sugars /g lignocellulosic waste when M5 and M6 was added, respectively (Fig. 5-11).

Fig. 5-11 The effect of other players on the combination of M1 and M4

The combination of M3 and M5 can obtain 34.9 mg reducing sugars /g lignocellulosic waste maximumly, which can be increased to 56.0 mg reducing sugars /g lignocellulosic waste when M1 was put into the combination. The combination of M3, M5 and M6 can only produce 50.9 mg reducing sugars /g lignocellulosic waste, which was further decreased to 44.9 mg /g lignocellulosic waste when M4 was put into the combination of M3 and M5. However, when M2 was added into the combination, the maximum reducing sugars yield was decreased to 22.4 mg /g lignocellulosic waste (Fig. 5-12).

Fig. 5-12 The effect of M1, M2, M4 and M6 on the combination of M3 and M5

Fig. 5-13 The effect of other players on the combination of M3 and M6

The combination of M3 and M6 can produce 47.4 mg reducing sugars /g lignocellulosic waste. When M1 and M5 were put into the combination, the reducing sugars yield can be increased to 81.0 mg /g lignocellulosic waste and 50.9 mg/g lignocellulosic waste, respectively. However, the reducing sugars yield was

decreased to 36.3 mg /g lignocellulosic waste and 9.2 mg /g lignocellulosic waste as M7 and M2 were added into the combination of M3 and M6, respectively (Fig.5-13).

Fig. 5-14 The effect of other players on the combination of M2 and M7

5.3.3 The negative roles of M2 and M7

To further verify that M2 and M7 were the negative factors for reducing sugars production during combination, the following experiments including both M2 and M7 have been done. The results show that the combination of M2 and M7 can produce 44.9 mg reducing sugars /g lignocellulosic waste maximumly, which is not very low and very high compared with the yield of other combinations. From Fig. 5-14, the results show that only M1 can improve the performance of the combination of M2 and M7, the reducing sugars yield can be increased to 57.0 mg /g lignocellulosic waste by the combination of M1, M2 and M7. M3, M4, M5 and M6 may not cooperate well with M2 and M7, the reducing sugars yield was dropped to 41.5, 17.3, 0 and 19.9 mg/g lignocellulosic waste when the above four strains involved in M2 and M7, respectively.

Fig. 5-15 shows that when M1 and M3, M1 and M5, and M1 and M6 were combined with M2 and M7, the reducing sugars yield was increased from 49.9 mg

/g lignocellulosic waste to 67.0, 55.6 mg/L and 59.4 mg/g lignocellulosic waste, respectively. However, either M1 and M4 or M3 and M4 were not commensally with M2 and M7, the maximum reducing sugars yield was slightly decreased. When M3 and M6 were combined with M2 and M7, the produced reducing sugars was consumed total, which indicated that these four strains may compete with each other and eat up all the products (Fig. 5-16). M4 and M5 can cooperate well with M2 and M7, the reducing sugars yield was slightly increased to 47.5 mg /g lignocellulosic waste. However, M3 and M5, M4 and M6, and M5 and M6 might not coexist with M2 and M7; the maximum reducing sugars yield was dropped to 17.8 mg, 39.4 mg, and 38.9 mg/g lignocellulosic waste, respectively (Fig. 5-16).

Fig. 5-15 The effect of other players on the combination of M2 and M7

Fig. 5-16 The effect of other players on the combination of M2 and M7

Fig. 5-17 The effect of other players on the combination of M2 and M7

Fig. 5-18 The effect of three of M1, M3, M4, M5 and M6 on the combination of M2 and M7

When combined three other strains with M2 and M7, M1, M5 and M6 can cooperate very well with them, the reducing sugars yield can be dramatically increased to 79.4 mg/g lignocellulosic waste; M3, M5 and M6 can improve the reducing sugars production yield to 71.6 mg/g lignocellulosic waste; M3, M4 and M5 can also work well with them to get much reducing sugars (Fig. 5-18). M1, M4, and M6 can slight improve the reducing sugars yield to 48.0 mg/g lignocellulosic waste. However, the reducing sugars yield was decreased to 36.0 mg, 32.5 mg, 22.9 mg and 41.7 mg/g lignocellulosic waste when M1, M3 and M4, M1, M3 and M5, M1, M3 and M6, and M1, M4 and M5 were combined with M2 and M7, respectively (Fig. 5-17).

Finally, six strains including M2 and M7 were combined together to investigate the role of each one on reducing sugars production. The results show that the reducing sugars yield was extremely decreased and even worse than individual strains. The combination of M1, M2, M3, M4, M5 and M7 can produce 6.0 mg reducing sugars/g lignocellulosic waste; the combination of M1, M2, M3, M4, M6 and M7 can produce 6.5 mg reducing sugars/g lignocellulosic waste; the combination of M1, M2, M4, M5, M6 and M7 can produce 6.6 mg reducing sugars/g lignocellulosic waste; the combination of M1, M2, M3, M5, M6 and M7

can produce 12.0 mg reducing sugars/g lignocellulosic waste; the combination of M2, M3, M4, M5, M6 and M7 can produce 32.5 mg reducing sugars/g lignocellulosic waste (Fig.5-19).

Fig. 5-19 The effect of four or five of M1, M3, M4, M5 and M6 on the combination of M2 and M7

5.3.4 Summary of reducing sugars yield within 24-h by some combination

Table 5-1 shows the summary of reducing sugars yield from lignocellulose by individual strains and best combinations within 24-h. All the original strains had the reducing sugars yield from 9.9 ± 1.1 to 18.1 ± 0.8 mg/g lignocellulosic waste except of M6. Significant reducing sugars production was only observed after 96 hours incubation (Figs. 5-1-5-19). Eight combinations showed at least 3 times higher production than the single strain. Among them, the best defined microbial cocktail consisting of M1, M3, M4 and M5, was identified. The reducing sugars yield of this defined microbial cocktail was 165.2 ± 1.4 mg/g-lignocellulosic waste, which is approximate ten times higher than the original consortia (Table 5-1) and individual strains (Fig. 5-1).

Table 5-1 Reducing sugars production from lignocelluloses by defined microbial strains

Microbial strain*	Reducing sugars concentration after
	24 hours incubation (mg/g-lignocellulosic waste)
M1	17.3 ± 2.1
M2	10.8 ± 1.0
M3	13.2 ± 1.0
M4	10.3 ± 1.4
M5	18.1 ± 0.8
M6	4.9 ± 1.7
M7	9.9 ± 1.1
M1+M2+M6	57.0 ± 2.3
M1+M2+M7	57.0 ± 0.9
M1+M2+M3+M5	55.9 ± 1.3
M1+M2+M3+M7	67.0 ± 0.7
M1+M2+M4+M5	65.0 ± 1.2
M1+M3+M4+M5	165.2 ± 1.4
M1+M3+M4+M6	122.2 ± 2.2
M1+M2+M5+M6+M7	51.8 ± 1.0

* M1: *Microbacterium* sp. F28; M2: *Cellulosimicrobium* sp. C10; M3: *Tsukamurella* sp. C35; M4: *Pseudallescheria* sp. D42; M5: *Bacillus* sp. F4; M6: *Strenotrophomonas* sp. B15b; M7: *Pseudomonas* sp. E8.

5.4 Discussions

Microbes can synthesize various enzymes to degrade a number of carbon sources. This delivers *in situ* a mixture of different depolymerizing enzymes to break down the stability of lignocelluloses (Béguin and Aubert, 1994). Extrapolating the results drawn from the experiments performed in this study to obtain the best combination is significant on reducing sugars production from lignocellulosic waste since there are three pieces of evidence that support the

contention that the synthetic cocktail is much better than the original microbial communities and single strain. First, the maximum reducing sugars produced by the combination of M1, M3, M4 and M5 can be increased over ten times than that by original consortia. Second, the species isolated and selected were unique and seldom reported in lignocellulose degradation and reducing sugars production. Third, the maximum reducing sugars yield was significant. One clear advantage of defined inoculums is that there is not natural contamination with reducing sugars producing-microorganisms, such as some unknown microorganisms in mixed microbial communities, which become abundant in the mixture but which remain insignificant in reducing sugars production and consume reducing sugars for survival.

Microbacterium sp. F28 (M1) played a positive role in the combination, which is an aerobic or facultative anaerobic bacterium commonly found in dairy products and some in soil (Speck, 1943). Some researchers have reported that this strain is capable of working under conditions of high temperature (Robinson, 1966; Park et al., 2006). *Tsukamurella* sp. C35 (M3) shows good coexistence during combinations, which is an aerobic actinomycete, a fungi-like bacterium. Actinomycete is known for its ability to degrade both lignin and cellulose in lignocelluloses that commonly can be found in soil (Pasti and Belli, 1985; Pasti et al., 1990). However, there is no study that proves *Tsukamurella* sp. C35 degrades lignin (Nam et al., 2004). *Tsukamurella pulmonis* and *Tsukamurella tyrosinosolvens* are two known species that are able to degrade xylose and cellobiose (Nam et al., 2004). Other than glycolipids, production of bioactive biosurfactants (Vollbrecht et al., 1998) were also found to be associated with *Tsukamurella* sp. biosurfactants enhance the emulsification of hydrocarbons, have the potential to solubilize hydrocarbon contaminants and increase their availability for microbial degradation. *Pseudallescheria* sp. D42 (M4) is the only fungi selected in this study and shows key role on obtaining high reducing sugars yield. This strain was recorded as a filamentous fungus found in soil, human, faecally contaminated cattle feed and plant material, which is a potential animal pathogen and can degrade a wide range of organic waste (Anastasi et al., 2005). This fungi species can produce hyphae, conidiophores, synnemata, conidia, and rhizoid-like

structure. Members of the genus are familiar to plant microbiologists because they are one of the few true pathogens of plants.

Bacillus sp. F4 (M5) also plays a positive role in the combination test, which is recognized as a strictly aerobic or facultatively anaerobic cellulolytic bacterium that can be found in soil environment (Johnsrud, 1997). Other than that, it also contains cellulolytic enzymes and hemicellulolytic enzymes which help degrade lignocellulosic materials (Varma et al., 1994). The isolate *Bacillus* sp. F4 was large and wide rod occurring singly or in pairs. Several reports have indicated xylanase can be induced by lignocelluloses such as wheat bran, rice straw, corncobs and sugarcane bagasse (Beg et al., 2001). *Stenotrophomonas* sp. B15b (M6) is good for achieving high reducing sugars yield, which belongs to aerobic bacteria ranging from common soil organism to opportunistic human pathogens. Cellobiose is recognized as a carbon source for *Stenotrophomonas maltophilia*, the first discovered species when genus *Stenotrophomonas* was defined (Palleroni and Bradbury, 1993). It is a Gram-negative bacterium with recorded minimal glycolytic acid production. *Stenotrophomonas*, which are cells straight to curved rods, do not belong to the cellulolytic species. However, in nature, cellulose utilization is carried out not by pure cultures of microorganisms but by multiple cellulolytic species coexisting with each other and with many noncellulolytic species (Chen and Weimer, 2001). While cellulolytic species may compete directly for cellulose, both cellulolytic and noncellulolytic species can compete for cellodextrin products of cellulose hydrolysis, in cross-feeding of nutrients, and in production of inhibitory compounds (Shi et al., 1997).

