Bio-fuel Production by Using Integrated Anaerobic Fermentation

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ABSTRACT

Saccharification is one of the most critical steps in producing lignocellulose-based bio-ethanol through consolidated bioprocessing (CBP). However, extreme pH and high ethanol concentrations are commonly considered potential inhibitors for the application of *Clostridium* sp. in CBP. The fermentation of several saccharides derived from lignocellulosics was investigated with a co-culture consisting of *Clostridium themocellum* and *Clostridium thermolacticum* with or without immobilization. Alkali environments proved to be more favorable for ethanol production. Fermentation inhibition was observed at high ethanol concentrations (>8g/L) and extreme pH (>10). However, low levels of initial ethanol addition resulted in an unexpected stimulatory impact on the final ethanol productions for all cultures under selected conditions. The co-culture was able to actively ferment glucose, xylose, cellulose and micro-crystallized cellulose (MCC). The ethanol yield observed in the co-culture was higher (up to two-fold) than in mono-cultures, especially in MCC fermentation. The highest ethanol yield (as a percentage of the theoretical maximum) observed were 75% (w/w) for MCC and 90% (w/w) for xylose. Immobilization technique using sodium alginate is efficient in improve the ethanol production during co-culture fermentation, although the immobilization is not able to change the ethanol sensitivity of this co-culture. The ethanol yield through the use of immobilized technique increased to 97% of the theoretical efficiency for glucose. For cellobiose and MCC under optimized condition, the ethanol yields were approaching 85% of the theoretical efficiency. In order to examine the feasibility of this immobilization co-culture on lignocellulosic biomass conversion, untreated and pretreated aspen fermentations were performed. The immobilization co-culture shows clear benefit in bio-ethanol production in CBP process. With a 3h, 9% NaOH pretreatment, the aspen powder fermentation yield approached 78% of the maximum theoretical efficiency, which is almost twice the yield of the untreated aspen fermentation.

Keywords: Consolidated bioprocessing, *Clostridium* sp., Fermentation, Co-culture, Lignocellulosic ethanol, Immobilization, Alginate gel

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INTRODUCTION

1.1 Problem Statement

Affordable bio-fuel has become an important topic due to an imbalance between supply and demand for existing energy sources as well as the problems associated with recent fuel refinery processes (1). It is generally accepted that renewable energy will be more sustainable and more reliable than current energy resources (2). In the past century, the US and other countries have started to develop alternative energy sources to support the world's energy consumption needs and to decrease environmental pressures (2, 3). However, one challenge of exploring bio-fuel resources is to balance the conflict between the food crisis and the energy crisis since most of the existing bio-fuel feed stocks are food-based. Lignocellulosic biomass offers a great potential as a bio-fuel resource. This is mainly because lignocellulosics are an abundant raw material and bypass the issue of utilizing food for fuel production.

Consolidated bioprocessing (CBP) is a promising solution offering the potential for higher efficiency and lower production cost compared to other current conversion processes (4). Different than traditional separate hydrolysis and fermentation (SHF) or newly developed simultaneous saccharification and co-fermentation (SSCF), the CBP fermentation merges cellulase production, hydrolysis and fermentation all together (5). This merging can decrease the contamination possibility during transmission and reduce a large portion of capital cost (6). The other advantage of

CBP is that it inherits the capability of fermenting both pentose and hexose from SSCF (7). However, as a relatively new technology, technical difficulties are unavoidable, especially since during the CBP cellulase is generated inside the reaction vessel, making this process more complex than simply adding enzymes.

Proper microbes with specific traits combined with suitable process modifications (8) can help deal with the extreme conditions and inhibitors present in CBP fermentation. Co-cultures have been widely studied (9). Although production of biofuels in co-culture systems usually is higher than in their separate mono-cultures, the overall ethanol production even in co-culture systems remains low. For wild strains, less than 70% theoretical ethanol yield was produced per glucose equivalent (10), and less than 66% was reported to be produced per xylose equivalent (11). In this study, attempts were made to evaluate the potential advantages of integrating *C. themocellum* and *C. thermolacticum* into a CBP fermentation process with respect to ethanol and acetate formation, as compared to their mono-cultures. The ability of this co-culture and their mono-cultures to saccharify major biomass polymers and their fragments under a wide pH range (5-10) was examined. This innovative co-culture containing *C. themocellum* and *C. thermolacticum* were selected because of their remarkable de-polymerization ability for polysaccharides (12). Both microbes can hydrolyze a wide range of saccharides and tolerate relatively high temperatures up to 60 °C (13, 14). *C. themocellum* ATCC 27405 which contains cellulosomes is proficient in converting both crystalline and amorphous cellulose efficiently into ethanol, acetate and hydrogen (14). *C. thermolacticum* ATCC 43739 can produce a variety of de-polymerization enzymes but is especially apt in degrading pentoses (13). Thus, integrating *C. themocellum* and *C. thermolacticum* into the CBP fermentation can theoretically digest lignocellulosics directly into bio-ethanol.

A secondary purpose of this research is the optimization of this co-culture fermentation for ethanol production, and potentially reducing side reactions towards unwanted products such as acetate. Although much research has been devoted to investigating the effect of pH and ethanol concentrations during *C. themocellum* or *C. thermolacticum* mono-culture fermentation processes as independent variables, less attention has been paid to the interactions of these fermentation parameters in any of these mono-culture fermentations. In addition, the influences of pH values and ethanol concentration in previous studies are inconsistent (15, 16) requiring further explorations. It is expected that the use of co-cultures influences these effects.

In addition, we explored the potential use of encapsulation to increase bio-ethanol production with *C. thermocellum* and C. *thermolacticum*. Specifically, our project compared the ethanol production of co-culture and mono-culture, non-encapsulated and encapsulated strains in response to the pH change, the ethanol inhibition, and their correlation. Ca-alginate encapsulation was applied to these microbes in this project. The formed micro-capsules had a liquid core, in which *C. themocellum* or *C. thermolacticum* cells were grown, surrounded by a spherical polymeric membrane. It could be shown that the composite membrane of the capsule was able to protect the sensitive microorganisms from the toxicity of pretreatment and fermentation (12). Few studies have been applied to *Clostridium* sp. encapsulation for bio-ethanol production. Thus, in this project, the encapsulation of *C. thermocellum* and *C. thermolacticum* by calcium alginate, a mild matrix for living cells, will be studied to prepare a high ethanol tolerance biocatalyst for the lignocellulosic biomass fermentation. The calcium alginate is used because several successful fermentation processes with alginate entrapment of *Zymomonas mobilis* (17-20), *Aspergillus niger* (19) and *Saccharomyces cerevisiae* (20, 21) have been described. By testing the performance of encapsulated co-culture, the immobilized co-culture fermentation process for aspen powder was optimized.

1.2 Objectives

The *overall objective* of this project is to meet the demand for an inexpensive and highly efficient integrated anaerobic *Clostrdium* sp. fermentation process to produce ethanol as an energy source directly from insoluble lignocellulosic substrate (aspen).

To complete the overall objective, the following specific aims focus on several aspects of the CBP fermentation process for *Clostridium* sp.

1) Test the hypothesis that the *C. themocellum*/*C. thermolacticum* co-culture provides a higher ethanol yield or a higher sugar to end-products conversion rate than their mono-cultures in CBP fermentation at extreme pH (5-10) and varying ethanol concentrations (0-4%).
2) Assess whether or not the application of encapsulation results in the improvement of ethanol tolerance, pH tolerance, sugar to end-products conversion rate, or ethanol production in the *C. themocellum* and *C. thermolacticum* CBP fermentation.
3) Optimize the CBP fermentation parameter (pH and wood/medium ratio) for untreated and pretreated aspen, utilizing the conditions that provide a higher ethanol yield in aim 1 and aim 2.

1.3 Significance

This work focused on several aspects of the CBP fermentation process by *Clostridium* sp. with the goal to meet a demand for an inexpensive and effective second generation bio-ethanol production. The main challenge of this fermentation process is the efficiency of ethanol production. We assessed, if an encapsulation and co-culture strategy is efficient in improving the *Clostridium* sp. CBP fermentation by quantifying the ethanol yield in this study on various substrates. By understanding the effect of the encapsulation and co-culture strategy, the integrated anaerobic microbial CBP fermentation for untreated or pretreated aspen was optimized. The condition for maximum ethanol production was defined.

The observations obtained from this study could provide practical information for applying encapsulation and/or a co-culture method for cellulolytic and thermophilic bacterium for a one-step bio-ethanol production.

Chapter 2

LITERATURE SURVEY

2.1 Bio-ethanol Potential

The liquid energy carriers produced biologically are considered to be promising alternative energy carries because of the well established storage, logistics, and applications methods (22). Available liquid energy carriers are mainly ethanol, butanol, mixture of ethanol, butanol and acetone (ABE), as well as biodiesel. Among all of these energy carriers, ethanol is beneficial in many aspects. First of all, the production of bio-ethanol is the most established process as compared to the processes used for other energy carriers (23). Especially, ethanol production from agronomic plants is well established. 12.5 billion liters of ethanol are produced in Brazil every year from sucrose (24). Almost one fourth of the cars in Brazil run on the alternative fuel called gasohol, which is the mixture of ethanol and petroleum (25). In the US, corn is the major substrate for bio-ethanol production. Every year, about 5 billion liters of ethanol are produced in the US from corn kernels (24). Furthermore, bio-ethanol is a cleaner fuel than fossil fuel. Burning ethanol made from plants is estimated to reduce greenhouse gas emissions by 86% (26). In addition, ethanol, as a petroleum gasoline additive, is safer than the methyl tertiary butyl ether (MTBE) which is currently used for cleaner combustion (27). MTBE is reported to be toxic and has a potential to contaminate ground water (27).

However, for bio-ethanol production, the transport cost per energy unit is still higher than for petroleum. Moreover, water needed for feeding the feedstock, as well as the fluctuations of feedstock availability due to weather conditions all influence the cost of bio-ethanol.

2.2 Feedstock

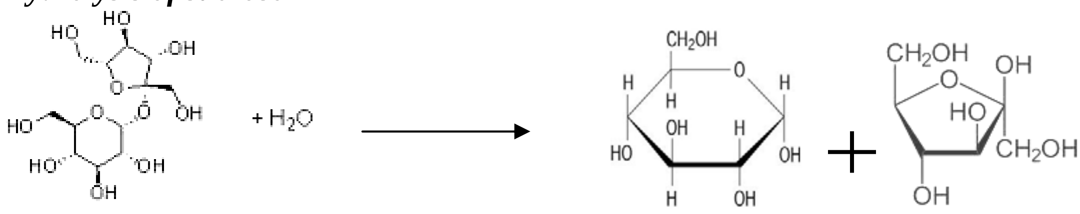
An appropriate, cost effective and reliable feedstock supply, an efficient process, and value-added by-products are all necessary to make this bio-fuel sustainable. Currently, the bio-ethanol is mainly produced from simple structure substrates including sugar (sucrose) and starch from agronomic plants like sugarcane and corn. The goal is to expand feedstock sources to lignocellulosic biomass with much more complicated structures (28).

2.2.1 Sugars

Most microorganisms possess the capacity to ferment simple hexoses (glucose, fructose). Thus, biomass composed of high concentrations of hexose or hexose precursors are easy to be employed for fermentation after hydrolysis, or even pretreatments. Today, the most widely used sugar for commercial ethanol production is sucrose in sugarcane, sugar beet, fruits, or sweet sorghum (29). Among these feedstocks, sugarcane juice, which is extracted from sugarcane fiber, is considered to be the main source of sucrose. During extraction, blackstrap molasses, containing 35 – 40% sucrose and 15 – 20% invert sugars (glucose and fructose), is produced as a by-product (30). Then the molasses can also be fermented to produce ethanol (Figure 2.1).

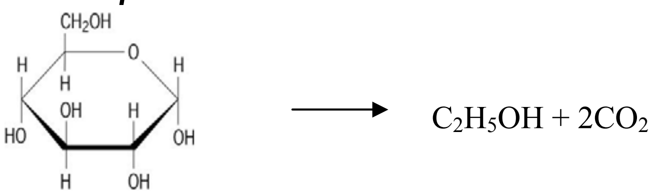
Lactose, a milk sugar existing in milk and whey permeates, is the other potential sugar for bio-ethanol production (31). This raw material has been used for bio-energy production. This is because the milk permeate is low in protein (0.5%) but high in lactose (5%) which makes it not suitable for animal feed (32). Lactose fermentation has been studied using *Clostridium thermolacticum*. Acetate is the major product in this process. In addition, ethanol, hydrogen and carbon dioxide are produced as by-products (31). (Figure 2.2)

Hydrolysis of sucrose

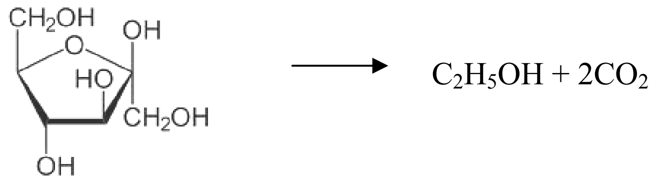


Sucrose Glucose Fructose

Ethanol formation

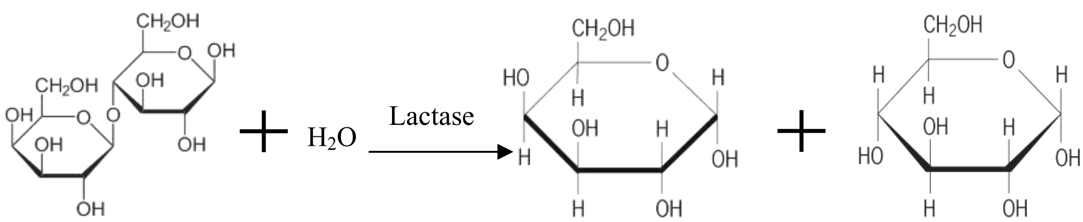


Glucose



Fructose

Figure 2.1 Sucrose hydrolysis and ethanol formation



Lactose Galactose Glucose

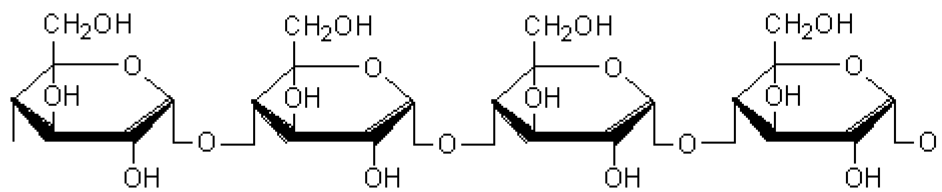
Figure 2.2 Lactose hydrolysis

2.2.2 Starch

At present, the vast majority of industrial ethanol and almost all bio-ethanol is made from grain (wheat, corn, barley). Starch, the key sugar component stored in the grain, can be used for ethanol production. Starch molecules are long chains of α-D-glucose monomers (Figure 2.3). Slightly more complex than the sugar fermentation process, starch needs to be broken down into glucose first through hydrolysis with amylase recovered from fungi, or diastase and maltase from sprouting grain (Figure 2.4). Then fermentation enzymes will ferment the fermentable sugars into ethanol and carbon dioxide. In the grain fermentation, distillers grain which is a high protein cattle feed including fiber, protein and ash is also produced (33).

These cheap starchy crop resources vary depending on the geographic locations. For example, cassava grows mainly in Africa. Potato is a typical European product and sweet potato is mainly an Asian crop. For the US, corn and wheat account for the majority of the cereal grains. In 1990, about 200 million tons of corn was produced in America, and 4% of them were used in ethanol production (34). However, both sugars and starchy materials are relatively expensive for bio-fuel production, since these materials are also important food ingredients.

Amylose



Amylopectin

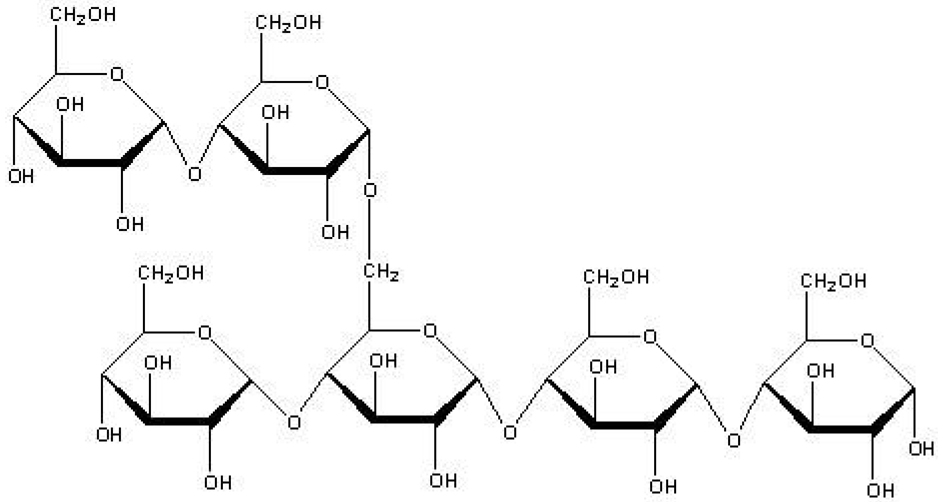


Figure 2.3 Starch molecular structures

Amylase hydrolysis

$$(C_6H_{10}O_5)_n + nH_2O \xrightarrow[\text{amylase}]{} n\ C_6H_{12}O_6$$

Diastase hydrolysis

$$\underset{\text{Starch}}{2(C_6H_{10}O_5)_n} + nH_2O \xrightarrow[\text{diastase}]{} \underset{\text{Maltose}}{n(C_{12}H_{22}O_{11})}$$

$$C_{12}H_{22}O_{11} + H_2O \xrightarrow[\text{Maltase}]{} 2C_6H_{12}O_6$$

Ethanol formation

$$C_6H_{12}O_6 \longrightarrow C_2H_5OH + 2CO_2$$

Figure 2.4 Starch hydrolysis and ethanol formation

2.2.3 Lignocellulosic biomass

The issue for starch and sucrose based ethanol is the high cost of using food crops, as well as some instability due to the competition between food and energy production (35). It is estimated, that this competition has the potential to increase food prices by 23-25% before 2013 (36). Thus, ethanol production from lignocellulosic biomass has become more attractive. Recent reports estimated that after overcoming the technical barriers, 270 billion gallon of ethanol will be produced using 2.45 billion metric tons of biomass (37). This estimated ethanol production is twice the current US gasoline consumption (37).

2.2.3.1 Lignocellulosic biomass structure and composition

The lignocellulosic biomass have the potential to be used in large-scale bio-ethanol production. A fundamental understanding of the cell wall structure is important to eliminate the cell wall's natural resistance to degradation through chemicals and enzymes. The utilization of lignocellulosic biomass is mainly depending on the composition of the biomass. Compositions of the available energy biomass resource are listed in Table 2.1 (27, 38). Biomass with high carbohydrate content and low lignin content is preferred.

Biomass	Cellulose %	Hemicellulose %	Lignin %
Corn cob	45	35	15
Grasses	25-40	35-50	10-30
Wheat straw	30	50	15
Leaves	15-20	80-95	0
Cotton seed hairs	80-95	5-20	0
Coastal Bermuda grass	25	35.7	6.4
Switchgrass	45	31.4	12
Cotton Stems	37.9	20.4	24
Spruce	42	6.7	26
Aspen	47	23	23
Bagasse	44	27	19
Rice Straw	39	23	20
Corn stover	37.5	22.4	17.6
Pine wood	46.4	8.8	29.4
Poplar	49.9	17.4	18.1
Water hyacinth	33.4	19.5	9.27

Table 2.1 Chemical composition of potential lignocellulosic biomass resources

2.2.3.1.1 Cellulose

Cellulose, hemicellulose and lignin are the main components of plant cell walls. Cellulose is a linear high molecular weight polymer composed of β-D-glucopyranose units which are linked by 1-O-4 glycosidic linkages (39) (Figure 2.5). It provides structural support and chemical resistance for the plant. Considering the uniform hydrolysable glucose building blocks, the cellulose molecule would be the best carbohydrate source for the fermentation process. Major functional groups, hydroxyl groups are able to form intermolecular and intramolecular hydrogen bonds. Intermolecular linkages which form between different molecules are responsible for the formation of microfibril structures and highly ordered crystalline areas (Figure 2.6) (39). Cellulose in plant materials consists of both, crystalline and amorphous areas. Because of the high energy of a large amount of hydrogen bonds, cellulose crystalline areas are hard to degrade with enzymes or chemicals. Under normal conditions, due to the existence of hydrogen bonds, the cellulose is relatively insoluble. An efficient cellulose degradation technique, including effective enzymes, high temperatures, concentrated acid or alkaline, is necessary for both amorphous and crystalline cellulose in the conversion process.

