Utilization of Cellulosic Materials by *Thermotoga petrophila*

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Thermotoga petrophila is a hyperthermophilic anaerobic bacterium that grows optimally at 80 ° C. It can utilize plant biomass to produce biofuels, including ethanol and hydrogen, which are alternative and renewable sources of energy. Xylan, microcrystalline avicel PH105, switchgrass, corn husks and wheat straw were used as growth substrates to determine *T. petrophila*'s capability to use different types of plant biomass for the production of ethanol and hydrogen. The metabolism of cellulosic substrates was analyzed by integrating proteomics analysis, gene identification, cellulase and xylanase activities, growth, metabolic products and cell adhesion. *T. petrophila* showed best growth on xylan, followed by corn husks, switchgrass, avicel PH105 and wheat straw. The optimal pH for higher biofuel yield was within the range of 8.0 to 8.5. The metabolic end products were H_2, CO_2, acetate, lactate, formate and succinate when *T. petrophila* grew on all the tested cellulosic materials. The highest yield of hydrogen (9.6 mM) and the highest yield of ethanol (0.95 mM) were both detected when *T. petrophila* was grown on xylan. No growth was observed on xylose, which was not expected because *T. petrophila* grew very well on xylan, a β-1, 4-xylopryranose polymer from which xylose can be produced upon hydrolysis. The possible reason for this phenomenon may be that *T. petrophila* has no specific sugar transporters for xylose, although it contains all the genes encoding xylose metabolizing enzymes.

The majority of exoglucanases and endoglucanases presented in *T. petrophila* were extracellular enzymes. The highest specific activities of exoglucanase (1361.3 mU/mg) and endoglucanase (1032.1 mU/mg) in *T. petrophila* were found in the supernatants of the growth culture with xylan as the sole substrate, indicating that xylan, not cellulose, is the best inducer to increase the expression of extracellular cellulases. Compared to the huge discrepancy of extracellular cellulases activities among different substrates (from 0 to 1361.3 mU/mg), intracellular cellulase (endoglucanase) activity was relatively steady (around 150 mU/mg). Xylanase activity was also detected in both the supernatant and the cell free extract; thus *T. petrophila* contains both extracellular and intracellular xylanase. The highest xylanase activity was detected in the cell free extract when *T. petrophila* was grown on cellobiose and xylan (3732.4 mU/mg and 3152.8 mU/mg, respectively), indicating that the majority of xylanase is an intracellular enzyme, and xylan and cellobiose are the best inducers to increase the expression of xylanase.

Adhesion of *T. petrophila* cells to xylan, with many filaments connecting all the cells, was observed using scanning electron microscope and fluorescent staining microscope, whereas there was no attachment between cells and cellulose. This difference may explain why *T. petrophila* grew much better on xylan than on cellulose because cell adhesion increases enzyme concentration near the substrate to improve the efficiency of cellulosic material utilization.

Furthermore, proteomics analysis was used to quantify all the expressed proteins in different growth media with various substrates. The proteomics data revealed that the most important enzymes for cellulose and hemicellulose utilization were ATP binding cassette (ABC) transporters, S-layer proteins and membrane binding proteins, which were up-regulated when *T. petrophila* was grown on cellulosic materials. The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) also indicated the up-regulated proteins from the media with cellulosic substrates were probably ABC transporters and S-layer proteins based on the size of proteins. Based on the gene identification, end product determination and proteomics analysis, the tentative cellulosic material metabolic pathways in *T. petrophila* were completely profiled.

Overall, our results suggest that the ability of *T. petrophila* to convert cellulosic materials into hydrogen and ethanol exceeds *T. maritima*, which is a model strain for studying hyperthermophiles. *T. petrophila* has great potential in applications of producing highly thermostable cellulases and biofuels from cellulosic materials. However, the mechanism of cell adhesion between *T. petrophila* and xylan and the regulation of the entire cellulosic materials metabolic pathway need further investigation.

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List of Abbreviations

ABC	ATP binding cassette
ACK	Acetate kinase
ADH	Alcohol dehydrogenase
AI	Arabinose isomerase
BSA	Bovine serum albumin
CMC	Carboxymethylcellulose
DAPI	4', 6'-diamidino-2-phenylindole
2D-HPLC-MS	Two dimensional liquid chromatograph-mass spectrometry analysis
2D-HPLC	Two dimensional high performance liquid chromatography
DNS	3, 5-Dinitrosalicylic acid
DTT	Dithiothreitol
ED	Entner-Doudoroff
EMP	Embden-Meyerhof pathway
Fase	Fumarase
FR	Fumarate reductase
GC	Gas chromatography
H_2ase	Hydrogenase
HPLC	High performance liquid chromatography
LDH	Lactate dehydrogenase
MDH	Malate dehydrogenase
MS	Mass spectrometry
NCBI	National Center for Biotechnology Information
PC	Pyruvate carboxylase
PFL	Pyruvate formate-lyase
PI	Propidium iodide

PK	Pyruvate kinase
POR	Pyruvate ferredoxin oxidoreductase
PPP	Pentose phosphate pathway
PTA	Phosphate acetyltransferase
RF	Ribulose 5-phosphate epimerase
RI	Ribulose-5-phosphate-isomerase
RK	Ribulokinase
RNF	NAD^+ Oxidoreductase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning electron microscope
TFA	Trifluoroacetic acid
TA	Transaldolase
TK	Transketolase
XK	Xylulokinase
XI	Xylose isomerase

Chapter 1
Introduction

1.1 Biofuels

Biofuels are derived from bio-organic materials such as oil seeds, animal fats, agricultural wastes and trees. This type of fuel is derived from biological carbon fixation. Although fossil fuels are derived from ancient carbon fixation, they are not considered biofuels by the generally accepted definition of biofuel. Biofuels are promising alternative sources of energy to replace the current petroleum-based fuels because not only can they be used as transportation fuels with minimal modification from current technologies, but also dramatically reduce greenhouse gas emissions (Levin *et al*., 2004 and 2006).

Science and engineering research aimed at efficient use of organic materials for biofuels has been very active due to predictions that energy consumption will continue to increase by 2% each year, along with the constant inflation in the price of fossil fuels (EIA, 2007). Today, the commonly used biofuels are liquid ones, such as bioethanol and biodiesel, which comprise about 1.2% of the global renewable energy supply. Liquid biofuels are classified under three major sources of large scale production: sugarcane ethanol from Brazil, corn ethanol from the United States, and rapeseed biodiesel from Germany (Rajagopal *et al*., 2007). Brazil and the United States together produce about 90% of the total 36 billion liters of ethanol produced globally. Germany produces more than 50% of the total 3.5 billion liters biodiesel (Rajagopal *et al*., 2007). These biofuels are derived from sugar and starch which are called the first generation of biofuels. However, current starch and sugar-based technologies are not competitive fuels compared to fossil fuels since some biomass sources are largely limited by human consumption needs, as they occupy scarce agricultural land and have relatively low transformation efficiency. Therefore, people are looking forward to explore biofuels, which do not demonstrate the drawbacks mentioned above; thus the second generation biofuels are posed and have become greater topics of interest than first generation biofuels.

The second generation biofuels are produced from cellulose, hemicellulose and lignin from trees and grasses that can grow on relatively marginal land, and they have been predicted to be the alternative raw materials for biofuel production. The final targets of utilization of the second generation biofuels are to reduce carbon emission, to increase energy efficiency, to reduce energy dependency and to use non-food based biomass as the substrate to overcome the limitations of first generation biofuels. Since cellulosic biomass is the most abundant biological energy source in the

world, it has a greater potential to supply cheaper, cleaner, and sustainable renewable energy than current petroleum-based fuel (Hertwich *et al*., 2009).

Currently, different biofuel technologies have already been applied to produce the second generation biofuels, including bioethanol, biodiesel, biomethane and hydrogen. The strategy is focused on the heat and chemical pretreatments and the enzymatic hydrolysis of cellulose and hemicellulose into sugar monomers that can be utilized by fermentative microorganisms to produce biofuels. However, the second generation biofuels are still in the demonstration stage with some impediments.

1.2 Current approaches and challenges of the second generation biofuels

1.2.1 Approaches to produce the second generation biofuels

Cellulose is one of the most foreseeable sustainable sources of fuels and materials available for humanity (Lynd *et al*., 1999) with an average of more than10^{12} tons of cellulose produced each year (Wilson, 2009). The second generation biofuels are generated from these abundant and inexpensive substrates, such as cellulose, hemicellulose and lignin from agricultural wastes, grasses and trees, which are plentiful in the nature.

Currently, the second generation bioethanol derived from cellulosic biomass has already been used in trial scale in many different countries, such as Brazil, Sweden, China, the United States (Tsai *et al*., 2008) and Canada (Natural Resources Canada, 2011). The main barrier of producing second generation biofuels is breaking down the crystalline structure of cellulose and lignin to amorphous cellulose before microbial fermentation (Kaplan *et al*., 1994), and these pretreatment techniques include thermal, chemical, biochemical and microbial approaches (Kaplan *et al*., 1994). By pretreatment, the main monosaccharides, xylose and glucose can be released from cellulose long chains and fermented by most microorganisms and. For example, corncobs are one agricultural by-product that can release large amounts of sugar in monomeric forms, which are broken down from polysaccharide chains using steam pretreatment. Similarly, the enzymatic pretreatment can partially hydrolyze the cellulose to oligomeric and monomeric sugars before the microorganisms are added into the fermenters. This pretreatment also decreases the viscosity of the hydrolysates and makes pumping and stirring the fermentation medium easier, resulting higher ethanol yield in the subsequent fermentation process (Kumakura, 1996).

Nowadays, bioethanol is the main focus in the current biofuel industry. The main processes of bioethanol production from cellulosic biomass are shown in **Figure 1-1** (Carroll *et al*., 2009). Firstly, the biomass is harvested and delivered to the biorefinery, which is defined as the facility integrating biomass conversion processes and equipment to produce fuels. The next step is to cut the biomass

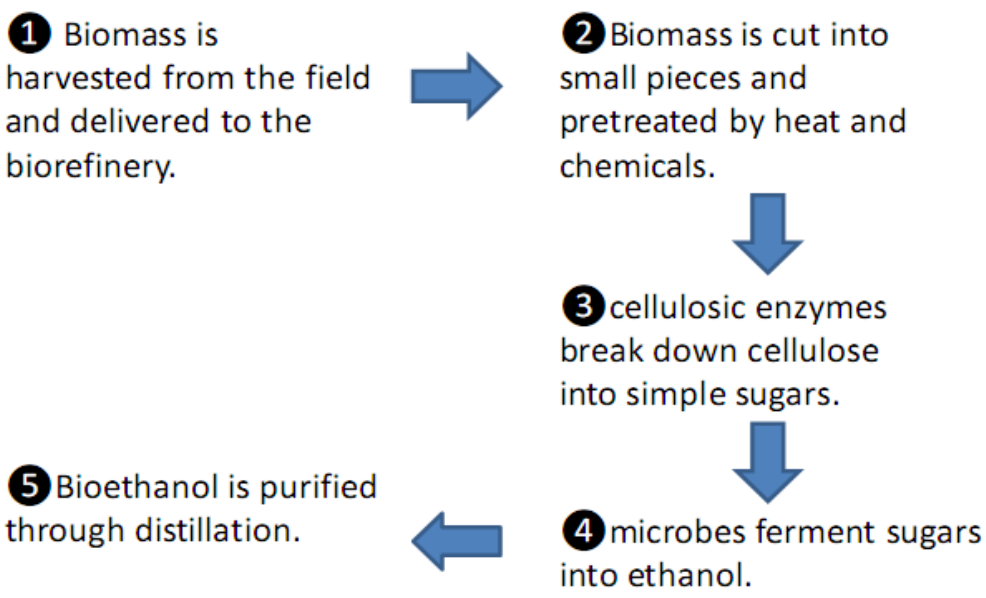

Figure 1-1. The current commercial bioethanol production strategies. The process of producing bioethanol was modified from Carroll (Carroll *et al*., 2009).

into small pieces and pretreat it with heat and chemicals to make its cellulose accessible for the enzymes. After pretreatment, the biomass is further mixed with cellulase enzymes and incubated at optimum reaction temperature in order to break down the cellulose chains into simple sugars. Finally, the microbes ferment sugars to produce ethanol or other biofuels depending on the capabilities of the microbes. The produced ethanol is then concentrated and purified by distillation before use.

1.2.2 Challenges of producing cellulosic biofuels in industry

During cellulosic biofuel production, efficiency and biofuel yield depend on many factors, including the source of cellulose, composition of cellulosic biomass, pretreatment method, reactor design, and whole refinery process. The major costs and impediments to widespread production of cellulosic biofuels are due to the difficulties of breaking down the anatomical structure of cellulose (Carroll *et al*., 2009). Therefore, many challenges and obstacles must be overcome in the production of cellulosic biofuels.

Cellulose is composed of linear chains of glucosyl residues. Approximately 30 cellulose molecules are assembled into larger units, which are elementary fibrils. Many fibrils are assembled into larger units called micro fibrils and further packed into cellulose fibers, which are the main components of the cell walls of plants (Moore *et al*., 1998). Cellulose is insoluble and has highly ordered crystalline regions, closely packed and inaccessible to enzymes. Furthermore, cellulose fibers are embedded in a matrix of other structural biopolymers, such as hemicellulose and lignin, which greatly impede cellulose hydrolysis. Therefore, pretreatment and cellulase hydrolysis steps are necessary before the microbe's fermentation on monosaccharides. The whole process is illustrated in **Figure 1-2**. However, the pretreatment takes large amounts of input energy and chemicals. The enzyme hydrolysis also requires costly commercial cellulases. Hence, about three or four times more investment is required to produce cellulosic biofuels than for biofuels produced from simple sugars (Carroll *et al*., 2009).

Recently, the development of pretreatment technologies to generate more digestible substrates and modification of cellulose hydrolytic enzyme cocktails has already improved the efficiency of utilizing cellulosic biomass and the yield of biofuels (Szijarto *et al*., 2008). However, the ideal solution is to integrate the pretreatment, cellulase reaction and microbe fermentation steps in one single fermenter, where cellulose is transformed to biofuels such as bioethanol and hydrogen. Many researches are searching for a microorganism that can decompose cellulosic materials as substrates without any pretreatment, and also can produce ethanol and hydrogen.

1.3 Enzymatic hydrolysis of cellulosic biomass

Native cellulose is highly resistant to enzyme hydrolysis because the highly crystalline structure and the presence of lignin effectively obstruct the degradation of cellulases and make the hydrolysis

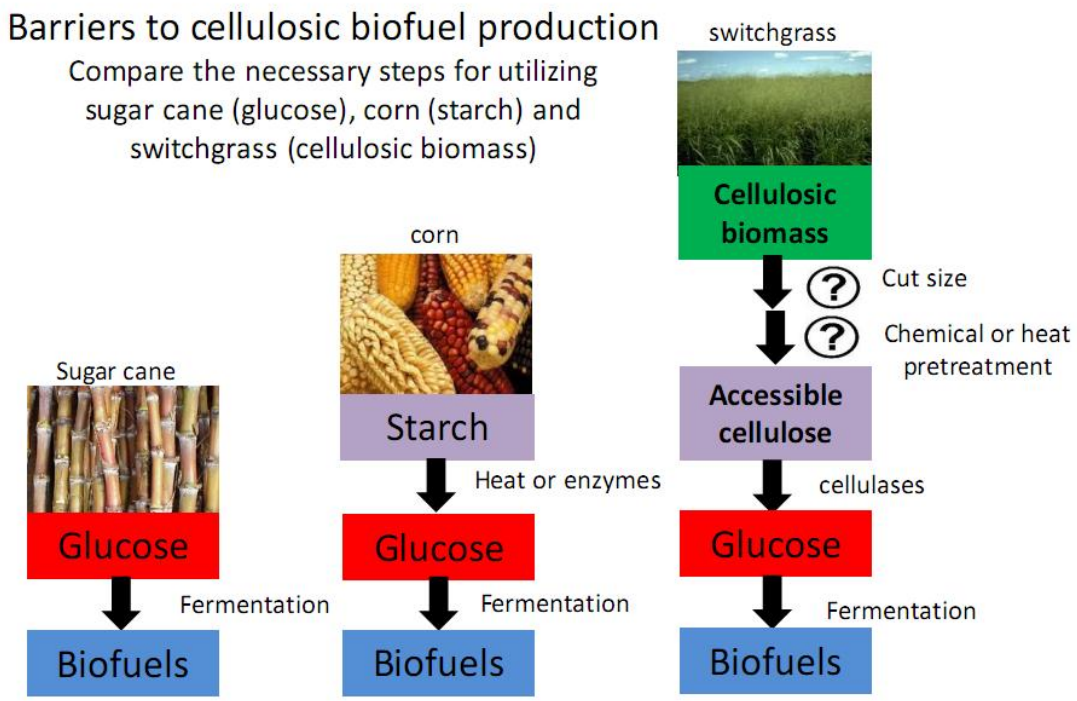

Figure 1-2. Schematic diagram of steps for cellulosic biofuel production. The biofuels from sugar cane require only one step microbial fermentation on simple sugars. The biofuels from starch require heat or enzymatic hydrolysis to degrade starch to simple sugars before microbial fermentation. The biofuels from cellulosic biomass such as switchgrass require physical, chemical and cellulolytic pretreatment to degrade cellulose and hemicellulose to simple sugars before microbial fermentation.

process very slow and incomplete. Thus, the biofuels from cellulosic materials require a treatment on the cellulosic material in some way prior to the use of enzymes. Compared to the chemical and physical pretreatments for degradation of cellulose, enzymatic hydrolysis of cellulose is more attractive and has its own prominent advantages: it is environment-friendly with relatively mild reaction condition. In addition, it has a great potential to decrease the cost of cellulosic ethanol competitive with that of fossil fuels.

The enzymes hydrolyzing cellulose to glucose are a synergistic enzyme system called glycoside hydrolases and cataloged into sequence-related families on the CAZy website: http://www.cazy.org/. This enzyme system contains endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21). Endoglucanases randomly cut the internal amorphous sites in the cellulose polysaccharide chain, thereby generating oligosaccharides of various lengths and producing new chain ends. Exoglucanases act on both reducing and non-reducing ends of cellulose polysaccharide chains, liberating either glucose or cellobiose. β-glucosidases hydrolyze soluble cellodextrin and cellobiose into glucose (Lynd *et al*., 2002).

Apart from the cellulases themselves, many other factors affect the hydrolysis of cellulosic materials, including type of substrates, pretreatment, characteristics of the enzyme preparation, temperature, time, pH, substrate concentration, reuse of enzymes and type of reactor (Linko, 1977). Many of these factors are interdependent and make the whole hydrolysis process more complicated. However, the strategy with the demand of economic feasibility is to design the hydrolysis process with lower requirements for enzyme concentration and higher reaction speed. The ideal reaction is rapid, complete and leads to a high glucose concentration without any substantial loss of enzymes. Therefore, hyperthermophilic cellulases become a good candidate because its tolerance to high temperature that gives high reaction speed, strong and complete cellulosic hydrolysis and little enzyme activity loss. Until now, the hydrolysis of cellulose has not approached the ideal situation, but many researchers are working in this area to encourage further development and optimize the process for commercial application.

Currently, cellulases are the third largest industrial enzyme, which are widely used in paper recycling, cotton processing, juice extraction, biobleaching and animal feed additives (Schaechter, 2009). If the production of ethanol, butanol, or some other fermentation products can be efficiently enhanced by the use of cellulases, it will become the largest volume industrial enzyme in near future.

1.4 Hyperthermophilic cellulolytic bacteria

Based on the crystal structure of cellulose, the characteristics of ideal enzymes involved in cellulolytic reactions are insensitive to fluctuation in substrates and very stable at the high temperatures that give high reaction speed. Thus, thermostable and hyperthermostable enzymes demonstrate the promising characteristics under such catalytic conditions. It is reasonable to utilize

hyperthermophilic cellulases (optimum temperature above 80^{o} C) with high enzyme activity and thermostability as biocatalysts in the biofuel industry (Yeoman *et al.*, 2010). Furthermore, hyperthermophilic cellulases allow easier mixing, better substrate solubility, higher biomass transfer rates and lower risk of contamination at the high temperatures (Turner *et al*., 2007). Therefore, hyperthermophiles and their enzymes have often been considered as the major sources of industrially relevant thermostable cellulolytic enzymes.

1.4.1 Hyperthermophiles

Hyperthermophiles are a group of microorganisms that grow optimally at temperatures of 80 ° C or above (Stetter, 1989). Some of them, such as *Methanopyrus kandleri*, are capable of growing at 122 ° C (Takai *et al*., 2008). They are normally found in extremely hot environments such as hot springs, deep sea hydrothermal vents, and sulphur-containing solfataric fields, and grow at a wide range of pH, from 0 to 9. Twenty-five years ago, the first hyperthermophile *Thermus aquaticus* was discovered and isolated, and exhibited an optimal growth temperature above 80 ° C, where mesophilic and thermophilic bacteria were killed within several seconds (Brock, 1978). So far, about 90 species, 1500 strains of hyperthermophiles are known, and according to their phylogeny and physiological properties, they are grouped into 34 genera and 10 orders (Stetter, 2006).

Hyperthermophiles are classified into two domains in the phylogenetic tree: *Bacteria* and *Archaea*, which are in the short deep branches close to the root of the phylogenetic tree. They may have already existed about 3.9 Gyr ago (Stetter, 2006). Usually, hyperthermophiles are considered to represent the most ancient phenotype of living organisms, and their metabolic pathway may reflect the situation at an early stage of evolution. Two types of hyperthermophiles discovered are now known based on their characteristics of metabolism: chemolithoautotrophy and chemoorganoheterotrophy. No extant phototrophic hyperthermophiles has been discovered. Most species are anaerobic microorganisms. Thus lithotrophic energy metabolism is mostly anaerobic or micro-aerophilic. They can use H_2 or S coupled to reduce SO_4^{2-}, CO_2 and NO^{3-}, but rarely O_2 (Stetter, 2006). The lithotrophic energy metabolism of hyperthermophiles appears to be similar to that of mesophiles. CO_2 fixation in hyperthermophiles proceeds via the reductive citric acid cycle, and then via the reductive acetyl-CoA/carbon monoxide dehydrogenase pathway (Kelly *et al*., 1994). The Calvin cycle has not been discovered in hyperthermophiles. Organotrophic metabolism in hyperthermophiles usually utilizes organic materials as the carbon and energy sources, such as sugars, peptides and starch, which are oxidized to CO_2 by external electron acceptors or fermented to acetate and other products (Kelly *et al*., 1994).

Because of its outstanding thermostable feature, cellulases in hyperthermophiles are highly attractive for contributing to biofuel production. Some cellulases have already been found in hyperthermophiles with high thermostability and activity on cellulose, which are listed in **Table 1-1**.

Table 1-1. Major hyper/thermophiles that can utilize cellulosic materials.

Microorganism	Species	pH	Temperature	Substrate
Bacteria	*Caldicellulosiruptor acetigenus*	5.2-8.5 (opt. 7.0)	50-78 (opt. 65)	CMC[b]
	Caldicellulosiruptor bescii	5.0-8.3 (opt. 7.3)	40-83 (opt. 75)	Avicel, filter paper, pretreated switchgrass, processed cardboard, pretreated populus
	Caldicellulosiruptor hydrothermalis	6.0-8.0 (opt. 7.0)	50-80 (opt. 65)	CMC[b], cellulose, filter paper
	Caldicellulosiruptor kristjanssoni	5.8-8.0 (opt. 7.0)	45-82 (opt. 78)	Avicel
	Caldicellulosiruptor kronotskyensis	6.0-8.0 (opt. 7.0)	45-82 (opt. 70)	CMC[b], cellulose, filter paper
	Caldicellulosiruptor lactoaceticus	5.8-8.2 (opt. 7.0)	50-78 (opt. 68)	Avicel
	Caldicellulosiruptor obsidiansis	6.0-8.0 (opt. 7.0)	55-85 (opt. 78)	Avicel, filter paper, pretreated switchgrass, processed cardboard, pretreated populus
	Caldicellulosiruptor owensensis	5.5-9.0 (opt. 7.5)	50-80 (opt. 75)	Cellulose, CMC[b]
	Caldicellulosiruptor saccharolyticus	5.5-8.0 (opt. 7.0)	45-80 (opt. 70)	Avicel, amorphous cellulose, CMC[b]
	Fervidobacterium islandicum	6.0-8.0 (opt. 7.2)	50-80 (opt. 65)	Cellulose
	Rhodothermus marinus	4.4-8.8 (opt.7.0)	54-77 (opt. 65)	CMC[b]
	Spirochaeta thermophila	5.9-7.7 (opt. 7.5)	40-73 (opt. 68)	Amorphous cellulose, avicel
	Thermotoga maritima	5.5-9.0 (opt. 6.5)	55-90 (opt. 80)	Avicel, CMC[b], switchgrass
	Thermotoga neapolitana	5.5-9.0 (opt. 7.0)	55-90 (opt. 80)	Avicel, CMC[b], switchgrass
	Thermotoga petrophila[a]	5.2-9.0 (opt.7.0)	47-88 (opt. 80)	Avicel, CMC[b], switchgrass
Archaea	*Desulfurococcus fermentans*	4.8-6.8 (opt. 6.0)	63-89 (opt. 82)	Filter paper, avicel, CMC[b]
	Thermococcus sibiricus	5.8-9.0 (opt. 7.5)	40-88 (opt. 78)	Filter paper, avicel, CMC[b]

[a]*Thermotoga petrophila* data were from the growth and cellulase enzyme activities measurement (Chen and Ma, unpublished data, 2010). Other strains refer to the published data (Bergquist *et al*., 1999; Hamilton-Brehm *et al*., 2010; Huang *et al*., 1998; Lynd *et al*., 2002; Mardanov *et al*., 2009; Miroshnichenko *et al*., 2008; Onyenwoke *et al*., 2006; Perevalova, 2005; Sukumaran *et al*., 2005). [b]CMC: Carboxymethylcellulose

1.4.2 *Thermotoga petrophila*

The *Thermotoga* genus is a member of *Thermotogales* that represents a very deep phylogenetic branch within the 16S rRNA gene tree. They are thermophilic, non-spore forming, rod-shaped cells with an outer sheath-like envelope ('toga'), Gram-negative, and strictly anaerobic fermentative bacteria. The optimal growth temperature is up to 80°C (Huber *et al*., 2006). *Thermotoga* species are considered as a model system for studying adaption to high temperatures, cellulosic metabolic pathways and microbial evolution (Frock *et al*., 2010). Three *Thermotoga* species have been found to have the ability to degrade cellulose to produce fermentative hydrogen and ethanol, and demonstrates the potential for biofuel production (Evan *et al*., 2000; Takahata *et al*., 2001; Nguyen *et al*., 2008). Furthermore, six genome sequences for the *Thermotoga* genus are already available, supplying more genetic information for profiling cellulosic metabolic ways and biotechnological application. Ten *Thermotoga* species are characterized to data and listed in **Table 1-2**.

