Utilization of Cellulosic Materials by *Caldicellulosiruptor* species

by

Xia Ling

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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.

I understand that my thesis may be made electronically available to the public.

Abstract

Caldicellulosiruptor is a genus of thermophilic, anaerobic, Gram-positive, non-spore-forming cellulolytic bacteria and is capable of fermenting a wide spectrum of carbohydrates. Both *Caldicellulosiruptor saccharolyticus* and *Caldicellulosiruptor kristjanssonii* were found to be able to use the raw cellulosic material，switchgrass，for producing H_2. Therefore, experiments were designed and conducted to select the optimal substrate and investigate the parameters involved in the fermentation process. *C. saccharolyticus* and *C. kristjanssonii* were cultivated on different sugars including glucose, xylose, cellobiose, xylan, avicel (PH105), and switchgrass. The highest H_2 production was obtained on glucose by *C. saccharolyticus* with 2.9 mol (H_2)/mol (glucose), whereas the yield by *C. kristjanssonii* was only 1.7 mol (H_2)/mol (glucose). Moreover, the sample of *C. saccharolyticus* on xylose gained higher cell density, compared with glucose. During the cell growth, there was a small decrease of the H_2/acetate ratio from 20 hour's data to 40 hour's along with the existence or significant increase of lactate. This suggested that a possible direction shift of metabolism happened in between the two time points, perhaps due to the H_2 end product inhibition. A scanning electronic microscope (SEM) and confocal microscope were used to detect the adhesion between cells of these two microorganisms and cellulose and hemicellulose substrates in real time. A series of microscope pictures revealed non-specific and specific

attachments between the cells and cellulosic materials, such as avicel and switchgrass, as well as the attachment behavior corresponding to the amount of H_2 production. Proteomic analysis of *C. saccharolyticus* showed the existence of proteins related to mobility of the microbes such as flagella and chemotaxis. Other proteins found , that contribute to the attachment between the cell and substrates, are the family 3 cellulose-binding module (CBM 3), the fibronectin-binding-A domain-containing proteins, the s-layer proteins, and a lysine motif protein. Groups of proteins like glycoside hydrolases (GHs), alcohol dehydrogenase (ADH) and hydrogenase that are responsible for the breakdown of cellulose and hemicellulose substrates, and the production of ethanol and H_2, were also found in the proteome.

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Chapter 1 Introduction

1.1 Biofuel production

According to the 2010 International Energy Report (U.S. Energy Information Administration, 2010), the consumption of world-marketed energy will grow by 49 % from 2007 to 2035. Total world energy use will rise from 495 quadrillion British thermal units (Btu) in 2007 to 590 quadrillion Btu by 2020, and to 739 quadrillion Btu in 2035, with a 43 % increase of world energy-related carbon dioxide emissions from 29.7 billion tonne in 2007 to 33.8 billion tonne in 2020 and 42.4 billion tonne in 2035. These increases are in progress, even though the recession that started December 2007 had a significant influence on energy consumption (U.S. Energy Information Administration, 2010). Among all the increased consumption of energy, with the assumption that world oil prices will remain relatively high through most of the projection period, liquid fuel consumption is expected to increase at an average annual rate of 0.9 % from 2007 to 2035, whereas total energy demand will increase by 1.4 % per year. Renewable resource are the fastest-growing source of world energy, with consumption increasing by 2.6 % per year; hydroelectricity and wind provide the largest shares in this market. The expected increase of nuclear energy use before this year's Fukushima Nuclear Power Plant accident was 2.6 trillion kilowatthours in 2035(U.S. Energy Information Administration, 2010).

Meanwhile, biohydrogen fuel offers us a brand new and attractive energy future, it is environmentally problem-free (water as the only end-product; hydrogen means "water-

former" in Greek), has the highest energy content of the known fuels (142 kJ/g or 61,000 Btu/lb) (Boyles, 1984), and is renewable. The ways of producing hydrogen include water electrolysis, thermochemical, radiolytic, and biological processes (Kothari *et al*., 2008), among which biological processes have significant advantages in energy cost, environmentally friendliness, and sustainability. Among the biological processes—biophotolysis, photo-fermentation, fermentative hydrogen production (FHP), and hybrid systems (Das and Veziroğlu, 2001)—fermentative H_2 production is attracting much attention because it can produce a wide variety and easily gained renewable resources. The materials containing cellulose and hemicellulose are the most extensively studied and popular resource for fermentative H_2 production.

One excellent example of a cellulosic material that could be used is a summer perennial grass called switchgrass (*Panicum virgatum*). Switchgrass is one of the three dominant native grasses found on the North American tallgrass prairie prior to settlement. It has been under investigation in Canada as a bioenergy crop since 1991(Samson, 2007), and was selected as a model for biomass-to-biofuel conversion by the Bioenergy Science Center (funded by the U.S. Department of Energy; http://bioenergycenter.org/). It was most commonly used for livestock forage in the south-central United States before, but there are plans to encourage its growth in Ontario. It is estimated that once fully established in Ontario, switchgrass can typically produce 8-12 tonne/ha of harvestable dry matter each year. Typically switchgrass and grain corn have similar energy contents on a dry matter basis of approximately 18.5 GJ/tonne. Assuming a harvested grain corn yield of 6.5 tonne/ha and switchgrass yield of 10 tonne/ha, switchgrass produces 185 GJ/ha of energy,

while grain corn produces only 120 GJ/ha. Thus, the net energy gain per ha is 73% higher for switchgrass than grain corn if the fossil energy inputs used for crop production are subtracted from energy output (Samson, 2007). Switchgrass is best grown as a one-cut per year crop, and can be harvested any time after fall, which is when grass is dried out and adequate nutrient and carbohydrate translocation to the root reserves has occurred for winter survival. Consequently, only the insoluble cellulose part is left in the grass when it is harvested. According to the owner of Nott Farm (Clinton, Ontario), switchgrass is capable of producing high yields with very low applications of fertilizer, and need no fertilizers or sewing after the first two years of growth. In addition, it is tolerant of poor soils, flooding and drought, which are widespread agricultural problems in the Southeast, making switchgrass an ideal crop for efficient use marginal and poor land that cannot be used for food-crop growth. Costs to grow and harvest switchgrass are approximately $40-$50/tonne in Ontario (Samson, 2007). Switchgrass' perennial nature, stand longevity, adaptation to marginal and non-fertilized farmlands, low input requirements and high productivity make it stand out as the lowest cost means to capture and store solar radiation in a field crop in Ontario (Samson, 2007).

The usual application scheme to transfer biomass into biofuel involves several steps: First the solar energy is collected by the plant and stored as forms of biomass (in cell walls); then the cell wall polymers are destructed into component sugars (pretreatment and saccharification), and then the sugars are converted to biofuels (fermentation) (Rubin, 2008) as shown in **Figure 1.1**.

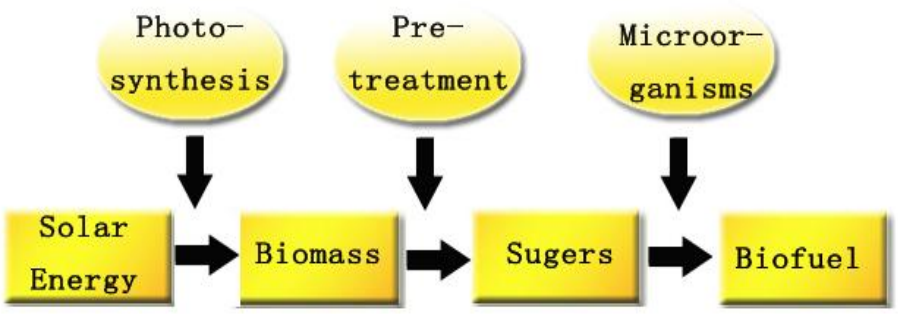

Figure 1.1The application scheme of biomass biofuel. The application scheme of biomass including several steps: plant stored the energy from the sun through photo-synthesis in the cellulose and hemicelluloses biomass; the sugar could be used by microbes was released from biomass raw materials through the pretreatment of the materials; then the biofuel such as H_2 and ethanol were produced by the microorganisms with sugars gained from last step.

During the transfer of the cellulosic and lignocellulosic biomass into the hydrogen or ethanol as fuel, hydrolysis is the rate-limiting step whole process. Cellulose is a linear, insoluble biopolymer composed of repeating β–D-glucopyranose residues linked by β-1, 4 glycosidic bonds (Desvaux, 2003). Enzymatic cellulose hydrolysis is generally a slow and incomplete process. In complex biological systems such as rotting trees and plant debris in soil, the decomposition of cellulose may last as long as months because plant cellulose always exists in a highly crystalline form (Schwarz, 2001). The significant factor of biofuel production from biomass is the efficient conversion of the biomass into sugar that can be used by microorganisms directly. This conversion remains a formidable challenge because of the high degree of polymerization of cellulose. The release of such long-chain polysaccharides as cellulose and hemicellulose, and the subsequent hydrolysis of these polysaccharides into their component 5- and 6-carbon chain sugars are involved in the breakdown of biomass (Rubin, 2008; Van Wyk, 2001). Usually, the conversion involves physical and chemical pretreatment before the biological hydrolysis step with certain special enzymes. As the approaches used in many biofuel production studies are costly and not very efficient, thermal and chemical pretreatments are applied to solubilize or release the sugars and thus increase the accessibility by microorganisms (Kostamo *et al*., 2004; Ranatunga *et al*., 1997). A typical chemical pretreatment of biomass materials involves diluted acid and heat, mainly as sulfuric acid at a temperature as high as 150-200˚C (Panagiotopoulos *et al.*, 2011). On the other hand, certain microorganisms can be used in third-generation biofuel production method that involve direct use of high efficiency cellulase and hemicellulase without particular chemical or thermo pretreatments. The

biologic scheme for these methods is to search for novel microorganisms that can take biomass as a nutrition source and to study the special characteristics of the enzymes involved. The free-living organisms and symbiotic animal–microbe consortia invariably present in biomass–rich environments are always the targets of researchers (Rubin, 2008). Potential Research and Development-driven improvements should offer large cost savings, in particular through increasing cellulose hydrolysis yield, halving cellulase loading, eliminating pretreatment and incorporating consolidated bioprocessing (CBP), hydrolysis and fermentation into one-step (Lynd *et al*., 2005 and 2008). The contributions of such improvements in reduced production costs are listed in **Figure 1.2**(data adapted from Lynd *et al.*, 2008).

The hydrolysis of cellulose requires the co-operation of three classes of cellulolytic enzymes: cellobiohydrolases (CBH, EC 3.2.1.91), endo-β-1, 4-glucanases (EG, EC 3.2.1.4), and β-glucosidases (BG, EC 3.2.1.21) (Viikari *et al*., 2007). Cellulase with high adsorption capabilities, high catalytic efficiencies, high thermal stability, and low end-product inhibition is always the "ideal" enzyme people look for in both fungi and bacteria. However, almost all cellulolytic strains identified so far are low in one or more types of glycoside hydrolases (GH) (Maki, 2009). Much research has been done on various kinds of fungi and bacteria. A cost-effective system built to produce hydrogen and ethanol from cellulosic biomass requires highly efficient hydrolysis activity enzymes. Several microorganisms have been found to harbor cellulose-related enzymes, including mesophilic, thermophilic, and hyperthermophilic ones. These microbes have potential to serve in biomass biofuel production.

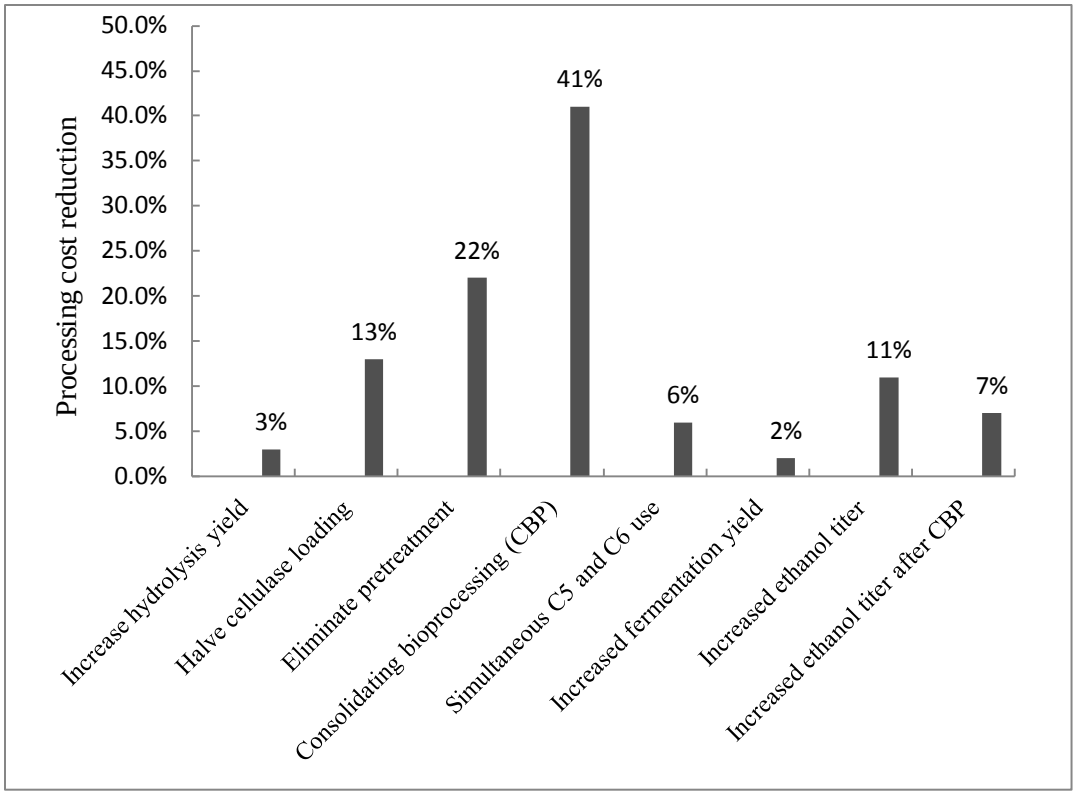

Figure 1.2 Reduction in processing costs for various technological advances. The percentage cost reduction of various techniques used to reduce the cost of biofuel production were shown in bars, the techniques includes: increasing cellulose hydrolysis yield, halving cellulase loading, eliminating pretreatment, incorporating consolidated bioprocessing, simultaneous C5 and C6 sugar use, increased fermentation yield, increased ethanol titer, and increased ethanol titer after Consolidated Bioprocessing (CBP) (data adapted from Lynd *et al.*, 2008).

Thermostability is usually defined as the retention of activity after heating at a chosen temperature for a prolonged period, but it is so far still a relative term without a clear definition (Viikari *et al*., 2007). Cellulases from hyper/thermophile have special capability to keep their catalysis activities at high temperature, and has higher specific activity that helps decrease the amount of enzyme needed for the cellulose degrading, and it also has higher stability that allows elongated hydrolysis time. There was less risk of contamination from most of the microorganisms could not survive at high temperatures (Adams, 1990; Claassen and Vrije, 2006; Levin *et al*., 2009; Viikari *et al*., 2007; Wal Van *et al*., 2002; Werken *et al*., 2008). Industrial bioconversions of lignocelluloses usually involve application of high temperature, and there are other problems such as cooling from high temperature, pumping of oxygen or stirring, and, neutralization from acidic or basic pH during the industry process, that always act as the barriers to economic-scaled application of cellolase to produce biomass biofuel (Grishutin *et al*., 2004; Maki *et al*., 2009). The unique characteristics of hyper/thermophlic cellulase perfectly meet the requirements of high temperature and its capability of keeping active under high temperatures increases flexibility for process configurations and cut the cost of cooling (Viikari *et al*., 2007).

The term "third generation of biofuel" was recently proposed by researchers. Unlike previous two generations' methods, which involved multiple-step process including cellulase production, substrate hydrolysis, and fermentation, this third generation uses only one step with the aid of cellulolytic (and hemicellulolytic) microorganisms (Demain *et al*., 2005; Lynd *et al*., 2002 and 2005). Which saves such steps as extensive pre-treatment of the cellulosic materials by steam-explosion and/or acid treatment and a following step to

add of exogenously produced cocktails of cellulolytic enzymes (Lynd *et al*., 2002 and 2005). Moreover, it has many advantages financially and environmentally, since the seeds or grains such as wheat used in the production of first generation biofuel will be saved, preventing possible food shortages and food price rises (Anonymous, 2000, A Framework for Decision Maker. UN Biofuel Report).

1.2 Cellulolytic microorganisms

Cellulolytic species have been found within the phyla Proteobacteria, Actinobacteria, Spirochaetes, Firmicutes, Fibrobacteres, and Bacteroids, among which approximately 80 % of the isolations are from Firmicutes and Actinobacteria (Bergquist *et al*., 1999; Hamilton-Brehm *et al*., 2010). The majority of the gram-positive cellulolytic bacteria has been found within Firmicutes and belongs to the class *Clostridia* and the genus *Clostridium* (Carere *et al*., 2008).

Lignocellulolytic enzyme-producing fungi come in many kinds and are widespread, such as species from the ascomycetes (e.g., *Trichoderma reesei*), basidiomycetes including white-rot fungi (e.g., *Proteobacteria chrysosporium*), brown-rot fungi (e.g., *Fomitopsis palustris*), and a few anaerobic species (e.g., *Orpinomyces sp.*) that degrade cellulose in the gastrointestinal tracts of ruminant animals as well (Dashtban *et al*., 2009; Wyman *et al*., 2005; Yoon *et al*., 2007).

Many studies on cellulase have focused on the mesophilic fungus *T. reesei*. Eight major cellulase genes have been identified from the *T. reesei* genome, among which there are two

cellobiohydrolases (CBH I and II, e.g., Cel7A and Cel6A), and six endoglucanases (EG I–VI, e.g., Cel7B, Cel5a, Cel12A, Cel45A, Cel61A, Cel74A) (Foreman *et al*., 2003). And Cel7A (CBHI) is the major cellulose of *T. reesei*; Cel7A constitutes about 60 % of the cellulases expressed and has a capability to hydrolyze solid cellulose (Nidetzky and Claeyssens, 1994; Stalberg, 1991). In addition to cellulolytic/hemicellolytic activities, higher fungi such as basidiomycetes (e.g., *Phanerochaete chrysosporium*) have unique oxidative systems that, together with lignolytic enzymes, are responsible for lignocellulose degradation (Dashtban *et al*., 2009). *P. chrysosporium* has also been found to be one of the most efficient lignin-degrading microorganisms that have been studied (Moredo *et al*., 2003). Fungi attract most of the attention for their capability to produce copious amount of extracellular cellulose, and this kind of cellulase is often less complex compared with cellulase from bacteria source. However, increasing attention is focused on the bacteria host cellulase because bacteria's higher growth rate allows higher recombinant production of enzymes, and this cellulase has better adapter capabilities to extreme environments and stress (there are many extremophiles in bacteria). Moreover, the bacteria can express multi-enzyme complex that has a co-operation effect of degrading cellulose. A number of bacteria strains from thermophilic or psychrophilic, alkaliphilic or acidiophilic, and halophilic bacteria can serve as novel and unique sources of cellulase and hemicellulase (Maki, 2009).

1.3 *Caldicellulosiruptor* genus

Caldicellulosiruptor is a genus of thermophilic, anaerobic, gram-positive, non-spore-forming cellulolytic bacteria and is capable of fermenting a wide spectrum of carbohydrates (Rainey *et al*., 1994).

So far, there are nine *Caldicellulosiruptor* organisms have been identified: *C. saccharolyticus* (Rainey *et al*., 1994), *C. lactoaceticus* (Mladenovska *et al*., 1995), *C. owensensis* (Huang *et al*., 1998), *C. acetigenus* (Yang *et al*., 2010), *C. bescii* (DSM6725S) (Yang *et al*., 2009), *C. kristjanssonii* (Bredholt *et al*., 1999), *C. kronotskyensis* (Miroshnichenko *et al*., 2008), *C. hydrothermalis* (Miroshnichenko *et al*., 2008) and *C. obsidiansis OB47* (Hamilton-Brehm *et al*., 2010). These bacteria have been isolated mostly from neutral or slightly alkaline geothermal springs in New Zealand, Iceland and California. Among them, *C. bescii* (DSM6725S) was renamed from *Anaerocellum thermophilum*, and was reclassified recently in 2010 (Yang *et al*., 2010), *C. saccharolyticus* was identified as a *Clostridium* species (Luthi *et al*., 1991; Onyenwoke *et al*., 2006; Rainey *et al*., 1994) and was reclassified in 1994; and *C. acetigenus* was reclassified from *Thermoanaerobium acetigenum* in 2006 as well (Onyenwoke *et al*., 2006).