Cellulosimicrobium sp. C10 (M2) is a chemo-organotrophic aerobic or facultative anaerobic cellulolytic genus of bacteria. This genus of bacteria is commonly found in the gut of termites (Bakalidou et al., 2002; Kim et al., 2009). *Cellulomonas* group, characterized as cellulose-degrading *Actinobacteria*, are small and thin rod-shaped bacteria, which are common in composting (Michael et al., 2008). The genera *Cellulomonas* are coryneform bacteria that produce at least six endoglucanases and one exoglucanase (Cex) (Chaudhary et al., 1997). Cellulosome-like protuberant structures have been noted on *Cellulomonas* cells grown with cellulose and cellobiose as carbon sources (Lamed et al., 1987).

Various strains of *Cellulomonas* have been reported to be able to produce high yields of cellulose on cellulosic substrates and lower yields on xylan, galactomannan, starch and sugars (Poulsen and Petersen, 1988). Which is coincident with this study, *Cellulosimicrobium* sp. plays a negative role on reducing sugars production during combination. *Pseudomonas* sp. E8 (M7) is recognized as aerobic cellulolytic bacteria which occur as free-living organisms in soil and aquatic habitats. *Pseudomonas* sp. E8 is a rod and motile by means of a single polar flagellum. This type of bacteria are very helpful in lignocellulose degradation (Abalos et al., 2001). The members of the *Pseudomonas* group were reported strict aerobes and commonly known for the presence of a variety of metabolic pathways that are capable of degrading complex organic compounds (Landy et al., 2008). It also shows negative effects on reducing sugars production when combined with others, as *Pseudomonas* species are commonly known for furfural degradation, which might not have cooperation and commensalism with other lignocellulose-degrading species (López et al., 2004).

Bacillus sp. F4 has played a much critical role than *Strenotrophomonas* sp. B15b, which can be verified in the following combinations. In the individual test, the pure culture of *Bacillus* sp. F4 can produce much more reducing sugars than *Strenotrophomonas* sp. B15b, although these two strains were not the most productive microorganisms among the seven selected strains, some microorganisms can work corporately to become stronger. When these two strains were cooperated with *Microbacterium* sp. F28, separately, they can produce almost the same quantity of reducing sugars. But when they were combined with *Tsukamurella* sp. C35, separately, the reducing sugars yield was slightly higher than the combination of *Bacillus* sp. F4 and *Tsukamurella* sp. C35. All these evidences proved that *Bacillus* sp. F4 is much more productive than *Strenotrophomonas* sp. B15b. However, when *Bacillus* sp. F4 was put into the combination of *Microbacterium* sp. F28 and *Tsukamurella* sp. C35, the reducing sugars yield was dropped a lot, which shows that the reducing sugars production capability can be affected by the combination members, some strains were working in mutualistic or commensalistic association (Drzyzga et al., 2001; Thomas et al., 2002). Eventually, the maximum reducing sugars yield, 165.3 mg/g

lignocellulosic waste was produced by the combination of *Microbacterium* sp. F28, *Tsukamurella* sp. C35, *Pseudallescheria* sp. D42 and *Bacillus* sp. F4.

Some researchers have noted that the addition of a third microorganism alters the relationships between two microorganisms (Dollhopf et al., 2003). It is interesting that the combination of *Microbacterium* sp. F28 and *Pseudallescheria* sp. D42 can produce 54.4 mg reducing sugars/ g lignocellulosic waste, but when *Bacillus* sp. F4 was added, the maximum reducing sugars yield was increased to 84.2 mg/g lignocellulosic waste; while, when *Strenotrophomonas* sp. B15b was taken part in it, the maximum reducing sugars yield was dropped to 38.8 mg/g lignocellulosic waste. These results further verified that *Bacillus* sp. F4 was much better than *Strenotrophomonas* sp. B15b, which would compete with their co players to hamper reducing sugars production.

However, *Bacillus* sp. F4 and *Strenotrophomonas* sp. B15b did not always expedite the reducing sugars yield, just as the combination of *Tsukamurella* sp. C35 and *Pseudallescheria* sp. D42 could produce 51.6 mg reducing sugars/g lignocellulosic waste; *Tsukamurella* sp. C35 and *Bacillus* sp. F4 could produce 56.0 mg reducing sugars/g lignocellulosic waste, but when these three strains were put together, they only could produce 49.9 mg reducing sugars/g lignocellulosic waste. Meanwhile, the cocktail of *Tsukamurella* sp. C35 and *Pseudallescheria* sp. D42 could produce 51.6 mg reducing sugars/g lignocellulosic waste maximumly, *Tsukamurella* sp. C35 and *Strenotrophomonas* sp. B15b could produce 47.7 mg reducing sugars/g lignocellulosic, but only 32.3 mg of reducing sugars/g lignocellulosic waste was produced by these three strains together. These results indicated that *Pseudallescheria* sp. D42 was the most important player to produce more reducing sugars. It is significant that *Pseudallescheria* sp. D42 was the only fungi during the combination, which means fungi played a key role and took charge of breaking down lignocellulose and then converted them to reducing sugars.

5.5 Conclusions

In this chapter, a defined microbial cocktail was successfully constructed. These combination experiments could explain various intertwined relationships not only by the detection of synergistic or commensalistic interactions but also by detecting inhibitory effects and competition.

Over ten times higher reducing sugars yield could be achieved by a defined microbial community, which was the combination of *Microbacterium* sp. F28, *Tsukamurella* sp. C35, *Pseudallescheria* sp. D42 and *Bacillus* sp. F4. The maximum reducing sugars yield was 165.3 mg/g lignocellulosic waste. *Microbacterium* sp. F28 (M1), *Tsukamurella* sp. C35 (M3), *Stenotrophomonas* sp. B15b (M6) and *Bacillus* sp. F4 (M5) played positive and critical roles in the combination, which showed good coexistence during combinations for achieving high reducing sugars yield. *Pseudallescheria* sp. D42 (M4), the only fungi, was the most important factor during conversion of lignocellulose to reducing sugars. *Cellulosimicrobium* sp. C10 (M2) and *Pseudomonas* sp. E8 (M7) showed negative roles during the combinations for reducing sugars production, although they have been reported they can secrete lignocellulosic enzymes.

Chapter 6. Optimization of Reducing Sugars Yield from Lignocellulosic Waste by the Defined Microbial Cocktail Using Response Surface Methodology

6.1 Introduction

During the previous study in Chapter 5, a defined microbial cocktail was successfully selected from seven pure strains, which could produce 165.3 mg reducing sugars/g lignocellulose. It is known that many factors affect reducing sugars yield produced by microorganisms, including substrate concentration (sole carbon source), temperature, pH and pretreatment methods. Therefore, optimization of environmental conditions is expected to improve reducing sugars yield further more.

In this chapter, the effects of substrate concentration, pH and temperature under three pretreatment methods have been conducted. The single-dimensional search is laborious, time consuming, and incapable of reaching the true optimum due to ignoring the interaction among variables. To solve this problem, response surface methodology (RSM) has been proposed in this study to determine the influences of individual factors and their interactive influences. With RSM, the interaction of possible influencing parameters on reducing sugars production from lignocellulose can be effectively evaluated. Therefore, the main objectives of this chapter were to investigate the effects of substrate concentration, pH and temperature under three types of pretreatment methods as well as their interactive effects on reducing sugars production from lignocellulose by the best defined microbial cocktail in Chapter 5.

6.2 Materials and methods

6.2.1 Inoculums preparation

Microbacterium sp. F28 (M1), *Tsukamurella* sp. C35 (M3), *Pseudallescheria* sp. D42 (M4) and *Bacillus* sp. F4 (M5) were isolated from natural sources and mealworm guts in chapter 3 and 4. In chapter 5, this combination had been approved to produce the maximum reducing sugars. Before inserting cell biomass into each culture medium, 20ml of cell solution with consistent cell concentration must be prepared. Firstly, cell biomass of each microbial strain were taken out from nutrient agar plate and deposited into de-ionized water. Secondly, the mixture of each strain was mixed to form homogenous cell solution by using vortex machine. Then, the cell concentration was measured by using spectrophotometer (OD 600), The ABS reading from spectrophotometer was expected to be 0.017 (± 0.001) to ensure same cell concentration for cell solution of each microbial strain. This trial and error approach can be done by concentration or dilution. The prepared volume of each cell solution must be enough for each cycle.

6.2.2 Central composite design experiments

A three-level-three factor central composite design (CCD) obtained by using the software MINITAB 15[®] (Minitab Inc., USA) was employed to find out the interactive effects of three variables, e.g., substrate concentration, pH and temperature. The levels of factors used for optimization is presented in Table 6-1.

The trial was essentially a full 2^3 factorial design augmented by six axial points coded $\pm\alpha$ and six replications of center points (all factors at level zero), resulting in a total number of 20 experiments. The center runs provided a means for estimating the experimental errors and a measure of lack of fit. The axial points were added to the factorial design to provide for estimation of curvature of the model. The distance from the centre point was given by $\alpha = 2^{n/4}$ (for three factors $n=3$, α

=1.682). The levels of pH (X_1), temperature (X_2), and substrate concentration (X_3) in coded units were calculated with the following equations:

$$x_i = \frac{X_i - X_i^*}{\Delta X_i}$$

Where x_i is the coded value of the i th test variable; X_i is the uncoded value of the i th test variable, X_i^* is the value of X_i at the centre point of the investigated area, and ΔX_i is the step size.

pH, temperature, and substrate concentration were chosen as three independent variables in this experiment design; the maximum reducing sugars yield (RSY) was dependent variable. The results corresponding to the CCD is shown in Table 6-2. The response variable (RSY) was fitted using a predictive polynomial quadratic equation in order to correlate the response variable to the independent variables. The general form of the predictive polynomial quadratic equation is

$$Y = A_0 + \sum_{i=1}^k A_i x_i + \sum_{i=1}^k A_{ii} x_i^2 + \sum_{i=1}^k \sum_{j=1}^k A_{ij} x_i x_j$$

Where Y is the predicated response, x_i are the input variables, which influence the response variable Y , A_0 the i th linear coefficient, A_{ii} the quadratic coefficient and A_{ij} is ij th interaction coefficient.

The quality of fit of the model equation was expressed by the coefficient of determination of R^2 . The significance of the regression coefficients was evaluated by p-value.

6.2.3 Pretreatment of lignocellulose

Uniform lignocellulosic waste was provided by a local landscape and construction firm in Singapore. The raw waste (leaves, uprooted weeds, twigs, grass clippings, and small branches) was collected and washed thoroughly with tap water to remove the soil and clay. Then it was milled to powder with a size of less

than 1 mm. Followed by three pretreatment methods. (1). Organosolv pretreatment (OS) . To remove the extractives (e.g., waxes, fats, some resins and portions of wood gums) and liberate cellulose and hemicellulose from lignin with toluene-ethanol (2:1, v/v) in a Soxhlet apparatus for 6 hours and drying at 60 °C until completely dry (Sun et al., 2004). (2) Liquid hot water pretreatment (LHW). First, 1 L of glass bottle was prepared to contain lignocellulosic waste at solid loading of 10% (w/w). The mixture was put into autoclave machine at 120 °C for 60 minutes. After the mixture had cooled down, it was centrifuged to separate lignocellulose and water. Next, the lignocelluloses were put in 60 °C oven until totally dry. (3). Diluted sulfuric acid pretreatment (DSA). Prepare one Liter glass bottles containing lignocellulosic waste loading of 10% (w/w), mixed with dilute sulfuric acid of 1% (w/w). Then put into autoclave machine at 120 °C for 60 minutes, splitted the lignocellulose concoction into 50ml tubes and centrifuged with 5000 rpm for 5 minutes. Finally, poured away acid, kept biomass substrate and washed lignocellulose with distilled water to neutralization, put in 60 °C oven until totally dry (Sun and Cheng, 2005).