Most studies were performed on both *T. maritima* and *T. neapolitana,* which can use cellulose as a sole carbon source. However, there were no reports on *T. petrophila* except one that stated *T. petrophila* was capable of growing on cellulose with no other details (Takahata *et al*., 2001). Therefore, *T. petrophila* was chosen as the object of this research to determine if *T. petrophila* has a higher ability to utilize cellulosic materials and compare the available data of *T. maritima* and *T. neapolitana.*

T. petrophila was isolated from the production fluid of the Kubiki oil reservoir in Niigata, Japan (Takahata *et al*., 2001). Cells of *T. petrophila* are rod-shaped anaerobic bacteria that range between 2 μm and 7 μm in length and 0.7 μm to1 μm in width. They possess an outer sheath-like structure (toga) and several subpolar and lateral flagella, and have the ability to grow at temperatures between 47 °C and 88 °C, and a pH between 5.2 and 9.0. However, the optimal growth condition of *T. petrophila* is at pH 7.0 and 80 °C, with a doubling time of 54 minutes when *T. petrophila* is grown on glucose. Moreover, this bacterium is capable of utilizing a wide range of substrates, including monosaccharides and polysaccharides such as yeast extract, peptone, glucose, galactose, fructose, ribose, arabinose, sucrose, lactose, maltose, starch or cellulose as its sole carbon and energy source. End products, including acetate, lactate, H_2, and CO_2, were detected during fermentation (Takahata *et al*., 2001). *T. petrophila* cells can reduce elemental sulfur or thiosulfate to H_2S. However, their growth rate and cellular yield decrease in the presence of these electron acceptors. *T. petrophila* is sensitive to 100 μg/mL rifampicin, streptomycin, vancomycin and chloramphenicol (Takahata *et al*., 2001).

Table 1-2. ***Thermotoga*** **species.**

Species	**Temperature (^{o}C)**	**Genome**	**Isolation site**	**Country**	**References**
T. lettngae TMO	50-75 (opt. 65)	CP000812	Sulphate-reducing bioreactor	Netherlands	Balk *et al*., 2002
T. elfii SEBR 6459	50-72 (opt. 66)	N/A	Oil field	Sudan	Ravot *et al*., 1995
T. hypergea SEBR 7054	56-90 (opt. 70)	N/A	Oil-producing well	Cameroon	Fardeau *et al*., 1997
T. subterranean SL1	50-75 (opt. 70)	N/A	Continental oil reservoir	France	Jeanthon *et al*., 1995
T. thermarum LA3	55-84 (opt. 70)	N/A	Continental solfataric springs	Djibouti	Windberger *et al*., 1989
T. neapolitana NS-E (DSM 4359)	55-90 (opt. 77)	CP000916	Shallow submarine hot springs	Italy	Jannasch *et al*., 1988
T. petrophila RKU-1	47-88 (opt. 80)	CP000702	Oil reservoir	Japan	Takahata *et al*., 2001
T. naphthophila RKU-10	48-86 (opt. 80)	CP001839	Oil reservoir	Japan	Takahata *et al*., 2001
T. maritima MSB8 (DSM 3109)	55-90 (opt. 80)	AE000512	Geothermally heated sea floors	Italy	Huber *et al*., 1986
T. sp. strain RQ2	76-82 (opt. 80)	CP000969	Geothermally heated sea floors	Azores	Huber *et al*., 1986

N/A means not applicable.

Since *T. petrophila* is an anaerobic bacterium, theoretically it is capable of producing higher hydrogen because the theoretical maximum yield of hydrogen production is from the anaerobic fermentation with four moles of hydrogen per mole of glucose or eight moles of hydrogen per mole of sucrose if all of the substrate would be converted to acetic acid (Sung *et al*., 2002). Moreover, *T. petrophila* does not need a cool down system after heat pretreatment and directly enters the next fermentation step. Consequently, pretreatment and fermentation are integrated in one step and can dramatically decrease the cost of biofuels production. *T. petrophila* even can grow on cellulosic materials without any pretreatment and produce H_2 and ethanol (Chen and Ma, unpublished data, 2010). Therefore, *T. petrophila* shows great potential as biofuel producer. However, *T. petrophila* is a strictly anaerobic bacterium, so a requirement of anaerobic environment might increase the cost of biofuel production. Nonetheless, *T. petrophila* still has great potential in biofuel industry applications.

1.5 Project objectives

The main goal of this research is to investigate the utilization of cellulosic materials for biofuel production by *T. petrophila* and compared *T. petrophila*'s capability with current various microorganisms, which have already been used in the biofuel industry.

A) Due to the potential application in commercial production of biofuels, it is very necessary to determine *T. petrophila*'s ability to utilize cellulosic materials in optimal growth conditions, including the best source of cellulose and hemicellulose, the optimal concentration of carbon source, and the ideal level of pH and fermentation temperature for achieving the highest biofuel yield and utilization efficiency.

B) In order to screen cellulases and hemicellulases with high activities for commercial use, activities of cellulases and hemicellulases in *T. petrophila* will be determined and compared to those of *T. maritima* and *T. neapolitana*. Different carbon sources will be added to the medium to determine the best inducer for the expression of cellulases and hemicellulases in *T. petrophila*. Determination of cellulases and hemicellulases as extracellular or intracellular will be indicative for purification of cellulases and hemicellulases in industry applications. The mechanisms of cellulosic materials utilization will be investigated by observing the appearance change of cells and cellulosic materials during growth.

C) By quantitative and qualitative analysis, the cellulosic metabolic pathway will be profiled to give a complete picture of cellulosic material utilization, which will help to clarify the whole metabolic process from cellulose to monosaccharide and to biofuels.

Chapter 2
Material and methods

2.1 Chemicals

In order to determine *T. petrophila*'s ability of utilizing cellulosic materials, different sources of celluloses and hemicelluloses were used for growing *T. petrophila* including avicel PH105, switchgrass, corn husks, wheat straw and xylan from beechwood. The simple sugars including cellobiose, glucose and xylose were also tested for comparison with cellulosic materials. Among all the cellulosic materials, switchgrass, corn husks and wheat straw were the native carbon sources harvested from fields, while others were purchased from companies. All the substrates for growth are listed in **Table 2-1**.

Nowadays, switchgrass (*Panicum virgatum*) is a very popular biomass resource for biofuel production. It is a perennial warm season bunchgrass native to North America. Compared to food based crop, switchgrass is a versatile plant growing on land considered unsuitable for agricultural products. Even on barren land, it grows very rapidly with an average height of 1.5 meters (**Figure 2-1**). Therefore, it is an ideal cellulosic substrate used as feedstock for energy production (Fike *et al*., 2005).

Other chemicals such as 4', 6'-diamidino-2-phenylindole (DAPI), propidium iodide (PI), 3, 5-Dinitrosalicylic acid (DNS) and vitamins, were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada).

2.2 Strains of hyperthermophiles

The target hyperthermophile in this research was *Thermotoga petrophila* RKU-1. The other two *Thermotoga* species: *T. maritima* MSB8 *and T. neapolitana* DSM 4359 were used to compare with *T. petrophila* RKU-1. All of the organisms were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunscheweig, Germany.

2.3 Growth of *T. petrophila*

2.3.1 The growth of *T. petrophila*, *T. maritima* and *T. neapolitana*

For culturing *T. petrophila*, 1 L medium (modified from Takahata *et al*., 2001) contained: 1 g NH_4SO_4, 20 g NaCl, 3 g $MgCl_2 \cdot 6H_2O$, 6 g $MgSO_4 \cdot 7H_2O$, 0.3 g $CaCl_2 \cdot 2H_2O$, 0.5 g KCl, 0.2 g

Table 2-1. Different carbon sources for the growth of *T. petrophila*.

Substrates	Description of substrates
Glucose	From EMD (Philadelphia, PA, USA)
D-(+)-cellobiose	≥98% from Sigma-Aldrich Canada (Oakville, ON, Canada) Canada
Xylan from beechwood	>90% xylose residues from Sigma-Aldrich Canada (Oakville, ON, Canada)
Avicel PH105	Microcrystalline cellulose from FMC Biopolymer Company (Philadelphia, PA, USA)
Switchgrass*	Harvested in Nott Farms - RR 4, Clinton, ON, milled to powder with a size of 0.25 mm
Corn husks*	Purchased from the local stores in Waterloo, milled to powder with a size of 0.25 mm
Wheat straw*	Provided by Dr. Duane Falk, Department of Plant Sciences, University of Guelph, milled to powder with a size of 0.25 mm

*Switchgrass, corn husks and wheat straw were milled into fine powder with a Cyclone milling machine (UDY Corporation, Fort Collins, Co, USA) equipped with copper screen (0.25 mm diameter openings).

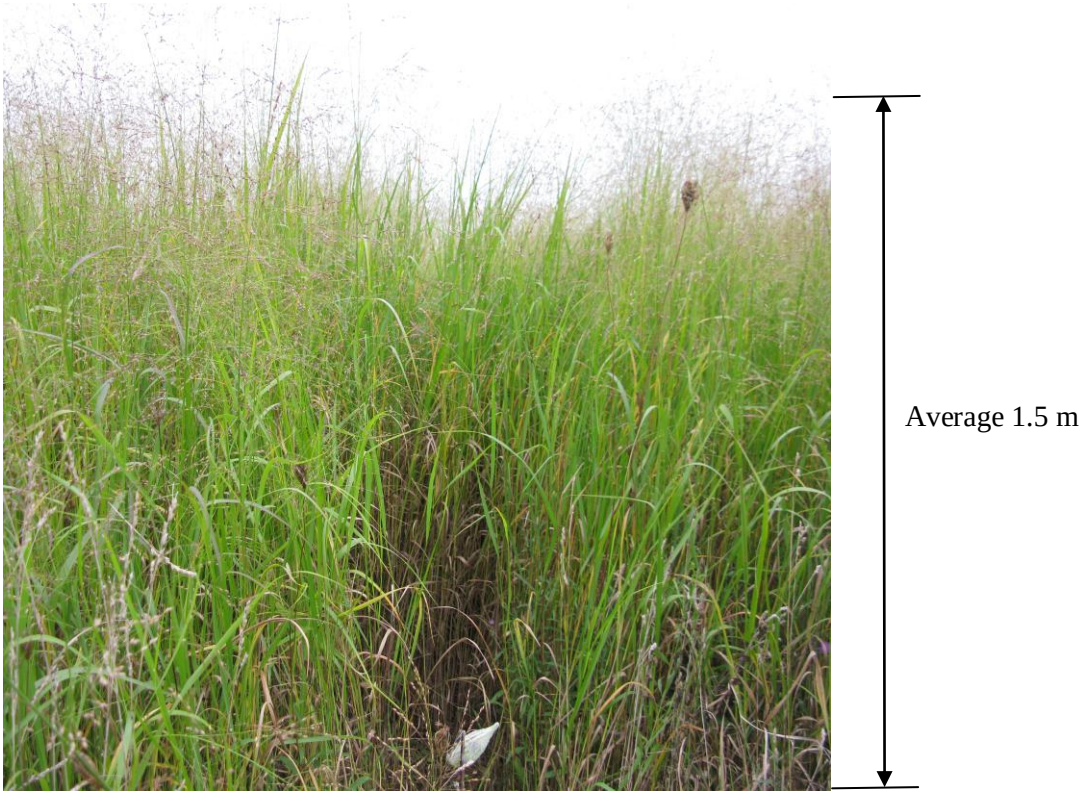

Figure 2-1. Switchgrass harvested on Oct 27th, 2010 at Nott Farms - RR 4, Clinton, ON, Canada.

KH_2PO4, 0.05 g NaBr, 0.025 g H_3BO_3, 0.02 g $SrCl_2$ $6H_2O$, 0.01 g sodium citrate, 2.25 g HEPES, 0.6 mg resazurin and 10 ml trace mineral solution (**Table 2-2**).Yeast extract (0.1% [w/v]) was added when preparing culture for inoculum. For other growth on different substrates, yeast extract was omitted. The soluble carbon sources (0.4% [w/v]) were added after autoclave. The insoluble carbon sources (0.4% [w/v]) were added before autoclave. The basic medium was adjusted to pH 7.0 with 2 M NaOH. The medium was autoclaved at 121 °C for 30 minutes and the bottle was sealed with a rubber stopper and an aluminum cap. After adding 8 mL of 5% (w/v) sugar stock solution (if applicable) and 10 mL vitamin stock solution (**Table 2-3**), the complete medium was degassed and gassed with nitrogen. An 8 mL degassed reductant Na_2S $9H_2O$ (3% [w/v]) were injected into the medium with gas-tight syringes. Then, the medium was incubated at 80 °C until the color of medium changed into colorless. The ratio of a 2% fresh inoculum (v/v) was added into the bottle and incubated at 80 °C.

For culturing *T. maritima*, 1 L medium (modified from Huber *et al*., 1986) contained: 15 g NaCl, 2 g Na_2SO_4, 2 g $MgCl_2$ $6H_2O$, 0.5 g $CaCl_2$ $2H_2O$, 0.25 g $NaHCO_3$, 0.1 g K_2HPO_4, 0.05 g KBr, 0.02 g H_3BO_3, 0.02 g KI, 0.003 g Na_2WO_4 $2H_2O$, 0.002 g $NiCl_2$ $6H_2O$, 5 g $Na_2S_2O_3$, 2.8 ml 15% Cysteine/HCl (w/v), 1 mg resazurin and 10 ml trace mineral solution (**Table 2-2**). Yeast extract (0.1% [w/v]) was added when preparing culture for inoculum. For other growth on different substrates, yeast extract was omitted. The insoluble carbon sources (0.4% [w/v]) were added before autoclaving. The soluble carbon sources (0.4% [w/v]) were added after autoclaving. The basic medium was adjusted to pH 7.0 with 2 M NaOH. The medium was autoclaved at 121 °C for 30 minutes and the bottle was sealed with a rubber stopper and an aluminum cap. After adding 8 mL of 5% (w/v) sugar stock solution (if applicable) and 10 mL vitamin stock solution (**Table 2-3**), the complete medium was degassed and gassed with nitrogen. An 8 mL degassed reductant Na_2S $9H_2O$ (3% [w/v]) were injected into the medium with gas-tight syringes. Then, the medium was incubated at 80 °C until the color of medium changed into colorless. The ratio of a 2% fresh inoculum (v/v) was added into the bottle and incubated at 80 °C.

For culturing *T. neapolitana,* 1 L medium (modified from Childers *et al*., 1992) contained: 10 g NaCl, 0.1 g KCl, 1 g NH_4Cl, 0.2 g $MgCl_2$ $6H_2O$, 1 g $CaCl_2$ $2H_2O$, 0.3 g KH_2PO_4, 0.3 g K_2HPO_4, 2.8 ml 15% Cysteine/HCl (w/v), 8 ml 3% $Na_2S \cdot 9H_2O$ (w/v), 1 mg resazurin and 10 ml trace mineral solution (**Table 2-2**). Yeast extract (0.1% [w/v]) was added when preparing culture for inoculum. For other growth on different substrates, yeast extract was omitted. The soluble carbon sources (0.4% [w/v]) were added after autoclaving. The insoluble carbon sources (0.4% [w/v]) were added before autoclaving. The basic medium was adjusted to pH 7.0 with 2 M NaOH. The medium was autoclaved at 121 °C for 30 minutes and the bottle was sealed with a rubber stopper and an aluminum cap. After adding 8 mL of 5% (w/v) sugar stock solution (if applicable) and 10 mL vitamin stock solution (**Table 2-3**), the complete medium was degassed and gassed with nitrogen. An 8 mL degassed reductant Na_2S $9H_2O$ (3% [w/v]) were injected into the medium with gas-tight syringes. Then, the

Table 2-2. Trace mineral solution.

Substances	**g/L**
Nitrilotriacetic acid	1.5
$MgSO_4 \cdot 7H_2O$	3
NaCl	1
$FeSO_4 \cdot 7H_2O$	0.1
$CoSO_4 \cdot 7H_2O$	0.18
$MnSO_4 \cdot 2H_2O$	0.5
$CaCl_2 \cdot 2H_2O$	0.1
$ZnSO_4 \cdot 7H_2O$	0.18
$CuSO_4 \cdot 5H_2O$	0.01
$KAl(SO_4) \cdot 12H_2O$	0.02
H_3BO_3	0.01
$Na_2MoO_4 \cdot 2H_2O$	0.01
$NiCl_2 \cdot 6H_2O$	0.025
$Na_2SeO_3 \cdot 5H_2O$	0.3 mg

Prepared with the protocol from Takahata (Takahata *et al.*, 2001). All the trace minerals were dissolved in deionized water. The stock solution was stored at 4^o C in darkness.

Table 2-3. Vitamin solution.

Substances	**mg/L**
Biotin	4
Folic acid	4
Pyridoxine-HCl	10
Thiamine-HCl $2H_2O$	10
Riboflavin	10
D-Ca-pantothenate	10
p-aminobenzoic acid	10
Cobalamin	10
Niacin	10
Thioctic acid	10

Prepared with the protocol from Takahata (Takahata *et al*., 2001). Vitamins were dissolved in deionized water and then filtered into autoclaved bottle by using Acrodisc 25 mm syringe filter. The stock solutions were stored at 4 °C in darkness.

medium was incubated at 78 °C until the color of medium changed into colorless. The ratio of a 2% fresh inoculum (v/v) was added into the bottle and incubated at 78 °C.

2.3.2 Procedures for removing carry-over nutrients from inoculum culture

A washing step before inoculation was necessary to minimize the transfer of nutrients to determine the growth of *T. petrophila* on different substrates. Otherwise, the nutrients carried over from the inoculum would support growth, which affects the accuracy of growth on tested substrates.

T. petrophila was grown on 0.4% (w/v) glucose in 50 mL serum bottles at 80^{o} C for 16 hours when the bacterial population was in log phase. Cells were then harvested by centrifuging those serum bottles at 1, 520 x *g* and 4^{o} C for 30 minutes. Subsequently, the serum bottles were carefully removed out of the centrifuge and placed in the fume hood. While constantly gassing with nitrogen at 2 psi, the supernatant was removed by using a 15 cm metal hub needle (Hamilton Company, Reno, NV, USA). A 20 mL of anaerobic growth medium without any carbon nutrients was injected and the bacterial biomass was resuspended and centrifuged again. This washing step was repeated twice. Pellets were then suspended with 2 mL of degassed culture medium without any carbon sources. The 2 mL resuspension was the inoculum for the next generation of growth. Normally, 0.5 mL inoculum from the 2 mL resuspension was added to each 50 mL growth medium to make the initial cell number around 2.4 $x10^{7}$ per ml of culture medium.

During the whole growth process, the cell numbers were monitored by using a Petroff Hausser counting chamber and Nikon Elipse E600 microscope.

2.4 Gene identification

In order to find all the genes coding cellulase, hemicellulases and other enzymes related to cellulose and hemicellulose utilization in *T. petrophila*, gene searching and gene identification were done based on the available genome of *T. petrophila* on the website of National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov).

All the annotated genes coding cellulase, hemicellulase and other enzymes related to cellulose and hemicellulose metabolism were searched by the names of enzymes and species. For the genes without annotations in the genome of *T. petrophila*, the same genes with annotation in other *Thermotoga* species were searched and then their gene sequences were compared to the whole genome of *T. petrophila* by using a "BLAST" function on NCBI. Genes in *T. petrophila* demonstrated high homology (above 80%) with the ones in other *Thermotoga* species were considered to encode the same enzymes. This method revealed all the unannotated genes encoding cellulase, hemicellulase and relative enzymes in the genome of *T. petrophila*. The genetic relationship of those enzymes between *T. petrophila* and other *Thermotoga* species were determined by the value of gene identity.

2.5 Determination of cellulase and hemicellulase activities of *T. petrophila*

The aromatic compound 3, 5-Dinitrosalicylic acid (DNS or DNSA) reacts with reducing sugars and other reducing molecules to form 3-amino-5-nitrosalicylic acid, which absorbs light strongly at 540 nm. The enzyme assay used in this project was modified from the DNS method of Wood (Wood *et al*., 1988). The final concentration of DNS in the reaction mixture was increased for the higher sensitivity of detection. Cellulase and xylanase activities were determined by DNS method to quantify the reduced sugar produced from cellulosic materials after the growth of *T. petrophila*.

In order to explore the mechanism of utilizing inaccessible cellulosic materials by *T. petrophila*, cellulase and hemicellulase activities were both measured in the cell free extract and culture supernatant. Cellulases and hemicellulases were determined as intracellular or extracellular enzymes from the same batch sample by using the DNS enzyme assay.

2.5.1 Preparation of cell free extract

When the bacterial population entered late log phase, cells were harvested by centrifuging 450 mL culture medium at 5, 000 x *g* and 4^{o} C for 30 minutes. The pellet was collected for making cell free extract. An eppendorf tube was placed into a 10 mL serum bottle with the lid cut off the tube and fixed at the bottom of the bottle with adhesive tape. A small stir bar was placed in the eppendorf tube. The bottle was sealed with a rubber stopper and a metal cap and then degased for 40 minutes with nitrogen by using an oil pump and manifold system. After 40 minutes of degasing, the bottle was opened and weighed for about 0.2 g of pellet into the eppendorf tube in the serum bottle. The bottle was resealed and degased for another 5 minutes using oil pump and pressurized for 5 minutes with 2 psi nitrogen. Subsequently, the bottle was removed from the manifold. An 800 µL EPPS buffer solution (100 mM, pH 8.6) was anaerobically added to the bottle using a 1 mL gas tight syringe (Hamilton Company, Reno, NV, USA). The bottle was placed on the stir machine until the homogenous suspensions of the cells were obtained. Then, DNase I solution (final concentration 0.01 mg/mL) and lysozyme solution (final concentration 0. 1 mg/mL) were anaerobically injected to the cell suspension. The bottle was incubated at 37^{o} C for 30 minutes and then centrifuged at 4, 000 x *g* and 4^{o} C for 5 minutes. The supernatant in this bottle was the cell free extract used for intracellular enzyme assays.

2.5.2 Preparation of concentrated supernatant from culture medium

In order to determine cellulase and hemicellulase activities in the culture supernatant and compare them to the ones in cell free extract, the supernatant from culture medium was concentrated by using Centricon Plus-70 (Millipore Corporation, Billerica, MA, USA) with 5 kDa pore size. The concentration step was operated according to the product manual of Centricon Plus-70 aerobically for about two hours.

2.5.3 Standard curve of DNS (3, 5-Dinitrosalicylic acid) method

A series of concentrations (0 mM, 2 mM, 4 mM, 6 mM and 8 mM) of glucose and xylose solutions were prepared, respectively. A 500 µL of 1% (w/v) DNS solution with 0.4 M NaOH, 500 µL of each different concentration of glucose or xylose solution were mixed together (Wood *et al*., 1988). The reaction mixture was incubated at 95^{o}C for 15 minutes and cooled to room temperature after incubation. Finally, the absorbances were measured at 540 nm and plotted in excel (Y is absorbance value, X is the concentration of glucose or xylose) to get the standard curves of glucose and xylose, separately.