So far, eight *Caldicellulosiruptor* organisms' genomes have been sequenced; they are *C. saccharolyticus* (Van de Werken *et al*., 2008), *C. obsidiansis OB47* (Elkins *et al*., 2010), *C. bescii* (Kataeva *et al*., 2009), *C. hydrothermalis* (Blumer-Schuette *et al*., 2011), *C. kristjanssonii* (Blumer-Schuette *et al*., 2011), *C. kronotskyensis* (Blumer-Schuette *et al*., 2011), *C. owensensis* (Blumer-Schuette *et al*., 2011), and *C. lactoaceticus* (Blumer-

Schuette *et al*., 2011). According to the results of both cross-species DNA-DNA hybridization and 16S rRNA analysis between the seven species from *Caldicellulosiruptor* (*C. saccharolyticus*, *C. bescii*, *C. hydrothermalis*, *C.owensensis*, *C. kronotskyensis*, *C. lactoaceticus*, and *C. kristjanssonii*), *C. saccharolyticus* showed significant phylogenetic divergence from genus *Caldicellulosiruptor* (94.4 to 96.6% rRNA identity), which may due to its origin in New Zealand (Blumer-Schuette *et al*., 2010).

All *Caldicellulosiruptor* species are able to grow on CMC, and there is at least one GH5 enzyme (potentially endo-acting cellulase) present in their genomes (Cantarel *et al*., 2009). Blumer-Schuette and co-workers also showed that *C. hydrothermalis* and *C. owensensis* did not grow well on microcrystalline cellulose (avicel) and had less diverse avicel-induced secretome profiles compared with other *Caldicellulosiruptor*s. In addition to the catalytic portion of cellulase enzymes, a particular crystalline cellulose-binding carbohydrate binding motif (CBM) family, CBM 3, is present in multi-domain enzymes from *C. bescii*, *C. kristjanssonii*, *C. kronotskyensis*, and *C. saccharolyticus*. However, CBM 3 was absent from the genomes of *C. hydrothermalis* and *C. owensensis*, and these two species also lack exo-acting cellulases, indicating that the CBM 3 family may be an additional determinant for a *Caldicellulosiruptor* species' ability to hydrolyze crystalline cellulose (Blumer-Schuette *et al*., 2010).

As to the capability of using hemi-cellulose (xylanases, mannanases and xyloglucanases), genes related to the GHs that hydrolyze the β-1,4-xyloside linkages of xylan, the β-1,4-mannoside linkages of mannan, and the β-1,4-glucan linkages were found in most of the

seven *Caldicellulosiruptor* species in the same study, but no two *Caldicellulosiruptor* species have the same GH profile, and the genes related to CBM families capable of binding to xylan (CBM 6, 9, 22, and 36) were found in all the seven species. Another mannan binding CBM family (CBM 27) was found in *C. hydrothermalis* and *C. lactoaceticus* (Blumer-Schuette *et al*., 2010).

C. bescii strain DSM 6725 is a thermophilic cellulose-degrading organism that can resist the highest temperature known in *Caldicellulosiruptor*, growing at up to 90 ˚C (pH 7.2) and degrades crystalline cellulose and xylan, as well as untreated plant biomass, including potential bioenergy plants such as poplar and switchgrass. However, it does not grow on xylose or pectin (Yang *et al*., 2009), and it has the most similar genome to *C. saccharolyticus* DSM 8903 (Kataeva *et al*., 2009).

1.4 *C. saccharolyticus* and *C. kristjanssonii*

C. saccharolyticus is the most extensively studied of all the *Caldicellulosiruptor* species known. It was originally isolated from a thermal spring in New Zealand. It is a strict, fermentative anaerobe, growing at temperature between 45 and 80˚C ($T_{optimum}$ =70 ˚C) and pH 5.5 to 8.0 (Optimum 7.0), nonmotile, nonflagellated, oval-ended straight rod, 0.4–0.6μm by 3.0–4.0μm, occurring singly and in pairs (Rainey *et al*., 1994). *C. saccharolyticus* is capable of fermenting all kinds of carbon sugars, including cellulose, hemicellulose, pectin, and starch (Van de Werken *et al*., 2008), and smaller sugars such as arabinose, fructose, galactose, glucose, mannose and xylose (Vanfossen *et al*., 2009). Moreover, it is one of the very few microorganisms that can utilize cellulose without pretreatment.

Hydrogen is one of the main fermentation end products of *C. saccharolyticus*. It is estimated that thermophilic bacteria and archaea can produce H_2 up to the theoretical maximum 4 mol /mol (-hexose) as the Thauer limit (Kengen *et al*., 2009） while generally mesophilic (co-)cultures reach only H_2 yields of less than two mol/mol(-hexose) (Kleerebezem and van Loosdrecht, 2007). *C. saccharolyticus'* highest hydrogen yields could reach 82 to 90 % of theoretical maximum when it is at low growth rates (De Vrije *et al*., 2007). When *C. saccharolyticus* grows on glucose, a relatively larger part of the consumed glucose is used for maintenance and the hydrogen yields are dependent on the growth rate of the bacteria (De Vrije *et al*., 2007). Other fermentation end products of *C. saccharolyticus* include acetate, lactate, and trace amounts of ethanol, but hydrogen sulfide is not produced (Rainey *et al*., 1994; Van Niel *et al*., 2003).

C. kristjanssonii, the other not so extensively studied *Caldicellulosiruptor* organism, first reported by Sylvia Bredholt (1999), was described as nonmotile rods cells with rounded ends and two sub-terminal flagella, 2.8-9.4 μm by 0.7-1.0 μm that occur singly, in pairs or in short chains, growing under temperature between 45 ˚C and 82 ˚C with an optimum 78 ˚C and in pH 5.8-8.0 with an optimum 7.0. *C. kristjanssonii* is able to grow on wide range of carbon substrates including avicel, cellobiose and a wide spectrum of monosaccharide. The whole genome of *C. kristjanssonii* has been sequenced but the detailed annotation information hasn't been available yet (Blumer-Schuette *et al*., 2011). There are very limited studies on *C. kristjanssonii* so far after the isolation of the organism many years ago. It is the difference of the gene information from well-studied *C. saccharolyticus* and great

capability on cellulose and hemicelluloses utilization that attract our attention to work further on this microorganism.

The outstanding hydrogen production capability of *C. saccharolyticus* is based on many factors. According to the available 2.97-Mb whole genome sequence (http://genome.ornl.gov/microbial/csac), within a 35.2 % GC content and initial annotated 2695 ORFs, approximately 20 % of the ORFs are involved in carbohydrate degradation, transport, and metabolism, suggesting that *C. saccharolyticus* will yield a plethora of industrially relevant GHs (Van de Werken *et al*., 2008; Vanfossen *et al*., 2008). NMR analysis of (13)C-labeling patterns showed that the Embden-Meyerhof (EM) pathway is the main route for glycolysis in *C. saccharolyticus* (De Vrije *et al*., 2007), and the genomic sequences showed the present of the components of a complete EM pathway as well, identifying an ROK family glucokinase (Csac_0778), 6-phosphofructokinase, (Csac_2366 and Csac_1830), a bifunctional phosphoglucose/phosphomannose isomerase (Csac_1187), fructose-1,6-bisphosphate aldolase (Csac_1189), and pyruvate kinase (Csac_1831), as well as pyruvate-phosphate dikinase (PPDK) (Csac_1955) (Van de Werken*et al*., 2008). There is, however, no evidence of the present of an Entner-Doudoroff pathway or oxidative branch of the pentose phosphate pathway (PPP) (Van de Werken *et al*., 2008). It was even suggested at the very beginning, when *C. saccharolyticus* was still named as *Clostridium thermocellum*, that it produce cellulosome to degrade cellulose and hemicellulose (Sissons *et al*., 1987), but the genome sequences did not show the presence of genes encoding the typical molecular components of a cellulosome such as dockerin domains and scaffolding proteins. Gene clusters (Csac_1076 to Csac_1081, Csac_1089 to Csac_1091) containing

genes encoding cellulase precursors and an extracellular cellulase (Csac_0678) which may assist the cellulose hydrolyzing were found as well (Van de Werken *et al*., 2008). Recent proteomic analysis have also detected the present of extracellular cellulases of *C. saccharolyticus* (Muddiman *et al*., 2010), among which, CelB (CelB was annotated as a bi-functional cellulase that has both cellobiohydrolase (GH10) and endoglucanase (GH5) activities (Saul *et al*., 1990)) was cloned and expressed in *E.coli*. A recombinant biocatalyst for hydrolyzing lignocellulosic biomass at as high a temperature as 80 ˚C was also obtained (Part *et al*., 2011). Multiple genes involved in xylan (hemicellulose) utilization were detected from the genome such as gene cluster Csac_2404 to Csac_2411 and Csac_0203 to Csac_0205, and the former one showed more significantly up-regulated level when the organism is grown on a xylan substrate (Van de Werken *et al*., 2008).

Phosphoenolpyruvate-dependent phophotransferase systems (PTS), ATP-binding cassette (ABC) transporters, and proton-linked transport systems are all important components in microbial uptake mechanisms for sugar transport (Koning *et al*., 2002; Nataf *et al*., 2009). Various distributions and numbers of these transport mechanisms in different microorganisms were detected from genome sequence information: for example some anaerobic, extremely thermophilic bacteria, such as *Thermotoga maritima* (Conners *et al*., 2005; Nelson *et al*., 1999) and *Carboxydothermus hydrogenoformans* (Wu *et al*., 2005), lack a PTS in lieu of ABC transporters for sugar uptake (Albers and Driessen, 2008; Conners *et al*., 2005; Wu *et al*., 2005), while other thermophiles, such as *Thermoanaerobacter tengcongensis*, contain one PTS in addition to multiple ABC transporters (Wang *et al*., 2004). *C. saccharolyticus* has most of the 24 putative

carbohydrates ABC transporters have been known and a single PTS for carbohydrate specificities for transport systems as well (Amy *et al*., 2009). Zsófia Herbel and co-workers' (2010) experiments showed that when *C. saccharolyticus* uses cellulose substrates, an induction of the cellulase enzyme complex(es) is needed, the cellulase enzyme complex(es) needed by *C. saccharolyticus* to degrade cellulose can only be triggered by various monomeric sugars or yeast extract, and it was found that the bacterium will use the monomeric sugar first within a few days when the culture is grown on both monosugar and cellulose, and it begins to use cellulose in the following period, and H_2 production resumed for an extended period of time. Thus the amount of H_2 production, to a considerable degree, depends on the level of induced cellulase, which depends on the initial concentration of the monomeric sugar. The result of another experiment also supported this conclusion as there was no growth detected when the cellulose was supplied as the sole carbon source from the beginning (Herbel *et al*., 2010). Moreover, Amy and co-workers (2009) showed that genes Csac_0692-0694 were up-regulated only in a monosaccharide mixture. We will get a better idea about the induction of cellulase if we dig deeper and compare the transcriptome results of *C. saccharolyticus* on monosaccharide and polysaccharide or cellulose substrates.

As mentioned, the H_2 yield of most studies so far was lower than two mol/mol glucose. It is understood that from the evolutionary perspective, the low yield is a result of the fact that H_2-producing microorganisms have developed their metabolic pathways preferentially for cell growth rather than H_2 synthesis. Nevertheless, people are expecting to significantly improve the H_2 yield by metabolic engineering with the help of a good understanding of

metabolic pathways (Cai *et al*., 2011). There are many factors that affect the production of H_2, such as the water content of biomass materials, the increasing volume of carbon dioxide gas during the fermentation, and especially the partial pressure of H_2. The mechanisms behind this are that the H_2 formation pathways are sensitive to the concentration of H_2 and other end-products. As a reaction to the increase of H_2 concentration, the H_2 synthesis will decrease, and the metabolic pathways will shift to the direction that will produce more reduced products. Examples are the synthesis of succinate and formate use CO_2, pyruvate, and reduced nicotinamide adenine dinucleotide (NADH) via the hexose monophosphate pathway (Cai *et al*., 2011; Das and Veziroğlu, 2001; Willquist *et al*., 2009). *C. saccharolyticus* is also a better candidate for a hydrogen producer than other hyper/thermophilic hydrogen producing species because of its greater tolerance of hydrogen inhibition. It is detected on species such as *Pyrococcus furiosus* and *T. maritima* that H_2 and acetate inhibit the growth of microorganisms (Van Niel *et al*., 2003; Verhaart *et al*., 2010). However, the same H_2 levels that were observed will inhibit the total grown of *P. furiosus* on pyruvate and *T. maritima* grown on glucose only diminish *C. saccharolyticus'* growth on sucrose by 7 % (Schafer and Schonheit, 1991; Schroder *et al*., 1994; Van Niel *et al*., 2003).

The adhesion between the cellulolytic organism cell and cellulose can be summed as non-specific adhesion and specific adhesion. The former one is the results of the van der Waal's forces that include hydrophobic, ionic, and electrostatic interaction with the solid substrate (Busscher and Weerkamp, 1987; Pell and Schofield, 1993). While the specific attachment is associated with the specific characteristics of the cell which could be summarized as

several structures such as fimbriae and pili adhesins, cellulosomes, carbohydrate epitopes of bacterial glycocalyx layer, and enzyme binding domains that as cellulose binding protein (CBP) or cellulose binding domain (CBD) (Miron *et al* ., 2001).

In a study of Morris and Cole (1987), bacterial adhesion to cellulose was measured for 13 cellulolytic and 10 non-cellulolytic, xylan utilizing strains of the ruminal bacterium *Rurninococcus albus*. The results show that although both cellulolytic and non-cellulolytic strains have the carboxymethylcellulase (CMCase) activity, only one non-cellulolytic strain showed some adhesion to the cellulose material, which may due to a specific enzyme activity whose function, is not yet known. Most cellulolytic strains bind to the substrate. Other studies by Jensen *et al*. (2009) prove that instead of being related to the concentration of total or planktonic biomass, the first-order hydrolysis rates of cellulose correspond to the concentration of attached bacteria with either enriched leachate or rumen fluid after inoculation.

It is interesting that the gene analysis mentioned above showed that the *C. hydrothermalis* and *C. owensensis* did not grow well on microcrystalline cellulose (avicel) and the cellulose-binding carbohydrate binding motifs (CBM) family, CBM3 happened to be absent from their genome sequences but was found in all other five *Caldicellulosiruptor* species (*C. saccharolyticus, C. bescii, C. kronotskyensis*, and *C. lactoaceticus, and C. kristjanssonii*) studied that grew better on avicel. The good avicel user *C. saccharolyticus* also contained a prominent S-layer protein that appears in the cellulolytic *Caldicellulosiruptor* species, suggesting the possible role of this protein in cell-substrate

interaction (Blumer-Schuette *et al*., 2010). Consequently, it is very possible that the *Caldicellulosiruptor*'s capability for cellulose utilization is related to its attachment capability with the substrates.

Unfortunately, there are only limited methods for demonstrating the adhesion between the cell and the cellulose, including direct and *in situ* microscope detection such as with a scanning microscope (Miron *et al*., 2001) and a confocal laser scanning microscope (Larsen *et al*, 2008). Obtaining good-quality pictures showing the adhesion of a batch sample of cellulolytic microorganisms other than Rumen organisms is difficult. However, a recent work by Dumitrache (2010) provided very clear confocal laser scanning micrographs showed the attachment between the *Clostridium thermocellum* biofilms and the solid cellulosic substratum.

Although many studies have worked on the *Caldicellulosiruptor* type strain *C. saccharolyticus*, many questions still need to be answered. For instance, how will the end products detection results on different substrates and the known genome sequences contribute to drawing an exact hydrogen production mechanism pathway? Will the proteomic identification and expression results of intercellular and extracellular proteins match the gene information gained from genome sequencing? Does an attachment exist between the cellulose substrates and *C. saccharolyticus* and *C. kristjanssonii*? Is the attachment a key factor in cellulose material use and their cellulose-degrading capability? Since *C. kristjanssonii* shows significant phylogenetical divergent from *C. saccharolyticus*, does *C. kristjanssonii*'s performance in hydrogen production on cellulose differs as well?

Further study of metabolism profiling of *C. saccharolyticus* will help us understand much better the hydrogen production mechanism and prepare us well for the manipulation of related genes and creation of an effective high-hydrogen-produced strain. Further study of one particular selected cellulosic material—a local and cheap species, switchgrass—will contribute to the large-scale biofuel production. Less is known about *C. kristjanssonii*, because of the limited studies and annotated genome information, but it is notable that *C. kristjanssonii* would not be inhibited at P_{H2} levels up to 50 kPa (Bredholt *et al*., 1999). Still, there are many blanks, such as about the metabolic pathway, that makes it worth our attention and further study.

1.5 Objectives

In order to develop an economical and environmentally favorable process for fermentative production of H_2, *C. saccharolyticus* and *C. kristjanssonii* were utilized as the microorganism candidates to ferment switchgrass. The following experiments have been carried out to investigate the parameters involved and optimize the fermentation process.

1> To optimize the growth of *C. saccharolyticus* and *C. kristjanssonii* on different substrates, including monosaccharide, disaccharide, hemicelluloses, microcrystalline cellulose, and untreated cellulosic material (switchgrass).

2> To measure the metabolic end products such as hydrogen, ethanol, carbon dioxide, and other end products of the two organisms and to examine their potential as biofuel produced strains.

3> To use 2D LC-MS/MS proteomic analysis for determining the translation level of the cellulase genes in *C. saccharolyticus* grown on different substrates.

4> To detect the attachment between the cellulolytic microorganism (*C. saccharolyticus* or *C. kristjanssonii*) and the cellulosic materials and to find the contribution of the attachment to the cellulose utilization capability.

Chapter 2 Methods and Materials

2.1 Chemicals and organisms

C. saccharolyticus DSM 8903 and *C. kristjanssonii* DSM 12137 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany). Switchgrass was gained from Nott Farm (Clinton, Canada). All the chemicals were commercially available. Cellobiose (D-(+)-cellobiose) and xylan (from Beechwood, and from oat) were from Sigma-Aldrich (Canada), Glucose (Dextrose) is from EMD (USA), xylose (D-(+)-xylose) was from Alfa Aesar (USA), avicel (PH105) was from FMC (USA). The fluorescent dye Syto 9 and Wheat germ agglutinin (WGA) - tetramethylrhodamine conjugate was purchased from Invitrogen (USA).

2.2 Growth conditions

C. saccharolyticus and *C. kristjanssonii* were grown on modified medium DSMZ 640 and DSMZ671 (http://www.dsmz.de/microorganisms/medium) without trypticase and yeast extract, and with addition of 2 ml/L vitamin solution (vitamin solution: biotin 2 mg/L, folic acid 2 mg/L, pyridoxine-HCl 10 mg/L, thiamine-HCl · 2 H_2O 5 mg /L, riboflavin 5 mg/L, nicotinic acid 5 mg/L, D-Ca-pantothenate 5 mg/L, vitamin B_{12} 0.1 mg/L, p-aminobenzoic acid 5 mg/L, lipoic acid 5 mg/L). Glucose, xylose, cellobiose, switchgrass, xylan (from Beechwood), and avicel were used as the sole carbon and energy source. The chopped switchgrass and filter paper were ground into 0.25 mm diameter powder with UDY Cyclone sample mill machine (UDY 3010-080P, USA). All substrates were finalized to a

concentration of 4 g/L. The growth temperatures for *C. saccharolyticus* and *C. kristjanssonii* were maintained at 70 ˚C and 78 ˚C respectively, and the growth of the microorganisms were monitored by cell number counting with a counting chamber (Petroff-Hausser 3900, Hausser, USA) under a microscope.

All the media were autoclaved at 121 ˚C for 30 min, and anaerobic environment inside the container was created by charging with pure nitrogen gas. All the media were reduced with 0.75 g/L cysteine-HCl · H_2O, and the reducing condition of medium was indicated by 0.50 mg/L resazurin.

Both *C. saccharolyticus* and *C. kristjanssonii* were grown on the medium with glucose and yeast extract first (followed the complete DSM 640 and DSM 671 recipe) overnight, the culture was then harvested and centrifuged at 3000 x *g* for 40 min at 4 ˚C to remove media, the pellet was resuspended in media without any carbon source (without any sugar, yeast extract, or trypticase), then the resuspension was centrifuged again at 3000 x *g* for 40 min at 4 ˚C, this step was repeated twice to remove as much residue carbon source as possible, and then the cell pellet was resuspended with medium without carbon source again, which resuspension was used for inoculation. For all the experiments involving different carbon sources, the treated seed inoculation prepared as described above was used, and be inoculated into the medium with only one particular substrate (glucose, xylose, cellobiose, xylan, avicel, and switchgrass) separately.