6.2.4 Basic nutrients for microorganisms

The inoculated flasks were fed with lignocellulosic waste as the sole carbon source with the following basic nutrient (g L⁻¹): (NH₄)₂SO₄ (2.0), CaCl₂ (0.1), KH₂PO₄ (0.5), K₂HPO₄ (2.0), MgSO₄·7H₂O (0.1), NaCl (6.0) and Yeast extract (1.0).

6.2.5 Operating procedure

The tests were operated for 96 hours per test. The batch experiments were conducted using 250 ml conical flasks. A total of 20 flasks of each pretreatment method with designated substrate concentration, pH and temperature were simultaneously operated. 5 ml of each microorganism (total 20ml inoculums) were added to individual bottle with 230 ml basic nutrients. 5 mL of broth was collected during regular time for analysis. The reaction flasks were operated on water bath

shaker according to the temperature required. The reducing sugars yield was measured after 12 hours and stopped once reducing sugars yield began to decrease.

Table 6-1 Levels of factors used for optimization of reducing sugars yield after three pretreatments

Variable	Label	Level				
		-1.682 ($-\alpha$)	-1	0	1	+1.682 ($+\alpha$)
X₁	pH	5.0	5.8	7.0	8.2	9.0
X₂	Temperature (°C)	25	33.1	45	56.9	65
X₃	Initial lignocelluloses	5	11.1	20	28.9	35
	Concentration (g/L)					

6.2.6 Analytic methods

Samples in the cultivated flasks were analyzed every 24 hours in the cultivated flasks. Before measuring, samples were centrifuged at 6000 rpm for 15 min. the supernatant was taken for measurement. Reducing sugars were measured by DNS method with glucose as the standard (Miller, 1959). Each sample was measured in triplicates. The absorbance of the cell suspension was determined by reading it at 600nm in a spectrophotometer. The concentration of the cell suspension was determined by using a standard curve of absorbance against bacteria concentration at 600nm.

6.3 Results and discussion

6.3.1 Response surface methodology (RSM)

The design matrix in actual terms and the experimental results of maximum reducing sugars yield under three types of pretreatment methods are presented in Table 6-1. The maximum reducing sugars yield was calculated by dividing the maximum reducing sugars produced concentration by the amount of lignocellulose waste added to each bottle. The obtained values were then subjected to the

response analysis to evaluate the relationship between pH (X_1), temperature (X_2) and substrate concentration (X_3).

6.3.2 Interactive effect of pH and substrate concentration under three types of pretreatment on maximum reducing sugars yield

Three dimensional response surface plots and two-dimensional contour lines to estimate maximum reducing sugars yield over independent variables were shown in Figs. 6-1-6-9. These figures depict the relative effects of two variables on the maximum reducing sugars production yield when the third variable was kept constant in their zero level. In fig. 6-1 (A) and (B), increasing lignocellulose concentration resulted in the significant increase of the maximum reducing sugars yield (RSY), however the RSY decreased rapidly when substrate concentration exceeded 28.9 g/L. The peak value of RSY increased with pH value increase from 5 to 5.8, decreasing for further high pH value. RSY reached the maximum of 173.1 and 96.5 mg reducing sugars/g lignocellulose as pH were at 5.8 and 8.2, respectively. The statistical difference of pH was found to be significant ($P < 0.01$). However, the interactive effect of pH and substrate concentration was insignificant ($P > 0.05$).

This phenomenon was possibly attributed to the fact that the utilization of substrate in any pH range was unlimited by the defined microbial cocktail, thus the increase of substrate concentration could significantly enhance the maximum reducing sugars yield. However when pH value was 9, the RSY was decreased to 10.2 mg reducing sugars/g lignocellulose, which indicated that the higher pH may inhibit the microorganism growth, thus decreased the RSY. Compared with another two pretreatment methods, the same trends were observed. However the RSY reached much lower value even at peak, which was 138.8 mg /g lignocellulose under liquid hot water pretreatment (Fig. 6-2 (A) and (B)), and 124.7 mg /g lignocellulose under diluted sulfuric acid pretreatment (Fig. 6-3(A) and (B)), substrate concentration was kept at 28.9 g/L at this time. The statistical difference was significant ($P < 0.05$). The response surface of RSY illustrated that obvious inhibition was observed when substrate concentration exceeded to 35 g/L (as shown in Figs. 6-1 (A), 2 (A), and 3 (A)), which was primarily caused by

overloading. The shock of high substrate concentration on methane production has been observed in literatures. It has been found that the methane yield decreased remarkably when substrate concentration was increased from 3 to 15 g VS/l (Tempest and Hunter, 1965). Margesin et al (2000) reported that high substrate concentration can to high organic loading rate (OLR), but resulted in the decrease of methane yield. There are also reported that the overloading resulted in the inhibition of the microorganisms due to the accumulation of VFAs (Hempfling and Mainzer, 1975).

Table 6-2 Maximum reducing sugars yield at various pH, temperature and substrate concentration under three pretreatment methods

Run	Code values			Real values			RSY under OS	RSY under LHW	RSY under DSA
	x ₁	x ₂	x ₃	X ₁	X ₂	X ₃	mg reducing sugars/ g lig	mg reducing sugars/ g lig	mg reducing sugars/ g lig
1	-1	-1	-1	5.8	33.1	11.1	35.9	17.5	28.3
2	1	-1	-1	8.2	33.1	11.1	20.1	13.6	17.2
3	-1	1	-1	5.8	56.9	11.1	75.0	36.7	58.3
4	1	1	-1	8.2	56.9	11.1	62.2	33.1	42.9
5	-1	-1	1	5.8	33.1	28.9	53.1	23.1	31.4
6	1	-1	1	8.2	33.1	28.9	21.4	9.1	13.3
7	-1	1	1	5.8	56.9	28.9	173.1	124.7	138.8
8	1	1	1	8.2	56.9	28.9	96.5	63.2	79.8
9	-1.682	0	0	5.0	45.0	20.0	54.2	35.9	43.4
10	1.682	0	0	9.0	45.0	20.0	10.2	8.5	10.4
11	0	-1.682	0	7.0	25.0	20.0	10.6	8.9	14.1
12	0	1.682	0	7.0	65.0	20.0	41.1	31.2	36.7
13	0	0	-1.682	7.0	45.0	5.0	60.3	40.5	56.4
14	0	0	1.682	7.0	45.0	35.0	95.7	58.0	75.3
15	0	0	0	7.0	45.0	20.0	140.6	117.1	130.0
16	0	0	0	7.0	45.0	20.0	140.4	116.9	128.6
17	0	0	0	7.0	45.0	20.0	140.6	116.8	130.2
18	0	0	0	7.0	45.0	20.0	140.5	116.9	129.5
19	0	0	0	7.0	45.0	20.0	140.6	117.7	128.7
20	0	0	0	7.0	45.0	20.0	140.5	117.2	129.3

Table 6-3 Estimated regression coefficients, T_{exp} and significance level of the model

Source	Organosolv pretreatment (OS)			Liquid hot water pretreatment (LHW)			Diluted sulfuric acid pretreatment (DSA)		
	Coefficient value	T_{exp}	P-value	Coefficient value	T_{exp}	P-value	Coefficient value	T_{exp}	P-value
A_0	-1725.82	-5.371	0.000	-1683.03	-6.836	0.000	116.521	18.464	0.000
A_1	343.91	5.177	0.000	342.77	6.733	0.000	327.62	7.299	0.000
A_2	24.01	4.19	0.002	22.72	5.175	0.000	21.42	5.534	0.000
A_3	10.44	1.488	0.167	8.59	1.599	0.141	11.10	2.342	0.041
A_{11}	-22.96	4.229	0.000	-23.04	-7.111	0.000	-21.48	-7.517	0.000
A_{22}	-0.25	-5.803	0.000	-0.23	-7.223	0.000	-0.22	-7.707	0.000
A_{33}	-0.2	-2.723	0.021	-0.24	-4.103	0.002	-0.26	-5.152	0.000
A_{12}	-0.37	0.568	0.529	-0.40	-0.918	0.380	-0.42	-1.092	0.301
A_{13}	-0.94	-1.24	0.243	-0.60	-1.032	0.326	-0.80	-1.569	0.148
A_{23}	0.13	1.776	0.106	0.14	2.402	0.037	0.14	2.696	0.022
R^2	0.9033			0.9324			0.9411		
$A_{\text{dj}}-R^2$	0.8162			0.8716			0.888		

(A)

(B)

Fig. 6-1 Response surface (A) and contour lines (B) for the interactive effects of initial concentration of lignocellulosic waste and pH on reducing sugars production yield after organosolv pretreatment.

(A)

(B)

Fig. 6-2 Response surface (A) and contour lines (B) for the interactive effects of initial concentration of lignocellulosic waste and pH on reducing sugars yield after hot liquid water pretreatment.

(A)

(B)

Fig. 6-3 Response surface (A) and contour lines (B) for the interactive effects of initial concentration of lignocellulosic waste and pH on reducing sugars yield after diluted acid pretreatment.

6.3.3 Interactive effect of pH and temperature under three types of pretreatment on maximum reducing sugars yield

Fig. 6-4 (A) and (B) illustrate the interactive effects of pH and temperature on maximum reducing sugars yield after organosolv pretreatment. Increasing pH significantly enhance the maximum reducing sugars yield under all three pretreatment methods. However when pH was increased to 9, the reducing sugars yield was dropped dramatically. Temperature also affected maximum reducing sugars yield which was increased from 10.6 to 173.1 mg reducing sugars/g lignocellulosic waste when temperature was increased from 25 °C to 56.9 °C. The statistical difference was significant ($P < 0.05$). However, high temperature could inhibit microorganisms; the observed decreasing of maximum reducing sugars yield at 65 °C indicated that too much high temperature may lead to no microorganism growth and less reducing sugars yield.

The same trends also were found in liquid hot water pretreatment and diluted sulfuric acid pretreatment (Fig. 6-5 (A) and (B)). The difference is that the interactive effects of pH and temperature under organosolv pretreatment and diluted sulfuric acid are not very significant ($P > 0.05$), however, it is significant for the liquid hot water pretreatment. The maximum and minimum reducing sugars yields were obtained by organosolv pretreatment and diluted sulfuric acid, which is 173.1 and 124.0 mg reducing sugars/g lignocellulose (Fig. 6-6 (A) and (B)), respectively. This phenomenon was possibly attributed to the fact that the combination of pH and temperature are not very critical on microorganism growth and they can mend each other to minimize the effect of single factor. In another aspect, this result also indicated that organosolv pretreatment was the best among the three pretreatment methods. Diluted sulfuric acid pretreatment can only obtain the lower reducing sugars yield due to the byproducts of furfural which can inhibit microorganism growth (Nguyen et al., 1999; Saha et al., 2005; Sun and Cheng, 2005).

(A)

(B)

Fig. 6-4 Response surface (A) and contour lines (B) for the interactive effects of temperature and pH on reducing sugars yield after organosolv pretreatment.