2.5.4 Cellulase and hemicellulase enzyme assay

Different substrates were used in the assay for determining different enzyme activities. Carboxymethylcellulose (CMC) is a cellulose derivative with carboxymethyl groups (-CH_2-COOH) bounded to some of the hydroxyl groups of the glucopyranose monomers that make up the cellulose backbone. Since CMC is modified and soluble cellulose, it is used to detect endo-1, 4-β-glucanase activity. Microcrystalline cellulose avicel PH105 is used to detect exoglucanase. Filter paper and switchgrass are used to detect the complete cellulase activity (Wood *et al*., 1988). Xylan is used to detect xylanase activity (Ghose *et al*., 1987).

A mixture of 800 µL 0.1 M sodium citrate buffer (pH 7.5), 0.5 mL enzyme sample (cell free extract or supernatant) and 1% (w/v) of each insoluble substrate (switchgrass, avicel PH105, filter paper or xylan) or 0.5% (w/v) soluble substrate CMC were mixed together. The reaction mixture with a total volume of 1.3 mL was incubated at 80^{o} C for 15 minutes, 30 minutes and 1 hour, respectively. After the enzyme reaction was done, a mixture of 500 µL of 1% (w/v) DNS solution with 0.4 M NaOH and 500 µL of reaction mixture was gently mixed and incubated at 95^{o}C for 15 minutes. The mixture was then centrifuged at 5, 520 x *g* for 5 minutes at room temperature. The supernatant was transferred into the cuvette and the absorbance was measured at 540 nm. Based on the enzyme activities within different reaction times, the time with the highest specific activity was chosen as the optimal reaction time for all the enzyme assays.

The amount of glucose or xylose released from cellulose or hemicellulose was quantified by using the standard curves of glucose and xylose (section 2.5.3). One unit of enzyme corresponded to the release of 1 µmol of glucose or xylose per minute. Protein concentration was determined by using the Bradford method with bovine serum albumin (BSA) as standard (Bradford, 1976).

2.6 Attachment of *T. petrophila* cells to cellulosic materials

2.6.1 DAPI and PI fluorescent staining

The florescent stain 4', 6'-diamidino-2-phenylindole (DAPI) binds strongly to A-T rich regions of the DNA. DAPI can pass through a bacterial intact cell membrane; therefore, it can be used to stain both live and fixed cells (Coleman *et al*., 1981). Propidium iodide (PI) is both an intercalating agent and a fluorescent molecule that appears red under the UV light in the range between 562 nm and 588 nm (Nicoletti *et al*., 1991). PI is unable to stain viable cells because it cannot penetrate the cell membranes of live cells. However, PI is able to stain dying or dead cells because it is capable of penetrating their damaged membranes (Nicoletti *et al*., 1991). Based on the characteristics of DAPI and PI, DAPI were used to stain both viable and dead cells, and PI was used to stain the dead cells. Viable cells with DAPI staining appeared blue under a UV filter or green under a B_2 filter. On the other hand, dead cells appeared red with PI staining under a green filter. Furthermore, both DAPI and PI stained on xylan with the same color of cells under different filters (Chen and Ma, unpublished data, 2011).

2.6.1.1 Filtration treatment of culture medium before DAPI and PI staining

By directly using culture medium with cells and insoluble substrates for fluorescent staining, the cells that appeared to have associated with the substrates may not be credible since some of the cells attached on insoluble substrates may be due to the fact that the cells adhered to the insoluble substrate by centrifugation during the staining procedure. Therefore, a filtration treatment of culture medium was operated to validate the attachment. The culture medium was filtered by passing through Whatman Grade 1 filter paper of 11 μm pore size. The free cells and attached cells with insoluble substrates were separated.

Firstly, a vacuum system was connected with a funnel which was installed to a Whatman Grade 1 filter paper with an 11 μm pore size. The vacuum was then turned on and culture was poured through the filter paper in the funnel. All the particles larger than 11 μm were accumulated on the filter paper and other finer particles smaller than 11 μm passed through. After all the culture media was filtered, about 10 mL of 1x phosphate buffered saline (PBS) buffer at pH 7.2 (**Table 2-4**) was poured to wash the filter paper to make sure there were no free cells attached on the filter paper. The washing step was repeated three times. The filter paper was then taken out from the funnel and gently dipped into 1 mL 1x PBS buffer. This 1 mL suspension was stained by DAPI and PI for attachment observation.

Table 2-4. Solutions prepared for DAPI and PI staining.

	Chemicals	Amount	Final concentration
PBS pH 7.2-7.4 (1 L)	NaH_2PO_4 (anhydrous)	0.23 g	1.9 mM
	Na_2HPO_4 (anhydrous)	1.15 g	8.1 mM
	NaCl	9 g	154 mM
Buffered Glycerol pH 8.8 (1 L)	Tris	2.422 g	20 mM
	Glycerol	900 mL	90% (v/v)

2.6.1.2 DAPI and PI staining

DAPI and PI staining was operated according to the protocol from Susanne Vesely in the Department of Biology of University of Waterloo (Coleman *et al*., 1981).

A 1 mL portion of the non-filtered culture medium or filtered culture medium (prepared according to the method described in section 2.4.1.1) was taken into a 1.5 mL eppendorf tube. The medium was then centrifuged at room temperature and 14, 000 x *g* for 30 seconds. The pellet was washed with 1 mL of 1x PBS (the recipe refers to **Table 2-4**) and then was centrifuged at 14, 000 x *g* for 30 seconds at room temperature. After removing the supernatant, a 300 μL of 5% (v/v) glutaraldehyde was added to resuspend the pellet and then incubated at room temperature for 30 minutes. Subsequently, the tube containing the pellet and glutaraldehyde was centrifuged at 14, 000 x *g* for 30 seconds at room temperature and the supernatant was discarded. The pellet was washed with 1 mL of 1x PBS. Another 50 μL of DAPI (10 μg/mL) was added and the pellet was resuspended again. After that, the eppendorf tube with the suspension was incubated for 10 minutes in darkness. After incubation, the eppendorf tube was centrifuged at 14, 000 x *g* for 30 seconds at room temperature and the supernatant was discarded. The pellet was washed with 1 mL of sterile dH_2O. Subsequently, 10 μL of 0.5 mg/mL PI and 90 μL of sterile dH_2O was added to the pellet and resuspended. The sample was incubated in the dark at room temperature for 5 to 10 minutes. It was then centrifuged at 14, 000 x *g* for 30 seconds at room temperature and the supernatant was discarded. T, the pellet was resuspended in 500 μL (or slightly less) of 0.5-5 mg/mL p-phenylenediamine in buffered glycerol (the recipe refers to **Table 2-4**). Finally, 5 μL of the suspension was added onto the glass slide. The slide was covered with a coverslip and sealed by transparent nail polish to prevent the sample from drying out. The slide was then observed using a Nikon microscope Labophot-2 with a Nikon mercury lamp.

2.6.2 Scanning electron microscope

Scanning electron microscope (SEM) is a high magnification microscope that is capable of making observations and characterizations of heterogeneous organic and inorganic materials on a nanometer (nm) to micrometer (μm) scale. One of its key features is its ability to construct a three-dimensional image of a very wide variety of materials. In the SEM, the specific area to be examined is irradiated with a finely focused electron beam, which may be swept in a raster across the surface of the specimen to form images or may be static to obtain an analysis at one position (Goldstein *et al*., 2003).

In this project, SEM was used to observe the attachment between *T. petrophila* cells and cellulosic substrates. The sample for SEM was prepared according to the protocol from Dale Weber in the Department of Biology of University of Waterloo (personal communication).

Firstly, the cells were rinsed with BP buffer (0.2 M sodium phosphate buffer pH 6.0) three times gently in order to separate them from the medium. The cells were then fixed by immersing them in BP buffer (pH 6.0) with 2.5% (v/v) glutaraldehyde at room temperature for one hour. The cells were washed with BP buffer three times and each time the cells were submerged in BP buffer for 20 minutes. The cells were then rinsed with 20% (v/v) ethanol once followed with 50% (v/v) ethanol once, 70% (v/v) ethanol twice, 95% (v/v) ethanol once and 100% (v/v) ethanol twice. Each time the sample was kept in ethanol solution at room temperature for 10 minutes. Then, the sample was dried by using dry ice (solid carbon dioxide) as a transition fluid. Then a sticky plastic membrane was placed on a tub using a gripper, and the sample (powder) was subsequently placed on the membrane for observation. For the observation under SEM, the specimen was required to be electrically conductive, at least at the surface, and electrically grounded to prevent the accumulation of electrostatic charge at the surface. Therefore, the tub with target materials was coated with sputter ultra-thin gold under vacuum evaporation. SEM Hitachi s-570 in the Department of Biology of the University of Waterloo was used in this experiment.

2.7 Determining of metabolic end products of *T. petrophila* by gas chromatography (GC) and high performance liquid chromatography (HPLC)

2.7.1 Gas chromatography (GC)

Gas chromatography (GC) is a common method used in biochemistry and analytic chemistry for separating and analyzing compounds. The metabolic products in the gas phase from the growth of *T. petrophila* were detected by GC. In this experiment, Shimadzu GC-2014 was used with Porapak Q 80/100 column (6' x 1/8" x .085" SS, Part No. #2701PC, Grace Davison Discovery Sciences, Deerfield, IL, USA). A 100 µL gas tight syringe (Cat No. #1710, Hamilton Company, Reno, NV, USA) was used for sample injection. The major parameters for the GC measurements are listed in **Table 2-5**.

The standard curves of CO_2 and H_2 were determined by measuring a series concentrations (0.01%, 0.1%, 1%, 2%, 20% [v/v]) of CO_2 and H_2, separately. Both standard curves of CO_2 and H_2 were integrated into the GC methods program, which would quantify CO_2 and H_2 produced from the growth of *T. petrophila*.

2.7.2 High performance liquid chromatography (HPLC)

Shimadzu HPLC with Alltech IOA-1000 organic acids column (300 nm x 7.8mm, Part No. 9646, Grace Davison Discovery Sciences, Deerfield, IL, USA) was used for the determination of metabolic

Table 2-5. Parameter settings for GC measurement.

Parameters	Value
Oven	40^{o}C
DINJ1	140^{o}C
DTCD1	140^{o}C
FID	140^{o}C
N_2 carrier gas	10 mL/min
Methanizer Temperature	380^{o}C
Running time	10 minutes

products in the liquid phase from the growth of *T. petrophila*. Some 9 mm auto sample vials (Cat No. 022410) and 400 μL (Cat No. 042111) inserts from Microliter Analytical Supplies, Inc. (Suwanee, GA, USA) were used for loading liquid samples. The major parameter settings for HPLC measurements are listed in **Table 2-6**.

The standard curves of the organic components were determined by measuring a series of master mixture with various concentrations (0.1 mM, 0.2 mM, 0.5 mM, 1 mM, 2 mM, 5 mM and 50 mM) of each organic component. The master mixture included cellobiose, glucose, xylose, arabinose, succinate, lactate, formate, acetate, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol and butyric acid. A 20 μL of each concentration of master mixture was injected for HPLC measurement. Finally, all the standard curves of the components were integrated into the HPLC methods program, which would quantify all the tested components in the unknown samples.

2.8 Quantitative and qualitative analysis of expressed proteins in *T. petrophila*

2.8.1 Proteomics analysis to qualify all the expressed proteins

Proteomics analysis on *T. petrophila* biomass quantified all the intracellular enzymes. The certain enzymes overexpressed from certain substrates were the crucial enzymes in the metabolism of that substrate. Those enzymes could be induced by certain substrates for increasing the efficiency of substrate utilization. In this experiment, cellobiose, switchgrass and xylan were used as substrates to grow *T. petrophila* biomass. Cellobiose was used as the control. All the expression levels of the enzymes from switchgrass and xylan media were compared to the expression level of the same enzymes from cellobiose medium. By using proteomics analysis, the most important cellulases and hemicellulases were found, which would be very helpful for producing recombinant cellulases and hemicellulases for commercial use. All the experiment protocols refer to Dwivedi (Dwivedi *et al*., 2008).

The protein extraction was done in our laboratory in the University of Waterloo. Other steps, including protein digestion, purification and 2D-Liquid chromatograph-mass spectrometry analysis (2D-HPLC-MS), were performed by collaborator Dr. Oleg V. Krokhin at the University of Manitoba.

2.8.1.1 Protein extraction

First, the cells were harvested by centrifuging a 500 mL medium at 4^{o}C and 6, 000 x *g* for 30 minutes. After 30 minutes, the cells were washed with 1x BPS buffer (the recipe refers to **Table 2-7**) three times. Every time after washing, the cells were centrifuged at 6, 000 x *g* and 4^{o}C for 30 minutes. The pellet was resuspended in a total of 600 μL of lysis buffer (the recipe refers to **Table 2-7**). The mixture was sonicated with 5 voltages for 5 rounds, each round lasting 15 seconds. Between

Table 2-6. Parameter settings for HPLC measurement.

Detector	**RID detector**
Solvent	0.0085 M H_2SO_4
Pump A pressure profile	Max: 1000 psi; Min: 0 psi
Flow rate	0.4 mL/min
Temperature	60^{o} C
Running time	60 minutes
Inject volume	20 μL

Table 2-7 Composition of PBS and lysis buffer.

	Chemicals	**Final concentration**
10x PBS pH 7.4	NaCl	80 g/L
	KCl	2 g/L
	Na_2HPO_4	14.4 g/L
	KH_2PO_4	2.4 g/L
Lysis buffer pH 7.4	Tris-HCl	10 mM
	$CaCl_2$	3 mM
	$MgCl_2$	2 mM
	0.5% (v/v) bacterial protease inhibitor	0.0025% (v/v)
	Igepal	0.1% (w/v)

rounds, the sample was cooled on ice. Subsequently, cell debris was removed by centrifugation at 4^{o} C and 14, 000 x *g* for 10 minutes and the supernatant was collected. The protein concentration in the supernatant was measured by the Bradford method with BSA as standard (Bradford, 1976). The protein sample was kept at -80^{o} C until the sample was ready for protein digestion.

2.8.1.2 Protein digestion and purification

A 100 mM ammonium bicarbonate and 10 µL of 100 mM freshly prepared solution of dithiothreitol (DTT) were mixed with 100 µg proteins from the protein extraction to make the final volume 100 µL. The mixture of protein sample was then incubated at around 57^{o} C for 30 minutes, and then brought to room temperature. A10 µL volume of 500 mM solution of iodoacetamide was added into 100 mM ammonium bicarbonate for 45 minutes of incubation at room temperature in the dark. Around 16 µL of 100 mM DTT was added to quench excess iodoacetamide. Another 65 µL of ammonium bicarbonate was added to make the final volume around 200 µL. An aliquot of 1-2 µg of trypsin (Sequencing grade, Promega) was added into the 200 µL mixture to make enzyme/substrate ratio around 1/100-1/50. The sample was incubated at 37^{o} C for 10 hours or overnight. Finally, the solution was acidified with a small volume of trifluoroacetic acid (TFA). Proteins were then digested by trypsin. This step was performed by collaborator Dr. Oleg V. Krokhin at the University of Manitoba.

2.8.1.3 2D-Liquid chromatograph-mass spectrometry analysis (2D-HPLC-MS)

2D-Liquid chromatograph-mass spectrometry analysis (2D-HPLC-MS) is an analytical chemistry technique which combines the physical separation capability of two dimensional high performance liquid chromatography (2D-HPLC) with mass analysis capabilities of mass spectrometry (MS). It is a prominent technique to identify proteins from complex proteome samples in many laboratories. Normally, the strong cation exchange chromatography is used for the first dimension, and the reversed phase separation is used for the second dimension. For better sensitivity, the reversed phase separation is usually operated in the nanoflow scale and mass spectrometry is utilized as the final step for detection. This step was performed by collaborator Dr. Oleg V. Krokhin at the University of Manitoba.

The method for 2D-HPLC-MS used for proteomics analysis in this experiment is illustrated generally in **Figure 2-2**. Firstly, the peptide sequence retention data sets for model optimization were collected by using micro LC-MALDI MS. The known amount of testing protein samples were reduced (DTT), alkylated (iodoacetamide), and trypsin digested. The 1x100 mm XTerra column with an Agilent 1100 Series HPLC system was used for the first-dimension HPLC separation. UV detection at 214 nm was used to monitor the elution profile. A splitless nanoflow Temp LC system (Eksigent, Dublin, CA) with 300 µm x 5 mm PepMap100 precolumn and a 100 µm x 150 mm

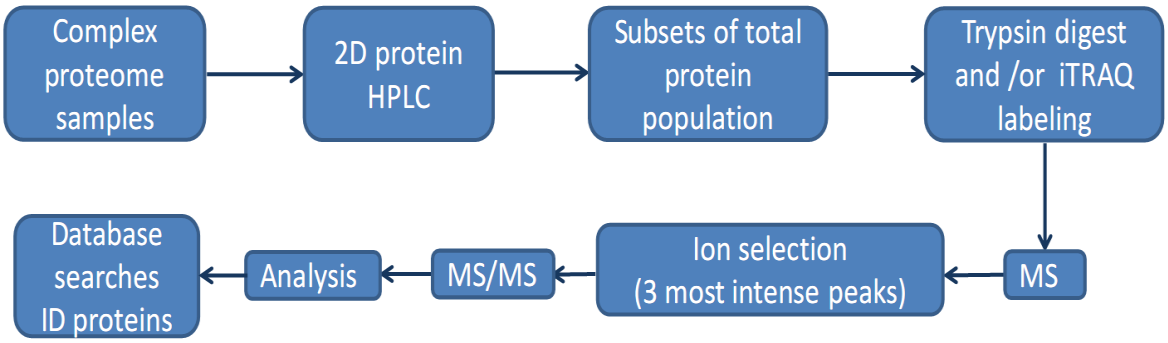

Figure 2-2. Outline of 2D-HPLC-MS method. 2D-HPLC-MS was performed by the collaborator Dr. Oleg V. Krokhin at the University of Manitoba. The method of 2D-HPLC-MS refers to Dwivedi (Dwivedi *et al*., 2008).

analytical column packed with 5 μm Luna C18 (Phenomenex, Torrance, CA) were used in the second-dimension separation prior to MS analysis. After two dimensional LC separations, a QStar Elite mass spectrometer (Applied Biosystems, Foster City, CA) was used in standard MS/MS data-dependent acquisition mode. More details about the experimental procedure refer to Dwivedi (Dwivedi *et al*., 2008).

Three protein samples from the growth culture of *T. petrophila* were tested by proteomics analysis. They were prepared from the biomasses of *T. petrophila* grown on 0.4% (w/v) of cellobiose, xylan and switchgrass, respectively. Cellobiose was used as the control. All the expression values of enzymes from *T. petrophila* grown on xylan and switchgrass were relative values and deducted from the expression values of the control. Therefore, all the positive values in xylan and switchgrass samples indicate that the corresponding enzymes were up-regulated compared to the cellobiose sample. In contrast, all the negative values indicate that the corresponding enzymes were down-regulated compared to the cellobiose sample. The values larger than 1.9 or lower than -1.9 represented significant up or down-regulation.

2.8.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a technique widely used in biochemistry and molecular biology to separate proteins based on their electrophoretic mobility. Each protein has identical charge per unit mass which results in different migrating speeds while moving in the SDS gel.

A separating gel (12.5%) and stacking gel (5%) were prepared following the protocol of Weber (Weber *et al*., 1969). a Hoefer Dural Gel Caster SE 245 (Hoefer, Inc., San Francisco, CA, USA) was used for making the gels. Each gel was run under 25 mA constant current with the supernatant proteins and cell free extract proteins from the same batch for 1-1.5 hours by using Hoefer mini-vertical SDS gel electrophoresis unit. The BLUeye Prestained protein ladder (FroggaBio, Inc., Toronto, ON, Canada) was used to determine the size of unknown protein proteins. After staining for 4 hours (or overnight) with the staining solution (0.3 mM Coomassie Blue R-250, 20% [v/v] methanol. 10% [v/v] glacial acetic acid), the gel was destained with the destaining solution (20%[v/v] methanol, 10% [v/v] glacial acetic acid). The gel was scanned with HP scanner (Precision Scanner 5000C) and the scanned image was further saved as bmp files. The molecular weight of each protein was calculated by using the standard curve of the commercial protein ladder plotted in a semi-log graph.

2.9 Metabolic profiling of the cellulose utilization by *T. petrophila*

According to the major carbohydrate pathways, including the Entner-Doudoroff pathway (ED pathway), glycolysis pathway and pentose phosphate pathway (PPP), a tentative scheme of the metabolic pathway was made based on the available genome sequences, proteomics analysis and all the end products detected from GC and HPLC. Firstly, based on the end products determination, all the carbohydrate pathways involved in the metabolism for producing those end products were tentatively identified. All the enzymes involved in each step of the metabolism scheme were filled in the profiling with relative expression values from proteomics data by using method 2.6.1. All the locus tags of the *T. petrophila* genes coding those enzymes by using method 2.2 were also integrated in the metabolic profiling.

The metabolic profiling shows a detailed explanation of cellulosic material utilization. It shows the overexpressed enzymes from cellulose and hemicellulose to simple sugars and all other end products in *T. petrophila*. Those enzymes are the crucial enzymes in the metabolism of cellulosic materials.

Chapter 3
Results

3.1 Identification of cellulase and xylanase genes in the genome of *T. petrophila*

3.1.1 Cellulase genes of *T. petrophila*

In order to determine if *T. petrophila* contained all the cellulase and hemicellulase genes in its genome, all the cellulase genes including exoglucanase, endoglucanase and β-glucosidase genes in *T. petrophila* were searched by using the method described in section 2.4. There were four endoglucanase genes in *T. petrophila*, six endoglucanase genes in *T. maritima* and three endoglucanase genes in *T. neapolitana* (**Table 3-1**).

There were three β-glucosidase genes in *T. petrophila, T. maritima* and *T. neapolitana,* respectively (**Table 3-1**). *T. petrophila* endoglucanase gene showed higher identities to *T. maritima* (95%-99%) than *T. neapolitana* (80%-84%). There were no exoglucanase genes found in *T. petrophila* and other *Thermotoga* species.

3.1.2 Xylanase genes of *T. petrophila*

Xylanase genes including endo-1, 4-β-xylanase, β-xylosidase, α-N-arabinofuranosidase and acetyl xylan esterase were searched in the genome of *T. petrophila* by using the method described in section 2.4. From the results shown in **Table 3-2**, *T. petrophila* contained two endo-1, 4-xylanase genes, one β-glucosidase gene, α-N-arabinofuranosidase gene and acetyl xylan esterase gene. *T. petrophila* genes showed higher sequence identities to *T. maritima* (96%-99%) than *T. neapolitana* (80%-85%).

3.2 Growth of *T. petrophila* on cellulose and hemicellulose

In order to determine *T. petrophila*'s ability to utilize cellulose and hemicellulose, different types of cellulosic materials, including xylan, avicel PH105, switchgrass, wheat straw and corn husks, were used as substrates for growth. All the substrates were described in section 2.1. The growth medium was prepared by using the method described in section 2.3.

T. petrophila was observed to be a rod shape, occurring singly and in pairs, about 2 to 10 μm long and 0.5 μm wide. Very rarely, up to four cells were enclosed in one sheath. From the optimal

Table 3-1. Homolog genes encoding cellulases present in *T. petrophila, T. maritima* and *T. neapolitana.*

	T. petrophila **(Locus Tag)**	***T. maritima*** **MSB8 (Locus Tag)**	***T. neapolitana*** **DSM 4359 (Locus Tag)**	**Gene Identities**	
				T. petrophila **and** ***T. maritima*** **MSB8**	***T. petrophila*** **and** ***T. neapolitana*** **DSM 4359**
Endoglucanase	Tpet_1703	TM1048	CTN_1519	90%	74%
	Tpet_1702	TM1049	CTN_1518	93%	75%
	Tpet_1701	TM1050	CTN_1517	93%	79%
	Tpet_1268	TM1524	-	96%	-
	-	TM1751	-	-	-
	-	TM1752	-	-	-
β-glucosidase	Tpet_0898	TM0025	CTN_0670	95%	80%
	Tpet_0848	TM0076	CTN_0616	97%	84%
	Tpet_0952	X74163 (Gene Accession No.)	CTN_0782	99%	83%

-means no gene was found. Gene searching and gene identification were done based on the available genome of *T. petrophila* on the website of National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The values of gene identities reveal the genetic relationship between *T. petrophila* and other *Thermotoga* species.

Table 3-2. Homolog genes encoding xylanases and relative enzymes in *T. petrophila, T. maritima* and *T. neapolitana.*

	T. petrophila **(Locus Tag)**	***T. maritima*** **MSB8 (Locus Tag)**	***T. neapolitana*** **DSM 4359 (Locus Tag)**	**Gene Identities**	
				T. petrophila **and** ***T. maritima*** **MSB8**	***T. petrophila*** **and** ***T. neapolitana*** **DSM 4359**
Endo-1,4-β-xylanase	Tpet_0863	TM0061	CTN_0632	97%	85%
	Tpet_0854	TM0070	CTN_0623	98%	81%
β- xylosidase	Tpet_0848	TM0076	CTN_0616	96%	84%
α-N- arabinofuranosidase	Tpet_0631	TM0281	CTN_0403	99%	86%
Acetyl xylan esterase	Tpet_0847	TM0077	CTN_0615	97%	82%

Gene searching and gene identification were done based on the available genome of *T. petrophila* on the website of National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The values of gene identities reveal the genetic relationship between *T. petrophila* and other *Thermotoga* species.

growth condition (**Table 3-3** and **Figure 3-1**), the best growth of *T. petrophila* was on cellobiose, followed by xylan, corn husks, glucose, switchgrass, avicel PH105 and wheat straw. From the generation time and specific growth rate constant in **Table 3-3**, the best growth of *T. petrophila* was on cellobiose, followed by xylan, avicel PH105, glucose, switchgrass, corn husks and wheat straw. *T. petrophila* did not grow on xylose, although it had all the genes involved in the xylose degradation, including xylose isomerase, xylulokinase, transaldolase, transketolase and L-ribulose-5-4 phosphate 4-epimerase (**Table 3-4**). *T. petrophila* also contained oligopeptide ATP binding cassette (ABC) transporters, which were candidate transporters for xylose transportation (Conner *et al*., 2005). But *T. petrophila* did not have the sugar ABC transporters that exist in *T. maritima*, which can grow on xylose (Huber *et al.*, 1986). This finding indicated a possible reason why *T. petrophila* cannot digest xylose.