2.3 Determination of end-products

2.3.1 GC and HPLC detection method

End-products measurement: Gas chromatography (GC, Shimadzu 2014, Japan) equipped with Grace Porapak Q 80/100 6' * 1/8" * .085" SS and SEG 054427 columns was used for the detection of gas product, mainly are H_2 and CO_2. The detectors used were thermal-conductivity (TCD) detector with helium and nitrogen gas (Flow rate as 25 ml/min) and flame-ionization (FID) detector with hydrogen and air (Flow rate as 25 ml/min); and GCsolution software from Shimadzu was equipped with the instrument and used as GC control and data process platform. High performance liquid chromatography (HPLC, Shimadzu, Japan) equipped with Grace IOA-1000, 7.8 x 300 mm Organic Acid Columns was used to detect the element in the liquid phase, including the end-products like ethanol, organic acid and the soluble sugar left (Mobile Phase as 0.0085M H_2SO_4; Flow rate as 0.3 ml/min; Column temperature as 60 ˚C; Detector as Refractive Index Detector [RID]), LCsolution software from Shimadzu was equipped and used as HPLC control and data process.

The samples were collected at different time points, and the pressure in the headspace of the sample container was detected directly with a pressure meter, a 100 µl volume gas sample from each sample was injected into GC instrument with gas tight syringe (VICI, USA) for gas composition detection, and 600 µl of liquid sample was collected for HPLC measurement. The pH of the liquid part was measured with pH paper (Baker pHIX, scaled

pH paper, Baker, USA); the samples were centrifuged at a high speed (14,500 x *g*, 10 min) twice and then were filtered through a 0.45 μm nylon syringe filter (National Scientific, USA) to remove the insoluble part.

The components that were tested by HPLC including acetate, lactate, formate, succinate, ethanol, butanol, glucose, xylose, cellobiose, and arabiose.

Experiment condition information such as room temperature, atmosphere pressure was recorded as well, and the standard curve was prepared for each compound with known concentration. The samples with unknown concentrations of the same compound would be detected using the HPLC or GC and automatically calculated and saved by the software LCSolution equipped with the HPLC and GCSolution with the GC respectively. For the concentration of H_2 in the sample, only the gas part was calculated because the solubility of H_2 is only 0.0016 g/kg (H_2/water) at room temperature (Greenwood, 1969), which is very low. For the concentration of CO_2 in the sample, both the CO_2 in the gas phase and the part dissolved in the liquid part were considered: the concentrations of CO_2 and H_2 in the gas phase were calculated based on the equation for the Ideal Gas Law: PV = nRT, where P is the partial pressure (in unit mmHg), V is volume (in unit L), n is number of moles, and T is temperature (in unit k), R is the gas constant, and R = 62.3637 L·mmHg/mol·K; The concentration of CO_2 in liquid part was calculated according to Henry's law ($p = k_H c$, where p is the partial pressure of the solute in the gas above the solution (in unit atm), c is the CO_2 concentration in the liquid (in M unit, multiplied by 1000 to be converted to mM), and k_H is a constant with the dimensions of pressure divided by concentration. In this study,

$c_{[CO2]liq} = p/k_H$ and the room temperature was 23 ˚C that equals to 296.15K, and $k_{H(296.15K)}$ = 31.2386 L*atm/mol (Francis *et al*., 2007), and the concentration of HCO_3 in the liquid was calculated using the equation $c_{[HCO3]} = 10^{(pH-pKa)} * c_{[CO2]liq}$ (pH was the pH of the liquid part sample, Ka = 4.21E-07, pKa= 6.376) according to the Henderson-Hasselbalch equation (Levy *et al.*, 1987).

2.3.2 Standard curves for GC and HPLC detection

For HPLC, calibrated curve for each component detected was drawn. A series solution of acetate, lactate, formate, succinate, ethanol, butanol, glucose, xylose, cellobiose, and arabiose were made with concentrations as 0.20, 0.50, 1.00, 2.00, and 5.00 mM separately, and the peak of each standard sample was detected by HPLC, using the same method as unknown sample used. And the standard curve and R^2 value for each curve was calculated and saved by the software.

For GC standard curves, the different concentrations of gas sample were prepared with 60 ml volume serum bottles; the concentrations were 0, 0.01, 0.1, 1, 2, and 20% (v/v) separately. The bottles were filled with 100% nitrogen gas, which were considered as 0% gas bottles (for different concentration of H_2 and CO_2); With the same method, 100% H_2 and 100% CO_2 bottles were prepared via manifold connected with different gas tanks; The positive pressure of all the bottles prepared was released by inserting a fine needle on the stop of the serum bottle, and the zero pressure was confirmed with a pressure meter; H_2 or CO_2 was injected into the N_2 bottle to make certain concentration of standard H_2 or CO_2 sample, same amount of N_2 was extracted in advance to balance the pressure; A 5 ml of

sterilized deionized H_2O was injected into each standard sample bottle to keep the positive pressure in the bottle. After all the standard sample bottles were prepared, 100 µL gas of each sample was tested by GC instrument with the same method used for unknown sample detection, and the standard curve was drawn according to the peak area and relative volume percentage(This part was carried out by my lab mate Cherry Chen).

2.4 Determination of attachment of *C. saccharolyticus* and *C. kristjanssonii* to insoluble substrates

A Confocal microscope and a Scanning Electronic Microscope (SEM) were applied to observe the attachment of bacteria to the surface of the cellulosic materials at different time points.

C. saccharolyticus and *C. kristjanssonii* were grown on insoluble substrates (avicel, switchgrass, xylan from oat, and Whatman (USA) NO.1 filter paper (0.25 mm powder made with the milling machine), and the samples at different time points were treated for SEM or confcocal microscope observation for the attachment observation after inoculation (for confocal microscope detection, only samples with switchgrass and avicel substrates were used).

2.4.1 SEM microscope detection

The cell sample was harvested on log phase and was centrifuged at 5,000 x *g* for 2 min at room temperature to get rid of medium; The pellet was then washed with phosphate buffer (0.175 M monobasic NaH_2PO_4, 2.46 mM dibasic Na_2HPO_4, 0.175 mM $NaH_2PO_4 \cdot 2H_2O$,

0.025 mM $Na_2HPO_4 \cdot 12H_2O$, pH 6.0) three times; Then the fixation buffer (includes 2.5 % glutaraldehyde and 4 % paraformaldehyde in 0.1M phosphate buffer, pH 7.2) was added to the samples, and the sample was kept at room temperature for one hour for fixation; the sample was then washed with phosphate buffer three times again; Increasing grade concentrated ethanol solutions were added for dehydration: 20%, 50%, 70%, and 95% ethanol was added in order with a 10 min inoculation in between, this step was repeated twice; then the sample was resuspended in 100% ethanol; after that, the sample was dried with CO_2 flow for Critical Point Dry; after drying, the sample was mounted onto metal stub with double sided carbon tape; a thin layer of gold was applied over the sample using an automated sputter coater; the coated sample was observed under SEM.

2.4.2 Confocal microscope detection

C. saccharolyticus and *C. kristjanssonii* samples on avicel and switchgrass were collected at different growth times, start from 0 h, and the culture on glucose and medium with avicel and switchgrass without inoculums were used as control.

Two kinds of fluorescent dyes—$2*10^{-7}$ mM Syto9 and $2.4*10^{-3}$ mg/ml Wheat germ agglutinin (WGA)-tetramethylrhodamine conjugate were used for this purpose. A confocal microscope (Zeiss LSM 510, Germany) was used to acquire imagine: the objective used was a 63x1.4 oil immersion lens, for syto9, 488 nm laser (Argon, strength 50%) was used to excite the fluorescence and a detector for the 500-530 nm band was used to detect the fluorescence, for WGA- tetramethylrhodamine, the exciting laser used was 543 nm (HeNe) and the detector was the one for 530-600 nm, two tracks for two channels configuration

was used, one channel for each dye. The cells were only dyed with dye Syto9, while the celluloses (avicel and switchgrass) were dyed with both Syto9 and WGA-tetramethylrhodamine, but more WGA- tetramethylrhodamine bound with the cellulose than Syto9. Two artificial colors were assigned for the dyes used in this study to distinguish them: the red was for Syto9 and green for WGA- tetramethylrhodamine. The photos were processed by ZEN 2009 equipped with the microscope (Zeiss, Germany).

The sample inoculation were collected at different time points, and kept in room temperature for around 20min to be cooled down to room temperature (the growth temperature above 70 °C), then the incubation serum bottle was gentle shaked by hand and 0.5ml of sample was quickly extracted and put into a Petri dish (Mat Tek 35mm glass bottom dish, USA). The stain directly was applied directly to final working concentration, and mixed gentle. All the staining steps were carried out in the safe cabinet without the lamp on and operated quickly. The sample with staining was kept in a box wrapped with aluminium foils, and inoculated at room temperature for 20 min and observed under confocal microscope. All the samples need to be observed under confocal microscope within an hour after the dye was applied.

2.4.3 Measurement of water content of switchgrass

The switchgrass samples were collected at different time of the year directly from the field of Nott Farm (Clinton, Ontario), the whole plant with seeds removed were kept in the 70 °C incubator to dry for 48 h to 72 h, depended on the amount of water contained (the grass was reweighted every 12 h, till the weight no longer changed). The weights of the grass before

and after drying were measured, based on which, the water contents of the grass samples were calculated. The grass samples were collected at three different times during the year such as late June (switchgrass was close to half to its highest height, not mature); late September (switchgrass was mature and reached to its highest height but not dry); and late December (switchgrass was mature, reached to its highest height, and dried naturally in the field and the insoluble sugar part were back to the field, the grass was collected by the farmer and stocked dried in garage).

2.4.4 Detection of sugar content in buffered media

Media with variety of sugar substrates were prepared separately and incubated without adding inoculums, the content of sugar in the medium at 0 h, 20 h and 40 h inoculation with HPLC with the procedure described in **section 2.3.1**.

The inoculation conditions were the same as the ones used in **section 2.2**, medium DSM640 and medium DSM671 without any other carbon source added except the selected substrates as glucose, xylose, cellobiose, xylan, avicel, and switchgrass separately, the concentration of each carbon source was 4 g/L, and the media were inoculated in 70 and 78 ˚C separately.

2.5 Proteomic sample preparation and result analysis

2.5.1 Protein extraction

Protein samples are extracted from log-phase *C. saccharolyticus* cells which grown on different substrates (glucose, xylose, cellobiose, xylan, avicel, and switchgrass). The cultures were harvested and centrifuged at 7,000 x *g* for 7 min at 4 ˚C to remove the medium, then the cell pellet was washed with PBS buffer (8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l Na_2HPO_4, and 0.24 g/l KH_2PO_4, pH 7.4) by three times. For sample on insoluble substrates, before the first centrifuging, the substrates were filtered out with cheesecloth (VWR International, Canada, 5 by 10 cm size, and stuff the cheesecloth into the neck of the funnel). The washed cell samples were broken by five rounds of sonication (Fisher Sonic Dismembrator Model 100, Canada) at level 5 (15 seconds for each round, on ice) with 100 µl lysis buffer(10 mM pH 7.4 Tris-HCl , 3mM $CaCl_2$, 2mM $MgCl_2$, 0.0025 % bacterial protease inhibitor (Sigma, USA), 0.1 % Igepal CA-630 (Fluka, Switzerland)) to release the protein. The protein concentrations of all samples were determined using the Bradford method, and bovine serum albumin served as the standard protein (Bradford, 1976).

2.5.2 Proteomic test

This process was carried by Dr. Krokhin's lab at the University of Manitoba. The method used was 2D LC-MS/MS proteomic test (Muddiman *et al*. 2010 A and B).

2.5.3 Proteomic result analysis

The proteins detected from proteomic analysis were cross-compared with the published genome sequence annotation information of *C. saccharolyticus* (http://www.ncbi.nlm.nih.gov/, genome NC_009437), as well as the information from DOE Joint Genome Institute website (JGI, http://www.jgi.doe.gov/), the function of identified enzyme proteins were estimated based on the enzyme information from Carbohydrate-Active Enzymes (CAZymes, http://www.cazy.org/).

2.6 Isolation of *C. kristjanssonii*'s plasmid and genome DNA

C. kristjanssonii was grown on DSM 671 medium with glucose substrate and harvested on mid-log phase (around 20 h) for the plasmid and genome DNA extraction. The plasmid was extracted with Bio Basic EZ-10 Spin Column Plasmid DNA kit (Bio Basic, Canada) and the result was confirmed with 1% agarose electrophoresis, and the concentration of the DNA was detected by Nano drop.

The same cell pellet from the culture as used in plasmid extraction was used for the extraction of genome DNA, the pellet was gently resuspended in 0.4 ml of 10 mM Tris-25 mM EDTA (TE buffer, pH 8.0); lysozyme solution was added to a final concentration as7.25 mg/ml in TE buffer; the solution was mixed by gently inverting the micro centrifuge tube roughly 25 times and was incubated at 37 ˚C for 30 min; then the sample was cooled to room temperature; a mixture was made by adding sodium dodecyl sulfate (SDS), Proteinase K, and NaCl solutions with final concentrations as 1.25 mg/ml, 0.25 mg/ml, and

0.4 M separately. The mixture was mixed by gently inverting the micro centrifuge tube roughly 25 times and was incubate at 55 ˚C for 15 min, followed by a 15 min's incubation at 75 ˚C; RNase was added to a final concentration as 0.18 mg/ml for the bacterial cell lysis after the sample was cooled to room temperature; then the sample was mixed by inverting the micro centrifuge tube 25 times and was incubated at 37 ˚C for 30 min; the ammonium acetate solution was added to the tube to a final concentration as 1.95 mg/ml and the sample was mixed by inverting the tube 25 times and was incubate on ice for 20 min. The function of the ammonium acetate is to precipitate membranes, lipids, and proteins; the sample was centrifuged at 14,000 x *g* for 15 min at 4 ˚C to pellet the protein; the supernatant was used for phenol: chloroform: isoamyl alcohol (at 25:24:1 ratio) extraction to help purify the DNA by removing non-polar proteins, and lipid residues, and the DNA sample was extracted by placing the sample on ice with roughly 0.8 times the volume of aqueous solution of chilled isopropanol; then the DNA sample was washed twice by adding one ml of cold 70 % ethanol to remove any remaining salt. The DNA sample was confirmed with 1 % agarose gel electrophoresis and the concentration of the DNA was detected by Nano drop.

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confirmed with 1 % agarose gel electrophoresis and the concentration of the DNA was detected by Nano drop.

Chapter 3 Results and Discussions

3.1 End-product Detection

Both *C. saccharolyticus* and *C. kristjanssonii* were able to grow on the medium with glucose, xylose, cellobiose, xylan (from beechwood), avicel(PH105), and switchgrass without chemical pretreatment as the sole carbon and energy source. No significant growth was detected on negative control (medium without any carbon source added). The growth experiments were carried out for three times, the results were reproducible, and one of the set of the data was used. As shown in **Figure 3.1** and **Figure 3.2**, there was a pH drop detected during the growth.

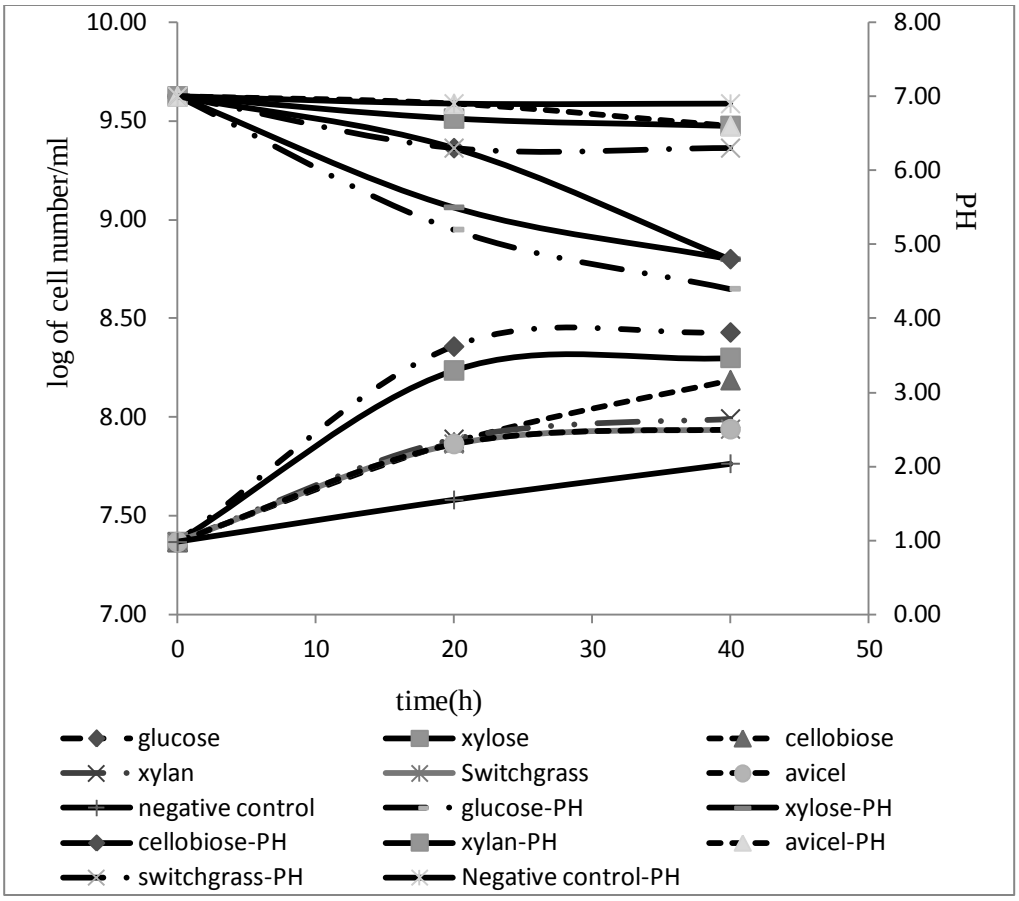


Figure 3.1 Growth curve and pH change of *C. saccharolyticus* on different substrates at 70 ˚C. All samples had growth compared with control, and a drop of pH was detected during the growth of the organisms.

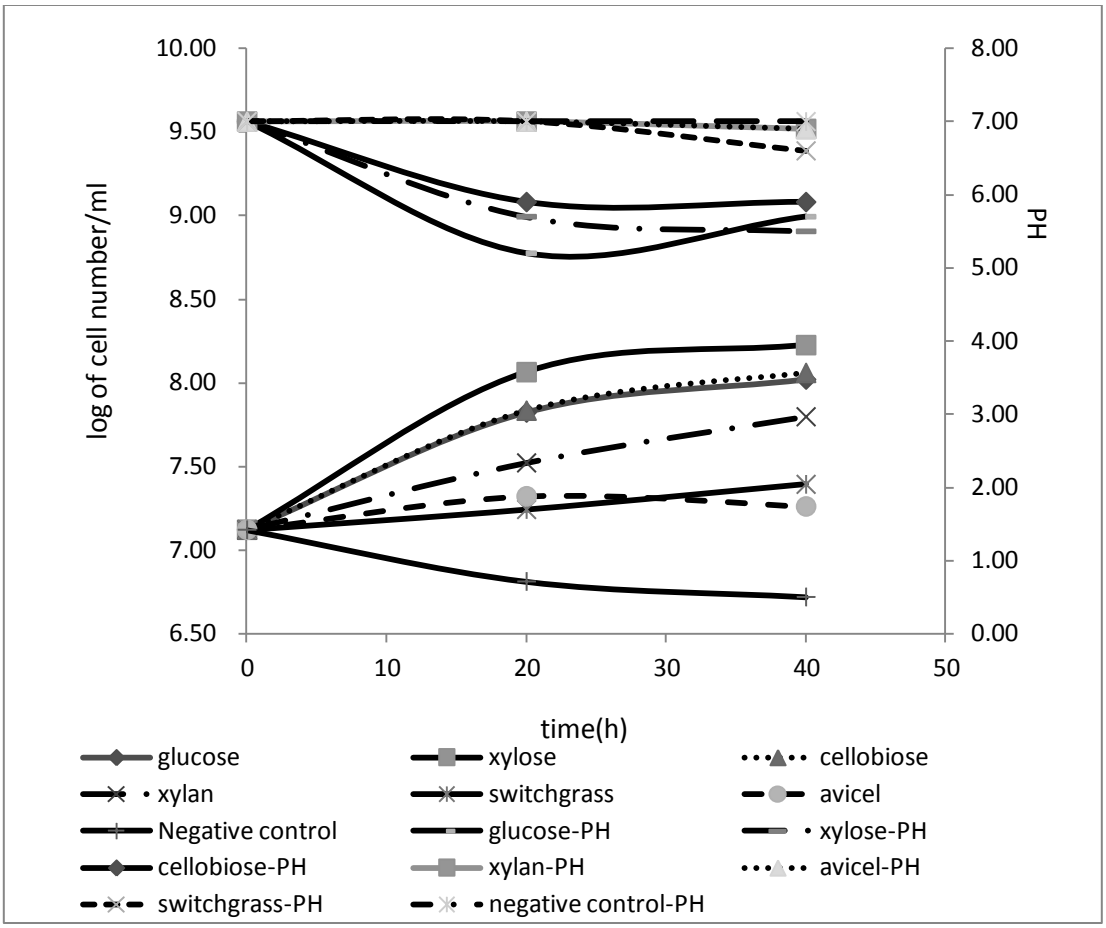


Figure 3.2 Growth curve and pH change of *C. kristjanssonii* on different substrates at 78 ˚C. All samples had growth compared with control, and a drop of pH was detected during the growth of the organisms.