(A)

(B)

Fig. 6-5 Response surface (A) and contour lines (B) for the interactive effects of temperature and pH on reducing sugars yield after hot liquid water pretreatment.

Fig. 6-6 Response surface (A) and contour lines (B) for the interactive effects of temperature and pH on reducing sugars yield after diluted acid pretreatment.

6.3.4 Interactive effects of substrate concentration and temperature under three types of pretreatment on maximum reducing sugars production yield

The maximum reducing sugars yield could be increased to the peak value with the increasing of substrate concentration and temperature under organosolv pretreatment (as shown Fig. 6-7 (A)), then declined when these two factors have been further increased. The contour plot showed a symmetrical mound shape with the maximum response occurring within the central contour (Fig. 6-7 (B)), further indicated that the interactive effects of temperature and substrate concentration on maximum reducing sugars yield was not significant ($P>0.05$). However the interactive effects of temperature and substrate concentration in the liquid hot water pretreatment (Fig. 6-8 (A)) and diluted sulfuric acid pretreatment (Fig. 6-9 (A)) are very significant ($P<0.05$). As shown in Fig. 6-8 (A), the variation of substrate concentration of diluted sulfuric acid pretreatment ($P<0.05$) is more important than that of organosolv pretreatment and liquid hot water pretreatment ($P>0.05$). The contour plot of liquid hot water pretreatment and diluted sulfuric acid pretreatment showed an elliptical ridge running diagonally on plot (Figs. 6-8 (B) and 6-9 (B)), suggested that the interactive effects of temperature and substrate concentration had an significant influence on maximum reducing sugars yield ($P<0.05$).

The main effect of substrate concentration was weaker than the other two variables, but its effect was still statistically significant ($P=0.041$) in diluted sulfuric acid pretreatment, indicating that the substrate concentration was also an important factor in reducing sugars production from lignocellulosic waste. However, how temperature enhanced reducing sugars yield was not obvious. It has been reported that lowering operational temperature generally leads to decrease in the maximum specific growth and substrate utilization rate. Specific maintenance rate has been shown to give linear Arrhenius plots over a limited temperature range (Conrad and Wetter, 1990). Most of the biohydrogen-producing reactors reported previously were operated mesophilically (30-50 °C) or thermophilically (50-60 °C) (Lay, 2000; Ginkel et al., 2001), which were coincident with the results of current

study, since the maximum reducing sugars yield was decreased dramatically when the temperature was increased to 65 °C.

Fig. 6-7 Response surface (A) and contour lines (B) for the interactive effects of initial concentration of lignocellulosic waste and temperature on reducing sugars yield after organosolv pretreatment.

(A)

(B)

Fig. 6-8 Response surface (A) and contour lines (B) for the interactive effects of temperature and initial concentration of lignocellulosic waste on reducing sugars yield after hot liquid water pretreatment.

(A)

(B)

Fig. 6-9 Response surface (A) and contour lines (B) for the interactive effects of temperature and initial concentration of lignocellulosic waste on reducing sugars yield after diluted acid pretreatment.

Table 6-4 Comparison of reducing sugars yield with literatures

Feedstock	Component	Reaction condition	Reducing sugars yield	Reference
Rice hull	35.6 ± 0.1% cellulose 12.0 ± 0.7% hemicellulose	Lime pretreatment (100 mg g ⁻¹ hulls, 121 °C, 1h); Enzyme at the dose level of 0.15 ml of each enzyme preparation g ⁻¹ hulls	154 mg/g-substrate	(Saha and Cotta, 2008)
Pinus densiflora (2-year-old)		Biological pretreatment: white rot fungus cultivated for 8 weeks; Crude enzymes from brown rot fungi for enzymatic hydrolysis	70.9 mg/g-substrate	(Lee et al., 2008)
Balsam (a soft wood)	47.09 wt.% glucan and 6.23 wt.% xylan	Dilute acid pretreatment (0.25-0.75 wt.%) and temperature (150-175 °C) enzymatic hydrolysis: spezyme CP and Novozyme 188	125 mg/g-substrate	(Jensen et al., 2010)
Lignocellulosic waste	54.6 ± 2.5% cellulose and hemicellulose	Organosolv pretreatment (ethanol: toluene) Microbial hydrolysis (the combination of four microorganisms)	Before optimization, 165.3 mg/g-substrate (pH 7, 30 °C, initial lignocellulosic waste concentration was 10 g/L); After optimization, 173.1 mg/g-substrate (pH 5.8, 56.9 °C, initial lignocellulosic waste concentration was 28.9 g/L)	In this study

The results of this chapter showed that the reducing sugars yield can be increased to 173.1 mg/g-lignocellulose by the defined microbial community, which was much higher than those of enzymatic hydrolysis using similar feedstocks (Table 6-4). These optimization results indicated that microbial hydrolysis can effectively convert lignocellulosic waste to reducing sugars and is an alternative hydrolysis method for fuels and chemicals production.

6.4 Conclusion

Of all the factors, the response surface methodology was successfully applied to optimize reducing sugars production from lignocellulosic waste by the defined microbial cocktail. The pH and temperature showed most significant individual effects. Increasing pH and temperature can significantly enhance maximum reducing sugars production yield; however the yield decreased when pH exceeded to 9 and temperature increased to 65 °C since the high pH and temperature may lead to the inhibition of microorganisms growth and decreased reducing sugars production yield. High substrate concentration could lead to overloading, thus decreased the reducing sugars yield, however the effect of substrate concentration is not significant in both organosolv pretreatment and liquid hot water pretreatment. Temperature and substrate concentration of liquid hot water pretreatment and diluted sulfuric acid pretreatment showed significant interactive effect, which demonstrated that the inhibition caused by high substrate concentration can be efficiently minimized by increasing temperature. The maximum reducing sugars production yield of 173.1 mg reducing sugars/g lignocellulose was estimated at the optimum conditions of temperature 56.9 °C, pH 5.8 and initial concentration of lignocellulosic waste 28.9 g/L. The best pretreatment method is organosolv pretreatment, and the liquid hot water pretreatment is slight better than diluted sulfuric acid pretreatment for reducing sugars production.

Chapter 7 Conclusions and Recommendations

7.1 Conclusions

After an intensive investigation, a unique microbial hydrolysis process for converting lignocellulosic waste into reducing sugars was developed. The three major tasks for microbial hydrolysis process were completed in this thesis: (1) a group of reducing sugars-producing microorganisms was isolated from natural sources and mealworm guts, which effectively convert lignocellulose to reducing sugars for fuels and chemicals production; (2) a defined microbial cocktail was found most favorable for much more reducing sugars production; (3) optimal environmental conditions were investigated and the best pretreatment method was selected, which promoted reducing sugars yield. The experimental results obtained enabled the following conclusions to be made:

The isolated microbial communities from the four natural sources e.g. 1-year compost (C1), 4-month compost (C4), aerated activated sludge (AAS) and garden soil (GS) under aerobic condition can degrade above 85% of polysaccharide in lignocellulosic waste. The highest reducing sugars yield, 12 mg/g lignocellulosic waste, was recorded in the microbial community in AAS. The isolated microbial communities from mealworm guts under aerobic condition can degrade above 58-78% of polysaccharides and produced more than 15 mg reducing sugars/g CMC.

Phylogenetic analysis of the microbial population during conversion of lignocellulose to reducing sugars showed that the isolates identified in this study belonged to bacterial groups that exist in soil or compost environments and utilize cellulose as an energy source, e.g., *Betaproteobacteria*, *Gammaproteobacteria*, and *Cellulomonas*. *Pseudomonas* sp. also exists in the mixed microbial community, which has often been reported to be able to degrade cellulose and hemicellulose of plant cell wall. Although there are some isolates, i.e. *Xenophilus* and *Stenotrophomonas*, which have not been verified to be related to

lignocellulose degradation in literature, their role in this study was under investigation. Furthermore, it was indicated that some high efficiency microorganisms were found in the microbial community, which played a key role in high lignocellulose degradation efficiency and reducing sugars production.

Over ten times higher reducing sugars yield could be achieved by a defined microbial community, which was the combination of *Microbacterium* sp. F28, *Tsukamurella* sp. C35, *Pseudallescheria* sp. D42 and *Bacillus* sp. F4. The maximum reducing sugars yield was 165.3 mg/g lignocellulosic waste. In the present study, the impact of the elimination of one member on the other members of the community was evaluated; it was concluded that *Microbacterium* sp. F28 (M1), *Tsukamurella* sp. C35 (M3), *Stenotrophomonas* sp. B15b (M6) and *Bacillus* sp. F4 (M5) played a positive and critical role in the combination, which showed good coexistence during combinations for achieving high reducing sugars yield. *Pseudallescheria* sp. D42 (M4), the only fungi, was the most important factor during conversion of lignocellulose to reducing sugars. *Cellulosimicrobium* sp. C10 (M2) and *Pseudomonas* sp. E8 (M7) showed negative roles during the combinations for reducing sugars production, although they have been reported they can secrete lignocellulosic enzymes.

Of all the factors, the pH and temperature were found to be the most significant individual effects. Increasing pH and temperature can significantly enhance maximum reducing sugars yield, however the yield decreased when pH exceeded to 9 and temperature increased to 65 °C since the high pH and temperature may lead to the inhibition of microorganisms growth and decreased reducing sugars production yield. High substrate concentration could lead to overloading, thus decreased the reducing sugars yield, however the effect of substrate concentration was not significant in both organosolv pretreatment and liquid hot water pretreatment. Temperature and substrate concentration of liquid hot water pretreatment and diluted sulfuric acid pretreatment showed significant interactive effect, which demonstrated that the inhibition caused by high substrate concentration can be efficiently minimized by increasing temperature. The

maximum reducing sugars yield of 173.1 mg/g lignocellulosic waste was obtained at the optimum conditions of temperature 56.9 °C, pH 5.8, and lignocellulose concentration 28.9 g/L. The best pretreatment method was organosolv pretreatment, and the liquid hot water pretreatment was slight better than diluted sulfuric acid pretreatment for reducing sugars production.

Surprisingly, it was found reducing sugars yield was increased from 12 to 173.1 mg/g lignocellulosic waste in 72 hr and 24 hr, respectively, after proper combination and optimization. It might be because of availability of right microbial players, understanding of interactions among these players, and environmental conditions. Overall, microbial hydrolysis could be an effective hydrolysis alternative.

7.2 Limitations and recommendations

It should be noted that all the preliminary studies of microbial hydrolysis process for converting lignocellulosic waste to reducing sugars were investigated only in laboratory scale; scale-up study is suggested to study in the future. In addition, during microbial hydrolysis, reducing sugars were consumed after the peak value. The challenge now is to separate the reducing sugars before they were consumed.

From this thesis, it has been shown that it should be possible to promote the reducing sugars yield by the defined microbial community, which including *Microbacterium* sp. F28, *Tsukamurella* sp. C35, *Pseudallescheria* sp. D42 and *Bacillus* sp. F4. The question arises as to whether or not these strains can be further improved by genetic engineering, e.g. combination of genes. Role of enzymes in metabolism need to be further investigated.

Although phylogenetic analysis in Chapters 3 and 4 showed that microbial diversity between natural sources and mealworm guts was quite similar and only

different in a strain level, microbial discrepancy in those microbial communities was not investigated in details. Microbial mutation may occur. Gene cloning and structural analysis plus physiological characterization may be conducive to identifying and distinguishing those isolated microorganisms.