3.3 Enzyme activities of the cellulase and xylanase of *T. petrophila*

3.3.1 DNS standard curves

In order to determine *T. petrophila*'s ability to utilize cellulose and hemicellulose, cellulase and hemicellulase activities were determined by using the method described in section 2.5. DNS standard curves were done by using the method described in section 2.5.3. The standard curves of glucose and xylose are shown in **Figure 3-2**.

3.3.2 Cellulase activities of *T. petrophila* grown on various substrates.

In order to compare the cellulase activities in *T. petrophila*, *T. neapolitana* and *T. maritima*, cell free extracts from the three microorganisms were prepared by using the method described in section 2.5.1. All the cell free extracts were obtained from biomass grown on 0.4% (w/v) glucose. The enzyme assays were done by using the method described in section 2.5.4. All the assays were tested at an optimum temperature of 80^{o} C. With 30 minutes incubation, all the protein samples showed their highest specific cellulase activity. Therefore, a period of 30 minutes was chosen as the optimal reaction time.

From the results in **Figure 3-3**, *T. neapolitana* grown on glucose showed the highest endoglucanase specific activity (43 mU/mg) and presented slightly lower endoglucanase specific activity (39.13 mU/mg) than *T. neapolitana*, but much higher activity than *T. maritima* (13.5 mU/mg). *T. neapolitana* and *T. petrophila* had very close exoglucanase specific activity (23.7 mU/mg and 22 mU/mg, respectively) which were both higher than *T. maritima* (9.9 mU/mg). The complete cellulase specific activities in *T. petrophila* (12.9 mU/mg and 8.1 mU/mg) were the highest among the three tested species.

Table 3-3. Growth of *T. petrophila* on 0.4% (w/v) of various substrates at 80° C and pH 7.0.

Time (h)	Growth (cell numbers/mL)								
	Control[a]	Xylose	Glucose	Cellobiose	Xylan	Avicel PH105	Switchgrass	Wheat straw	Corn husks
0	2.4×10^7	2.2×10^7	2.5×10^7	2.0×10^7	1.7×10^7	1.7×10^7	2.1×10^7	2.2×10^7	2.1×10^7
5	2.1×10^7	1.8×10^7	5.3×10^7	4.9×10^7	4.6×10^7	3.0×10^7	4.6×10^7	4.1×10^7	4.8×10^7
19	1.1×10^7	1.3×10^7	9.4×10^7	2.6×10^8	9.4×10^7	6.1×10^7	6.4×10^7	4.8×10^7	9.4×10^7
25	1.3×10^7	9.2×10^6	1.1×10^8	3.2×10^8	1.3×10^8	8.0×10^7	7.9×10^7	5.6×10^7	1.3×10^8
44	1.5×10^7	8.7×10^6	7.9×10^7	1.7×10^8	8.2×10^7	5.8×10^7	4.9×10^7	3.1×10^7	1.0×10^8
Generation time (hour)	No growth	No growth	16.9	5.9	13.6	13.6	28.0	60.0	47.9
***k* (specific growth rate constant, h^{-1})**	N/A	N/A	0.04	0.12	0.05	0.05	0.02	0.01	0.01

[a]Control was the medium without any carbon sources. N/A means not applicable. Xylose, glucose and cellobiose were used as substrates to compare the growth on cellulosic materials including xylan, avicel PH105, switchgrass, wheat straw and corn husks. All the details of substrates were described in section 2.1. The growth medium was prepared by using the method described in section 2.3.

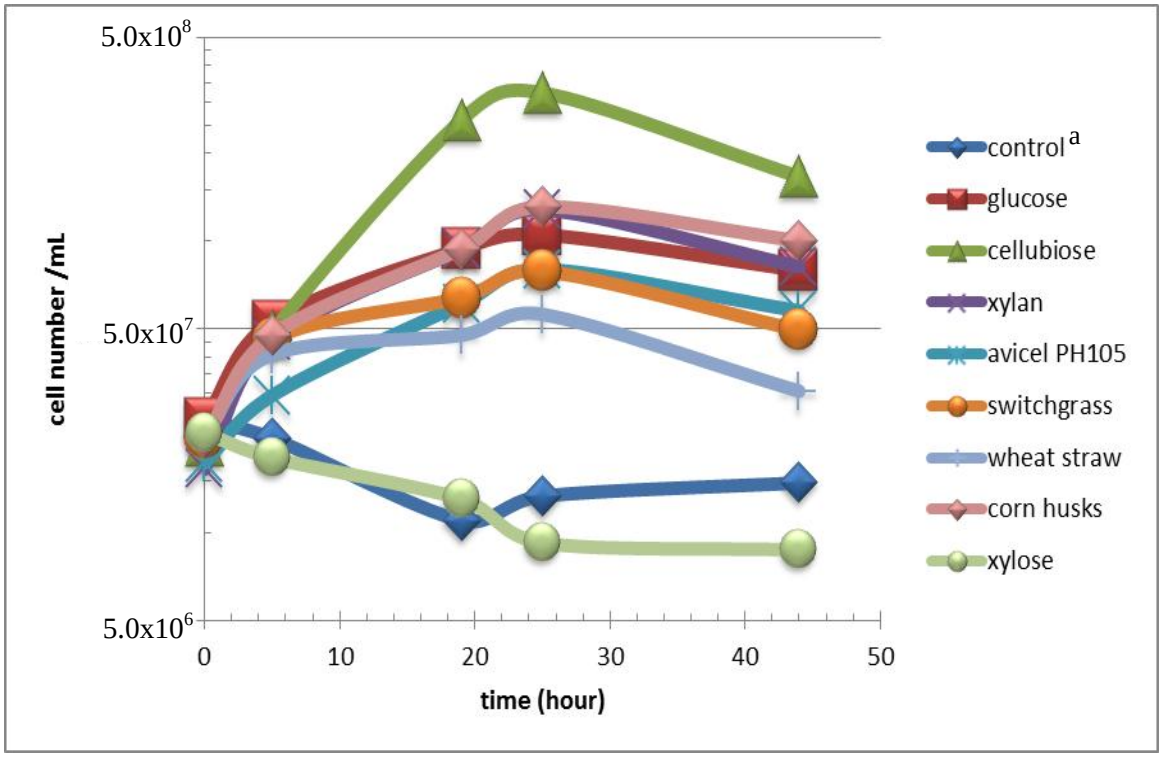

Figure 3-1. Growth of *T. petrophila* on 0.4% (w/v) of various substrates at 80° C and pH 7.0. [a]Control was the medium without any carbon sources. Xylose (green dot), glucose (red square) and cellobiose (green triangle) were used as substrates to compare the growth on cellulosic materials including xylan (purple cross), avicel PH105 (blue asterisk), switchgrass (orange dot), wheat straw (light blue line) and corn husks (pink diamond). All the details of substrates were described in section 2.1. The growth medium was prepared by using the method described in section 2.3.

Table 3-4. All the genes related to metabolism of xylose in the genome of *T. petrophila*, *T. maritima* and *T. neapolitana*.

	Proteins	*T. petrophila* Locus Tag	*T. maritima* MSB8 Locus Tag	*T. neapolitana* DSM 4359 Locus Tag
Oligopeptide ABC family cluster 1	periplasmic oligopeptide-binding protein	Tpet_0853	TM0071	CTN_0622
	permease protein	Tpet_0852, Tpet_0851	TM0072, TM0073	CTN_0621, CTN_0620
	ATP-binding protein	Tpet_0488, Tpet_0489, Tpet_0849, Tpet_0850	TM0074, TM0075	CTN_0619, CTN_0618
Oligopeptide ABC family cluster 2	periplasmic oligopeptide-binding protein	Tpet_0868	TM0309	CTN_0638
	permease protein	Tpet_0865, Tpet_0864	TM0059, TM0060	CTN_0635, CTN_0634
	ATP-binding protein	Tpet_0867, Tpet_0866	TM0057	CTN_0636
Sugar ABC family		N/A	TM0955, TM0956, TM0958, TM0959	CTN_1621, CTN_1620, CTN_1618, CTN_1617
Related enzymes for xylose metabolism	xylose isomerase	Tpet_1124, Tpet_0504, Tpet_0140, Tpet_0499	TM1667, TM0414, TM0788, TM0423	CTN_0719, CTN_0253, CTN_1788, CTN_0427
	xylulokinase	Tpet_0808	TM0115	CTN_0574
	transaldolase	Tpet_0617	TM0295	CTN_0388
	transketolase	Tpet_1050	TM1762, TM0953,	CTN_0952, CTN_1623
	L-ribulose-5-4 phosphate 4-epimerase	Tpet_0629	TM0283	CTN_0401

N/A means no gene was found.

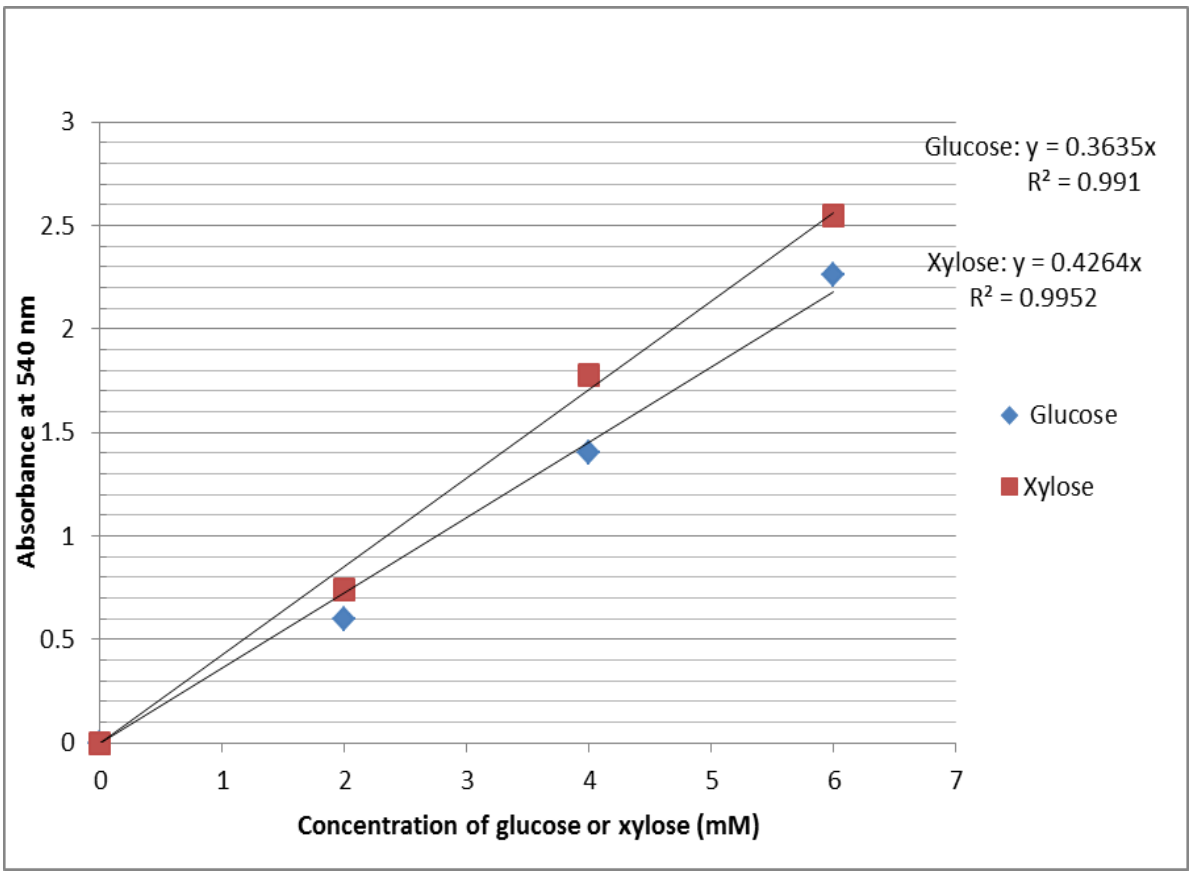

Figure 3-2. Standard curves for determination of glucose and xylose. The standard curves were obtained using the DNS method described in section 2.5.3. The absorbance was measured at 540 nm. Each point was repeated twice and the mean value was used to plot the standard curves. The standard deviations of all the points were below 0.003.

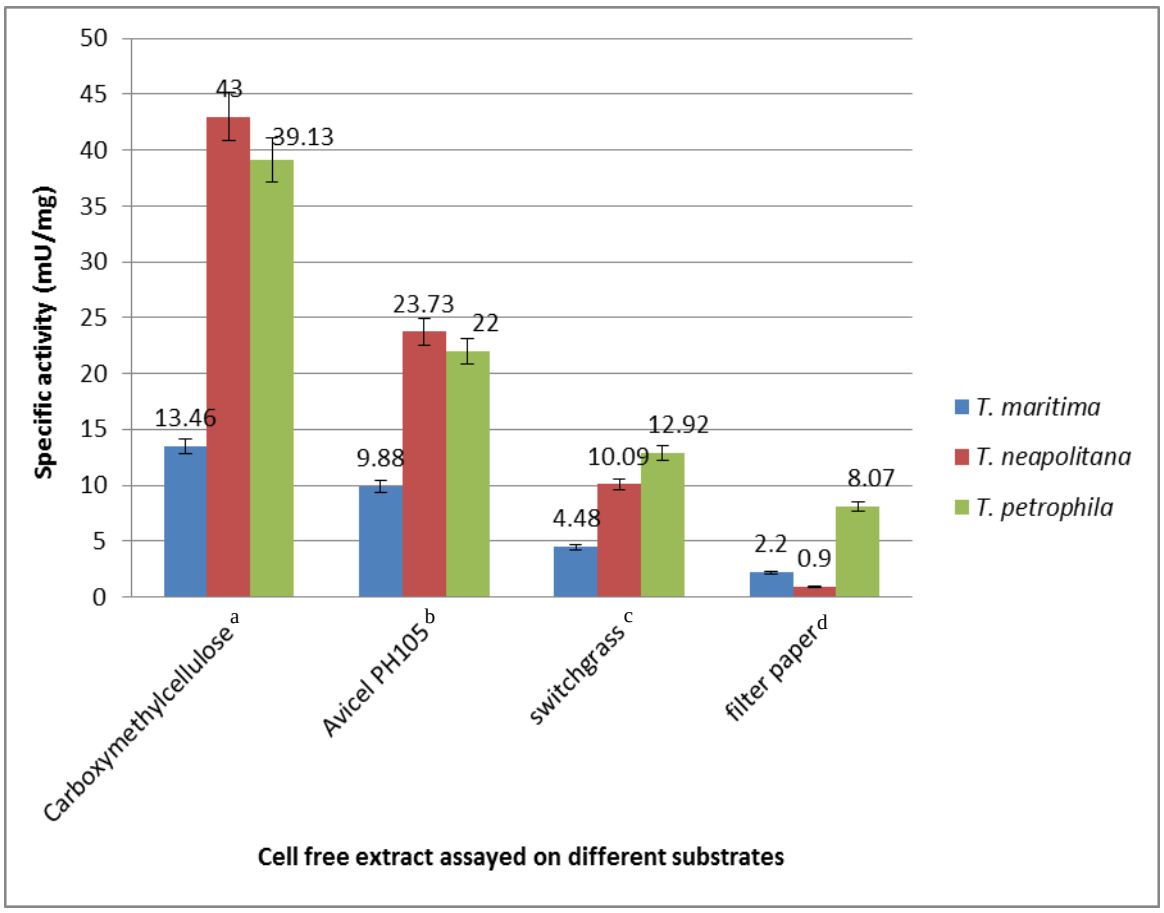

Figure 3-3. Comparison of cellulase specific activities in *T. petrophila, T. maritima* and *T. neapolitana* grown on glucose. Green bars represent *T. petrophila*. Blue bars represent *T. maritima*. Red bars represent *T. neapolitana*. The assays were conducted using the method described in section 2.5.4. [a]Carboxymethylcellulose (CMC) was used for determination of endoglucanase activity. [b]Avicel PH105 was used for determination of exoglucanase activity. [c]Switchgrass and filter paper[d] were used for the determination of complete cellulase activity.

In order to determine which substrate was the best inducer to increase the expression of cellulase, *T. petrophila* was grown on different substrates including simple sugars and cellulosic materials according to the method described in section 2.1. In order to determine whether the cellulases were intracellular or extracellular enzymes, the enzyme assays were done by using the cell free extract with all intracellular proteins and the culture supernatant with all extracellular proteins. The cell free extract was prepared by using the method described in section 2.5.1. The culture supernatant was concentrated by using the method described in section 2.5.2. The enzyme assays were done by using the method described in section 2.5.4. All the biomass was harvested from different media with various substrates after 22 hours incubation at 80^{o} C, while all the bacterial populations were in the late log phase.

The specific and total cellulase activities are shown in **Figure 3-4** and **Figure 3-5**. The exoglucanase activity was only found in the culture supernatant when *T. petrophila* grew on different substrates except glucose (47.3 mU/mg). The highest exoglucanase specific activity was found in the supernatant when *T. petrophila* grew on xylan (1361.3 mU/mg) followed by cellobiose (291.4 mU/mg). Surprisingly, the supernatant from the media with switchgrass and avicel PH105 did not indicate any exoglucanase activity both in the supernatant and the cell free extract. One possibility is that *T. petrophila* grew worse on cellulose than xylan, less cellulase from fewer cells in the culture medium were expressed. In addition, all the cellulase were concentrated anaerobically that may reduce the cellulase activity. From the cellulase activities from different media, xylan is the best inducer to increase the expression of cellulase, not cellulose.

Endoglucanase activity was found both in the culture supernatant and the cell free extract. The highest endoglucanase specific activity was detected from the supernatant of xylan medium (1032.1 mU/mg) followed by cellobiose medium (822.3 mU/mg) and switchgrass medium (119.2 mU/mg). There was no endoglucanase activity detected in the supernatant from avicel PH105 and glucose medium. All the cell free extract from different substrates had endoglucanase activity except the switchgrass medium. Other than endoglucanase activity from avicel PH105 being significantly lower (19 mU/mg), endoglucanase activity from cell free extract when *T. petrophila* grew on xylan, cellobiose and glucose, respectively, were very similar (166.7 mU/mg, 141.5 mU/mg and 182.4 mU/mg, respectively). It was concluded that compared to the extracellular endoglucanase, the intracellular endoglucanases remained relatively constant since they were not significantly induced by any substrate.

In order to compare the total activities of cellulase in the cell free extract and supernatant, either the biomass or culture supernatant could be mainly used for the extraction and purification of cellulases. **Figure 3-5** showed the total activities of all the exoglucanase and endoglucanase in cell free extract and supernatant when *T. petrophila* was grown on different substrates. The highest total activity of exoglucanase was detected in the supernatant from xylan medium (628.9 mU) followed by

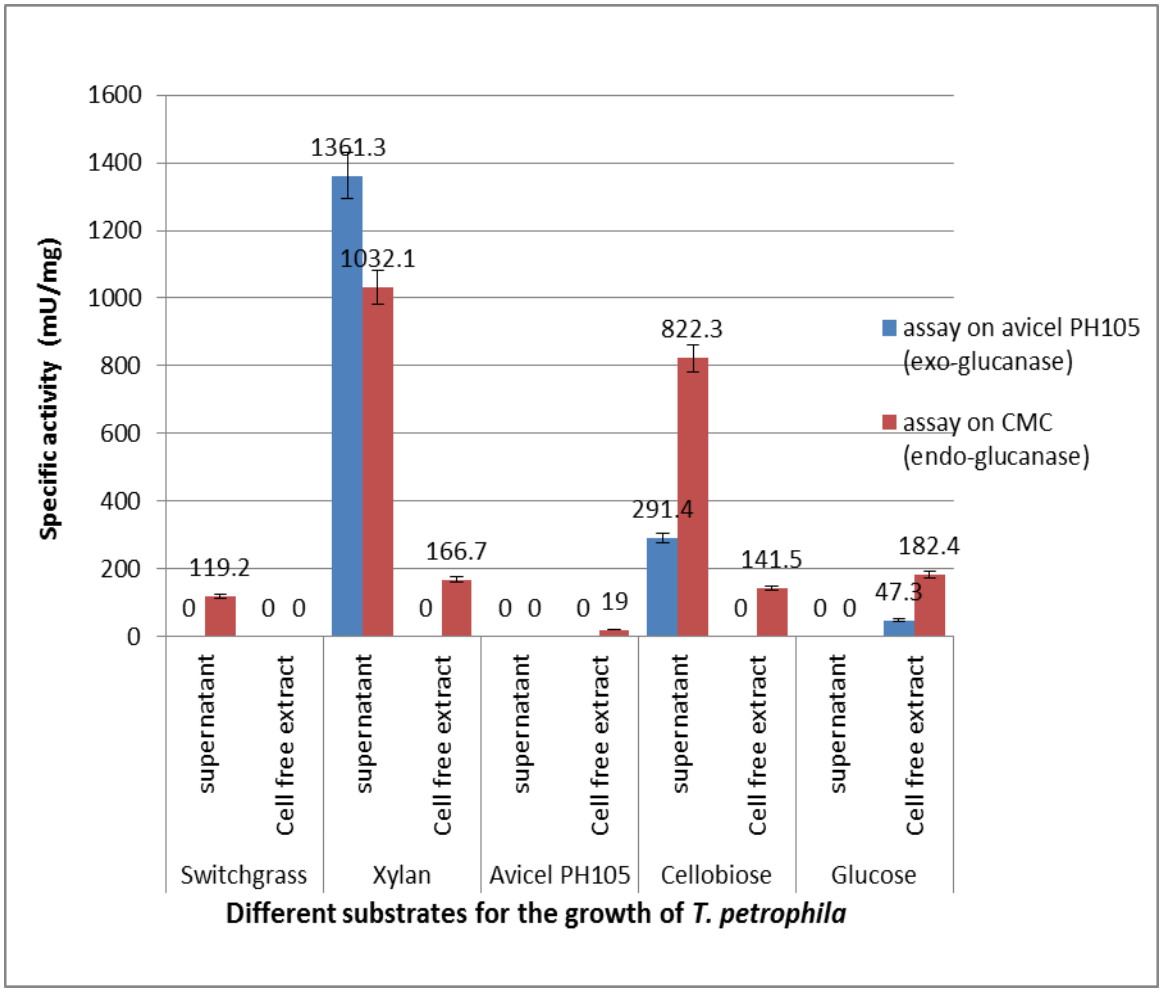

Figure 3-4. Specific activities of cellulases from both culture supernatants and cell free extracts of *T. petrophila* grown on 0.4% (w/v) of various substrates for 22 hours at 80° C and pH 7.0. The assays were conducted using the method described in section 2.5.4. The red bars represent the specific activities of endoglucanase using CMC as substrate in the assays and the blue bars represent the specific activities of exoglucanase using avicel PH105 as substrate in the assays.