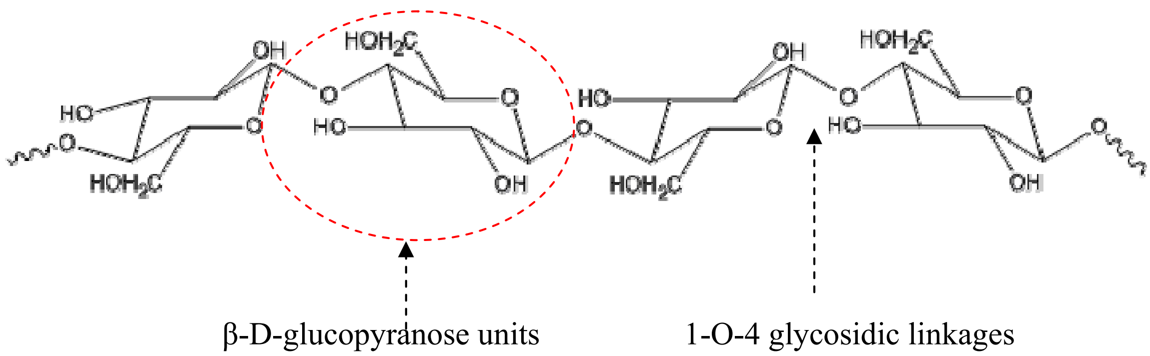


Figure 2.5 Cellulose molecular structure

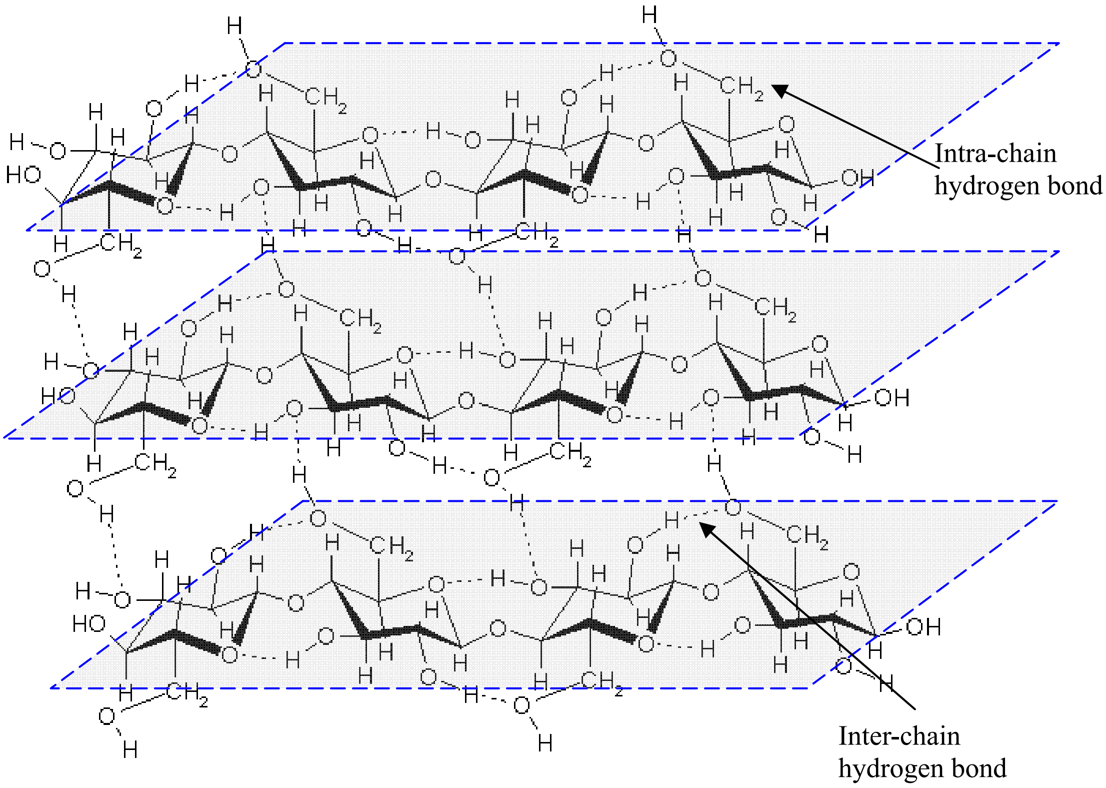
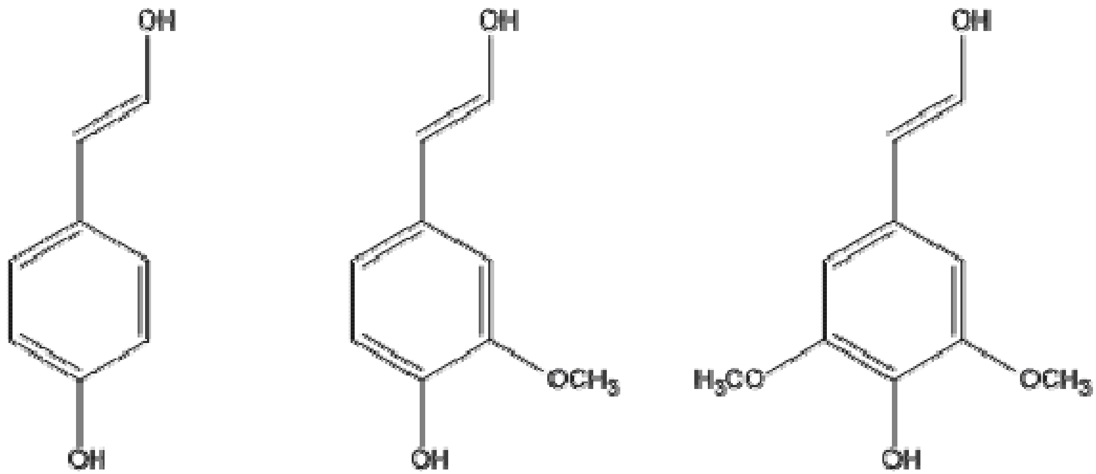


Figure 2.6 Cellulose crystalline arrays

2.2.3.1.2 Lignin

As the second most abundant material in the plant cell wall, lignin provides additional strength and protection against fungi and insect attack (40). The high molecular weight (in excess of 10,000 Da) phenylpropan structure of lignin leads to high insolubility in most solvents. Lignin significantly adds to the rigidity and moisture resistance of the biomass (41). Furthermore, the existence of the lignin has been considered as an inhibitor for cellulase, due to the non-productive binding of lignin and cellulase depending on the lignin types (42). As a result, chemicals like surfactants (43), $MgSO_4$, and $CaCl_2$ (44), and exogenous proteins like bovine serum albumin (BSA) (45) are added prior to enzyme or microbe loading to reduce the reaction between lignin and cellulase. In addition, many pretreatment methods have been developed to diminish the obstacle of lignin. Minimizing the effect of lignin inhibition depends on the source of lignocellulosic biomass, since lignin within the lignocellulosic materials is not uniform and varies in different plants and even in different locations of the plant. For example, in the middle lamella and primary cell wall, lignin content is higher than in the secondary cell wall (39). There are three monolignol building blocks for lignin: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (39) (Figure 2.7). Guaiacyl lignin is commonly found in softwood while a mixture of guaiacyl and syringyl together with small amounts of *p*-hydroxyphenyl are found in hardwood. For grass or non-wood plants, all three units are abundant (39).



p-Coumaryl alcohol Coniferyl alcohol Sinapyl alcohol

Figure 2.7 The structures of lignin building blocks

Functional groups in lignin including methoxyl groups, phenolic hydroxyl groups, benzyl hydroxyl group, carbonyl groups and small amount of terminal aldehyde groups are the other factors that will affect the degradation of lignin. Also the hydroxyl and methoxyl groups in lignin precursors will interact with cellulose fibrils (46) (Figure 2.8). The lignin carbohydrate complexes formed by the interactions are also considered to be restricting factor for biomass accessibility.

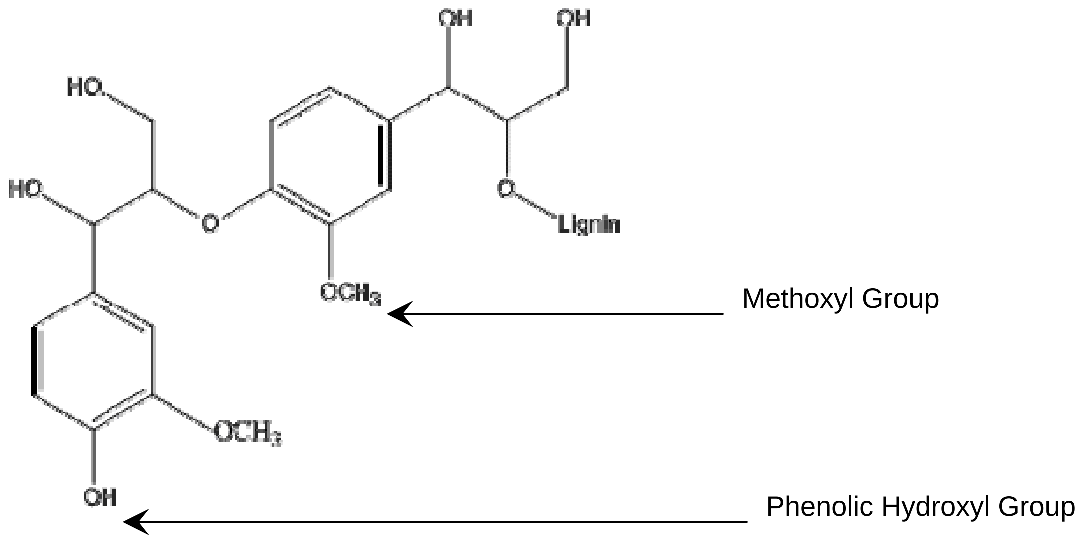


Figure 2.8 Functional groups of lignin

Although lignin is harmful for lignocellulosic biomass conversion, it is considered to be a valuable by-product in the bio-refining process. Products like filter material, expanded polyurethane foam and thermoplastic have been successfully produced from lignin extracted from plants (47, 48). The overall efficiency of lignocellulosic biomass conversion would be maximized if the recovery of lignin would become a part of the overall biomass conversion process.

2.2.3.1.3 Hemicellulose

Hemicellulose, another important polysaccharide type in plant cell walls, is a branched hetero-polysaccharide consisting of various pentose and hexose units including xylose, mannose, galactose, rhamnose, and arabinose. Besides these units, they may also contain acetic, uronic acid as well as 4-O-methyl ether (Table 2.2). They are shorter chain molecules (around 200 DP) with covalent bonds to lignin to form cross-linked structures. Due to the linkages among hemicellulose, cellulose and lignin, removal of hemicellulose which is easier than removal of lignin and cellulose, is considered to be an efficient way to increase cellulose utilization. Xylose is the most important type of sugar within hardwood and grass type hemicellulose. In hardwood, it comprises most of the hemicellulose main chains (49). Thus, conversion of xylose is the key to utilize hardwood hemicellulose. Due to the structural diversity, a wide range of enzymes including endoxylanase, exoxylanase, mannanase, arabinosidase, acetylesterase, and glucoronisidase are needed (50). Pretreatments, especially alkaline pretreatment, also were found to be effective in separating hemicellulose from lignin and cellulose.

Plant species	**Hemicellulose type**	**Percentage**
Grasses	Arabinoxylan	20-40%
Hardwood	O-Acetyl-4-Omethylglucuronoxylan	10-35%
	Glucomannan	3-5%
Softwood	Galactoglucomannan	15-25%
	Arabino-4-O-methylglucuronoxylan	10-15%

Table 2.2 Hemicelluloses composition (24)

2.2.3.1.4 Aspen

Different types of lignocellulosic biomass have different proportions of components. This is not only observed in structural molecules like cellulose, hemicellulose and lignin, but also in nonstructural components including proteins and extractives. The ratio of structural molecules will change the hydrolysis efficiency and will impact the cost efficiency of the conversion technology. Nonstructural components, which may cause the production of inhibitors during biochemical conversion, are also critical for conversion processes.

Aspen, a hardwood tree in the Salicaceae (Willow) family, is well understood in its structure and chemical composition because it has been widely used in paper industry as well as in wood industry as raw material of fiber, lumber, cut stock, particleboard, and plywood. As a fast growing species, aspen covers over 5 million acres in Minnesota, which is about 1/3 of the commercial forest land in the US (51, 52). However, this wide application of aspen leads to the formation of large amounts of cutting waste, sawdust, under-utilized of remains. As large amount of aspen waste saw dusts are available from industries, more and more researchers in the bio-renewable energy industry have shown interest in this material.

2.3 Pretreatments techniques

As mentioned above conversion of lignocellulosic materials into ethanol has considerable limitations because of their physical and chemical properties. Plant cell walls have a natural resistance to chemical, physical and enzymatic degradations. Lignin, which is the second most abundant natural polymer and currently not directly used in ethanol production, is partially linked to the polysaccharides in the cell walls (53-56). In addition, main component - cellulose will form crystals which are barriers for complete sugar conversion for specific microbes (55). Therefore, an efficient pretreatment process altering the lignocellulosics structural complex is necessary. Possible alteration includes the removal or modification of the lignin, degrade or removal of the hemicellulose, decrystallization of the cellulose and increase of the accessible surface area (57, 58). The goal is to increase the accessibility for enzymes or microbes without undesirable fractionation of carbohydrates or formation of fermentation inhibitors, and consequently improvement of the efficiency of polysaccharides degradation as well as the production of ethanol and other value added co-products during biomass conversion. An ideal pretreatment is performed with minimum cost and energy demand (6).

Pretreatment processes were invented as early as 1932 (59). Physical, chemical, and biological methods are the major current pretreatment processes (1). Combination methods which utilize at least two of these methods above to increase pretreatment effectiveness have also been developed (59). In this section, promising cost-efficient pretreatment methods are reviewed, followed by a brief discussion of the pros and cons of each technology with the aim of discussing ways to integrate the pretreatment process with consolidate bioprocessing (CBP) for bio-ethanol production.

2.3.1 Physical pretreatment

Physical pretreatments are methods without addition of chemicals or micro-organisms. They use external forces to reduce the lignocellulosic materials into fine particles in order to increase the surface area of the materials. According to the forces used, the physical pretreatment can be further divided into two sub-catalogs: mechanical (dry, wet, vibratory ball milling) (60) and non-mechanical method (steam explosion, irradiation and pyrolysis) (2).

2.3.1.1 Mechanical pretreatment

Mechanical pretreatments use shearing force to reduce biomass' particle size, change the lignocellulose structure, and reduce degree of polymerization and crystallinity of cellulose (2). Depending on the final size of the material, the mechanical pretreatment consists of milling, grinding or chipping. Chipping leads to 10 to 30 mm particles, and milling and grinding lead to 0.2 to 2 mm (27). Milling includes ball milling, two roll milling, hammer milling, compression milling agitation bead milling, pan milling, fluid energy milling, and colloid milling (60, 61). For aspen, vibratory ball milling is reported to be a more effective method as compared to ordinary ball milling (60). However, according to the research performed by Cadoche et al, the energy input to reduce the biomass to fine particle is higher than the theoretical energy content held in biomass, which makes the milling not economical for most lignocellulosic biomass, especially for high moisture biomass (62-64). Furthermore, these methods are species selective. Improper application of mechanical pretreatment will lead to carbohydrate losses, in which case the final fermentable sugars and ethanol yield will be reduced (65). Therefore, mechanical pretreatment is considered to be impractical to be applied exclusively. Combination of mechanical pretreatment and chemical size-reduction is commonly used to make the pretreatment more cost-efficient (66).

Extrusion, which utilizes heating, mixing and shearing to increase accessibility of the materials, is a novel promising pretreatment technology. Both physical and chemical modifications occur as the lignocellulosic biomass passes through the extruder (67). High efficiency makes this pretreatment method appealing (67).

2.3.1.2 Non-mechanical method

Irradiation pretreatment can be performed by Gamma-ray, microwave, ultrasound, pulsed electrical filed, UV and electron-beam. Irradiation will cause the disruption of beta-1,4-glycosidic bonds and cellulose crystalline structures (2). In addition, the high energy of these radiations will lead to the formation of free radicals, which leads to a further degradation of the lignocellulosic materials (68). This method is widely used in waste water sludge pertreatment (69, 70). For the application for lignocellulosic biomass, including rice straw, bagasse, sawdust, chaff, corn stalk, peanut hust, and oil palm empty fruit bunch, was studied in pervious experiments (68) by using ultrasonic irradiations. Dramatic enhancements of the enzymatic hydrolysis efficiency were achieved (71, 72). Unfortunately, irradiation pretreatments are reported to consume high levels of energy and require long process time with expensive high quality equipment. Irradiation pretreatment methods by themselves are currently limited to laboratory scale, and not considered as a feasible solution for industrial applications.

Steam explosion is exposing biomass to steam under high pressure and temperature followed by a decompression at the end (38). Liquid hot water (LHW) pretreatment (co-current, counter-current, and flow through) is a pretreatment similar to steam explosion, except that, in LHW pretreatment, instead of steam, biomass is merged into hot water with certain pressure and temperature (6). Both these processes are able to cleave the acetyl groups and uronic acid groups from hemicellulose and consequentially acidify the medium. Furthermore, water at high temperature acts as acid (73). As a result, acidic condition will cause partially hydrolysis of hemicellulose and amorphous cellulose to oligosaccharides and to fermentable sugars, also resulting in a more accessible material for the following hydrolysis or fermentation steps (61). Moreover, for steam explosion, the lignin structure is partially modified which also leads to higher digestibility of the biomass (74). In addition, the shear forces caused by expansion contribute to the structural modification (75). Both mechanisms are similar to acid pretreatment. However, the steam explosion is able to offer higher fermentable sugar concentrations due to the lower water content (60). There are no chemicals added in both methods. In addition, size reduction is not required for lignocellulosic biomass since breakage of particles will occur during pretreatments (6, 6, 73, 73), these methods are more economic feasible in lignocellulosic conversion application. Steam explosion has been applied on aspen in pervious papers. The total reducing sugars yield after hydrolysis is increased over seven times with the pretreatment as compared to non treated aspen (76).

LHW was applied on yellow poplar wood sawdust with and without pH control (77). The pretreatment proved to involve the partial conversion of cellulose and hemicellulose into oligosaccharides and monosaccharides. Nevertheless, during steam explosion and LHW, high temperature is reported to increase the lattice structure of the cellulose, and consequentially increase the cellulose crystalline (78, 79). In addition, higher pretreatment temperature also leads to increased material solubility adding to the pretreatment yield loss (77). High severities also causes the production of various fermentation inhibitors, includes furfural, 5-hydroxymethylfurfural (5-HMF), phenolic compounds, and aliphatic acid, (6, 41), making this method not suitable for pretreatment with CBP. Monitoring and controlling the pH is considered to be an efficient improvement for LHW pretreatment (77). With controlled pH, cellulose solubility, and formation of HMF, levulinic and formic acid are minimized (77). As a result, the pretreatment yield loss will be minimized.

2.3.2 Biological pretreatment

Because of the environmental issues, more and more studies turn to biological pretreatment methods, which use fungi, bacteria or their enzymes. Fungi are applied as delignification agent to digest or change the lignin structure, and hydrolyze the hemicellulose (27). As a result, enzymes used for the hydrolysis process are able to approach polysaccharides such as cellulose and other hemicelluloses much easier. In the present literature, various species of brown rot fungi, white rot fungi, and soft rot fungi have been studies and summarized (27, 80). White rot fungi only attacks lignin, but brown rot and soft rot fungi mainly attack cellulose while slightly modifying the lignin structure (1). Among these species, white rot fungi are more commonly used in pretreatment since they produce lignin-degrading enzymes including laccases and peroxidases (81). *Pleurotus ostreatus* is reported to be able to convert over 35% of wheat straw cellulose into reducing sugars in five weeks (82). *Stereum hirsutum* was found to be very efficient in pretreated *Pinus densiflora*, a type of softwood (83). Other white rot fungi like *Cyathus stercolerus*, *Phanerochaete chrysosporium*, *Phanerochaete sordida* and *Pycnoporus cinarbarinus* were also tested on several substrates for their delignification efficiency (81).

Researchers agree that there are significant advantages to the biological pretreatment methods, especially since these procedures are completely environmentally friendly and require low energy input (67). However, the drawback of these methods outweighs their advantages. Almost all those fungi need more than one week to react with biomass. In addition, lignin degradation of white rot fungi requires a carbon source, mainly cellulose and hemicelluloses, since it is a co-oxidative process.

Thus, while reacting with lignin, these fungi will reduce cellulose and hemicelluloses concentration in biomass, thereby decreasing the yield of fermentable sugars (84). As a result, although plenty of experiments have been done at lab-scale, none of the biological pretreatments have been applied to industrial-scale production (60). Thus these methods still need to be improved before they can be used for large-scale production. Development of genetically modified strains with high lignin degradation capacity and high cellulase activity are necessary (60).

2.3.3 Chemical pretreatment

Chemical pretreatments have been studied dated back to the early 1900s and several reports have been published since 1980s comparing response of enzymatic hydrolysis after different chemical pretreatment methods. Different than physical methods, chemical pretreatments are mainly used for modifying the lignin in the biomass, removing hemicellulose, and to change cellulose polymerization as well as cellulose crystalline structure (85). Acids, alkali, salts, organic solvents as well as oxidizing agents are all considered to be effective pretreatment agents (58).

2.3.3.1 Alkali Pretreatment

Alkali and acid pretreatments are very commonly used methods. During this alkali pretreatment, biomass is soaked in the dilute alkali solution (0.5-2%) and treated for varying periods of time temperature. Neutralization and removal of lignin fragments and other inhibitors is usually involved at the end of the pretreatment. Generally alkali conditions are less severe than other pretreatments and prevent lignin condensation reactions. Due to the mild conditions, alkali pretreatments usually require longer pretreatment time, pressure or addition of oxygen which will facilitate some delignification (1, 86). This milder condition allows alkali pretreatments to remove some lignin fraction without leading to severe carbohydrate lose or inhibitor formations (79). Under alkali condition, peeling reactions, which start from the reducing end-group of the polysaccharides, will happen and will reduce the overall sugar yield. At high temperature, random chain cleavage will break some of the 1-4-β-glycosidic bonds between glucose building blocks to lower the degree of polymerization and the crystallization of cellulose (87). In this process some lignin and hemicellulose is removed. Ether linkages between carbohydrates and lignin are deconstructed by forming intermediate epoxide structure. This intermediate structure allows the nucleophilic substitution (88).

In addition to the cleavage of ether bonds between carbohydrates and lignin, alkaline can cause the cleavage of some ether bonds in lignin, which results in lignin fragmentation (89). Ester saponification forming carboxyl and hydroxyl groups is involved in hemicellulose, and between lignin and carbohydrates (89). The pore size of the materials, the swelling capacity and surface area are increased (89), so that the enzymes or microbes can penetrate easier and digest the cellulose structure (61). Furthermore, alkali will remove the acetyl and uronic acid groups from hemicellulose to enhance the accessibility of the enzymes (90). NaOH (91-93), Na_2CO_3 (94), $Ca(OH)_2$ (lime) (90, 95-98), KOH (87), NH_4OH (99) and aqueous ammonia (87, 100-102) were used to hydrolyze wheat straw (103), spruce wood (92), sugarcane, cassava, peanuts wastes (94), bagasse (90), switchgrass (102, 104), (95)corn cob (91), corn stover (96, 97), corn husk (105) and municipal solid waste (95) (Table 2.3).

2.3.3.2 NaOH pretreatment and Lime pretreatment

NaOH is the most commonly used chemical among all these alkaline pretreatment agents (1). The major reaction involved in these two pretreatments is the intermolecular ester bonds saponification. Hemicellulose is dissolved, and cellulose II is formed which will lead to extended swelling of the native cellulose structure and increase of the internal surface area (61). In addition, lignin structure, cellulose crystallinity, acetyl groups on hemicellulose, and linkage between lignin and carbohydrates are also disrupted (58). In this case, the biomass will become more accessible for the following hydrolysis steps. Besides NaOH, $Ca(OH)_2$ (Lime) pretreatment is also widely studied due to its low unit cost, safe handling and easy recovery using CO_2 (6, 87, 106). However, the equipment scaling issue makes the commercial operation of lime pretreatment challenging (66).

