Gluconic and xylonic acid production from lignocellulosic biomass by *Gluconobacter oxydans*

by

Lu Lu

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
August 3, 2013

Keywords: lignocellulosic biomass; gluconic acid; xylonic acid; fermentation; enzyme

Approved by

Maobing Tu, Chair, Assistant Professor of Forestry and Wildlife Sciences
Tom Gallagher, Co-chair, Associate Professor of Forestry and Wildlife Sciences
Xinyu Zhang, Assistant Professor of Polymer and Fiber Engineering

Abstract

To address the growing concerns over national energy security and climate change, significant amount of efforts have been made for developing sustainable biofuels and chemicals from renewable lignocellulosic biomass. Gluconic acid and xylonic acid are two important chemicals in food and pharmaceutical industries, which can be produced through fermentation by the microorganism *Gluonobacter oxydans*. The value-added chemicals production (such as gluconic acid and xylonic acid) from lignocellulosic biomass can generate extra revenue in the forest biorefinery process, which has the potential to significantly improve the viability of biofuels production from biomass.

In this study, we first investigated the gluconic acid and xylonic acid fermentation on glucose and xylose respectively without pH control. A nearly 100% conversion of glucose was obtained resulting in a gluconic acid production yield of 100%. However, for xylonic acid fermentation, only 61% conversion of xylose was reached after 48 h with a xylonic acid production yield of 57%. The low conversion yield of xylose probably was caused by the quick pH dropping and pH-sensitive enzymes. Subsequently, the effects of pH control (addition of $CaCO_3$, NaOAc buffering, NaOH neutralization) on gluconic acid and xylonic acid fermentation were further studied. Higher pH (>5.5) resulted in further oxidation of gluconic acid to 2-keto gluconic acid (2KGA) and 5-ketogluconic acid (5KGA) in the gluconic acid fermentation on glucose. With the addition of $CaCO_3$ at 6 g/L, xylose was completely converted into xylonic acid in the 48 h fermentation by *G. oxydans*.

Finally, we studied the gluconic acid and xylonic acid fermentation on pretreated woody biomass. Sweetgum (wood chips) was first pretreated with 50% ethanol and 1% H_2SO_4 at 170 ^{0}C for 1 h. The pretreated substrate was washed and collected for gluconic acid production through SHF (separated hydrolysis and fermentation) and SSF (simultaneous saccharification and fermentation) with 2.5, 5.0 and 10.0 FPU/g enzyme loading. In SHF process, the hydrolyzed glucose was quickly converted to gluconic acid within 10 h, and considerable amount of KGA (up to 20 g/L) was also produced with the further extension of fermentation to 48 h. In the SSF process, the hydrolyzed glucose was accumulated during the fermentation, which indicated the fermentation conditions (pH 4.8 and 37 ºC) in the SSF process was not suitable for gluconic production by *G. oxydans*. In conclusion, we believe that pH plays a very important role in gluconic acid and xylonic acid fermentation by *G. oxydans*. For gluconic acid fermentation, relatively high pH (>5.5) results in the accumulation of ketogluconic acid. For xylonic acid production, pH should be governed under neutral condition.

Acknowledgments

Foremost, I would like to express my deepest appreciation to my major advisor Dr. Maobing Tu for his remarkable guidance of my research work in past two years. Without his detailed and constructive advice, generous support and warm encouragement, this work would not have been possible. I am deeply motivated by his enthusiastic and professional approach to research, which will always inspire me in the future. Furthermore, I would express my sincere gratitude to my co-advisor Dr. Tom Gallagher and committee member Dr. Xinyu Zhang for their time and attention on my research and valuable advices for my thesis. My warm thanks also go to members in our research team (Rui Xie, Mi Li, Jing Li, Chenhuan Lai, Daihui Zhang, Zhiqiang Shi and Pixiang Wang) for their help and collaboration during my study at Auburn University. Finally, I would like to thank my parents and family for all their encouragement and support during my entire academic career. Without their consistent help and love, I cannot get this far.

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List of Abbreviations

GA	Gluconic acid
XA	Xylonic acid
KGA	Keto-gluconic acid
2KGA	2-keto-gluconic acid
5KGA	5-keto-gluconic acid

Chapter 1 Introduction

Our world depends heavily on non-renewable energy such as fossil fuel. Since millions of years are required to form these natural resources, their reserves are finite and subject to depletion while being consumed, not to say the consequential environmental problems like green gas effects (Hill, Nelson et al. 2006; Goldemberg 2007; Tomás-Pejó 2008; Mohan 2010). Based on these considerations, enormous attentions and efforts have been paid in biorefinery to produce biofuels and chemicals as potential alternatives for sustainable development (Pan, Gilkes et al. 2006; Hu, Heitmann et al. 2008; Thomsen, Thygesen et al. 2009).

Lignocellulosic material, the most abundant renewable organic resource on earth, provides a potential alternative to reduce our dependence on non-renewable resources and meanwhile, to mitigate the negative effects on global climate change (Demirbas 2007; Tomás-Pejó 2008; Chandel, Singh et al. 2010; Chandel and Singh 2011). Lignocellulosic biomass can be generally grouped into four categories: agricultural residues (including corn stover and sugarcane bagasse), dedicated energy crops, wood residues (including saw mill and paper mill discards), and municipal paper waste. Among which, forest feedstocks contribute approximately 370 million tons of total lignocellulosic biomass in the U.S per year (30%) (Limayem and Ricke 2012).

Despite significant efforts in the past years, the utilization of lignocellulosic biomass has not been commercialized in large scale. One of the primary challenges for reaching its commercialization is the relatively high cost compared to petroleum industry. To make biorefinery more economically viable, studies have been focused on: 1), generating valuable bioproducts during biofuel refinery; and 2), fully utilizing both cellulose and hemicellulose in

lignocellulosic hydrolysates (Hill, Nelson et al. 2006; Pan, Gilkes et al. 2006; Thomsen, Thygesen et al. 2009; Zhang, Tu et al. 2011).

Gluconic acid and xylonic acid are two chemicals which can be widely applied in various industries according to their properties (Meiberg and Spa 1983; Tomotani, Neves et al. 2005). *Gluconobacter oxydans* is a gram-negative bacterium belonging to the acetic acid bacteria family which is featured for capability to rapidly oxidize a variety of organic compounds to corresponding acids and ketones (Silberbach, Maier et al. 2003; Elfari, Ha et al. 2005). Obligate-aerobic strain *G. oxydans* is of great importance in industry for it can, not only quantitatively conduct microbial conversion through fermentation process, but also almost completely excrete the products into the medium (Olijve and Kok 1979; Herrmann, Merfort et al. 2004; Schweiger, Gross et al. 2010). The ability of oxidizing hexose as well as pentose sugars enables *G. oxydans* to convert both glucose and xylose to valuable acids from cellulose hydrolysates and hemicellulosic prehydrolysates, generating high economic effectiveness thus providing potentials to make lignocellulosic biomass commercially applicable.

Chapter 2 Literature Review

2.1 Lignocellulosic biomass

Driven by energy crisis and environment issue, since late 1970s, dramatic interests have been raised in production of biofuels and chemicals to replace petroleum-derived products (Sheehan and Himmel 1999; Gray, Zhao et al. 2006). In recent years, motivated by high oil price, this interest has gained more and more pace and has been viewed to be mainstreamed in the future (Rosillo-Calle and Walter 2006).

A primary challenge which hinders biofuels and bioproducts from industry application is the relatively high production cost (Hill, Nelson et al. 2006; Kumar, Singh et al. 2008; Binder and Raines 2009). The first generation biofuels and chemicals are produced from high-value sugars and oils. The current process of using crops such as sugar cane and corn is well-established. Relatively mature technology which utilizes enzymatic liquefaction and saccharification can produce clean glucose stream. This stream is then fermented to ethanol by *Saccharomyces* yeasts. However, the first generation bioethanol has a limitation in raw material supplication since it is food-competitive. The more promising second generation biofuel is based on cheaper and more abundant substrate lignocellulosic biomass. Utilization of forest residues in US would make a vital contribution in stimulating the valorization of local biomass to secure the energy supply, reduce the emissions of fossil CO_2, and support the rural economy (Zaldivar, Nielsen et al. 2001; Schell, Riley et al. 2004; Gray, Zhao et al. 2006; Lange 2007).

Different from corn grain whose major carbohydrate is starch, lignocellulose (Table 2.1), the most abundant renewable organic resources on earth, is mainly composed of carbohydrate polymers (cellulose and hemicellulose) and the aromatic polymer lignin (Pérez, Muñoz-Dorado et al. 2002; Zhao, Cheng et al. 2009; Scheller and Ulvskov 2010; Agbor, Cicek et al. 2011).

These carbohydrate polymers contain different sugar monomers (pentose and hexoses sugars) and are tightly bound to lignin (Wyman, Spindler et al. 1992; Foyle, Jennings et al. 2007; Moxley, Zhu et al. 2008).

Table 2.1. Chemical compositions in softwood and hardwood.

Chemical composition	Softwood (%)	Hardwood (%)
Cellulose	40-50	40-50
Hemicellulose	15-20	20-35
Lignin	23-33	16-25
Extractive	1-5	1-2
Inorganics	0.2-0.5	0.2-0.5

The wood cell is composed by several different layers, which are: the middle lamella (M), primary wall (P), secondary wall (S), and warty layer (W). These layers differ from each other in structures and chemical compositions (Wise 1944; Nikitin 1966; Sjöström 1993; Rowell 2012). Cellulose remains a basic skeleton structure surrounded by other substances which act like a matrix (hemicellulose) and encrusting material (lignin). These three main components are tightly associated. Lignin and polysaccharides are cross-linked, forming lignin-carbohydrate complex (LCC) (Walker and Wilson 1991; O'sullivan 1997; Klemm, Heublein et al. 2005).

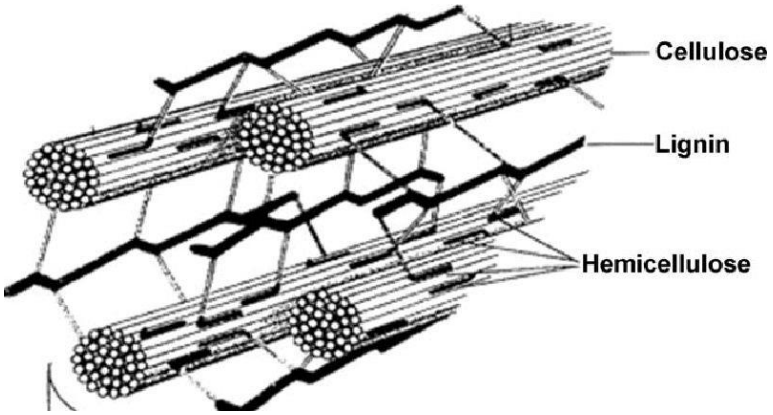

Figure 2.1. Proposed model for the structure of lignocellulose (Yinghuai, Yuanting et al. 2013).

The chemical compositions of agricultural residues and woody biomass vary among species, ages, growth conditions, and processing methods. The example of compositions of different lignocellulosic material is listed in Table 2.2 (Nidetzky, Steiner et al. 1993; Agblevor, Besler et al. 1996; Ragauskas, Nagy et al. 2006; Hallac 2011; Li, Tu et al. 2012).

Table 2.2. Chemical compositions of different lignocellulosic materials.

Lignocellulose	Glucan	Xylan	Mannan	Galactan	Arabinan	Lignin
Spruce wood	41.9	6.1	14.3	NA	1.2	27.1
Pine wood	37.7	4.6	7.0	NA	NA	27.5
Birch wood	38.2	18.5	1.2	NA	NA	22.8
Poplar wood	49.9	17.4	4.7	1.2	1.8	18.1
Sweetgum	41.2	16.2	3.3	1.9	0.8	25.8
Corn stover	36.4	18.0	0.6	1.0	3.0	16.6
Wheat straw	38.2	21.2	0.3	0.7	2.5	23.4
Switchgrass	31.0	20.4	0.3	0.9	2.8	17.6

Its compactness and complexity make lignocellulosic biomass difficult to be converted, which results in a high cost. Since biomass processing and utilization is complicated and expensive, value added chemicals production associated with biofuels production lignocellulosic biomass plays a critical role to lower the cost and to make the biorefinery process more economically competitive (Sun and Cheng 2002; Zielinski 2006; Lange 2007; Chandel and Singh 2011).

2.1.1 Cellulose

Cellulose is the main constitute of plant cell walls (Figure2.1). It is the most abundant carbon source on earth which contributes to about 50% weight of woods. Cellulose is composed of thousands of anhydroglucose molecules linked by β (1,4)-glycosidic bonds. The basic building unit of this chain polymer is cellobiose, a glucose-glucose dissacharide (Zaldivar, Nielsen et al. 2001).

The successive glucose units are rotated and repeated in cellobiose units. These unites are linked together through an oxygen which is covalently bounded to C1 and the adjoining C4 of glucose ring. The intra-chain hydrogen bindings between hydroxyl functional groups and oxygens stabilize the linkages and form the linear configuration of the cellulose chain. Van der waals and intermolecular hydrogen bonds between hydroxyl groups and oxygens enhance parallel stacking of multiple chains, therefore result in elementary fibrils which can further aggregate to form larger microfibrils (5-50 nm in diameter and several microns in length).

These intra- and inter- chain hydrogen bonding networks make cellulose a relatively stable polymer and give high axial stiffness to cellulose fibrils. In these fibrils, there are crystalline regions where cellulose chains are highly-ordered and arranged, and amorphous-like regions which are disordered (Nakagaito and Yano 2005; Ouajai and Shanks 2005; Ioelovich 2008; Pääkkö, Vapaavuori et al. 2008; Moon, Martini et al. 2011). The average DP (degree of

polymerization) of plant cellulose ranges from 7000 to 15000 glucose units according to different sources (Wise 1944; Fengel and Wegener 1983; Fengel and Wegener 1983).

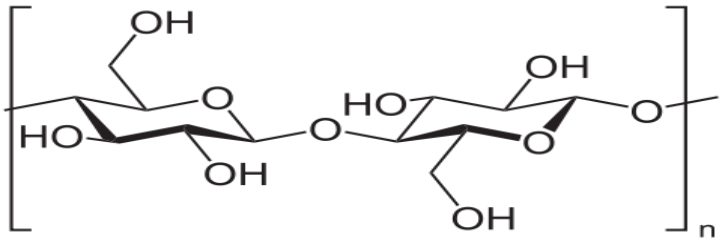

Figure 2.2. The basic structure of cellulose.

The structural characteristics and the encapsulation by lignin make cellulose highly crystalline and compact. It can be hydrolyzed to fermentable sugar glucose by acid hydrolysis or enzymatic hydrolysis (Zaldivar, Nielsen et al. 2001; Gray, Zhao et al. 2006; Demirbas 2007; Zhao, Zhang et al. 2012) .

2.1.2 Hemicellulose

Compared with cellulose, the structure of hemicellulose is more complicated. Hemicellulose is a highly branched heteropolymer containing both pentose sugars (D-xylose, D-arabinose) and hexose sugars (D-mannose, D-glucose and D-galactose) (Chandel, Singh et al. 2010). Most biomass hemicelluloses consist of either xylan or glucomannan as backbones and with acetyl group, arabinose, galactose, and methyl glucuronic acid on side chains (Zhang, Tu et al.). They serve as a connection between lignin and cellulose fibers and provide rigidity of the whole cellulose–hemicellulose–lignin network. The degree of branching and identity of minor sugars in hemicellulose depends primarily on the source of raw materials. Hemicellulose has better solubility than cellulose hence can be isolated from wood by extraction. The average degree of polymerization (DP) of hemicellulose is between 70 and 200 depending on different wood species.

Hemicellulose in hardwood is mainly composed by xylan with a weight percentage around 15-30%. In softwood, the primary existence is galactoglucomannan which accounts for 15-20% and xylan for 7-10%. Hardwood xylans are made by β-D-xylopyranosyl units which have 4-O-methyl-α-D-glucouronic acid linked to xylan backbone by O-(1→2) glycosidic bonds and acetyl side groups esterified at the C2 and/or H3 group. In softwood, xylans exist in the formation of arabino-4-O-methylglucuronoxylans which are not acetylated but substituted at C2 and C3 with 4-O-methyl-α-D-glucuronic acid and α-L-arabinofuranosyl residues respectively (Fengel and Wegener 1983).

Hardwood hemicellulose, which is rich in xylan (Figure1.2), is fit to produce xylonic acid (Fengel and Wegener 1983; Saha 2003; Laureano-Perez, Teymouri et al. 2005; Zielinski 2006).

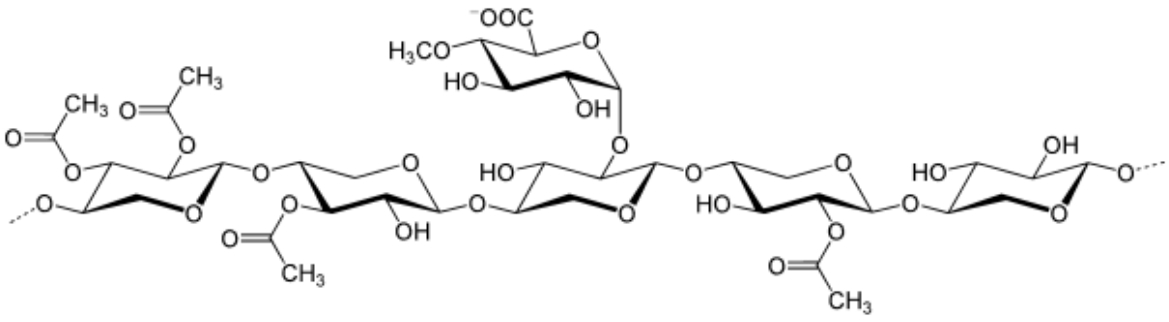

Figure 2.3. The basic structure of xylan in hemicellulose.

2.1.3 Lignin

Lignin is a complex, hydrophobic, and cross-linked aromatic polymer which is naturally found as an integral part of plant cell wall. It is embedded in a carbohydrate matrix of cellulose and hemicellulose polymers (Pearl 1967; Sarkanen and Ludwig 1971; Lin and Dence 1992).

Lignin includes several polymers of phenylpropene units: guaiacyl (G) units from the precursor *trans*-coniferyl-alcohol, syringyl (S) units from *trans*-sinapyl-alcohol, and p-

hydroxyphenyl (H) units from *trans*-p-coumaryl alcohol. The exact composition of lignin differs a lot in different species. Basically, lignin can be classified into guaiacyl lignin groups and guaiacyl-syringyl lignin groups. The structure of lignin is not homogenous. It consists of amorphous regions, structured particles and globules therefore is essentially changed under high temperature and acidic conditions (Wise 1944; Atalla and Agarwal 1986; Sjöström 1993; Novikov, Novikova et al. 2002).

2.2 Bioconversion process

Bioconversion of lignocellulosic biomass to biofuels and chemicals is primarily composed of four steps: pretreatment, hydrolysis, fermentation, and product separation/purification (Mosier, Wyman et al. 2005; Balat 2011). Figure 2.3 shows the basic features of this process in a block diagram.

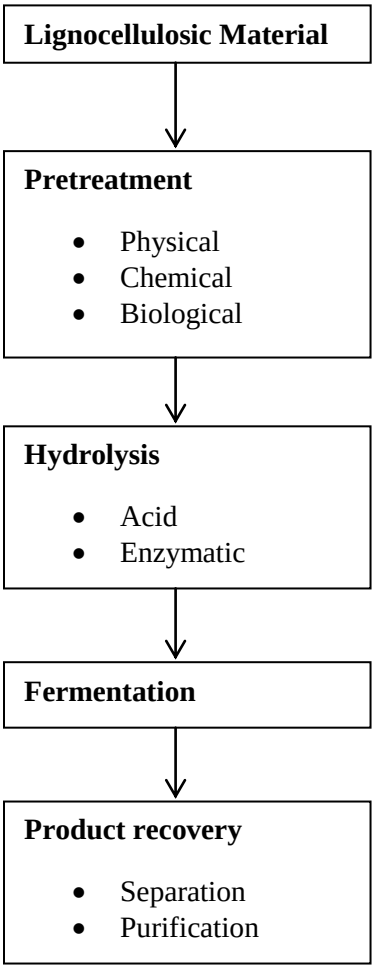

Figure 2.4. Schematic diagram of lignocellulosic bioconversion process.