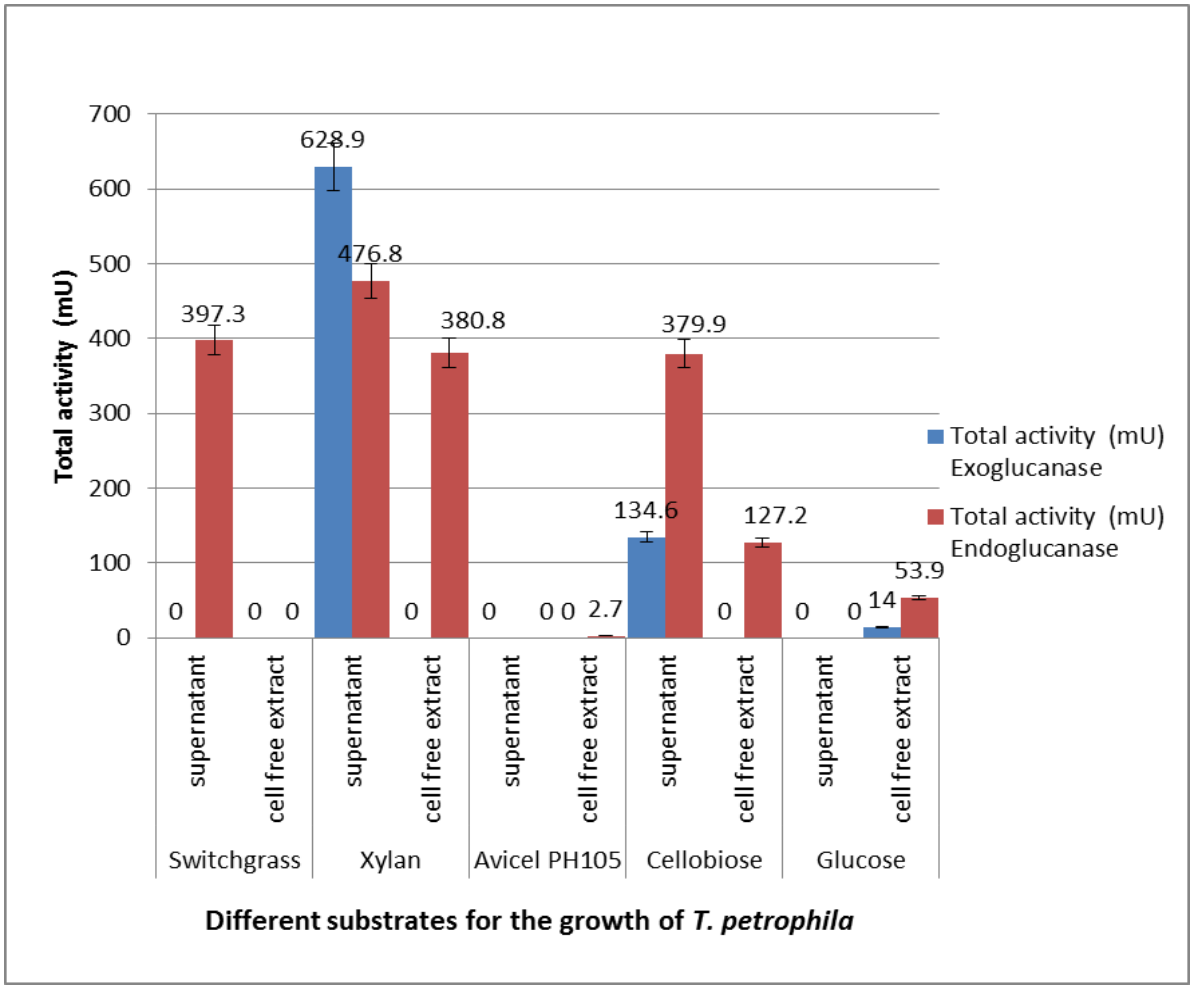

Figure 3-5. Total activities of cellulases from both culture supernatants and cell free extracts of *T. petrophila* grown on 0.4% (w/v) of various substrates for 22 hours at 80° C and pH 7.0. The assays were conducted using the method described in section 2.5.4. The red bars represent the total activities of endoglucanase using CMC as substrate in the assays and the blue bars represent the total activities of exoglucanase using avicel PH105 as substrate in the assays.

the supernatant from cellobiose medium (134.6 mU) and cell free extract from glucose medium (14 mU). The highest total activity of endoglucanase was found in the cell free extract from xylan medium (476.8 mU) followed by the supernatant from switchgrass medium (397.3 mU), cell free extract from xylan medium (380.8 mU), supernatant from cellobiose medium (379.9 mU), cell free extract from cellobiose medium (127.2 mU) and cell free extract from glucose medium (53.9 mU).

3.3.3 Xylanase activities of *T. petrophila* grown on various substrates.

In order to determine which substrate was the best inducer to increase the expression of xylanase, *T. petrophila* was grown on different substrates including simple sugars and cellulosic materials according to method 2.1. In order to determine whether the xylanase was an intracellular or extracellular enzyme, the enzyme assays were done by using cell free extract with all intracellular proteins and culture supernatant with all the extracellular proteins. The cell free extracts were prepared by using the method described in section 2.5.1. Supernatant samples were concentrated by using the method described in section 2.5.2. The enzyme assays were done by using the method described in section 2.5.4. All the biomass was harvested on different substrates after 22 hours when all the bacterial populations were in the late log phase, which was determined by the growth curve in **Figure 3-1**.

The results of xylanase specific activities are shown in **Figure 3-6**. Xylanase specific activity was detected in both cell free extract and supernatant from all growth with different substrates tested except the cell free extract from switchgrass. The highest xylanase specific activity was found in the cell free extract from cellobiose medium (3732.4 mU/mg), followed by the cell free extract from xylan medium. For the supernatant samples, the highest xylanase specific activity was from avicel PH105 (2681.3 mU/mg) followed by xylan (1836.3 mU/mg), glucose (1133.6 mU/mg), switchgrass (916.34 mU/mg) and cellobiose (750.44 mU/mg).

From the results of total activities of xylanase shown in **Figure 3-7**, the highest total activity of xylanase was found in the cell free extract from xylan medium (7203 mU). For the same growth medium with switchgrass, avicel PH105 or glucose, the total activity of xylanase from the supernatant was higher than the cell free extract. In contrast, when *T. petrophila* was grown on xylan and cellobiose, the total activity of xylanase was higher in the cell free extract than the supernatant.

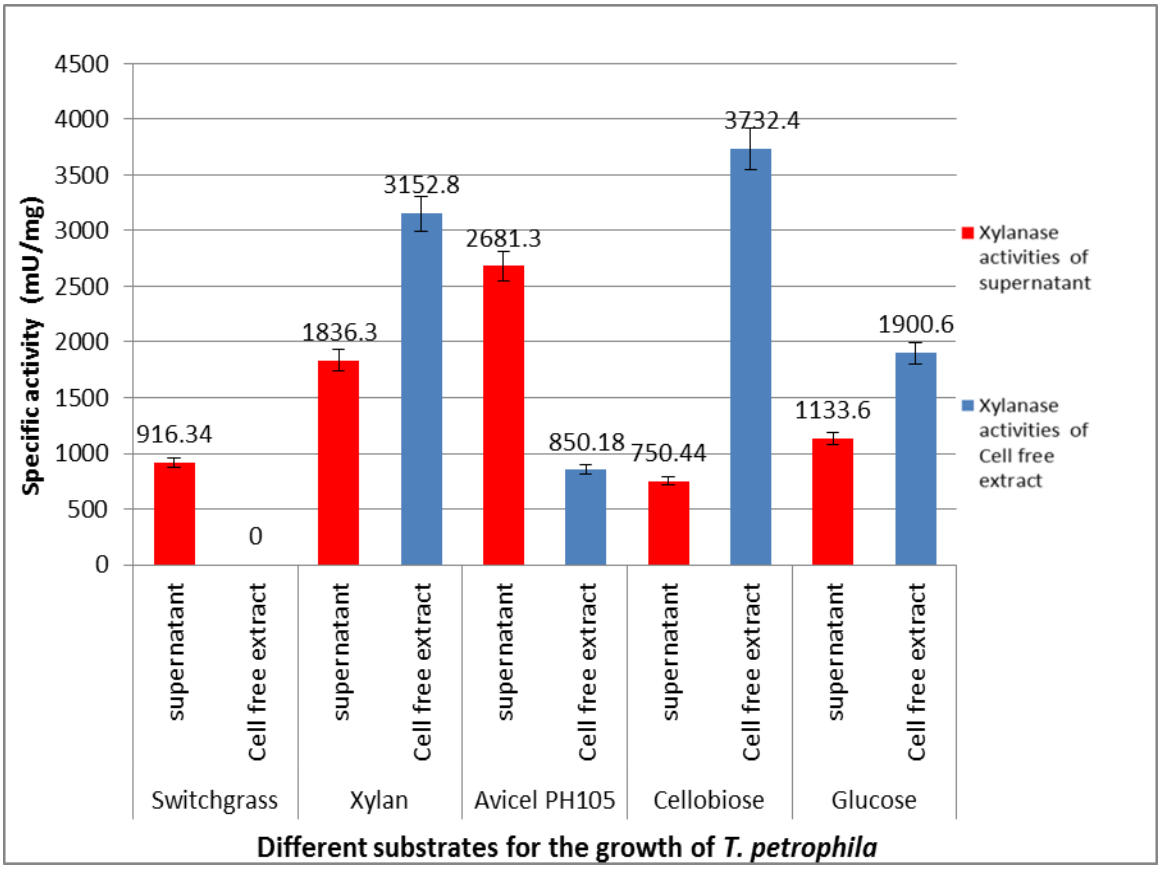

Figure 3-6. Specific activities of xylanase from both culture supernatants and cell free extracts of *T. petrophila* grown on 0.4% (w/v) of various substrates for 22 hours at 80° C and pH 7.0. The assays were conducted using the method described in section 2.5.4. The red bars represent the specific activities of xylanase in the culture supernatants. The blue bars represent the specific activities of xylanase in the cell free extracts. The culture supernatants and the cell free extracts were prepared using the methods described in section 2.5.1 and 2.5.2.

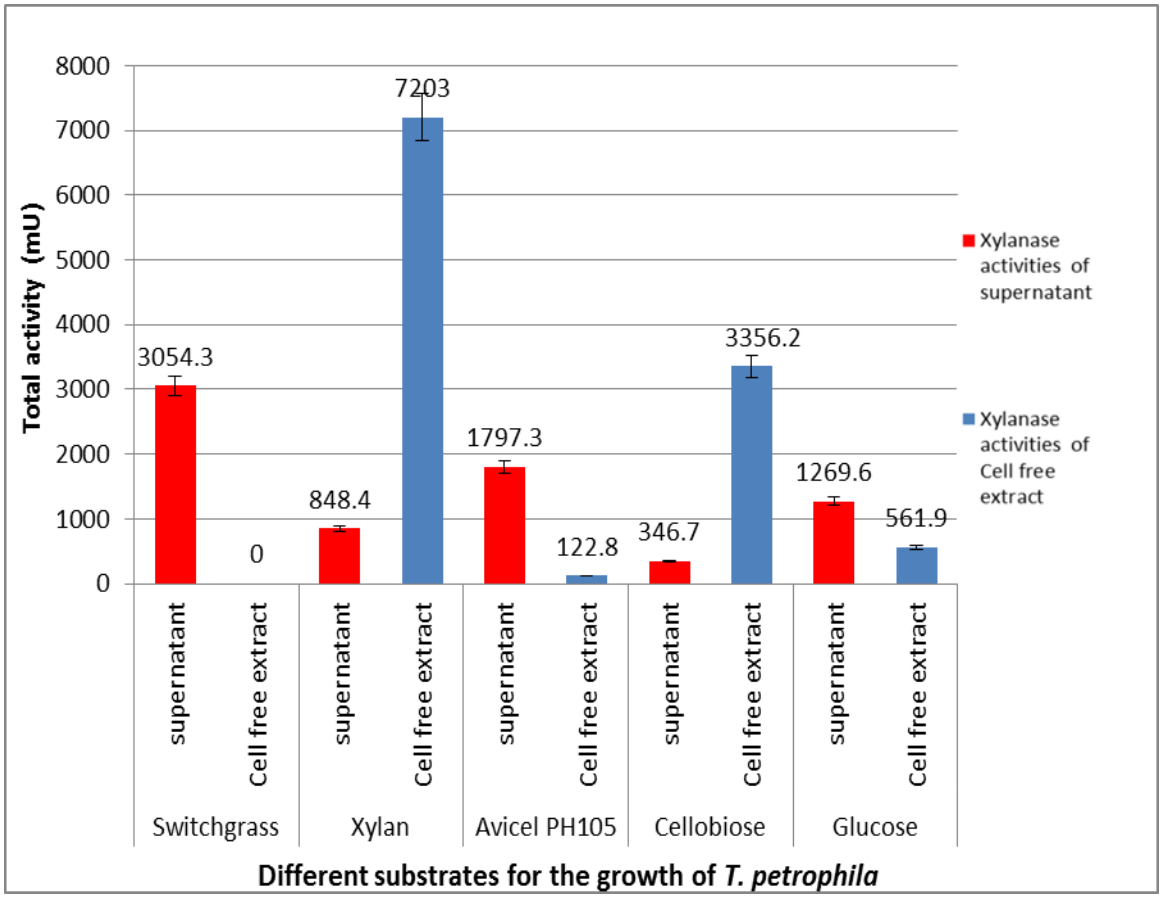

Figure 3-7. Total activities of xylanase from both culture supernatants and cell free extracts of *T. petrophila* grown on 0.4% (w/v) of various substrates for 22 hours at 80° C and pH 7.0. The assays were conducted using the method described in section 2.5.4. The red bars represent the total activities of xylanase in the culture supernatants. The blue bars represent the total activities of xylanase in the cell free extracts. The culture supernatants and the cell free extracts were prepared using the methods described in section 2.5.1 and 2.5.2.

3.4 Attachment of *T. petrophila* cells to cellulosic materials

3.4.1 DAPI and PI staining

3.4.1.1 DAPI and PI staining of *T. petrophila* grown on xylan

T. petrophila was grown on 0.4% (w/v) xylan according to the method described in section 2.3 and harvested after 25 hours of incubation at 80^{o}C and pH 7.0. The bacterial population was in the early stationary phase which had the highest number of cells. The medium without filtration was used as the staining target to do fluorescent staining by using the method described in section 2.6.1. The results are shown in **Figure 3-8**.

From **Figure 3-8 A-B**, all the staining samples were the culture medium with cells and xylan particles without any pretreatment. The culture medium was harvested after 25 hours of incubation at 80^{o}C and pH 7.0 when the bacterial population was in the early stationary phase with highest cell density. The free cells and attached cells to xylan particles were both observed. **Figure 3-8 A** was observed under a B_2 filter which gave the living cells a green color and dead cells a red color. The xylan particles were stained green under B_2 filter, so it was difficult to distinguish the live attached cells with green color even if there were some. However, there were a large number of dead attached cells observed. **Figure 3-8 B** was under a green filter which only showed dead cells with a red color. From **Figure 3-8 B**, dead cells attached on xylan particles were observed. Because xylan particles and cells were overlapped and showed the same color, it was not possible to see the structure of attachment.

In order to confirm the attachment structure, a filtration treatment was done according to using the method described in section 2.6.1.1. After filtration, xylan particles were washed with PBS buffer. By this method, all the free cells were filtered out from xylan particles. The xylan particles were then stained by DAPI and PI by using the method described in section 2.6.1.2. The attached cells were still observed (**Figure 3-8 C-D**) which confirmed the attachment between cells and xylan particles, and those cells tightly adhered on xylan particles after filtering and washing. **Figure 3-8 E-F** show the xylan particles without any inoculum as the control to demonstrate the absence of attachment without inoculation.

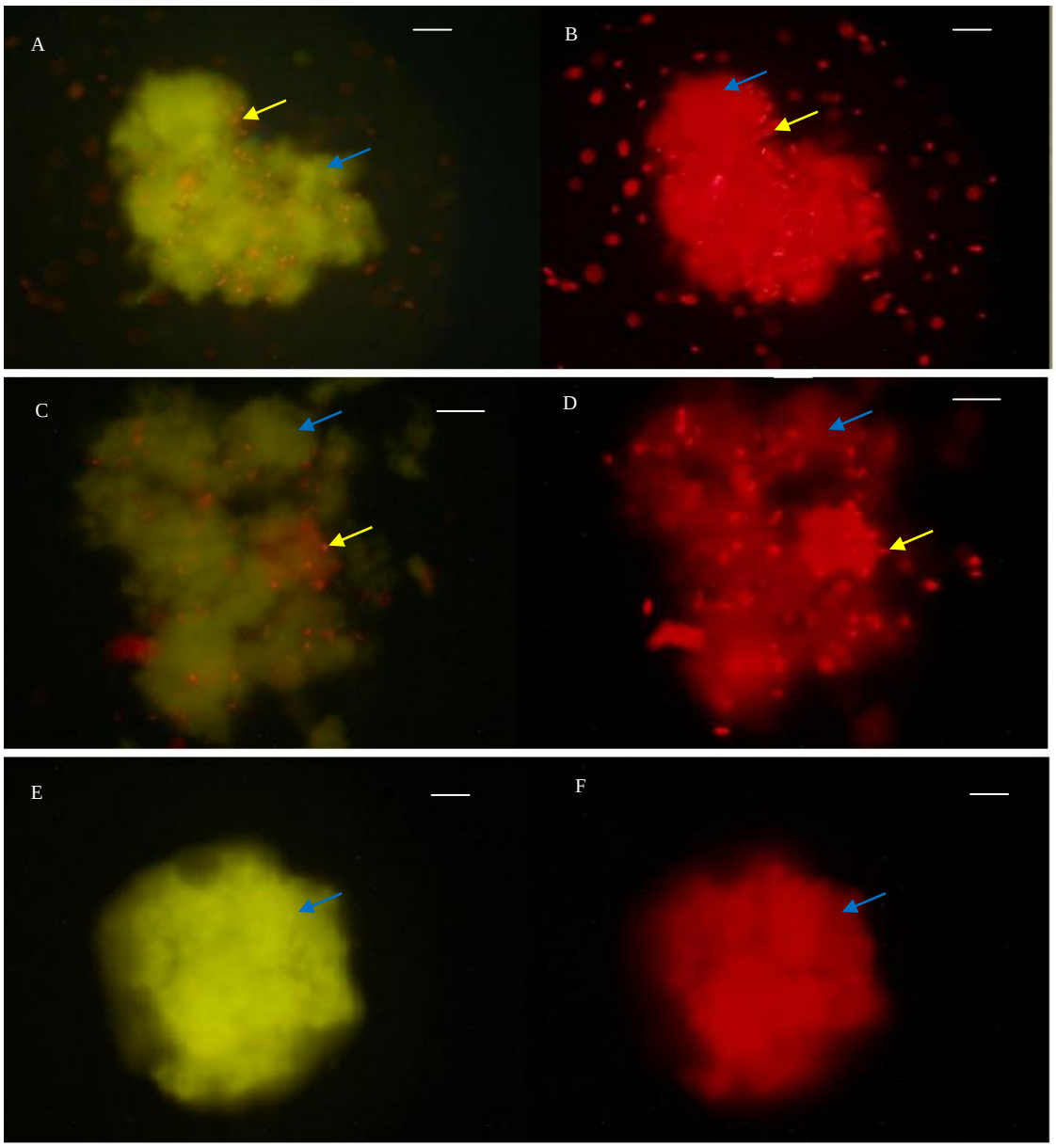

Figure 3-8. DAPI and PI staining on *T. petrophila* cells and xylan particles in the early stationary phase (25 hours of incubation at 80° C and pH 7.0). A-B: *T. petrophila* grown on xylan without filtration under B_2 filter and green filter, respectively. **C-D:** *T. petrophila* grown on xylan with filtration under B_2 filter and green filter, respectively. **E-F:** The control (xylan without *T. petrophila*) under B_2 filter and green filter, respectively. Magnification: 350 x. White scale bar is 10 μm. Yellow arrows show the cells (rod); blue arrows show the xylan particles.

3.4.1.2 DAPI and PI staining of *T. petrophila* grown on switchgrass

T. petrophila was grown on 0.4% (w/v) switchgrass according to the method described in section 2.3. The medium was harvested after 25 hours of incubation at 80° C and pH 7.0, when the cell population was in the early stationary phase with the highest amount of cell number. The results are shown in **Figure 3-9**. However, no cells were observed, indicating that there was no attachment between cells and switchgrass particles.

3.4.1.3 Attachment determination at different growth phases when *T. petrophila* was grown on 0.4% (w/v) xylan at 80° C, pH 7.0.

Since the attachment structure between cells and xylan particles was found in the growth of *T. petrophila* in the early stationary phase from results described in section 3.4.1.1, the mechanisms of how cells attached on xylan and when cells started to attach on xylan were required for further research. Therefore, during the growth of *T. petrophila* on 0.4% (w/v) xylan at 80° C and pH 7.0, several time points were picked to monitor the change in the number of attached cells in different growth phases. All the media samples were filtered and washed by using the method described in section 2.6.1.1. The fluorescent staining was done by using the method described in section 2.6.1.2. From results of the cell counting before filtering and after, the free (non-attached) cells were about one to two times more than the attached cells in lag phase, late log phase and late stationary phase.

Figure 3-10 shows the growth curve of *T. petrophila* grown on 0.4% (w/v) xylan. Three time points (5 hours, 19 hours and 50 hours) were picked for fluorescent staining which represented the lag phase, late log phase and late stationary phase, respectively.

The fluorescent staining results are shown in **Figure 3-11**. In the lag phase, a small amount of cells were already attached on xylan (**Figure 3-11 A-C**). With further incubation, the culture entered the end of the log phase where the maximum cell number was achieved and the number of attached cells increased along with growth (**Figure 3-11 D-F**). When the culture entered the late stationary phase, the cell population stopped increasing and the attached cells were barely seen (**Figure 3-11 G-I**). It was concluded that as the cells were growing they needed to break down xylan as a carbon source and as a result, they were attached on xylan to help the degradation of xylan. Once the cell population stopped increasing and was ready to enter death phase, the attached cells were gradually released from the xylan particles since they did not require the utilization of xylan at that stage of growth. Therefore, the adhesion structure was probably disassembled because no attached cells with filaments observed in late stationary phase.

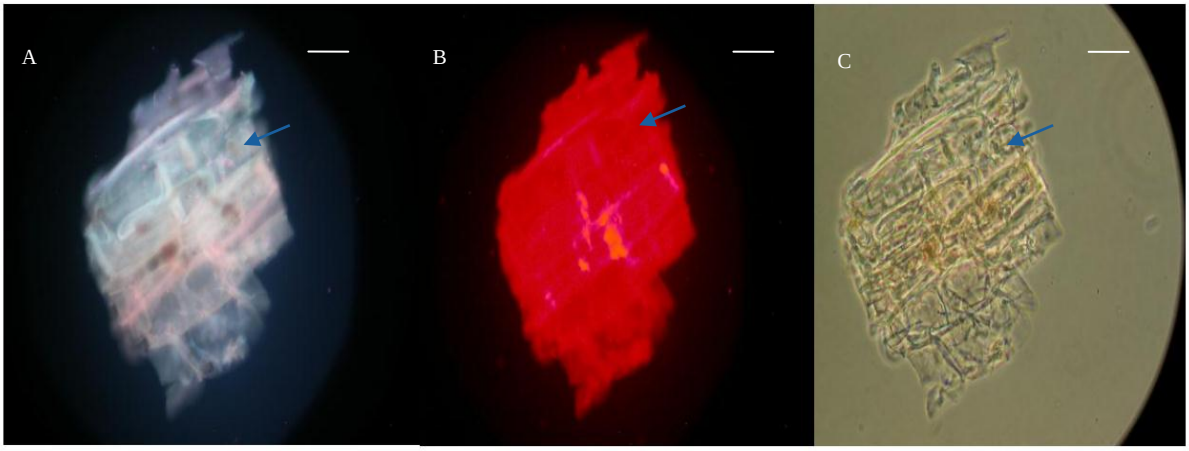

Figure 3-9. DAPI and PI staining on *petrophila* cells and switchgrass in the early stationary phase (25 hours of incubation at 80° C and pH 7.0 with filtration). A: under UV filter and green filter. **B**: under green filter. **C**: under phase contrast. Magnification: 350 x. White scale bar is 10 μm. Blue arrows show the switchgrass particles.

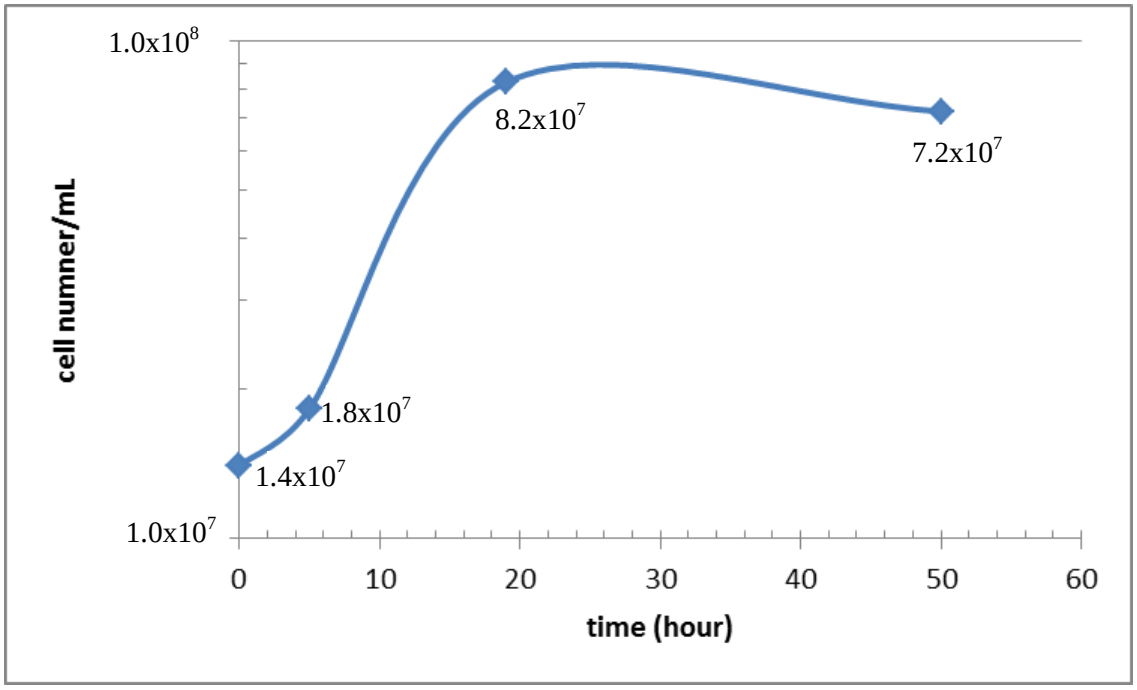

Figure 3-10. Growth of *T. petrophila* grown on 0.4% (w/v) xylan at 80° C and pH 7.0. The growth medium was prepared by using the method described in section 2.3.