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Appendix A Bacterial Sequences

1. E8 [Organism=*Pseudomonas* sp.]a [isolate=E8] *Pseudomonas* sp. E8 16S ribosomal RNA gene, partial sequence (Accession number: GU183385)

ATGCAAGTCGAGCGGATGAAGGGAGCTTGCTCCCGGATTCAGCGGCGG
ACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTT
CCGAAAGGAGCGCTAATACCGCATACGTCCTACGGGAGAAAGTGGGG
GATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTTCGGATTAGCTAGT
AGGTGGGGTAATGGCTCACCTAGGCGACGATCCGTAACCTGGTCTGAGA
GGATGATCAGTCACACTGGAAGTACGACACGGTCCAGACTCCTACGGG
AGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCC
ATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTG
GGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACA
GAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGG
GTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGT
TTGGTAAGATGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATC
CATAACTGCCTGACTAGAGTACGGTAGAGGGTGGTGGAAATTTCTGTG
TAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGC
GACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCA
AACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTA
GCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTC
GACCGCCTGGGGAGTACGGCCGCAAGGTTAAACTCAAATGAATTGAC
GGGGGCCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGC
GAAGAACCTTACCTGGCCTTGACATGTCCGGAATCCTGCAGAGATGCG
GGAGTGCTTCGGGAATCGGAACACAGGTGCTGCATGGCTGTCGTCAG
CTCGTGTGCTGAGATGTTGGGTAAAGTCCCGTAACGAGCGCAACCCTTG
TCCTTAGTTACCAGCACGTTAAGGTGGGCACTCTAAGGAGACTGCCGG
TGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTT
ACGGCCAGGGCTACACACGTGCTACAATGGTTCGGTACAGAGGGTTGCC
AAGCCGCGAGGTGGAGCTAATCCCAGAAAACCGATCGTAGTCCGGATC
GCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGA
ATCAGAATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCC
GTCACACCATGGGAGTGGGTGCTCCAGAAGTAGCTAGTCTAACCGCA
AGGGGACG

2. C10 [Organism=*Cellulosimicrobium* sp.] [isolate=C10], *Cellulosimicrobium* sp. C10 16S ribosomal RNA gene, partial sequence (Accession number: GU183386)

TGCAGTCGAACGATGATGCCCAGCTTGCTGGGTGGATTAGTGGCGAAC
GGGTGAGTAACACGTGAGTAACCTGCCCTTGACTTCGGGATAACTCCG
GGAAACCGGGGCTAATACCGGATATGAGCCGTCCTCGCATGGGGGTGG
TTGGAAAGTTTTTCGGTCAGGGATGGGCTCGCGGCCTATCAGCTTGTTG
GTGGGGTGATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAG
GGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA
GGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGA
CGCCGCGTGAGGGATGAAGGCCTTCGGGTGTAAACCTCTTTCAGCAG

GGAAGAAGCGCAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACT
 ACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAA
 TTATTGGGCGTAAAGAGCTCGTAGGCGGTCTGTTCGCGTCTGGTGTGAA
 AACTCGAGGCTCAACCTCGAGCTTGCATCGGGTACGGGCAGACTAGAG
 TGCGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGA
 TATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCCGCAACTG
 ACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATAACCCTG
 GTAGTCCATGCCGTAAACGTTGGGGCACTAGGTGTGGGGCTCATTCCAC
 GAGTTCCGTGCCGCAGCAAACGCATTAAGTGCCCCGCCTGGGGAGTAC
 GGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCCGCACAAGC
 GGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAG
 GCTTGACATGCACGGGAAGCCACCAGAGATGGTGGTCTCTTTGGACAC
 TCGTGCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCTGTGAGATGTT
 GGGTTAAGTCCCGCAACGAGCGCAACCCTCGTCCCATGTTGCCAGCGG
 GTTATGCCGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAG
 GTGGGGATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGC
 ATGCTACAATGGCCGGTACAAAGGGCTGCGATACCGTAAGGTGGAGCG
 AATCCCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACC
 CCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTG
 AATACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGTCACGAAAGTC
 GGTAACACCCGAAGCCCATGGCCCAACCGTTCGCGGGGGAG

3. G10 [Organism=*Bacillus* sp.][isolate=G10] *Bacillus* sp. G10 16S ribosomal RNA gene partial sequence (Accession number: GU183387)

GGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGAT
 AACTTCGGGAAACCGGAGCTAATACCGGATAATCCTTTTCTCTCATGA
 GGAAAAGCTGAAAGATGGCTTCGGCTATCACTTACAGATGGGCCCGCG
 GCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCG
 TAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGC
 CCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGA
 AAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGT
 AAAACTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTA
 CCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCACCAG
 CCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTA
 AAGCGCGCGCAGGCGGTCTTTTAAGTCTGATGTGAAAGCCCCCGGCTC
 AACCGGGGAGGGTCATTGGAACTGGGAGACTTGAGTGCAGAAAAGG
 AGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGA
 ACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAAGTACGCTGAGGCG
 CGAAAGCGTGGGGAGCGAACAGGATTAAATACCCTGGTAGTCCACGCC
 GTAAACGATGAGTGCTAATTGTTAAAGGGTTTCCGCCCTTTATTGCTGC
 AGCAAACGCATTAAGCACTCCGCC

4. B2a [Organism=*Xenophilus* sp.][isolate=B2a] *Xenophilus* sp. B2a 16S ribosomal RNA partial sequence (Accession number: GU183388)

TGCAGTCGAACGGTACAGGCCTTCGGGTGCTGACGAGTGGCGAACGGG
 TGAGTAATACATCGGAACGTGCCAGTCGTGGGGGATAGCTCGGCGAA
 AGCCGGATTAATACCGCATAACGATCCACGGATGAAAGCGGGGGACCTT

CGGGCCTCGCGCGATTGGAGCGGCCGATGGCAGATTAGGTAGTTGGTG
GGGTAAAGGCTCACCAAGCCGACGATCTGTAGCTGGTCTGAGAGGACG
ACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCA
GCAGTGGGGAATTTTGGACAATGGGCGCAAGCCTGATCCAGCCATGCC
GCGTGCAGGATGAAGGCCTTCGGGTTGTAAACTGCTTTTGTACGGAAC
GAAAAGGCTCTGGTTAATACCTGGGGCTCATGACGGTACCGTAAGAAT
AAGCACC GGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGC
GAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTGATG
TAAGACAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGTG
ACTGCATTGCTGGAGTGCGGCAGAGGGGGATGGAATTCCGCGTGTAGC
AGTGAATGCGTAGATATGCGGAGGAACACCGATGGCGAAGGCAATC
CCCTGGGCCTGCACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACA
GGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTGGTTGT
TGGGAATTTGCTTTCTCAGTAACGAAGCTAACGCGTGAAGTTGACCGCC
TGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGGGGAC
CCGCACAAGCGGTGGATGATGTGGTTTAATTCGATGCAACGCGAAAAA
CCTTACCCACCTTTGACATGTACGGAACCTTGCCAGAGATGGCTTGGTG
CTCGAAAGAGAACCGTAACACAGGTGCTGCATGGCTGTCGTCAGCTCG
TGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCAT
TAGTTGCTACATTGAGTTGGGCACTCTAATGAGACTGCCGGTGACAAAC
CGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATAGGTGG
GGCTACACACGTCATACAATGGCCGGTACAAAGGGTTGCCAACCCGCG
AGGGGGAGCTAATCCCATAAAGCCGGTCGTAGTCCGGATCGCAGTCTG
CAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGGATCAGAAT
GTCACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCA
TGGGAGCGGGTTCTGCCAGAAGTAGTTAGCCTAACCGCAAGGAGGGCG

**5. G7 [Organism=*Pseudomonas* sp.][isolate=G7]*Pseudomonas* sp. G7 16S
ribosomal RNA gene partial sequence (Accession number: GU183389)**

GAAGGGAGCTTGCTCCCGGATTCAGCGGCGGACGGGTGAGTAATGCCT
AGGAATCTGCCTGGTAGTGGGGGACAACGTTCCGAAAGGAGCGCTAAT
ACCGCATACGTCCTACGGGAGAAAGTGGGGGATCTTCGGACCTCACGC
TATCAGATGAGCCTAGGTCGGATTAGCTAGTAGGTGGGGTAATGGCTC
ACCTAGGCGACGATCCGTAACCTGGTCTGAGAGGATGATCAGTCACACT
GGAAGTGAAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAA
TATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAA
GAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTA
AGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACC GGCTA
ACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCG
GAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGGTAAGATGGATGT
GAAATCCCCGGGCTCAACCTGGGAACTGCATCCATAACTGCCTGACTA
GAGTACGGTAGAGGGTGGTGGAAATTCCTGTGTAGCGGTGAAATGCGT
AGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACTGGACTGAT
ACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATAC
CCTGGTAGTCCACGCCGTAAACGATGTGCGACTAGCCGTTGGGATCCTTG
AGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTA
CGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGGCCCGCACAAGC
GGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGG

CCTTGACATGTCCGGAATCCTGCAGAGATGCGGGAGTGCCTTCGGGAA
TCGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCTGAGATGT
TGGGTAAAGTCCCGTAACGAGCGCAACCCTTGTCTTAGTTACCAGCAC
GTTATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAG
GTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACA
CGTGCTACAATGGTTCGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGC
TAATCCCAGAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGAC
TGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTG
AATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTG
GGTTGCTCCAGAAGTAGCTAGTCTAACCGCAAGGGGAC

**6. F28 [Organism=*Microbacterium* sp.][isolate=F28] *Microbacterium* sp. F28
16S ribosomal RNA gene, partial sequence (Accession number: GU183390)**

AACGGTGAACCAGGTGCTTGCTCTTGGGGATCAGTGGCGAACGGGTGA
GTAACACGTGAGCAACCTGCCCCCTGACTCTGGGATAAGCGCTGGAAAC
GGCGTCTAATACTGGATATGAACCACAAACGCATGGTTACTGGTTGGA
AAGATTTTTTGGTTGGGGATGGGCTCGCGGCCTATCAGCTTGTTGGTGA
GGTAATGGCTCACCAAGGCGTCGACGGGTAGCCGGCCTGAGAGGGTG
ACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCA
GCAGTGGGGAATATTGCACAATGGGCGGAAGCCTGATGCAGCAACGCC
GCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTTAGCAGGGAA
GAAGCGAAAGTGACGGTACCTGCAGAAAAAGCGCCGGCTAACTACGT
GCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTAT
TGGGCGTAAAGAGCTCGTAGGCGGTTTGTCTGCGTCTGCTGTGAAATCCC
GAGGCTCAACCTCGGGCCTGCAGTGGGTACGGGCAGACTAGAGTGCGG
TAGGGGAGATTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCA
GGAGGAACACCGATGGCGAAGGCAGATCTCTGGGCCGTAACTGACGCT
GAGGAGCGAAAGGGTGGGGAGCAAACAGGCTTAGATACCCTGGTAGT
CCACCCCGTAAACGTTGGGAACTAGTTGTGGGGTCCATTCCACGGATTC
CGTGACGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGC
AAGGCTAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGCGGA
GCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGA
CATATACGAGAACGGGCCAGAAATGGTCAACTCTTTGGACACTCGTAA
ACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCTGAGATGTTGGGTGA
AGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTAATG
GTGGGAACTCATGGGATACTGCCGGGGTCAACTCGGAGGAAGGTGGGG
ATGACGTCAAATCATCATGCCCTTATGTCTTGGGCTTCACGCATGCTA
CAATGGCCGGTACAAAGGGCTGCAATACCGTGAGGTGGAGCGAATCC
CAAAAAGCCGGTCCCAGTTCGGATTGAGGTCTGCAACTCGACCTCATG
AAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATAC
GTTCCCGGGTCTTGTACACACCGCCCGTCAAGTCATGAAAGTCGGTAAC
ACCTGAAGCCGGTGGCCT