2.3.3.3 Ammonia pretreatment

Ammonia will lead to a cellulose structure change from cellulose I to III (61). Ammonilysis of esters, acetals, lactone groups also hemicellulose removal, cellulose decrystallization and delignification will occur (61). Three techniques are involved in using ammonia: soaking in aqueous ammonia (SAA), ammonia recycle percolation (ARP), and ammonia fiber explosion-method (AFEX).

ARP and SAA are pure chemical pretreatment. ARP is using aqueous ammonia (5-15%), but instead of soaking, it is performed using a flow-through reactor packed with biomass called percolation at high temperature (80-180ºC) (107). Low pressure is applied to the system to prevent evaporation (108). During this pretreatment, the structure swelling and delignification are achieved by high temperature. Ammoniolysis reactions result in delignification, with carbohydrates left behind. Upon finishing the reaction, the solids are flashed to atmospheric pressure, then washed and filtered to separate the carbohydrates. The liquid is collected for ammonia recycling, lignin and remaining carbohydrates separations (107). As a result, ARP is able to separate the biomass into three major components including cellulose, pentosans or pentoses, and lignin (109). For SAA, biomass is soaked with aqueous ammonia at low temperatures to maximize the delignification and minimize the carbohydrate losses (110). Other than SRP, the SAA reduces the liquid loading which reduces the energy cost of this process (109). AFEX is considered to be a combination of chemical pretreatment and physical pretreatment which will be discussed in the following section.

However, during the alkali pretreatments, the formation or incorporation of irrecoverable salts leads to sever issues. The removal and lack of recyclability of these salts limits the used of this method (6). In addition, the efficiency of alkali pretreatment method depends on the biomass lignin content. It is proved to be more efficient in low-lignin material pretreatments like hardwood and agricultural residues than in high-lignin materials like softwood with lignin content over 26% (111). Yield loss is another issue that hinders the acceptance of alkali pretreatment. During most alkali pretreatments, hemicellulose is dissolved within alkali solutions, and the recovery of the hemicellulose is expensive (112).

Ref.	Feedstock	Chemical	Time	Temp °C	Increase bioconversion	Effect
(96)	Corn stover	0.5 g $Ca(OH)_2$/g raw biomass	4 weeks	55	overall yields of glucose and xylose were 93.2% and 79.5% at 15 FPU/g cellulase	Glucan (91.3%) and xylan (51.8%) were converted to glucose and xylose respectively. Of the initial lignin, 87.5% was maximally removed
(87)	Sugar Cane bagasse	NaOH, NH_3 (aqueous), NaOH +NH_3, $Ca(OH)_2$, and $Ca(OH)_2$. + Na_2CO_3	18h	20	Enhanced the enzymatic digestibility from 20 to 72%	(*Combine with steam explosion at 200C, 6.9 MPa, and 5 min)
(100)	Corn cobs/stover mixture (CCSM)	10wt% ammonia	15-60 mins	170	The ethanol yield reached 83% by SSF	Delignification of 74-80%, 50-56% of the total hemicellulose but <8% of the total glucan was solubilized
(100)	switchgrass	10wt% ammonia	30-60 mins	170	The level of ethanol yield reached 84% by SSF	Delignification of 71-84%, 50-56% of the total hemicellulose. 10% of the total glucan was solubilized
(97)	Corn stover	0.075 g $Ca(OH)_2$/g raw biomass	4 hours	120	Polysaccharide conversions approaching 100%.	Loses 32% of the lignin.
(101)	corn cobs/stover mixture (CCSM) and switchgrass	0.28 g H_2O_2/g biomass+10 wt% ammonia	90 mins	170	Digestibility of CCSM was 95%. 3-4 times higher than untreated one. and 93%,	Delignification was 94-99% (101)
(101)	hybrid poplar	0.28 g H_2O_2/g biomass+10 wt% ammonia	90 mins	170	Digestibility of switchgrass was 93%	yielding 50% hemicellulose removal and 80% delignification
(105)	Bagasse	ammonia water (25-28% ammonia)	20 mins	120	Enzymatic hydrolysis was effectively improved. The carbohydrate yields from cellulose and hemicellulose were 72.9% and 82.4%	
(105)	corn husk	ammonia water (25-28% ammonia)	20mins	120	The carbohydrate yields from cellulose and hemicellulose were 86.2% and 91.9%	
(102)	Switchgrass	30% aqueous ammonium hydroxide	5 or 10 days	Room tempe rature	The percentage of maximum theoretical ethanol yield achieved was 72 with SSF	40-50% delignification. Hemicellulose content decreased by approximately 50%
(104)	Switchgrass	0.1g $Ca(OH)_2$/g dry biomass	2 hours	100	The 3-d reducing sugar yield was five times that of untreated switchgrass, the 3-d total sugar (glucose + xylose) yield was seven times, the 3-d glucose yield was five times, and the 3-d xylose yield was 21 times.	About 10% glucan, 26% of xylan and 29% of lignin became solubilized

(93)	wheat straw	1.5% sodium hydroxide	144 hours	20		60% lignin release and 80% hemicellulose release
(92)	spruce	3% NaOH/12% urea	24 hours	-15	about 80% theoretical xylose yield and 60% mannose yield were obtained	Over 60% glucose conversion
(107)	Oak Wood	15 wt% ammonia	40mins	170	The enzymatic digestibility of ARP-treated waste oak wood yielded 86.1% with 60 FPU/g glucan and 82.3% with 10 FPU/g glucan	82% of delignification, but retains more than 90% of the glucan content
(108)	corn stover	15 wt% ammonia	60mins	170	Enzymatic digestibility of ARP-treated corn stover is 93% with 10 FPU/g-glucan enzyme loading. The ethanol yield from the SSF of low-liquid ARP-treated corn stover using Saccharomyces cerevisiae reached 84% of the theoretical maximum.	ARP process solubilizes about half of xylan, but retains more than 92% of the cellulose content.
(110)	corn stover	15 wt% ammonia	12hours	60	The treated corn stover retained 100% glucan and 85% of xylan, but removed 62% of lignin. 77% of the maximum theoretical yield based on glucan and xylan for SSCF	The treated corn stover retained 100% glucan and 85% of xylan, but removed 62% of lignin
(113)	hybrid poplar	10 wt% ammonia solution	1hour	180	The enzymatic digestibility of the biomass pretreated at this condition (measured with enzyme loading of 30 IFPU/g dry biomass) was consistently above 90%, and the overall glucose yield (on the basis of glucose content in the original biomass) was also high at 85-90%.	The extent of delignification in the ARP process was in the range of 23-63%. solubilized significant amounts of xylan into the pretreatment effluent, yet left most of the glucan fraction intact.
(98)	Hybrid poplar wood	0.4g $Ca(OH)_2$/g dry biomass	2hours	140 or 160	72 h of enzymatic hydrolysis, the overall yields attained is 94-95 g glucan/100 g of glucan in raw biomass and 73 g xylan/100 g xylan in raw biomass	94-96% glucan and 70-74 % xylan recovered. *21.7bar

Table 2.3 Summary of alkaline pretreatments of lignocellulosic biomass

2.3.3.4 Acid Pretreatment

Acid pretreatment is one of the oldest and most commonly used methods. 72% sulfurous acid was considered to be the first diluted acid used in the pretreatment process in the US, and then 42% (w/w) hydrochloric acid hydrolysis was applied in Germany (114). Organic acids including maleic acid and fumaric acid (115) are also beeing studied. Both concentrated and diluted acids are applied in these acids pretreatment procedure. Concentrated acids disturb the hydrogen bonds in crystalline cellulose and convert crystalline cellulose into amorphous cellulose. Furthermore, these pretreatment methods decrease the DP of cellulose and degrade the pentoses (6). The advantage of applying concentrate acid pretreatment is that it is not specific to biomass type. In addition, mild temperature condition and high monosaccharide yield (over 90%) are the characteristics that made this method appealing for decades (114). The issue with concentrated acid pretreatment is that concentrated acids especially at higher temperatures (200-500ºC) lead to the formation of furfural or hydroxymethyl furfural, which reduces the sugars yield (116). The other drawback for concentrated acid pretreatment is high corrosion of the equipments and high acid recycling cost (67). Facing these issues, diluted acid pretreatment became more favorable in the bio-fuel industry. There are typically two types of diluted acid pretreatments: high solid loading (10-40%) low-temperature (less than 160ºC) batch pretreatment and low solid loading (5-10%) high temperature (more than 160ºC) continuous-flow pretreatment (1). Besides reactions with cellulose, diluted acid pretreatment also cause hemicelluloses dissolution. This will release water soluble sugar monomers and oligomer from cell wall matrix, so that the porosity of the cell wall and enzyme digestibility are both increased. In addition, although diluted acid is not able to remove lignin, researchers suggested that the lignin in biomass will be modified (109). As a result, the diluted acid pretreatment is less flexible in choosing feedstock. Biomass with lower lignin content is preferred. In addition, due to the mild condition, extension of time or increase of temperature is required (114).

2.3.3.4.1 Sulfuric acid

Environmental toxicity and equipment corrosion leads to the limitation of applying this pretreatment (117). The sulfuric acid pretreatment has been used to treat a wide variety of lignocellulosic biomass, including switchgrass, aspen, spruceetc. (Table 2.4). Pretreatment is usually done by applying sulfuric acid (0.5%-2%) on biomass at high temperature (130-210ºC) for a few minutes to hours (Table 2.4). Although the sulfuric acid is appealing because of its low cost, the loss of sugars, high energy input, restricting equipment requirements, and high disposal cost all impacts the overall pretreatment cost. Furthermore, the by-products of this pretreatment process, includeing furfural (from pentoses), 5-hydroxymethylfurfural (from hexoses), acetic acid, and metal ions, are all considered to be inhibiting the subsequent fermentation. These inhibitors are known to decrease the growth rate of microorganisms and ethanol production during the fermentation process (118). Cleaning processes like stream stripping are necessary prior to fermentation (119).

Ref	Feedstock	Chemical	Time	Temp	Enzyme Loading	Effect
(112)	Poplar wood (Sunds Reactor)	2.0% Sulfuric acid	1.1 min	190°C	15FPU/g Cellulase	82.8% total sugar recovery
(120)	Eucalyptus	1.84% sulfuric acid	30min	180°C	20 FPU cellulase & 30 CBU beta-glucosidase	Over 80% cellulose conversion
(121)	spruce chips	8-10% bisulfite and 1.8-3.7% sulfuric acid	30min	180°C	14.6 FPU cellulase plus 22.5 CBU beta-glucosidase	more than 90% cellulose conversion
{{402 Monavari, S. 2009}}	Spruce chips	3% SO2	3min→5 min	190°C then 210°C	65 FPU/ -glucosidase IU/g g cellulase and 17-	Glucose recovery of about 80% theoretical (122)
(123)	Olive tree	1.4% sulfuric acid	10 min	210°C	5 FPU/g cellulase and 15UI/g β-glucosidase	Obtain 75% of all sugars in the raw material
(124)	Apple bagasse	0.8 mol L−1 Sulfuric acid	15min	121°C	15 FPU/g cellulase	0.47g/g ethanol yield
(125)	Wheat straw	0.75% v/v sulfuric acid	1 hour	121°C	recombinant Escherichia coli strain FBR5	74% saccharification yield
(126)	Corn stover	2.0% sulfuric acid	43 min	120°C	22 FPU/g cellulase	77% xylose yield but only 8.4% glucose yield
(127)	Aspne, balsam, fir, basswood, read maple, switchgrass	0.25%-1.0% w/v sulfuric acid	over approximately a 70 min	160-190°C	N/A	Maximum yields range from 70% (balsam) to 94% (switchgrass) for xylose, from 10.6% to 13.6% for glucose, and from 8.6% to 58.9% for other minor sugars
(128)	Rice straw	0.5% sulfuric acid	10min → 3min	201-234°C	N/A	78.9% of xylan and 46.6% of glucan were converted to xylose and glucose
(129)	Switchgrass (Panicum virgatum L.) and reed canarygrass	0 to 100 g (kg DM)−1 sulfuric acid	30-180 days	Amber temperature	Saccharomyces cerevisiae D5A	Conversion of glucose to ethanol for reed canarygrass ranged from 22% to 83%, switchgrass conversions ranged from 16% to 46%
{{418 Li,Chenlin 2010}}	switchgrass	1.2% (w/w) sulfuric acid	20min(130)	160ºC	cellulase (NS50013) concentration of 50 mg protein/g glucan and β-glucosidase (NS50010) concentration of 5 mg protein/g glucan	85% glucose yield

Table 2.4 Summary of sulfuric acid pretreatments on lignocellulosic biomass

2.3.3.4.2 Organic acid

Maleic acid and fumaric acid have been studied and compared with sulfuric acid pretreatment (115). They proofed to be efficient alternatives for sulfuric acid pretreatment due to their low furfural and HMF formation which results in less impact at the downstream process (131-134), although this downstream process is considered to be a low-value by-product stream (109). Oxalic, salicylic and acetylsalicylic acid are also used as catalysts for the organosolv process together with solvents like methanol, acetone, ethanol, tetra-hydrofufuryl alcohol or triethylene glycol (27).

2.3.4 Combination pretreatment

On the foundation of well established pretreatment methods, researchers become focused on combination pretreatment methods which combine at least two methods to maximize the utilization of biomass by overcoming the disadvantages of the single methods (135).

Several combination pretreatments have been summarized by Charles E. Wyman (59). He listed five catalogs of examples including two or more physical pretreatments in sequence, two or more chemical pretreatment in sequence, physical pretreatment followed by chemical pretreatment, chemical pretreatment followed by physical pretreatment, chemical pretreatment followed by biological pretreatment (136). More detailed examples are listed in the following table (Table 2.5).

Although these combination methods have better conversion rates compare to single methods, they might require higher expenses because of the complexity of the pretreatment equipments. Since the improvement is not dramatic and a large amount of additional expense is needed for large scale combination methods, the single methods are still considered to be the most economic feasible option for biomass pretreatment.

Combinations	Examples	Pros	Cons	Ref
Physical pretreatments in sequences	Irradiation and mechanical crushing	Shorten reaction time, no need to neutralize, lower energy input	Higher cost	(137)
Physical pretreatment followed by chemical pretreatment	Ammonia fiber explosion	No inhibitor to hydrolysis microbes, completely lignin separation	Incompletely lignin separate, inhibitor to hydrolysis microbes, low yield, high cost	(119)
Chemical pretreatment followed by physical pretreatment	Acid Steam explosion	Higher hemicelluloses Removal. Better yield than AFEX, no inhibitor to hydrolysis microbes	High cost	(138, 139)
Chemical pretreatment followed by biological pretreatment			Inhibitor to hydrolysis microbes, low yield, high cost	(140)

Table 2.5 Combination pretreatment used

2.4 Hydrolysis techniques

Hydrolysis is the method by which glycosidic bonds are cleaved in lignocellulosic substrates. The hydrolysis conditions influence the recovery of neutral sugars (141). During hydrolysis the xylan hemicelluloses sould be completely hydrolyzed to D-xylose (50-70% w/w) and L-arabinose (5-15% w/w), and the cellulose sould be completely converted to glucose (142).

2.4.1 Acid hydrolysis

Feather and Harris found that hydrolyzed sugars are very stable in acid (143). Sulfuric acid has been used most frequently both in diluted or concentrated solutions for acidic hydrolysis. It is suitable for most species including wood chips, rice straw, sugar beet pulp (144), wheat straw (145) spruce chips (146), waste cellulose Saline crops, aspen, basswood, balsam, red maple, and switch grass (147) and so on. Other acids such as phosphoric acid and HCl have also been used.

For diluted acid hydrolysis, high pressure and high temperature are required. Extremely low concentrations of sulfuric acid (0.05-0.2 wt %) and high temperatures (200 -230°C) are proven to be effective for hydrolysis of biomass (148).

Concentrated acid hydrolysis proved to provide a complete and rapid conversion of cellulose to glucose and hemicellulose to five-carbon sugars with little degradation. Zhou used a two-step concentrated sulfuric acid process to hydrolyze wood chips (35°C, 80% sulfuric acid 120min for the first part, 30%, 95°C, 180 min for the second part) and(149) reached a yield of above 90% (150). The low temperatures and pressures employed will allow the use of relatively low cost materials such as fiberglass tanks and piping {{500 Yat, S.C. 2006}}. However, this process involves two reactions. First, the cellulosic material is converted to sugar which could be used for fermentation and as the reaction continues, a second reaction takes place. The sugars from the first reaction will convert into other chemicals such as furfural, which will inhibit microbial reaction.

2.4.2 Biological hydrolysis

Considered to be a more environmentally friendly and efficient hydrolysis method, biology technologies are also been used for the hydrolysis process. There are two technologies being developed for hydrolysis: enzymatic conversion and direct microbial conversion (151). Between these two methods, enzymatic conversion is the most common one.

Enzymatic hydrolysis of cellulose to glucose is usually catalyzed by cellulase (EC 3.2) and β-glucosidase (EC 3.2.1.21) enzymes. However, due to the highly crystalline structure of cellulose, the main problem of this reaction is the long reaction period.

In order to get over this issue, many revisions have been done. For example, Ionic liquid (IL) which contain 1-n-butyl-3-methylimidazolium chloride are used by Dadi et al to disrupt the cellulose structure (152); Yanpin Lu used an "enzyme–microbe synergy" which requires the presence of metabolically active cellulolytic microbes to increase hydrolysis efficiently (153).

2.4.3 Enzymes for hydrolysis of lignocellulosic materials

More than fifty years ago, Reese et al. postulated that the enzymatic hydrolysis consists of two steps (154). First of all, the active C_1 site is initiating the hydrolysis by opening up unspecified crystalline cellulose structures to produce soluble cello-oligosaccharides. This step provides a better access for C_x sites. Consequently, C_x will de-polymerize oligosaccharides – cellobiose to the glucose (155). In this original model, C_x is a cellobiase, however, the precise action of C_1

remains unknown.

With the later clarification of cellulose crystallinity structure, the *Trichoderma reesei* system has proved to be a better way to interpret cellulase actions than the C_1 - C_x system (156). *Trichoderma reesei* consists of three major components: endo-ß-glucanase (EC 3.2.1.4), exo-ß-glucanase (EC 3.2.1.91) and ß-glucosidase (EC 3.2.1.21). Each of these components consists of two domains which are the catalytic domains (CD) and cellulose binding domains (CBD) (119). These components of *Trichoderma reesei* are integrated with each other in an ordered way (Figure 2.9). Endo- ß -glucanase also called 1, 4-ß-D-glucan glucanohydrolase form a complex with cellulose and then breaks internal bonds to disrupt the crystalline structure of cellulose and expose individual cellulose polysaccharide chains. This reaction yields glucose as well as cello-oligo saccharides and releases plenty of reducing ends by random scission of cellulose chains. This is the rate-limiting step for the utilization of cellulose. The second step is random adsorption of exo- ß -glucanase to the cellulose surface (157). The exo- ß –glucanases' tunnel-shaped active sites attacks on the non-reducing end of amorphous cellulose fractions to hydrolyze them into D-glucose and D-cellobiose (158). At last, the small pieces of cellobiose are hydrolyzed by ß-glucosidase to glucose (159).

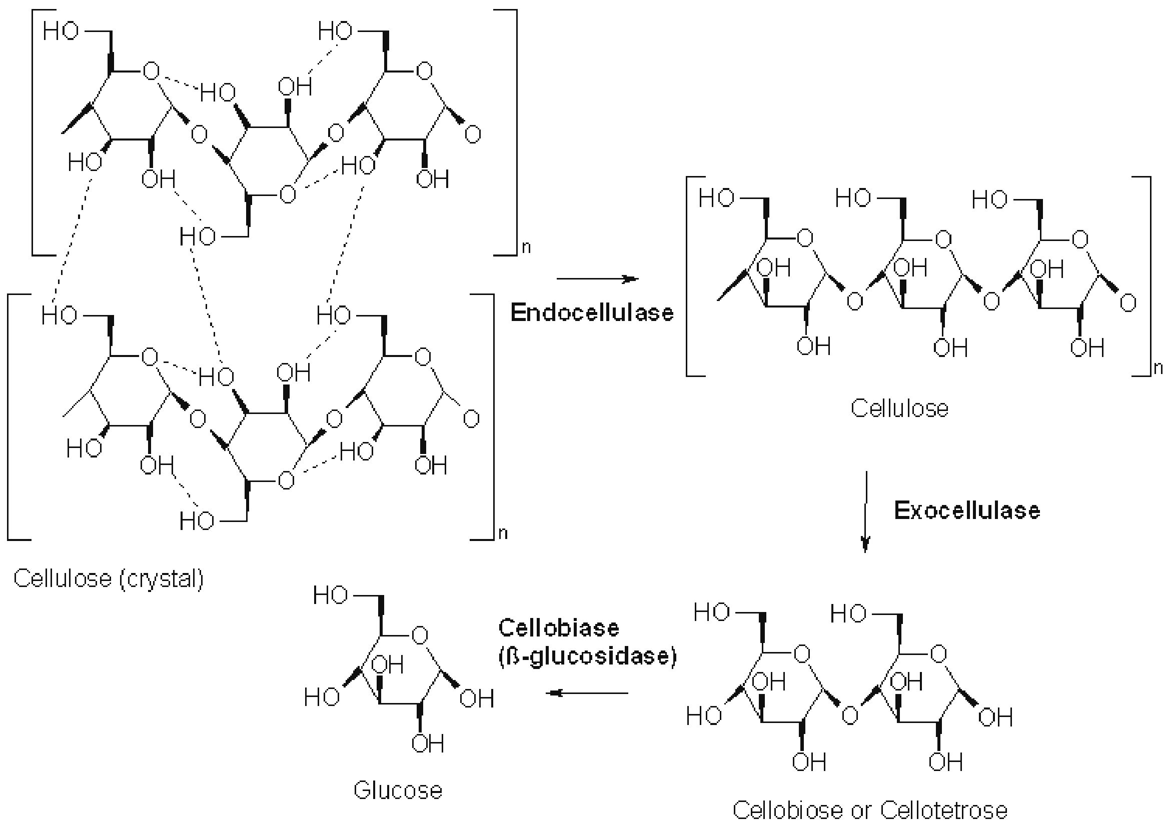


Figure 2.9 Hydrolysis reactions of Trichoderma reesei (112)

The adsorption of enzyme is considered to be a very important step (160). According to previous studies, the factors that will increase the availability of enzyme binding sites includes: increase surface area, decrease particle size of cellulose as well as removal of most of the hemicellulose (161). Application of a proper pretreatment method has a dramatic effect on modifying the enzyme adsorption step which will enhance enzymatic hydrolysis efficiency (161).