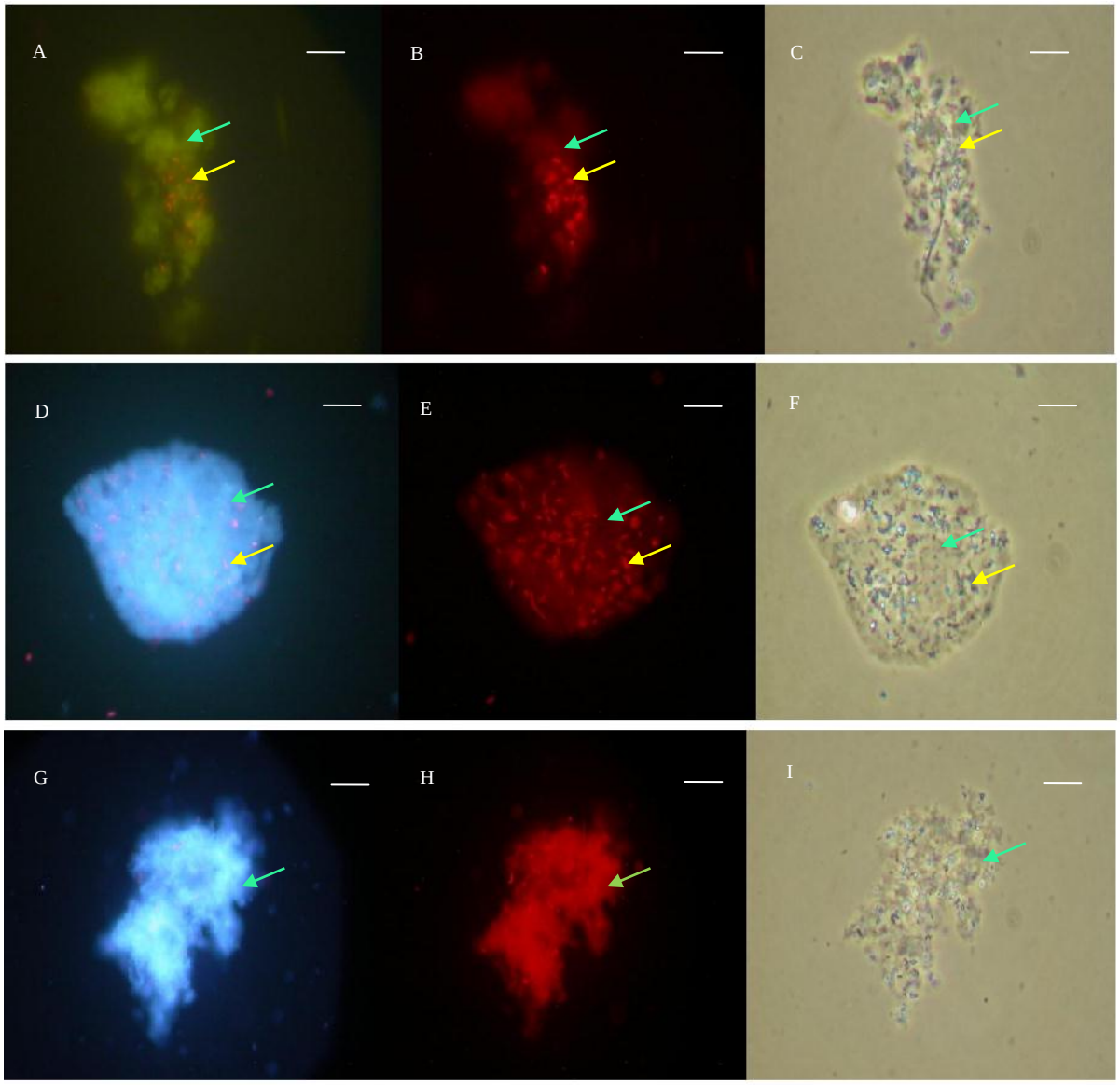

Figure 3-11. DAPI and PI staining of *T. petrophila* grown on 0.4% (w/v) xylan in different growth phases at 80° C and pH 7.0 after filtration. A-C: the lag phase, under B_2 filter, green filter and phase contrast, respectively. **D-F**: the late log phase, under UV filter, green filter and phase contrast, respectively. **G-I**: the late stationary phase, under UV filter, green filter and phase contrast, respectively. Magnification: 350 x. White scale bar is 10 µm. Yellow arrows show cells; green arrows show the xylan particles.

3.4.2 Scanning electron microscope (SEM)

In order to confirm the attachment structure between cells and xylan or switchgrass particles with more details and higher resolution, a scanning electron microscope (SEM) was used for attachment determination based on the method described in section 2.6.2. All of the culture media were harvested after 19 hours incubation at 80^{o} C and pH 7.0, and filtered by using the method described in section 2.6.1.1 to collect xylan or switchgrass particles for SEM sample preparation according to the method described in section 2.6.2. With the help from Dale Weber of the Department of Biology of the University of Waterloo, the SEM observation was done. The results are shown in **Figure 3-12**, **Figure 3-13**, **Figure 3-14** and **Figure 3-15**.

From **Figure 3-12**, the attachment between cells and xylan particles was observed and shown by many filaments connecting all the cells and xylan particles. The size of the cells was shorter (1-2 μm) than when it grew on monosaccharides (average 4-7 μm). The control was the medium with xylan, but without inoculum. The result is shown in **Figure 3-13**. There were no cells observed in the control sample. However, there were no cells attached on switchgrass (**Figure 3-14**), which was consistent with fluorescent staining result (**Figure 3-9**).

Normally cells secret more cellulase and xylanase into the culture media for increasing the utilization of inaccessible cellulosic materials. When grown on xylan, *T. petrophila* used attachment structures to increase the enzyme concentration around xylan. But when grown on switchgrass, there was no such attachment observed. A speculation was raised that *T. petrophila* might secrete more free cellulase and relevant enzymes to the culture medium to degrade cellulose instead of adhesion structure. Therefore, the protein concentration in the culture medium was detected by the Bradford method (Bradford, 1976). After the biomass samples from different substrates were harvested by centrifugation, the protein concentrations in the supernatants were determined. The results are listed in **Table 3-5**. The protein concentration of the supernatant from switchgrass culture was five times higher than that from xylan and cellobiose culture that meant *T. petrophila* secreted five times more proteins outside of the cells to help degrade cellulose.

3.5 Metabolic end products of *T. petrophila*

3.5.1 Metabolic end products of *T. petrophila* grown on cellulose and hemicellulose

In order to determine the metabolic end products from fermentation of cellulosic materials by *T. petrophila* during growth, all the end products generated in gas phase were monitored by GC according to the method described in section 2.7.1 and all the end products in liquid phase were monitored by HPLC according to the method described in section 2.7.2. All the end products achieved highest yield after 25 hours of incubation at 80^{o} C and pH 7.0. The results are listed in

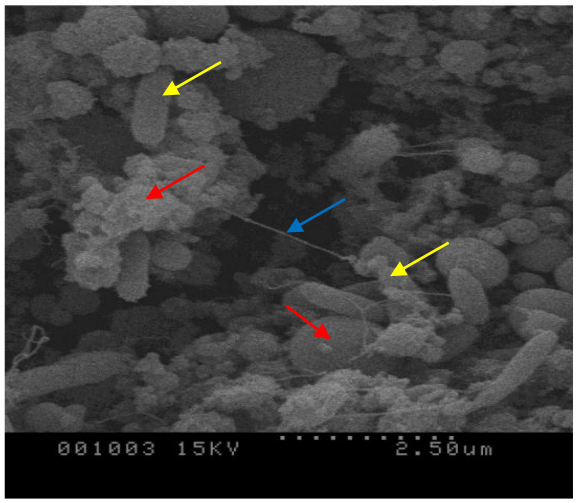

Figure 3-13. SEM of *T. petrophila* grown on 0.4% (w/v) xylan in the late log phase (19 hours of incubation at 80° C and pH 7.0). The SEM was conducted using the method described in section 2.6.2. Magnification: 9200 x. Yellow arrows show cells; red arrows show xylan particles; blue arrows show filament.

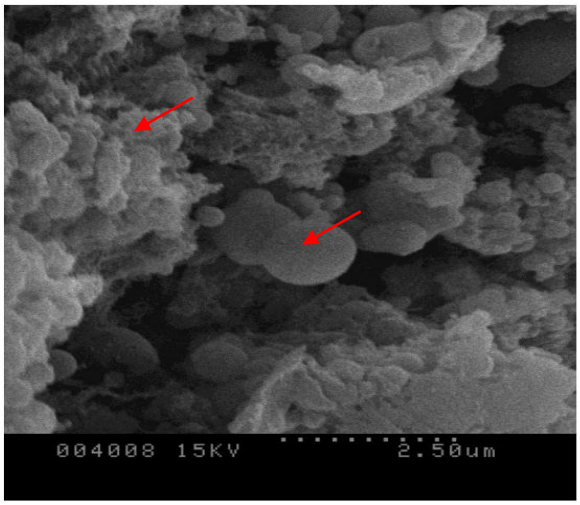

Figure 3-12. SEM of xylan without cells as control (19 hours of incubation at 80° C and pH 7.0). The SEM was conducted using the method described in section 2.6.2. Magnification: 9200 x. Red arrows show xylan particles.

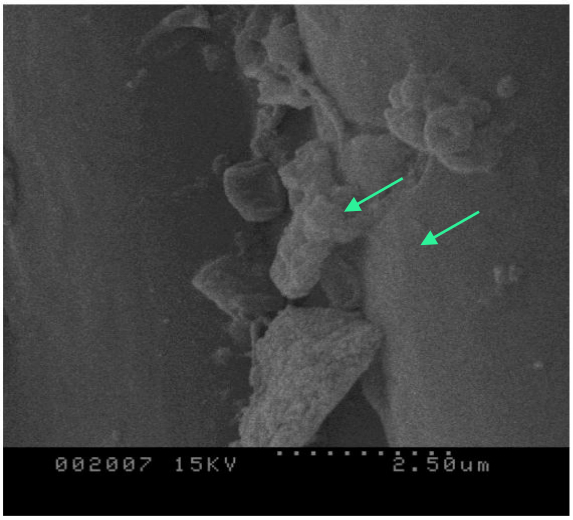

Figure 3-15. SEM of *T. petrophila* grown on 0.4% (w/v) switchgrass in the late log phase (19 hours of incubation at 80° C and pH 7.0). The SEM was conducted using the method described in section 2.6.2. Magnification: 9200 x. Green arrows show switchgrass particles.

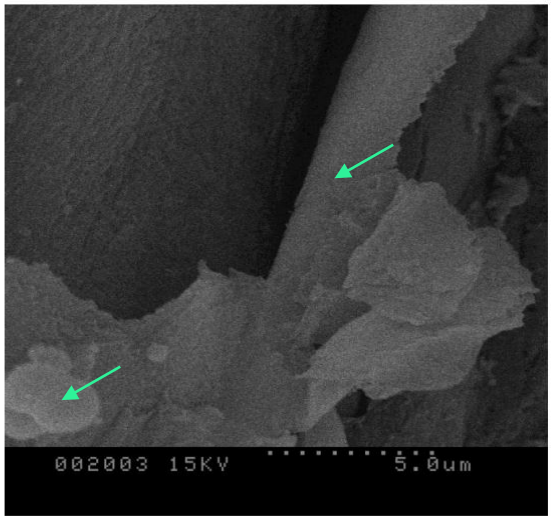

Figure 3-14. Switchgrass without cells as control (19 hours of incubation at 80° C and pH 7.0). The SEM was conducted using the method described in section 2.6.2. Magnification: 9200 x. Green arrows show switchgrass particles.

Table 3-5. Protein concentrations of the different culture supernatants of *T. petrophila* grown on switchgrass, xylan and cellobiose, respectively.

Substrates	Mean of Protein concentrations (µg/mL)	Variance* (Standard deviation)
Switchgrass	13.6	0.91
Xylan	2.7	0.02
Cellobiose	2.7	0.006

Protein concentrations were determined by using the Bradford method with bovine serum albumin (BSA) as standard (Bradford, 1976). *The average of the three repeats for each sample was calculated and recorded in the above table.

Figure 3-16, **Figure 3-17** and **Figure 3-18**.

Biofuels including ethanol and H_2 were both detected as end products when *T. petrophila* was grown on cellulose and hemicellulose. Also CO_2 and organic acids, including acetic acid, lactic acid, formic acid and succinic acid, were detected as well. When *T. petrophila* grew on glucose and cellobiose, the carbon balance was around 90%. The insoluble substrates were not detectable by HPLC; therefore, the carbon balances when *T. petrophila* grew on cellulosic materials were not calculated.

The highest yield of H_2, CO_2 and lactate were found when *T. petrophila* was grown on cellobiose (10.13 mM, 7.27 mM and 0.66 mM, respectively). The highest yield of ethanol, acetate and fructose (0.95 mM, 4.77 mM and 1.50 mM, respectively) were found when *T. petrophila* grew on xylan. The highest yield of formate and fructose (0.32 mM and 1.57 mM, respectively) were found when *T. petrophila* grew on corn husks. Succinate was only found when *T. petrophila* grew on glucose (0.03 mM). In conclusion, the yield of all the end products was consistent with the growth condition. Cellobiose and xylan supported the best growth of *T. petrophila* and also gave the highest yield of biofuel products and other end products.

Surprisingly, fructose was produced during the growth of *T. petrophila* on all substrates. The reason could be due to the conversion of glucose into fructose. In order to explore the transformation between glucose and fructose either from fermentation by *T. petrophila* or by thermal reaction, H_2O (pH 5.0), 0.45% (w/v) HEPES buffer (pH 7.0) and medium (pH 7.0) was each added with 0.4% (w/v) glucose and incubated at 80^{o} C for 25 hours in the absence of *T. petrophila*. Each sample was prepared in duplicate. After 25 hours of incubation at 80^{o}C in the absence of *T. petrophila*, fructose was detected in all the solutions which confirmed that fructose came from glucose. The amount of fructose produced from 5 mM glucose medium without *T. petrophila* (0.359 mM) (**Table 3-6**) was very close to the one with *T. petrophila* (0.29 mM) (**Figure 3-17**). This result proved the transformation from glucose to fructose was due to a thermal reaction and not an enzymatic function from *T. petrophila*. Also HEPES buffer and medium, which had a stable pH 7.0, produced 0.314 mM and 0.359 mM fructose, respectively, which were much lower than H_2O at pH 5.0 (**Table 3-6**). This data proved that this thermal reaction was relative to pH. When the pH was stable, glucose was also relevantly steady at high temperatures.

3.5.2 Determination of the optimum pH for biofuel production from *T. petrophila*

In order to determine the optimal pH for the highest yield of biofuel, *T. petrophila* was grown on 0.4% (w/v) cellobiose at 80^{o} C with different initial pHs according to using the method described in section 2.3. pHs from 5.5 to 9.0, which were able to support the growth of *T. petrophila* were adjusted by using pH meter AB15 from Fisher Scientific International, Inc. The cell number was counted by

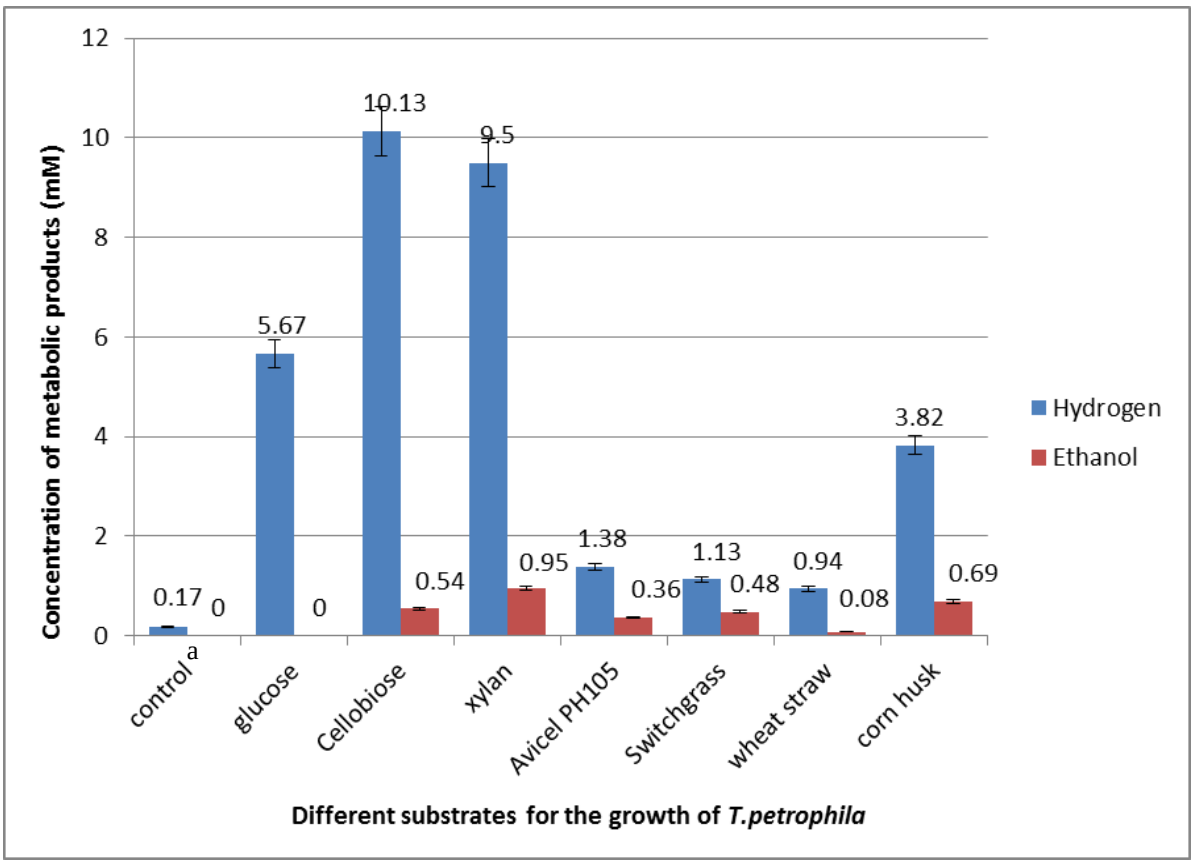

Figure 3-16. The concentrations of hydrogen and ethanol produced by *T. petrophila* grown on 0.4% (w/v) of various substrates at 80° C and pH 7.0 for 25 hours. [a]Control was the medium without any carbon sources. Hydrogen (blue bar) was measured by GC described in section 2.7.1. Ethanol (red bar) was measured by HPLC described in section 2.7.2.

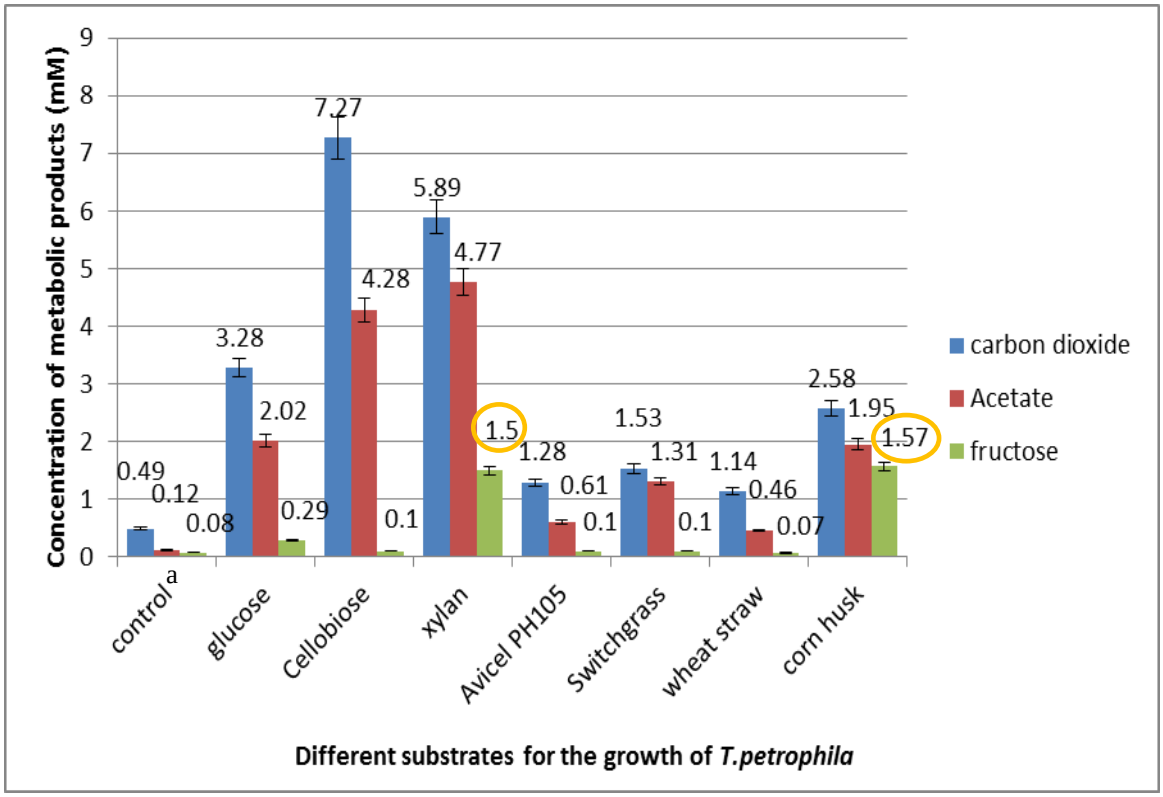

Figure 3-17. The concentrations of carbon dioxide, acetate and fructose concentration produced by *T. petrophila* grown on 0.4% (w/v) of various substrates at 80° C and pH 7.0 for 25 hours. [a]Control was the medium without any carbon sources. Carbon dioxide (blue bar) was measured by GC described in section 2.7.1. Acetate (red bar) and fructose (green bar) were measured by HPLC described in section 2.7.2. Fructose was produced from glucose by Keto-enol tautomeric transformations at high temperatures. Fructose and xylose showed the peaks at the similar retention time in HPLC; therefore, xylose from xylan and corn husks might contribute to the significant production of fructose (orange circle).

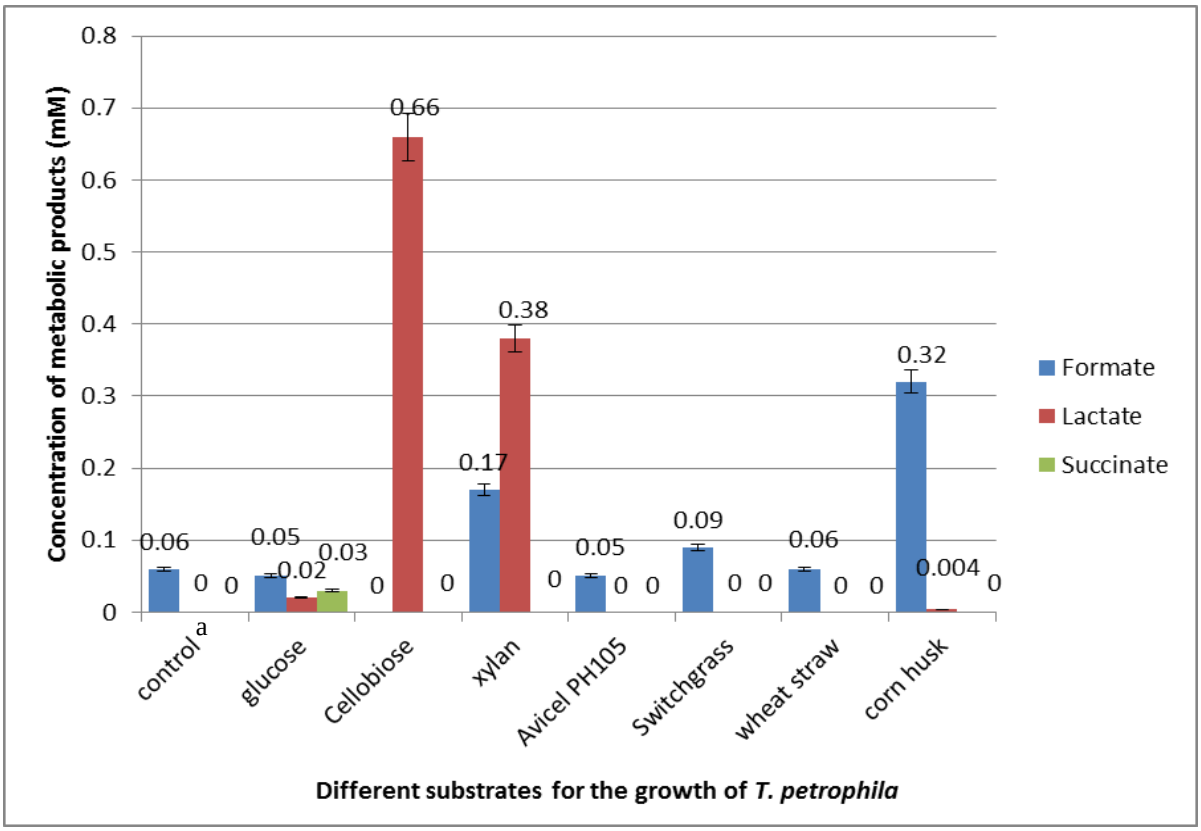

Figure 3-18. The concentrations of formate, lactate and succinate concentration produced by *T. petrophila* grown on 0.4% (w/v) of various substrates at 80° C and pH 7.0 for 25 hours. [a]Control was the medium without any carbon sources. All the components including formate (blue bar), lactate (red bar) and succinate (green bar) were measured by HPLC described in section 2.7.2.