The predominant end-products were hydrogen, carbon dioxide, acetate, lactate, and a small amount of ethanol. Both *C. saccharolyticus and C. kristjanssonii* showed a better hydrogen production capability on switchgrass than on pure microcrystalline cellulose (avicel), but showed even much better on hemicellulose (xylan). As shown in **Figure 3.3** and **Figure 3.4**, the orders of the hydrogen production capability of two organisms were similar. A small amount of ethanol was detected on all kinds of substrates except avicel and switchgrass (<0.02 mM), and the order of the quantity of ethanol matched the order of hydrogen production (data shown in **Table 3.1** and **Table 3.2**). Comparisons of the end-products production were shown in **Figure 3.3** to **Figure 3.6**.

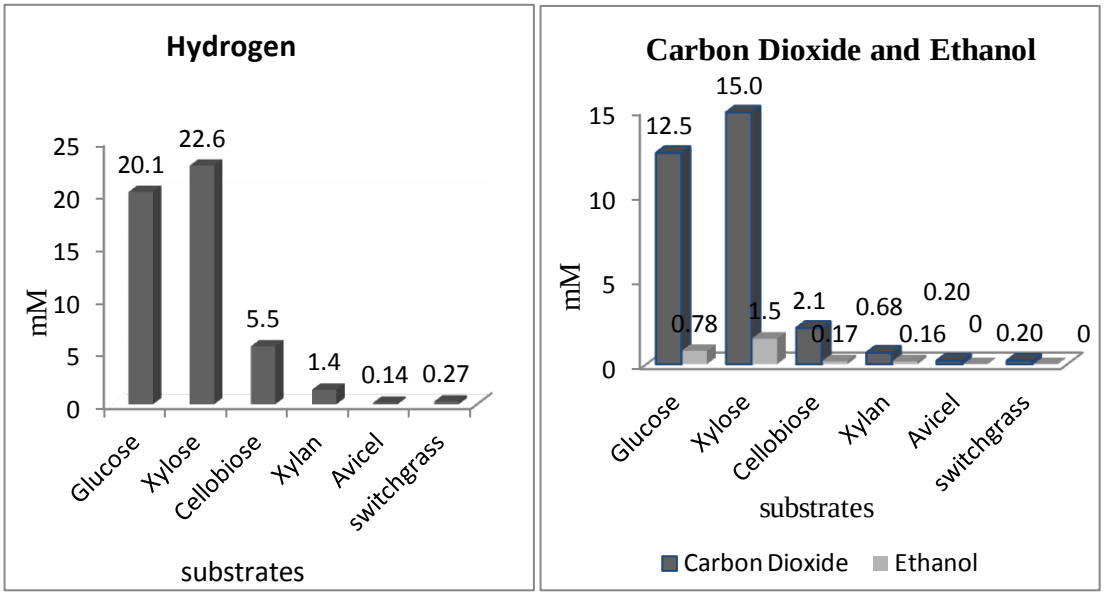


Figure 3.3 Hydrogen, carbon dioxide, and ethanol production of *C. saccharolyticus* on different substrates at 20 h. There's no ethanol detected on samples on avicel and switchgrass sample, which may due to the amount of ethanol produced by these samples were less than the detection limit (0.02mM).

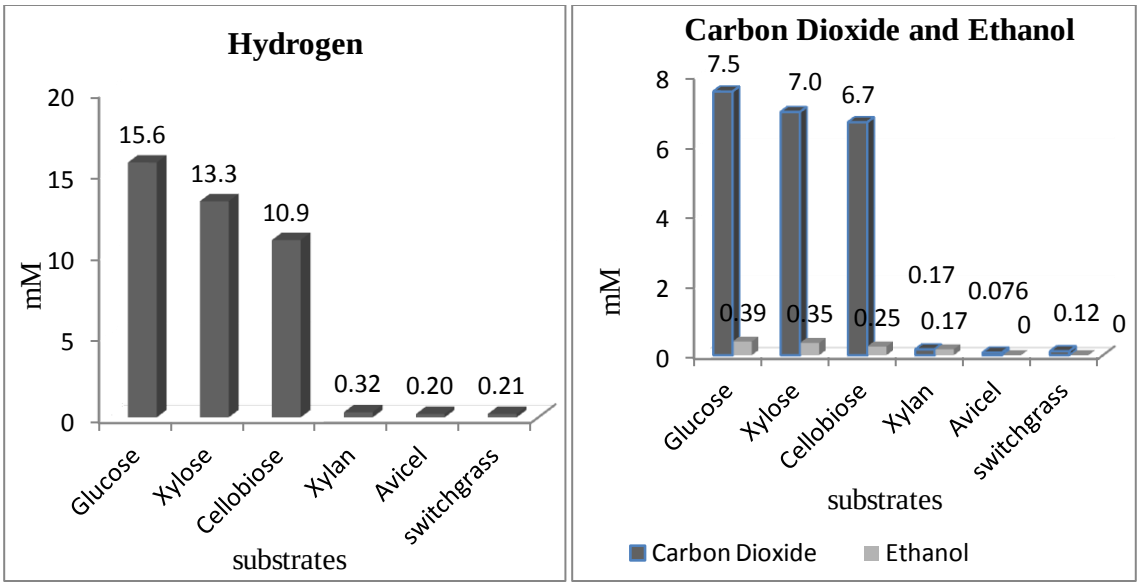


Figure 3.4 Hydrogen, carbon dioxide, and ethanol production of *C. kristjanssonii* on different substrates at 20 h. There's no ethanol detected on samples on avicel and switchgrass sample, which may due to the amount of ethanol produced by these samples were less than the detection limit (0.02mM).

Table 3.1 Gaseous end products, ethanol, acetate, and lactate product of *C. saccharolyticus* on different substrates at 20 h and 40 h.

End products (mM)	Substrates (4g/L)						
	Glucose	Xylose	Cellobiose	Xylan	Avicel	switchgrass	Control
				After 20 h			
H_2	20.1	22.6	5.5	1.4	0.14	0.27	0
CO_2	12.5	14.9	2.1	0.68	0.20	0.20	0.003
ethanol	0.78	1.5	0.17	0.16	0	0	0
acetate	6.3	7.7	1.8	1.4	0.04	0.34	0
lactate	0	2.8	0	0	0	0	0
				After 40 h			
H_2	25.2	26.3	15.9	2.1	0.31	0.34	0
CO_2	13.5	15.1	7.58	0.78	0.21	0.22	0.003
ethanol	1.2	2.1	0.82	0.26	0	0	0
acetate	8.3	9.5	2.0	1.5	0.09	0.76	0
lactate	3.1	6.6	5.3	0	0	0	0

Table 3.2 Gaseous end products, ethanol, acetate, and lactate product of *C. kristjanssonii* on different substrates at 20 h and 40 h.

End products (mM)	Substrates（4g/L）						
	Glucose	Xylose	Cellobiose	Xylan	Avicel	switchgrass	Control
				After 20 h			
H_2	15.6	13.3	10.9	1.3	0.20	0.21	0
CO_2	7.5	6.9	6.7	0.18	0.076	0.11	0.002
ethanol	0.39	0.35	0.25	0.17	0	0	0
acetate	4.7	4.3	3.4	1.2	0.08	1.3	0
lactate	7.0	4.9	7.0	0	0	0	0
				After 40 h			
H_2	13.7	13.6	11.6	1.6	0.33	0.49	0
CO_2	8.4	8.0	5.6	0.69	0.18	0.13	0.004
ethanol	0.34	0.56	0.19	0	0	0	0
acetate	4.6	4.6	3.7	1.3	0.10	2.0	0
lactate	6.9	9.1	7.9	0	0	0	0

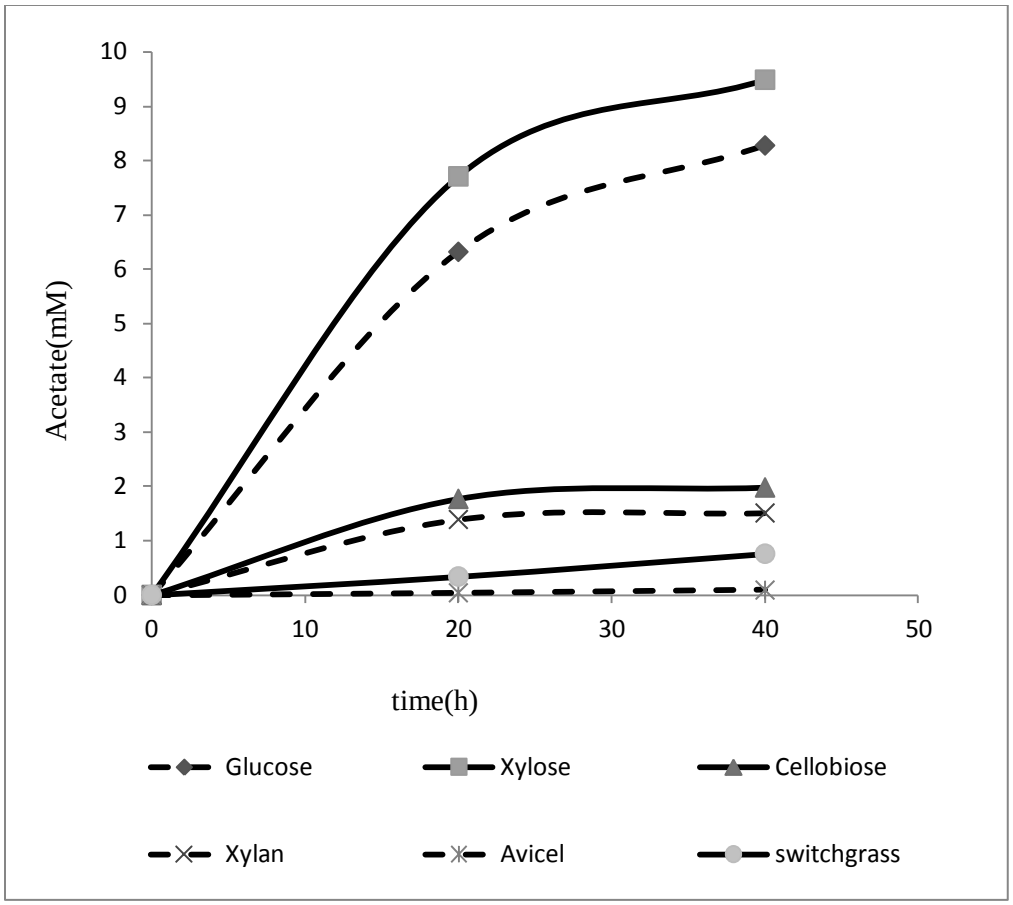


Figure 3.5 Acetate production of *C. saccharolyticus* on different substrates. The order of acetate production amount matches the order of hydrogen production and growth curve.

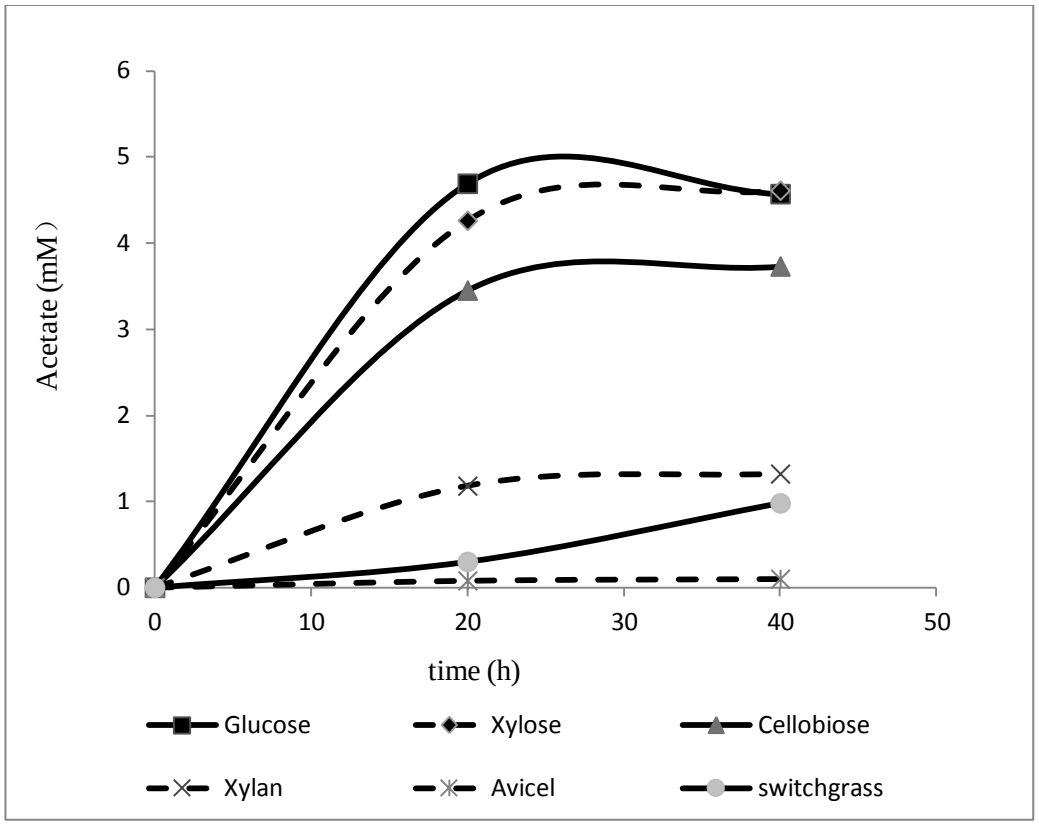


Figure 3.6 Acetate production of *C. kristjanssonii* on different substrates. The order of acetate production amount matches the order of hydrogen production and growth curve.

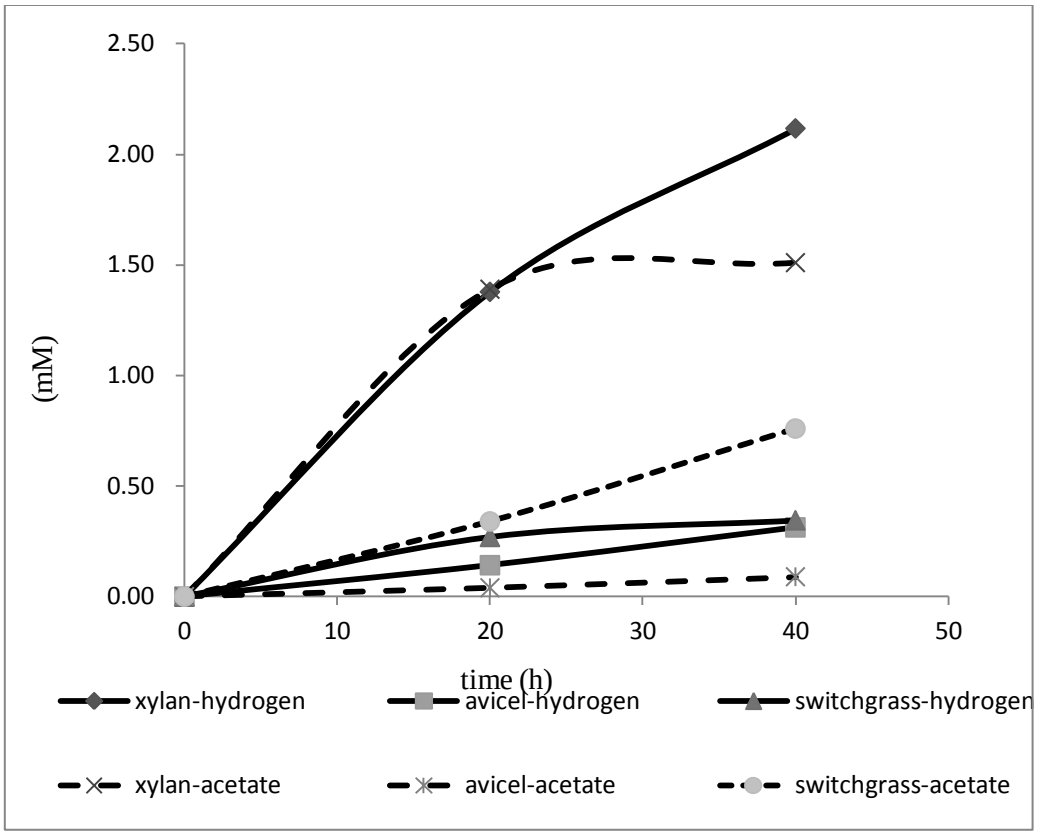


Figure 3.7 Hydrogen and acetate production of *C. saccharolyticus* on different substrates. The hydrogen/acetate ratio of avicel and xylan sample was decreased from 20h to 40h, while the one for switchgrass was increased.

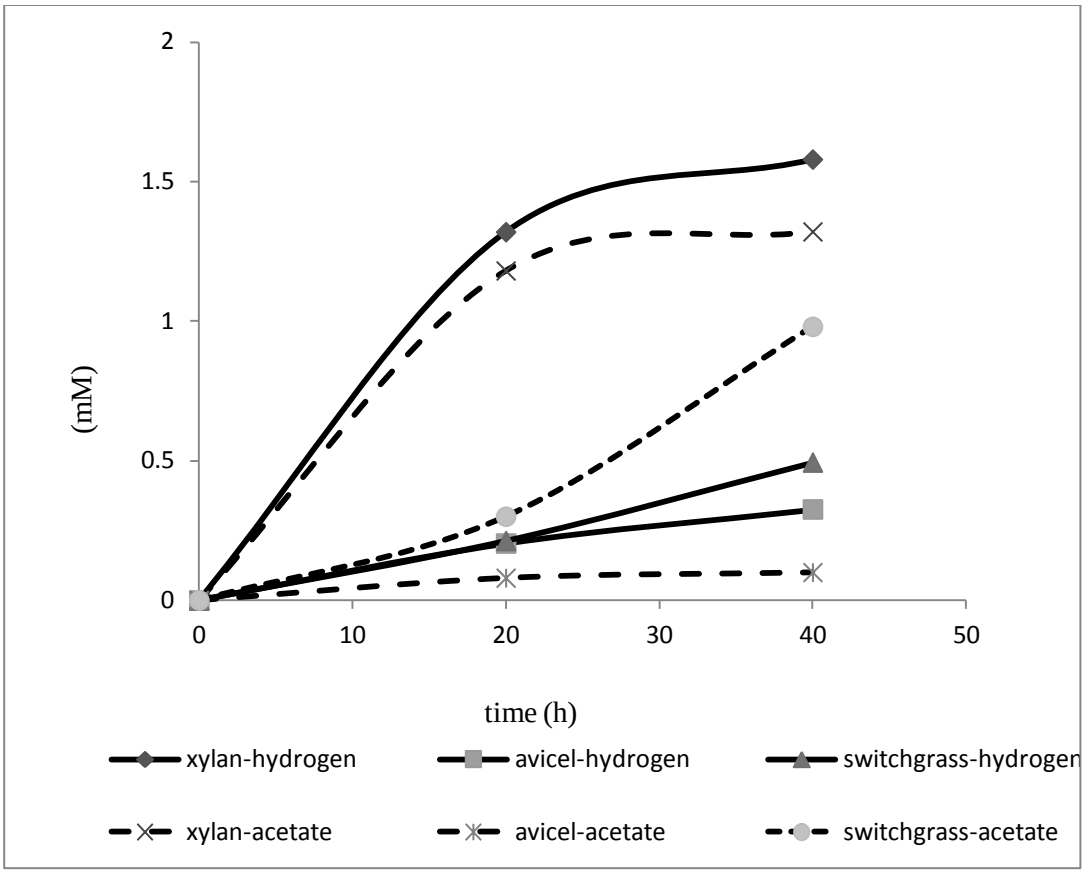


Figure 3.8 Hydrogen and acetate production of *C. kristjanssonii* on different substrates. The hydrogen/acetate ratio of avicel and xylan sample was decreased from 20h to 40h, while the one for switchgrass was increased.