2.4.4 Common Barriers and Inhibitions for Hydrolysis

Many researches have proven that several structural and chemical factors will affect the enzymatic hydrolysis efficiency. The chemical barriers are the composition of cellulose, hemicellulose, lignin and acetyl groups connected to hemicelluose (162). The physical and structural barriers include cellulose crystallinity, surface features, particle size, moisture content of materials, cellulose degree of polymer (DP), and carbohydrate-lignin complex structures. These factors are correlated with each other, if one of them changes, other barriers will change as well. As a result, a better understanding of all these factors separately as well as the interaction of these factors would be helpful for the optimization of pretreatment in order to get a maximum biomass conversion rate.

2.4.4.1 Cellulose crystallinity

Natural cellulose contains crystalline regions (50%-90%) as well as amorphous regions (163). Crystalline areas of cellulose have been considered to play a critical role in inhibiting enzymatic hydrolysis (164). Pure crystalline forms of the cellulose were found to be more difficult to digest than the amorphous forms. This is because the crystalline regions are closely packed and have a strong supporting strength caused by internal and external hydrogen bonds (160). Extreme reduction of particle size or dramatic enlargement of the surface area will give more chance to form crystal barrier (165). However, crystallinity index is not correlated to sugar conversion rates for all species. For example, as pointed out by Han et al., the correlation between crystallinity index and release of fermentable sugars for rice straw and sugarcane bagasse are quite insignificant (161).

2.4.4.2 Surface feature

Cellulose is a heterogeneous porous substrate with both internal and external surfaces (166). Internal surface refers to the capillary structure of cellulose fiber while the external surface is the area of cellulosic particles (166). Increasing the internal or external surfaces has a direct correlation to the improvement of lignocelluolsic biomass degradation (167). Nevertheless, both internal and external surface accessibility are related to cellulose crystallinity and lignin presence. Decreasing crystallinity, particle size or degree of polymerization will lead to extension of the cellulose surface area for enzymes' binding sites (168). As a result, surface area is generally not considered to be an individual factor for promoting higher sugar yield (169). For example, different conversion rates caused by different surface areas might be because of the required surface size for the enzyme used, because of the biomass used in the experiments, or the sensitive difference among surface area measurement technologies rather than surface features themselves (170).

2.4.4.3 Moisture content of cellulose

It has been determined in previous studies that higher moisture content leads to better sugar conversion rates during enzymatic hydrolysis (160). With less water content in the biomass, capillary structure of cellulose collapse, degree of swelling decrease and hydrolysis rates goes down as a result (171).

2.4.4.4 DP value of the cellulose

During the synthesis of the cellulose molecules, their lengths vary over a wide range. These lengths are described by degree of polymerization (DP). It can be also considered as the number of glycosylic residues per cellulose chain (172). During the decrease of DP, amorphous ends have the tendency to recrystallize (6, 85, 136). Only if the DP drops to very low values, such as 10 or 15 units, the molecules will not be able to form new hydrogen bonds among each other, so that no new crystalline areas will be re-formed (6). As a result, rate of hydrolysis will be enhanced. However, since altering the DP usually is accompanied with the change of crystallinity and surface properties of the biomass, it is hard to separate out the correlation between DP and lignocellulosic biomass utilization efficiency exclusively.

2.4.4.5 Nature of association

Avgerinos and Wang reported that altered native cellulose-hemicellulose-lignin association would make the carbohydrates more accessible to be attacked by enzymes (173). Because lignin and hemicellulose are deposited in the space between amorphous regions of cellulose molecules, they will prevent chemical or biological agents from contacting sufficient number of glucosidic links in the cellulose to permit significant hydrolysis.

As the support material for biomass, lignin is the most recognized factor that slows down the enzymatic hydrolysis. Several reports have shown a positive correlation between enzyme accessibility and delignification (174). Lignin removal or proper lignin modification causes increase of internal surface area and median pore volume which will increase biomass digestibility (173). On the other hand, modification and lignin removal will release more cellulose that is being trapped within the lignin structure which will also improve the enzymatic hydrolysis efficiency (175). However, the issue for delignification is the high process cost. Thus, extensive delignification is not economically option in bio-ethanol production from woody biomass.

Hemicellulose, which is abundant in biomass, blocks the access of cellulase to the surface of cellulose (165). The extraction of hemicellulose in some of the initial preparation methods for biomass will significantly increase the cellulose digestibility (176).

2.4.4.6 Acetyl group

Acetyl groups on the xylan background will limit swellability of biomass and reduce its digestibility (177). The removal of acetyl groups or the lowering of acetyl group content would lead to better digestibility. Thus, acetyl group content for different raw materials are tested for illustrating the effects on pretreatment processes. However, removal of acetyl group would initiate the formation of acetic acid which lowers the pH and the cell activities for enzymes.

2.4.4.7 Inhibitor by-products

Some inhibitors will be produced during pretreatment or chemical hydrolysis procedure. For example, under oxidative condition, some glucose will be oxidized to glucuronoside, which is more resistant to hydrolysis. The mechanism of this inhibition is not clear yet.

During the presence of acid, both hydrolysis and condensation will happen under vigorous condition. A small amount of compounds like hydroxymethyl furfural (HMF) and furfural will be generated from cellulose and hemicellulose degradation (116) (Figure 2.16). The furan ring of these compounds is provides high resistance to chemicals and enzymes. As a result, the cell growth and respiration are negatively affected. Comparing these two, the HMF is generally less toxic and lower in concentration than furfural (178).

Furfural Hydroxymethyl furfural

Figure 2.10 Structure of furfural and hydroxymethyl furfural

Other product inhibitors are released through lignin degradation. For example, phenolic compounds, especially low molecule weight phenolic compounds, are considered to be a severe issue in this process. These compounds are considered to be more toxic than furfural; they will lower the cell growth and sugar assimilation of the microorganisms by causing the cell membrane integrity loss (179). This degradation only occurs at high temperatures (over 180 ºC) or at the existence of strong acid and base.

2.4.4.8 Extractives

Extractives are containing resin, tannin, and etc. They are a group of inhibitors with lower toxicity than furfural (178). Since they are naturally occurring compounds, it is hard to prevent their formation. The only way to deal with it is to remove or reduce the extractives before hydrolysis and fermentation.

2.4.4.9 Heavy metal ions

Heavy metal ions caused by equipment corrosion is another issue for hydrolysis. Although the existence of certain ions is beneficial to microbes due to their nutrient requirements, for other organisms or enzymes, these compounds are toxic to the metabolism.

2.5 Fermentation process

Traditionally, lignocellulosic materials can be first converted to fermentable sugars consisting of mainly glucose, followed by fermentation to ethanol by using special yeasts. This method is called separate hydrolysis and fermentation (SHF) (170). In order to simplify the process and increase the conversion rate sugar to fuel, simultaneous saccharification and fermentation (SSF), which is hydrolysis and fermentation of pretreated biomass at the same time (170, 180) has been developed.

For the SHF method, yeast is used for ethanol and CO_2 productions in the absence of oxygen. Large amount of CO_2 are collected with scrubbers, and used in other industries, for example carbonating beverage productions. The main product - ethanol has been distilled from solutions containing yeast cells and biomass residues to reach a higher concentration (around 96%). The residues from distillation are used for livestock feed due to high protein content (181). This traditional yeast fermentation can be used as a standard method to show the pretreatment effects on hydrolysis processes for future research.

2.5.1 Fermentation Micro-organisms

2.5.1.1 Bacteria

Many bacteria can perform ethanol formation by Embden-Meyerhof pathway (i.e. *Enterobacteriaceas, Spirochaeta, Bacteroides*, etc.) (182) and Entner-Doudoroff pathway (i.e. *Zymomonas,* etc.) (183). Besides ethanol as the major product, certain species also produce other alcohols (butanol, isopropylalcohol, 2,3-butanediol), organic acid (acetic acid, formic acid, and lactic acids), polyols (arabitol, glycerol and xylitol), ketones (acetone) and gases (methane, carbon dioxide, hydrogen) as by-products (85). Due to their rapid growth rate, the bacterial ethanol-production is conventionally used in commercial production.

Microbial species for lignocellulosic biomass degradation fall into several main genera: *Bacillusm, Clostridium, Thermoanaerobacter*, *Zymomona, Neocallimastrix, Piromonas, and Sphaeromonas* (14). These microbes can be further divided into two groups, aerobic and anaerobic, by the different cellulose degradation mechanisms. For aerobic microbes, the cellulose degradation is involving the non-complexed cellulase that secrets and releases into the environment (184). But for anaerobic microbes, the degradation process is utilizing the membrane bonded cellulase complex called cellulosomes (185-187). By combining various enzymes in a complex, the degradation loss of intermediates have been dramatically reduced. Thus, the anaerobic microbes proved to be highly efficient degradation agent compared to the aerobes. Among these anaerobic species, *Clostridium* sp. stands out due to their excellent polysaccharides degradation abilities. Detailed applications are summarized in Table 2.6.

A combination of *C. thermocellum* and *C. Thermolacticum* has been chosen in this project due to the following unique growth conditions and efficient degradation capacities: **a)** This combination can degrade crystalline cellulose which is considered to be one of the barriers for biomass enzyme hydrolysis (14). **b)** This combination can utilize the majority of lignocellulosic biomass efficiently. *C. thermocellum* can efficiently ferment hexoses and *C. Thermolacticum* can proficiently ferment pentoses

(13, 14). **c)** It diminishes the substrates competitions of the co-culture because two strains are proficient in using different substrates. **d)** Both of these microbes require growth at around 60 °C. At this temperature, some of the contaminations can be omitted and a smaller temperature increase is needed for further ethanol recovery (13, 14). **e)** Fermentation at the anaerobic condition will reduce the expensive oxygen transfer step (206). Detailed information of these two microbes will be covered later in this review.

Genus	Species	Hexose/ Cellulose	Pentose/ Hemicellulose	Ref.
Bacillus	*Bacillus macerans*		√	(188)
Clostridium	*Clostridium autoethanogenum*		√	(189)
Clostridium	*Clostridium carboxidivorans*	√	√	(190)
Clostridium	*Clostridium acetobutylicum*	√	√	(191)
Clostridium	*Clostridium thermocellum*	√		(14)
Clostridium	*Clostridium thermohydrosulfuricum*	√		(192)
Clostridium	*Clostridium thermosaccharolyticum*	√		(193)
Clostridium	*Clostridium beijerinckii*	√	√	(194)
Clostridium	*Clostridium stercorarium*	√	√	(195)
Clostridium	*Clostridium aurantibutyricum*	√	√	(196)
Enterobacteriaceae	*Erwinia amylovora*	√	√	(197)
Enterobacteriaceae	*Leuconostoc mesenteroides*	√	√	(198)
Enterobacteriaceae	*Sarcina ventriculi*	√		(199)
Enterobacteriaceae	*Streptococcus lactis*	√		(200)
Enterobacteriaceae	*Spirochaeta aurantia*	√		(201)
Enterobacteriaceae	*Spirochaeta stenostrepta*	√		(202)
Enterobacteriaceae	*Spirochaeta litoralis*	√		(203)
Thermoanaerobacter	*Thermoanaerobacter mathranii*	√	√	(204)
Zymomonas	*Zymomonas mobilis*	√		(205)

Table 2.6 Performance of lignocelluosic biomass fermenting bacteria

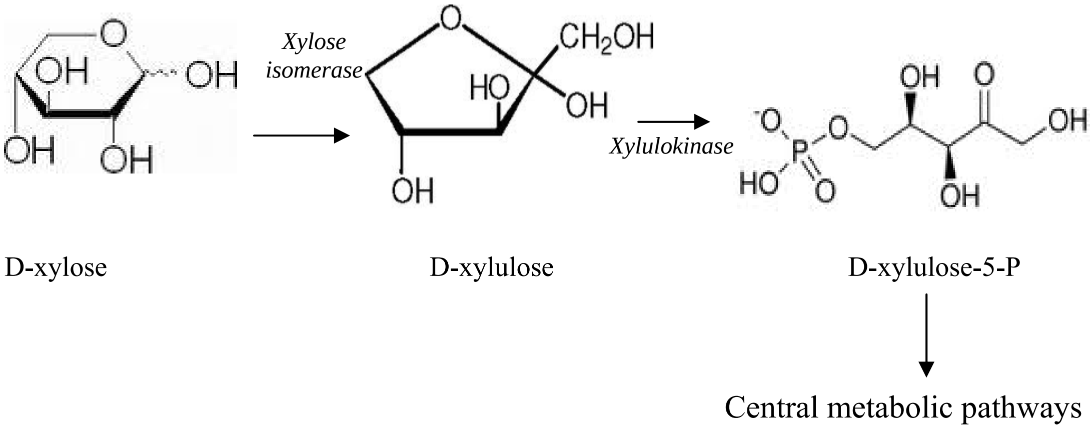


Figure 2.11 Bacteria xylose ulilization

2.5.1.1.1 *C. thermolacticum* (ATCC 43739)

Thermophilic anaerobic bacteria, *C. Thermolacticum,* can ferment a wide range of substrates to produce acetate, hydrogen, or ethanol at optimum growth conditions: 60-65 °C under pH=6.0-7.8 (207). These substrates include natural materials like lignocelluosic biomass or paper pulp sludge, and pure saccharides such as glucose, cellobiose, or galactose (16, 208). For centuries, this organism has been broadly studied in hydrogen production from milk permeate or kitchen waste (16, 31, 209-211), in acetic acid production from lactose (209), and in the production of xylanase that can tolerate high temperatures (80 °C) and a wide pH range (pH=3-11) (212, 213)

This strain also has potential in ethanol production since changing the growth conditions will shift the *Clostridium thermolacticum*'s metabolism pathway to produce a significant amount of ethanol (16, 214). Fardeau et al. found that *C. thermolacticum* has the ability to produce ethanol (13). Similar observations also were found in the preliminary test of my project. The preliminary results show the cellulolytic ability of ATCC 43739 since this strain can produce ethanol when growing it in a medium with various carbon sources. However, unlike my own preliminary results, Le Ruyet et al. noted that no cellulose is degraded and ethanol is only observed as a minor product (215). These differences are expected because cultivation conditions and end-product concentrations will all shift the metabolism path of this organism (13, 216).

2.5.1.1.2 *C. thermocellum* (ATCC 27405)

C．thermocellum is one of the most powerful cellulose degraders (217, 217). Cellulosomes possessed by *C．thermocellum* lead to itsexcellent cellulose digestion ability (185-187). We found that highly concentrated *C. thermocellum* can completely digest 0.5 g/L of No.1 Whatman filter paper within 5 days (data not shown).

The cellulosomes are mainly responsible for cellulose saccharification, particularly crystalline cellulose saccharification. These complexes, which are18nm in diameter, range from 200kDa to 600kDa (218). A typical cellulosome in *C. thermocellum* mainly consists of scaffolding subunits, dockerins (70 residues), cohesins (140 residues), and a carbohydrate binding module (CBM) (185). The scaffolding subunits are the backbone of the whole cellulosome complex. Dockerins, cohesins, and CBM bind to this backbone to promote the adherence of *C. thermocellum* to cellulose and catalyze the hydrolysis of both amorphous and crystalline cellulose (219).

In addition to cellulosome various other enzymes secreted by *C. thermocellum* also contribute to its efficient biomass degradation ability. *C. thermocellum* can produce cellobiose phosphorylase and cellodextrin phosphorylase to initiate the cleavage of glucans into cellodextrins (cello-oligosaccharide) (220, 221) which will also provide extra ATP for cellulase synthesis (222). *C. thermocellum* also expresses enzymes containing dehydrogenase, galactouronase and pectin lyase (220, 221). Thus, *C. thermocellum* exhibits the ability to produce ethanol, acetic acid and lactic acid from lignocellulosic biomass through the Embden-Meyerhof Glycolysis Pathway (220, 223).

C. thermocellum has been used to investigate the use of direct cellulosic biomass fermentation since 1978 (196, 224). For instance, a high ethanol amount (0.92 g/l) was obtained from the cellulose culture containing 10 g urea/l by wild type ATCC 27405 (225). This strain has also been used to ferment paper pulp sludge, sugar cane bagasse, wheat bran, and corn stover in solid substrate cultivations. High ethanol concentration was also observed in paper pulp sludge fermentation (208).

The issue for *C. thermocellum*, however, is that xylanases synthesis. *C. thermocellum* cellulosomes dose not has the capacity to hydrolyze pentose, because no β-xylosidase activity, which is critical for xylan metabolism, is observed (226). Instead, these xylanases are responsible for opening the access for cellulose degradation enzymes (226). The inability of this organism to completely ferment hemicellulose limits its utility in lignocellulosic biomass de-polymerization. We hypothesize that the co-culture of *C. thermocellum* and *C. thermolacticum* organisms would widen the substrate range and increase the productivity (16).

2.5.1.1.3 *Ethanol Sensitivity in C. thermocellum and C. thermolacticum*

Limited data are available on applying the wild type cultures of *C. thermocellum and*

C. thermolacticum in bio-ethanol production. To date, these wild type strains have been depressed by one of the major products - ethanol (16). For *C. thermolacticum*, as reported in Talabardon et al., when ethanol production reaches less than 2% v/v, the cell growth of *C. thermolacticum* will completely stop and inhibit further ethanol production (16). For *C. thermocellum*, an ethanol concentration of 3% v/v (207, 227) is high enough to inhibit the cell growth, due to the irreversible cell structural changes caused by the existence of ethanol.

Research to date has tended to explore the area of microbes modifications. Although those modifications like direct mutations have been performed to upgrade their tolerances and to increase their enzyme activities, the stability information of these mutated strains is lacking. In addition, those mutated strains are designed only for one specific method, so that they are less valuable when used for other applications. Thus, a systematic improvement - immobilization, could be a better approach to protect cells against the ethanol inhibitions than changing the microbes themselves.

2.5.1.1.4 ***Lack of Proper Regulations in C. thermocellum and C. thermolacticum***

Very little is known about proper regulations of fermentation which favor ethanol production, although numerous factors including pH, substrates and end-products concentration, nutrients and gaseous products have shown the impacts on the cell growth and final ethanol and acetate yield of these strains (16, 31). In this study, pH and substrates are the two major factors investigated.

The pH of a medium is the first factor studied since the influence of the pH value on biomass conversion and ethanol production is inconsistent in pervious studies. Take *C. thermocellum* for instance, fermentation performed at pH=7.2 is reported to increase the ethanol yield but to decrease the production of lactate or acetate (15, 16). However, a conflicting finding claims that ATCC 27405 grew better when the pH was between 7 and 10 (229). Another study demonstrated PH=10 to be the optimum pH for ethanol production and pH=7 for glucose accumulation (229). This inconsistency suggests that the effect of the pH value on ethanol yield may be associated with other fermentation parameters. Thus, the correlation between the pH value and ethanol inhibition will be examined in this study. Through a set of experiments we will investigate the pH value, ethanol concentration, and their interaction on ethanol yield or biomass conversion for the strains used.

The second factor that influences the cell growth is the type and concentration of the substrates and end-products. Generally, *C. thermocellum* is a long cellolextrin degrader. The conversion rate on β-1, 3-glucan, β-1, 4-glucan or longer cellolextrins (DP > 4) is almost 2-5 times that of the degradation of sugars such as glucose and fructose (230, 231), although the effect of substrates is different among *C. thermocellum* strains (232). Certain substrates or end-products like glucose (233) are not only unfavorable for this strain, but also inhibit or depress metabolism (234). Three reasons are involved in this situation: 1) utilizing longer cellolextrins will provide more ATP per glucose during phosphrolytic cleavage (230, 235-237); 2) The existence of insoluble substrates might trigger the cellulase activity (230, 235-237); and 3) If cellobiose is compared to longer cellolextrins, cellobiose will cause the substrate competition between B-glucosidase and phosphorylase to decrease the enzyme activities (233). For example, the amount of cellulase synthesized by *C.*

thermocellum is higher when using avicel as a substrate rather than cellobiose (233, 237). For *C. thermolacticum*, although most saccharides can be degraded by this strain, the amount of ethanol or acetate produced varies according to the substrates as shown in our preliminary study.

The substrate or end-product amounts also play a critical role for the strains, particularly for *C. thermocellum*. A lower initial substrate amount and a lower accumulated sugar concentration are favorable for ethanol production and the total conversion rate (238). For example, *C. thermocellum* can only grow within 0.2%-5% of cellobiose (217, 239). Higher cellobiose does not lead to a higher ethanol yield (240). In other words, for *C. thermocellum* a limited carbon source does not appear to be a limitation of production yield, but excess carbon does (217, 241).

To improve the ethanol yield of both strains, analysis of the effects of the pH and the substrates on the fermentation process will be done in this project. Detailed information revealing the factors that are favorable for the ethanol-production of these two strains will provide various strategies for a high efficient fermentation process.

2.5.1.1.5 *Co-culture Fermentation*

Co-culture fermentation proved to be more efficient than mono-culture fermentation in previous studies because of the different enzymes produced by different microbes, so the scope of substrate utilization will be widening using proper combinations. In this research, *C. thermocellum* is a strain that can produce endoglucanase and xylanase. However, a large amount of sugar remains unutilized, particularly the pentose. As a result, *C. thermolacticum* which can ferment pentose is applied in this research to utilize the remaining sugars produced by *C. thermocellum*.

In previous papers, co-culture fermentation with one of these two microbes, particularly *C. thermocellum* can be divided into two groups. The first group is the sequential co-culture fermentation in which fermentation is performed by various microbes one after another (242). In this case, two microbes usually require different growth conditions. Available examples contain the co-culture of *C. thermocellum* and *C. acetobutylicum* ATCC824 (242), *C. thermosaccharolyticm* HG-2 (231), *Aectogenium kivui* LKT-1 or *C. paraputrificum* (243).