Table 3-6. Detection of fructose produced from different solutions in the presence of 5 mM glucose and absence of *T. petrophila* at 80° C for 25 hours.

Solutions with 5 mM glucose	Fructose produced (mM)	Variance
H_2O (pH 5.0)	1.952	0.00002
0.45% (w/v) HEPES (pH 7.0)	0.314	0.00026
Medium[a] (pH 7.0)	0.359	0.00065

[a]Medium was prepared according to the method described in section 2.3.1. Fructose was measured by HPLC described in section 2.7.2.

using a Petroff Hausser counting chamber and Nikon Elipse E600 microscope. End products were determined by GC and HPLC by using the method described in section 2.7.

The result of cell numbers during growth is recorded in **Table 3-7**. The growth curve is shown in **Figure 3-19**. From **Table 3-7** and **Figure 3-19**, when the initial pH was above 8.0 (inclusive), it took a longer time for *T. petrophila* cell population to enter stationary phase (72 hours). However, a higher cell density was obtained. When pH was from 6.0 to 7.5 (inclusive), cell population was in stationary phase after only 48 hours, but the cell number was lower.

Table 3-8 and **Figure 3-20** showed all the end products after 48 hours of incubation at 80^{o} C when the cell population was in the late log phase. Except glucose and fructose, the production of all other end products including H_2, CO_2, ethanol, acetate, lactate and succinate increased with the increase of initial pH value until 8.5. Therefore, considering the biofuel yield and time consumption, the optimal pH was 8.5.

3.6 Quantitative and qualitative analysis of expressed proteins when *T. petrophila* grew on cellulose and hemicellulose

3.6.1 Proteomics analysis

In order to find all the expressed enzymes involved in the metabolism of cellulosic materials and help finish the profiling of the cellulosic material metabolic pathway, proteomics analysis was done by using the method described in section 2.8.1. All the proteins from the biomass harvested from cellobiose, xylan, avicel PH105 and switchgrass media, respectively, were qualified and quantified by proteomics analysis. The certain enzymes up-regulated by certain substrates were found as the crucial enzymes in the metabolism of that substrate which would be very helpful for producing recombinant cellulases and hemicellulases for commercial use. Cellobiose-containing medium was used as the control. All the expression levels of enzymes from avicel PH105, switchgrass and xylan media were compared to the expression level of the same enzymes from cellobiose medium. Therefore, all the values in **Table 3-9**, **Table 3-10** and **Table 3-11** were relative values and not absolute values. If the value was greater than 1.96, those proteins were up-regulated (color in red). In contrary, if the value is less than -1.96, those proteins are significantly down-regulated (color in blue).

From the proteomics data, 930 proteins were quantified when *T. petrophila* was grown on cellobiose, avicel PH105, switchgrass and xylan. **Table 3-9** summarizes the highly expressed proteins in the degradation of cellulose. Many ABC transporters (Tpet_0489, Tpet_0866, Tpet_0488, Tpet_0867, and Tpet_0895) and relative proteins such as monosaccharide-transporting ATPase and Tpet_0867, and Tpet_0895) and relative proteins such as monosaccharide-transporting ATPase and binding membrane proteins were up-regulated when *T. petrophila* was grown on cellulose. Another

Table 3-7. Growth of *T. petrophila* on 0.4% (w/v) cellobiose with various initial pHs at 80° C.

Time (hour)	Cell number / mL								
	Initial pHs								
	Control[a]	**5.5**	**6.0**	**6.5**	**7.0**	**7.5**	**8.0**	**8.5**	**9.0**
0	1.5×10^7	1.8×10^7	1.9×10^7	1.6×10^7	1.3×10^7	2.0×10^7	1.4×10^7	1.5×10^7	1.8×10^7
5	1.9×10^7	2.1×10^7	3.3×10^7	3.5×10^7	4.8×10^7	3.8×10^7	1.8×10^7	1.6×10^7	1.8×10^7
48	1.5×10^7	2.9×10^7	9.2×10^7	1.1×10^8	2.5×10^7	4.5×10^8	2.6×10^8	3.9×10^8	2.7×10^8
72	7.0×10^7	6.9×10^7	7.1×10^7	1.0×10^8	1.9×10^8	3.7×10^8	5.4×10^8	7.9×10^8	6.5×10^8
80	6.8×10^7	7.0×10^7	6.9×10^7	1.0×10^8	2.0×10^8	3.5×10^8	4.9×10^8	7.5×10^8	6.6×10^8

[a]Control was the medium without any carbon sources. The growth media were prepared by using the method described in section 2.3. The initial pHs were adjusted by using pH meter AB15 from Fisher Scientific International, Inc.

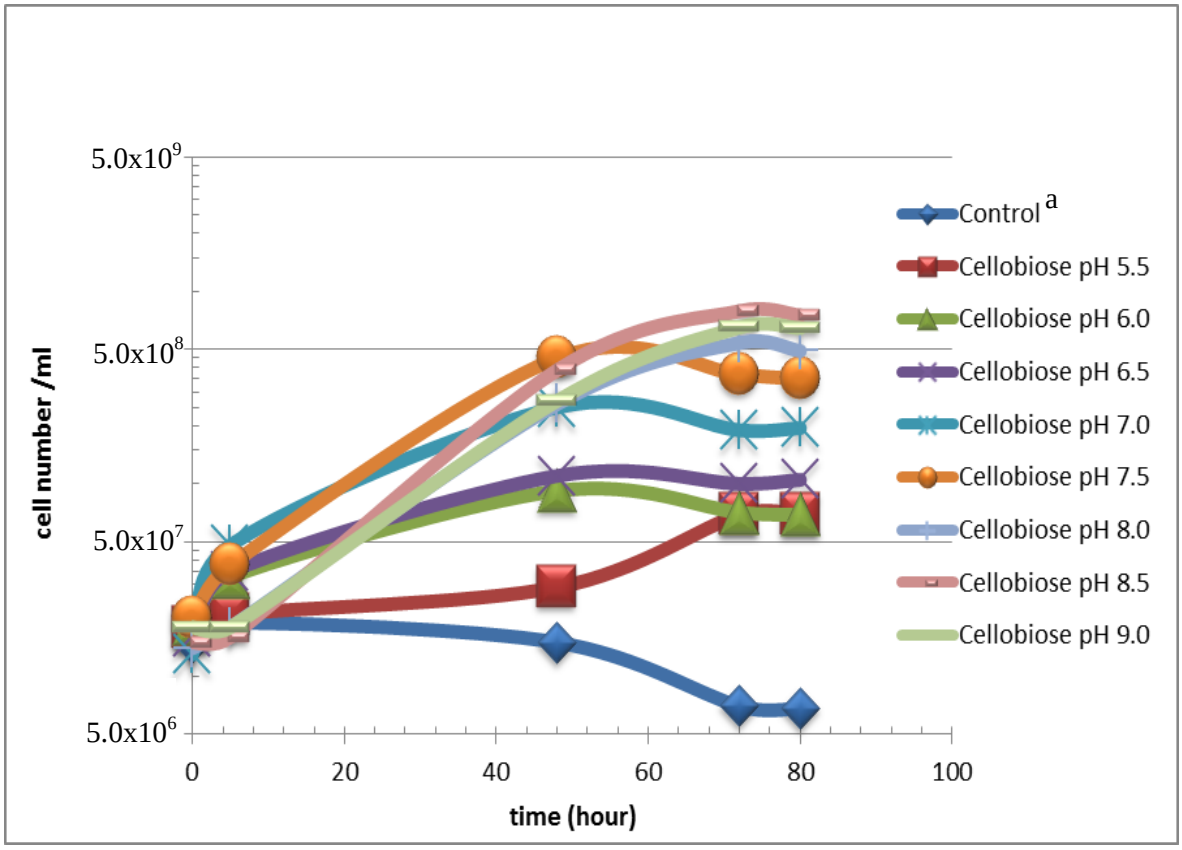

Figure 3-19. Growth of *T. petrophila* on 0.4% (w/v) cellobiose with various initial pHs at 80° C. [a]Control was the medium without any carbon sources. The growth media were prepared by using the method described in section 2.3. The initial pHs were adjusted by using pH meter AB15 from Fisher Scientific International, Inc. The different initial pHs of media were used to determine the optimal pH for the growth of *T. petrophila*. (control, blue square; pH 5.5, red diamond; pH 6.0, green triangle; pH 6.5, purple cross; pH 7.0, blue asterisk; pH 7.5, orange dot; pH 8.0, light blue line; pH 8.5, pink line; pH 9.0, light green line)

Table 3-8. Metabolic end products of *T. petrophila* grown on 0.4% (w/v) cellobiose with various initial pHs at 80° C in the late log phase.

Metabolic products	Concentrations of metabolic products (mM)								
	Initial pHs								
	Control[a]	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0
H_2	0.07	0.01	1.30	1.80	7.20	12.00	16.00	16.00	12.00
CO_2	0.12	0.07	0.81	1.00	4.80	8.34	11.00	10.40	8.50
Ethanol	0	0	0.02	0.02	0.02	0.20	0.43	0.51	0.21
Acetate	0	0.1	0.62	0.76	3.50	6.20	8.30	8.40	6.90
Formate	0	0.04	0	0.13	0.18	0.30	1.40	2.50	6.40
Lactate	0	0	0.66	0.02	0.11	0.57	1.00	1.70	2.00
Succinate	0	0.01	0.03	0.03	0.02	0.01	0.13	0.33	0.31
Fructose	0.01	0.04	0.11	0.38	0.82	1.20	1.42	1.27	1.95
Glucose	0.03	2.50	3.00	3.30	3.30	3.40	3.60	3.90	4.70

The growth media were prepared by using the method described in section 2.3. The initial pHs were adjusted by using pH meter AB15 from Fisher Scientific International, Inc. All the end products were measured by GC and HPLC described in section 2.7 after 48 hours incubation at 80° C when the cell population was in the late log phase. [a]Control was the medium without any carbon sources.

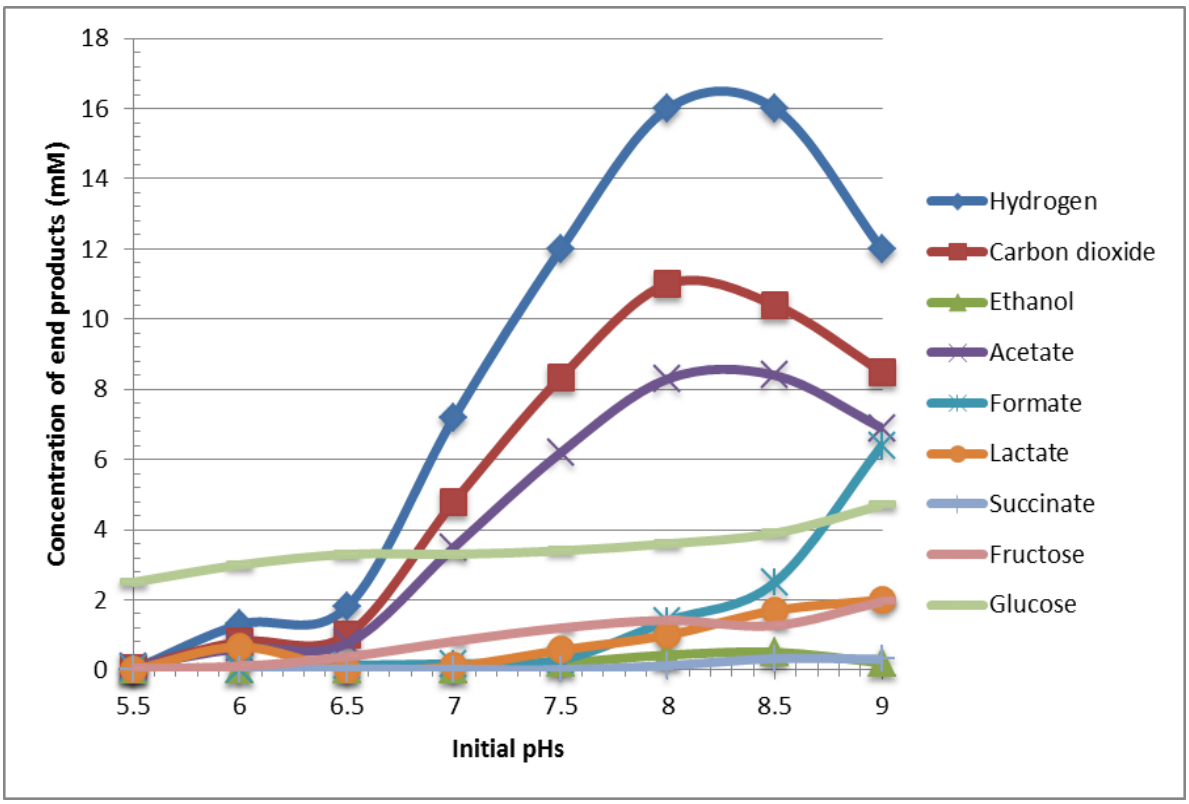

Figure 3-20. Metabolic end products of *T. petrophila* grown on 0.4% (w/v) cellobiose with various initial pHs at 80° C in the late log phase. The growth media were prepared by using the method described in section 2.3 and incubated at 80° C for 48 hours when the cell population was in the late log phase. The initial pHs were adjusted by using pH meter AB15 from Fisher Scientific International, Inc. Hydrogen (blue diamond) and carbon dioxide (red square) were measured by GC described in section 2.7.1. Other products including ethanol (green triangle), acetate (purple cross), formate (light blue asterisk), lactate (orange dot), succinate (light blue line), fructose (pink line) and glucose (light green line) were measured by HPLC described in section 2.7.2.

Table 3-9. Highly expressed proteins for the catabolism of cellulose.

Proteins and functions		Protein expression value		
		Avicel PH105	**Switchgrass**	**Xylan**
Overexpressed ABC transporters and relative proteins for transporting cellulose fragments into cells				
Tpet_0489	Oligopeptide/dipeptide ABC transporter	2.466113166	1.9885121	2.46703945
Tpet_0866	Oligopeptide/dipeptide ABC transporter	2.921511334	1.81863761	2.60221389
Tpet_0488, 0850	Oligopeptide/dipeptide ABC transporter	2.272267959	1.68768854	2.54303673
Tpet_0867	Oligopeptide/dipeptide ABC transporter	2.062688976	1.45825936	2.61679188
Tpet_0895	Oligopeptide/dipeptide ABC transporter	2.646422893	1.57630088	-0.4720328
Tpet_1495	ABC transporter related protein	2.732068	1.84381767	2.68843341
Tpet_1722	ABC transporter related protein	4.363487067	2.32941623	1.05245316
Tpet_0896	ABC transporter related protein	3.79819554	1.94144779	-0.1444467
Tpet_0568	ABC transporter related protein	2.814964655	0.77485529	1.10622122
Tpet_0812	Monosaccharide-transporting ATPase	3.889490933	2.94638115	2.61679188
Tpet_0569	Macrolide transporter ATP-binding/permease protein FtsX	2.371008288	2.98889049	0.9975033
Tpet_1510	Phosphate ABC transporter ATP-binding protein	2.134454445	1.79313264	1.15885723

Tpet_0852	Binding-protein-dependent transport systems inner membrane component	3.779542818	2.79769393	2.82583712
Tpet_0893	Binding-protein-dependent transport systems inner membrane component	2.434798955	1.45825936	-0.1444467
S-layer proteins for assembling attachment structure of cells on cellulose				
Tpet_0443	S-layer domain-containing protein	2.787626932	2.56873984	2.04798111

All the expression values of enzymes from *T. petrophila* grown on xylan and switchgrass were relative values and deducted from the expression values of the control (cellobiose as substrate for the growth of *T. petrophila*). Therefore, all the positive values in xylan and switchgrass samples indicate that the corresponding enzymes were up-regulated. Reversely, all the negative values indicate that the corresponding enzymes were down-regulated. The values larger than 1.9 (red color in the above table) represent significant up-regulation.

Table 3-10. Highly expressed proteins for the catabolism of hemicellulose.

Proteins and functions		Protein expression value		
		Avicel PH105	**Switchgrass**	**Xylan**
Remove hemicellulose side groups				
Tpet_0607	β-glucuronidase, galactosidase	0.308150875	1.576300876	1.973898111
Tpet_0490	β-glucuronidase, galactosidase	0.378933132	0.448557334	3.209321362
Tpet_0869	α-glucuronidase, galactosidase	-0.162856952	0.290344277	2.420306904
Tpet_0648	L-arabinose isomerase	0.706382319	0.641550316	3.397056368
Tpet_0631	α-N-arabinofuranosidase	-0.162856952	0.234661495	2.372684164
Tpet_0847	Acetyl xylan esterase	0.884617866	0.939668049	2.497716128
Overexpressed ABC transporters and relative proteins for transporting xylan fragments into cells				
Tpet_0489	Oligopeptide/dipeptide ABC transporter	2.466113166	1.9885121	2.46703945
Tpet_0866	Oligopeptide/dipeptide ABC transporter	2.921511334	1.81863761	2.60221389
Tpet_0488, 0850	Oligopeptide/dipeptide ABC transporter	2.272267959	1.68768854	2.54303673
Tpet_0867	Oligopeptide/dipeptide ABC transporter	2.062688976	1.45825936	2.61679188
Tpet_1495	ABC transporter related protein	2.732068	1.84381767	2.68843341
Tpet_0809	ABC transporter related domain	1.834594669	1.71458158	2.06616387

Tpet_0812	Monosaccharide-transporting ATPase	3.889490933	2.94638115	2.61679188
Tpet_0852	Binding-protein-dependent transport systems inner membrane component	3.779542818	2.79769393	2.82583712
Tpet_0864	Binding-protein-dependent transport systems inner membrane component	0.884617866	0.73141161	3.35413483
Tpet_0851	Binding-protein-dependent transport systems inner membrane component	0.514932467	0.54740833	2.34042413
Tpet_0865	Binding-protein-dependent transport systems inner membrane component	0.160488356	0.73141161	3.92281975
Tpet_0485	DdpA, ABC-type dipeptide transport system	0.826561698	1.05484284	3.27720204
Tpet_0636	Extracellular solute-binding protein. Domains: UgpB, ABC-type sugar transport system periplasmic component	0.308150875	0.54740833	3.31046353
S-layer proteins for assembling attachment structure of cells on xylan				
Tpet_0443	S-layer domain-containing protein	2.787626932	2.56873984	2.04798111
Tpet_1024	S-layer domain-containing protein	1.670514723	1.45825936	2.18986986

All the expression values of enzymes from *T. petrophila* grown on xylan and switchgrass were relative values and deducted from the expression values of the control (cellobiose as substrate for the growth of *T. petrophila*). Therefore, all the positive values in xylan and switchgrass samples indicate that the corresponding enzymes were up-regulated. Reversely, all the negative values indicate that the corresponding enzymes were down-regulated. The values larger than 1.9 (red color in the above table) represent significant up-regulation.

Table 3-11. The expected proteins up-regulated in the catabolism of cellulose and xylan, which were not shown as up-regulated from the proteomics data.

Proteins and functions		Protein expression value		
		Avicel PH105	**Switchgrass**	**Xylan**
Cellulose				
Hydrolysis of cellulose to cellodextrins				
Tpet_1701	endoglucanase	0.826561698	1.33271825	-0.0162074
Tpet_1702	endoglucanase	0.706382319	1.89323544	0.02511781
Tpet_1703	endoglucanase	-0.078148026	0.59503975	-0.4225239
Hydrolysis of cellodextrins				
Tpet_0898	β-glucosidase	-0.250437908	-0.070033974	-0.279621148
Tpet_0848	β-glucosidase	0.003871268	0.234661495	1.737490476
Tpet_0952	β-glucosidase	0.160488356	-0.070033974	-0.626728673
Hemicellulose				
Hydrolysis xylan to xylosaccharides				
Tpet_0863	1, 4-β-D-xylanase	0.996956903	0.978810081	1.588503188
Xylosaccharides to xylose				

Tpet_0848	1, 4-β-xylosidase	0.003871268	0.234661495	1.737490476

Remove hemicellulose side groups

Tpet_1559	β-glucuronidase, galactosidase	1.794541297	3.111193908	1.778476938
Tpet_1167	β-glucuronidase, galactosidase	-0.078148026	0.177364498	0.671583343
Tpet_1557	β-glucuronidase, galactosidase	-0.435044336	-0.511101307	-0.32635369
Tpet_1676	α-glucuronidase, galactosidase	-0.250437908	-0.511101307	0.06577103

All the expression values of enzymes from *T. petrophila* grown on xylan and switchgrass were relative values and deducted from the expression values of the control (cellobiose as substrate for the growth of *T. petrophila*). Therefore, all the positive values in xylan and switchgrass samples indicate that the corresponding enzymes were up-regulated. Reversely, all the negative values indicate that the corresponding enzymes were down-regulated. The values larger than 1.9 (red color in the above table) represent significant up-regulation.

type of protein that was up-regulated in the cellulose sample was S-layer protein (Tpet_0443) which served to attach extracellular proteins. However, no functional proteins such as exoglucanase, endoglucanase and β-glucosidase were significantly up-regulated.

All the highly expressed proteins for the degradation of hemicellulose are summarized in **Table 3-10**. Some ABC transporters (Tpet_0498, Tpet_0866, Tpet_0488 and Tpet_0867) and related enzymes such as monosaccharide-transporting ATPase and inner membrane binding proteins were up-regulated. Besides that, there were some enzymes up-regulated to remove xylan side groups when *T. petrophila* was grown on xylan such as β-glucuronidase (Tpet_0607 and Tpet_0490), α-glucuronidase (Tpet_0869), L-arabinose isomerase (Tpet_0648), α-N-arabinofuranosidase (Tpet_0631) and Acetyl xylan esterase (Tpet_0847). Some other functional enzymes such as 6-phosphogluconate dehydrogenase (Tpet_0482), dehydrogenase/reductase (Tpet_0594), carbohydrate kinase (Tpet_0628), β-galactosidase (Tpet_0607), alcohol dehydrogenase (Tpet_0484), and mannitol dehydrogenase domain-containing protein (Tpet_0856) were up-regulated as well. In addition, S-layer proteins (Tpet_0443 and Tpet_1024) were overexpressed as well.

Table 3-11 lists all the important enzymes which were expected to be up-regulated for breaking cellulose and hemicellulose when *T. petrophila* was grown on cellulose and hemicellulose, but they were not up-regulated based on the proteomics data. For degradation of cellulose, endoglucanase (Tpet_1701, Tpet_1702 and Tpet_1703) and β-glucosidase (Tpet_0898, Tpet_0848 and Tpet_0952) were supposed to be up-regulated. For degradation of hemicellulose, 1, 4-β-D-xylanase (Tpet_0863), 1, 4-β-xylosidase (Tpet_0848), β_glucuronidase (Tpet_1559, Tpet_1167 and Tpet_1557) and α-glucuronidase (Tpet_1676) were supposed to be up-regulated.

3.6.2 Identification of extracellular and intracellular protein by SDS-PAGE

Since very different activities of cellulases and xylanases were detected from the supernatant and cell free extract, proteins of supernatant and cell free extract were identified by SDS-PAGE according to using the method described in section 2.8.2. All the extracellular and intracellular proteins are shown in **Figure 3-21**.

The supernatant from culture medium with cellobiose had a unique protein band which was about 60 kDa. The supernatant from culture medium with xylan had three unique bands with sizes of 65 kDa, 63 kDa and 41 kDa, respectively. The bands around 65 kDa corresponded to ABC transporters and the bands around 41 kDa may be S-layer proteins based on the size of proteins detected in proteomics data.

Figure 3-21. SDS-PAGE (12.5%) of expressed proteins in culture supernatants and cell free extracts from various growth media. Lane 1: marker; **Lane 2**: cell free extract from cellobiose medium, 10 µg; **Lane 3**: culture supernatant from cellobiose medium, 5 µg proteins; **Lane 4**: cell free extract from xylan medium, 10 µg; **Lane 5**: culture supernatant from xylan medium, 5 µg proteins; **Lane 6**: cell free extract from switchgrass medium, 10 µg; **Lane 7**: culture supernatant from switchgrass medium, 5 µg proteins; **Lane 8**: cell free extract from avicel PH105 medium, 10 µg; **Lane 9**: culture supernatant from avicel PH105 medium, 5 µg proteins; **Lane 10**: marker. Red arrows show the uniquely expressed proteins in culture supernatant, but not in cell free extract.