2.2.1 Pretreatment

Pretreatment is the fundamental step for optimal hydrolysis and downstream operations (Wyman 1994; Wyman 1999). Since hydrolysis process may be limited by several factors: 1) degree of polymerization; 2) crystallinity; 3) accessible surface area; 4) particle size /specific surface area; and 5) lignin content and distribution. Desirable pretreatment is required: 1) to liberate the cellulose from rigid lignin and hemicellulose protection surrounding the cellulose microfibrils; and 2) to reduce the cellulose crystallinity by changing the physicochemical structural and compositional factors hinder the hydrolysis of cellulose (Mansfield, Mooney et al. 1999; Kumar, Barrett et al. 2009).

The pretreatment process contains mainly physical and thermochemical processes, which leads to the disruption of recalcitrant material in biomass. This provide maximal exposure of cellulases to cellulose surface area by increasing substrate porosity and lignin redistribution, therefore reaching effective hydrolysis with minimal energy consumption and maximal sugar recovery (Mosier, Wyman et al. 2005; Pan, Gilkes et al. 2006; Yang and Wyman 2008; Zhu and Pan 2010; Agbor, Cicek et al. 2011; Kumar, Hu et al. 2012).

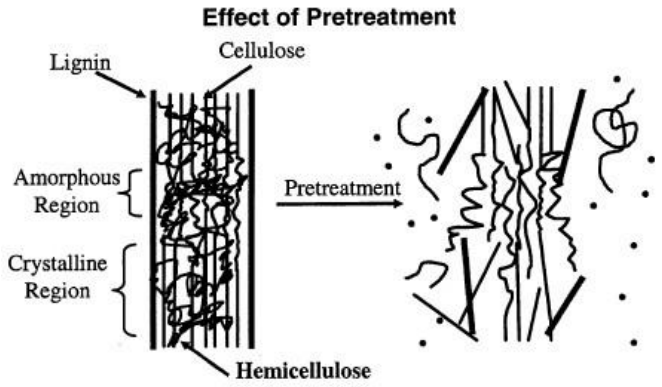

Figure 2.5. Schematic goals of pretreatment on lignocellulosic biomass (Hsu, Ladisch et al. 1980).

Pretreatment is regarded as having the most potential to lower the overall cost. Different technologies can be mainly summarized into three groups, namely, physical (uncatalyzed steam-explosion, liquid hot water pretreatment, mechanical comminution, high energy radiation, etc.); chemical (catalyzed steam-explosion, acid pretreatment, alkaline pretreatment, ammonia fiber expansion/AFEX, organosolv pretreatment, etc.); and biological pretreatment (Balan, Bals et al. 2009; Zhao, Peng et al. 2009; Zheng, Pan et al. 2009; Zhu and Pan 2010).

An ideal pretreatment should be: minimum size reduction, preserving hemicellulose, less inhibitors generated from degradation, low energy input, and cost-effective (Press 2000). Although it has been extensively studied to process various biomasses, each of the methods has their advantages and disadvantages. Table 2.3 lists the advantages and limitations of some typical processes (Parveen, Diane M. et al. 2009; Balat 2011).

Table 2.3. Advantages and limitations of various pretreatment processes for lignocellulosic biomass.

Pretreatment process	Advantages	Limitations
Mechanical comminution	Reduces cellulose crystallinity	Power consumption usually higher than inherent biomass energy
Steam explosion	Causes hemicellulose degradation and lignin transformation; cost-effective	Destruction of a portion of the xylan fraction; incomplete disruption of the lignin-carbohydrate matrix; generation of compounds inhibitory to microorganisms
AFEX	Increases accessible surface area, removes lignin and hemicellulose to an extent; does not produce inhibitors for down-stream processes	Not efficient for biomass with high lignin content
CO2 explosion	Increases accessible surface area; cost-effective; does not cause formation of inhibitory compounds	Does not modify lignin or hemicelluloses
Ozonolysis	Reduces lignin content; does not produce toxic residues	Large amount of ozone required; expensive
Acid hydrolysis	Hydrolyzes hemicellulose to xylose and other sugars; alters lignin structure	High cost; equipment corrosion; formation of toxic substances
Alkaline hydrolysis	Removes hemicelluloses and lignin; increases accessible surface area	Long residence times required; irrecoverable salts formed and incorporated into biomass
Organosolv	Hydrolyzes lignin and hemicelluloses	Solvents need to be drained from the reactor, evaporated, condensed, and recycled; high cost
Biological	Simple equipment degrades lignin and hemicelluloses; low energy requirements	Rate of hydrolysis is very low

The selection of pretreatment methods may affect the cost and performance in subsequent hydrolysis and fermentation (Ren, Wang et al. 2009). Based on different features of various pretreatment methods, it is necessary to choose proper method according to the substrate and objectives. The organosolv pretreatment is a delignification process similar to organosolv pulping— an organic or aqueous organic solvent mixture with or without acid or alkali catalysts is used to break down the internal lignin and hemicellulose bounds (Chum, Johnson et al. 1988; Pan, Arato et al. 2005; Pan, Xie et al. 2008; Zhao, Cheng et al. 2009; Zheng, Pan et al. 2009). For economic and safety reasons, ethanol is favored.

The advantages of organosolv pretreatment include: 1) solvent is easy to recover by distillation and recycle, 2) lignin can be isolated as solid and carbohydrates as syrup which result in convenient separation. These features is beneficial to fully use all biomass components and for downstream hydrolysis.

2.2.2 Detoxification

After pretreatment, lignin and partial hemicellulose are dissolved in liquid prehydrolysates. The free hemicellulose polymer is hydrolyzed to a mixture of monomeric and oligomeric sugars, including xylose, mannose, arabinose, galactose and glucose released from the cellulose hydrolysis. The residual solid part, cellulose and lignin can be filtered and washed (Ren, Wang et al. 2009).

During thermo-chemical pretreatment, inhibitory compounds such as weak organic acids (acetic, formic and levulinic acids), furan derivatives, i.e. furfural, 5-hydroxymethylfurfural (HMF) and phenolic compounds (phenol, vanillin, p-hydroxybenzoic acid) will be generated. These compounds are toxic to microbial fermentation and will decrease the fermentation yield.

These toxic compounds can be categorized into four groups: 1) sugar and lignin degradation products, 2) compounds released during pretreatment, 3) fermentation products and 4) heavy metal ions (Olsson and Hahn-Hägerdal 1996). The category and concentration of toxic compounds are mainly associated with feedstock species and pretreatment conditions. Other fermentation variables, such as pH conditions, temperatures and dissolved oxygen concentrations will also affect the inhibition level (Clark and Mackie 1984; Lopez, Nichols et al. 2004; Mussatto and Roberto 2004; Liu and Blaschek 2010; Zhao, Peng et al. 2012).

Detoxification step is needed to remove the toxic compounds before fermentation. Several methods, including biological, physical, and chemical ones, have been proposed to transform inhibitors into inactive compounds or to reduce their concentration (Larsson, Reimann et al. 1999; Palmqvist and Hahn-Hägerdal 2000; Klinke, Thomsen et al. 2004; Lopez, Nichols et al. 2004; Mussatto and Roberto 2004; Park, Yun et al. 2005; Feng, XueBing et al. 2009; Pienkos and Zhang 2009; Parawira and Tekere 2011).

Table 2.4. Advantages and limitations of different detoxification methods.

Detoxification	Methods	Advantage& Limitation
Biological	Oxidative polymerization of low molecular weight phenolic compounds; Degrade inhibitors of acetic acid, furfural and benzoic acid derivatives	Complete removal of phenolic compounds.
Physical	Evaporation(different vapor points of inhibitors and water); Extraction(different solubility between water and solvent); Adsorption(activated charcoal and ion exchange resins)	Evaporation may relatively increase the concentration on non-volatile inhibitors; Glucose loss as being adsorbed.
Chemical	Overliming (precipitation of toxic components and the instability of some inhibitors at high pH)	Lower concentration of furfural and HMF.

2.2.3 Enzymatic hydrolysis

After pretreatment, the pretreated lignocellulosic biomass need to be converted to soluble and fermentable sugar through chemical hydrolysis (such as diluted acid hydrolysis and acid hydrolysis) or enzymatic hydrolysis (Ladisch, Lin et al. 1983; Taherzadeh and Karimi 2007). Products after hydrolysis are illustrated in Figure 2.6 (Balat 2011).

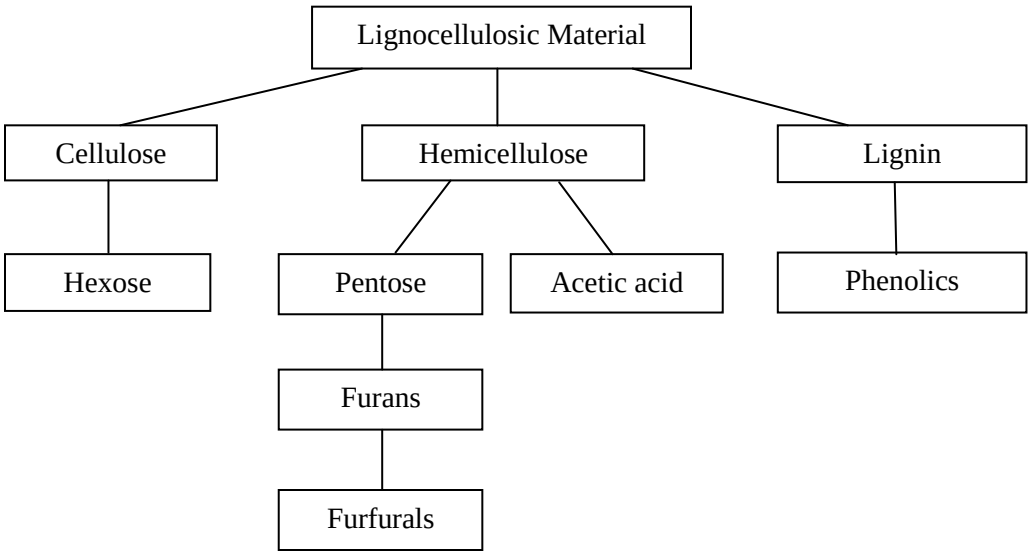

Figure 2.6. Main degradation products occurring during hydrolysis of lignocellulosic material (Balat 2011).

Cellulose from lignocellulosic biomass will be hydrolyzed to monosaccharide glucose; the hemicellulose can be mainly hydrolyzed to xylose and small amount of mannose, acetic acid, galactose, and glucose.

A significant disadvantage of acid hydrolysis is that under high temperature and high pressure, sugar will be degraded to undesirable compounds: xylose is further degraded to furfural and similarly, hexose degradation will generate 5-hydroxymethyl furfural (HMF) (Dunlop 1948;

Ulbricht, Northup et al. 1984; Gullu 2003; Binder and Raines 2009; Capuano and Fogliano 2011; Rosatella, Simeonov et al. 2011).

Using enzymatic hydrolysis can get rid of these degradations with 100% selective conversion of cellulose to glucose. Over the past half century, significant researches have been conducted to assess the ability of highly specific cellulolytic enzymes to depolymerize the cellulosic component of lignocellulosic substrates. However, efficient, rapid and complete enzymatic hydrolysis with low protein loadings still remains one of the major technical and economical bottlenecks in the overall bioconversion process (Walker and Wilson 1991; Xia and Cen 1999; Chang and Holtzapple 2000; Berlin, Maximenko et al. 2007; Arantes and Saddler 2010; Zhao, Song et al. 2011). Limitation factors relate to: 1) substrates aspects including lignin/hemicellulose association, degree of cellulose crystallinity and polymerization, extent of surface area and etc.; and 2) enzymes aspects like end-product inhibition, need for synergism, irreversible enzyme adsorption and etc. (Coughlan 1985; Zhang and Lynd 2004; Mansfield, Mooney et al. 2008; Sannigrahi, Miller et al. 2010).

Enzymatic saccharification of cellulose is generally viewed as a heterogeneous reaction system. Cellulases in aqueous environment react with the insoluble, macroscopic and structured cellulose. This process is typically catalyzed by cellulases from bacteria and fungi (Ryu, Kim et al. 1984; Himmel, Ruth et al. 1999; Lee, Evans et al. 2000). The widely accepted mechanism for enzymatic cellulose hydrolysis (Figure 2.7) involves synergistic actions by endoglucanses (EG, endo-1,4-b-D-glucanases, or EC 3.2.1.3.), exoglucanases or cellobiohydrolases (CBH, 1,4-b-D-glucan cellobiohydrolases, or EC 3.2.1.91.), and β-glucosidases (BGL, cellobiases or EC 3.2.1.21).

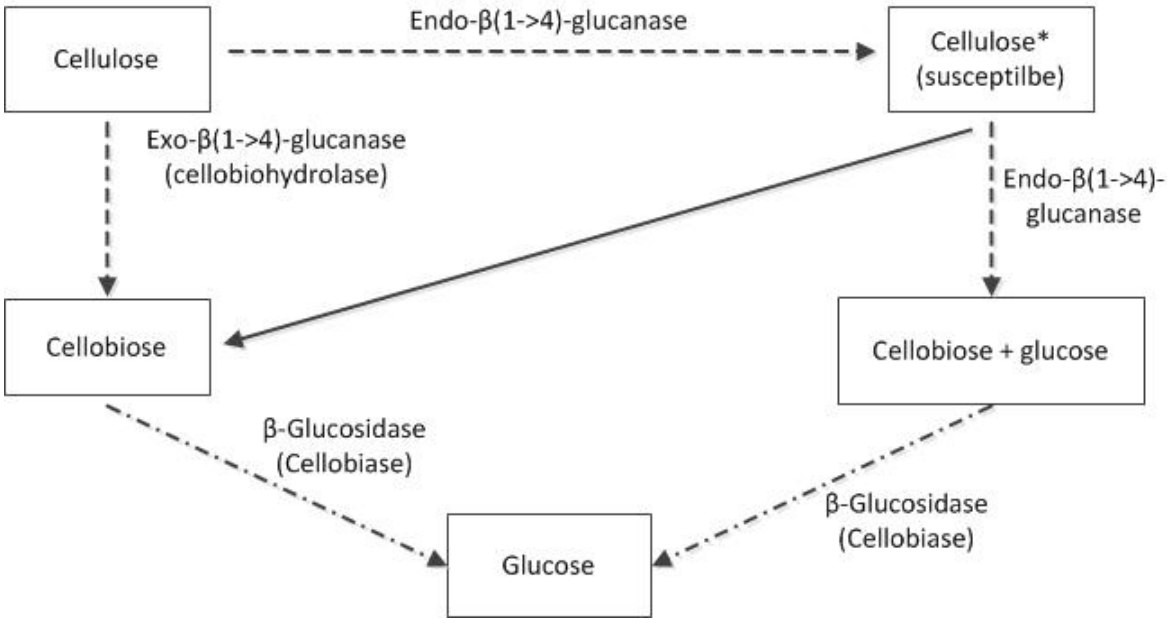

Figure 2.7. Model of actions in enzymatic cellulose hydrolysis.

2.2.4 SHF and SSF

Two principal configurations that use enzymes for saccharification are separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) (Wingren, Galbe et al. 2008; Ask, Olofsson et al. 2012).

In SHF process, enzymatic hydrolysis and fermentation are carried out separately, which makes it possible to run each process under their optimal conditions. In addition, SHF offers the possibility of cell recycling, whereas in SSF it is not possible to separate cells and solid raw material particles. Its primary limitation is the end-product inhibition of the cellulolytic enzymes (Alfani, Gallifuoco et al. 2000; Tomás-Pejó, Oliva et al. 2008).

SSF integrates the enzymatic hydrolysis of cellulose with microbial fermentation for target biofuels or chemicals production. This allows released sugars from the hydrolysis to be rapidly consumed by microorganisms, thereby minimizing end-product inhibition on the cellulolytic

enzymes. SSF is attractive for lower capital costs, lower process time and minimum loss of sugars by using same reaction vessel. One disadvantage is that since conditions for both enzymatic hydrolysis and fermentation have to be the same, therefore, typically suboptimal for both (Wyman, Spindler et al. 1992; Hari Krishna, Janardhan Reddy et al. 2001; Öhgren, Bengtsson et al. 2006; Ask, Olofsson et al. 2012).

Choice of configuration should be determined by a tradeoff of advantages and drawbacks associated with motivations.

2.2.5 Gluconic acid production

Gluconic acid (GA, penta-hydroxycaproic acid; Figure 2.8) is a naturally existed polyhydroxycarboxylic acid which can be produced from glucose (Figure 2.9) through dehydrogenation catalyzed by glucose oxidase (Ramachandran, Fontanille et al. 2006; Singh and Kumar 2007).

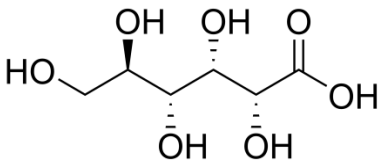

Figure 2.8. Structure of gluconic acid

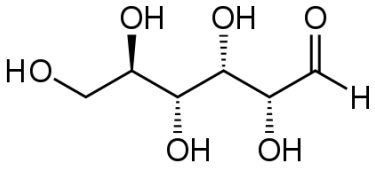

Figure 2.9. Structure of glucose

The general characteristic of gluconic acid has been listed in Table 2.5 (Ramachandran, Fontanille et al. 2006). As a noncorrosive, nonvolatile and nontoxic mild organic acid, gluconic acid, together with its derivates, have been reported of significant applications in various industries, including food, construction, pharmaceutical and hygienic industry etc.

Table 2.5. Characteristics of gluconic acid

Gluconic acid	
Nature	Noncorrosive, mild acidid, less irritating, nonodorous, nontoxic, easily biodegradable, nonvolatile organic acid
Relative molecular mass	196.16
Chemical formula	$C_6H_{12}O_7$
Synonym	2,3,4,5,6-pentahydroxyhexanoic acid
pKa	3.7
Melting point(50% solution)	Lower than 12 ^{0}C
Boiling point(50% solution)	Higher than 100 ^{0}C
Density	1.24 g/mL
Apperance	Clear to brown
Solubility	Soluble in water
Sourness	Mild, soft, refreshing taste

Gluconic acid has been used as additive in food industry to impart a refreshing sour taste in many products i.e wine and fruit juice etc. In virtue of its excellent chelating capacity in alkaline condition, gluconic acid has also been applied in cleaning and construction industries to increase the resistance and stability of cement during extreme climates (Seiskari, Linko et al. 1985; Shiraishi, Kawakami et al. 1989; Perfettini, Revertegat et al. 1991; Kim and Kim 1992; Marcincinova-Benabdillah, Boustta et al. 2001; Anastassiadis, Aivasidis et al. 2003). Sodium salt of gluconic acid, based on its excellent capability of chelating calcium and other di-/trivalent ions, helps sequestering iron in textile industry and derusting in metallurgy.

To meet the market demand of gluconic acid, various approaches, including biological, electrochemical and biochemical, have been developed to manufacture gluconic acid and its salts

(Isbell, Frush et al. 1932; Tomotani, Neves et al. 2005). Considering the process cost and efficience, microbial fermation is most favored for manufacturing gluconic acid.