The other group of co-culture fermentation is simultaneous co-culture fermentation. This category is considered to be a better process than sequential fermentation due to its lower capital cost. Thus, this process is also the one that will be used in this project. Microbes chosen in our study require similar or the same growth conditions. Previous co-cultures of *C. thermocellum* were performed by applying *C. thermohydrosulfuricum* 39E (240), *Zymomonas mobilis* (244), *Zymomonas anaerobia* (245), *C. thermohydrosulfuricum* (246, 247) or even various *C. thermocellum* strains (9). Similar to simultaneous *C. thermocellum/C. thermolacticum* co-culture fermentation, the theory applied in order to achieve complete utilization of biomass by the co-culture of *C. thermocellum* S-7 and *C. thermosaccharolyticum* HG-4 is to ferment pentose by *C. thermosaccharolyticum* and ferment hexose through *C. thermocellum* (216). For this co-culture fermentation on corn stover, ethanol yield reached 9.7 g/l which is higher than mono-culture fermentation (216). Nevertheless, in that study, the ethanol adapted strain S-7 was applied which made it possible for this method to produce a high ethanol yield. If the wild type *C. thermocellum* were used, the improvement is expected to be less dramatic. For using wild type *C. thermocellum* in co-culture development, the co-culture of *C. thermocellum* ATCC 27405 and *Thermoanaerobacter sp.* stain B6A was studied (248). This co-culture was applied on woody biomass anaerobically; 1.32g/l of ethanol was produced by fermenting white birch (248). This yield is about 1g/l higher than the fermentation performed by *C. thermocellum* ATCC 27405 (248). However, similar to other co-cultures, using wild type ATCC 27405, low ethanol production yield is to low to be feasible for industrial production.

Although the previous co-culture improved the ethanol yield compared to their mono-cultures, issues including ethanol inhibition remain unsolved for wild type microbes. In addition, no co-culture containing wild type *C. thermolacticum* or *C. thermocellum* is reported to be capable of tolerating high ethanol concentrations and leading to a high ethanol yield. In this case, technological improvement of wide type *C. thermolacticum*/*C. thermocellum* co-culture or their mono-cultures is required to further improve the fermentation efficiency

2.5.1.1.6 Modification for Strain Improvement

Although some microbes are able to ferment lignocellulosic biomass and produce ethanol efficiently, the industrial production processes are not optimized for most cases (249). As a result, strain improvement techniques are necessary. Improving strains by mutations are an old but successful process in the lab.

Mutagenesis by applying UV light (250, 251), ethyl-methane-sulfonate (EMS) (252), fluoropyruvate (253) or by other mutation agents, will result in bacteria strains able to produce higher concentration of ethanol and being much more tolerant to the end-products (254, 255). Similar to mutagenesis, directed evolution, which involved selecting and expending the organisms with the greatest expression of the desired traits is the other classic way of improving the strains (256). For example, if increasing level of ethanol are applied during *C. thermocellum* culturing stains with increased ethanol tolerance are selected. After this strain improvement, the mutant strains have been investigated for increased ethanol tolerance, ethanol yield and catabolite selectivity (216, 228). However, the issue with these traditional methods is that the strains with new traits formed at the laboratory level are quite instable after fermentation reactions (257). The distinguishing benefits might get lost.

Protoplast fusion is another conventional technique for strains enhancement. Fusing two strains lead to genome restoration which results in higher frequencies of genetic recombination (257).

Currently, molecular based genetic and protein engineering seems to be more appealing to researchers. DNA transformation by using plasmids, like pIMK1 plasmid (258), or knocking out the genes involving the by-product formation by ClosTron (259), are both promising ways of improving bio-ethanol conversion with *Clostridium* sp. For example, in order to efficiently utilize the xylose by S.cerevisiae, expression of xylose reductase (XYL1) and xylitol dehydrogenase

(XYL2), together with the over expression of xylulokinase (XYL3) will lead to a 84% theoretical yield on 1:1 glucose/xylose mixture (260). Also, *E.coli* has been used by several laborateries as a host for genetic manipulation for bio-ethanol productions. When pyruvate decarboxylase (*Pdc*) and the alcohol dehydrogenase genes (*adhB*) in Z.mobilis got expressed in *E.coli*, nearly maximum theoretical ethanol yield was achieved from sugar mixtures (261). The scaffolding protein in *C. thermocellum* (Cip A) family 3 Carbohydrate-binding modules (CBMs) is also fused to antimicrobial peptides (AMPs) and expressed in E.coli for the directing the binding of the AMPs (262).

2.5.1.2 Fungi

2.5.1.2.1 Yeast

Saccharomyces uvarum (263), *Schizosaccharomyces pombe* (264), and *Kluyueromyces sp.* (265), *Pachysolen tannophilus, Candida Shehatae, Pichia stipitis,* and especially *Saccharomyces cerevisiae,* are the major yeast species currently used for industrial ethanol fermentation processes (266). For yeast-mediated ethanol production, small amount of oxygen (0.05 – 0.10 mm Hg) is reqired during the Embden-Meyerhof pathway. Besides the oxygen, nutrients are also required as other microbial bio-fuel process to maintain the cell-related ractions (267).

Saccharomyces cerevisiae is budding yeast that can tolerate up to 20% v/v ethanol during fermentation which makes this strain appealing to ethanol production industry (268). In addition, better than bacterial fermentations, yeast fermentations don't usually result in various by-products such as acetic acid (Figure 2.10). However, this strain only utilizes hexoses, and no sufficient crystalline cellulose degradation has been reported for this strain (269). The other drawback of using yeast in bio-ethanol production is the acid sensitivity of yeasts. Acidic hydrolysates, even diluted hydrolysates, are reported to inhibit the fermentation process (270). Thus, modifications for gaining xylose-utilizing and arabinose-utilizing functions and higher acid tolerance are developed (271).

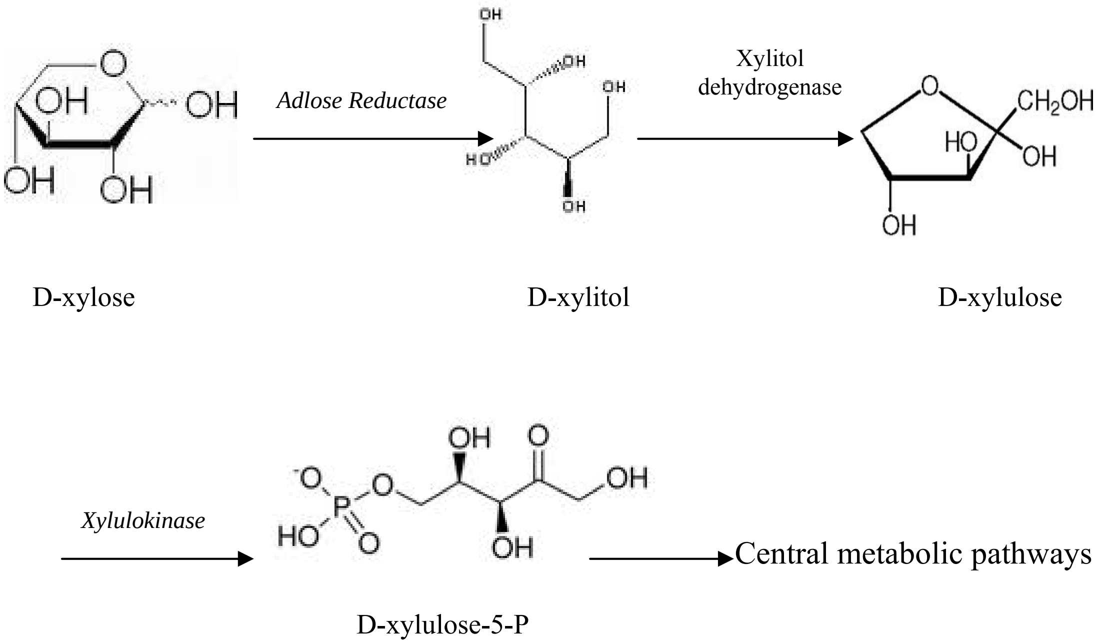


Figure 2.12 Yeast xylose utilization

2.5.1.2.2 Mold

Aspergillus niger is a soil dwelling black mold that has been used in citric acid production (272) and cellulases and hemicellulases productions (273). Co-culture of *A.niger* and *S.cerevisiae* are reported to have 96% of the theoretical ethanol yield on potato starch fermentation (274).

2.5.2 Consolidated Bioprocess

CBP, also known as direct microbial conversion (DMC), is a process combining cellulase production, hydrolysis and fermentation. The establishment of this process is a potential way to lower the capital cost and optimize the bio-conversion process of SSCF (Figure 2.13) (5, 275). For example, using *C. thermocellum* on diluted acid pretreated hardwood will provided a higher conversion yield compared to SSF (241). The DOE also applied both modified *C. thermocellum* and *Thermoanaerobacterium saccharolyticum* strains to ferment both hexoses and pentoses in CBP for ethanol production (276).

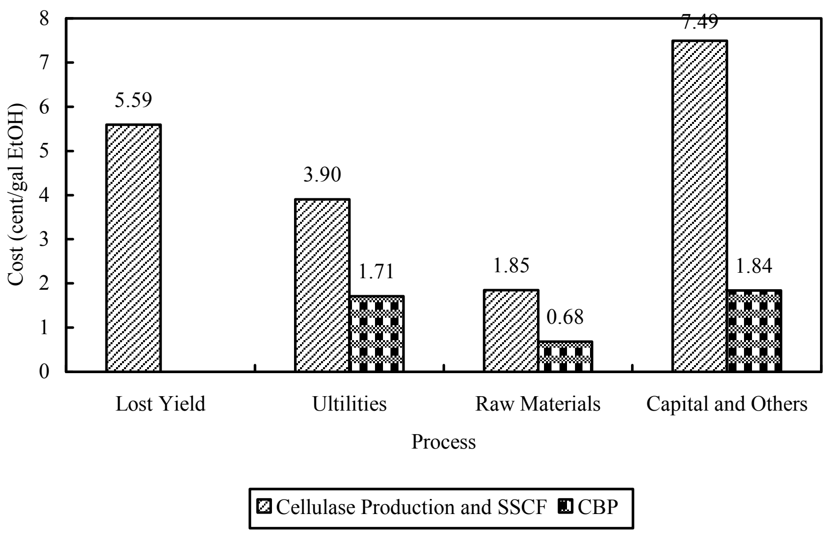


Figure 2.13 Ethanol Production Cost Comparison between SSCF and CBP (5)

As a relatively immature technology, however, CBP has unavoidable pitfalls mainly due to the operation process. In the CBP, microorganisms that produce the enzymes will be treated as living microbial systems rather than enzymes, since the cellulase production is inside the reaction vessel. This adds up to more technical difficulties for process engineering and also requires some more specified traits of the microorganisms themselves (4). The other issues for CBP is, even if some bacteria and fungus lead to better yields than SSCF, this process requires a much longer reaction time, and produces numerous by-products with low value (277).

To improve this situation, some researchers have focused on microorganisms engineering or metabolism engineering to facilitate the enzymatic expression of microorganisms (278). Others are working mainly on process engineering methods to optimize the microorganisms' working conditions (279). With all these modifications that have been made for the CBP, it has become a potential alternative pathway with many excellent properties compared to other methods to manufacture bio-energy although more improvements are needed.

This study is mainly focused on process development of *Clostridium* sp. CBP fermentation by applying cell immobilization. Thus, I will examine a fundamental understanding of the fermentation process including material balance and the effect of fermentation parameters on the final product yield.

2.5.3 Immobilization

The two strains chosen for this study are sensitive to ethanol (280). One to three percent of ethanol will depress or inhibit the fermentation process (280). Optimizing the fermentation temperature makes the cells even more sensitive to ethanol (281). As a result, DNA transformation (282), protein engineering (283), and direct metabolism engineering (185) has been performed for these strains in an attempt to upgrade its tolerance and to increase its enzyme activity.

Whole cell immobilization is another potential solution. Cell immobilization is achieved by entrapping the cells in polymers or a porous surface, attaching the cells on a surface or behind a barrier, or self-aggregating so that the cells can be reused several times and prevent poisoning (284, 285) (Figure 2.14). For *Clostridium* sp., entrapment of cells in a porous matrix is the most common method (286). Horne and Hsu trapped *C. thermocellum* ATCC27405 on bituminous coal for cellobiose fermentation (286). Fix-bed bioreactors packed with polyurethane foam as the support matrix can be applied for *C. tyrobutyricum* JM1 for an efficient and stable hydrogen production system (287). However, weak entrapment strength, small growth space and leakage usually limit the final immobilized cell concentration (288). Therefore, encapsulation, a modified version of entrapment, will be used in this project to ensure the final cell loading.

Encapsulation is a technique used with composite membranes to protect sensitive microorganisms from being poisoned (12). Existing capsulation methods have been summarized in Park & Chang's paper (288). Generally, this technique is accomplished by incorporating the active ingredients in the matrix, emulsifying the mixture into microcapsules, and then stabilizing these microcapsules (288). A formed microcapsule has a liquid core, in which *Clostridium* sp. cells are grown, surrounded by a spherical polymeric membrane. Compared to the entrapment, this method enhanced the stability of the membrane which provides the cells with sufficient protection. Smaller droplets will improve the mass transport of the cell, increase the cell loadings and enhance the control of the working parameters.

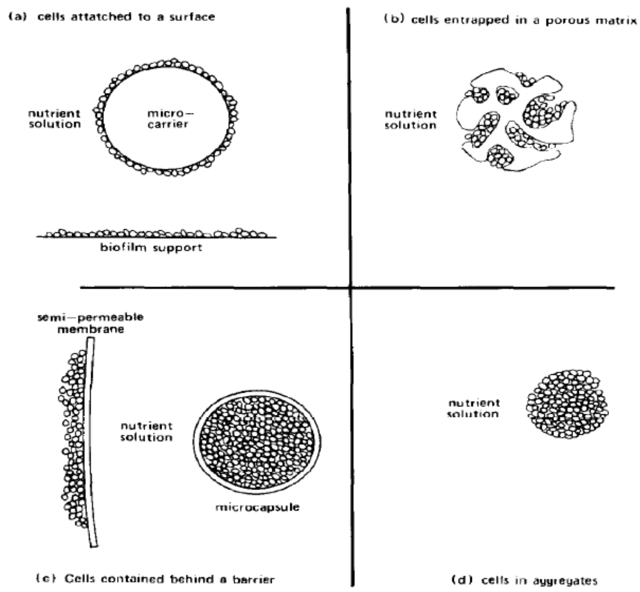


Figure 2.14 Immobilization of cells (285)

Chapter 3

MATERIALS AND METHODS

3.1 Microbial Species and Media

Both the *C. thermocellum* (ATCC 27405) and *C. thermolacticum* (ATCC 43739) used for this study were obtained from the American Type Culture Collection (ATCC). Media was prepared as described by He et al. (2009). The basic media was used for both strains unless otherwise noted. All media were prepared using standard anaerobic techniques under controlled conditions (80%N_2/20%CO_2). The redox potential decrease in media was tested using resazurine which changes from reddish pink to light yellow if reduced.

Cultures were maintained in standard media containing either a paper strip (Whatman No.1) or sucrose (Sigma-Aldrich). The cultures were stored in the refrigerator and were subcultured once every month. The purity of the microbes was regularly checked under the microscope.

3.2 Substrate

Sterilized anaerobic fermentation substrate solutions containing glucose (Sigma-Aldrich), xylose (Sigma-Aldrich), cellobiose (Sigma-Aldrich), microcrystal cellulose (Sigma-Aldrich), or hemicellulose (Kaufert Lab, University of Minnesota, Saint Paul, MN) were added after autoclaving the medium.

3.3 Growth of Organisms

A low initial substrate/medium ratio (1% w/v) was used and a 5 mL homogeneous microbe culture with a cell weight of 5 g/L was transferred into 100 mL serum bottles containing 50 mL of medium prepared under anaerobic conditions unless otherwise noted. For the co-culture, the inoculation was performed by adding 2.5 mL of 5 g/L *C. thermocellum* and 2.5 mL of 5 g/L *C. thermolacticum* per 50 mL prepared medium. Cultures incubated for 10 days at 57 °C without shaking were analyzed for the end-products (ethanol and acetate). Fermentations on each substrate without adjusting the pH and without adding exogenous ethanol were used as controls and mixtures with only medium and substrates were used as blanks. Triplicates were conducted for each treatment.

3.4 Determination of Cell Mass

The turbidities of the cultures were determined with a spectrophotometer (Sequoia-Turner Model 340) at 575 nm using a 13mm path length test tube. The dry cell density was determined by the relationship between dry cell weight and opacity density (O.D.). Based on this test (original data not shown), the relationships were found to be:

Clostridium thermolacticum →1 unit of O.D. change = 1.1 g dry cell/L

Clostridium thermocellum →1 unit of O.D. change = 0.5 g dry cell/L

3.5 Biomass Characterization

A characterization of aspen wood powder was done to provide fundamental information about the structure of the substrates for this project. The substrates, one of the major factors, during CBP fermentation for this project can be determined according to a standard characterization test (289). Aspen wood chips were commercial chips received from the SAPPI Mill in Cloquet, MN. Wood chips were air dried and ground using a Thomas-Wiley Mill. The fractions pass through an 80-mesh screen to ensure uniform surface area. Prepared materials are stored in sealed serum bottle for further use. The composition of biomass was determined according to the NREL standard method (289).

3.6 Encapsulation

The encapsulation process was performed according to Klein's method (290, 291). 2.5% (w/v) microbe cells were mixed with calcium chloride in a beaker and then the cell mixture was dropped into 1 L 0.25 M sodium alginate solutions through a 23 gauge syringe needle (3 mm) at the rate of 1.5 drop/sec (292). Calcium alginate capsules were formed immediately by ionic interaction. After 5 minutes the gelatin pellets were removed and washed. During this project, concentration of alginate, calcium and other gel-forming polymers were adjusted if the pore size, wall thickness and mechanical strength needed to be changed. The entire encapsulation process was conducted under anaerobic and sterile conditions.

3.7 Lignin preparation (Organosolv pulping)

The organosolv pulping was performed in a 2 L Parr Bench Top Reactor (Series 4520). In each batch, 100 g aspen was pulped with an ethanol and water mixture. The ratio of ethanol and water was 6:4. The ratio of liquor to aspen was 13:1. 0.1% of MgSO4 was added to the liquor to promote lignin removal. After the aspen chips were placed inside the reactor, the ethanol/water mixture was added, and the lid of the reactor was sealed. Then, the temperature of the reactor was increased to and maintained at 190^{o}C for three hours. Afterward, the reactor vessel was cooled down to room temperature. The reactor was opened and the aspen chips drained in a Buchner funnel. The remaining material was rinsed with the same ratio ethanol/water mixture. Bundles of the fiber were separated in a British disintegrator. The separated mixture and washing liquid was collected, ethanol was removed by storing the liquid in a chemical hood for two days allowing the participation the organosolv lignin. Participated organosolv lignin was filtered through a British disintegrator and air dried.

3.8 Analytical Methods

End products (ethanol and acetate yield) in the water phase were determined using a Waters high performance liquid chromatograph unit (HPLC) with a Bio-Rad Aminex HPX-87H column with a de-ashing BIO-RAD Micro-guard refill cartridge filter. The column temperature was set to 30 °C and the detector temperature was set to 50 °C. Waters 2414 refractive index detector and Waters 2478 dual λ absorbance detector are used. Distilled water was used as the mobile phase delivered at the flow rate of 0.5 mL/min, with a sample injection volume of 5 μL. Calibration curves were generated using pure ethanol and acetate (Sigma-Aldrich) and the culture medium. Percentages of yields for final products were calculated in relation to original saccharides addition.

Weights of the samples (oven dried) were recorded as the inputs of the samples. After fermentation under certain conditions, the fermented wood samples were weighed (oven dried) again as output weights. The yield of each sample was calculated as:

$$\text{Yield (\%)} = (\text{input weight} / \text{output weight}) \times 100\%.$$

3.9 Calculation of theoretical ethanol yield

Based on hydrolysis and fermentation reaction equations of pentose:

$$(C_5H_8O_4)_n + nH_2O \rightarrow n\ C_5H_{10}O_5$$

$$3\ C_5H_{10}O_5 \rightarrow 5\ C_2H_5OH + 5\ CO_2$$

$$3\ C_5H_{10}O_5 \rightarrow 4\ C_2H_5OH + 7\ CO_2$$

The theoretical ethanol yields of pentoses were calculated as (10):

% minimum theoretical yield = % of xylan x 1.136 x 0.40 (g ethanol/ g biomass)

% maximum theoretical yield= % of xylan x 1.136 x 0.51 (g ethanol/ g biomass)

% maximum theoretical yield= % of xylose x 0.51 (g ethanol/ g biomass)

Based on hydrolysis and fermentation reaction equations of hexoses:

$$(C_6H_{10}O_5)_n + nH_2O \rightarrow n\ C_6H_{12}O_6$$

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$$

The theoretical ethanol yield of hexose was calculated as (10):

% maximum theoretical yield = % of cellulose x 1.11 x 0.51 (g ethanol/ g biomass)

% maximum theoretical yield= % of glucose x 0.51 (g ethanol/ g biomass)

Chapter 4

RESULTS AND DISSCUSSION

4.1 Preliminary test for parameters determination

The preliminary study was performed before the main research to gain a fundamental knowledge of the strains and substrates used. Although other investigators have reported the effect of pH on the ethanol yield for these strains using several substrates, the correlations changes caused by the chosen medium and culture conditions are barely studied. The other focus of these preliminary experiments was to determine the pH range and substrate types for this study based on the culture collections obtained and cultivation condition applied.

4.1.1 Biomass Characterization and Fermentation of various Substrates

The chemical composition of the prepared aspen sample is shown in Figure 4.1. In this figure, the glucan mainly originates from cellulose degradation and xylan from hemicellulose degradation. As shown in the figure, glucan and xylan comprise over 60% of the aspen which indicates that cellulose and hemicellulose are the major components within the aspen sample. Since the ethanol production in lignocellulosic microbial fermentation in this study is based on conversion of saccharides, the degradation of the major sacchrides is most critical. Thus, in order to analyze the effect of major fermentation parameters, such as pH and ethanol or substrate concentration on the process, these effects were studied using cellulose, hemicellulose and their mono- or di-saccharides intermediates. The major intermediates include glucose, cellobiose, and xylose. The effect of minor sacchrides like arabinan, mannan and galactan, however, were not considered in this study.