3.7 Metabolic profiling of the cellulose utilization by *T. petrophila*

Based on gene identification, end product measurement and proteomics analysis, the whole metabolic pathway, from cellulose and hemicellulose to simple sugars and other end products, was profiled. The entire profiling of metabolic pathway is illustrated in **Figure 3-22**. All the enzymes involved in the pathway were followed with expression values when *T. petrophila* was grown on switchgrass and xylan, respectively.

In this metabolic pathway, all the enzymes were detected by proteomics analysis and their coding genes were also found in *T. petrophila*'s genome except exoglucanase, fumarate reductase and ribulokinase. Exoglucanase was not anotated, so it was not found in both the proteomics data and genome of *T. petrophila*. Ribulokinase did not exist in both the proteomics data and genome of *T. petrophila* either. Fumarate reductase was found in the genome sequences, but it was not detected by proteomics.

From cellulose to glucose in **Figure 3-22**, endoglucanase (Tpet_1701, Tpet_1702 and Tpet_1703) and β-glucosidase (Tpet_0989 and Tpet_0848) were up-regulated when *T. petrophila* was grown on switchgrass. Interestingly, β-glucosidase (Tpet_0848) was expressed more in xylan medium (1.737) than switchgrass medium (0.235).

From xylan to single sugar residues, endoxylanase (Tpet_0863), xylosidase (Tpet_0848), arabinofuranosidase (Tpet_0631) and acetyl xylan esterase (Tpet_0847) were up-regulated when T. *petrophila* was grown on xylan. Also those enzymes degrading xylan were also up-regulated when *T. petrophila* was grown on switchgrass, but less than the expression values when *T. petrophila* was grown on xylan.

Since *T. petrophila* could not use xylose as a substrate, when xylan was broken down to xylose, glucose and arabinose, only glucose and arabinose were further broken down in the metabolic pathway. However, xylan might be hydrolyzed into oligo-xylose that might be transferred from outside to inside of cells, which was then hydrolyzed to xylose that will be further metabolized via PPP pathway. Glucose released from xylan went through EMP pathway. Arabinose entered into PPP pathway. It was transferred to ribulose, then to ribulose-5-phosphate which was an intermediate product in PPP pathway. The end product of PPP pathway was glyceraldehyde-3- phosphate and fructose-6- phosphate which entered the EMP for further catabolism. In the PPP pathway, arabinose isomerase (Tpet_0648) and transketolase (Tpet_1050) were up-regulated, especially arabinose isomerase (Tpet_0648) which was significantly up-regulated (3.397) when *T. petrophila* was grown on xylan confirming that arabinose from xylan was really transferred to ribulose which entered the PPP for further degradation.

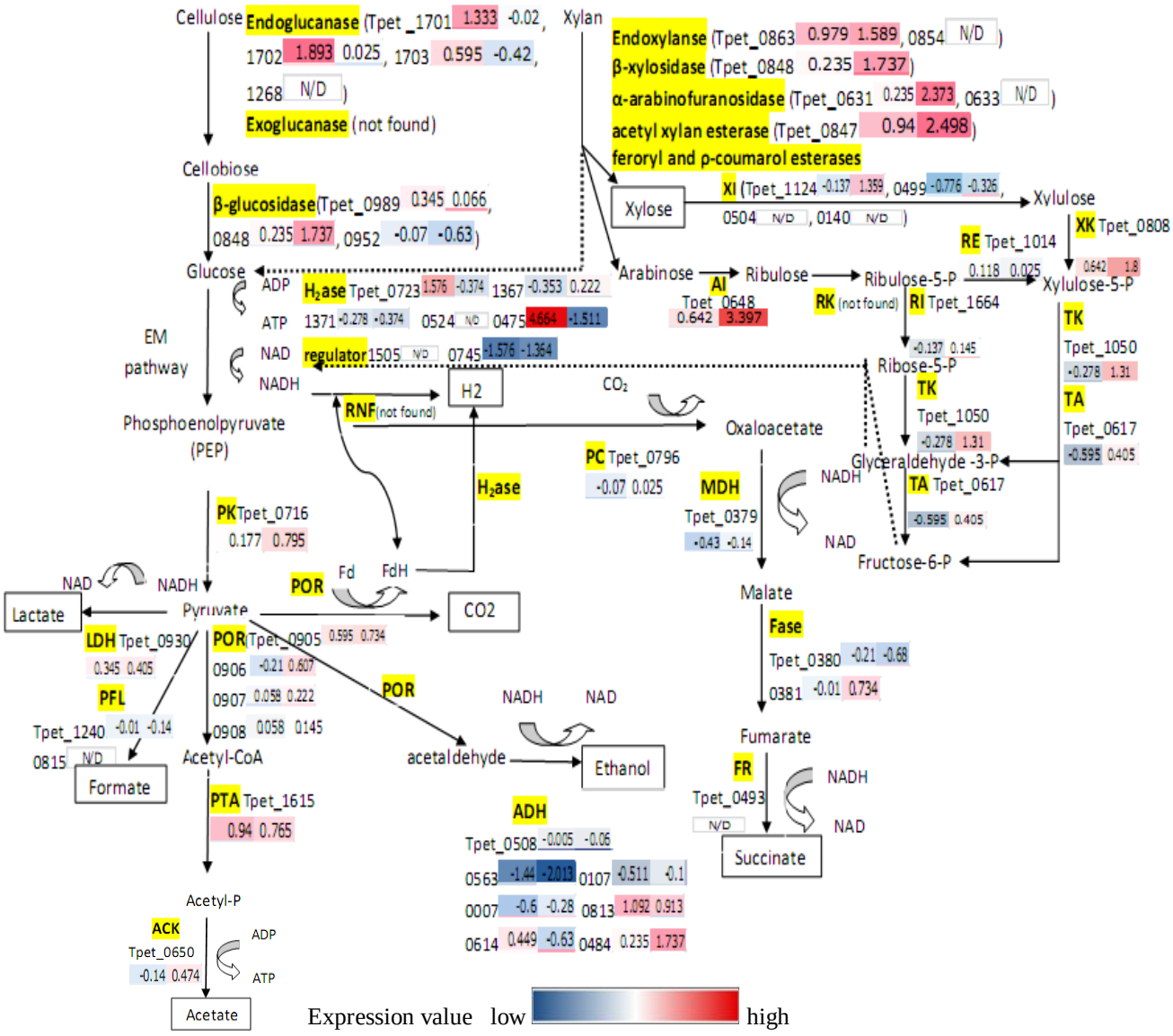

Figure 3-22. Tentative metabolic pathways for the utilization of cellulose and xylan in *T. petrophila*. There were two values (colored from blue to red from the lowest to highest value) for each enzyme (highlighted in yellow). The number on the left represented the expression level of the corresponding protein from cells grown on switchgrass. The number on the right represented the expression level of the corresponding protein from the cells grown on xylan. A positive or a negative value indicated more or less proteins expressed in the cells respectively than grown on cellobiose. Any value larger than 1.9 or smaller than -1.9 is treated as significantly up-regulated or down-regulated, respectively.(ACK: Acetate kinase; ADH: Alcohol dehydrogenase; AI: Arabinose isomerase; Fase: Fumarase; FR: Fumarate reductase; H_2ase: Hydrogenase; LDH: Lactate dehydrogenase; MDH: Malate dehydrogenase; PC: Pyruvate carboxylase; PFL: Pyruvate formate-lyase; PK: Pyruvate kinase; POR: Pyruvate ferredoxin oxidoreductase; PTA: Phosphate acetyltransferase; RF: Ribulose 5-phosphate epimerase; RI: Ribulose-5-phosphate-isomerase; RK: Ribulokinase; RNF: NAD^+ Oxidoreductase; TA: Transaldolase; TK: Transketolase; XI: Xylose isomerase; XK: Xylulokinase)

Chapter 4
Discussion

4.1 Identification of genes coding cellulase and xylanase of *T. petrophila*

T. petrophila contains four endoglucanase genes based on the genome annotation, while *T. maritima* has six and *T. neapolitana* has three. Each bacterial has three β-glucosidase genes. No exoglucanase gene was identified in *T. petrophila*, even in the entire *Thermotoga* genus. However, exoglucanase activity was detected in the DNS assay. Thus, exoglucanase could exist in *T. petrophila*. Therefore, *T. petrophila* has a full capability to utilize cellulose. By gene homolog, *T. petrophila* had much higher identity with *T. maritima* (96-99%) than *T. neapolitana* (80-85%), which meant *T. petrophila* enzymes might have a closer genetic relationship with *T. maritima* than *T. neapolitana.*

T. petrophila possesses all the genes encoding xylanase proteins, including two endo-1, 4-β-xylanase genes, one β-xylosidase gene, one α-N-arabinofuranosidase gene and one acetyl xylan esterase gene. This finding is also consistent with the fact that *T. petrophila* can utilize xylan as the sole carbon source. *T. petrophila* has higher identity with *T. maritima* (96-99%) than *T. neapolitana* (82-86%).

4.2 Growth of *T. petrophila* on cellulose and hemicellulose

T. petrophila grew best on cellobiose and xylan, followed by corn husks, glucose, switchgrass, avicel PH105 and wheat straw. However, *T. petrophila* could not grow on xylose, which was consistent with the findings in the published paper (Takahata *et al*., 2001), although *T. maritima* and *T. neapolitana* can grow on xylose (Huber *et al*., 1986; Jannasch *et al*., 1988). Based on the genome annotation in *T. petrophila* and gene homolog with *T. maritima* and *T. neapolitana, T. petrophila* contained all the genes involved in degradation of xylose, including four xylose isomerase genes, one xylulokinase gene, one transaldolase gene, one transketolase gene and one L-ribulose-5-4 phosphate 4-epimerase. It also contained oligopeptide ABC transporters, including periplasmic oligopeptide-binding proteins, permease proteins and ATP-binding proteins. Those oligopeptide ABC transporters were closely related to archaeal sugar transporters, and they proved to help xylose bind and enter cells (Conner *et al*., 2005). But *T. petrophila* did not have the specific sugar ABC transporters for xylose that existed in *T. maritima* and *T. neapolitana* (**Table 3-4**). This is a probable reason that *T. petrophila* cannot use xylose as a carbon source.

The best growth of *T. petrophila* was on simple sugars (cellobiose and glucose), followed by hemicellulose (xylan) and cellulose (avicel PH105, switchgrass, corn husks and wheat straw) **(Table 3-3)**. This finding was consistent with published paper (Tolonen *et al*., 2010) because cellulose and xylan are glucose or xylose polymer that should be catabolized to glucose or xylose, so the specific growth rate constant on cellulosic materials was supposed to be smaller than that of the simple sugars. However, the highest specific growth rate constant was 0.12 (with the generation time 5.9 hours) when *T. petrophila* grew on cellobiose (**Table 3-3),** which was still much smaller than the value of the published data with the generation time of 54 minutes when *T. petrophila* grew on glucose (Takahata *et al*., 2001). A possible reason was that the published data were obtained when its growth on rich medium in the presence of 0.2% (w/v) yeast extract, which can be utilized by microorganisms. In contrast, the medium used in **Table 3-3** did not contain yeast extract that might help the growth.

However, after 25 hours incubation, all the cultures with different substrates entered the stationary phase, and the cell density in the different culture media achieved maximum value (**Figure 3-1**). The growth of *T. petrophila* on xylan and avicel PH105 was slower than cellobiose, but higher than glucose. The growth on switchgrass was almost the same with glucose (**Figure 3-1**). This phenomenon indicated *T. petrophila* had efficient cellulase and xylanase, which broke cellulose and xylan to supply enough simple sugar residues for the growth of *T. petrophila*. Therefore, the cellulase and xylanase hydrolysis steps would not dramatically decrease the growth rate of *T. petrophila*. This capability in *T. petrophila* is beneficial in reducing the time for cellulosic biofuel production when using utilizing cellulosic materials as the substrates.

In conclusion, *T. petrophila* was able to utilize cellulose and hemicellulose without any pretreatment. It shows great potential as biofuel producer. The best growth was found from cellobiose and xylan followed by corn husks, glucose, switchgrass, avicel PH105 and wheat straw.

4.3 Cellulase and xylanase activities

T. petrophila had much higher cellulase activities compared to *T. maritima* and *T. neapolitana*. In *T. petrophila*, exoglucanase was only found in the supernatant of the culture media, except in the glucose medium, which had very little activity in the cell free extract. This finding indicates that exoglucanase is an extracellular enzyme which is secreted outside of cells to degrade the end of cellulose polysaccharide chains. The highest exoglucanase activity was found when *T. petrophila* was grown on xylan and cellobiose; thus xylan and cellobiose, not cellulose, were the best inducers to increase the expression of exoglucanase. Also, the end product cellobiose did not have the inhibition of cellulolysis. This result was not consistent with *Clostridium thermocellum*, *Trichoderma reesei* and other fungi (Holtzapple *et al*., 2004, Johnson *et al*., 1982 and Gusakov *et al*., 1992). This result shows that *T. petrophila* is an ideal microorganism for biofuel production as it can constantly produce biofuel during fermentation without end product inhibition and even induce cellulase expression.

Endoglucanase was both found in the cell free extract and culture supernatant. This result indicated that endoglucanase was both an extracellular and intracellular enzyme. The highest specific activity of endoglucanase was detected from the cellobiose and xylan cell free extracts. However, the total activities of endoglucanase in the culture supernatant were significantly higher than that from the cell free extract, indicating that the majority of endoglucanase was intracellular enzyme. Compared to the huge discrepancy of exoglucanase and extracellular endoglucanase activities among different cells grown on different substrates, intracellular endoglucanase activity was steady. Endoglucanase activity was also detected in the supernatant from avicel PH105, which proved that cellulose also can stimulate the expression of xylanase. No exoglucanase and endoglucanase activities detected when *T. petrophila* grew on avicel PH105. One possibility would be that endoglucanase and exoglucanase might be induced by xylan and cellobiose, but not cellulose. Avicel is microcrystalline cellulose, which does not contain any xylan or cellobiose, therefore, there was less or no cellulase secreted from avicel PH105 medium.

Xylanase activity was found both in the cell free extract and supernatant, indicating that it is an extracellular and intracellular enzyme. The highest xylanase activity was found in the cell free extract when *T. petrophila* was grown on cellobiose followed by xylan. Generally, xylanase activity from the cell free extract was much higher than in the supernatant, which was not consistent with the reference (Kim *et al.*, 2010), which stated that extracellular xylanase was the primary enzyme responsible for xylan degradation in ecosystems. The reason for this discrepancy may be because the filament structure from the attachment between *T. petrophila* cells and xylan was probably the primary interaction to transport xylanase to xylan. Those xylanases either bound on xylan particles and the cells or stayed inside of the cells. There was no free extracellular xylanase secreted to the culture medium. This result was confirmed with protein concentration determination in the supernatant in which there were five times less proteins detected in the xylan medium than in the cellulose medium. In conclusion, all the samples from xylan, cellobiose and glucose medium had generally higher xylanase activity than the one from the cellulose medium, except the avicel PH105 supernatant. Therefore, xylan and simple sugars can be added into a fermentation medium as inducers to stimulate the expression of xylanase genes.

In summary, in order to purify cellulases and hemicellulases from *T. petrophila*, xylan and cellobiose should be added into the culture medium as inducers to increase the yield of cellulases and hemicellulase. The majority of cellulases exist in the culture supernatant, while the majority of hemicellulases exist inside of the cells. Therefore, the culture supernatant should be targeted to harvest cellulase and biomass should be harvested for hemicellulase.

4.4 Attachment of *T. petrophila* cells to cellulose and hemicellulose

Many cells were observed on the surface and embedded in the xylan particles, indicating that adhesion between *T. petrophila* cells and xylan particles. There were filament structures between cells and xylan particles, which were probably the microtubules for transporting xylanase to xylan (Tolonen *et al*., 2010). However, only dead cells were observed in the attachment. One possibility is that, as the cells that were alive appeared to be the same color as xylan particles, when live cells attached to xylan, they overlapped and proved to be difficult to differentiate. Another possibility is that *T. petrophila* is a strictly anaerobic bacterium, and during the staining process, the cells were washed three times, which may have exposed them to oxygen before glutaraldehyde fixed the cells and killed the cells in the process.

During monitoring of the mechanism of attachment throughout the whole growth process, cells started to attach to xylan in the early lag phase. With further incubation, the number of attached cells increased along with the culture growth. The number of attached cells was at maximum when the culture entered its stationary phase. Once the culture stopped growing, the attached cells were released gradually from the xylan particles and no cells with filaments attached on xylan anymore. Adhesion to plant substrates is an important adaptation in xylanolytic bacteria that enhances xylan hydrolysis (Tolonen *et al*., 2010).

Unfortunately, there was no attachment observed when *T. petrophila* was grown on switchgrass, even though *T. petrophila* secreted more than five times the amount of proteins into the switchgrass medium than into the xylan medium. This may be due to the mechanism of utilizing cellulose by increasing the concentration of cellulases.

4.5 Metabolic end products of *T. petrophila*

H_2, CO_2, ethanol, acetate, lactate, succinate, formate and fructose were detected as end products during the growth of *T. petrophila*. The highest yields of H_2 and ethanol were found when *T. petrophila* was grown on xylan and cellobiose. Therefore, xylan and cellobiose were the best substrates for biofuel production. In all the cellulose substrates, *T. petrophila* produced the highest H_2 and ethanol when grown on corn husks, followed by switchgrass. Therefore, corn husks and switchgrass were the best biofuel feedstocks among all the celluloses tested in this project. The optimal pH for obtaining the highest yield of H_2 and ethanol is 8.0 to 8.5.

In the experiment, fructose was detected as the end product because glucose was not thermostable at high temperatures. It was transferred to fructose at 80^{o}C. This transformation has been confirmed by published data (Slimestad *et al*., 2006). D-glucose is transferred to enediol and further changed to D-fructose. The reaction also works from D-fructose to D-glucose (**Figure 4-1**). pH is found to be an

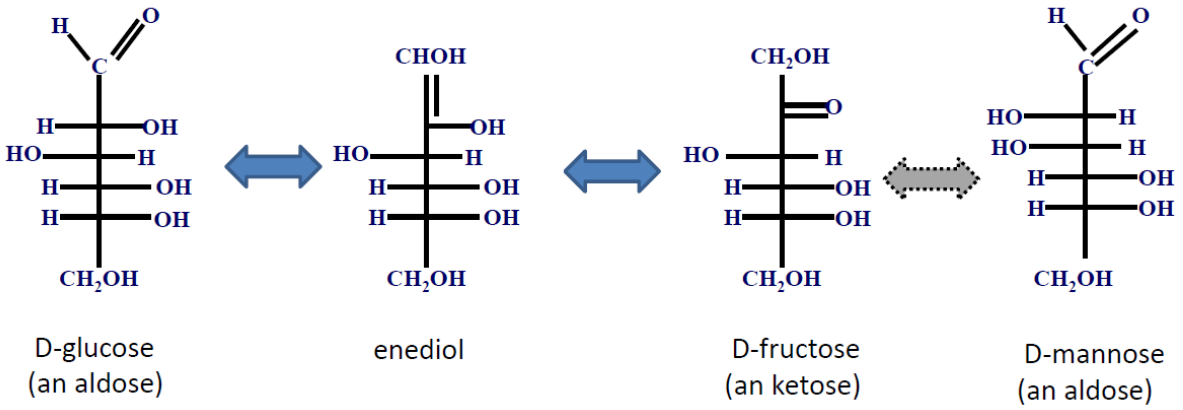

Figure 4-1. Non-enzymatic keto-enol tautomeric transformations, a start reaction in the decomposition of glucose (modified from Slimestad *et al*., 2006). The epimerization reactions between the aldoses (D-glucose and D-mannose) and the ketose (D-fructose) are dependent on temperature, pressure and pH of solvents. Fructose was formed from glucose (5mM) in water (pH 5.0), HEPES buffer (pH 7.0) and medium (pH 7.0) at 80^{o} C by using HPLC (**Table 3-6**), but no mannose was detected.

important factor to affect the reaction rate. Without a stable pH, the reaction would occur much more easily and faster.

4.6 Proteins expression when *T. petrophila* grew on cellulose and hemicellulose

From the proteomics result, 930 proteins were quantified when *T. petrophila* was grown on avicel PH105, switchgrass and xylan. The up-regulated enzymes for cellulose utilization were certain ABC transporters, S-layer proteins and membrane binding proteins. There were no functional proteins up-regulated inside the cells when *T. petrophila* was grown on cellulose. This result may be because most of the cellulases are extracellular enzymes that cannot be found by using biomass for proteomics analysis. Therefore, the future experiment plan is to do supernatant proteomics analysis. When *T. petrophila* was grown on xylan, not only the ABC transporters, S-layer proteins and membrane binding proteins, but also the functional proteins such as arabinofuransidase and acetyl xylan esterase were up-regulated.

Furthermore, the unique bands from xylan and cellobiose supernatant shown on SDS-PAGE were consistent with their higher cellulase and xylanase activity from the xylan and cellobiose medium. Based on the size of protein bands shown on SDS-PAGE, those bands were suspected to be S-layer proteins and ABC transporters, which were acknowledged to be responsible for attachment assembling and transporting a wide range of substrates to the inner membrane (Tolonen *et al*., 2010; Conner *et al*., 2005), respectively. But further experiment will be required to confirm those protein bands.

4.7 Metabolic profiling of the cellulose utilization by *T. petrophila*

The entire metabolic pathway included two stages: hydrolyzing down cellulose and hemicellulose into simple sugars and further metabolizing simple sugars into end products, including H_2, CO_2, ethanol, succinate, lactate, acetate and formate. During the second stage, glucose from cellulose went to the Embden-Meyerhof pathway (EMP). Since *T. petrophila* cannot use xylose as a substrate, when xylan was hydrolyzed down to xylose, glucose and arabinose, glucose went to the EMP pathway, and arabinose entered the pentose phosphate pathway (PPP). The end products of PPP were glyceraldehyde-3-phosphate and fructose-6-phosphate, which still entered EMP.

In this metabolic pathway, all the enzymes were detected by proteomics analysis, and their coding genes were also found in the genome sequence of *T. petrophila* except exoglucanase, fumarate reductase and ribulokinase. Exoglucanase was not anotated in the *Thermotoga* genus, so it was not found in either the proteomics data or genome of *T. petrophila*. Ribulokinase also did not exist in either the proteomics data or the genome of *T. petrophila*. Fumarate reductase was found in the

genome sequences, but it was not detected by proteomics, perhaps because fumarate reductase is an intergral membrane protein not detectable by proteomics analysis from cell biomass (Iverson *et al*., 1999). Therefore, proteomics analyis using a culture supernatant is really needed to confirm the metabolic pathway.

The key enzymes related to degrading cellulose, including endoglucanase and β-glucosidase, were up-regulated when *T. petrophila* was grown on cellulose. The key enzymes related to degrading xylan, including endoxylanase, β-xylosidase, arabinofuranosidase, acetyl xylan esterase and arabinose isomerase, were up-regulated when *T. petrophila* was grown on xylan. However, endoxylanase and acetyl xylan were still up-regulated when *T. petrophila* was grown on switchgrass, because switchgrass was a material that naturally contains xylan; thus, those genes were also induced in some degree.

4.8 Recommendations for future research

4.8.1 Proteomics of culture supernatant

From the proteomics analysis on biomass of *T. petrophila*, cellulases, xylanases, S-layer proteins, ABC transporters and inner proteins of binding proteins were up-regulated under cellulosic material growth condition. However, from the cellulase and xylanase enzyme activities from the supernatant and cell free extract, majority of cellulase and xylanase are extracellular enzymes. Therefore, the proteomics analysis on the culture supernatants from different growth media with different carbon sources, including cellobiose, xylan, switchgrass and xylan will be more indicative to quantify the overexpressed cellulase and xylanase. It may also reveal the unique extracellular proteins in the culture media, which may not exist in the cell free extract. Those enzymes might be the crucial ones to break down insoluble cellulosic substrates and their expression level can indicate the capability to utilize cellulosic materials among all the tested microorganisms.

4.8.2 Microarray

A microarray should be used for detecting the transcription level of all enzymes under various media with cellobiose, xylan, switchgrass and xylan as carbon sources, respectively. The microarray result can reveal all the expressed genes when *T. petrophila* grows on cellulosic materials and be compared to the up-regulated enzymes involved in utilization of cellulosic materials from proteomics result. Also microarray is used to verify the consistency of transcription levels with the translation levels from proteomics result, which will help to understand the entire cellulosic metabolism and the mechanisms of regulation under different growth conditions.

4.8.3 qPCR verification of proteomics and microarray

Based on the tentative cellulosic metabolic pathway in *T. petrophila* obtained by using genome sequence, end product measurement and proteomics analysis, key enzymes were identified and their expression and functions need to be further confirmed by qPCR. According to the available genome sequence, the primers of genes encoding those key enzymes will be designed for qPCR to verify their transcription levels observed from the microarray data. The expression level of mRNA of key enzymes can also reveal the ability to utilize different cellulosic materials.

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