Certain studies have been done with various microorganisms to produce gluconic acid throgh fermentation process. Related enzymes and fermentation kinetics of gluconic acid by *Aspergillus niger* upon various substrates including glucose, cellulose microfiber and whey etc. have been widely studied (Perfettini, Revertegat et al. 1991; Buzzini, Gobbetti et al. 1993; Vassilev, Vassileva et al. 1993; Roukas 2000; Sankpal and Kulkarni 2002; Znad, Markoš et al. 2004; Ikeda, Park et al. 2006; Sharma, Vivekanand et al. 2008). Apart from *A.niger*, some other specises of this genera such as *Penicillium*, *Gliocadium*, *Scopulariopsis* and *Gonatobotrys* have been tested for gluconic acid production (Milson and Meers 1985; Ramachandran, Fontanille et al. 2006).

In addition to fungal species, bacteria, which are most frequently used, play a central role in gluconic acid production. *Zymomonas mobilis* has been investigated to convert glucose and fructose to gluconic acid and sorbitol. The gluconic acid accumulated was further metabolized to ethanol. After permeabilizing the cells using cationic detergents, maximum sorbitol and gluconic acid concentrations were determined by Rehr of 295 $g \cdot L^{-1}$ with no ethanol production. Since the production of gluconic acid and sorbitol were simultaneously when using glucose and Jerusalem artichoke as substrate via glucose-fructose oxidoreductase from *Zymomonas mobilis* and inulinase, cells of *Z. mobilis* need to be permeabilized. The optimum amounts of both chitin-immobilized inulinase and permeabilized cells for coimmobilization were determined by Kim as well as the operational conditions (Rehr, Wilhelm et al. 1991; Kim and Kim 1992).

The capability to produce gluconic acid by *Gluconobacter oxydans* was identified by Olijve in late 70s when analyzing the growth of it in glucose containing medium. Result shows that

G. oxydans was able to grow successively in glucose and nitrogen-limited chemostat cultures. Construction of mass balances of organisms growing at increasing dilution rates in glucose-limited cultures, at pH 5.5, indicated a major shift from extensive glucose metabolism through pentose phosphate pathway to the direct glucose oxidation yielding gluconic acid (Olijve and Kok 1979; Olijve and Kok 1979). Continuous production has been achieved with a highest glucose conversion of 96%. Other fermentation products (2-keto-gluconic acid, 5-keto-gluconic acid and 2,5-diketogluconic acid) which are derived from further oxidation of gluconic acid were characterized according to multiple pH level (Seiskari, Linko et al. 1985; Shiraishi, Kawakami et al. 1989; Silberbach, Maier et al. 2003; Keliang and Dongzhi 2006). Following are the patterns on gluconic acid production in recent years (Table 2.6).

(Information based on: http://www.freepatentsonline.com).

Table 2.6. Recent patents on gluconic acid microbial production.

Patent number	Year	Author	Patented process
BR9403981	1996	Jonas, R.H.H.H.; Moura-de-Silveira M. and Castilho-Lopes-de-Costa, J.P.	Gluconate production by Zymomonas sp. with controlled ethanol production and selective precipitation
WO9635800	1996	Vroemem, A.J. and Beverini, M	High yield enzymatic conversion of glucose to GA
WO9724454	1997	Lantero, O.J and Shetty J.K	An enzymatic method that allows high conversion rates of glucose to GA without expensive down stream recovering procedures
5962286	1999	Anastassiadis, S.; Aivasidis, A. and Wandrey, C.	Process for the production of GA with a strain of *Aureobasidium pullulans* (de bary) Arnaud
6828130	2004	Chatterjee, C.; Chatterjee, N. P. and Furtado, E. D.	Production of gluconate salts
6942997	2005	Lantero, Oreste J. and Shetty, J.K.	Process for the preparation of GA and GA produced thereby
7618664	2009	Vorage, M. J. A. W. ; Kremer, D. R. ; Sloots, B. and Meiberg, J. B. M.	Method for preparing calcium gluconate

2.2.6 Xylonic acid production

Xylose (Figure 2.10) is the main building block for hemicellulose. It represents up to 30% of many lignocellulosic feedstocks such as agricultural residues and hardwoods, which could be mostly released in the biomass prehydrolysate after pretreatment and ready to be utilized by bio-catalysts for biofuels and chemicals (van Maris, Winkler et al. 2007). As the monomer of xylan, which is most abundant hemicellulose in hardwoods, xylose can be metabolized to various products by microorganism. Possible products include ethanol, butanol, isopropanol, acetone, 2,3- butanediol, itaconic acid, xylitol and xylonic acid. Among various products converted microbiologically from xylose, xylonic acid (Figure 2.11) is the most advantageous one in regard of the yield (Buchert, Viikari et al. 1986).

Xylonic acid, derived from the five-carbon hemicellulose sugar xylose, is a multifunctional versatile platform chemical. Identified by the US Department of Energy (DOE) as one of the top 30 high-value chemicals that can be produced via biomass conversion, xylonic acid has been widely applied in numerous industries (Liu, Valdehuesa et al. 2011; Nygård, Toivari et al. 2011). It has been effectively utilized as a cement dispersing agent to improve concrete dispersion with approximately twice effectiveness as lignosulfonate in cement hydration retardation (Chun, Dair et al. 2006). It has also been used as a precursor for large-scale synthesis of 1,2,4-butanetriol (Jokic, Ristic et al. 1991; Niu, Molefe et al. 2003).

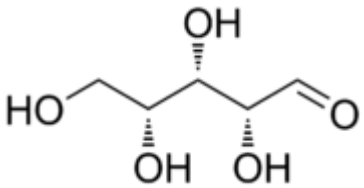

Figure 2.10. Structure of xylose

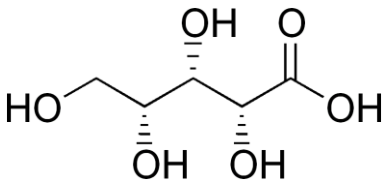

Figure 2.11. Structure of xylonic acid

Production of xylonic acid from hemicellulose hydrolyzates will dramatically lower the cost while accelerating the industrialization of xylonic acid and increasing the additional value of hemicellulose by better use of woody biomass.

Currently, two methods, chemical synthesis and microbial synthesis, are mostly used in xylonic acid production. As there are many active hydroxyl groups in sugar, when making sugar related derivate, block groups are usually used to protect the hydroxyl group during the process. In strong base condition, using methanol as solvent to oxidize xylose with iodine (I) can oxidize aldehyde to carboxyl group as xylonate, without oxidizing hydroxyl group. After adding concentrated sulfuric acid to methanol solvent, xylonic acid can be obtained. In addition to iodine catalyst, palladium (Pd) catalyst can also be used. The process for chemical production is complicated, the reaction condition is severe and the contamination is heavy (Niu, Molefe et al. 2003; Werpy, Petersen et al. 2004; Frost 2009).

Compared to chemical method, the reaction condition for microbial synthesis is mild. With lower cost and less contamination, it has wider potential applications. The production of xylonic acid from xylose has been reported by: *Acetobacter sp.* (Bernauer and Riedl-Tumova 1950)*; Pseudomonas sp.* (Nelson 1946); *Aerobacter sp.* (Masuo and Nozaki 1956)*; Fusarium* (Hayasida 1938), *Penicillium corylophilum* (Ikeda and Yamada 1963), *Pullularia pullulans* (Kiessling, Lindberg et al. 1962)*; Pichia quercuum* (Suzuki and Onishi 1967; Suzuki and Onishi 1973), *Micrococcus sp.*(Ohsugi, Tochikura et al. 1970), *Enterobactercloacae* (Ishizaki, lhara and Yoshitake, 1973), *Erwinia sp.* (Suzuki and Uchida 1965; Yamada 1977), *Arthrobacter sp.* (Yamanaka, Gino et al. 1977) and *Gluconobacter sp.* (Buchert, Puls et al. 1988; Buchert and Viikari 1988; Buchert 1991).

Buchert et al. have tested 11 microbial strains for xylonic acid formation, finding that *Acetobacter* sp. ATCC 8303, *Gluconobacter oxydans* subsp *suboxydans* ATCC 621, *Pseudomonasaeruginosa* subsp *erythrogenes* ATCC 19582 and *Pseudomonas fragi* ATCC 4973 can product xylonic acid. Among them, *Pseudomonas fragi* ATCC 4973 can ferment on xylose as the only carbon source (Buchert, Viikari et al. 1986). However, comparing to *Gluconobacter oxydans* subsp *suboxydans* ATCC 621, although both could produce xylonic acid from 100g·L^{-1} xylose with yields of over 95%, the tolerance of the *G. oxydans* towards potential inhibitors in hemicellulose hydrolyzates is better than the former one.

Thus, *Gluconobacter oxydans* subsp *suboxydans* ATCC 621 is regarded as better in producing xylonic acid from hemicellulose hydrolyzates (Buchert, Puls et al. 1988).

2.2.7 Analysis methods

Concentration of gluconic acid and xylonic acid can be determined by modified hydroxylamine methods according to Lien (Lien Jr 1959). It is a kind of chemical analysis methods similar to DNS method. Hydroxylamine reacts with carbonyl group to form hydroxamic acids which will generate colored complexes when react with iron (Ⅲ) in acidic solution (Bryant and Smith 1935; Hestrin 1949; Musser and Ortigoza 1966). However, this method has limitations in analyzing mixed sugar fermentation as well as ketogluconic acid. It cannot separately determine the concentration of neither gluconic acid, xylonic acid nor further oxidation products (Cori and Lipmann 1952; Klein, Rosenberg et al. 2002; Önal, Schimpf et al. 2004; Rodriguez, Gonzalez et al. 2004).

HPLC method for sugars, organic acids and other products have also been reported to be widely used. Rodriguez used HPLC (Knauer, Berlin, Germany) equipped with a diode array

detector (DAD) and a Hyperfil RP-18 column at a flow rate of 0.6 mL/min to analyze organic acid (Rodriguez, Gonzalez et al. 2004). The products in the reaction mixture at different reaction periods can also be quantitatively analyzed by HPLC (Bio-Rad, Aminex HPX-87H) (Önal, Schimpf et al. 2004). In a research from Nygård et al. , HPLC on a fast acid analysis column linked to an Aminex HPX-87H column (Bio-Rad, Hercules, CA) with 2.5 mM H_2SO_4 as eluent and a flow rate of 0.5 mL/min was used for chemical analysis. The column temperature was maintained at 55 °C. Peaks were detected using a Waters 410 differential refractometer and a Waters 2487 dual wavelength UV (210 nm) detector (Nygård, Toivari et al. 2011). Other HPLC methods for quantitatively determination have also been reported (Suzuki and Onishi 1967; Buchert, Viikari et al. 1986; Buchert, Puls et al. 1988; Buchert and Viikari 1988; Büchert, Puls et al. 1989; Liu, Valdehuesa et al. 2011). An existing problem in using HPLC in products analysis is peak co-elution and overlap, therefore calculation and the combination of multiple HPLC analysis may be needed to get accurate results.

2.3 Enzyme systems in *G. oxydans* ATCC621

G. oxydans is an obligate aerobic Gram-negative bacterium. It has a respiratory metabolism characterized by incomplete oxidation of sugars, alcohols and acids to corresponding products (aldehyde, ketone and organic acid). These partially oxidized organic compounds are excreted almost completely into the medium (White and Claus 1982; Seiskari, Linko et al. 1985; Shiraishi, Kawakami et al. 1989; Silberbach, Maier et al. 2003; Prust, Hoffmeister et al. 2005).

Because of its incomplete oxidation over a wide range of substrates, *G. oxydans* is well adapted for industrial use. Some of the current applications of *G. oxydans* strains have been summarized in Table 2.7 (Gupta, Singh et al. 2001).

Table 2.7. Industrial application of *G. oxydans*.

Application	References
2,5-Diketogluconic acid formation from D-Glucose	(Stubbs, Lockwood et al. 1940; Aida, Kojima et al. 1955; Adachi, Shinagawa et al. 1979; Qazi, Parshad et al. 1991)
5-Ketogluconic acid formation from D-Glucose	(Adachi, Shinagawa et al. 1979; Klasen, Bringer‐Meyer et al. 1992; Klasen, Bringer-Meyer et al. 1995; Elfari, Ha et al. 2005)
Free D-Gluconic acid formation from D-Glucose	(Meiberg and Spa 1983; Velizarov and Beschkov 1994)
L-Sorbose formation from D-Sorbitol	(Pomortseva and Krasil'nikova 1983; Stefanova, Koseva et al. 1987; Kosseva, Beschkov et al. 1991)
Aldehyde formation from Alcohol	(Ganadu, Andreotti et al. 2002; Kroutil, Mang et al. 2004)

G. oxydans ATCC621 contains two different enzyme systems for glucose oxidization (Figure 2.12) (Ye, Haemmerle et al. 1993; Matsushita, Toyama et al. 1994; Klasen, Bringer-Meyer et al. 1995; Toivari, Nygård et al. 2012): one is located in the cytosol and consists of the soluble NADP+-dependent enzymes glucose-dehydrogenase (GDH), and gluconate:NADP 5-oxidoreductase (Roukas and Harvey 1988). Another glucose oxidation system is located in the cytoplasmic membrane oriented towards the periplasm. It is made up of pyrroloquinoline quinone (PQQ)- and flavin-dependent enzymes: a PQQ-dependent GDH catalyzing D-gluconic acid (GA) formation (Pronk, Levering et al. 1989; Oubrie, Rozeboom et al. 1999; Merfort, Herrmann et al. 2006), a flavin-dependent gluconate-2-dehydrogenase catalyzing 2-KGA formation, and a PQQ-dependent gluconate-5-dehydrogenase (GA5DH), leading to the formation of 5-KGA (Olijve and Kok 1979; Weenk, Olijve et al. 1984; Levering, Weenk et al. 1988; Buse, Qazi et al. 1990; Qazi, Parshad et al. 1991; Silberbach, Maier et al. 2003; Herrmann, Merfort et al. 2004; Schweiger, Gross et al. 2010). Enzymes in Figure 2.12 are: (1) membrane-bound GDH; (2) membrane-bound GA2DH; (3) membrane-bound 2-diketo-D-gluconate-

dehydrogenase; (4) membrane-bound GA5DH; (5) soluble GA5DH; (6) soluble GDH (2,5-DKGA, 2,5-di-keto-gluconic acid).

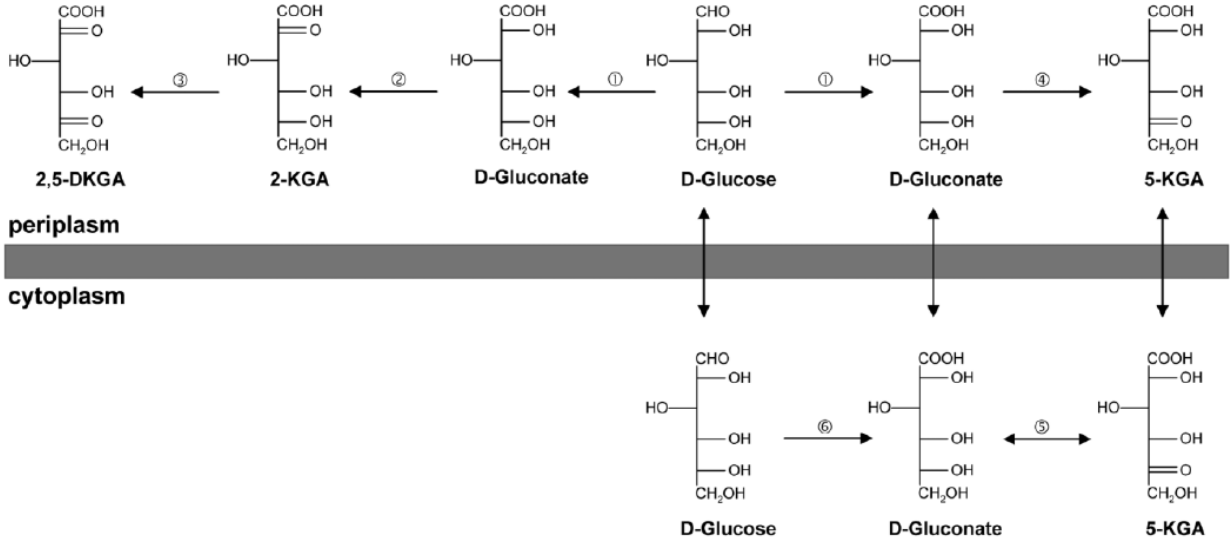

Figure 2.12. Schematic representation of glucose oxidation in *G. oxydans* (Merfort, Herrmann et al. 2006).

Comprehensive researches have proven that the xylose conversion to xylonic acid in *G. oxydans* ATCC621 is also catalyzed by the membrane-bound glucose-dehydrogenase (Buchert and Viikari 1988; Buchert and Viikari 1988; Nygård, Toivari et al. 2011; Toivari, Nygård et al. 2012). The proposed scheme of xylose and glucose oxidation is illustrated in Figure 2.13.

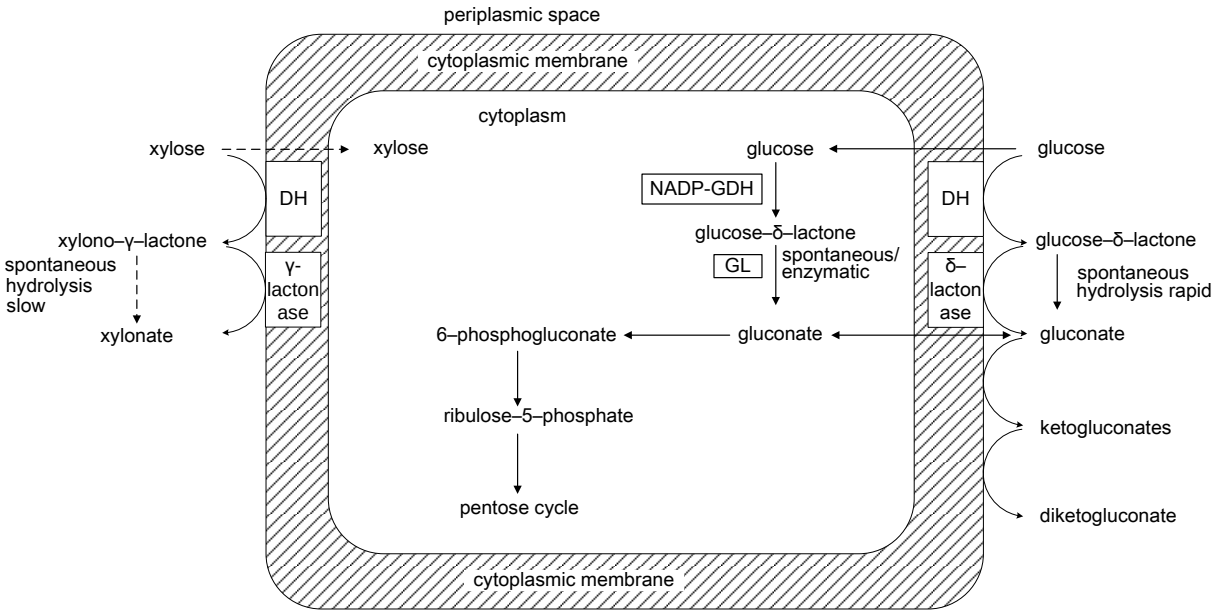

Figure 2.13. Proposed scheme of oxidative metabolism of glucose and xylose in *G. oxydans*, adapted from *(Buchert and Viikari 1988)*.

Chapter 3 Materials and Methods

3.1 Microorganism and cultivation

Gluconobacter oxydans subsp. *suboxydans* ATCC621 was used for sugar fermentation in this study. *G. oxydans* was maintained on mannitol agar plates (5.0 g/L yeast extract, 3.0 g/L peptone, 25 g/L mannitol and 15 g/L agar). Colony of *G. oxydans* was cultivated in a liquid medium containing: 73.0 g/L sorbitol, 18.4 g/L yeast extract, 1.50 g/L $(NH_4)_2SO_4$, 1.52 g/L KH_2PO_4 and 0.47 g/L $MgSO_4 \cdot 7H_2O$. All media were sterilized by autoclaving at 121 ^{0}C for 15 min. After incubated in an orbital shaker at 220 rpm, 30 ^{0}C for around 22 h until exponential growth phase, the liquid medium with bacterium inside was centrifuged and washed by sterile water 3 times. The dry weight of the *G. oxydans* biomass was measured at 600 nm with UV-Vis spectrometer (Genesys10, Thermo Fisher).