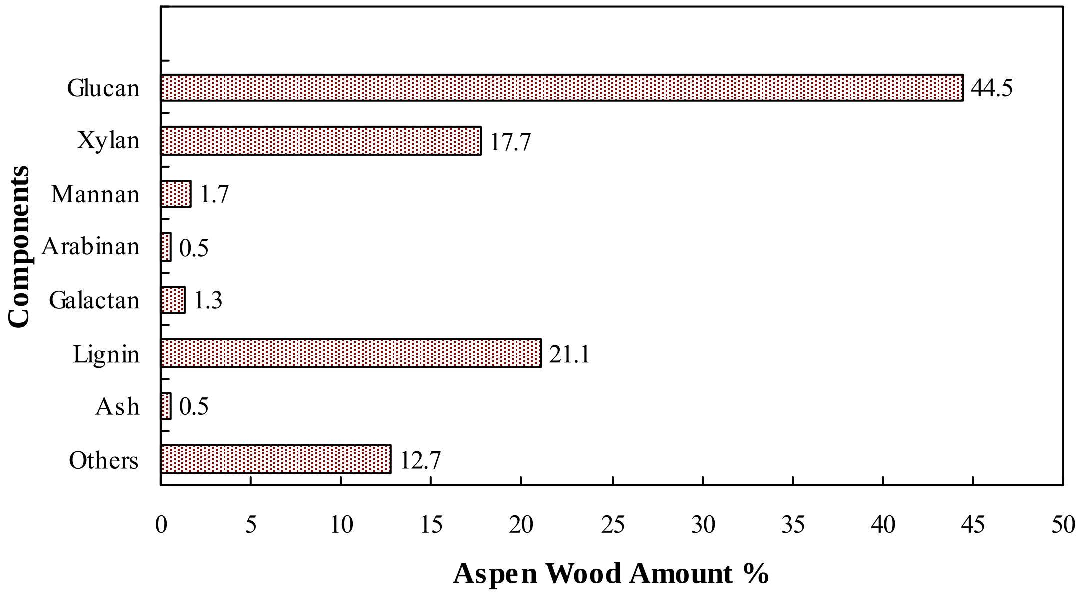


Figure 4.1 Chemical compositions of aspen

The reported capability to digesting the five chosen substrates (glucose, cellubiose, xylose, xylan, microcrystalline cellulose) for *C. thermocellum* and *C. thermolacticum* varies in previous reports due to the lack of uniform cultivation conditions (13, 216). Therefore, in this project, the wild type *C. thermocellum* strains ATCC 27405 and *C. thermolacticum* strain ATCC 43739 were grown in the standard medium provided by ATCC on filter paper and sucrose separately at 60 °C for 10 days. Figure 4.2 and Figure 4.3 show the average amount of ethanol and acetate produced by fermenting cellulose and xylan in this study. As can be seen, *C. thermolacticum* and *C. thermocellum* are both able to utilize crystalline cellulose, commercial cellulose powder (both crystalline and amorphous cellulose) and part of the xylan to produce ethanol. For both fermentations, the ethanol yield is extremely low when xylan is used as the sole carbon source. This difference in ethanol yield can be explained by the differences in enzymatic activities in these two strains. The average amounts of ethanol and acetate produced by fermenting cellobiose, xylose, and glucose are reported in Figure 6 and Figure 4.4. Both strains *C. thermolacticum* and *C. thermocellum* can properly convert these substrates directly into a considerable amount of ethanol and acetate, and the amounts of end-products are considerable for these fermentations.

These results all imply that *C. thermocellum* and *C. Thermolacticum* can utilize all the chosen substrates. Since the fermentation for each substrate leads to considerable amounts of ethanol or acetate, understanding the degradation mechanisms and growth limitations for each strain on each substrate is necessary to best utilize these strains to enhance the efficiency of CBP fermentation.

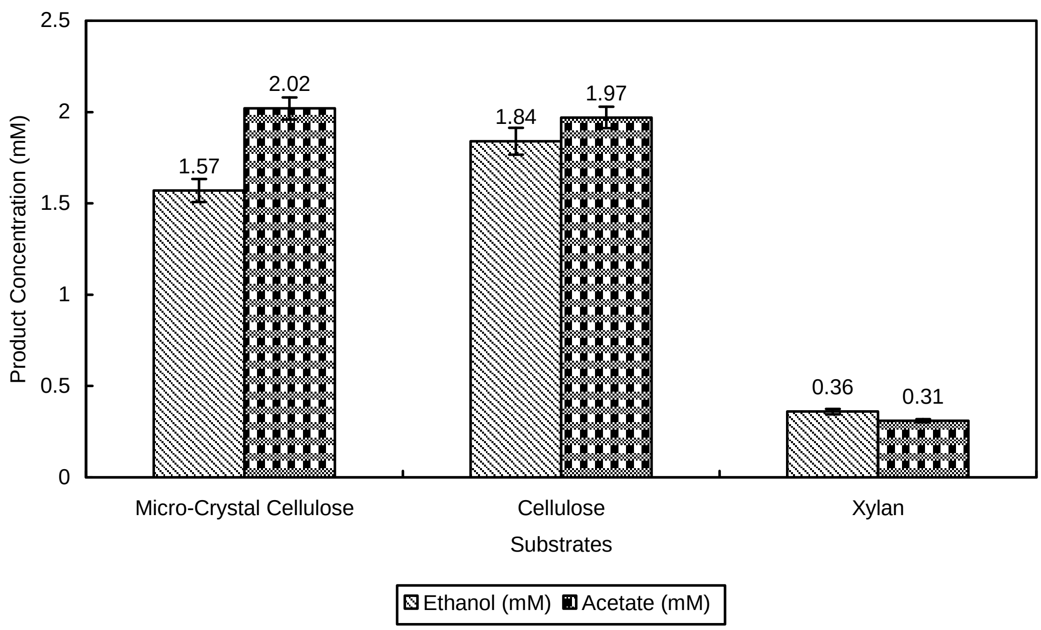


Figure 4.2 Ethanol and acetate productions from C. thermolacticum (ATCC 43739) fermentation of various polysaccharides at 60ºC for 10 days

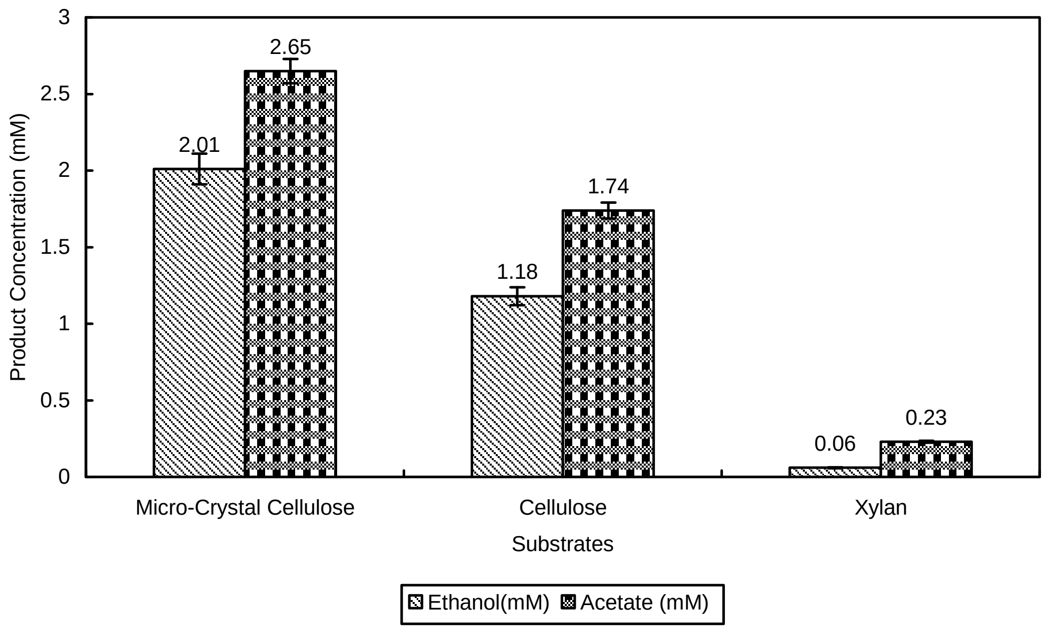


Figure 4.3 Ethanol and acetate productions from C. thermocellum (ATCC 27405) fermentation of various polysaccharides at 60ºC for 10 days

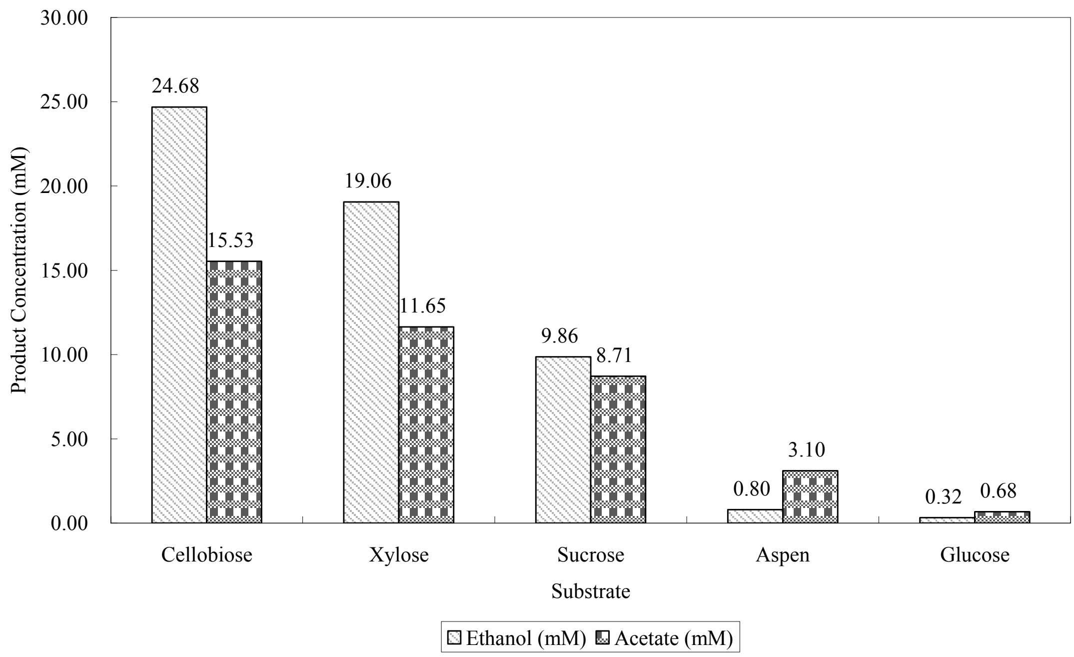


Figure 4.4 Ethanol and acetate productions from C. thermolacticum (ATCC 43739) fermentation of various sole carbon sources at 60ºC for 10 days

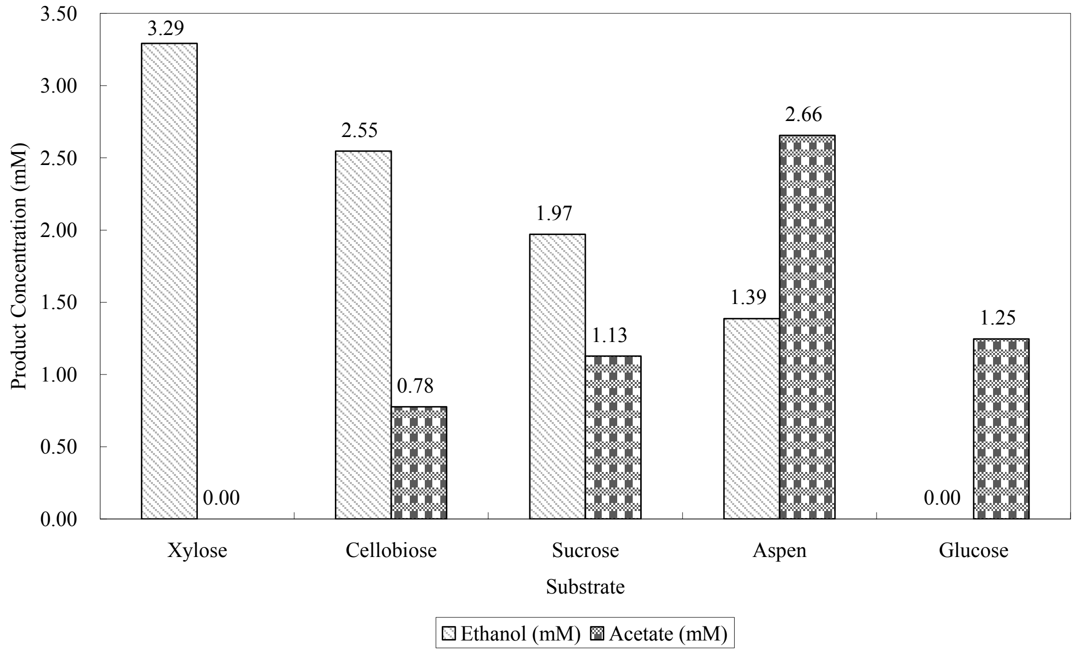


Figure 4.5 Ethanol and acetate productions from C. thermocellum (ATCC 27405) fermentation of various sole carbon sources at 60ºC for 10 days

4.1.2 Co-culture Ratio

Application of the co-culture was examined in this study. However, the ratio of *C. thermocellum* and *C. thermolacticum* are critical for the fermentation efficiency due to the different enzymatic activities between these two strains. By adjusting the ratio of these two co-culture components, the portion of enzymes like cellulase and xylase are dramatically changed. As a result, in order to improve the synchronization effect of these two microbes, optimum portion of *C. thermocellum* and *C. thermolacticum* added during fermentation was determined. Various ratios of *C. thermocellum* and *C. thermolacticum* co-cultures were added for fermentations at 57 ºC on a mixture of filter paper and sucrose solution. After ten days reaction time, a 1:1 ratio for *C. thermocellum* and *C. thermolacticum* led to the highest ethanol production. The ethanol production efficiency was significantly lower in the *C. thermocellum*/*C. thermolacticum* co-cultures at all other ratios tested (1:9, 2:8, 3:7, and 4:6) (Figure 4.6). Even with 10% change of the microbes' ratio, about 28% of the ethanol production decrease was detected. The more these addition rates of these two strains varied from the 1:1 ratio, the less ethanol production efficiency was observed. These results show that the promotion of co-culture fermentation efficiency requires relatively equal amount of *C. thermocellum* and *C. thermolacticum*. When one of the strain is notably domain in the co-culture (over 80%), extremely low ethanol production concentrations were obtained, which were close to the mono-culture fermentation efficiencies. To determine whether the ethanol production efficiency recessions were a result of the death of the minority strain, the microorganisms remaining in fermentation mixture were subcultured into the standard medium with sole carbon source (paper or xylose) that can only support one species to survive. The growth of the minority strains were significantly halted in those severely unbalanced-ratio co-cultures as compared to balanced-ratio co-culture and mono-cultures. This result suggests the decreased ethanol production was most likely due to the suppression of the minority microbes' growth possibly causing by the competitions between these strains at the beginning of the fermentations.

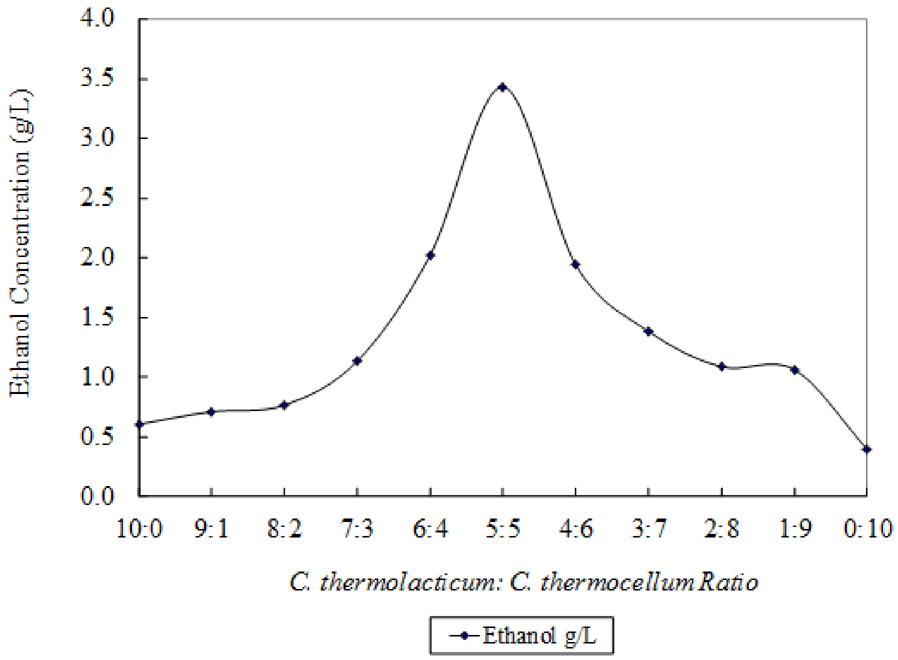


Figure 4.6 Determination on the optimum synchronization effect of the C. thermolacticium and C. thermocellum co-culture composition on ethanol production efficiency

4.1.3 Range of Fermentation Biomass Loading Rate and Substrate Inhibition

Low loading rate (1% w/v) of the carbohydrates has been applied in this study in order to minimize any potential inhibition, either through substrate or end-products during the change of fermentation conditions. However, the effect of loading rate (0.1%, 0.5%, 1%, 2%, 4%, 8%) and the potential substrate inhibition on chosen carbohydrates have been tested on the co-culture. According to the results, increasing the loading rate resulted in nearly proportional improvement of ethanol productions for xylose at all chosen loading rates, for glucose and cellobiose below 1% loading rate, and for MCC below 2% loading rate (Figure 4.7). Further increasing the substrate loading rate led to a plateau for MCC, and dramatically declined for glucose and cellobiose, indicating substrate inhibitions for cellobiose and glucose. These inhibition effects agree with the substrates inhibitions found in pervious research about *C. thermocellum* YM4. Glucose works as the competitive inhibitor of Alpha-D-Glucose-1-Phosphate (G-1-P), Cellobiose works as the competitive inhibitor of phosphate (Pi), and these two pairs of competences competes with the Cellobiose Phosphorylase (CBP) active site (293). In this case, the existence of excessive amount of glucose or cellobiose will disturb the hexose metabolism pathway, so that less ethanol will be produced. (293)

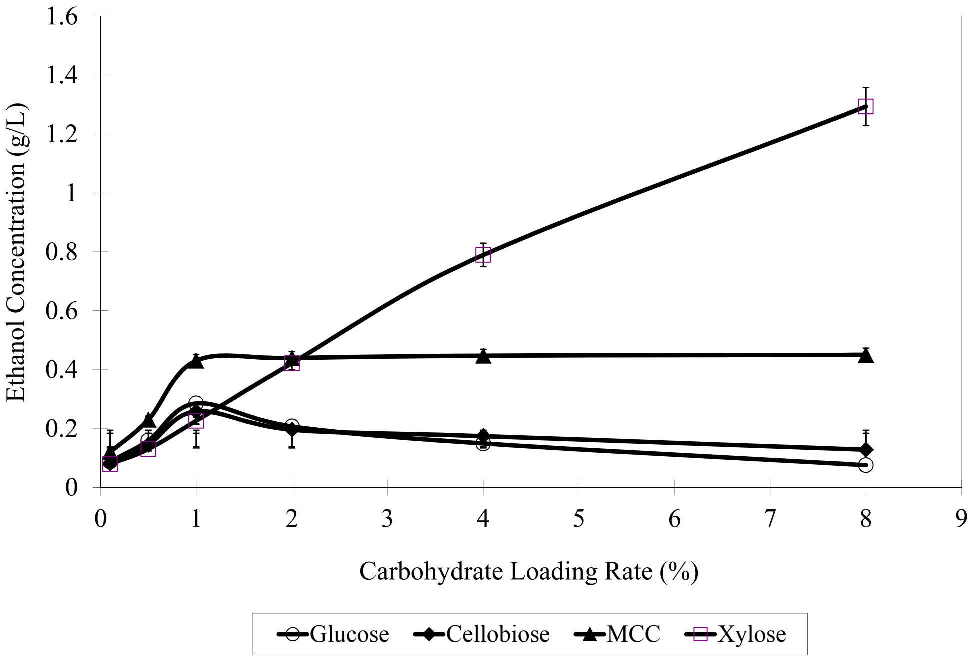


Figure 4.7 Effect of biomass loading rate on ethanol production efficiency

4.1.4 Range of Initial Ethanol Concentration

The pH and the ethanol concentrations are the major influencing factors during the fermentation process. Extremely high ethanol concentrations in the fermentation cultures have been proven to inhibit or depress the fermentation process. A growth inhibition at ethanol levels of less than 3% v/v (207, 227) has been reported for *C. thermocellum*. Ten grams per liter was the highest ethanol tolerance reported for *C. thermolacticum* (ATCC 43739) (16), although these sensitivities depend on the way experiments are completed. Nevertheless, no higher ethanol tolerance has been reported for these wild type strains. Thus, in this research, the maximum ethanol tolerance was examined for the fermentation processes using these two strains. We found that initial ethanol equivalent of 8 g/L and 16 g/L are the highest ethanol concentration *C. thermocellum* and *C. thermolacticum* can tolerate separately (Figure 4.8). Any sample exposed to an ethanol level over 16 g/L, showed no strain growth because of the irreversible change of cell membrane lipids through ethanol, as discussed by Baskaran (294).