Table 3.3 Sugar content in medium of inoculation of *C. saccharolyticus* on different substrates at 0 h, 20 h, and 40 h.

Sugar content (mM)	Substrates (4g/L)						
	Glucose	Xylose	Cellobiose	Xylan	Avicel	switchgrass	Control
				0 h			
xylose	0	29.4	0	0	0	0	0
arabinose	6.1	0	5.8	6.1	6.1	5.9	0
glucose	21.9	0	0	0	0.08	0	0
cellobiose	0	0	10.7	0	0	0	0
				After 20 h			
xylose	0	20.1	0	0	0	0	0
arabinose	6.03	0	5.7	6.0	0.02	6.2	6.0
glucose	15.0	0.05	0	0	0.02	0.09	0
cellobiose	0	0	9.7	0.02	0	0	0
				After 40 h			
xylose	0	17.2	0	0	0	0	0
arabinose	5.9	0	5.3	6.0	6.0	6.2	5.9
glucose	13.1	0	0.78	0	0	0	0
cellobiose	0	0	6.3	0.08	0	0	0

Table 3.4 Sugar content in medium of inoculation of *C. kristjanssonii* on different substrates at 0 h, 20 h, and 40 h.

Sugar content (mM)	Substrates (4g/L)						
	Glucose	Xylose	Cellobiose	Xylan	Avicel	switchgrass	Control
				0 h			
xylose	0	24.5	0.39	0.36	0.35	0.43	0.36
arabinose	0	0	0	0	0	0	0
glucose	22.5	0	0	0	0.02	0.09	0
cellobiose	0	0	11.2	0	0	0	0
				After 20 h			
xylose	1.2	16.0	0.34	0.37	0.37	0.33	0.28
arabinose	0	0.10	0	0	0	0	0
glucose	13.2	0	0.02	0.02	0.10	0	0
cellobiose	0	0	7.7	0	0	0	0
				After 40 h			
xylose	1.2	1.4	0.62	0.35	0.40	0.42	0.40
arabinose	0	0.14	0	0	0	0	0
glucose	13.1	0	4.9	0	0	0.11	0.01
cellobiose	0	0	3.3	0	0	0	0

The drop of pH was caused by the produce of organic acid (acetate and lactate) and CO_2; the acetate production on different substrates was shown in **Figure 3.5** and **Figure 3.6**, which proportional to the hydrogen produced. From most *C. saccharolyticus*' data, the ratio of hydrogen and acetate was similar but showed a little decrease (by 0.3-0.1) from 20 h to 40 h, and there was an increase of lactate produced amount, showing there may be a slight end-product inhibition, and the similar changes also happened on *C. kristjanssonii.* But the samples on switchgrass showed a different story: there were increases of acetate from 20 h to 40 h (**Figure 3.5** and **Figure 3.6**).

At 20 h, the cell number of *C. saccharolyticus* sample on glucose reached $2.3*10^8$ cell/ml, produced 20.1 mM hydrogen, 0.78mM ethanol, and *C. kristjanssonii* sample on glucose reached $1.32*10^7$ cell/ml, produced 15.6mM hydrogen and 0.39mM ethanol, but the cell numbers gained only considered the free cells in the medium, so, it is still hard to say that the growth curves drawn on **Figure 3.1** and **Figure 3.2** represent the exact cell growth of both organisms. *C. saccharolyticus* could produce maximum 2.9 mol hydrogen per mol simple sugar (glucose) in batch culture; and *C. kristjanssonii* was able to produce 1.7 mol hydrogen per mol simple sugar (glucose) at 20 h. This amount of H_2 produced was very high because the maximum H_2 producing amounts of most H_2 producing microorganisms are less than two mol of H_2 per mol sugar even in continuous fermentation (Kleerebezem and van Loosdrecht, 2007). Another end-product was ethanol. *C. saccharolyticus* could use xylose as growth substrate and had higher cell density at 20 h compared to glucose, but produced less H_2 than the glucose sample. A paper sludge growth experiment of *C. saccharolyticus* done by Donnison *et al* (2003) also showed that when both glucose and

xylose were co-fermented, the xylose consumption was higher than the glucose; however, less H_2 was produced from xylose than glucose (Donnison *et al*., 2003). Moreover, Zsófia Herbel found that *C. saccharolyticus* used the monomeric sugar first within a few days when the culture was grown on both monosugar and cellulose, and it began to use cellulose in the following period, and the H_2 production resumed for an extended period of time. Thus, the optimum substrates scheme for producing H_2 from *C. saccharolyticus* was to use xylose-cellulose co-fermentation. The limited amount of xylose in the medium will effectively help both the cell reproduction and the induction of cellulose and hemi-cellulase needed for the degrading of cellulose and hemi-cellulose materials.

Because of the lack of annotation information of the genome sequence and other metabolism studies on *C. kristjanssonii,* it was not known about its metabolism pathway. However, the growth experiments showed the metabolism of *C. saccharolyticus* and *C. kristjanssonii* were similar: they used similar carbon sources, both grew on all the carbohydrates substrates selected in this study, including glucose, xylose, cellobiose, xylan, avicel, and switchgrass. Moreover, their sugar preference orders were close, but *C. saccharolyticus* had much higher H_2 production capability than *C. kristjanssonii.* One reason might be that the much higher production of CO_2 of *C. kristjanssonii* provided more osmotic pressure and more acidic pH for the production of H_2 and cell reproduction (growth pH for *C. kristjanssonii* as 5.8-8.0, optimum 7.0). The sugar content of media are shown in **Table 3.3** and **Table 3.4**.

C. kristjanssonii cells in a liquid medium could be more easily autolysed if kept at room temperature. It could usually only be kept viable for about one week, while *C. saccharolyticus* culture would still survive for as long as at least two months under the same conditions (the test did not carried on further after two months).

3.2 Cell attachment on insoluble substrates

3.2.1 Observation of the attachment with SEM

The SEM was used to detect the attachment of the cellulose materials to the bacterial surface. Both *C. saccharolyticus* and *C. kristjanssonii* cells grown on substrates xylan, avicel, switchgrass, and filter paper were prepared for the SEM observation. In addition, one sample of *C. saccharolyticus* grown on xylan at 40 h was added.

Two samples showed the attachment of the cellulosic material to the bacteria. **Figure 3.9** shows the cells of *C. kristjanssonii* grown at 20 h on filter paper fiber, the cells were rods with rounded ends, 2.8-9.4 μm by 0.7-1.0 μm. While **Figure 3.10** shows that cells of *C. saccharolyticus* grown 40 h on xylan, *C. saccharolyticus* cells were straight rods 0.4-0.6 μm by 3.0-4.0 μm. Both pictures provided the evidence of the existence of the attachment between the *C. saccharolyticus* and *C. kristjanssonii* cells and insoluble substrates.

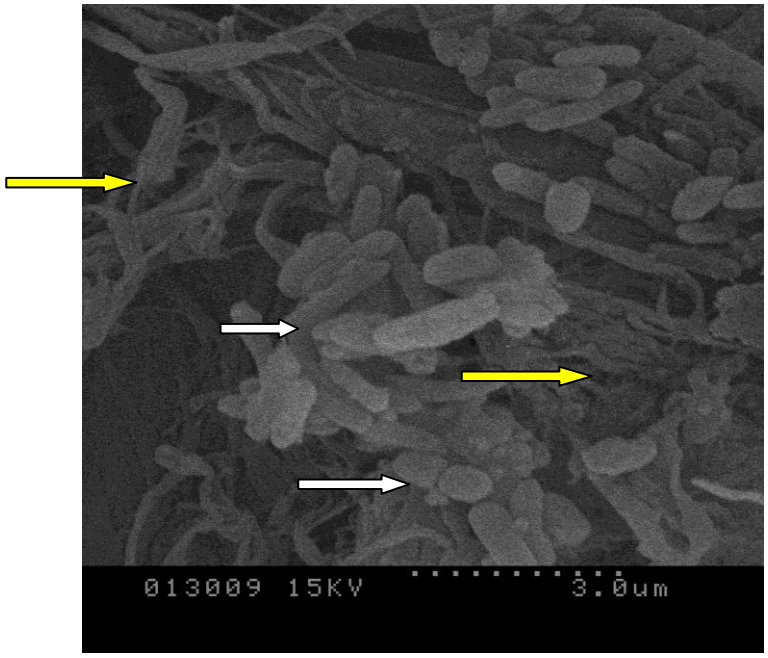


Figure 3.9 Attachment of cells of *C. kristjanssonii* grown on filter paper at 20 h. *C. kristjanssonii* cells were rods with rounded ends, 2.8-9.4 μm by 0.7-1.0 μm. White arrows pointed the cells; yellow arrows pointed the filter paper fibers.

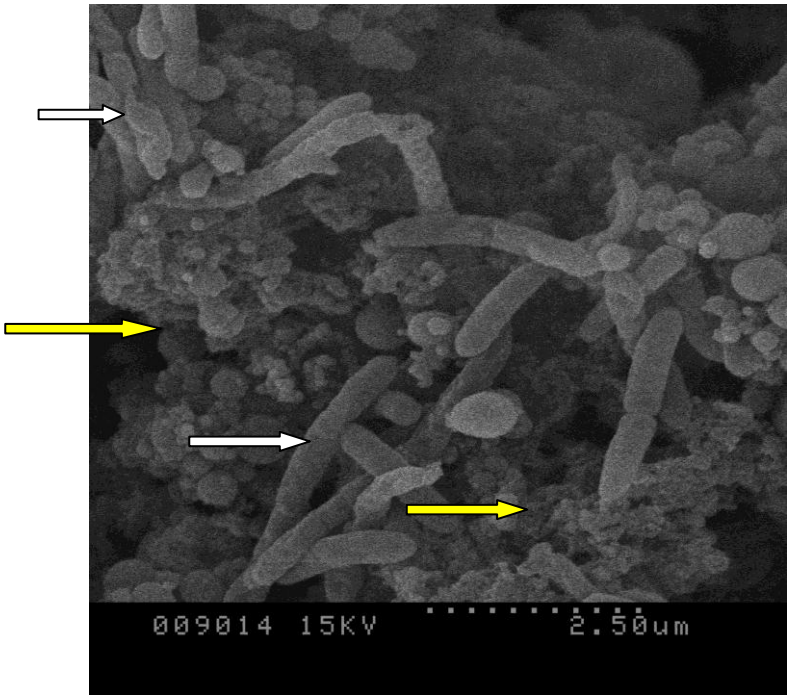


Figure 3.10 Attachment of cells of *C. saccharolyticus* grown on xylan at 40 h. *C. saccharolyticus* cells were straight rods 0.4-0.6 μm by 3.0-4.0 μm. White arrows pointed the cells, while the yellow arrows pointed the xylan fibers.

3.2.2 Observation of the attachment with confocal microscope

Both *C. saccharolyticus* and *C. kristjanssonii* were grown on substrates avicel and switchgrass, and the attachment of cells on the substrates were detected by using confocal microscope with the methods described in the Materials and Methods **section 3.2.2**. The artificial color for dye Syto9 was red, and for dye WGA- tetramethylrhodamine conjugate, it was green. The pictures of the substrates and the microorganisms were obtained respectively, and the cells were shown in red color, the celluloses showed the overlay colors of both green and red (vessel part of the grass was shown in red). **Figure 3.11** to **Figure 3.14** showed the three dimensional (3D) pictures that were taken as controls: *C. saccharolyticus* cells, *C. kristjanssonii* cells, avicel particles, and switchgrass fibers respectively., The size and shapes of both organisms were relatively uniform, while, the size and shape of avicel showed a variety but were not as much various as switchgrass fibers, which were prepared by using the milling machine in our laboratory. All 3D movie videos of the attachment gave 360˚ view of the samples on different time points are available in the enclosed CD.

Figure 3.15 to **Figure 3.21** provided evidence of the existence of attachments behaviors of *C. saccharolyticus* and *C. kristjanssonii* to cellulosic substrates avicel and switchgrass.

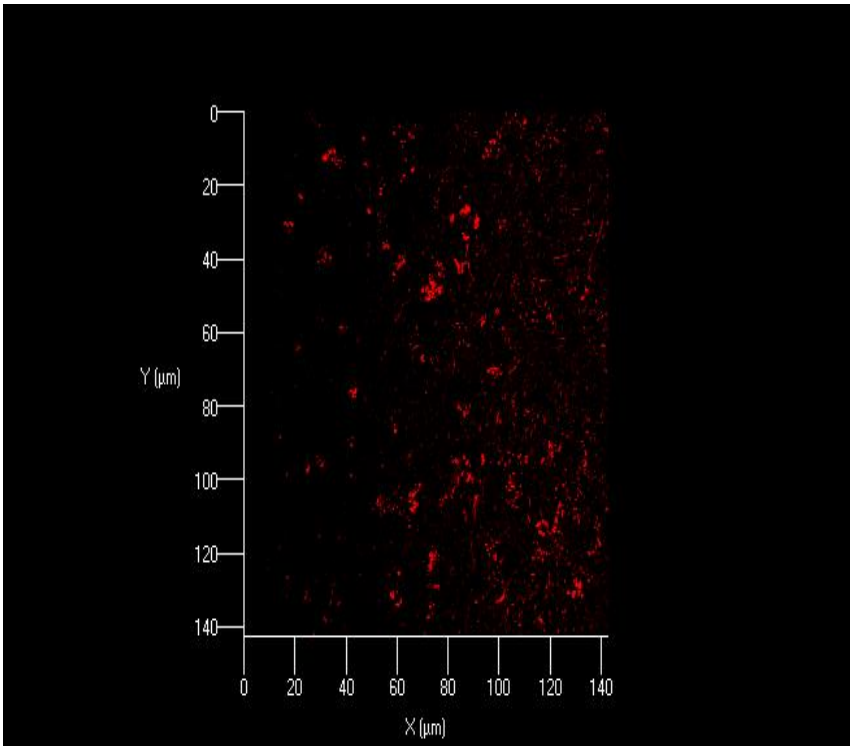


Figure 3.11 3D picture of *C. saccharolyticus* under confocal microscope. The cells were dyed in red, which was the artificial color of Syto9 dye. The cells were rod shape, had a relatively uniform size.

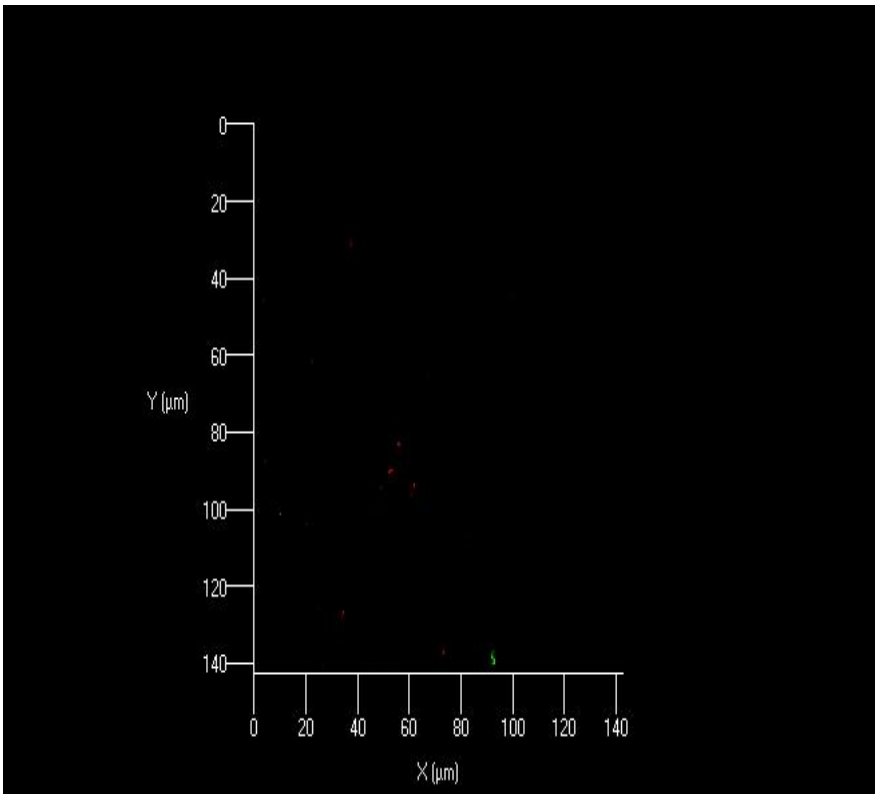


Figure 3.12 3D picture of *C. kristjanssonii* under confocal microscope. The cells were dyed in red, which was the artificial color of Syto9 dye. The cells were rod shape, had a relatively uniform size.

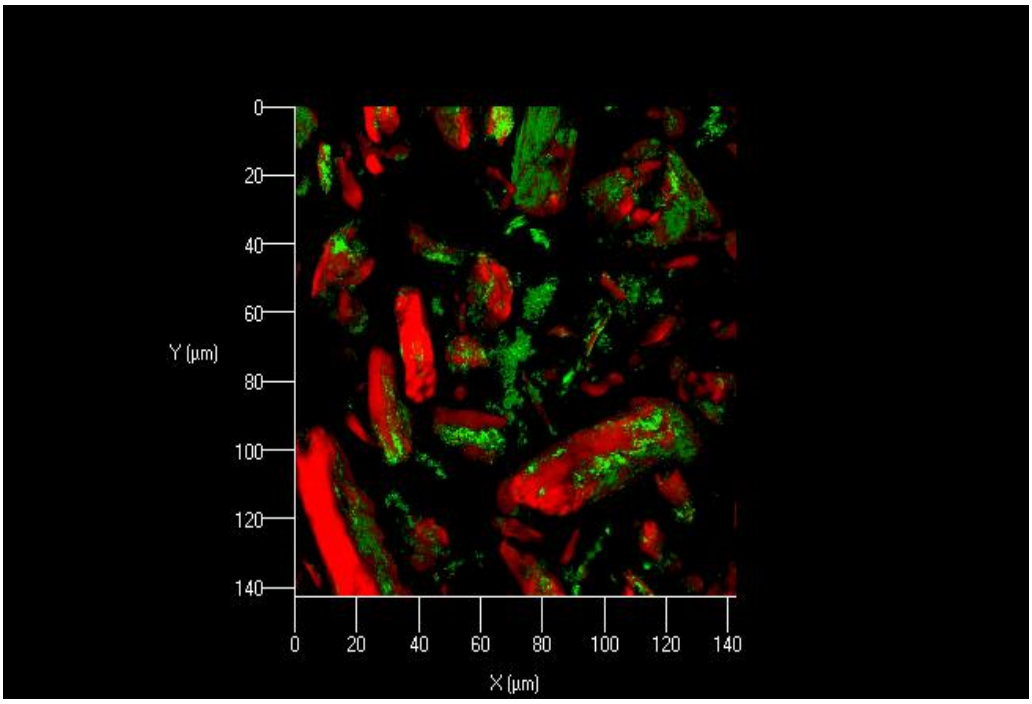


Figure 3.13 3D picture of avicel under confocal microscope. The avicel were dyed in both red and green, which were the artificial colors of dye Syto9 and WGA-tetramethylrhodamine conjugate separately. The size of avicel was not very uniform, both small and big particles were detected.