3.2 Feedstock preparation

3.2.1 Woody biomass substrates

Sweetgum (*Liquidambar styraciflua*) wood chips were collected from Forest Products Laboratory at Auburn University. The initial moisture content of these wood chips was approximately 9.0 wt %. Wood chips were ground by a Wiley mill (Thomas Scientific, Philadelphia, PA), and the wood powder between 20 and 40 mesh was collected for chemical composition analysis. The wood chips were reduced to an average size of 1.0 × 2.0 × 0.3 cm^3 (L × W × H) by a Waring commercial blender (Dynamics Corporation of America, New Hartford, CT) prior to organosolv pretreatment.

3.2.2 Ethanol organosolv pretreatment

The ethanol organosolv pretreatment procedure was modified from National Renewable Energy Lab (NREL) (Templeton and Ehrman 1995; LAP, LAP et al. 1998; Sluiter, Ruiz et al.

2005; Sluiter, Hames et al. 2008) and Pan (Pan, Gilkes et al. 2006). The flowchat of the procedure is shown in Figure 3.1.

Bark-free sweetgum chips were selected and screened through 7 mm griddle. Chips were cooked in aqueous 50% (v/v) ethanol with 1% (w/v) sulfuric acid as catalyst (w/v=1:7) in a 1 L parr batch reactor. An 80 g batch of chips was cooked each time in 560 mL liquid at 170 $^{\circ}$C for 60 min. After cooking, vessels were cooled to room temperature in a water bath. Pulp and liquor were then separated using metal mesh. The prehydrolysates were collected and stored at 4 $^{\circ}$C for later use. The pulp was washed three times with 300 mL aqueous 50% ethanol (the same concentration of cooking liquor) at 50 $^{\circ}$C and washes were combined with the spent liquor. The pulp was then washed three times with warm water and the washes were discarded. The washed pulp was homogenized in a commercial blender for 5 min and passed through a flat screen with slits to remove rejects (non-defiberized woodchips and knots). The screened pulp comprised the solids fraction.

The spent liquor and ethanol washes were combined and mixed with three volumes of water to precipitate the dissolved lignin. The lignin precipitate, described as ethanol organosolv lignin (EOL), was collected on Whatman No.1 filter paper. The filtrate and water washes were combined to give a water-soluble fraction containing monomeric and oligomeric hemicellulosic sugars, depolymerized lignin, and other unidentified components.

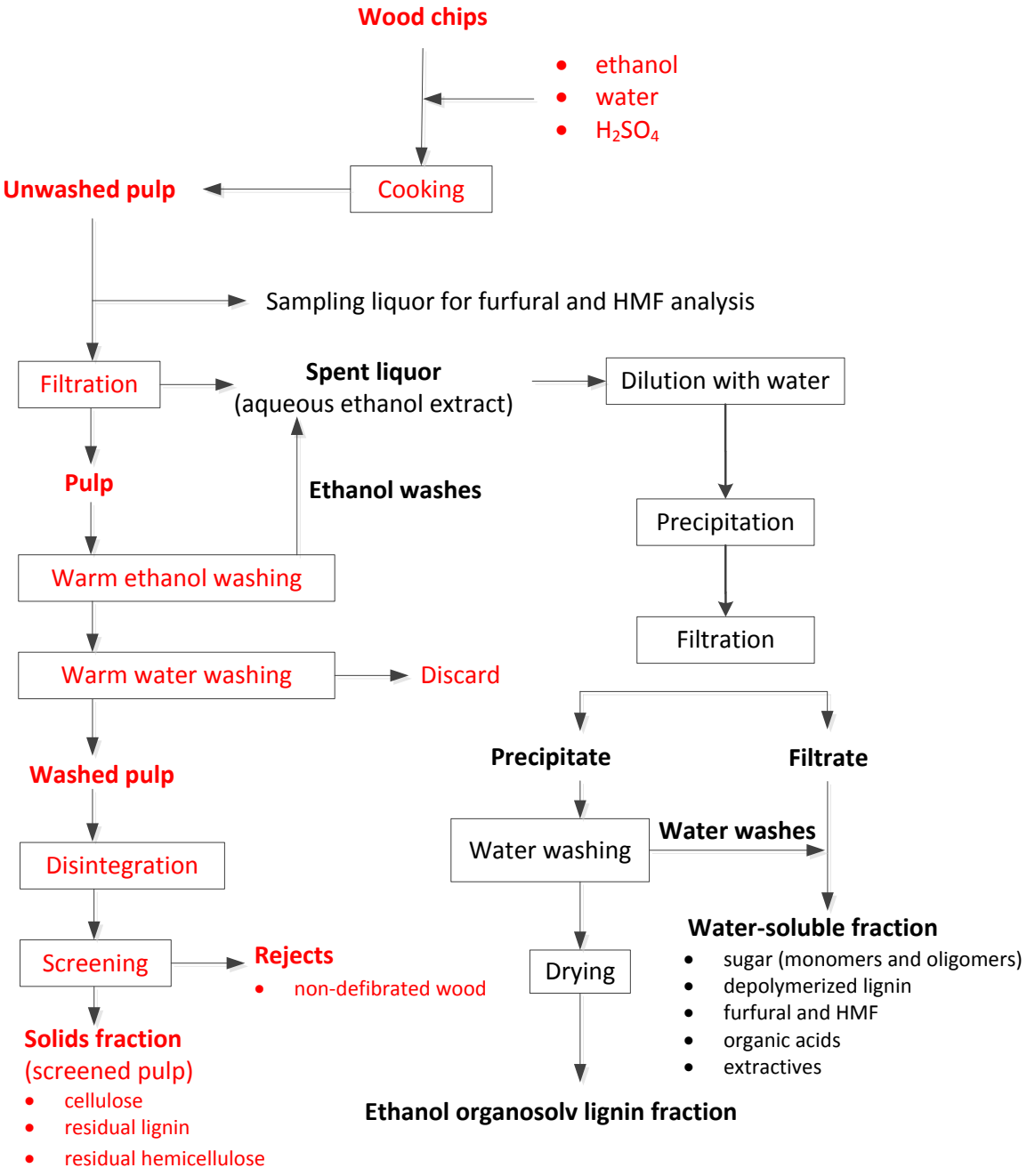

Figure 3.1. Flowchart of the laboratory-scale ethanol organosolv process, reproduced from (Pan, Gilkes et al. 2006).

3.2.3 Enzymatic hydrolysis

Commercial cellulase preparation (Novozym 22C) was obtained from Novozymes (Franklinton, NC). Cellulase activity was determined using the filter paper assay recommended by the International Union of Pure and Applied Chemists (Ghose 1987) and is expressed in filter paper units (FPU).The enzyme activity of the Novozym 22C (100 FPU/mL) was determined using Whatman #1 filter paper as the substrate.

Batch hydrolysis reactions contained solids (2% cellulose w/v) in 50 mM sodium citrate buffer, pH 4.8. Cellulase was used at loading of 2.5, 5.0 and 10 FPU /g cellulose in the solids. Reaction mixtures (50 mL) were incubated at 150 rpm, 50 $^{\circ}$C, in a rotary shaker and sampled periodically (0, 1, 3, 6, 9, 12, 24, 36, 48 and 72 h) for monosaccharides (glucose, xylose and mannose) determination, as described above. All enzymatic hydrolysis experiments were carried out in duplicates Average results and corresponding errors are given.

For SSF (simultaneous saccharification and fermentation), batch reactions were conducted at the same condition as enzymatic hydrolysis. Both monosaccharides concentrations and products concentrations were determined in duplicates.

3.2.4 Detoxification

Hemicellulose prehydrolysates were first nutreulized to pH around 5 by adding NaOH and then evaporated at 45 $^{\circ}$C until no ethanol existed. The ethanol presence was analyzed using HPLC. After that, alkaline detoxification treatment was carried out. By adding NaOH, adjusted the hydrolysates pH to 10 and heated the liquid in a 60 $^{\circ}$C water bath for 2 h. During this process, check the pH and change it back to 10 by adding NaOH every 30 min, mixed vigorously. After detoxification, pH of detoxified prehydrolysates was changed to 6.5 by adding sulfuric acid.

In untreated control group, prehydrolysates were adjusted to pH 6.5 by adding NaOH at room temperature.

3.2.5 Fermentation

Gluconobacter oxydans was added into each conical flask with the fermentation medium. The flasks were then covered with sponges. Flasks were incubated in an orbital shaker at 30 °C and 220 rpm for 48 h. Samples of fermentation liquid were taken out periodically in a Biological Safety Cabinet under aseptic condition. 0.3 mL of the aliquot mixed with *G. oxydans* were transferred into a 1.5 mL centrifuge tube and centrifuged at 12,000 rpm for 5 min. After centrifuge, supernatant was withdrawn and mixed with DI water to be diluted properly for chemical analysis.

Glucose and xylose consumption rate (g $g^{-1}h^{-1}$) was estimated from the change of sugar concentration over the first 3 h during the fermentation. Initial biomass concentration was 5 g/L. It was assumed that the biomass concentration did not change in the first 3 h fermentation. The gluconic acid and xylonic acid yield were calculated as % of the theoretical yield by using the following formulas:

$$\%\, Yield = \frac{[gluconic\ acid]}{0.92 \times [glucose]} \times 100\%$$

$$\%\, Yield = \frac{[xylonic\ acid]}{0.90 \times [xylose]} \times 100\%$$

3.2.6 Analytical procedures

Oven-dried weights were determined by drying to constant weight at 105 °C oven. Extractives were determined according to the ASTM Standard Test Method E1690-01. Acid-soluble lignin

was determined from absorbance at 205 nm (Lin and Dence 1992). Bacterium growth was measured by optical density at 600 nm (OD_{600}) by using a Geneys10 UV-Vis spectrometry.

A Shimadzu (LC-20A) HPLC system consisting of a degasser, SIL-20AC HT autosampler, LC-20AD gradient pump, and RID-10A detector equipped with a 300 mm × 7.8 mm i.d., 9 μm, Aminex HPX-87P column and a 30 mm × 4.6 mm i.d. guard column of the same material (Bio-Rad, Hercules, CA) was used to separate and quantitate individual sugars. Water was used as mobile phase at an isocratic flow rate of 0.6 mL/min to separate sugars. Column temperature was maintained at 85 °C during elution. Aliquots (20 μL) were injected after passing through a 0.45 mm nylon syringe filter. Monosaccharides were quantified with reference to standards.

Gluconic acid, xylonic acid, 2-keto-gluconic acid (2KGA), 5-keto-gluconic acid (5KGA) and other products were qualitatively determined with an Ultra Performance LC Systems (ACQUITY, Waters Corp.) coupled with a quadrupole time-of-flight mass spectrometer with electrospray ionization (ESI) in negative ESI-MS operated by the Masslynx software. Concentrations of these compounds were determined by using a Shimadzu HPLC system equipped with a Bio-Rad HPX-87H column. When conducting mixed sugar fermentation, as the peaks of gluconic acid and xylose were co-eluted, the concentration of each was determined with the combination of two HPLC results by subtracting the xylose concentration calculated from Bio-Rad Aminex® HPX-87P column.

Chapter 4 Results and Discussions

4.1 Gluconic acid and xylonic acid fermentation on glucose and xylose by *G. oxydans*

4.1.1 Background

As mentioned in the introduction part, *G. oxydans* is well adapted for industrial use since it can perform the incomplete oxidation of a wide range of substrates including sugars, alcohols and acids even in the presence of excess oxygen (Gupta, Singh et al. 2001). This kind of direct incomplete oxidation is preceded through one or two discrete steps and results in nearly quantitative yield of the oxidation products.

Previous studies have indicated that there are two enzymes systems in *G. oxydans* ATCC621 oxidizing glucose (Ye, Haemmerle et al. 1993; Matsushita, Toyama et al. 1994; Klasen, Bringer-Meyer et al. 1995; Toivari, Nygård et al. 2012). One is at the cytosol and consists of the soluble NADP+-dependent enzymes glucose-dehydrogenase (GDH), and gluconate:NADP 5-oxidoreductase (Roukas and Harvey 1988). The other glucose oxidation system is located in the cytoplasmic membrane oriented towards the periplasm. It is made up of pyrroloquinoline quinone (PQQ)- and flavin-dependent enzymes: a PQQ-dependent GDH catalyzing D-gluconic acid (GA) formation (Pronk, Levering et al. 1989; Merfort, Herrmann et al. 2006), a flavin-dependent gluconate-2-dehydrogenase catalyzing 2-KGA formation, and a PQQ-dependent gluconate-5-dehydrogenase (GA5DH), leading to the formation of 5-KGA (Olijve and Kok 1979; Weenk, Olijve et al. 1984; Levering, Weenk et al. 1988; Buse, Qazi et al. 1990; Qazi, Parshad et al. 1991; Silberbach, Maier et al. 2003; Herrmann, Merfort et al. 2004; Schweiger, Gross et al. 2010).

The direct incomplete oxidation of glucose by *G. oxydans* is generally viewed through a membrane-bound glucose-dehydrogenase (Matsushita, Shinagawa et al. 1989; Pronk, Levering et

al. 1989; Silberbach, Maier et al. 2003). The glucose is first converted to glucono-δ-lactone which is subsequently hydrolyzed to gluconic acid either spontaneously or by a glucono-δ-lactonase (Lien Jr 1959; Mitchell and Duke 1970; Buchert and Viikari 1988; Rehr, Wilhelm et al. 1991; Chun, Dair et al. 2006; Kaur, Macleod et al. 2006).

Previous researches have indicated that xylose is oxidized via the membrane-bound xylose-dehydrogenase. Similar to fermentation on glucose, in xylonic acid fermentation, an intermediate product xylono-γ-lactone is first produced and then converted to xylonic acid. The complete oxidation pathway located towards periplasmic space. Two major differences between these two pathways are: 1), hydrolysis of intermediate product glucono-δ-lactone to gluconic acid is quicker than that in xylose fermentation; 2), in glucose conversion, gluconic acid may be further oxidized to ketogluconate and diketogluconate while in xylose conversion, xylonic acid is the final products and will not be further oxidized (Ikeda and Yamada 1963; Ohsugi, Tochikura et al. 1970; Buchert, Puls et al. 1988; Buchert and Viikari 1988; Buchert and Viikari 1988; Büchert, Puls et al. 1989; Niu, Molefe et al. 2003).

Significant researches have shown pH is a remarkable factor of sugar fermentation and ketogluconate generation by *G. oxydans* (Ohsugi, Tochikura et al. 1970; Nygård, Toivari et al. 2011; Toivari, Nygård et al. 2012). Other factors, including substrate concentrations and usages of different buffer and salts will also differ the final proportion of composition (Stubbs, Lockwood et al. 1940; Katznelson, Tanenbaum et al. 1953; Levering, Weenk et al. 1988). Weenk et al. found that only when glucose concentration is lower than a threshold of 15 mM at an input of 100 mM will initiate the formation of ketogluconate (Weenk, Olijve et al. 1984). The lower solubility of crystalline calcium 5-keto-gluconate results in the accumulation of 5KGA when

using $CaCO_3$ as pH control (Stubbs, Lockwood et al. 1940; Kheshgi, Roberts et al. 1954; Moghimi and Tate 1978).

Studies of enzymes in sugar oxidation have shown that xylose and glucose are catalyzed by the same glucose-dehydrogenase located at membrane towards periplasm (Olijve and Kok 1979; Olijve and Kok 1979; Weenk, Olijve et al. 1984; Buchert and Viikari 1988; Levering, Weenk et al. 1988; Buchert 1990; Buchert 1991; Qazi, Parshad et al. 1991; Deppenmeier, Hoffmeister et al. 2002; Hölscher, Schleyer et al. 2009; Rauch, Pahlke et al. 2010). However, no research has been done to figure out why fermentations on glucose and xylose have different rates and response to pH. Therefore, further work is needed to explore the metabolism pathway in *G. oxydans* and affecting factors of enzyme activity.

4.1.2 Effects of pH on gluconic acid fermentation

Efficient glucose oxidation by *G. oxydans* was first investigated on 20 g/L (Figure 4.1) and 100 g/L pure glucose (Figure 4.2). 6 g/L $CaCO_3$ (Figure 4.3) was used as pH control in 20 g/L pure glucose medium, keeping the pH constant during the whole fermentation time according to Weenk (Weenk, Olijve et al. 1984). pH value of the fermentation medium was determined by using accurate pH paper.

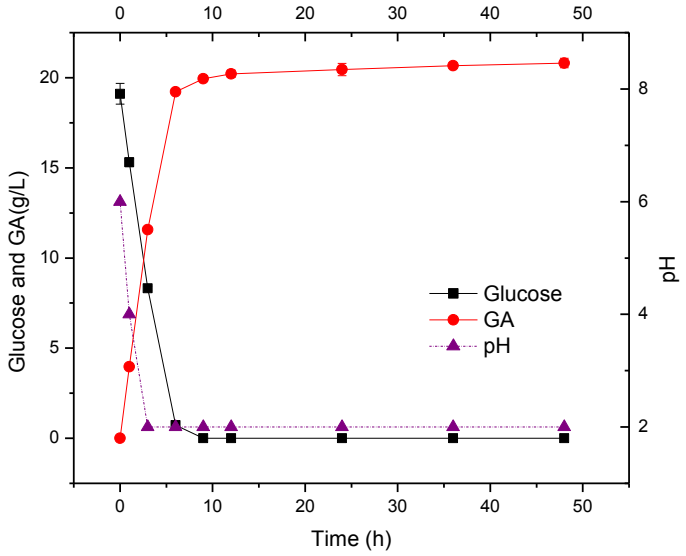

Figure 4.1. Gluconic acid fermentation on glucose by *G. oxydans* without pH control (20 g/L glucose).

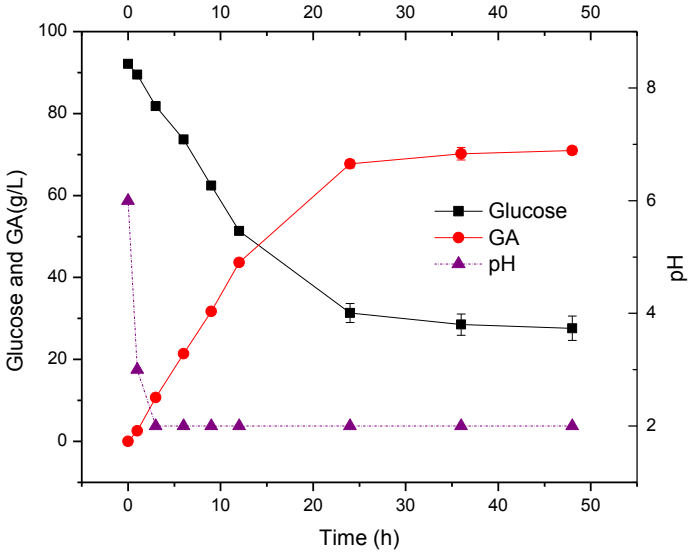

Figure 4.2. Gluconic acid fermentation on glucose by *G. oxydans* without pH control (100 g/L).