**7. B15b [Organism=*Stenotrophomonas* sp.][isolate=B15b] *Stenotrophomonas*
sp. B15b 16S ribosomal RNA gene, partial sequence (Accession number:
GU183393)**

CGGCAGCACAGTAAGAGCTTGCTCTTACGGGTGGCGAGTGGCGGACGG
 GTGAGGAATGCATCGGAATCTACTCTGTCGTGGGGGATAACGTAGGGA
 AACTTACGCTAATACCGCATACGACCTACGGGTGAAAGCAGGGGATCT
 TCGGACCTTGCGCGATTGAATGAGCCGATGCCCCGATTAGCTAGTTGGC
 GGGGTAAGAGCCCACCAAGGCGACGATCGGTAGCTGGTCTGAGAGGAT
 GATCAGCCACACTGGAAGTGAAGACACGGTCCAGACTCCTACGGGAGGC
 AGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATAC
 CGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTGTTGGGAA
 AGAAAAGCAGCCGGTTAATACCCGGTTGTTCTGACGGTACCCAAAGAA
 TAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAAATACGAAGGGTG
 CAAGCGTTACTCGGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTTG
 TTTAAGTCTGTCGTGAAAGCCCTGGGCTCAACCTGGGAATGGCGATGG
 AAAGTGGGCGACTAGAGTGTGGCAGAGGGTAGTGGAATTCCTGGTGTA
 GCAGTGAAATGCGTAGAGATCAGGAGGAACATCCGTGGCGAAGGCGA
 CTGCCTGGGCCAACACTGACACTGAGGCACGAAAGCGTGGGGAGCAA
 ACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGG
 ATGTTGGGTGCAATTTGGCACGCAGTATCGAAGCTAACGCGTTAAGTTC
 GCCGCCTGGGGAGTACGGTCGCAAGACTGAACTCAAAGGAATTGACG
 GGGGCCCCGCACAAGCGGTGGAGTATGTGGTTTAATTTCGATGCAACGCG
 AAGAACCTTACCTGGCCTTGACATGCACGGAACCTTCCAGAGATGGAT
 TGGTGCCTTCGGGAACCGTGACACAGGTGCTGCATGGCTGTCGTCAGCT
 CGTGTGCTGAGATGTTGGGTAAAGTCCCGCAACGAGCGCAACCCTTGTC
 CTTAGTTGCCAGCACGTAATGGTGGGAACTCTAAGGAGACCGCCGGTG
 ACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTAC
 GGCCAGGGCTACACACGTACTACAATGGTAGGGACAGAGGGCTGCAA
 GCCGGCGACGGTGAGCCAATCCCAGAAACCCTATCTCAGTCCGGATTG
 GAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCAGA
 TCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCG
 TCACACCATGGGAGTTTGTGTCACCAGAAGCAGGTAGC

8. C35 [Organism=*Tsukamurella* sp.][isolate=C35] *Tsukamurella* sp. C35 gene for 16S rRNA partial sequence (Accession number: GU183394)

GTACTTCGGGATAAGCCTGGGAACTGGGTCTAATACCGGATATGACC
 TTCTCCTGCATGGGGGTGGTGGAAAGTTTTTGCGGTACAGGATGGGCC
 CGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGA
 CGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACA
 CGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGG
 GCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGG
 TTGTAAACCTCTTTCAGTAGGGACGAAGCGCAAGTGACGGTACCTACA
 GAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAAATACGTAGG
 GTGCGAGCGTTGTCCGGATTTACTGGGCGTAAAGAGCTCGTAGGCGGT
 TTGTCACGTCGTCTGTGAAAACCCGAGGCTTAACCTCGGGCCTGCAGGC
 GATACGGGCAGACTTGAGTACTGTAGGGGAGACTGGAATTCCTGGTGT
 AGCGGTGGAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCG
 GGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGTAGCGA
 ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGTACTAG
 GTGTGGGTTTCCTTCCACGGGATCCGTGCCGTAGCTAACGCATTAAGT

ACCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGA
CGGGGGCCCCGCACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACG
CGAAGAACCTTACCTGGGTTTGACATATAGAGGATCGCCGGAGAGATT
CGGTTTGCCTTGTGCCTTCTATACAGGTGGTGCATGGCTGTCGTCAGCT
CGTGTCGTGAGATGTTGGGTAAAGTCCCGCAACGAGCGCAACCCTTGTC
TCATGTTGCCAGCACGTAATGGTGGGGACTCGTGAGAGACTGCCGGGG
TCAACTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTAT
GTCCAGGGCTTCACACATGCTACAATGGCGCGTACAGAGGGCTGCGAT
ACCGTGAGGTGGAGCGAATCCCTTAAAGCGCGTCTCAGTTCGGATTGG
GGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGAT
CAGCAACGCTGCGGTGAATACGTTCCC

9. F4 [Organism=*Bacillus* sp.][isolate=F4] *Bacillus* sp. F4 16S ribosomal RNA gene, partial sequence (Accession number: GU183397)

GCAGTCGAGCGANGGTTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGA
CGGGTGAGTAACACGTGGGTAACTGCCATAAGACTGGGATAACTCC
GGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCTGA
AATTGAAAGGCGGCTTCGGCTGTCATTATGGATGGACCCGCGTCGCA
TTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCC
GACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGAC
TCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCT
GACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCTGTA AAACT
CTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGA
CGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGG
TAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGC
GCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCACGGCTCAACCGT
GGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTG
GAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCA
GTGGCGAAGGCGACTTTCTGGTCTGTA ACTGACACTGAGGCGCGAAAG
CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAA
CGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCA
AAGGAATTGACGGGGGGCCCCGCACAAGCGGTGGAGCATGTGGTTTAATT
CGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACC
CTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATG
GTTGTCGTCAGCTCGTGTCGTGAGA

10. B18b, [Organism=*Bacillus* sp.] [isolate=B18b] *Bacillus* sp. B18b 16S ribosomal RNA gene, partial sequence (Accession number: GU394953)

GGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACA
ACGTTTCGAAAGGAACGCTAATACCGCATAACGTCCTACGGGAGAAAGC
AGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCCGATTAG
CTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTA ACTGGTC
TGAGAGGATGATCAGTCACACTGGA ACTGAGACACGGTCCAGACTCCT
ACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGAT

CCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTT
AAGTTGGGAGGAAGGTCAGTAAGTTAATACCTTGCTGATTTGACGTTA
CCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATA
CGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTA
GGTGGTTTGGTAAGATGGATGTGAAATCCCCGGGCTCAACCTGGGAAC
TGCATCCATAACTGCCTGACTAGAGTACGGTAGAGGGTGGTGGAAATTT
CCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCG
AAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGG
GAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTC
GACTAGCCGTTGGAATCCTTGAGATTTTAGTGGCGCAGCTAACGCGAT
AAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAA
TTGACGGGGGCCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGC
AACGCGAAGAACCTTACCTGGCCTTGACATGTCCGGAACCTTGCAGAG
ATGCGAGGGTGCCTTCGGGAATCGGAACACAGGTGCTGCATGGCTGTC
GTCAGCTCGTGTCTGTGAGATGTTGGGTAAAGTCCCGTAACGAGCGCAA
CCCTTGTCTTAGTTACCAGCACCTCGGGTGGGCACTCTAAGGAGACTG
CCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGC
CCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGT
TGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCG
GATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATC
GTGAATCAGAATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACC
GCCCGTCACACCATGGGA

**11. C26, [Organism=*Pseudomonas* sp.] [isolate=C26] *Pseudomonas* sp. C26
16S ribosomal RNA gene, partial sequence (Accession number: GU394954)**

TGCAAGTCGAGCGGATGAGTGGAGCTTGCTCCATGATTCAGCGGCGGA
CGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTT
CGAAAGGAACGCTAATACCGCATAACGTCCTACGGGAGAAAGTGGGGG
ATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTT
GGTGAGGTAAAGGCTACCAAGGCGACGATCCGTAACCTGGTCTGAGAG
GATGATCAGTCACACTGGAACCTGAGACACGGTCCAGACTCCTACGGGA
GGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCA
TGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGG
GAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACAG
AATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGG
TGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTT
CGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACCTGCATCC
AAAACCTGGCGAGCTAGAGTATGGCAGAGGGTGGTGGAAATTTCTGTGT
AGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCG
ACCACCTGGGCTAATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAA
ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAG
CCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCATTAAGTCGA
CCGCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGG
GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGA
AGAACCTTACCAGGCCTTGACATGCAGAGAACTTTCCAGAGATGGATT
GGTGCCTTCGGGAACCTCTGACACAGGTGCTGCATGGCTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTAAAGTCCCGTAACGAGCGCAACCCTTGTCC
TTAGTTACCAGCACGTTAAGGTGGGCACTCTAAGGAGACTGCCGGTGA

CAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACG
GCCTGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCCAAG
CCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCA
GTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATC
AGAATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCCGTC
ACACCATGGGAGTGGGTTGCTCCAGAAGTAGCTAGTCTAAC

12. B3a, [Organism=*Stenotrophomonas* sp.] [isolate=B3a] *Stenotrophomonas* sp. B3a 16S ribosomal RNA gene, partial sequence (Accession number: GU394955)

TGCAGTCGAACGGCAGCACAGTAAGAGCTTGCTCTTACGGGTGGCGAG
TGGCGGACGGGTGAGGAATGCATCGGAATCTACTCTGTCTGTTGGGGAT
AACGTAGGGAACTTACGCTAATACCGCATAACGACCTACGGGTGAAAG
CAGGGGATCTTCGGACCTTGCGCGATTGAATGAGCCGATGCCCCGATTA
GCTAGTTGGCGGGGTAAGAGCCCACCAAGGCGACGATCGGTAGCTGGT
CTGAGAGGATGATCAGCCACACTGGAAGTGAAGACACGGTCCAGACTCC
TACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGAT
CCAGCCATAACCGCGTGGGTGAAGAAGGCCTTCGGGTGTAAAGCCCTT
TTGTTGGGAAAGAAAAGCAGCCGGTTAATACCCGGTTGTTCTGACGGT
ACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAAT
ACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGT
AGGTGGTTGTTTAAGTCTGTCTGTAAGCCCTGGGCTCAACCTGGGAAT
GGCGATGGAACTGGGCGACTAGAGTGTGGCAGAGGGTAGTGGAATTC
CTGGTGTAGCAGTGAAATGCGTAGAGATCAGGAGGAACATCCGTGGCG
AAGGCGACTGCCTGGGCCAACACTGACACTGAGGCACGAAAGCGTGG
GGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGC
GAACTGGATGTTGGGTGCAATTTGGCACGCAGTATCGAAGCTAACGCG
TTAAGTTCGCCGCCTGGGGAGTACGGTTCGCAAGACTGAACTCAAAGG
AATTGACGGGGGCCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGAT
GCAACGCGAAGAACCCTTACCTGGCCTTGACATGCACGGAACCTTCCAG
AGATGGATTGGTGCCTTCGGGAACCGTGACACAGGTGCTGCATGGCTG
TCGTCAGCTCGTGTCTGTGAGATGTTGGGTAAAGTCCCGCAACGAGCGC
AACCCTTGTCCTTAGTTGCCAGCACGTAATGGTGGGAACTCTAAGGAG
ACCGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATC
ATGGCCCTTACGGCCAGGGCTACACACGTACTACAATGGTAGGGACAG
AGGGCTGCAAGCCGGCGACGGTGAGCCAATCCCAGAAACCCTATCTCA
GTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAG
TAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTAC
ACACCGCCCGTCACACCATGGGAGTTTGTGTCACCAGAAGCAGGTAGC
TT