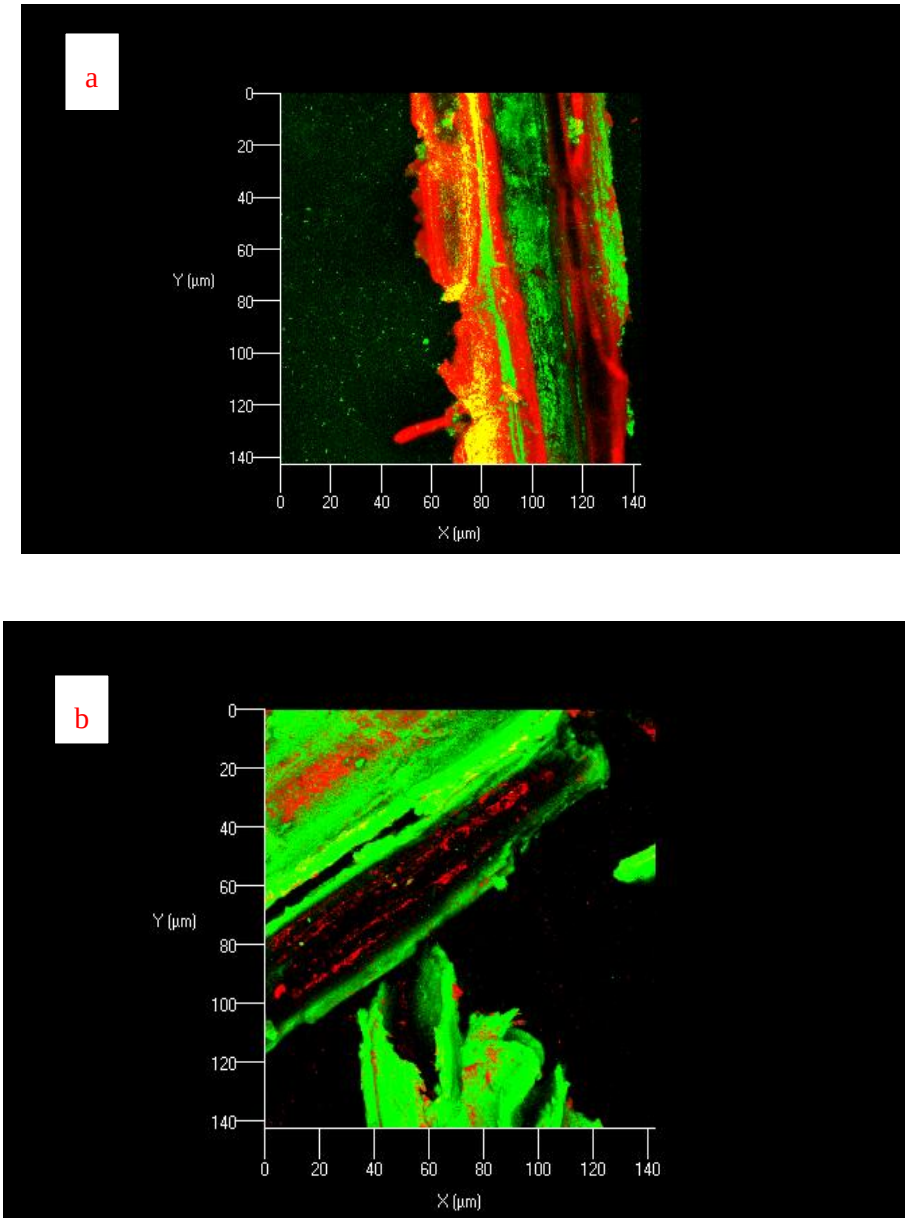
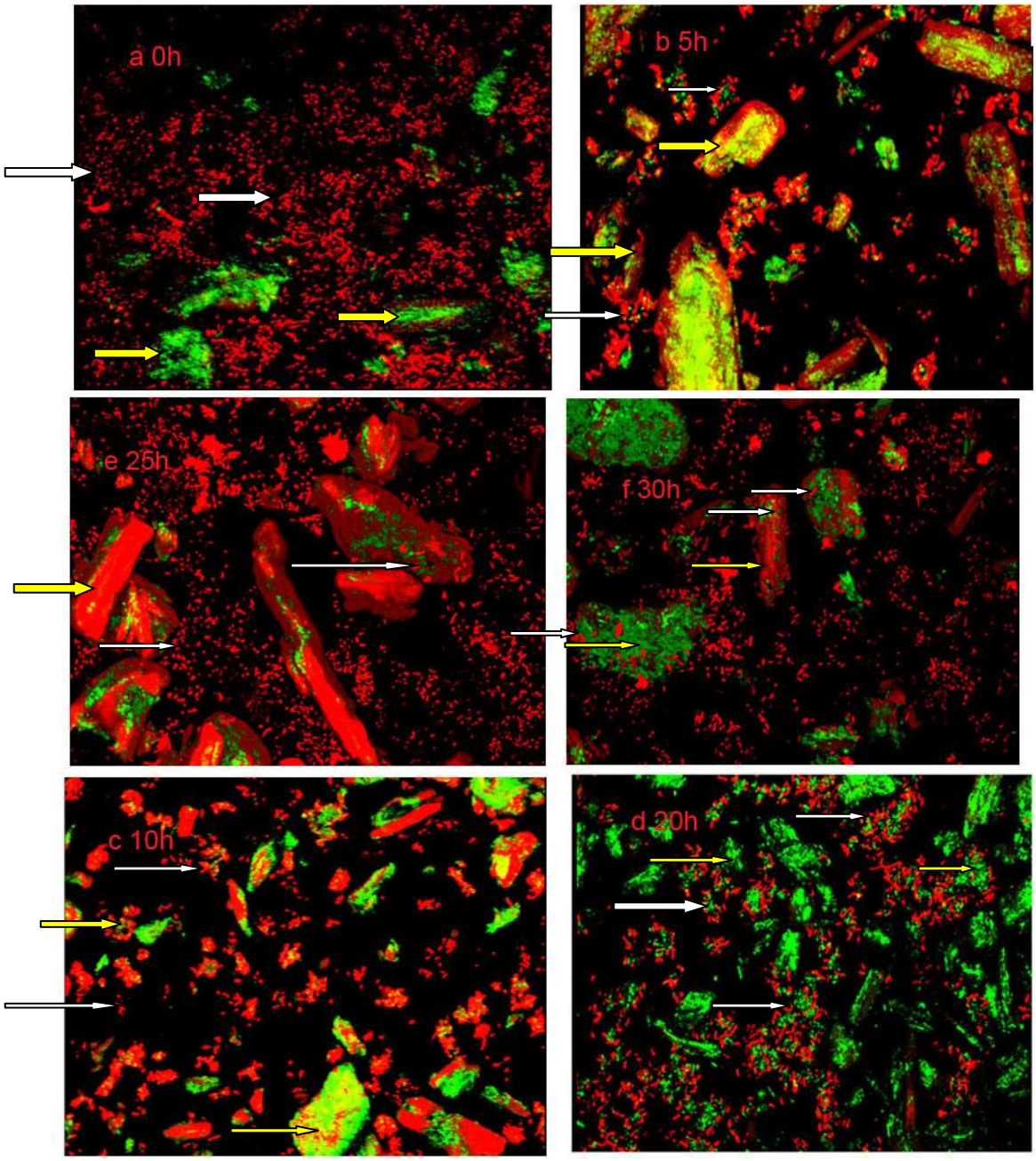


Figure 3.14 3D picture of switchgrass under confocal microscope (a and b). The switchgrass were dyed in both red and green, which were the artificial colors of dye Syto9 and WGA- tetramethylrhodamine conjugate separately. The size of switchgrass varied significantly; both small and big particles were detected.


a 0h
b 5h
e 25h
f 30h
c 10h
d 20h

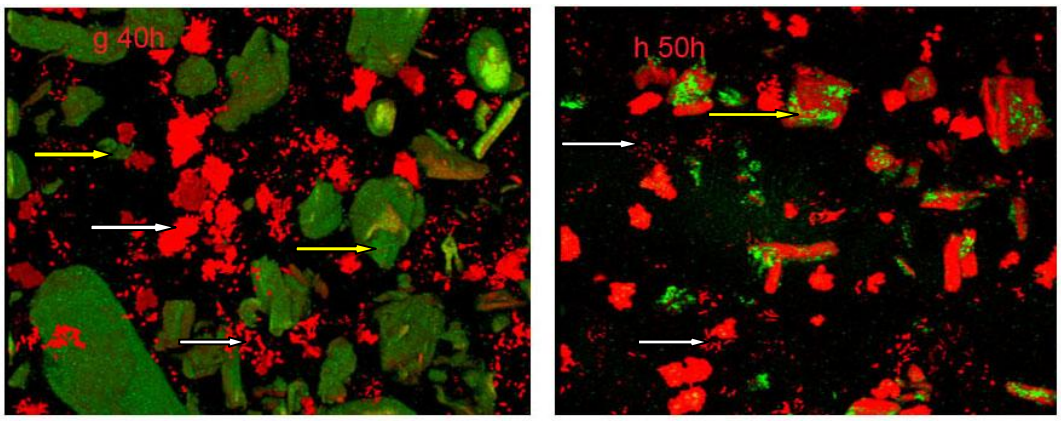


Figure 3.15 3D pictures of *C. saccharolyticus* on avicel at different time points of growth under confocal microscope. a: 0 h inoculation, depth of sample as10.05µm; b: 5 h inoculation, depth of sample as 5.10µm; c: 10 h inoculation, depth of sample as 0.45µm; d: 20 h inoculation, depth of sample as 5.10µm; e: 25 h inoculation, depth of sample as 5µm; f: 30 h inoculation, depth of sample as 3.90µm; g: 40 h inoculation, depth of sample as 5.05µm; h: 50 h inoculation, depth of sample as 2.00 µm. The cells were in red, while the avicel were dyed by both red and green. The white arrows point the cells, while the yellow arrows pointed the avicel.

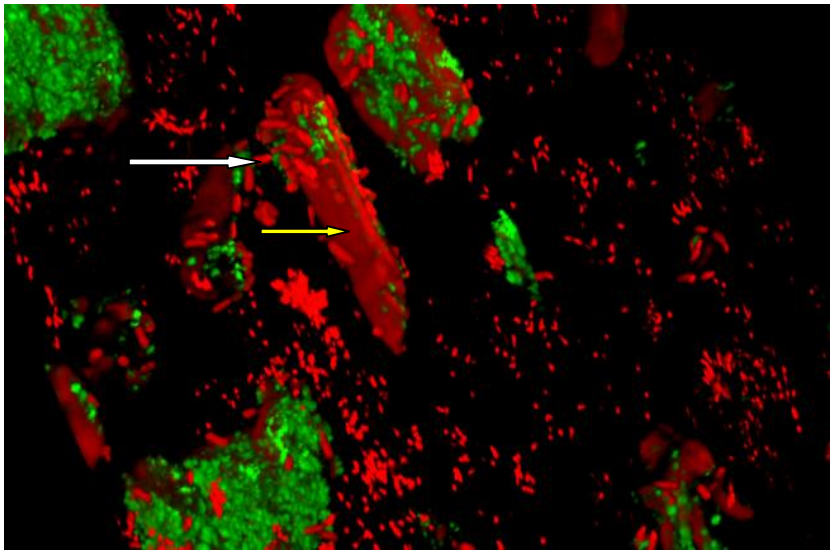


Figure 3.16 3D pictures of *C. saccharolyticus* on avicel when grew at 30 h. Depth of sample as 3.90 µm zoomed in for details of attachment. The cells were in red, while the avicel were dyed by both red and green. The white arrows pointed the cells, while the yellow arrows pointed the avicel. The cells were attached with both big and small particles of avicel, and the free cells were gathering together instead of spread evenly in the medium.

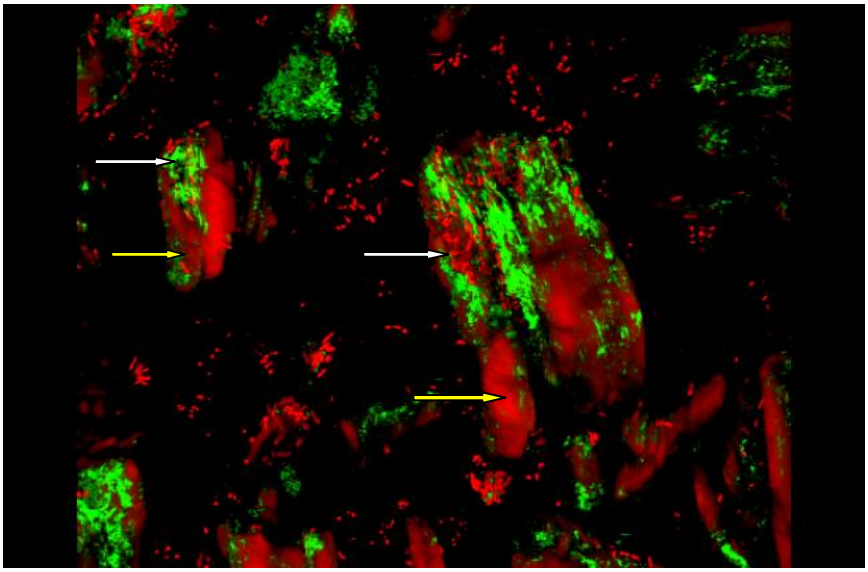
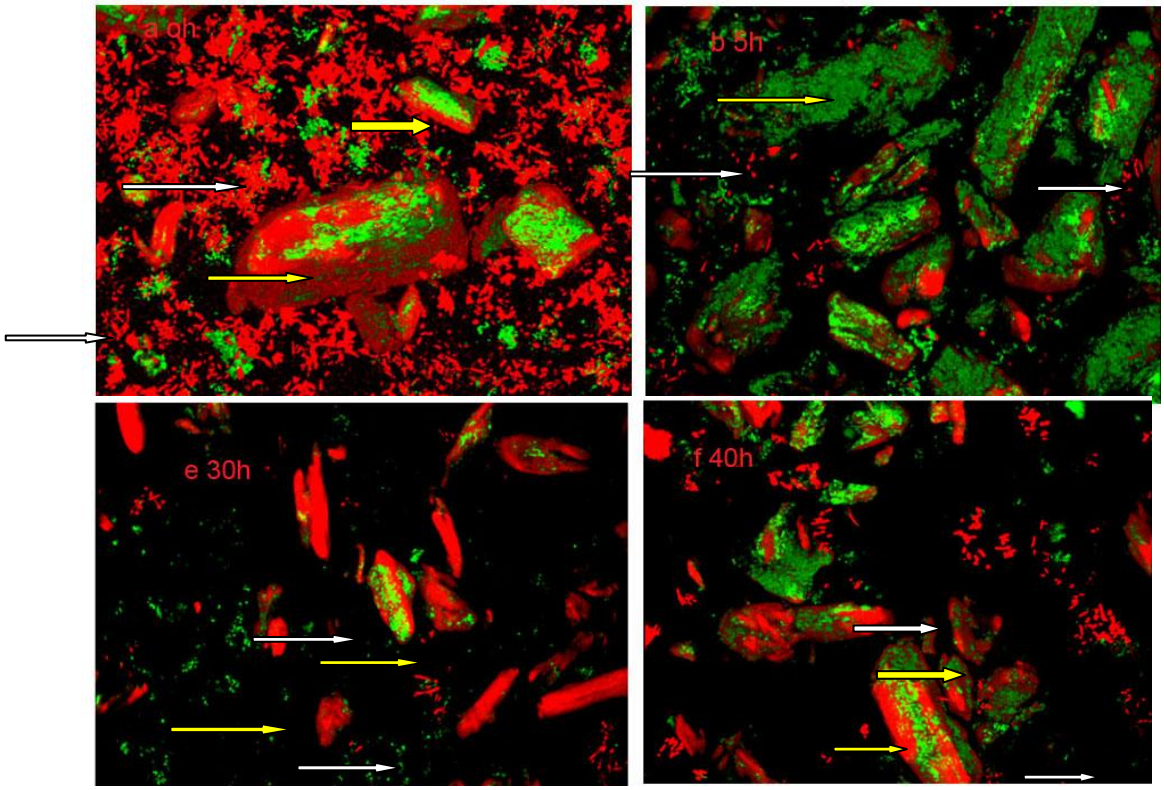


Figure 3.17 3D pictures of *C. saccharolyticus* on avicel when grew at 30 h under confocal microscope. Depth of sample as2.05µm zoomed in for details of attachment. The cells were in red, while the avicel were dyed by both red and green. The white arrows pointed the cells, while the yellow arrows pointed the avicel. The cells were attached with both big and small particles of avicel, and the free cells were gathering together instead of spreading evenly in the medium.


a 0h
b 5h
e 30h
f 40h

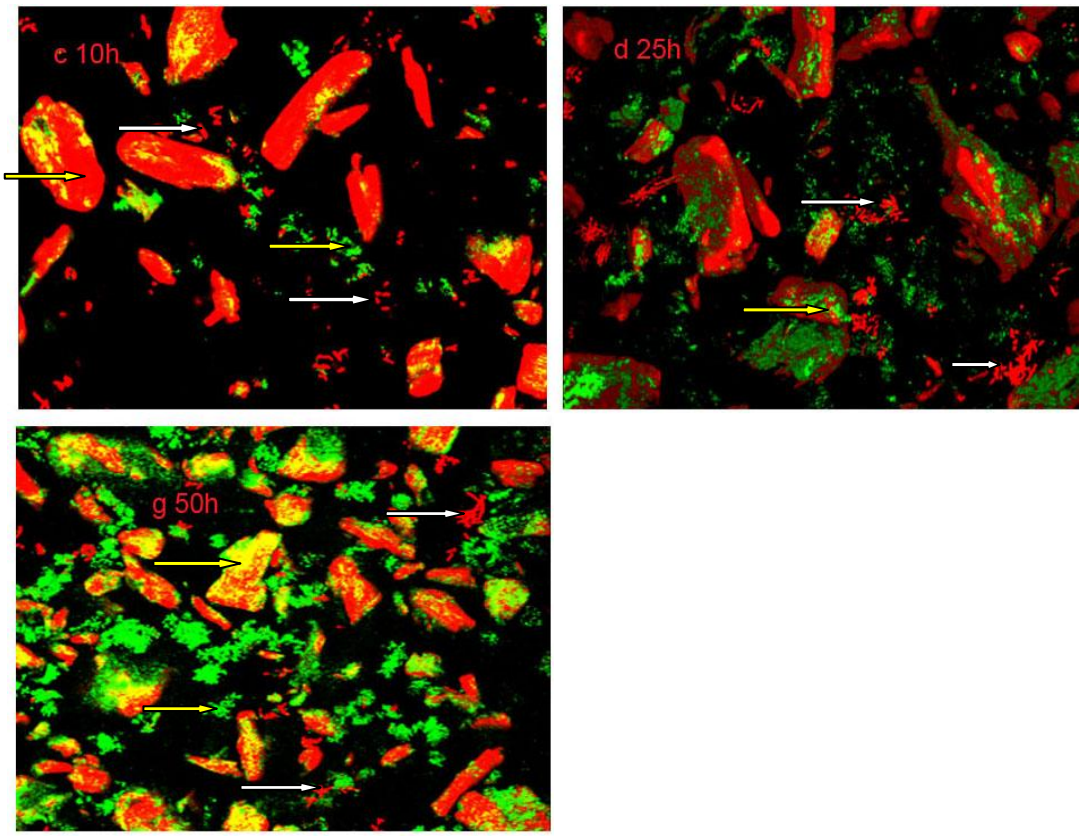


Figure 3.18 3D pictures of *C. kristjanssonii* on avicel at different time points of growth under confocal microscope. a: 0 h inoculation, depth of sample as12.00μm; b: 5 h inoculation, depth of sample as 1.05μm; c: 10 h inoculation, depth of sample as 0.25μm; d: 25 h inoculation, depth of sample as 0.75μm; e: 30 h inoculation, depth of sample as 0.20μm; f: 40 h inoculation, depth of sample as 0.80μm; g: 50 h inoculation, depth of sample as 0.85μm. The cells were in red, while the avicel were dyed by both red and green. The white arrows pointed the cells, while the yellow arrows pointed the avicel.