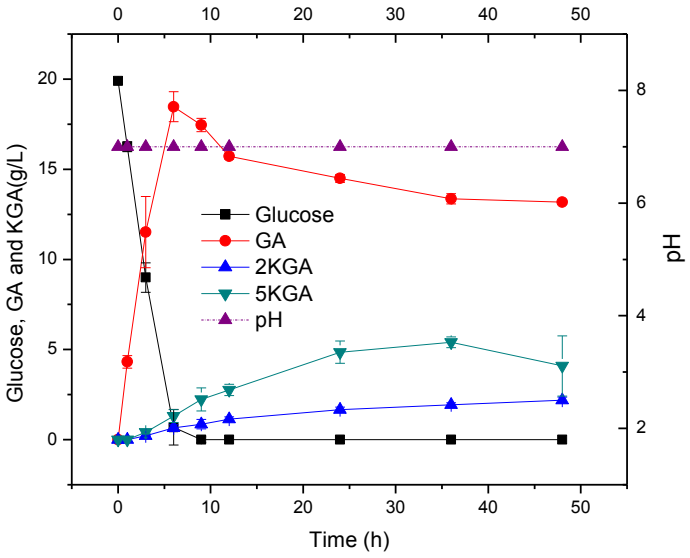

Figure 4.3. Gluconic acid fermentation on glucose by *G. oxydans* with pH control (6 g/L CaCO3, 20 g/L glucose).

Without pH control, 20 g/L of glucose was consumed completely by *G. oxydans* after 6 h fermentation and the gluconic acid production yield is about 100%. During this fermentation process, pH dropped quickly from 6.0 to 2.0, the glucose consumption rate was 0.72 g g^{-1} h^{-1}, and gluconic acid production rate was 0.77 g $g^{-1}h^{-1}$. For the fermentation with higher substrate concentration (100 g/L glucose), 66% glucose was consumed by *G. oxydans* within 24 h, and then consumption was nearly stopped afterwards. The pH dropped from 6.0 to 2.0 within 3 h. The production yield of gluconic acid was 70.1%. The termination of glucose consumption after 24 h was probably caused by the extensive pH drop. The consumption rate was 0.68 g $g^{-1}h^{-1}$ and production rate was 0.71 g $g^{-1}h^{-1}$.

With pH control at 7.0 (6 g/L $CaCO_3$), 20 g of glucose was quickly consumed by *G. oxydans* within 6 h and the gluconic acid reached 18.5 g/L with a conversion yield of 85.3%. After 6 h fermentation, gluconic acid started converting to 2 KGA and 5 KGA. At the end of 48 h fermentation, Gluconic acid dropped to 13.2 g/L and the production of 2KGA and 5 KGA was 2.1, and 4.1 g respectively. The fermentation with pH control resulted in a similar glucose consumption rate of 0.73 g $g^{-1}h^{-1}$ and GA production rate of 0.77 g $g^{-1}h^{-1}$.

Mass Spectrometry was used to identify the molecular weight of products (Figure 4.4). The results revealed that after 48 h fermentation, 2-ketogluconate (2KGA), 5-ketogluconate (5KGA) and gluconic acid (GA) were present while no glucose was found in the medium. The gluconic acid yield produced by *G. oxydans* in 20 g/L (2.0%) glucose medium in 48 h was 100% of theoretical yield.

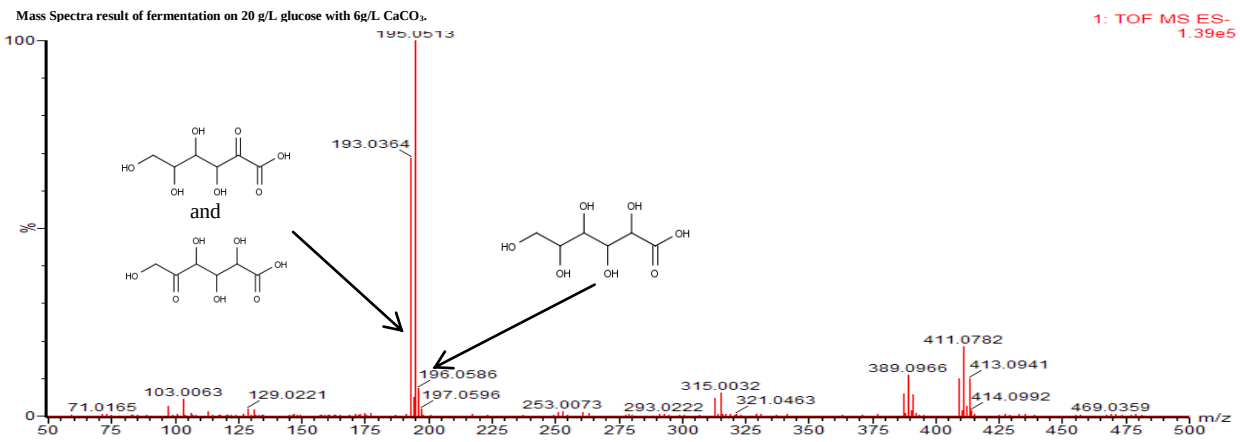

Figure 4.4. Mass spectra of fermentation samples on 20 g/L glucose with 6 g/L $CaCO_3$.

The pH curve revealed that without neutralization, the pH value of fermentation medium quickly dropped to 2 within 3 h fermentation. Both with and without pH control, 20 g/L glucose as substrate were totally consumed within 12 h and almost quantitatively converted to gluconic acid. However, without pH control, 100 g/L glucose was partially consumed and had around 29.3% left in the fermentation medium. These results indicated the fermentation capacity of *G. oxydans* may be limited within in a certain range without pH control. The potential reason involved the optimal pH range and tolerance of glucose-dehydrogenase at low pH value. More researches have been done to further explore the mechanism and would be discussed later.

Early study of Stubbs et al. (Stubbs, Lockwood et al. 1940) concluded that gluconic acid and 5-keto-guconic acid was produced from glucose in media containing $CaCO_3$. According to Levering (Levering, Weenk et al. 1988) and Weenk (Weenk, Olijve et al. 1984), in the pH-controlled fermentation, a sequential accumulation of gluconic acid and ketogluconic acid was observed.

Another series experiments have been designed to explore pH effects of the formation of ketogluconic acid. Gluconic acid fermentation has been conducted under four different conditions: 1) without pH control; 2) adding 6 g/L $CaCO_3$ in the fermentation media; and 3) controlling the pH at 5.5 during the 48 h fermentation. pH values were determined by a pH meter. The glucose consumption and GA production (Figure 4.5), 2KGA production (Figure 4.6) and 5KGA production (Figure 4.7) were illustrated as following.

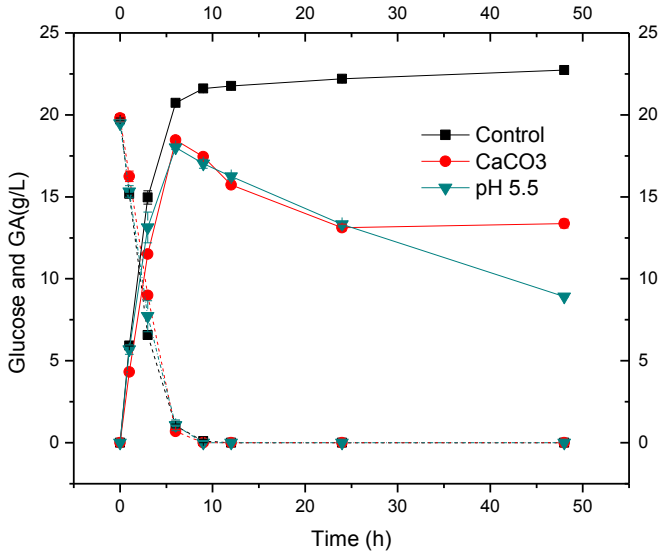

Figure 4.5. Effects of pH on gluconic acid fermentation by *G. oxydans* (20 g/L glucose, 6 g/L $CaCO_3$, pH 5.5 and without pH control).

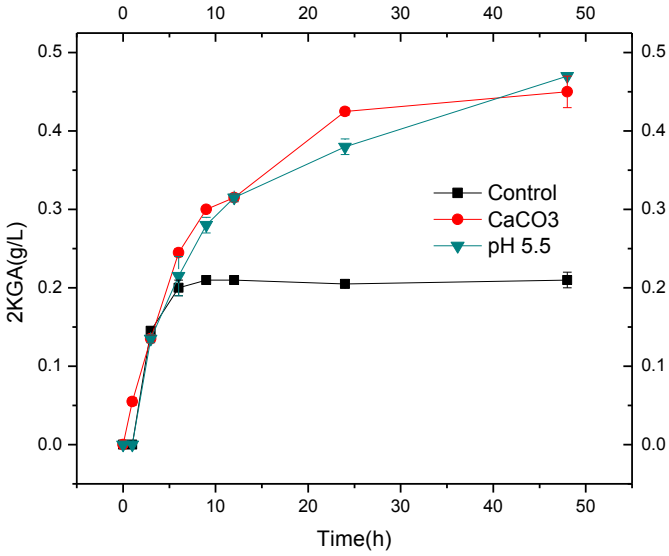

Figure 4.6. 2KGA production by *G. oxydans* on glucose (20 g/L) with 6 g/L $CaCO_3$, pH 5.5 and without pH control.

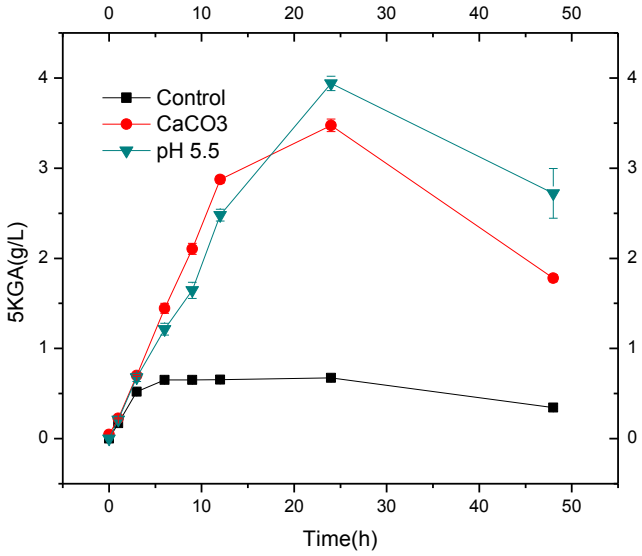

Figure 4.7. 5KGA productions by *G. oxydans* on glucose (20 g/L) with 6 g/L $CaCO_3$, pH 5.5 and without pH control.

In all these three conditions, 20 g/L of glucose was consumed completely by *G. oxydans* after 6 h fermentation. Without pH control, the glucose was almost quantitatively converted to gluconic acid with a consumption rate of 0.87 g $g^{-1}h^{-1}$ and gluconic acid production rate of 0.98 g $g^{-1}h^{-1}$. After 48 h, the gluconic acid production yield was nearly 100%. With the addition of $CaCO_3$, the glucose consumption rate was 0.72 g $g^{-1}h^{-1}$ and the gluconic acid production rate was 0.77 g $g^{-1}h^{-1}$. Gluconic acid was further converted to 2-keto-gluconic acid and 5-keto-gluconic acid. Similar patterns have also been observed in the fermentations in which pH were controlled by NaOH. When pH was controlled constant at 5.5 during 48 h fermentation process, the glucose consumption rate was 0.79 g $g^{-1}h^{-1}$, and the gluconic acid production rate was 0.88 g $g^{-1}h^{-1}$.

The comparison of the three fermentation curves indicated that pH higher than 5.5 favored the production of ketogluconic acid. Without pH control, glucose was nearly all converted to

gluconic acid. The other two showed considerable 2KGA and 5KGA production. The yield of 5KGA was much higher than that of 2KGA. This agreed well with previous report of Weenk and Levering (Weenk, Olijve et al. 1984; Levering, Weenk et al. 1988) on the strain ATCC621. The decrease of GA concentration and the increase of KGA concentration in the fermentation suggested that glucoses were first converted to gluconic acid and then to ketogluconic acid. This glucose oxidization pathway has been proposed previously by Merfort et al. (Merfort, Herrmann et al. 2006).

4.1.3 Effects of pH on xylonic acid fermentation

In this part, fermentation on xylose by *G. oxydans* was investigated. Fermentation media containing 20 g/L xylose were used for xylonic acid production without and with pH control: 1) 20 g/L xylose without pH control (Figure 4.9); 2) 20 g/L xylose with 6 g/L $CaCO_3$ (Figure 4.10); 3) 20 g/L xylose fermentation with 50 mM pH5.6 sodium acetate (NaOAc) /acetic acid buffer (Figure 4.11); and 4) 20 g/L xylose fermentation with 50 mM pH6.0 sodium citrate/citric acid buffer (Figure 4.12). pH values of the fermentation medium were determined using accurate pH paper.

Mass Spectrometry as well as High Performance Liquid Chromatography (HPLC) was combined to qualitatively and quantitatively determine the products of fermentation on xylose by *G. oxydans*. The result of mass spectra has been shown in Figure 4.8.

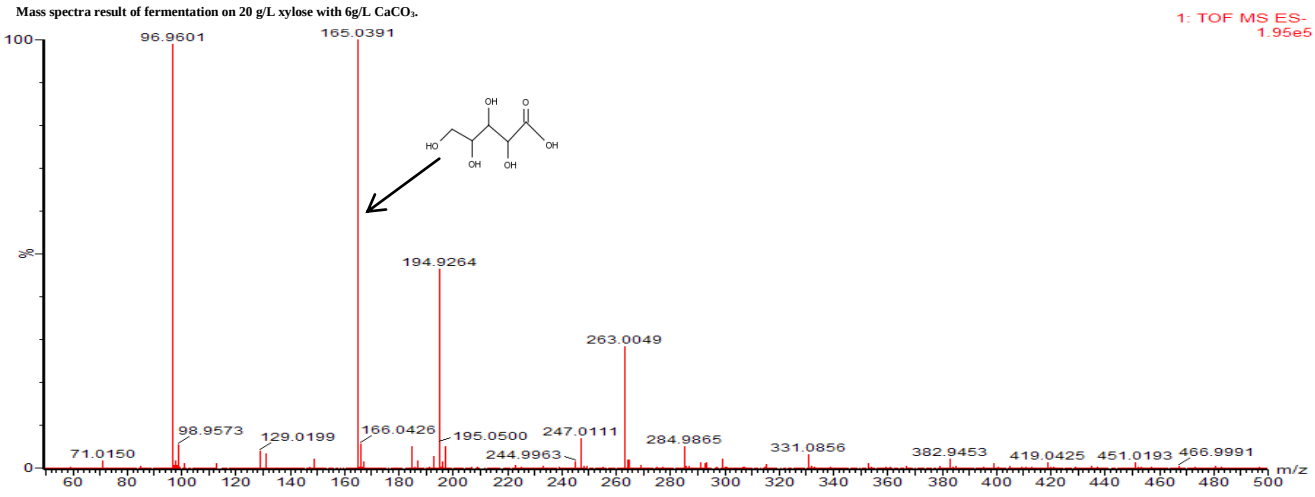

Figure 4.8. Mass spectra of fermentation samples on 20 g/L xylose with 6.0 g/L $CaCO_3$.

LC/MS analysis showed a high intensity peak of xylonic acid (165.0391 m/z $[M-H]^-$) as sole product. No further oxidization product of xylonic acid was found in the fermentation medium. This was different from that on glucose, in which more than one final product was produced under pH controlled condition.

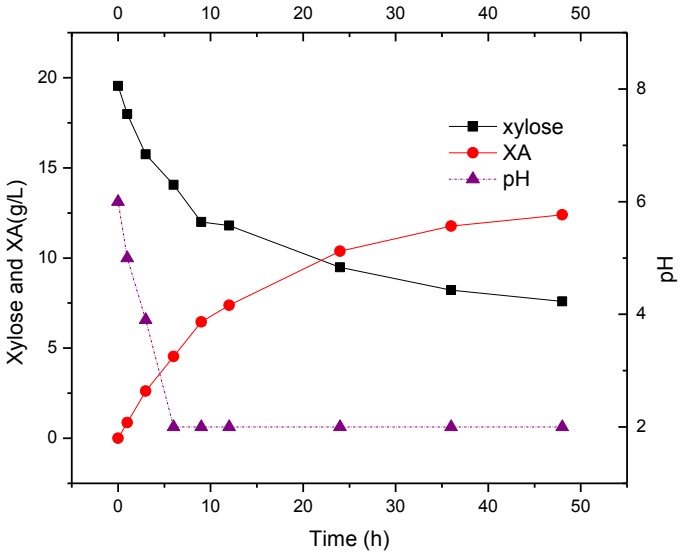

Figure 4.9. Xylonic acid fermentation on xylose by *G. oxydans* without pH control (20 g/L).

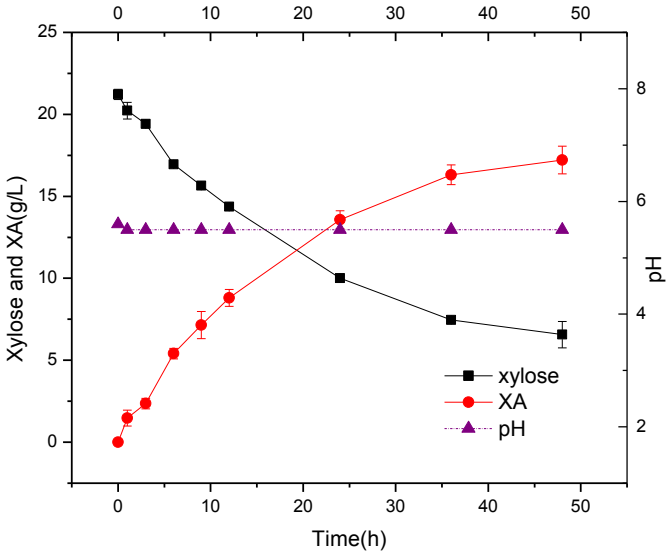

Figure 4.10. Xylonic acid fermentation on xylose (20 g/L) by *G. oxydans* (50 mM pH 5.6 sodium acetate buffer as pH control).

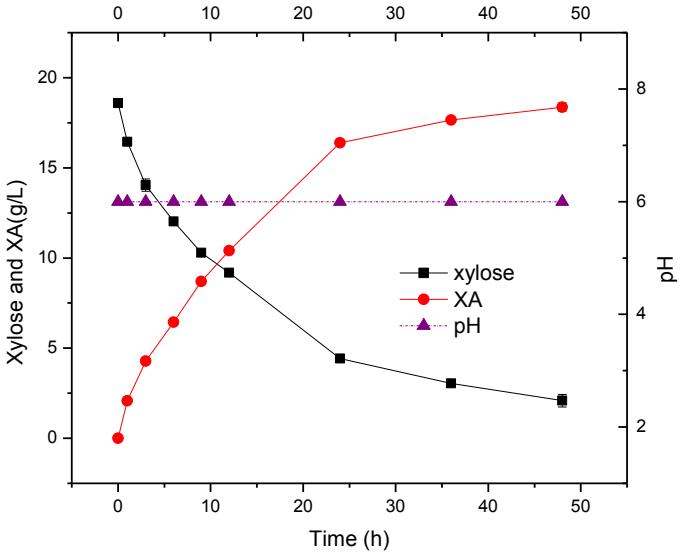

Figure 4.11. Xylonic acid fermentation on xylose (20 g/L) by *G. oxydans* (50 mM pH 6.0 sodium citrate buffer as pH control).

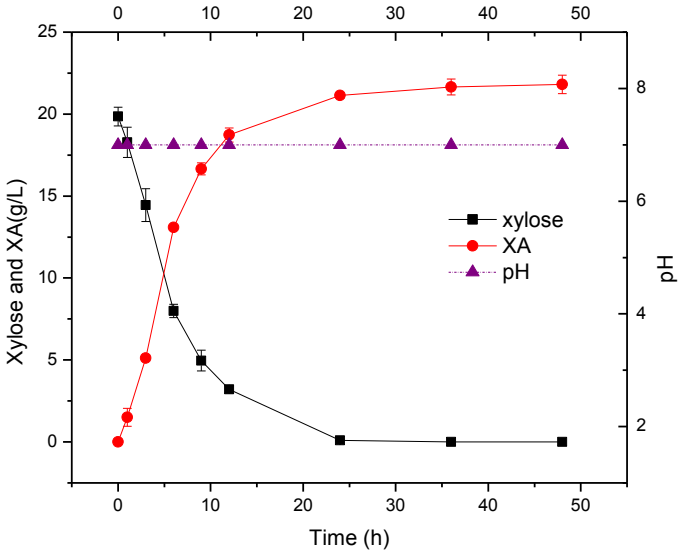

Figure 4.12. Xylonic acid fermentation on xylose by *G. oxydans* with 6 g/L $CaCO_3$ as pH control.