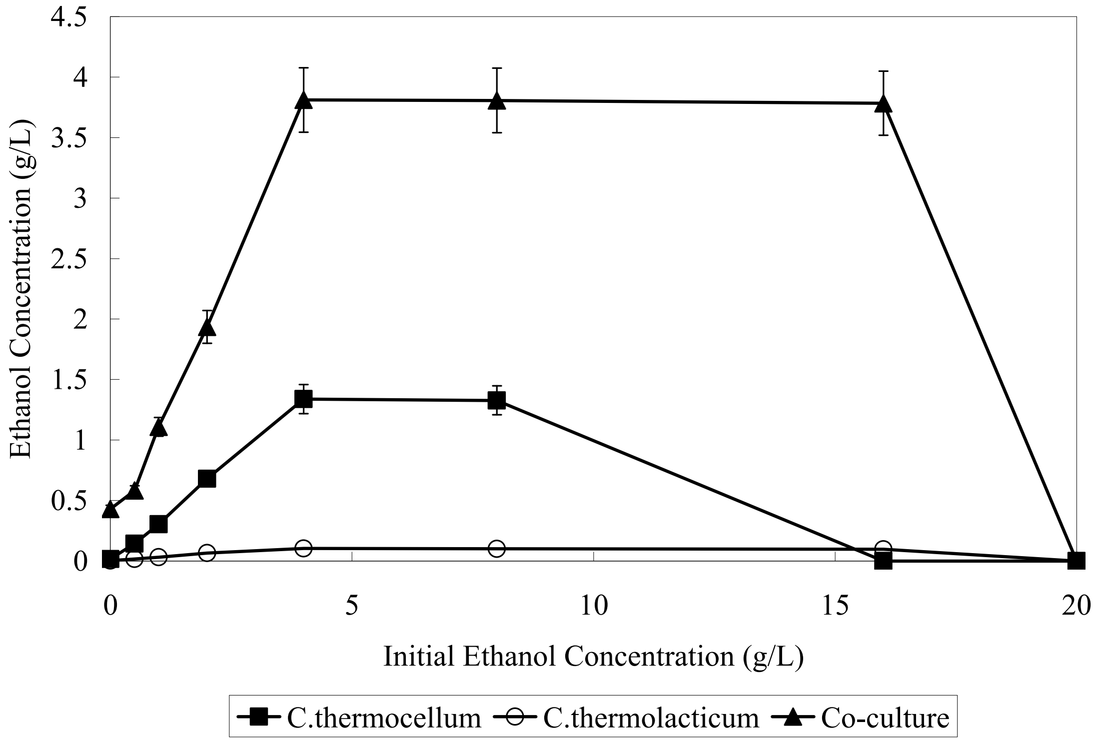


Figure 4.8 Effect of initial ethanol concentration on ethanol production efficiency

4.1.5 Range of Initial pH Value

To narrow down the pH range for this project, the wild type *C. thermolacticum* strain ATCC 43739 was grown in our preliminary tests on the standard medium provided by ATCC and on sucrose at 60 °C for 10 days. The pH of the medium was adjusted from 2.0 to 12.5. Figure 4.9 summarizes the pH effect on ethanol and acetate production for *C. thermolacticum* fermentation using sucrose as the sole carbon source (standard culture substrate). As shown in this figure, the ethanol and acetate yields are dramatically influenced by the medium pH. PH=7 is the optimum pH for the ethanol production from sucrose. At both acidic conditions and alkaline conditions, the yield of ethanol and acetate increase as the pH approaches 7.0. Although the ethanol and acetate yields are lower at other pH values, between pH=5 to pH=9, a considerable amount of ethanol and acetate are still detected. Few conversion products are observed under extremely high or low pH conditions because these conditions lower the viability of the microbes' cells and inhibit the growth of the cell.

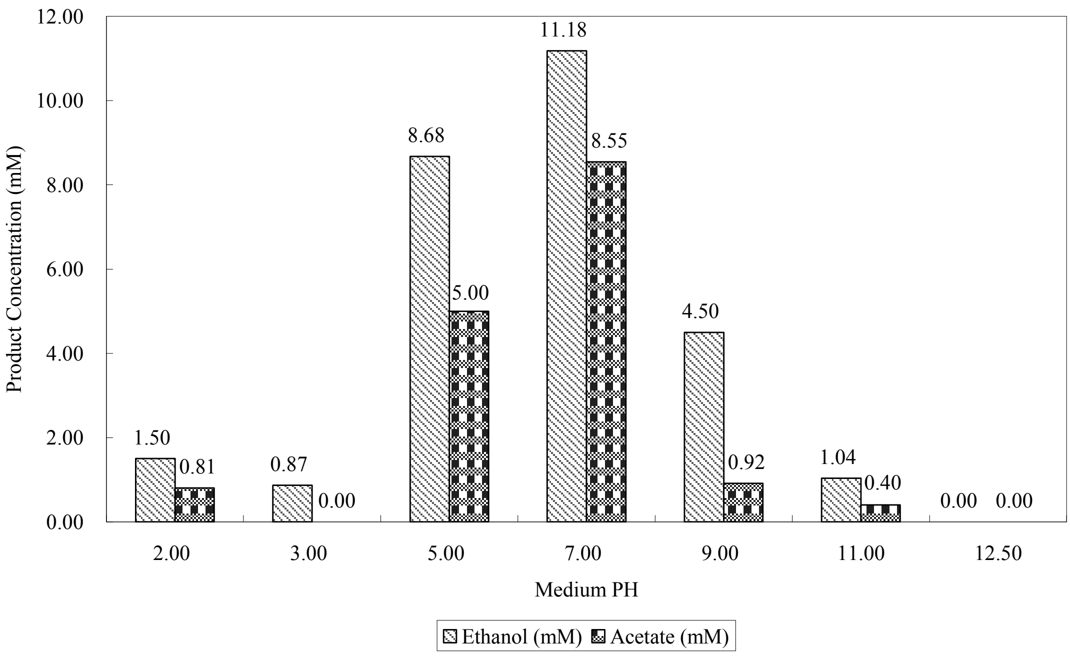


Figure 4.9 Ethanol and acetate yield from C. thermolacticum (ATCC 43739) fermentation at different pH value at 60ºC for 10 days

For the *C. thermocellum* strain ATCC 27405, previous papers have reported that wild type *C. thermocellum* grows better when the pH is between 7 and 10 (229). When the pH value was over 11, acetate and lactate productions decreased dramatically and when the pH value was lower than 6, no growth was detected (229). As a result, taking both strains into account, the pH range of this research was set to pH=5-10.

4.1.6 Concentration of Alginate and Calcium Chloride

4.1.6.1 Concentration of Alginate and Beads Leakage

As described in the introduction section, we propose to use encapsulation of *C. thermocellum* and *C. thermolacticum* by calcium alginate, a mild matrix for living cells, to attempt to achieve high ethanol tolerance for the lignocellulosic biomass fermentation. The porosity of the gel is determined by the concentration of sodium alginate, calcium chloride, as well as other chemicals within the medium. It is reported that entrapment of chemicals including flavin, nicotinamide, and cytochrome in the medium will diffuse 1% (w/v) of gel out of the alginate gel (295). In this case, more extensively polymerized alginate gel is necessary; this can be achieved by increasing the concentration of sodium alginate or calcium chloride. In order to determine the appropriate concentration of sodium alginate, addition of 0.5%, 1%, 2%, 3%, 4%, and 5% of sodium alginate were examined for encapsulation. According to the fermentation results, 0.5% and 1% of sodium alginate were not able to form appropriate alginate gels due to lack of polymerization. These formless gels were not able to hold microbes within the structures, leading to considerable amount of leakage of cells during fermentation. 2% of the sodium alginate was the minimum alginate concentration which resulted in the appropriate shape of alginate beads. However, at 2% concentration, the polymerization is still not extensive enough. A small amount of leakage of *Clostridium spp* spores was detected in the fermentation systems. By increasing the alginate concentration over 4%, cell leakages were no longer observed but the fermentation reactions were significantly reduced. The ethanol production dropped about 20% when the alginate concentration is increased from 3% to 4%. Further increase of the alginate concentration led to further loss of cell actives, due to the increase of internal diffusion limitations. If larger pellets were formed, the substrate equilibrium was reached slower than when higher alginate concentration was used.

4.1.6.2 Calcium Chloride Toxicity

Calcium chloride concentration is another critical factor in adjusting the polymerization of alginate pellets. Nevertheless, high calcium ion concentration has been shownto affect the growth of *Clostridium* co-cultures (293). As a result, the effect of calcium chloride concentration was tested on ethanol production during immobilized cells fermentation on cellobiose at pH=7. Figure 4.10 indicates that within the chosen concentration (1% -10%), the co-culture is not inhibited. Contrarily, by increasing the calcium chloride concentration from 1% to 3%, the ethanol production was increased by over 30%, suggesting that addition of calcium chloride is beneficial for *Clostridium* co-culture fermentation. This effect might be caused by the alkalinity of calcium chloride which would keep the medium pH relatively stable. In addition, the existence of Ca^{2+} would also improve the metabolism of the co-culture (296). Further increase of the calcium chloride concentration past 3% is not able to enhance the solvent production significantly. Thus, the lowest calcium chloride concentration (3%) leading to the higher solvent production improvement during fermentation is chosen for the encapsulation in this study.

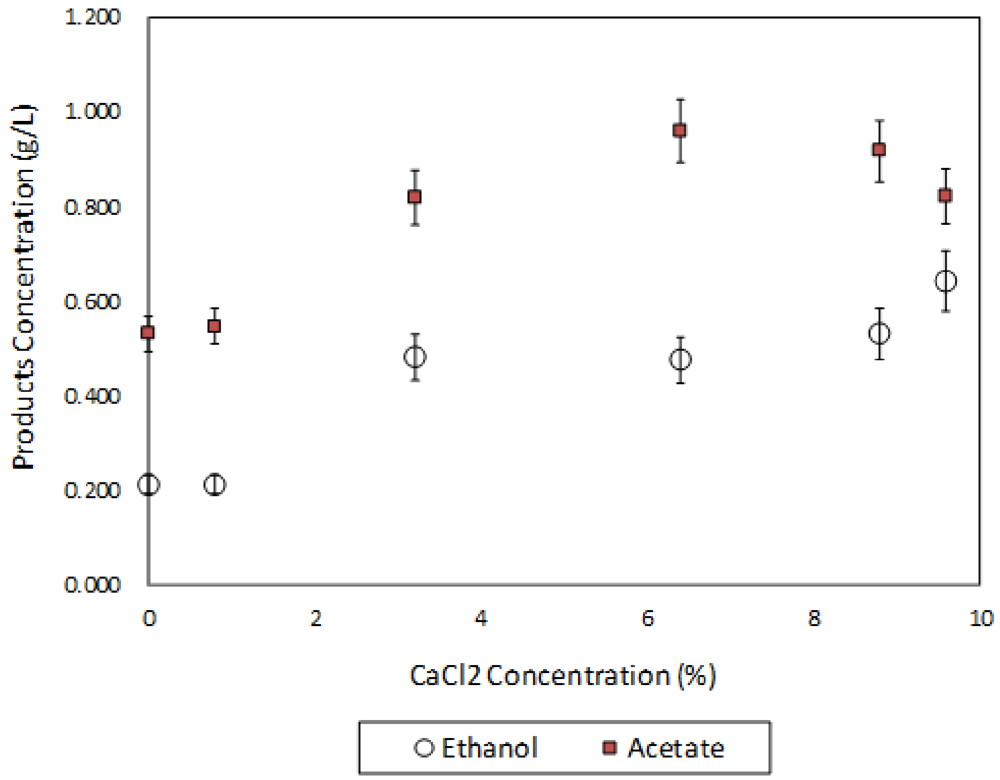


Figure 4.10 Effect of calcium chloride concentrations on fermentation production concentrations on cellobiose fermentation at pH=7 57ºC for 10 days

4.1.7 Organosolv Lignin toxicity

Lignin and lignin fragments are the other potential inhibitors within the lignocellulosic complex. Thus, in this study, the effects of lignin on the ethanol production efficiency were examined.

Organosolv lignin is closer to the native lignin structure than other types of lignin, such as Kraft lignin (297). In addition it does not contain sulfur in any form. Similar functional groups in organosolv lignin lead to similar response towards reactants than naturally occurring lignin. As a result, organosolv lignin is used to exam the effect of lignin on fermentation efficiency. Figures 4.11-4.18 show the ethanol and acetate production changes caused by addition of several levels of organosolv lignin on carbohydrate fermentations (glucose, xylose, cellobiose and

MCC). Both, ethanol and acetate productions for all substrates were not significantly changed through addition of lignin regardless of the addition rate, suggesting that the organosolv lignin is not a significant inhibitor for the co-culture fermentation process. However, although the structure and properties of organosolv lignin is close to naturally occurring lignin, lignin modifications still occurred during lignin extraction. Thus, naturally occurring lignin still has the potential to influence the fermentation process.

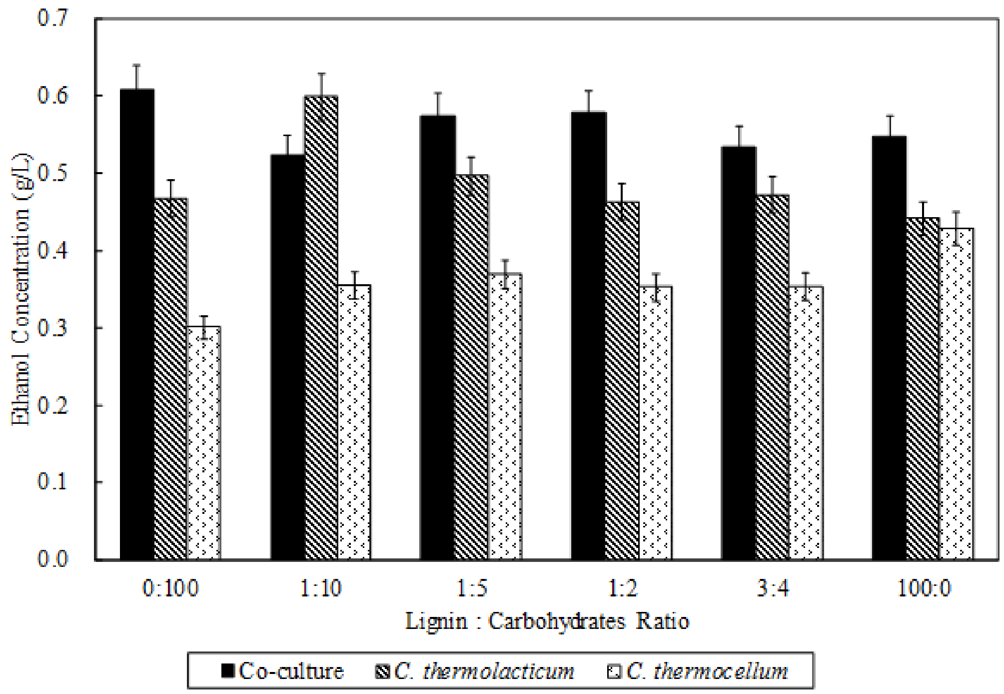


Figure 4.11 Effect of organosolv lignin on ethanol production during cellobiose fermentation at 57°C pH=7 for 10 days

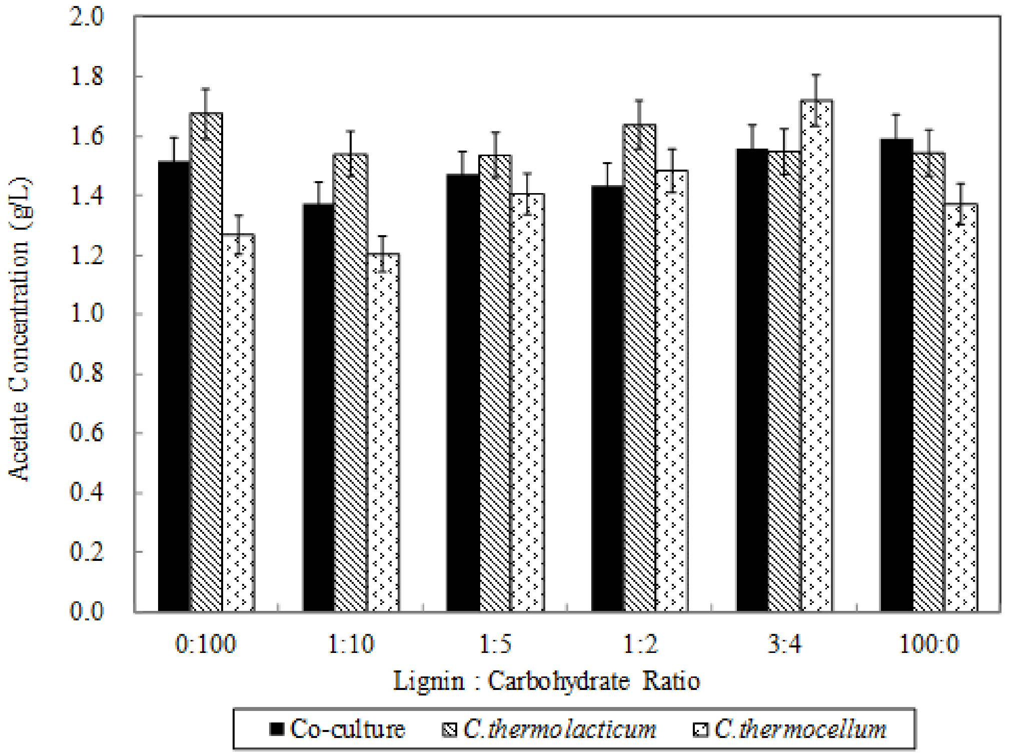


Figure 4.12 Effect of organosolv lignin on acetate production during cellobiose fermentation at 57ºC pH=7 for 10 days

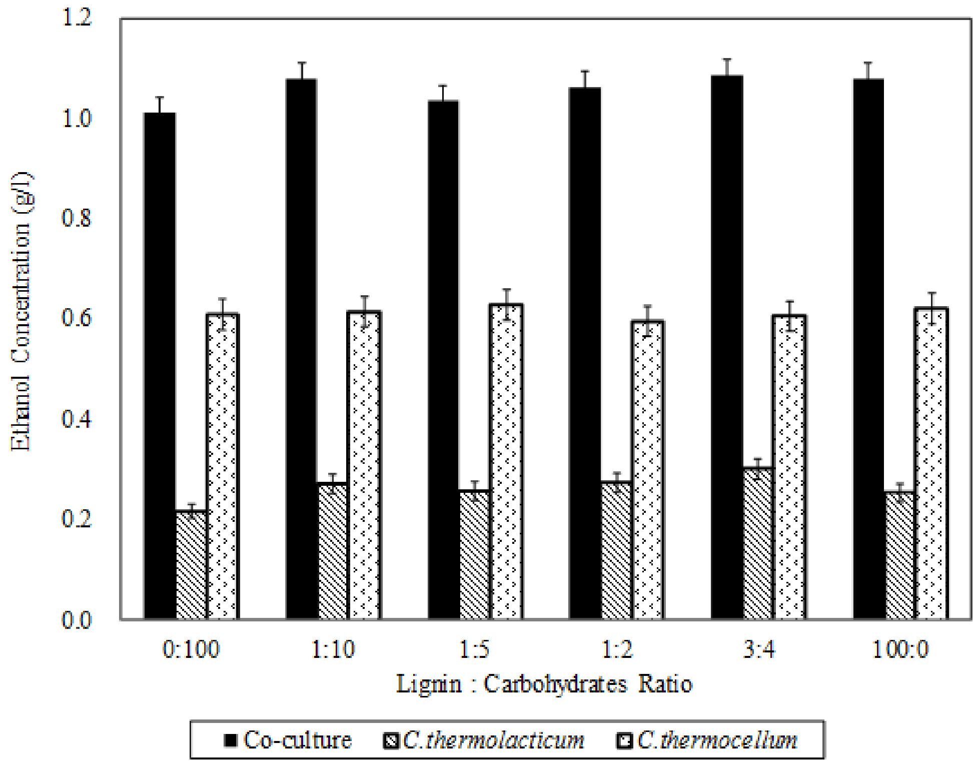


Figure 4.13 Effect of organosolv lignin on ethanol production during micro crystal cellulose fermentation at 57ºC pH=7 for 10 days

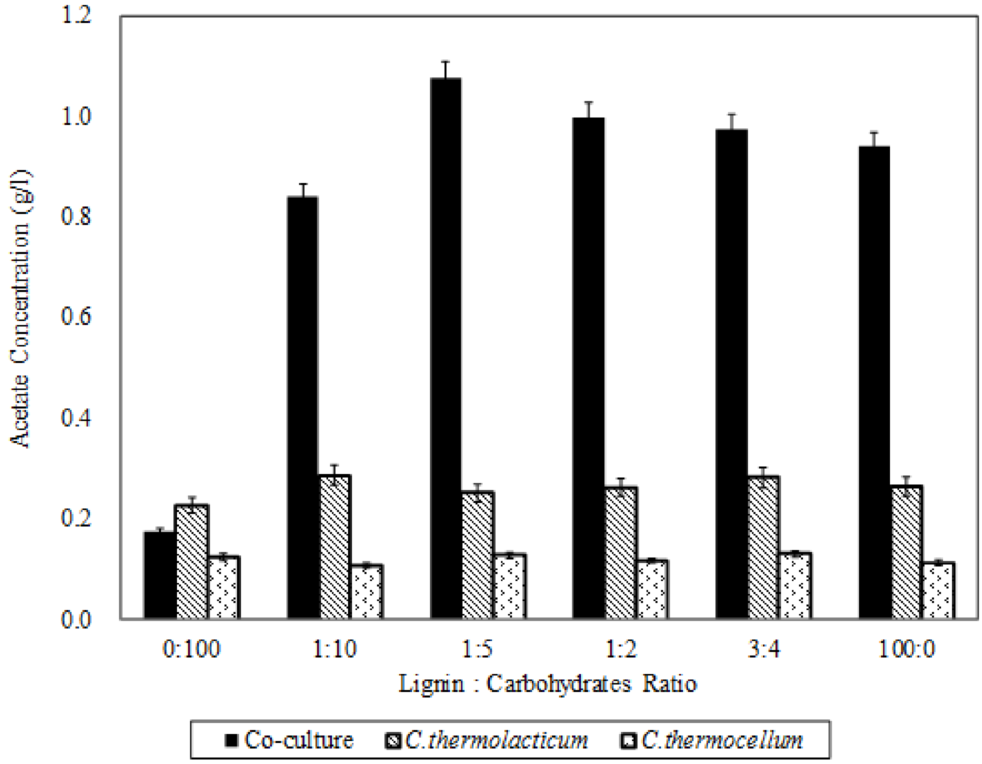


Figure 4.14 Effect of organosolv lignin on acetate production during micro crystal cellulose fermentation at 57ºC pH=7 for 10 days