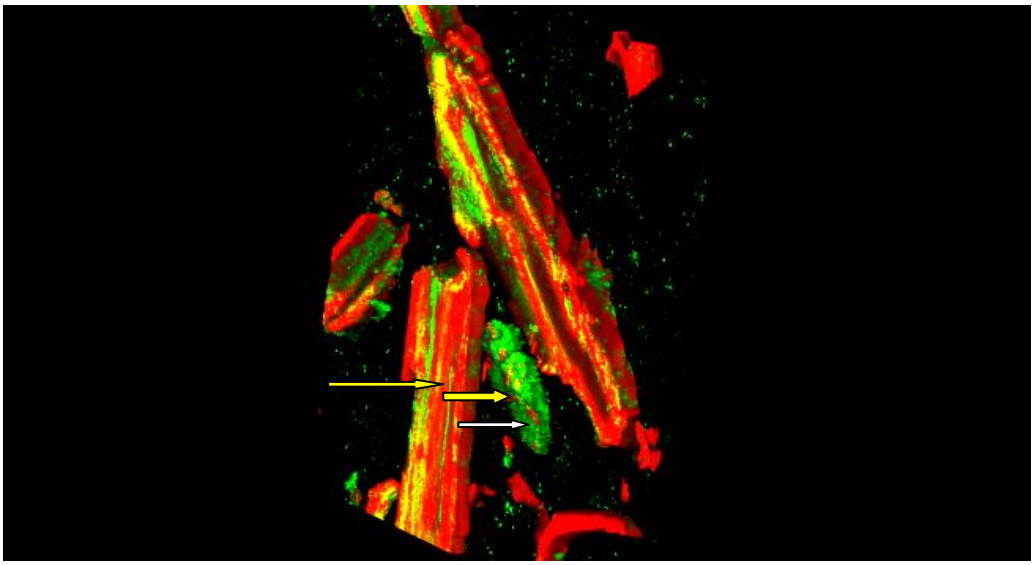
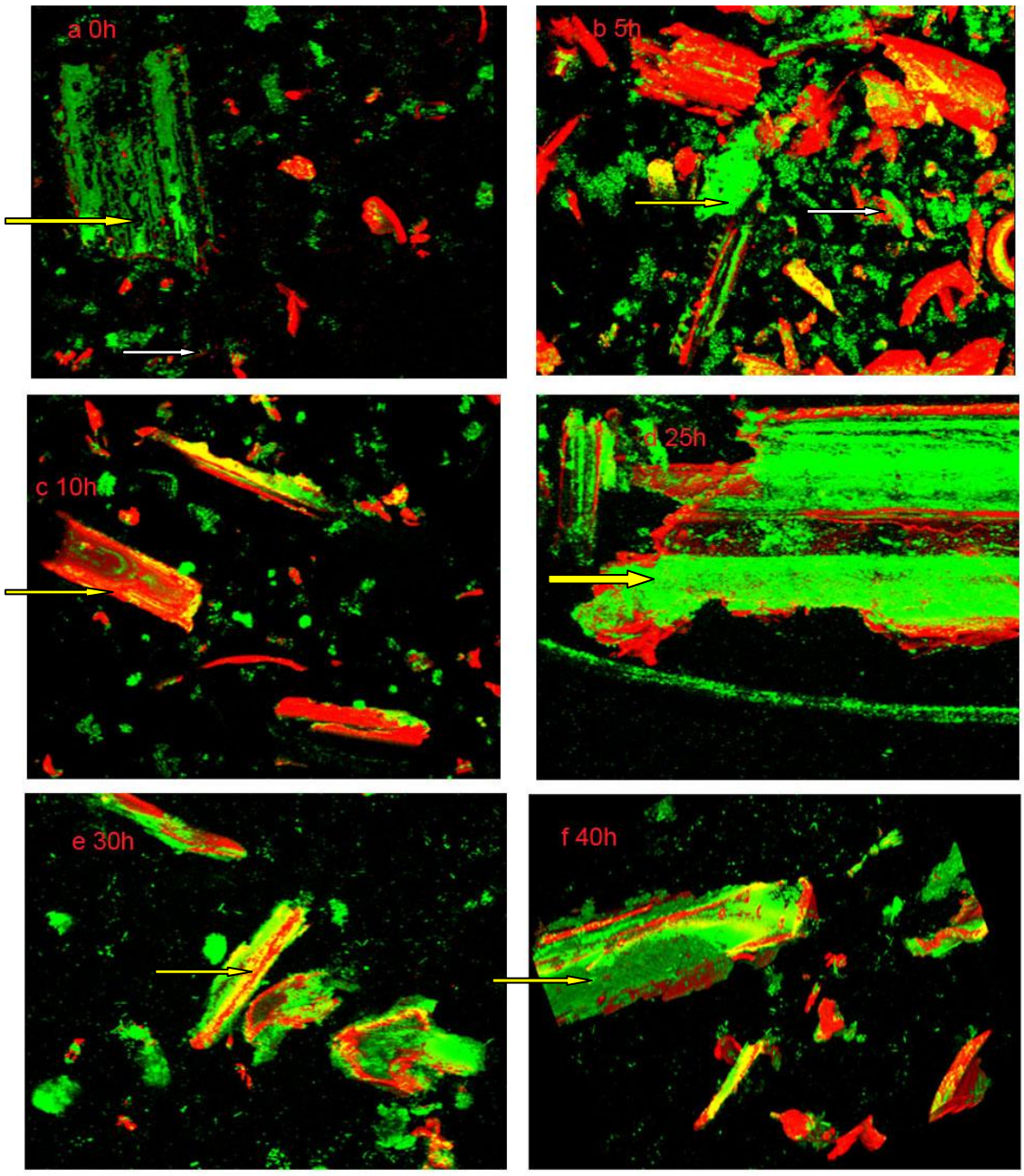


Figure 3.19 3D pictures of *C. kristjanssonii* on avicel at 50 h under confocal microscope. Depth of sample as 0.75µm zoomed in for details of attachment. The cells were in red, while the swichgrass were dyed by both red and green. Cells were detected to attach to the surface of switchgrass. The different sizes of particles of fibers with overlay color were avicel. The white arrows point the cells, while the yellow arrows pointed the avicel.


a 0h
b 5h
c 10h
d 25h
e 30h
f 40h

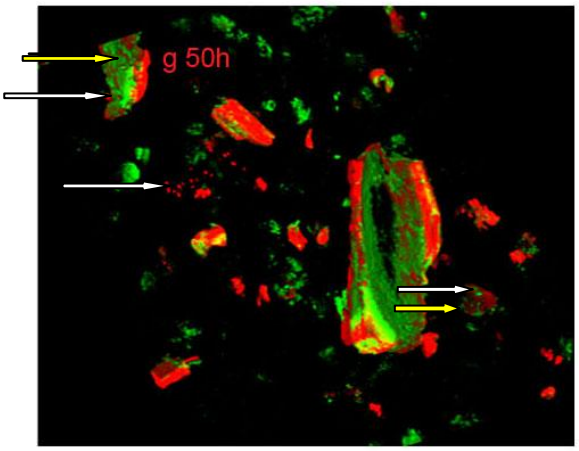


Figure 3.20 3D pictures of *C. saccharolyticus* on switchgrass on different time points of growth under confocal microscope. a: 0 h inoculation, depth of sample as 4.00μm; b: 5 h inoculation, depth of sample as 0.50μm; c: 10 h inoculation, depth of sample as 0.60μm; d: 25 h inoculation, depth of sample as 1.05μm; e: 30 h inoculation, depth of sample as 2.05μm; f: 40 h inoculation, depth of sample as 1.05μm; g: 50 h inoculation, depth of sample as 2.25μm. The cells were in red, while the switchgrass were dyed by both red and green. The white arrows pointed the cells, while the yellow arrows pointed the switchgrass.

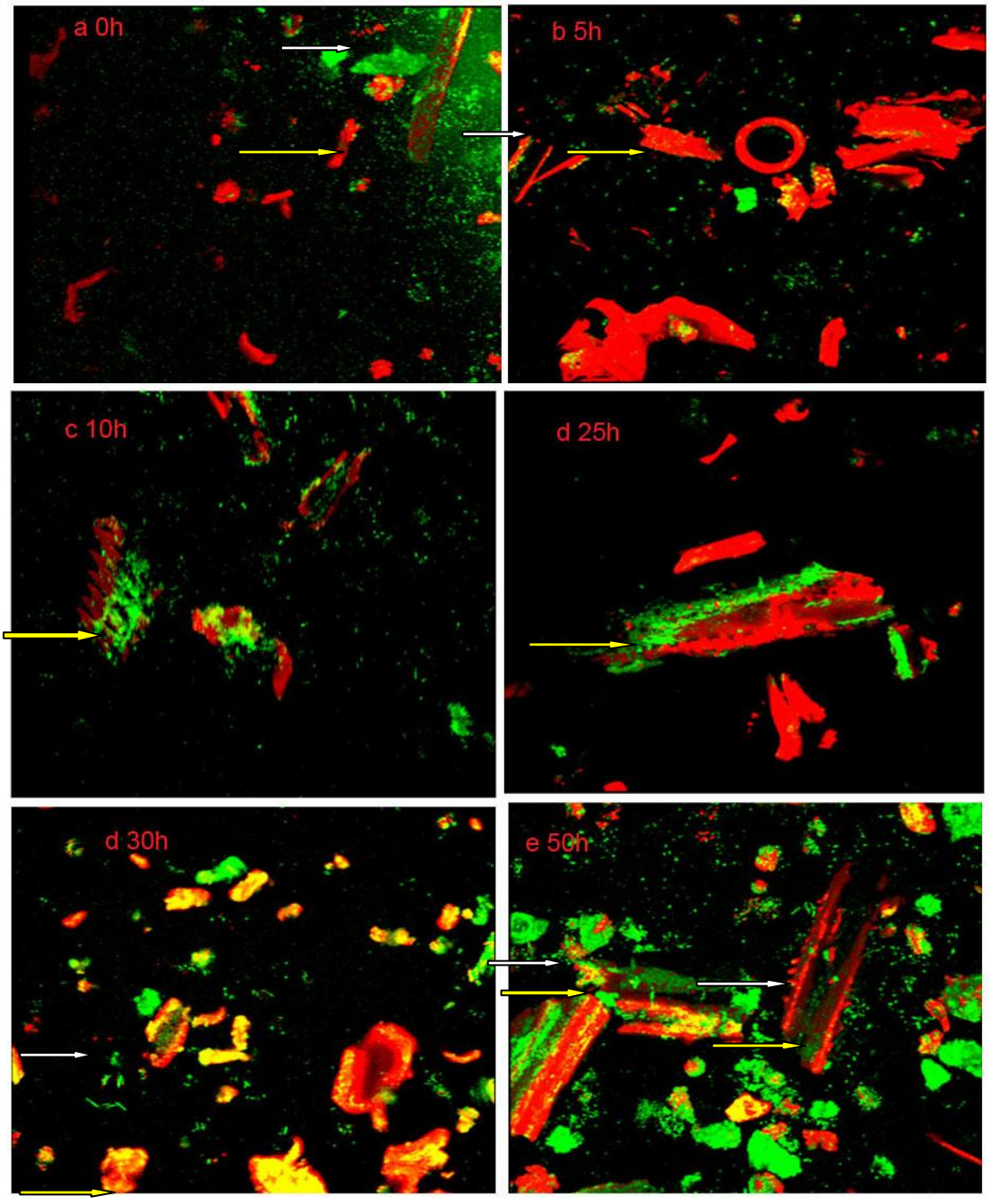


Figure 3.21 3D pictures of *C. kristjanssonii* on switchgrass on different time points of growth under confocal microscope. a: 0 h inoculation, depth of sample as 6.10μm; b: 5 h inoculation, depth of sample as 2.05μm; c: 10 h inoculation, depth of sample as 1.00μm; d:

25 h inoculation, depth of sample as 1.05μm; e: 30 h inoculation, depth of sample as 0.45μm; f: 50 h inoculation, depth of sample as 9.45 μm. The cells were in red, while the switchgrass were dyed by both red and green. The white arrows pointed the cells, while the yellow arrows pointed the switchgrass.

Figure 3.15a-h showed clear pictures of the cells of *C. saccharolyticus* on avicel at different time points of growth time under confocal microscope, in **Figure 3.15**a, 0 h sample showed that there was no obvious attachments between the cells and avicel, and the cells spread evenly around the avicel particles; by 5 h, as shown in **Figure 3.15**b, the cells started to attach to small avicel particles, but there was no obvious attachments detected between the big particles and cells; and more cells were detected that attached to the small particles from 10 h sample as shown in **Figure 3.15**c, and some cells started to gather together around the bigger particles; while in 20 h sample in **Figure 3.15**d, clear and nice pictures were obtained that showed the attachments between the cells and almost all kinds of sizes of particles; in **Figure 3.15**e, we can see that there were a large number of cells gathered around the avicel particles after 25 h's inoculation; by 30 h, there were still a lot of cells attached to variety sizes of avicel particles as well as many free cells lost attachments, as shown in **Figure 3.15**f; When the inoculation time researched 40 h, most of the cells left the avicel particles, but the cells did not spread evenly in the medium either, many cells aggregated together as shown in **Figure 3.15**g; the last picture was taken at 50 h, the cells did not re-attach with avicel, instead, more cells were binding with each other, and limited number of single cells could be found (**Figure 3.15**h).

For a clear vision of the attachment between *C. saccharolyticus* cells and avicel particles, zoomed pictures as **Figure 3.16** and **Figure 3.17** were taken to show the attachments at 30 h.

Figure 3.18a-g showed clear pictures of the *C. kristjanssonii* on avicel at different time points of inoculation under confocal microscope, the attachments behaviors were very similar to the sample of *C. saccharolyticus* on avicel. In **Figure 3.18**a, 0 h sample showed that there was no obvious attachments between the cells and avicel, and the cells spread relatively evenly around the avicel particles; by 5 h, as shown in **Figure 3.18**b, the cells started to attached to small the avicel particles and the end position of bigger avicel particles; significantly increased number of cells were attached to small particles as detected from 10 h sample from **Figure 3.18**c; while in 25 h and 30 h samples in **Figure 3.18**d and **Figure 3.18**e, there were similar attachment performances of cells detected—relatively more cells were attached to avicel, but there was not many attached cells found on bigger particle; in **Figure 3.18**f, the attached cell number did not increase, but the cells started to bind with each other; while in 50 h sample as shown in **Figure 3.18**g, the attachment performance of *C. kristjanssonii* was not very different from 40 h samples except the cells number started to decrease.

For a clear vision of the attachment between *C. kristjanssonii* cells and avicel particles, zoomed pictures as **Figure 3.19**were taken show the attachment at 50 h.

Figure 3.20a-g showed clear pictures of the *C. saccharolyticus* on switchgrass at different time points of inoculation under confocal microscope. In **Figure 3.20**a, 0 h sample showed no obvious attachments between the cells and switchgrass, and the cells spread relatively evenly around the grass particles; by 5 h, as shown in **Figure 3.20**b, some attachments between the cells and the small fibers were detected, but there was no obvious attachments

detected between cells and the big particles; there was no increase number of attached cells found from samples collected from 10, 25, 30, and 40 h inoculation, as shown in **Figure 3.20**c-f; however, a significantly increased number of cells attached to the relatively small switchgrass fibers were detected from 50 h sample as shown in **Figure 3.20**g.

Figure 3.21a-e show pictures of the *C. kristjanssonii* on switchgrass at different time points of inoculation under confocal microscope, and the attachment behaviors between *C. kristjanssonii* cells and switchgrass was similar to the one between *C. saccharolyticus* and switchgrass as shown in **Figure 3.20**. In **Figure 3.21**a, 0 h sample shows that there was no obvious attachments between the cells and switchgrass; by 5 h, as shown in **Figure 3.21**b, some attachments between the cells and the small fibers were detected, but there was no obvious attachments detected between the big particles and cells; And there was no increase number of attached cells found from samples collected from 10, 25, and 30 h inoculation till 50 h, when there was a increase numbers of attached cells to the relatively small switchgrass fibers detected, as shown in **Figure 3.21**c-e.

According to the confocal microscope observation results, *C. saccharolyticus* and *C. kristjanssonii* on two celluloses (avicel and switchgrass), the adhesion was more related to substrate type than microorganism strain. Both samples of the microbes on avicel showed similar adhesion performances, so did the samples of the two microbes on switchgrass. For the samples grown on avicel, the attachments started very soon after the inoculation of culture were transferred to the medium (5-10 h). And the process began on the microbes with the smaller particles of avicel and spread to the bigger ones. This phenomenon is

understandable: smaller particles have a bigger surface area, and the adhesion most probably was the result of simple van der Waal's forces as non-specific adhesion. The attachments between the avicel and two microorganisms were closely related to the growth and end-product production of the organisms. Between 20 h and 40 h, most cells were attached to the substrates, and many of them attached to the surface of big particles, while the attachment degree started to decrease with the 50 h sample, which corresponded to the growth and H_2 production peak time point of the microorganisms. These findings also proved that the adhesion between the cellulolytic cells and cellulosic substrates could have a significant meaning and a direct relation of the H_2 production. Increasing the amount of the attached cells would help improve H_2 production performance further, which could be achieved by increasing the cellulose substrate's surface area via reducing the particle size of the cellulosic materials used for fermentation.

The way to monitor the growth of the microorganism in this study was direct cell counting; however, this method only counts the cells that spread in the liquid part of the media, not attached to the substrates; The attachment detection experiments, including both SEM and confocal microscope observation, proved that a large number of cells attached to the insoluble substrates during inoculation. According to the rough estimate of the proportion of attached cells from the confocal microscope pictures, the exact cell numbers of the two microorganisms on avicel should be larger than the one described in the growth curve, based on free cell counting (**Figure 3.1** and **Figure 3.2**) during the late exponential stage. As to the samples on switchgrass, the adhesions were not as numerous as those on avicel samples at 20-40 h, and there were only a very small number of cells attached to the grass

fibers, but the adhesion started to enhance in 50 h sample, at a level proportional to the increase of H_2/acetate ratio of the switchgrass sample after 40 h.

As a methodology consideration, the SEM method was applied for the observation of the attachments of the sample first; however, the method did not show good reproducibility: only a couple of attached pictures were gained, and results were not constant. The reason may be the many centrifuge steps involved in preparing the SEM sample before the fixation step. Many steps were needed, and there were many chances that the original attachment status would be changed before it was fixed. The conductive properties of the sample variety increase the difficulty for continuous real-time observation of the samples. The confocal microscope method applied later proved to be a good method for the experiments similar to those in this study, once the proper type and concentration of dye and configuration of microscope were confirmed, the preparation time of the sample was very short. No step as centrifuges or repeating resuspending steps were involved, which helped to keep the original status of sample for a real-time observation. The confocal microscope method also gave access to colorful 3D pictures, from which, we could see the object from different directions and a movie of the sample showing in 360˚ view.

3.3 Proteomic test result

The *C. saccharolyticus* protein sample on cellobiose substrate was analyzed by proteomic method and more than 1500 proteins were successfully indentified, among which, protein Fli I, F, M, S, G,W, and P were found, shown the possible existence of flagella structure in *C. saccharolyticus*. But it has been suggested that *C. saccharolyticus* has no flagella

structure (Rainey *et al*., 1994). Other mobility related proteins found in the test were the methyl-accepting chemotaxis proteins (**Table 3.5**). The behavior of aggregating together around the cellulose, a process that was observed that from evenly spread at the beginning of growth showed the possible that *C. saccharolyticus* is a mobile microorganism, a fact also contradicted by the original description of the organism as a nonmobile bacterium (Rainey *et al*., 1994). Furthermore, microbe *C. kristjanssonii* showed the similar aggregating behaviors, but there was report about the existence of sub-terminal flagella structure in *C. kristjanssonii* (Bredholt *et al*,. 1999).

Besides, a CBM 3 protein which contributes to the attachment of the cell and fiber was found, as well as a fibronectin-binding-A domain-containing protein and a lysine motif protein (**Table 3.5**). A series of GHs protein were detected in the sample too as shown in **Table 3.5**, also the proteins as alcohol dehydrogenase (ADH) and hydrogenase that are responsible for the production of ethanol and H_2, respectively. The CBM 3 protein found in proteomic detection of *C. saccharolyticus* sample was believed to play a significant role in the specific attachment that occurs during the degrading of cellulose and is a part of extracellular cellulase in the degrading of cellulose (Park et al,. 2011). The reason we detected in our intracellular protein sample may come from the residual of secreted proteins. But since the sample have already been washed three times before the cell breaking step, the reason is not clear, and another possibility is that the cellulose with CBM 3 won't be secreted outside the cell without the induction of cellulose substrates(the sample was on cellobiose substrate) .

Table 3.5 Selected proteins identified in proteomic test of *C. saccharolyticus*.