13. C12b, [Organism=*Xenophilus* sp.] [isolate=C12b] *Xenophilus* sp. C12b 16S ribosomal RNA gene, partial sequence (Accession number: GU394956)

TGCAGTCGAACGGTAACAGGCCTTCGGGTGCTGACGAGTGGCGAACGG
GTGAGTAATACATCGGAACGTGCCAGTCGTGGGGGATAGCTCGGCGA
AAGCCGGATTAATACCGCATAACGATCCACGGATGAAAGCGGGGGACCT
TCGGGCCTCGCGCGATTGGAGCGGCCGATGGCAGATTAGGTAGTTGGT

GGGGTAAAGGCTCACCAAGCCGACGATCTGTAGCTGGTCTGAGAGGAC
GACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGC
AGCAGTGGGGAATTTTGGACAATGGGCGCAAGCCTGATCCAGCCATGC
CGCGTGCAGGATGAAGGCCTTCGGGTTGTAAACTGCTTTTGTACGGAA
CGAAAAGGCTCTGGTTAATACCTGGGGCTCATGACGGTACCGTAAGAA
TAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTG
CGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTGAT
GTAAGACAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGT
GACTGCATTGCTGGAGTGCGGCAGAGGGGGATGGAATTCCGCGTGTAG
CAGTGAAATGCGTAGATATGCGGAGGAACACCGATGGCGAAGGCAAT
CCCCTGGGCCTGCACTGACGCTCATGCACGAAAGCGTGGGGAGCAAAC
AGGATTAGATAACCCTGGTAGTCCACGCCCTAAACGATGTCAACTGGTT
GTTGGGAATTTGCTTTCTCAGTAACGAAGCTAACGCGTGAAGTTGACCG
CCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGGGG
ACCCGCACAAGCGGTGGATGATGTGGTTTAATTTCGATGCAACGCGAAA
AACCTTACCCACCTTTGACATGTACGGAACCTTGCCAGAGATGGCTTGGT
GCTCGAAAGAGAACCGTAACACAGGTGCTGCATGGCTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTAAAGTCCCGCAACGAGCGCAACCCTTGTC
ATTAGTTGCTACATTCAGTTGGGCACTCTAATGAGACTGCCGGTGACAA
ACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATAGGT
GGGGCTACACACGTCATAAATGGCCGGTACAAAGGGTTGCCAACCCG
CGAGGGGGAGCTAATCCCATAAAGCCGGTCGTAGTCCGGATCGCAGTC
TGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGGATCAGA
ATGTCACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACAC
CATGGGAGCGGGTTCTGCCAGAAGTAGTTAGCCTAACCGCAAGGAGGG

14. A10b, [Organism=*Bacterium* sp.] [isolate=A10b] *Bacterium* sp. A10b 16S ribosomal RNA gene, partial sequence (Accession number: GU394957)

GCTCTCTGGGTGGCGAGTGGCGGACGGGTGAGGAATGCATCGGAATCT
ACTCTGTCGTGGGGGATAACGTAGGGAACTTACGCTAATACCGCATA
CGACCTACGGGTGAAAGCAGGGGACCTTCGGGCCTTGCGCGATTGAAT
GAGCCGATGCCCCGATTAGCTAGTTGGCGGGGTAAAGAGCCACCAAGGC
GACGATCGGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAAGTGA
GACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACA
ATGGGCGCAAGCCTGATCCAGCCATACCGCGTGGGTGAAGAAGGCCTT
CGGGTTGTAAAGCCCTTTTGTGGGAAAGAAAAGCAGCCGGTTAATAC
CCGGTTGTTCTGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTG
CCAGCAGCCGCGGTAAACGAAGGGTGCAAGCGTTACTCGGAATTACT
GGGCGTAAAGCGTGCGTAGGTGGTTGTTAAGTCTGTCGTGAAAGCCC
TGGGCTCAACCTGGGAATGGCGATGGAAACTGGACGACTAGAGTGTGG
CAGAGGGTAGTGGAATTCCTGGTGTAGCAGTGAAATGCGTAGAGATCA
GGAGGAACATCCGTGGCGAAGGCGACTGCCTGGGCCAACACTGACACT
GAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATAACCCTGGTAGT
CCACGCCCTAAACGATGCGAACTGGATGTTGGGTGCAATTTGGCACGC
AGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGC
AAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAAGCGGTGGA
GTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCCTTGAC
ATGCACGGAACCTTCCAGAGATGGATTGGTGCCTTCGGGAACCGTGAC

ACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCTGAGATGTTGGGTTA
AGTCCCCGAACGAGCGCAACCCTTGTCTTAGTTGCCAGCACGTAATG
GTGGGAACTCTAAGGAGACCGCCGGTGACAAACCGGAGGAAGGTGGG
GATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTAC
TACAATGGTAGGGACAGAGGGCTGCAAGCCGGCGACGGTGAGCCAAT
CCCAGAAACCCTATCTCAGTCCGGATTGGAGTCTGCAACTCGACTCCAT
GAAGTCGGAATCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATA
CGTCCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTG

15. C42, [Organism=*Agromyces* sp.] [isolate=C42] *Agromyces* sp. C42 16S ribosomal RNA gene, partial sequence (Accession number: GU394958)

CACGTGAGTAACCTGCCCTGGACTCTGGGATAACTTCGAGAAATCGGA
GCTAATACCGGATAGGACCTTGCACCGCATGGTGTGGGGTGGAAAGTT
TTTCGGTTTGGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTAAT
GGCTCACCAAGGCGTCGACGGGTAGCCGGCCTGAGAGGGTGACCGGCC
AACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGG
GGAATATTGCACAATGGGCGCAAGCCTGATGCAGCAACGCCGCGTGCG
GGATGACGGCCTTCGGGTTGTAAACCGCTTTTAGTAGGGAAGAAGCCT
TCGGGTGACGGTACCTGCAGAAAAAGGACCGGCTAACTACGTGCCAGC
AGCCGCGGTAATACGTAGGGTCCGAGCGTTGTCCGGAATTATTGGGCG
TAAAGAGCTCGTAGGCGGTTTGTGCGCTCTGCTGTGAAAAGTAGAGGC
TCAACCTCTAGCCTGCAGTGGGTACGGGCAGACTTGAGTGGTGTAGGG
GAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGG
AACACCGATGGCGAAGGCAGGTCTCTGGGCACTTACTGACGCTGAGGA
GCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGC
CGTAAACGTTGGGCGCTAGATGTGGGGACCTTTCCACGGTTTCCGTGTC
GTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCT
AAAAGTCAAAGGAATTGACGGGGGCCCCGCACAAGCGGCGGAGCATGC
GGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATACA
CGAGAACGGGCCAGAAATGGTCAACTCTTTGGACACTCGTGAACAGGT
GGTGCATGGTTGTCGTCAGCTCGTGTCTGAGATGTTGGGTAAAGTCCC
GCAACGAGCGCAACCCTCGTCGCATGTTGCCAGCACGTTATGGTGGGG
ACTCATGTGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACG
TCAAATCATCATGCCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGG
CCGGTACAAAGGGCTGCGATGTCGTAAGGCGGAGCGAATCCCCAAAA
GCCGGTCTCAGTTCGGATTGAGGTCTGCAACTCGACCTCATGAAGTCGG
AGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCG
GGCCTTGTACACACCGCCCGTCAAGTCATGAAAGTCGGTAACACCCGA
AGCCGGTGGCCTAACCTT

16. C43a, [Organism=*Mycobacterium* sp.] [isolate=C43a] *Mycobacterium* sp. C43a 16S ribosomal RNA gene, partial sequence (Accession number: GU394959)

TCGAGTGGCGAACGGGTGAGTAACACGTGGGTGATCTGCCCTGAACTC
TGGGATAAGCCTGGGAACTGGGTCTAATACCGGATAGGACTTCGCAT
CGCATGGTGTGGGGTGGAAAGCTTTTGCGGTTTGGGATGGGCCCCGCG
CCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGT

AGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAA
GCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAA
ACCTCTTTCGCCAGGGACGAAGCGTAAAGTGACGGTACCTGGAGAAGAA
GCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGA
GCGTTGTCCGAATTACTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCTG
CGTCGTCTGTGAAATTCTGCAACTCAATTGCAGGCGTGCAGGCGATAC
GGGCAGACTTGAGTACTACAGGGGAGACTGGAATTCCTGGTGTAGCGG
TGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTC
TGGGTAGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGG
ATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGTACTAGGTGTG
GGGCTCATTTACGAGTTCCGTGCCGTAGCTAACGCATTAAGTACCCCG
CCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGG
GCCCCGACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACGCGAAG
AACCTTACCTGGGTTTGACATACACCAGACGTATGTAGAGATGCATATT
CCCTTGTGGTTGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTC
GTGAGATGTTGGGTAAAGTCCCGCAACGAGCGCAACCCTTGTCTGTAT
TGCCAGCGGGTTATGCCGGGGACTTGACAGGAGACTGCCGGGGTCAACT
CGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCCTTATGTCCAG
GGCTTCACACATGCTACAATGGCGCGTACAGAGGGCTGCGAGACCGTG
AGGTGGAGCGAATCCCTTAAAGCGCGTCTCAGTTCGGATTGGGGTCTG
CAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAA
CGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCTCCCGTCACGTC
ATGAAAGTCGG

Appendix B Fungal Sequences

1. D42 [Organism=*Pseudallescheria* sp.][isolate=D42] *Pseudallescheria* sp. D42 18S ribosomal RNA gene partial sequence (Accession number: GU183392)