The addition of $CaCO_3$ was used to control the fermentation pH (Buchert, Puls et al. 1988). The amount was calculated based on the theoretical concentration of complete conversion from xylose to xylonic acid. To further study the effects of different pH control methods on xylonic acid oxidation by *G. oxydans*, 50 mM pH 5.6 sodium acetate (NaOAc) buffer and 50 mM pH 6.0 sodium citrate buffer were used to control the pH value in the fermentation medium.

Without pH control, 20 g/L xylose was not completely converted to xylonic acid but gradually stopped at 48 h with 7.6 g/L xylose left. During the fermentation, pH dropped quickly from 6.0 to 2.0 within 6 h. The xylose consumption rate was 0.25 g $g^{-1}h^{-1}$, and the xylonic acid production rate was 0.17 g $g^{-1}h^{-1}$. The consumption yield of xylose was 61.2% and the production yield of xylonic acid was 57.3%.

With 50 mM sodium acetate (NaOAc) /acetic acid buffer which kept the pH at 5.5 during the fermentation process, a higher conversion yield of 73.0% was obtained. The xylose consumption rate was 0.12 g $g^{-1}h^{-1}$ and the xylonic acid production rate was 0.16 g $g^{-1}h^{-1}$. With 50 mM sodium citrate/citric acid buffer which kept the pH at 6.0 during fermentation, 2 g/L xylose was left after 48 h. The consumption rate was 0.30 g $g^{-1}h^{-1}$ and xylonic acid productiviion rate was 0.29 g $g^{-1}h^{-1}$. The production yield of xylonic acid was 88.9%.

With 6 g/L $CaCO_3$ which kept the pH at 7.0 during the fermentation process, 20 g/L xylose was completely converted to xylonic acid with nearly 100% conversion yield. Both xylose consumption rate (0.36 g $g^{-1}h^{-1}$) and xylonic acid production rate (0.34 g $g^{-1}h^{-1}$) were higher than the other three groups.

Previous research indicated that the optimal pH for *G. oxydans* to oxidize xylose to xylonic acid was ~ 6.5 (Buchert and Viikari 1988; Buchert 1990). The fermentation pH lower than 5.5

would dramatically decrease the enzyme activity and consequently slow or stop fermentation process completely.

Our results showed that both glucose and xylose can be fermented to corresponding organic acid at nearly 100% conversion yield when pH was properly controlled. Fermentation rates on glucose were quicker than that on xylose. Without pH control, the production of acids (gluconic acid or xylonic acid) would lead to a quick drop of pH to 2.0 at the early stage of fermentation. Although glucose can be completely consumed and converted to gluconic acid, the xylose consumption was retarded, leading to incomplete conversion of xylonic acid. This suggested the enzyme for gluconic acid and xylonic acid fermentation favored different optimal pH and the tolerance to low pH was different. pH-controlled fermentation (>5.5) on glucose resulted in the formation of ketogluconic acid while for fermentation on xylose without and with pH control, xylonic acid was the only products. This indicated the different metabolic pathway for further oxidization of gluconic acid and xylonic acid. In this case, pH control was probably not desirable for gluconic acid production since KGA formation would decrease the final production yield of gluconic acid.

4.1.4 Effects of glucose supplementation on xylonic acid fermentation

Previous study suggested that glucose (0.5%) is required for xylonic acid fermentation on xylose(Buchert, Puls et al. 1988). This supplement was required as energy source and also for the induction of enzyme. Therefore in this section, we added different concentrations of glucose to examine the effects of glucose supplementation on xylonic acid fermentation. 2.5, 5.0 and 20 g/L glucose was supplemented into xylonic acid fermentation on xylose (20 g/L). An experiment without glucose supplementation was also conducted as control. No pH control method was sued

in this case. The xylose consumption (Figure 4.13), XA production (Figure 4.14), added glucose consumption (Figure 4.15), and GA production (4.16) have been illustrated as following.

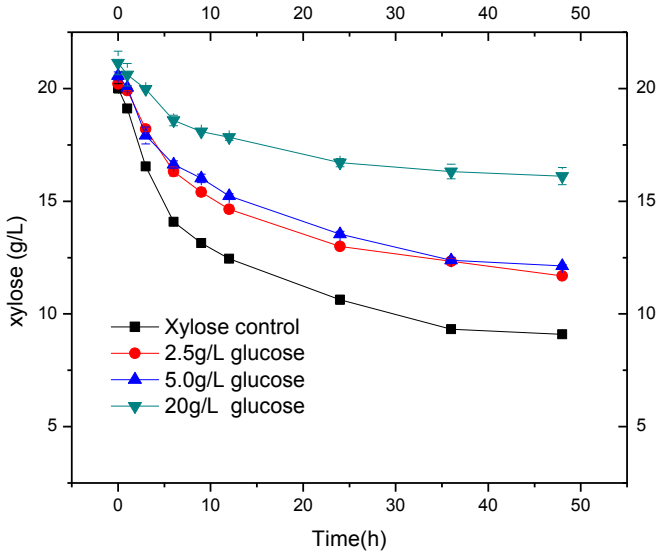

Figure 4.13. Effects of glucose supplementation on xylose consumptions by *G. oxydans* (20 g/L xylose with the addition of 0, 2.5, 5.0, 20 g/L glucose).

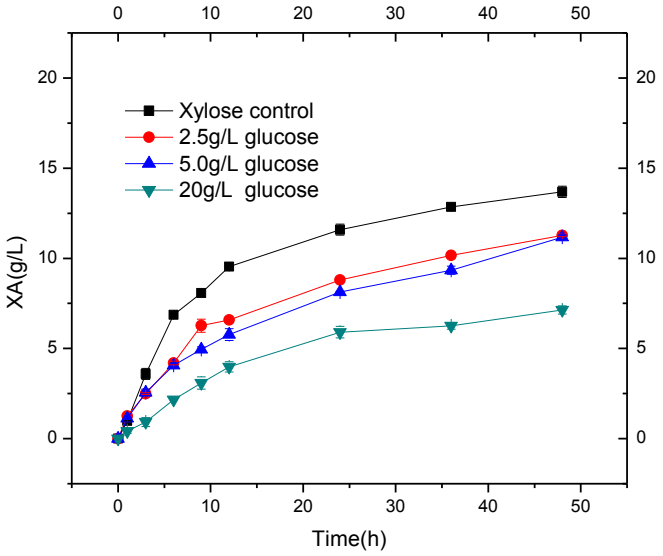

Figure 4.14. Effects of glucose supplementation on XA production by *G. oxydans* (20 g/L xylose with the addition of 0, 2.5, 5.0, 20 g/L glucose).

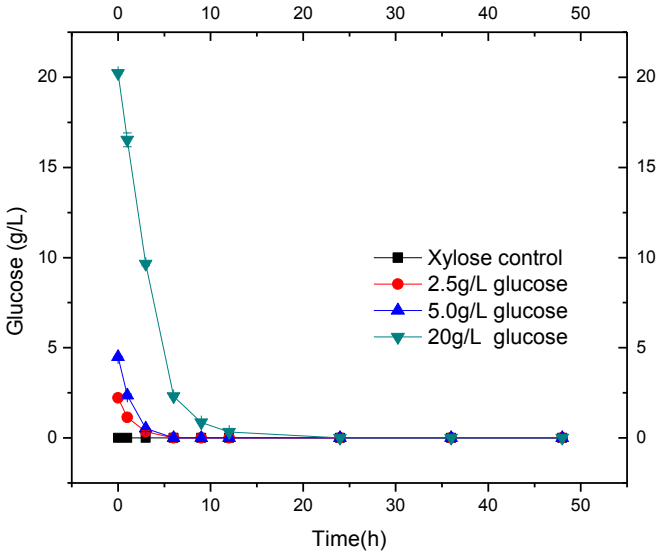

Figure 4.15. Glucose consumptions by *G. oxydans* (20 g/L xylose with the addition of 0, 2.5, 5.0, 20 g/L glucose).

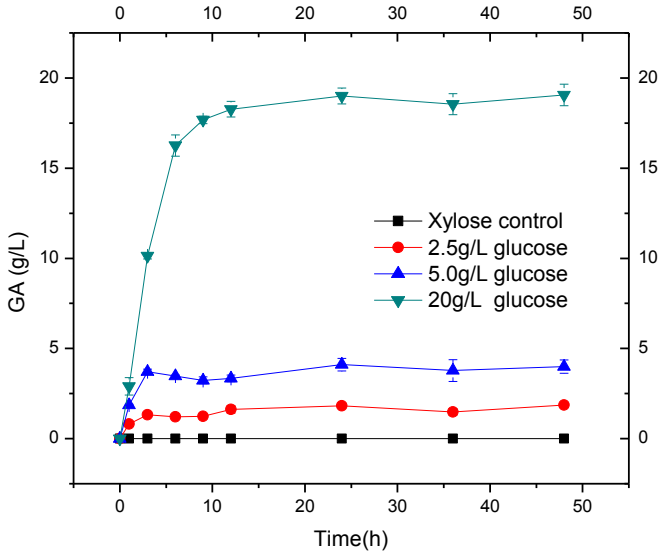

Figure 4.16. GA productions by *G. oxydans* (20 g/L xylose with the addition of 0, 2.5, 5.0, and 20 g/L glucose).

Xylose was consumed quickly without the addition of glucose, and higher amount of glucose added resulted in lower xylose consumption. With different amount of glucose (0, 2.5, 5.0 and 20 g/L), xylose consumption rates were 0.23 g $g^{-1}h^{-1}$, 0.13 g $g^{-1}h^{-1}$, 0.18g $g^{-1}h^{-1}$, and 0.08 g $g^{-1}h^{-1}$ respectively. Gradually slower production rates of 0.24 g $g^{-1}h^{-1}$, 0.17 g $g^{-1}h^{-1}$, 0.17 g $g^{-1}h^{-1}$ and 0.14g $g^{-1}h^{-1}$. The consumption yields were 54.5%, 42.2%, 41.0% and 23.8% respectively.

Different from xylose, glucose as a preferred carbon source was consumed faster than xylose in the fermentation regardless of the concentrations. Glucose was completely consumed and converted to gluconic acids during fermentation. The ketogluconic acids were negligible here. The glucose consumption rates of 2.5, 5 and 20 g/L were 0.13 g $g^{-1}h^{-1}$, 0.26 g $g^{-1}h^{-1}$, and 0.71 g $g^{-1}h^{-1}$ respectively. The production rates of gluconic acid were 0.09g $g^{-1}h^{-1}$, 0.25 g $g^{-1}h^{-1}$, and 0.68

g $g^{-1}h^{-1}$ respectively. Fermentation on 20 g/L glucose added resulted in a similar consumption rate to fermentation on 20 g/L glucose.

These results indicated that glucose did not facilitate xylose consumption in the fermentation by *G. oxydans*. It existed as a completing carbon source in the fermentation which was favored by the microorganism. The presence on glucose in the media, to a large extent, inhibited xylose consumption. Further, since the increase in glucose concentrations could result in faster gluconic acid production rates, the pH value in the fermentation with higher glucose supplementation concentrations could be expected to drop at a quicker speed (especially compared to that without glucose supplement), thus presumably interfered with xylose consumption.

The fact that glucose supplement retarded xylose fermentation by *G. oxydans* in terms of both consumption rate and final conversion yield raised the question that if it was the glucose that retarded xylose consumption or it was the pH dropping caused by gluconic acid oxidation that actually decreased xylose consumption.

To understand the question, experiment was further conducted on mixed sugars (20 g/L glucose and 20 g/L xylose) with 12 g/L of CaCO3 to control the pH constant at 7.0 during the fermentation.

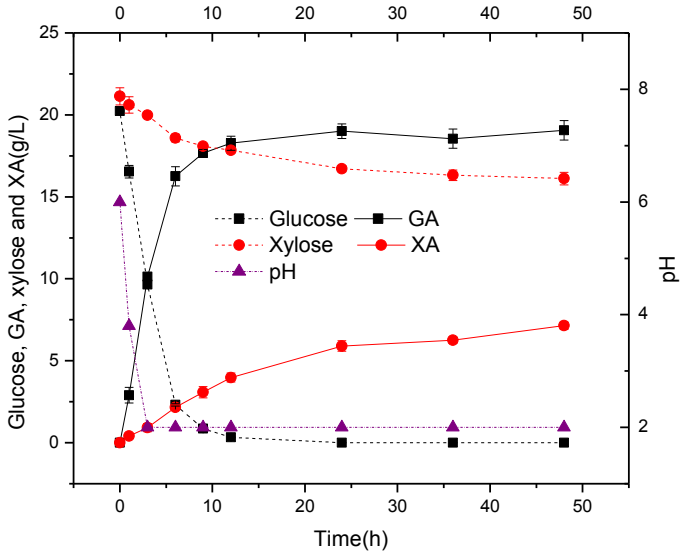

Figure 4.17. Mixed sugar fermentation by *G. oxydans* (20 g/L glucose and 20 g/L xylose without pH control).

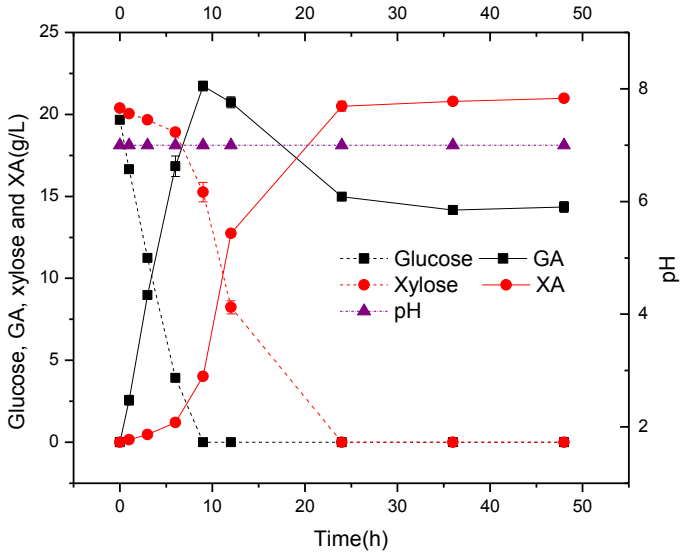

Figure 4.18. Mixed sugar fermentation by *G. oxydans* (20 g/L glucose and 20 g/L xylose with 12 g/L $CaCO_3$ as pH control).

Without pH control, the glucose was completely consumed at 6 h. pH dropped quickly from 6.0 to 2.0 at 3 h. The glucose consumption rate was 0.71 g $g^{-1}h^{-1}$, the xylose consumption rate was 0.08 g $g^{-1}h^{-1}$, and the gluconic and xylonic acid production rate was 0.68 g $g^{-1}h^{-1}$ and 0.06 g $g^{-1}h^{-1}$ respectively. With the addition of $CaCO_3$ (12g/L) to keep the pH constant at 7.0 in fermentation, the glucose consumption rate was 0.56 g $g^{-1}h^{-1}$, the xylose consumption rate was 0.05g $g^{-1}h^{-1}$, and the gluconic and xylonic acid production rate was 0.6 g $g^{-1}h^{-1}$ and 0.03 g $g^{-1}h^{-1}$ respectively.

The presence of glucose inhibited xylonic acid production from xylose (Figure 4.10 and 4.18). Without glucose (pH7.0), the xylose consumption rate was 0.36 g $g^{-1}h^{-1}$ (Figure 4.10). With 20 g/L of glucose, the xylose consumption rate dropped to 0.08 g $g^{-1}h^{-1}$. This indicated a strong inhibition of glucose on xylose consumption.

4.1.5 Effects of $CaCO_3$ concentrations on xylonic acid fermentation

Different concentrations of $CaCO_3$ were added to investigate the effects of low pH on enzyme activity. Xylonic acid fermentation on xylose was conducted with the addition of $CaCO_3$ at different concentrations: 0, 2, 4, and 6 g/L. After 48 h fermentation, 20 g/L glucose was added into the fermentation to examine the gluconic acid fermentation after xylonic acid fermentation. The fermentation results have been reported here with xylose consumption (Figure 4.19), xylonic acid production (Figure 4.20), glucose consumption from 48 to 72 h fermentation (Figure 4.21), and gluconic acid production (4.22). Fermentation pH was also recored from 0 to 72 h in Figure 4.23.

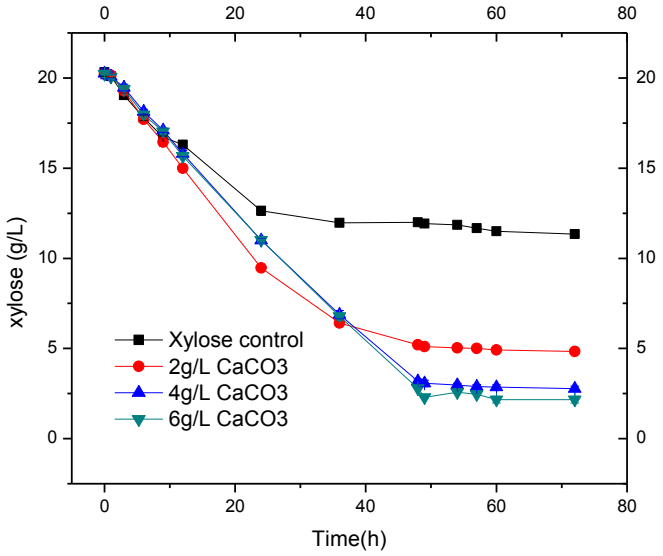

Figure 4.19. Effects of $CaCCO_3$ concentrations on xylose consumption by *G. oxydans* (20 g/L xylose with the addition of 0, 2, 4 and 6 g/L $CaCO_3$).

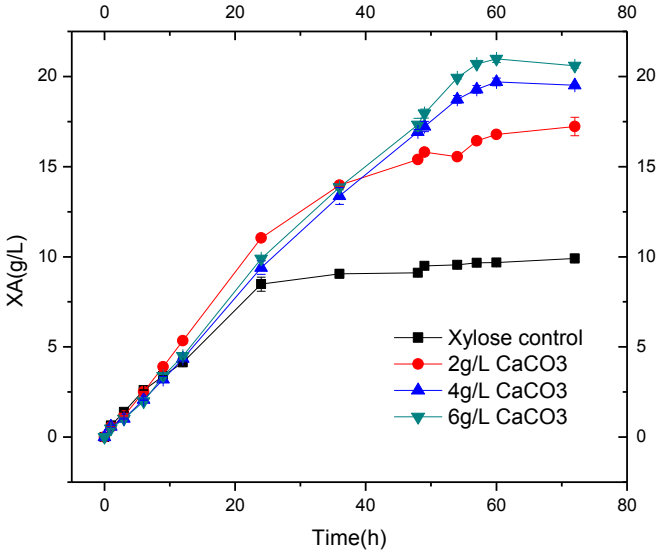

Figure 4.20. Effects of $CaCCO_3$ concentrations on XA production by *G. oxydans* (20 g/L xylose with the addition of 0, 2, 4 and 6 g/L $CaCO_3$).