Accession	Description
	Flagella related proteins
gi|146296283|	flagellar protein export ATPase FliI
gi|146296690|	flagellin domain-containing protein
gi|146296293|	flagellar motor switch protein
gi|146296280|	flagellar M-ring protein FliF
gi|146296281|	flagellar motor switch protein G
gi|146296707|	flagellar protein FliS
gi|146296292|	flagellar motor switch protein FliM
gi|146296680|	flagellar assembly protein FliW
gi|146296296|	flagellar biosynthetic protein FliP
	Chemotaxis related proteins
gi|146295857|	methyl-accepting chemotaxis sensory transducer
gi|146297449|	methyl-accepting chemotaxis sensory transducer
gi|146295474|	methyl-accepting chemotaxis sensory transducer
gi|146297486|	methyl-accepting chemotaxis sensory transducer
gi|146295457|	methyl-accepting chemotaxis sensory transducer
	Cellulose and fiber binding proteins
gi|146297363|	fibronectin-binding A domain-containing protein

gi|146296119|	type 3a cellulose-binding domain-containing protein
gi|146295299|	lysine 2,3-aminomutase MOTIF (LysM)
gi|146297448|	S-layer domain-containing protein
gi|146297442|	S-layer domain-containing protein
gi|146297710|	S-layer domain-containing protein
gi|146295275|	S-layer domain-containing protein
gi|146296043|	S-layer domain-containing protein
	GHs related proteins
gi|146297407|	cellulose 1,4-beta-cellobiosidase
gi|146296112|	cellulose 1,4-beta-cellobiosidase
gi|146297406|	xylan 1,4-beta-xylosidase
gi|146295735|	endo-1,4-beta-xylanase
gi|146297405|	endo-1,4-beta-xylanase
gi|146297433|	Acetyl xylan esterase
gi|146297733|	glycoside hydrolase family protein
gi|146297719|	glycoside hydrolase family protein
gi|146295272|	glycoside hydrolase family protein.
gi|146295632|	glycoside hydrolase family 3 protein
gi|146295423|	glycoside hydrolase family protein
gi|146295202|	glycoside hydrolase family protein
gi|146296574|	glycoside hydrolase family protein
gi|146295704|	glycoside hydrolase family protein

gi|146297675|	glycoside hydrolase family protein
gi|146295203|	glycoside hydrolase 15-related
gi|146295420|	glycoside hydrolase family protein
	Hydrogenases related proteins
gi|146296557|	hydrogenase formation HypD protein
gi|146296556|	hydrogenase assembly chaperone hypC/hupF
gi|146296553|	hydrogenase nickel insertion protein HypA
gi|146296554|	hydrogenase accessory protein HypB
gi|146296555|	(NiFe) hydrogenase maturation protein HypF
gi|146296869|	hydrogenase, Fe-only
	ADH related proteins
gi|146296249|	alcohol dehydrogenase
gi|146295750|	iron-containing alcohol dehydrogenase
gi|146296513|	iron-containing alcohol dehydrogenase
gi|146295802|	alcohol dehydrogenase
gi|146295466|	alcohol dehydrogenase
gi|146295905|	alcohol dehydrogenase
gi|146295666|	iron-containing alcohol dehydrogenase

The other two proteins found, fibronectin-binding -A domain-containing protein and a lysine motif protein, also contribute to the non-specific attachment between the cells and substrates. The former protein has binding sites for the cell surface (http://pfam.sanger.ac.uk), and the lysine motif protein is involved in bacterial cell degrading and has a general peptidoglycan-binding function (Joris *et al*,. 1992).

2D LC-MS/MS is a proteomic method routinely used to sequence peptides and identify proteins directly from complex mixtures. It uses a process known as tandem mass spectrometry (MS/MS), which because some samples present a complexity beyond the separation capacity of a 1D LC technique (Delahunty and Yates, 2007). Muddima *et al*. (2010 A and B) probed the extracellular proteome of *C. saccharolyticus* on glucose (medium contained yeast extract and peptides ≤1 kDa) as well as the intracellular proteome of the two thermophiles *C. saccharolyticus* and *T. maritima* in different environmental conditions, supplementing the former secretome information (only approximately 10% of the *C. saccharolyticus* proteome were identified). Only a handful of solid data is available for hyper/thermopile transcriptome so far (Muddiman *et al.*, 2010 A).

C. saccharolyticus' genome sequence is 2.97Mb, and initial annotation revealed 2695 ORFs and 2,679 predicted proteins. *C. saccharolyticus*' genome contains more than 60 GHs, the largest number of carbohydrate transport and metabolism genes in this group when compared with the genomes of *T. maritima, P. furiosus,* and *T. tengcongensis* (Van de Werken *et al*., 2008; Vanfossen *et al.*, 2008). Over 1500 proteins were identified from the intracellular proteome experiment on *C. saccharolyticus* on a cellobiose substrate

(strictly removed yeast extract and trypticase from the medium) in this study, while 71 proteins were identified in the secretome of *C. saccharolyticus* in Muddiman's study (2010A). Although there was only one set of data available so far in our study, it provided much information to compare with the published results. The genome sequences did not show the present of the genes encoding typical molecular components of a cellulosome such as dockerin domains and scaffolding proteins (Van de Werken *et al*., 2008), nor did the proteomic data. The genome of *C. saccharolyticus* encodes a great number of GHs, as mentioned above, and only a very small part of them were found in the intracellular proteome in this study, as shown in **Table 3.5**, Most of them were found in the extracellular proteome data by Muddiman's studies (2010A), as well as the CBM proteins that are responsible for the specific attachment between the substrates and cells, as discussed previously. A handful of non-specific attachment related proteins were detected in the extracellular proteome, such as the cell wall hydrolyses autolysin, etc. Very few hyper/thermopiles can grow on cellulose materials, even fewer can utilize cellulose raw materials such as switchgrass without chemical pretreatment. The reason that the many *Caldicellulosiruptor* strains have the capability of using raw grass substrates is the presence of GHs that can use complex β-linked glycans as well as α-linked ones (Van de Werken *et al*., 2008).

C. saccharolyticus has attracted considerable attention for its extraordinary capability to produce H_2. It is able to produce almost four mol of H_2 from each mol of glucose in continuous fermentation, and 2.9 mol of H_2 from each mol of glucose in batch culture, as shown in this study. A NiFe-hydrogenase and a Fe-only hydrogenase found in the

proteomic results as well as in the genome sequence information and the various proteins that required for maturation of hydrogenase such as HypC/HupF, HypA, and HypB were also found in its proteome (**Table 3.5**).

Although the NAD: ferredoxin oxidoreductase (Nfo) redundant that related to directly H_2 production from NADH were found in the genomes of *C. thermocellum, T. maritima,* and *T. ethanolicus,* but it could not been found in the genome of *C. saccharolyticus* (Van de Werken *et al.*, 2008), nor in the proteome information gained in this study. There was also a small amount of ethanol that found in the end products of both *Caldicellulosiruptor* microorganisms' cultures. Even though it has been suggested that there was no obvious ADH genes can be detected in the genome of *C. saccharolyticus*(Van de Werken *et al.*, 2008), but proteins of ADH enzymes were found in the proteomic results as shown in **Table 3.5**, so was an iron-containing ADH, but the function of this ADH has not been extensively studied yet.

3.4 Substrates analysis

3.4.1 Water and sugar content of switchgrass

As expected, there was an significant decrease of water content of switchgrass sample collected from the same field at different time from Jun to September, further to the dried sample collected on December, and the pictures (**Figure 3.22**) showed how the grass look like. The water contents detected were 78.3%, 45.2%, and 20.1% respectively (**Table 3.6**), indicating that when the dried grass was collected and stocked by the farmers, it was

relatively very dry after the nutrient transferring back to the fields, detailed data were in sugar conversion in buffered medium(**Table 3.7** and **Table 3.8**).

The HPLC results of sugar conversion in buffered medium test showed that there were simple sugar accumulation from insoluble substrates sample, cellobiose, arabinos, xylose, and glucose were detected, and the data of each sugar composition were shown in **Table 3.7** and **Table 3.8**. HPLC analysis of the sugar content of the switchgrass sample showed that little monosaccharide residues left in the dried sample, which confirmed the theory of the owner of Nott Farm, after maturing and drying, the switchgrass harvested by farmers has already given most of the nutrient and soluble mono- and disaccharides to the field, left the insoluble part such as cellulose. The very small amount of soluble sugar detected in switchgrass sample was possibly from the sugar left on the surface or vessel of the plants. The avicel and xylan samples also have the similar stories. On the other hand, both microorganisms produced more H_2 on switchgrass than on avicel (**Figure 3.3** and **Figure 3.4**). This may be due to the higher concentration of soluble sugar content in the switchgrass sample. Zsófia Herbel *et al* (2010) have stated that cellulase enzyme complex (es) needed by *C. saccharolyticus* to degrade cellulose could only be triggered by yeast extract or various monomeric sugars. In this study, all the potential carbon and energy source (yeast extract and trypticase) other than our target carbon source were removed from the media to exclude the possible effects, thus, the only soluble sugars in the media were from the residues of the materials themselves.

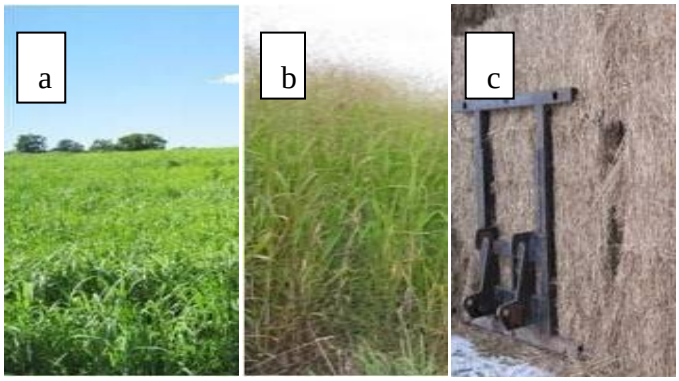


Figure 3.22 The switchgrass field of Nott Farm at different times. The samples were collected on Jun30, 2010, Sep 27, 2010, December 23, 2010(from a to c). Late June's switchgrass was close to half to its highest height, not mature; late September's switchgrass was mature and reached to its highest height but not dry; and late December's switchgrass was mature, reached to its highest height, and dried naturally in the field and the insoluble sugar part were back to the field, the grass was collected by the farmer and stocked dried in garage.

Table 3.6 Water content of switchgrass sample

Sample collected date	grass weight before drying (kg)	grass weight after drying(kg)	Water content (%)
Jun 30	2.81	0.61	78.3
Sep 27	1.24	0.68	45.2
Dec 23	2.49	1.99	20.1

Table 3.7 Sugar transfer in medium DSM 640 (for *C. saccharolyticus*) without inoculums.

Sugar content (mM)	Substrates (4g/L)						
	Glucose	**Xylose**	**Cellobiose**	**Xylan**	**Avicel**	**switchgrass**	**Control**
				0 h			
xylose	0	31.4	0	0	0	0	0
arabinose	6.4	0	5.8	5.8	6.2	6.1	0
glucose	23.8	0	0	0.11	0	0.10	0
cellobiose	0	0	12.5	0.02	0.07	0	0
				After 20 h			
xylose	0	31.3	0	0	0	0	0
arabinose	6.5	0	5.8	6.0	6.1	6.1	0
glucose	22.6	0	0.03	0.17	0.01	0.12	0
cellobiose	0	0	11.9	0	0	0	0
				After 40 h			
xylose	0	31.2	0	0	0	0	0
arabinose	5.5	0	5.7	5.8	6.2	6.1	0
glucose	20.8	0	0.04	0.18	0	0.13	0
cellobiose	0	0	11.9	0	0	0	0

Table 3.8 Sugar transfer in medium DSM 671(for *C. kristjanssonii*) without inoculums.

Sugar content (mM)	Substrates (4g/L)						
	Glucose	Xylose	Cellobiose	Xylan	Avicel	switchgrass	Control
				0 h			
xylose	0	26.6	0	0.30	0.30	0.28	0
arabinose	0	0	0.43	0	0	0	0
glucose	26.9	0	0	0.06	0	0.11	0
cellobiose	0	0	12.9	0	0	0	0
				After 20 h			
xylose	2.1	24.0	0.21	0.28	0.42	0.40	0
arabinose	0	0	0	0	0	0	0
glucose	23.4	0	0	0.11	0	0	0
cellobiose	0	0	11.8	0	0	0	0
				After 40 h			
xylose	4.0	20.7	0.31	0.26	0.31	0.44	0
arabinose	0	2.12	0	0.06	0	0	0
glucose	21.6	0	0.37	0	0.02	0.12	0
cellobiose	0	0	11.7	0	0	0	0

3.4.2 Sugar accumulation of the medium without inoculums

As shown in **Table 3.7** and **Table 3.8,** the sugar content of medium with all kinds of sugar substrates did not have obvious changes, thus there were no sugar conversions of insoluble sugar substrates in buffered medium. These results excluded the effect of sugar conversions of the whole study.

3.5 *C. kristjanssonii*'s plasmid and genome DNA

The existence of the plasmid in *C. kristjanssonii* was confirmed; the 1% agarose gel showed the existence and the approximate size of the plasmid DNA, the concentration and purity of the DNA sample were gained by a Nano drop test. The approximate size of the plasmid was 15 Kb, the concentration of the isolated plasmid DNA was 33.5 ng/µl, the 260/280 ratio was 1.83; the gel picture is shown in **Figure 3.23**.

The genome DNA was extracted from *C. kristjanssonii;* the 1% agarose gel showed the existence and approximate size of the genome DNA, the concentration and purity of the DNA sample were measured by using a Nano drop test. The approximate size of the genome DNA band showed on the agarose gel (%1) at about 25 Kb, and the concentration of the isolated genome DNA was 934.5 ng/µl with a 260/280 ratio 1.86, the gel picture was shown in **Figure 3.24.**

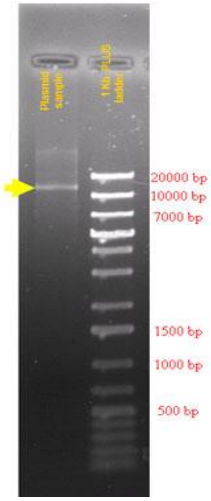


Figure 3.23 Plasmid isolated from *C. kristjanssonii*. The target band was shown by arrow. Approximate size of the plasmid was 15 Kb. The DNA ladder marker used was 1kb plus (Fermentas, Canada).

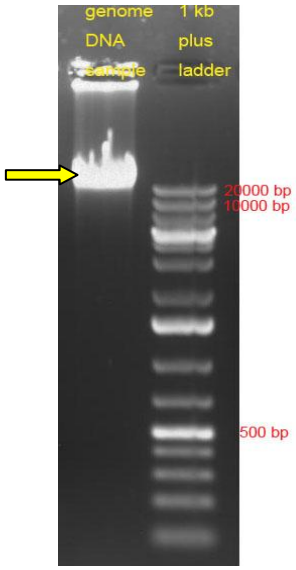


Figure 3.24 Genome DNA isolation from *C. kristjanssonii*. The target band was shown by arrow. Approximate size of the genome DNA was 25 Kb. The DNA ladder marker used was 1kb plus (Fermentas, Canada).

Chapter 4 Conclusion

In this project, contributions were made to develop a "one-step" fermentation process for biohydrogen production by using *C. saccharolyticus* and *C. kristjanssonii* as microorganisms and switchgrass as materials. The following conclusions could be drawn from the work conducted:

1. The growth of *C. saccharolyticus* and *C. kristjanssonii* on different substrates showed that both microorganisms were capable of utilizing substrates glucose, xylose, cellobiose, hemicellose xylan, and microcrystalline cellulose avicel, as well as cellulosic materials such as switchgrass without chemical pre-treatment.

2. Switchgrass was a good candidate of raw materials used for the production of hydrogen. The switchgrass sample harvested and dried by farmers contained a low concentration of water and a very small amount of soluble sugar, which was constant with the theory that switchgrass would give the nutrition back to field after mature and dried, which would help the field keep the fertility and encourage switchgrass' winter survivor.

3. Compared to other common-used H_2 producing microorganisms, both *C. saccharolyticu* and *C. kristjanssonii* produced a very high amount of H_2 when grown on glucose substrate (2.9 and 1.7 mol (H2)/mol (glucose) separately at 20 h growth). Moreover, *C. saccharolyticus* could use more xylose with a higher cell density at 20 h compared to glucose. A decrease of the H_2/acetate ratio was detected from 20 to 40 h

along with the increase of lactate production, indicating a shift of metabolism during this growth phase, which might be resulted from H_2 end product inhibition.

4. Both SEM and confocal microscope observations showed the existence of attachment of *C. saccharolyticus* and *C. kristjanssonii* cells' attachment to the insoluble cellulosic substrates—avicel and switchgrass during the growth. All samples showed the non-specific adhesion at the beginning after the inoculation, followed by the specific attachment. And the change in the approximate proportion of the attached cells to the insoluble substrates correlated to the rate of the hydrogen production. These findings showed the importance of the substrates adhesion to the H_2 production of cellulolytic microorganisms.

5. The aggregating behaviors of *C. saccharolyticus* observed via confocal microscope and the flagellar and chemotaxis proteins found in proteomics analysis suggested that *C. saccharolyticus* may be a mobile microorganism with flagella structure, which disagree with the original description of this microorganism. Furthermore, a 2D LC-MS/MS proteomic test on *C. saccharolyticus* identified more than 1500 proteins, which includes a series of proteins that may contribute to the process of attachment between the cells and substrates such as CBM3, fibronectin-binding-A domain-containing protein, s-layer proteins, and a lysine motif. Groups of proteins like GHs, ADH, and hydrogenase that should be responsible for the catabolism of cellulose and hemicellulose substrates, the production of ethanol and H_2 respectively were also

detected. Even though ADH was found in the proteomic sample of *C. saccharolyticus*, the ethanol producing capabilities of both microorganisms were not significantly high.

This study showed the great potential of *C. saccharolyticus* and *C. kristjanssonii* to be great candidate of biohydrogen producers.

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Appendix
Confocal video of samples

This appendix includes a list of video files of confocal videos of different samples at different time points:

1. This file is the confocal video of the sample *C. saccharolyticus* on avicel at 0 h. The file name of this video file is "*C. saccharolyticus* on avicel 0 h.mov".
2. This file is the confocal video of the sample *C. saccharolyticus* on avicel at 10 h. The file name of this video file is "*C. saccharolyticus* on avicel 10 h.mov".
3. This file is the confocal video of the sample *C. saccharolyticus* on avicel at 25 h. The file name of this video file is "*C. saccharolyticus* on avicel 25 h.mov".
4. This file is the confocal video of the sample *C. saccharolyticus* on avicel at 30 h. The file name of this video file is "*C. saccharolyticus* on avicel 30 h.mov".
5. This file is the confocal video of the sample *C. saccharolyticus* on avicel at 30 h. The file name of this video file is "*C. saccharolyticus* on avicel 30 h-2.mov".
6. This file is the confocal video of the sample *C. saccharolyticus* on avicel at 30 h. The file name of this video file is "*C. saccharolyticus* on avicel 30 h-3.mov".
7. This file is the confocal video of the sample *C. saccharolyticus* on switchgrass at 0 h. The file name of this video file is "*C. saccharolyticus* on switchgrrass 0 h.mov".
8. This file is the confocal video of the sample *C. saccharolyticus* on switchgrass at 10 h. The file name of this video file is "*C. saccharolyticus* on switchgrrass 10 h.mov".

9. This file is the confocal video of the sample *C. saccharolyticus* on switchgrass at 25 h. The file name of this video file is "*C. saccharolyticus* on switchgrrass 25 h.mov".
10. This file is the confocal video of the sample *C. saccharolyticus* on switchgrass at 30 h. The file name of this video file is "*C. saccharolyticus* on switchgrrass 30 h.mov".
11. This file is the confocal video of the sample *C. saccharolyticus* on switchgrass at 50 h. The file name of this video file is "*C. saccharolyticus* on switchgrrass 50 h.mov".
12. This file is the confocal video of the sample *C. saccharolyticus* on switchgrass at 50 h. The file name of this video file is "*C. saccharolyticus* on switchgrrass 50 h-2.mov".
13. This file is the confocal video of the sample *C. saccharolyticus* on switchgrass at 50 h. The file name of this video file is "*C. saccharolyticus* on switchgrrass 50 h-3.mov".
14. This file is the confocal video of the sample *C. kristjanssonii* on avicel at 0 h. The file name of this video file is "*C. kristjanssonii* on avicel 0 h.mov".
15. This file is the confocal video of the sample *C. kristjanssonii* on avicel at 10 h. The file name of this video file is "*C. kristjanssonii* on avicel 10h.mov".
16. This file is the confocal video of the sample *C. kristjanssonii* on avicel at 25 h. The file name of this video file is "*C. kristjanssonii* on avicel 25 h.mov".
17. This file is the confocal video of the sample *C. kristjanssonii* on avicel at 30 h. The file name of this video file is "*C. kristjanssonii* on avicel 30h.mov".
18. This file is the confocal video of the sample *C. kristjanssonii* on avicel at 50 h. The file name of this video file is "*C. kristjanssonii* on avicel 50h.mov".

19. This file is the confocal video of the sample *C. kristjanssonii* on avicel at 50 h, the avicel was a relatively bigger fiber. The file name of this video file is "*C. kristjanssonii* on avicel 50 h bigger fiber.mov".
20. This file is the confocal video of the sample *C. kristjanssonii* on swichgrass at 0 h. The file name of this video file is "*C. kristjanssonii* on switchgrass 0 h.mov".
21. This file is the confocal video of the sample *C. kristjanssonii* on swichgrass at 25 h. The file name of this video file is "*C. kristjanssonii* on switchgrass 25 h.mov".
22. This file is the confocal video of the sample *C. kristjanssonii* on swichgrass at 30 h. The file name of this video file is "*C. kristjanssonii* on switchgrass 30 h.mov".
23. This file is the confocal video of the sample *C. kristjanssonii* on swichgrass at 50 h. The file name of this video file is "*C. kristjanssonii* on switchgrass 50 h.mov".
24. This file is the confocal video of the sample *C. kristjanssonii* on swichgrass at 50 h. The file name of this video file is "*C. kristjanssonii* on switchgrass 50 h-2.mov".