TGTCTAAGTATAAGCAATTATACCGCGAAACTGCGAATGGCTCATTATA
TAAGTTATCGTTTATTTGATAGCACATTACTACATGGATAACTGTGGTA
ATTCTAGAGCTAATACATGCTAAAAATCCCGACTTCGGAAGGGATGTA
TTTATTAGATTAAAAGCCAACGCCCTTCGGGGCTTCGTGGTGATTCATG
ATAACTTTTTCGAATCGCATGGCCTTGCGCCGGCGATGGTTCATTCAAAT
TTCTTCCCTATCAACTTTTCGATGCGAAGGTCTTGTCTTCGCATGGTTGCA
ACGGGTAACGGAGGGTTAGGGCTCGACCCCGGAGAAGGAGCCTGAGA
AACGGCTACTACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAAT
CCCGACACGGGGAGGTAGTGACAATAAATACTGATACAGGGCTCTTTT
GGGTCTTGTAATCGGAATGAGTACAATTTAAATCCCTTAACGAGGAAC
AATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCA
ATAGCGTATATTAAAGTTGTTGTGGTTAAAAAGCTCGTAGTTGAACCTT
GGGCTGGCTGGCCGGTCCCCCTCACCGGGTGCACTGATCCAGCCGGG
CCTTTCCCTCTGTGGAACCCCATGGCCTTCACTGGCCGTGGCGGGGAAA
CAGGACTTTTACTTTGAAAAAATTAGAGTGCTCCAGGCAGGCCTATGCT
CGAATACATTAGCATGGAATAATAGAATAGGACGTGTGGTTCTATTTTG
TTGGTTTCTAGGACCGCCGTAATGATTAATAGGGACAGTCGGGGGCAT
CAGTATTCAATTGTCAGAGGTGAAATTCCTTGGATCTATTGAAGACTAAC
TACTGCGAAAGCATTTGCCAAGGATGTTTTTCATTGATAAGGAAAGAA
AGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACTATAA
ACTATGCCGACTAGGGATCGGACGATGTTATTTCTTGACGCGTTCGGCA
CCTTTTCGAGAAATCAAAGTGCTTGGGCTCCAGGGGGAGTATGGTCGCA
AGGCTGAACTTAAAGAAATTGACGGAAGGGCACCACCAGGGGTGGA
ACCTGCGGCTTAATTTGACTCAACACGGGGAACTCACCAGGTCCAGA
CACAGTGAGGATTGACAGATTGAGAGCTCTTTCTTGATTCTGTGGGTGG
TGGTGATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGCTTAATTGC
GATAACGAACGAGACCTTAACCTGCTAAATAGCCCGTACTGCTTTGGC
AGTTCGCCGGCTTCTTAGAGGGACTATCGGCTCAAGCCGATGGAAGTTT
GAGGCAATAACAGGTCTGTGATGCCCTTAGATGTCCTGGGCCGCACGC
GCGTTACACTGACAGAGCCAGCGAGTATTTCTTGGCCGGAAGGCCCG
GGTAATCTTGTTAAACTCTGTCTGCTGGGGATAGAGCATTGCAATTAT
TGCTCTTCAACGAGGAATCCCTAGTAAGCGCAAGTCATCAGCTTGCGTT
GATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTACTACCGATTG
AATGGCTCAGTGAGGCCTCCGGACTGGCCCGAGAGAGGTGGGCAACTAC
CACTCAGGGCCCGGAAAGTTGTCCAAACTCGGTCATTAGAGGAA

2. E41 [Organism=*Pseudallescheria* sp.][isolate=E41] *Pseudallescheria* sp. E41 18S ribosomal RNA gene partial sequence (Accession number: GU183395)

CCATGCATGTCTAAGTATAAGCAATTATACTGCGAAACTGCGAATGGC
TCATTATATAAGTTATCGTTTATTTGATAGCACTTTACTACATGGATAA
CTGTGGTAATTCTAGAGCTAATACATGCTAAAAATCCCGACTTCGGAA

GGGATGTATTTATTAGATTAAAAGCCAAACGCCCTTCGGGGCTTCGTGGT
GATTCATGATAACTTTTCGAATCGCATGGCCTTGCGCCGGCGATGGTTC
ATTCAAATTTCTTCCCTATCAACTTTCGATGCGAAGGTCTTGTCTTCGCA
TGGTTGCAACGGGTAAACGGAGGGTTAGGGCTCGACCCCGGAGAAGGA
GCCTGAGAAACGGCTACTACATCCAAGGAAGGCAGCAGGCGCGCAAA
TTACCCAATCCCGACACGGGGAGGTAGTGACAATAAATACTGATACAG
GGCTCTTTTGGGTCTTGTAATCGGAATGAGTACAATTTAAATCCCTTAA
CGAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTC
CAGCTCCAATAGCGTATATTAAGTTGTTGTGGTTAAAAAGCTCGTAGT
TGAACCTTGGGCCTGGCTGGCCGGTCCCCCTCACCGGGTGCACTGATCC
AGCCGGGCCTTTCCCTCTGTGGAACCCCATGGCCTTCACTGGCCGTGGC
GGGGAAACAGGACTTTTACTTTGAAAAAATTAGAGTGCTCCAGGCAGG
CCTATGCTCGAATACATTAGCATGGAATAATAGAATAGGACGTGTGGT
TCTATTTTGTGGTTTCTAGGACCGCCGTAATGATTAATAGGGACAGTC
GGGGGCATCAGTATTCAATTGTCAGAGGTGAAATTCTTGGATCTATTGA
AGACTAACTACTGCGAAAGCATTGCGCAAGGATGTTTTTCATTGATAAG
GAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTA
ACTATAAACTATGCCGACTAGGGATCGGACGATGTTATTTCTTGACGCG
TTCGGCACCTTTCGAGAAATCAAAGTGCTTGGGCTCCAGGGGGAGTAT
GGTCGCAGGCTGAAACTTAAAGAAATTGACGGAAGGGCACCACCAGG
GGTTAACGTCTGACTGTAAAGCCACAGAAGAAATCTGCTCCTGAAAGC
CGCCCGAAAGGGTGAGGTGGTGGTCCAGGGCTCCAGAGCCCGAACTT
AAAGGCTAGTCCTCCACTGCGGAGGGCGACATCCTCAAATTGCGGGGA
ACTCCTAAAGCTTCCACTCCAAAGCCGGTTCGCGAAAGCCGCCGGTGG
CCAGGTTAAAAGCCTCGGGTACTGGAAAAACGTGGAAGATGAGACAAT
GGACAATCCGCAGCCAAGCCCCTAAAGGCCTCCCCTGAAGAGGGACGC
CCACGGGGAAAGGTTTCAGAGACTTGACGGGGATGGGTATGCGCCGAGA
GGCGCCTGCTTAAGATAAAGTCCGGCCCTGCGCGAGAGCGCGGGGGTA
GCAGGGAACCTGCGGCTTAATTTGACTCAACACGGGGAACTCACCAG
GTTAACTACTTTTCGCGGTTTGTACGCGTCTGGGCCTGGAAAAGTAAGG
GCCGCCTAAAGGCCGTCTTGCTAGTGCCGACTTTACCTACGCGGGGGA
AGCTTAGATGCCCCCTACTCAGGGCGTGGGAGCAGCCTTTACGGGGCT
GCGTGTCGGTGCGACACCAGCTGGTACAGGGAACGCCGACCTGCCCAT
CACGGGGCAGAGGCCGATCCTGTGGCGAGTCCGGGTGCGCCGGACCG
TCGCAACGCACGCTAAGCGGTGGGCTGGCAGAAATGCCGGCTTAAGGT
ACGTGCTAATCCCACGGGGTAACCGTGCCCGTCCTGAATAGTTCCGAG
TTTACGAAGCAGGGCGGGGGTGGGCCGTTCGCGGCCTGCTCGTTCAGT
TACAGCGGGTTGAAAACCGCCGTAACGTAAGTGCCAGACACAGTGAGG
ATTGACAGATTGAGAGCTCTTTCTTGATTCTGTGGGTGGTGGTGCATGG
CCGTTCTTAGTTGGTGGAGTGATTTGTCTGCTTAATTGCGATAACGAAC
GAGACCTTAACCTGCTAAATAGCCCGTACTGCTTTGGCAGTTCGCCGGC
TTCTTAGAGGGACTATCGGCTCAAGCCGATGGAAGTTTGAGGCAATAA
CAGGTCTGTGATGCCCTTAGATGTCCTGGGCCGCACGCGCGTTACACT
GACAGAGCCAGCGAGTATTTCTTGGCCGGAAGGCCCGGGTAATCTTG
TTAACTCTGTCTGTGCTGGGGATAGAGCATTGCAATTATTGCTCTTCAA
CGAGGAATCCCTAGTAAGCGCAAGTCATCAGCTTGCGTTGATTACGTCC
CTGCCCTTTGTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAG
TGAGGCCTTCGGACTGGCCCGAGAGAGGTGGGCAACTACCACTCAGGGC
CGGAAAGTTGTCCAACTCGGTCATTTAGAGGAAGTAAAA

**3. G35 [Organism=*Pseudallescheria* sp.][isolate=G35] *Pseudallescheria* sp.
G35 18S ribosomal RNA gene partial sequence (Accession number:
GU183396)**

TGCATGTCTAAGTATAAGCAATTATACTGCGAAACTGCGAATGGCTCAT
TATATAAGTTATCGTTTATTTGATAGCACTTTACTACATGGATAACTGT
GGTAATTCTAGAGCTAATACATGCTAAAAATCCCGACTTCGGAAGGGA
TGTATTTATTAGATTAAAAGCCAACGCCCTTCGGGGCTTCGTGGTGATT
CATGATAACTTTTCGAATCGCATGGCCTTGCGCCGGCGATGGTTCATTC
AAATTTCTTCCCTATCAACTTTCGATGCGAAGGTCTTGTCTTCGCATGGT
TGCAACGGGTAACGGAGGGTTAGGGCTCGACCCCGGAGAAGGAGCCT
GAGAAACGGCTACTACATCCAAGGAAGGCAGCAGGCGCGCAAATTAC
CCAATCCCGACACGGGGAGGTAGTGACAATAAATACTGATACAGGGCT
CTTTTGGGTCTTGTAATCGGAATGAGTACAATTTAAATCCCTTAACGAG
GAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGC
TCCAATAGCGTATATTAAAGTTGTTGTGGTTAAAAAGCTCGTAGTTGAA
CCTTGGGCCTGGCTGGCCGGTCCCCCTCACCGGGTGCACTGATCCAGCC
GGGCCTTTCCCTCTGTGGAACCCCATGGCCTTCACTGGCCGTGGCGGGG
AAACAGGACTTTTACTTTGAAAAAATTAGAGTGCTCCAGGCAGGCCTA
TGCTCGAATACATTAGCATGGAATAATAGAATAGGACGTGTGGTTCTA
TTTTGTTGGTTTCTAGGACCGCCGTAATGATTAATAGGGACAGTCGGGG
GCATCAGTATTCAATTGTCAGAGGTGAAATTCTTGGATCTATTGAAGAC
TAACTACTGCGAAAGCATTTGCCAAGGATGTTTTCAATTGATAAGGAAC
GAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAATA
TAACTATGCCGACTAGGGATCGGACGATGTTATTTCTTGACGCGTTTCG
GCACCTTTTCGAGAAATCAAAGTGCTTGGGCTCCA

Appendix C Gram Staining Pictures of All Isolates

***Pseudallescheria* sp. D42**

***Pseudallescheria* sp. E41**

***Pseudallescheria* sp. G35**

All pictures of suspected mould were taken under 400X magnification

***Pseudomonas* sp. E8**

***Cellulosimicrobium* sp. C10**

***Bacillus* sp. G10**

***Xenophilus* sp. B2a**

***Pseudomonas* sp. G7**

***Microbacterium* sp. F28**

***Stenotrophomonas* sp. B15b**

***Tsukamurella* sp. C35**

***Bacillus* sp. F4**

***Bacillus* sp. B18b**

***Pseudomonas* sp. C26**

***Stenotrophomonas* sp. B3a**

***Xenophilus* sp. C12b**

***Bacterium* sp. A10b**

***Agromyces* sp. C42**

***Mycobacterium* sp. C43a**

All the gram staining pictures of bacteria were taken under 1000X magnification with oil emission