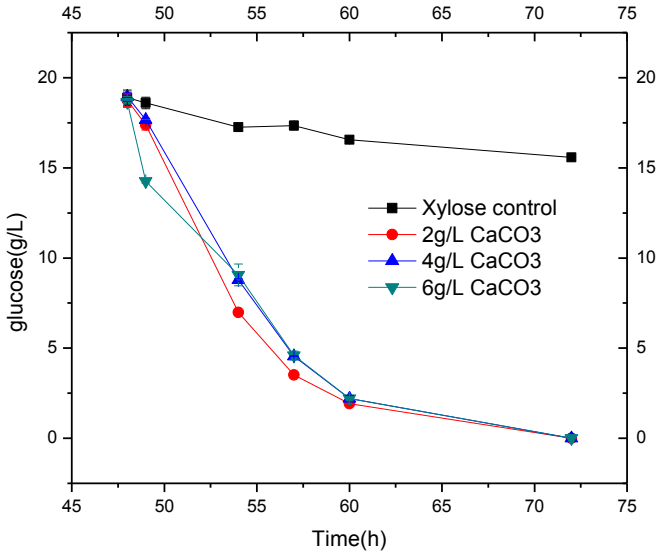

Figure 4.21. Glucose consumptions by *G. oxydans* (20 g/L glucose was added after 48 h fermentation with 20 g/L xylose, pH was controlled by adding various amount of $CaCO_3$: 0, 2, 4 and 6 g/L).

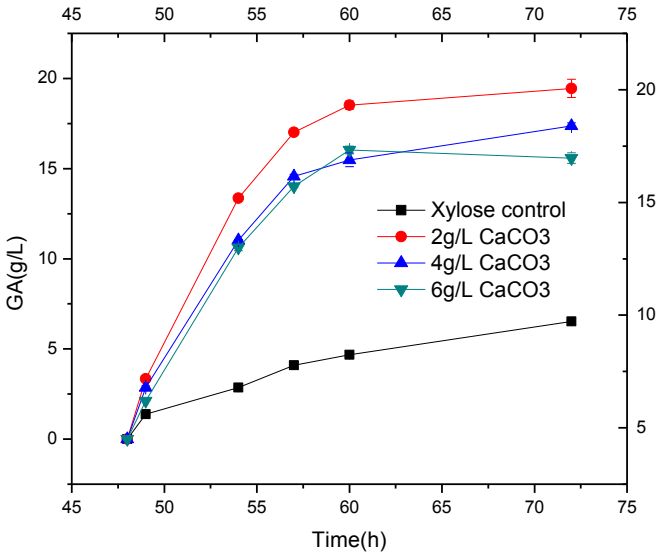

Figure 4.22. GA productions by *G. oxydans* (20 g/L glucose was added after 48 h fermentation with 20 g/L xylose, pH was controlled by adding various amount of $CaCO_3$: 0, 2, 4 and 6 g/L).

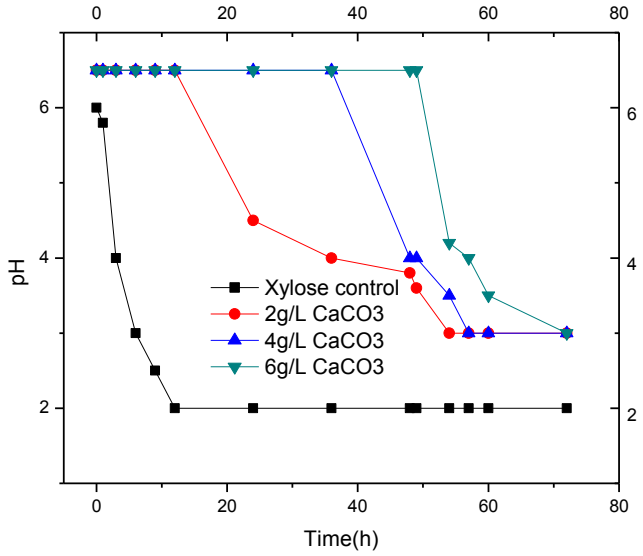

Figure 4.23. pH value of xylose fermentation by *G. oxydans* (20 g/L glucose was added after 48 h fermentation with 20 g/L xylose, pH was controlled by adding various amount of $CaCO_3$: 0, 2, 4 and 6 g/L).

As mentioned previously, without pH control, the fermentation pH dropped quickly to 2.0 with 12 h and less than 50% of xylose was consumed. The xylose consumption was stopped at 24 h (Figure 4.19). With the addition of 2 g/L $CaCO_3$, pH dropped to 4.5 at 24 h, and then to 3.8 at 48 h. Around 75% of xylose was consumed and the uptake of xylose was ceased at 48 h probably due to the low pH. With the addition of 4 and 6 g/L $CaCO_3$, more than 85% of xylose was consumed in 48 h fermentation. pH was dropped to 4 at 48 and 52 h respectively. With the addition of different amount of $CaCO_3$ (2, 4, and 6 g/L), the sequential fermentation of glucose was interestingly shown with similar consumption curves (Figure 4.21). This indicated the gluconic acid fermentation by *G. oxydans* can tolerate low pH at 4. However, lower pH (~2.0) will inhibit the fermentation.

4.1.6 Effects of low pH on xylonic acid fermentation

To investigate the effects of low pH (< 7.0) on xylonic acid fermentation, pH has been controlled at 2.0, 4.0, and 6.0 by 50 mM sodium citrate/citric acid buffer. Fermentation on 20 g/L xylose with no buffer solution has been used as control. The xylose consumption (Figure 4.24), xylonic acid production (Figure 4.25) and pH value (Figure 4.26) were reported below.

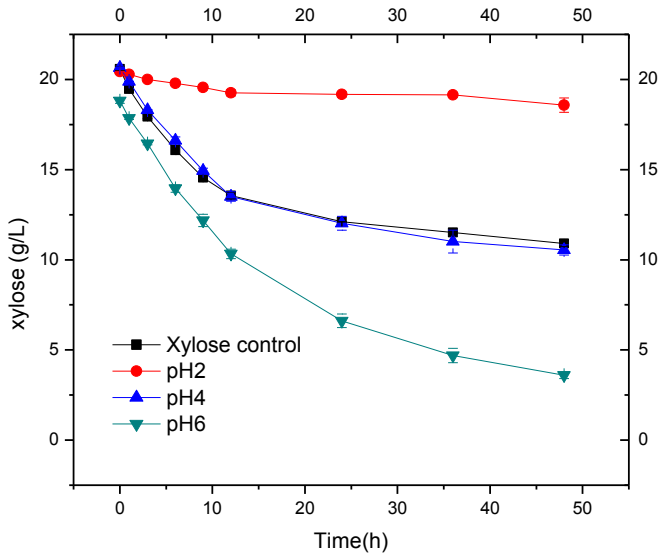

Figure 4.24. Xylose consumption by *G. oxydans* (20 g/L xylose with sodium citrate buffer at pH 2, 4, 6).

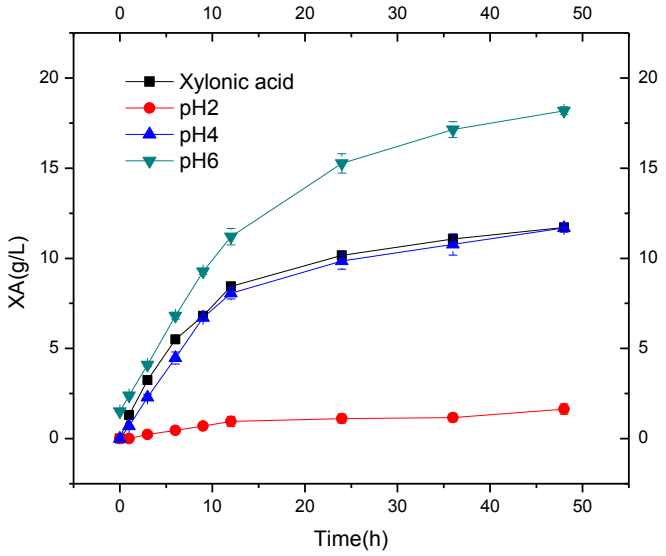

Figure 4.25. XA production by *G. oxydans* (20 g/L xylose with sodium citrate buffer at pH 2, 4, 6).

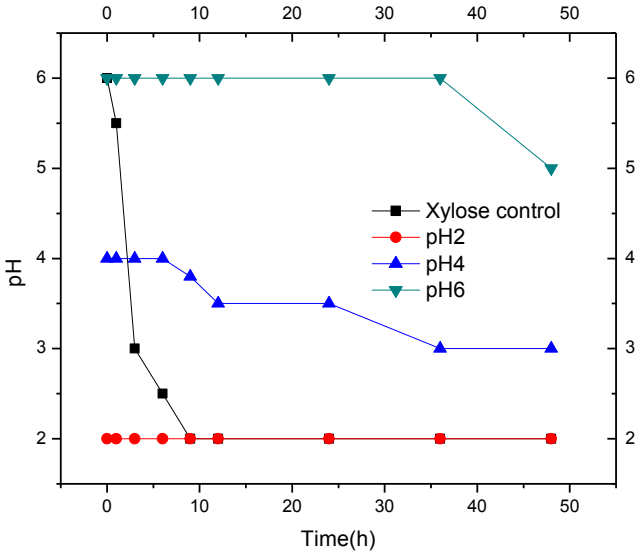

Figure 4.26. pH value of xylose fermentation by *G. oxydans* (20 g/L xylose with sodium citrate buffer at pH 2, 4, 6).

Higher pH resulted in high xylose consumption yield. The initial xylose consumption rates in the fermentation with pH control at 2.0, 4.0 and 6.0 were 0.03 g $g^{-1}h^{-1}$, 0.16 g $g^{-1}h^{-1}$, and 0.16 g $g^{-1}h^{-1}$, respectively. Without pH control, the initial xylose consumption rate was 0.18 g $g^{-1}h^{-1}$. The corresponding xylonic acid production rate was 0.02 g $g^{-1}h^{-1}$, 0.15 g $g^{-1}h^{-1}$, 0.27 g $g^{-1}h^{-1}$, and 0.22 g $g^{-1}h^{-1}$ respectively. The results indicated that xylose caould not be consumed at pH 2.0. The fermentation at pH 4.0 showed very similar xylose consumption rate to the control. The fermentation at pH 6.0 showed highest conversion yield of 90%, but did not consume the xylose completely. The incompleteness of fermentation at pH 6.0 was because the pH, although controlled, was still lower than neutral which was the optimal.

The different fermentation rates of glucose and xylose may relate to several factors: 1) different enzyme affinities because of structure differences; 2) hydrolysis of intermediate products glucono-δ-lactone to gluconic acid is quicker than hydrolysis of xylono-γ-lactone to xylonic acid; 3) in glucose conversion, gluconic acid may be further oxidized to ketogluconate and diketogluconate while in xylose conversion, xylonic acid is the final products. The H^+ concentration within *G. oxydans* cell may be lower during glucose fermentation than that during xylose fermentation therefore had less end-product inhibition.

Further study at enzymatic and cellular level can be done to further test the enzyme affinity to structures and the metabolism pathway to better understand this interesting point.

4.2 Gluconic and xylonic acid fermentation on lignocellulosic biomass by *G. oxydans*

4.2.1 Background

Lignocellulosic biomass can be used to produce valuable platform chemicals gluconic acid and xylonic acid through three fundamental steps: pretreatment, hydrolysis and fermentation, and product separation/purification (Mosier, Wyman et al. 2005; Balat 2011).

Pretreatment is the fundamental step for optimal hydrolysis and downstream operations (Wyman 1994; Wyman 1999). An effective pretreatment process is characterized by following criteria: avoiding size reduction, preserving hemicellulose fractions, limiting formation of inhibitors due to degradation products, minimizing energy input, and being cost-effective (Press 2000). Currently, numerous pretreatment methods are available, including steam explosion, Ammonia Fiber Expansion (AFEX), acid pretreatment and organosolv pretreatment etc. Different methods have their own advantages and limitations, selection should be made according to a comprehensive consideration of the features of methods, types of substrates and operation objectives. Organosolv pretreatment have some distinct advantages in this project. Firstly, it has better lignin removal. Lignin can be isolated as solid and washed from cellulose pulp. Secondly, solvent used in the pretreatment is easy to recover by distillation and can be recycled. From economic perspective, these features provide the optimal full use of all biomass components and enable the organosolv pretreatment method beneficial for downstream hydrolysis.

In order to use cellulose hydrolysates to produce gluconic acid by *G. oxydans*, enzymatic hydrolysis is required before fermentation process to break the cellulose chain into glucose molecules. Despites decades of efforts, rapid and complete enzymatic hydrolysis of lignocellulosic biomass with low protein loadings still remains one of the major technical and

economical bottlenecks in the overall bioconversion (Walker and Wilson 1991; Xia and Cen 1999; Chang and Holtzapple 2000; Berlin, Maximenko et al. 2007; Arantes and Saddler 2010; Zhao, Song et al. 2011).

The two principal configurations that use enzymes for saccharification are separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) (Wingren, Galbe et al. 2008; Ask, Olofsson et al. 2012). In SHF process, enzymatic hydrolysis and fermentation are carried out separately which makes it possible to run each process under optimal conditions. Its limitation is the end-product inhibition of the cellulolytic enzymes (Alfani, Gallifuoco et al. 2000; Tomás-Pejó, Oliva et al. 2008). SSF integrates the enzymatic hydrolysis of cellulose to glucose, catalyzed by the synergistic action of cellulase and β-glucosidase, with the fermentative synthesis of objective products. This allows the released sugars from hydrolysis to be rapidly consumed by microorganisms, thereby minimizing end-product inhibition. By using same reaction vessel, SSF is attractive for the lower capital costs, lower process time and minimum loss sugars. The primary disadvantage is that the conditions for both enzymatic hydrolysis and fermentation have to be the same, therefore, typically suboptimal for both of them (Wyman, Spindler et al. 1992; Hari Krishna, Janardhan Reddy et al. 2001; Öhgren, Bengtsson et al. 2006; Ask, Olofsson et al. 2012). Choice of configuration should be determined by a tradeoff of advantages and drawbacks associated with motivations.

During the thermo-chemical pretreatment, inhibitory compounds such as weak organic acids, furan derivatives and phenolic compounds may be generated. These compounds are toxic to fermenting microorganisms and thus will decrease the fermentation yield. Therefore, detoxification is needed to remove the toxic compounds before using hemicellulose prehydrolysates to produce xylonic acid by *G. oxydans*. Utilization of *G. oxydans* to produce

xylonic acid from Birchwood hemicellulose hydrolysates have been studied by Buchert (Buchert, Puls et al. 1988). The hydrolysates were steamed and hydrolyzed by *Trichoderma reesei*. Treatments including activated charcoal, ether extraction, mixed bed resin and CaO have been applied to improve the fermentability on xylose. However, none of them could increase the fermentability on 56 g/L xylose medium and some even acted negatively, despite lignin removal. The reason remains unclear and need more study to reveal the mechanism behind it.

The features of *G. oxydans* to convert a broad range of sugar substrates and to withhold inhibitory compounds make it outstanding in reaching the full utilization of lignocellulosic substrates. Based on the chemical composition of woody biomass, hardwood has a higher hemicellulose composition and lower lignin than softwood, therefore is better to be used as feedstock. The glucose oxidization pathway has been studied on pure sugar while no research has been done to investigate the utilization of *G. oxydans* on cellulose hydrolysates and hemicellulose prehydrolysates. Study in this area will offer a potential opportunity for industrial operation to generate gluconic acid and xylonic acid at low cost for extra revenue.

4.2.2 Gluconic acid and xylonic acid production from pretreated biomass via SHF.

In the experiments described below, sweetgum wood chips were pretreated using ethanol organosolv with 50% (v/v) ethanol and 1% (w/v) sulfuric acid as catalyst (w/v=1:7) in a 1 L parr batch reactor. The cooking condition was at 170 °C for 60 min. Pretreatment process has been conducted according to Figure 3.1. After washing and screening, solid fraction pulp, which was mainly composed of cellulose, has been collected for subsequent enzymatic hydrolysis. The moisture content has been analyzed after overnight oven dry using a 105 ^{0}C oven. The moisture content was 69.67±0.04% after balancing at 4 ^{0}C overnight. The chemical compositions (Table

4.1) of pulp have been analyzed according to laboratory analytical procedures (LAPs) for standard biomass analysis from NREL (National Renewable Energy Lab).

Table 4.1. Chemical composition of washed pulp of sweetgum after ethanol organosolv pretreatment

Extractives	Acid- insoluble Lignin	Glucan	Xylan	Mannan	Total
5.77±0.12%	6.84±0.06%	80.88±0.07%	6.18±0.22%	1.67±0.05%	101.3%

The sweetgum pulp after pretreatment contained 80% of glucose as the main composition. It would subsequently be used as substrates to produce gluconic acid from glucose by G. oxydans. Cellulase enzyme activity has been measured according to LAPs from NREL. Three enzyme loadings, 2.5, 5.0 and 10 FPU/g cellulose have been added for hydrolysis. The glucose, xylose and mannose release patterns during 72 h enzymatic hydrolysis have been shown as following (Figure 4.27-29).

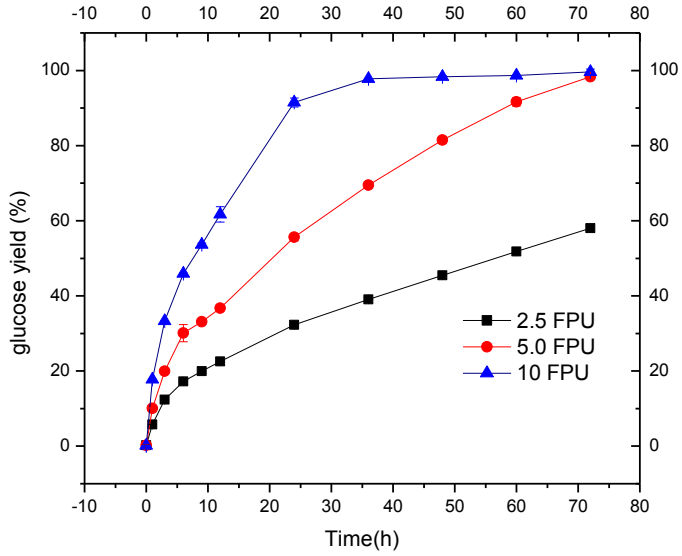

Figure 4.27. Effect of enzyme loading on the enzymatic hydrolysis of glucan in OPSG substrates.

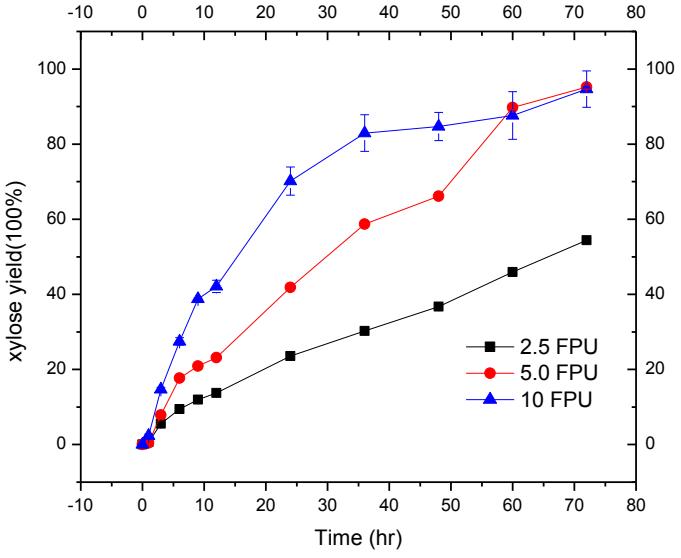

Figure 4.28. Effect of enzyme loading on the enzymatic hydrolysis of xylan in OPSG substrates.

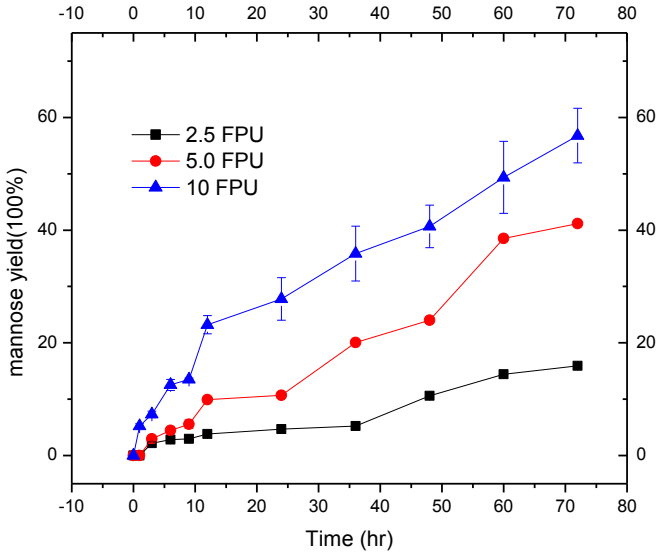

Figure 4.29. Effects of enzyme loading on the enzymatic hydrolysis of mannan in OPSG substrates.

Similar patterns have been found in glucose, xylose and mannose hydrolysis. The glucan-to-glucose yield reached 100% at 72 h under the 5.0 FPU of enzyme loading per gram of glucan. Increasing the enzyme loading to 10 FPU/g accelerated the hydrolysis rate and reached 100% yield at 36 h. Similarly, xylan-to-xylose yield reached 100% at 72 h under both 5.0 and 10 FPU of enzyme loading per gram of glucan. The mannose, even at 10 FPU enzyme loading, can only reach 55% yield.

After 72 h hydrolysis, enzymatic hydrolysates have been centrifuged at 3,000 rpm for 10 min. The supernatant was collected and used for gluconic acid production by *G. oxydans.* The pH of cellulose hydrolysates were first neutralized to 7.0 by adding NaOH and then adjusted to 6.0 by 50 mM sodium critate/citric acid buffer. Fermentation was performed in 250 mL flask at 30 ^{0}C, 220 rpm. Since *G. oxydans* was obligate aerobic, shaking speed was essential to guarantee enough oxygen supply. Fermentation on 20 g/L glucose has been conducted as control group.

The glucose consumption, gluconic acid production, ketegluconic acid production and xylose consumption have been showed as following (Figure 4.30-4.33).

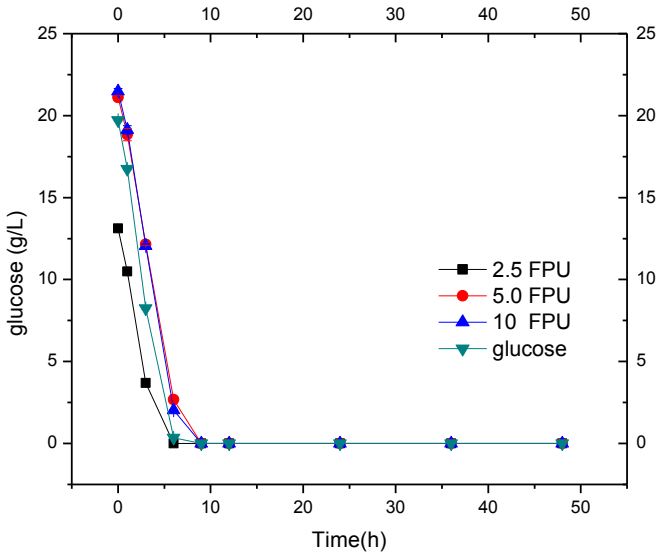

Figure 4.30. The hydrolyzed glucose consumption by *G. oxydans* on enzymatic hydrolysates.

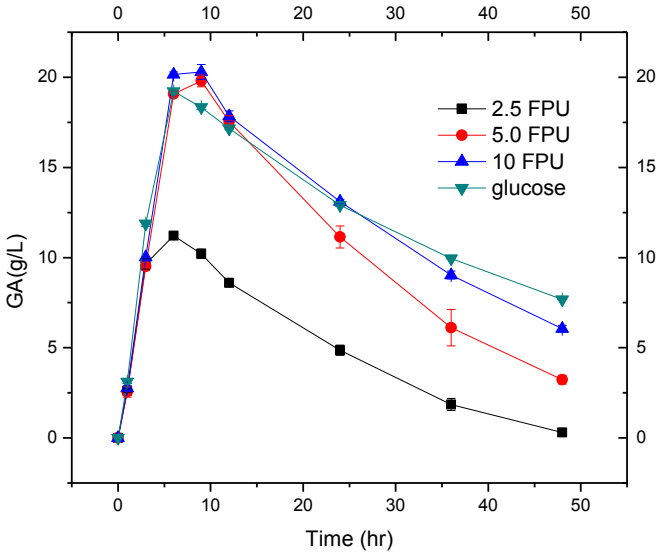

Figure 4.31. GA production by *G. oxydans* on enzymatic hydrolysates.

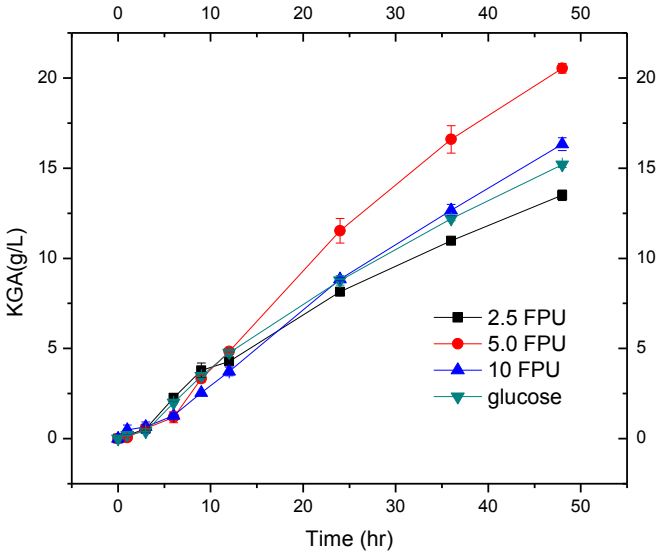

Figure 4.32. KGA production by *G. oxydans* on enzymatic hydrolysates.

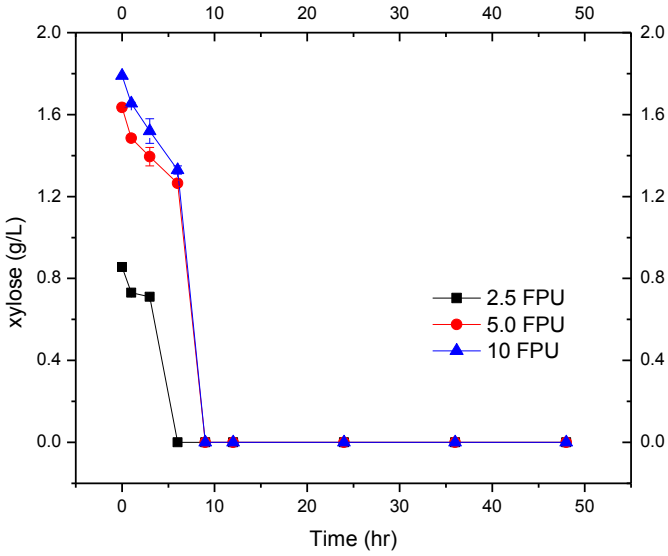

Figure 4.33. The hydrolyzed xylose consumption by *G. oxydans* on enzymatic hydrolysates.

The glucose consumption rate on enzymatic hydrolysates with 2.5, 5.0 and 10 FPU was 0.63, 0.6, and 0.63 g $g^{-1}h^{-1}$. The rates were slightly slower than glucose consumption rate on pure glucose fermentation which was 0.76 g $g^{-1}h^{-1}$. The production rate was 0.64 g $g^{-1}h^{-1}$, 0.63 g $g^{-1}h^{-1}$, and 0.69 g $g^{-1}h^{-1}$ compared to 0.79 g $g^{-1}h^{-1}$ in pure glucose fermentation. In all these four fermentation process, glucose can be completely consumed to reach 100% conversion yield. The amount of xylose in fermentation medium was small comparing with around 20 g/L of glucose. All xylose were consumed within 12 h.

4.2.3 Gluconic acid and xylonic acid production from pretreated biomass via SSF

SSF (simultaneous scarification and fermentation) has also been evaluated. Enzymatic hydrolysis and fermentation had different optimal condition—the former was at 50 ^{0}C, 220 rpm, and pH controlled at 4.8 by 50 mM sodium citrate/citric acid buffer; the latter was at 30 ^{0}C, 220 rpm, and pH 6.5. Since previous studies have concluded that enzymatic hydrolysis was the time-limited step in SSF process, the SSF condition was chosen at 37 ^{0}C, 220 rpm and pH 4.8. The glucose consumption, gluconic acid production, ketegluconic acid production, and xylose consumption (Figure 4.34-4.37) have been illustrated as follow.

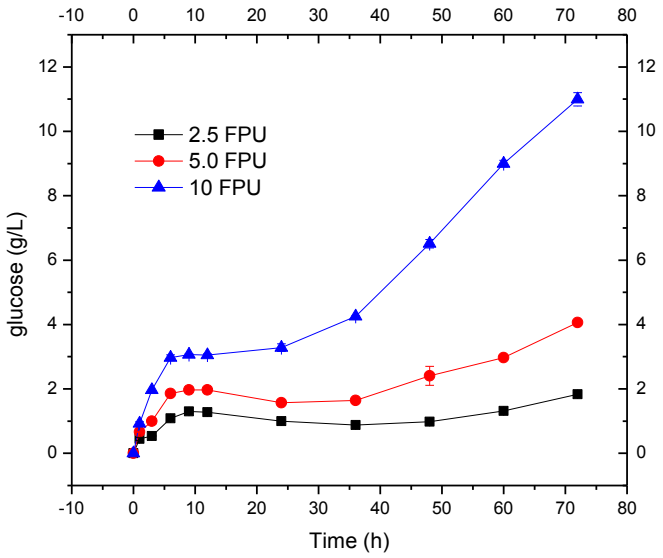

Figure 4.34. Effects of enzyme loading on glucose yield in SSF process.

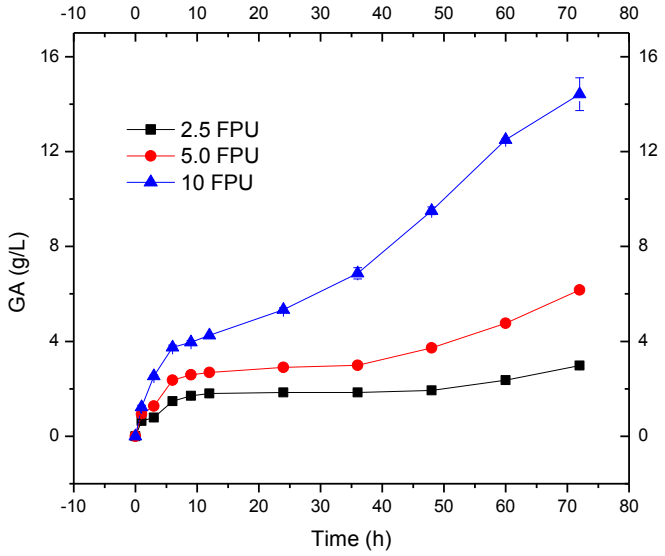

Figure 4.35. Effects of enzyme loading on GA yield in SSF process.

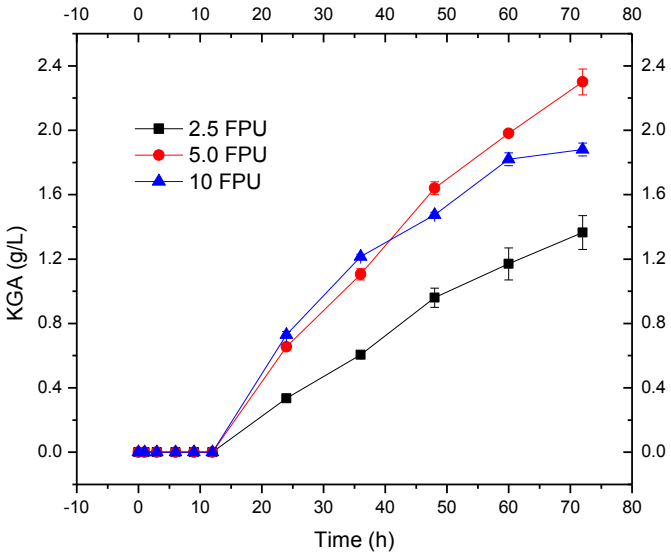

Figure 4.36. Effects of enzyme loading on KGA yield in SSF process.

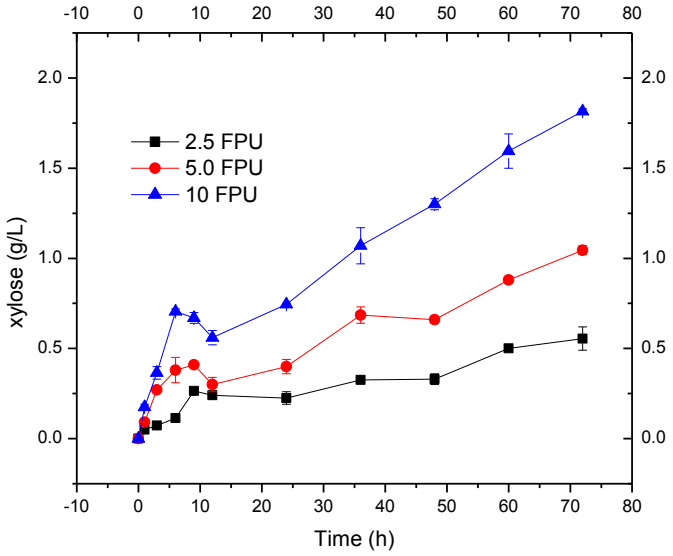

Figure 4.37. Effects of enzyme loading on xylose yield in SSF process.

Results above indicated that after 72 h SSF process, with 10 FPU enzyme loading, around 10 g/L glucose left in the medium while 14 g/L gluconic acid was generated. With 2.5 FPU and 5.0 FPU adding, less than 6 g/L glucose and gluconic acid were detected in the medium. These figures demonstrated that the fermentation was a limiting step while the released glucose was not totally converted to corresponding acid and ketogluconic acid. The glucose concentration and gluconic acid production curves manifested that the hydrolysis was much slower than at the optimal condition where it finished at 24 h with 10 FPU/g loading. Since bacterium *G. oxydans* was sensitive to medium environment, the residue of glucose may be caused by the deactivation of glucose-dehydrogenase in *G. oxydans* which was affected by the low pH and high temperature. The optimal temperature for *G. oxydans* was 28-30 ^{0}C while the used temperature was 37 ^{0}C. In addition to this, the pH at 4.8 was suboptimal for this strain. The xylose concentration profile

provided an evidence for this hypothesis: compared with SHF, the xylose was released from xylan but hardly consumed.

For SHF (separated hydrolysis and fermentation), the enzymatic hydrolysis was performed at 50 ^{0}C, 150 rpm, pH 4.8; the fermentation process was performed at 30 ^{0}C, 220 rpm, pH 6.0. After 72 h enzymatic hydrolysis, at 5 FPU and 10 FPU enzyme loading, glucose yield reaches around 100%. After 48 h fermentation, conversion yield reaches around 100%. For SSF (simultaneous saccharification and fermentation), the process was performed at 37 ^{0}C, 220 rpm, pH 4.8. After 72 h, around 10 g/L glucose was left at 10 FPU/g enzyme loading. The SHF process can achieve favorable yield within reasonable time and has potential for industrial use. The effects of temperatures, pH values and reaction time need to be further investigated to optimize the SSF rate and yield.

4.2.4 Fermentation on hemicellulose prehydrolysates

After pretreatment, the liquid prehydrolysates have been filtered, collected and stored at 4 ^{0}C. The liquid was first evaporated to remove ethanol. To minimize the effects of inhibitory compounds, detoxification was conducted following the method mentioned in Materials and Methods part. The detoxified prehydrolysates were then diluted to proper concentration for fermentation by *G. oxydans* (Figure 4.38). Un-detoxified prehydrolysates were used as a control group to see the effects of detoxification (Figure 4.39).

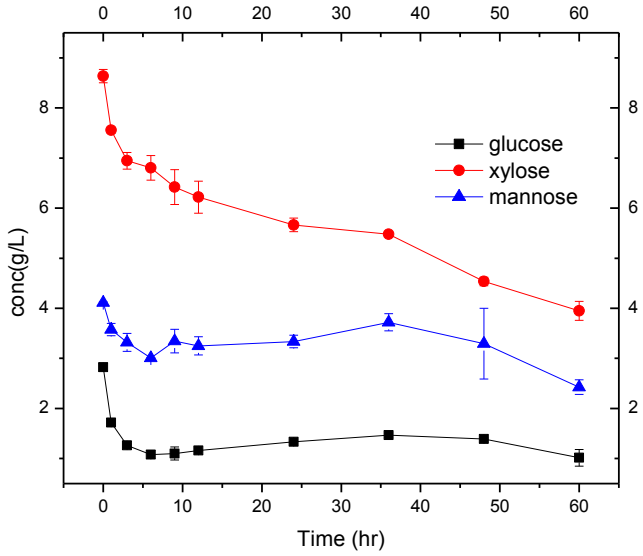

Figure 4.38. Sugar consumption by *G. oxydans* on hemicellulose prehydrolysates.

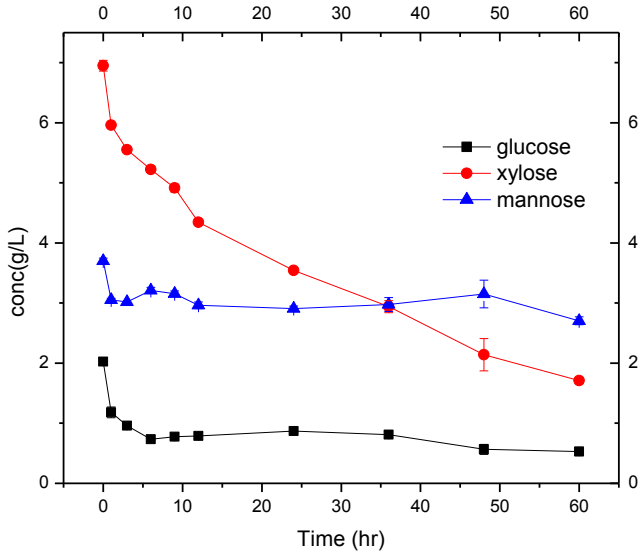

Figure 4.39. Sugar consumption by *G. oxydans* on alkaline detoxified hemicellulose prehydrolysates.

The high temperature and high pressure condition in pretreatment process may generate inhibitory compounds such as weak organic acids, furan derivatives and phenolic compounds. From the figures above, we can see that both glucose and xylose were not totally consumed after 60 h fermentation. This may be caused by toxic compounds in prehydrolysates liquids. In the treatment with alkaline detoxification, less glucose was left than in the control. Both glucose and xylose consumption was improved with detoxification.

Conclusion

In this thesis, we conducted two major parts of research work. Results of these two works were described in Chapter 4. In the first part, a series of works has been finished to investigate the gluconic acid and xylonic acid fermentation on glucose and xylose by *G. oxydans*; the major difference compared with the previous research is the supplementations of different concentrations of glucose and $CaCO_3$ in the fermentation on xylose. The results showed pH was a key factor affecting fermentation by *G. oxydans*. The role of pH control in ketogluconic acid formation during gluconic acid fermentation process has also been studied. The yield of ketogluconic acid revealed that relative high pH (> 5.5) was favored for the formation of further oxidization products.

In the second part, we compared the fermentation on pretreated biomass via SHF (separated hydrolysis and fermentation) and SSF (simultaneous saccharification and fermentation) process. The results proved that gluconic acid and xylonic acid can be produced from lignocellulosic biomass through biorefinery process including pretreatment, enzymatic hydrolysis and fermentation. These research works did contributions to the bioconversion of lignocellulosic biomass to valuable chemicals.

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