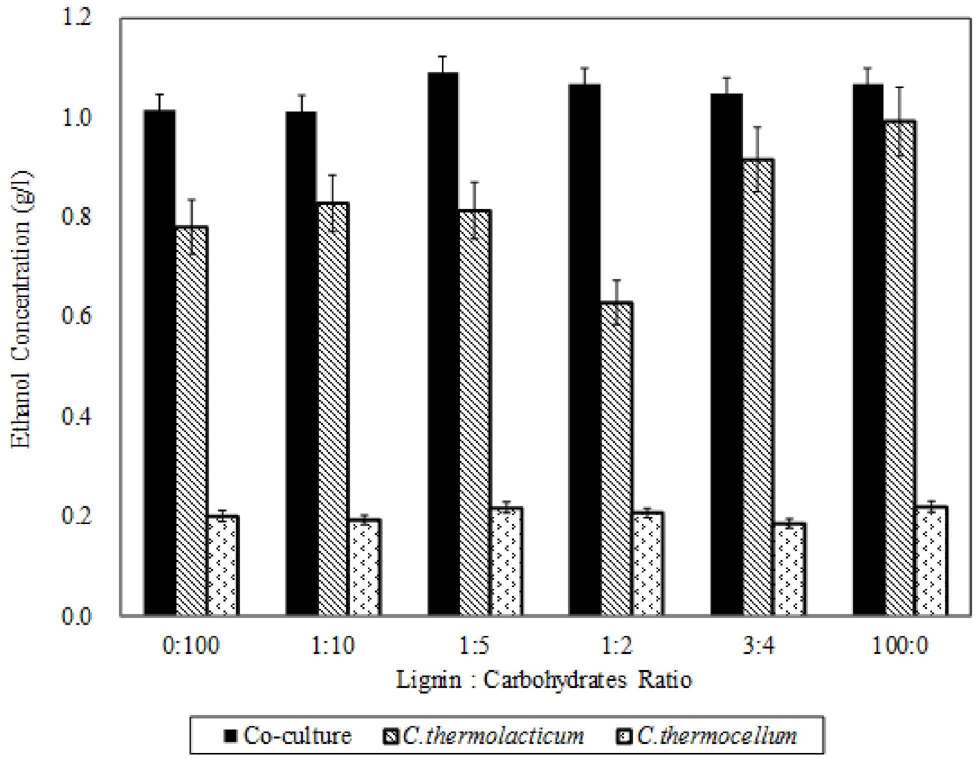


Figure 4.15 Effect of organosolv lignin on ethanol production during glucose fermentation at 57ºC pH=7 for 10 days

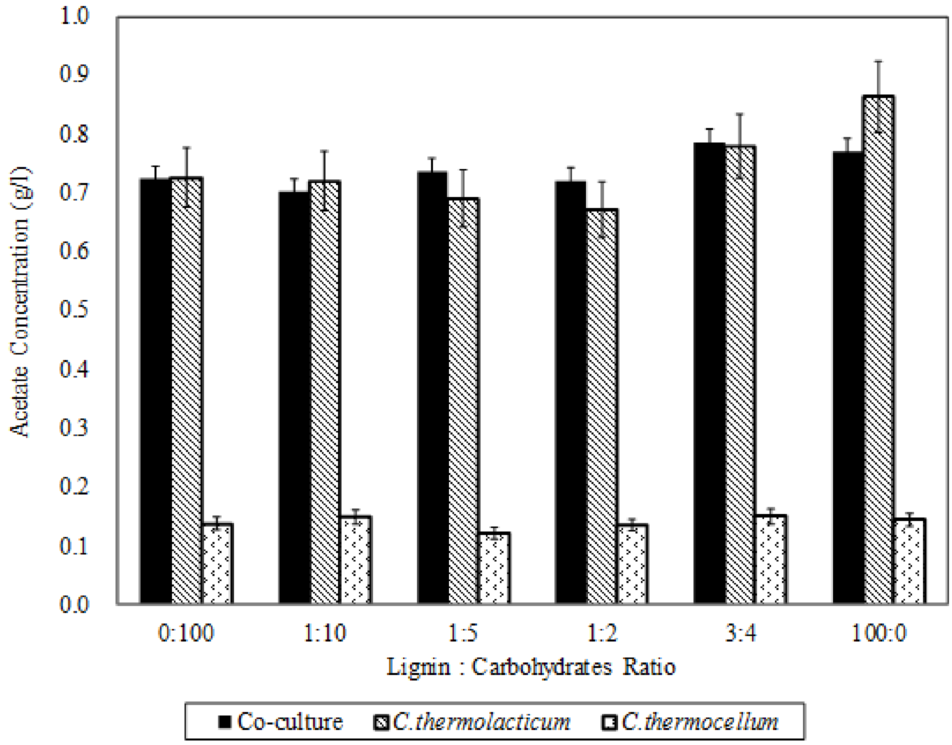


Figure 4.16 Effect of organosolv lignin on acetate production during glucose fermentation at 57ºC pH=7 for 10 days

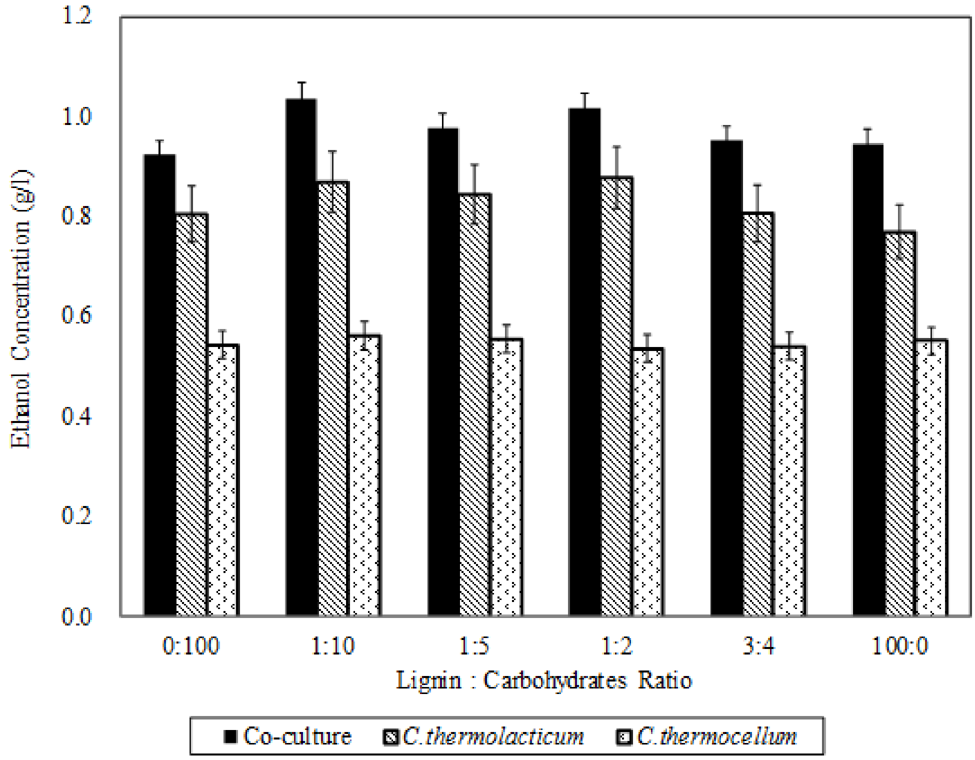
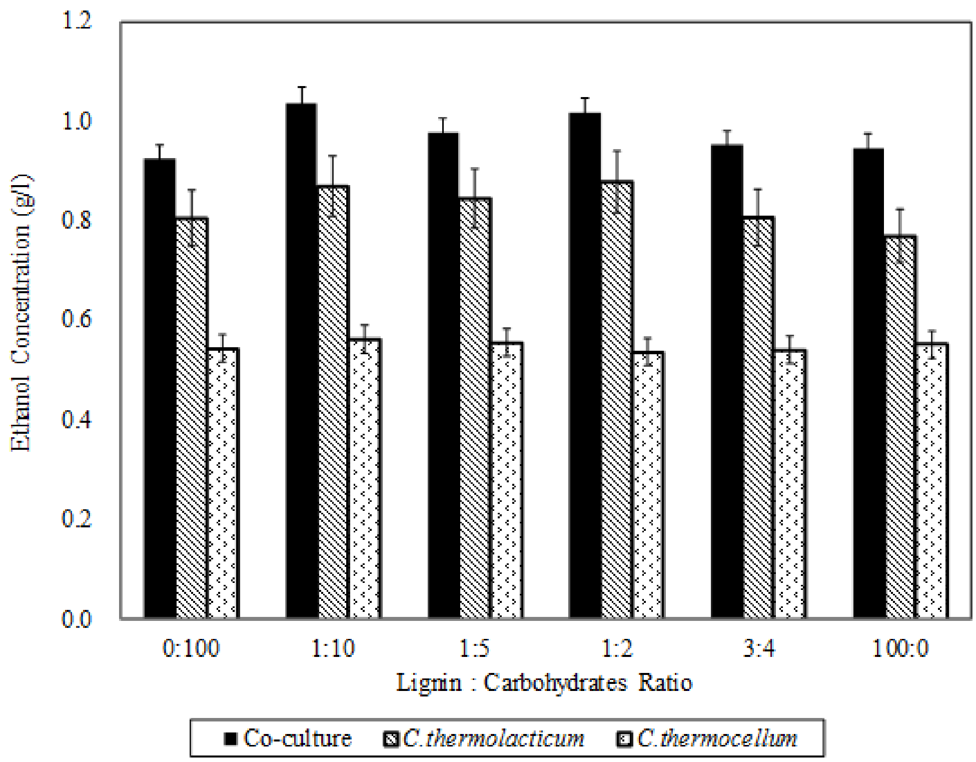


Figure 4.17 Effect of organosolv lignin on ethanol production during xylose fermentation at 57ºC pH=7 for 10 days



4.18 Effect of organosolv lignin on ethanol production during xylose fermentation at 57ºC pH=7 for 10 days

4.2 Free cell fermentation

In order to analyze the effect of major fermentation parameters on the CBP fermentation, the conversions of selected materials such as cellulose, xylan and their mono- or di-saccharide intermediates are investigated. The major saccharides include glucose, cellobiose, and xylose. The effect of minor saccharides such as arabinose mannose and galactose, however, are not considered in this study. Both strains produce ethanol, acetate and hydrogen. But depending on the conditions, the product distribution varies. Preliminary studies were performed with both mono-cultures at different substrate concentrations, initial ethanol concentrations, and pH values. 1% w/v substrate loading proved to be the most suitable for fermentation for both strains, which agrees with earlier studies (298). Initial ethanol equivalent of 16 g/L is the highest ethanol concentration both strains can tolerate. Any sample exposed to an ethanol level over 16 g/L, showed no strain growth because of the irreversible change of cell membrane lipids through ethanol, as discussed by Baskaran (294). It is also observed that a pH between 5 and 10 leads to the production of ethanol and acetate, any pH below or above appears to be unsuitable for the viability of the cells. The influences of pH, ethanol concentration and substrate types were further compared between *C. thermocellum/C. thermolacticum* mono-cultures and co-culture fermentation.

4.2.1 Benefit of co-culture fermentation

This study clearly demonstrated the advantage of applying a co-culture in CBP fermentations to improve the ethanol production for selected substrates (299). It demonstrated the potential of using simultaneous *C. thermocellum/C. thermolacticum* co-culture to convert components of lignocellulosics directly into ethanol and acetate. To examine tolerance towards ethanol concentration varying amounts of ethanol were added at the beginning of the fermentation step. Initial ethanol levels of up to 4 g/L were not only tolerated, but actually resulted in improved ethanol production. Although this co-culture is unable to shift the heterofermentative pathway into a homofermentative pathway or at least decrease the acetate production, ethanol production is significantly increased compared to the mono-cultures. Almost all the ethanol yields obtained from co-culture fermentation are above any of its mono-culture fermentation yields, often almost tripled as compared to the mono-cultures under identical conditions. The only exceptions are the experiments at pH=7 to pH=9 for the substrate glucose. The optimum acetate by-product generation, which occurs in smaller concentrations of mostly below 1 g/L, turns out to lie in the same narrow range irrespective of the substrate or pH value used. Although acetate is not desirable as a final product, it has been shown that the formation of acetate can stimulate ethanol production during fermentation indirectly contributing to final ethanol yield (300). Thus, small amounts of acetate production are not considered to have a negative effect on ethanol production in this study. The absence of the correlation between ethanol productivity improvement and increased E/A ratios suggests that the changes caused by co-culture application are caused by both mass action effects and membrane fluidity change. In addition, this co-culture exhibited a shorter lag period (48 h) than any of their mono-culture before the initiation of the selected sugar fermentations.

We hypothesize that our approach is effective because *C. thermocellum* can produce active cellulotyic and xylanolytic enzymes (301, 302), and *C. thermolacticum* is able to utilize the degraded substrates, which are less favorable for *C. thermocellum* (15). The improvement of MCC degradation clearly demonstrates this synergistic effect.

4.2.2 Impact of ethanol concentration on fermentation

Tables 4.1 and 4.2 compare the product formation during saccharide fermentations applying *C. thermocellum*, *C. thermolacticum* or *C. thermocellum/C. thermolacticum* co-cultures. Similar to mono-culture fermentations, ethanol and acetate were found to be the major end-products in *C. thermocellum/C. thermolacticum* co-culture fermentation.

The impact of the ethanol production and accumulation within the medium on total solvent production was studied by adding various levels of ethanol at the beginning of the fermentation. The addition of 0 g/L of ethanol represents the beginning stage of the fermentation when no ethanol has been produced. By adding ascending amounts of ethanol from 0.5 g/L to 4 g/L at the start, this study simulated the ethanol accumulation progress as a sequence of the continuous fermentation. Compared to the mono-culture fermentations, *C. thermolacticum*/*C. thermocellum* co-culture fermentation lead to higher ethanol production when the original ethanol concentration at the start of the fermentation is around 4 g/L for all substrates used under all pH values (Table 4.1, 4.2, Figure 4.19-4.22). However, the increase of ethanol yield caused by applying a co-culture was observed not to correlate to ethanol/acetate ratio improvement.

		C. thermocellum		*C. thermolacticum*		Co-culture	
	Initial Ethanol g/L	Ethanol g/L	SD	Ethanol g/L	SD	Ethanol g/L	SD
Glucose	0	0.476	0.004	0.028	0.001	0.285	0.005
	0.5	0.839	0.029	0.038	0.001	0.194	0.004
	1	1.229	0.005	0.043	0.004	0.965	0.003
	2	2.000	0. 019	0.071	0.003	1.189	0.001
	4	2.638	0.050	0.116	0.004	1.358	0.001
Cellobiose	0	0.399	0.001	0.036	0.003	0.258	0.003
	0.5	0.738	0.010	0.046	0.002	0.503	0.001
	1	1.128	0.030	0.058	0.006	1.427	0.008
	2	1.615	0.037	0.072	0.004	1.750	0.002
	4	1.673	0.033	0.112	0.003	3.163	0.001
MCC	0	0.016	0.009	0.004	0.001	0.430	0.001
	0.5	0.144	0.004	0.016	0.001	0.582	0.001
	1	0.304	0.006	0.032	0.001	1.109	0.006
	2	0.680	0.004	0.066	0.008	1.935	0.005
	4	1.339	0.006	0.104	0.002	3.811	0.032
Xylose	0	0.000	0.000	0.026	0.001	0.226	0.003
	0.5	0.000	0.000	0.033	0.001	0.200	0.007
	1	0.000	0.000	0.037	0.001	0.217	0.001
	2	0.000	0.000	0.064	0.004	0.754	0.010
	4	0.000	0.000	0.097	0.083	4.539	0.011
Hemicellulose	0	0.000	0.000	0.001	0.000	0.007	0.000
(Xylan)	0.5	0.000	0.000	0.002	0.000	0.008	0.001
	1	0.000	0.000	0.001	0.001	0.006	0.001
	2	0.000	0.000	0.002	0.001	0.021	0.001
	4	0.000	0.000	0.000	0.000	0.053	0.001

Table 4.1 Effect of initial ethanol addition (0-4 g/L) and substrate types on ethanol production (g/L) at pH=9 for C. thermocellum mono-culture, C. thermolacticum mono-culture and co-culture fermentations. The ethanol and acetate productions are the average value of triplicates runs under each chosen condition. SD is the standard deviation of the replicates of each condition.

		C. thermocellum		*C. thermolacticum*		Co-culture	
	Initial Ethanol g/L	Acetate g/L	SD	Acetate g/L	SD	Acetate g/L	SD
Glucose	0	0.689	0.007	0.028	0.001	0.533	0.001
	0.5	0.672	0.001	0.026	0.001	0.666	0.006
	1	0.583	0.003	0.025	0.001	0.557	0.006
	2	0.472	0.002	0.024	0.001	0.437	0.002
	4	0.514	0.009	0.022	0.001	0.357	0.005
Cellobiose	0	0.433	0.005	0.027	0.002	0.649	0.003
	0.5	0.403	0.006	0.029	0.001	0.643	0.003
	1	0.307	0.006	0.028	0.002	0.616	0.008
	2	0.424	0.003	0.026	0.002	0.526	0.002
	4	0.468	0.001	0.027	0.001	0.491	0.008
MCC	0	0.040	0.001	0.009	0.001	1.117	0.001
	0.5	0.043	0.002	0.007	0.002	1.026	0.002
	1	0.042	0.001	0.006	0.002	0.976	0.001
	2	0.041	0.002	0.005	0.002	0.651	0.001
	4	0.041	0.001	0.007	0.002	0.648	0.002
Xylose	0	0.000	0.000	0.029	0.002	0.453	0.001
	0.5	0.000	0.000	0.028	0.001	0.397	0.001
	1	0.000	0.000	0.027	0.002	0.374	0.001
	2	0.000	0.000	0.027	0.002	0.373	0.004
	4	0.000	0.000	0.028	0.002	0.332	0.001
Hemicellulose (xylan)	0	0.000	0.000	0.065	0.009	0.010	0.001
	0.5	0.000	0.000	0.064	0.006	0.011	0.001
	1	0.000	0.000	0.055	0.008	0.010	0.001
	2	0.000	0.000	0.054	0.003	0.010	0.001
	4	0.000	0.000	0.000	0.002	0.009	0.001

Table 4.2Effect of initial ethanol addition (0-4 g/L) and substrate types on acetate production (g/L) at pH=9 for C. thermocellum mono-culture, C. thermolacticum mono-culture and co-culture fermentations. The ethanol and acetate productions are the average value of triplicates runs under each chosen condition. SD is the standard deviation of the replicates of each condition.

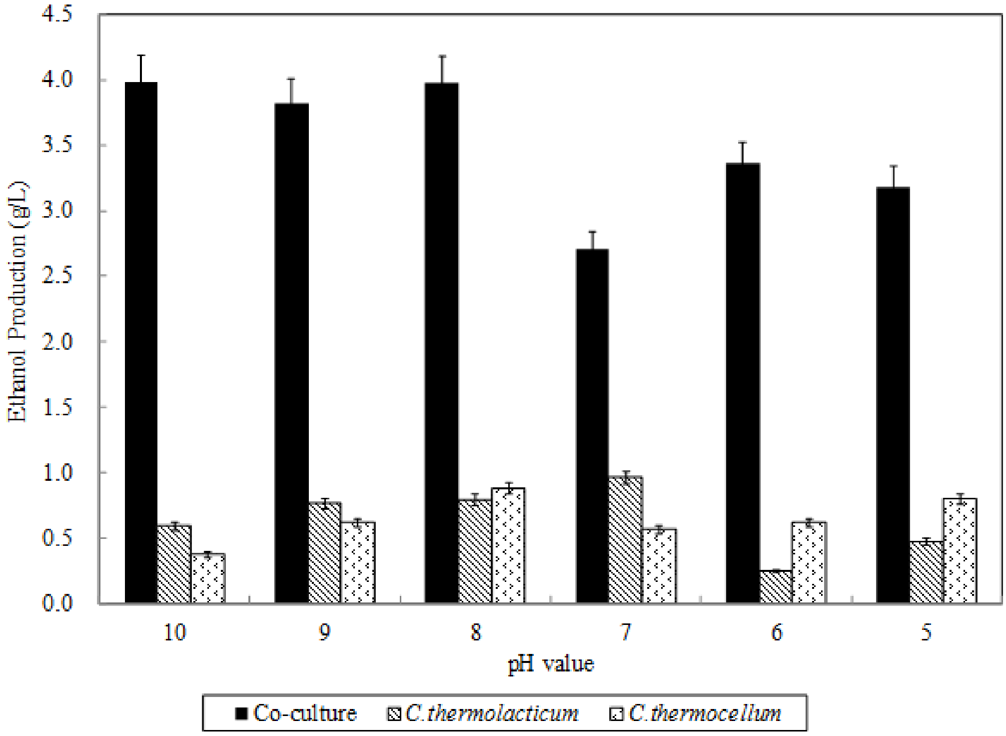


Figure 4.19 Effect of pH on ethanol production at 4 g/L initial ethanol level for MCC fermentation at 57ºC for 10 days

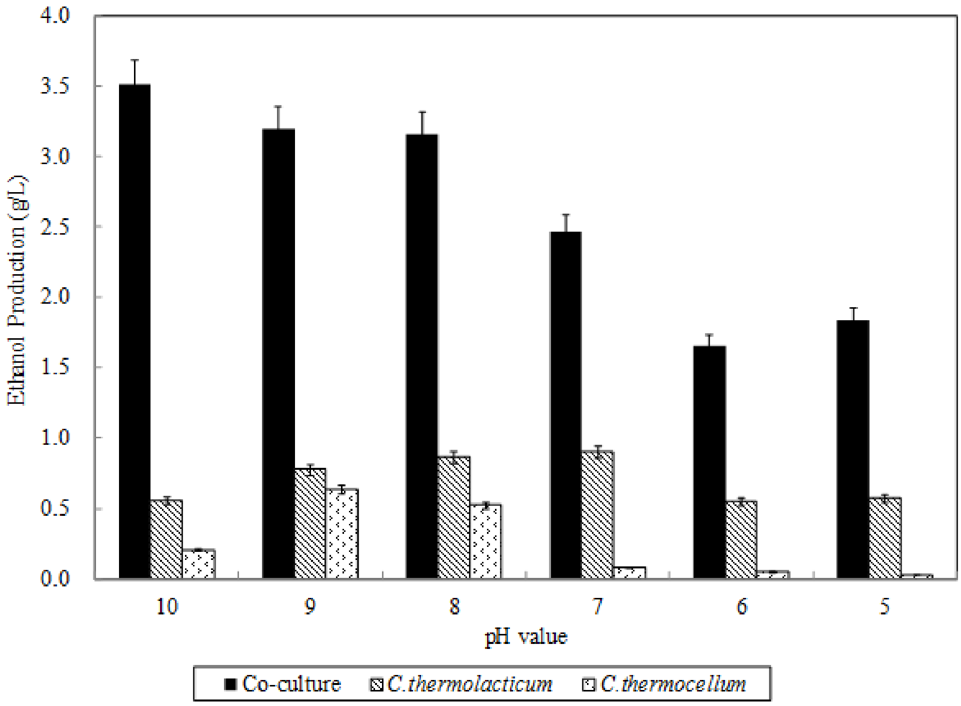


Figure 4.20 Effect of pH on ethanol production at 4 g/L initial ethanol level for cellobiose fermentation at 57ºC for 10 days

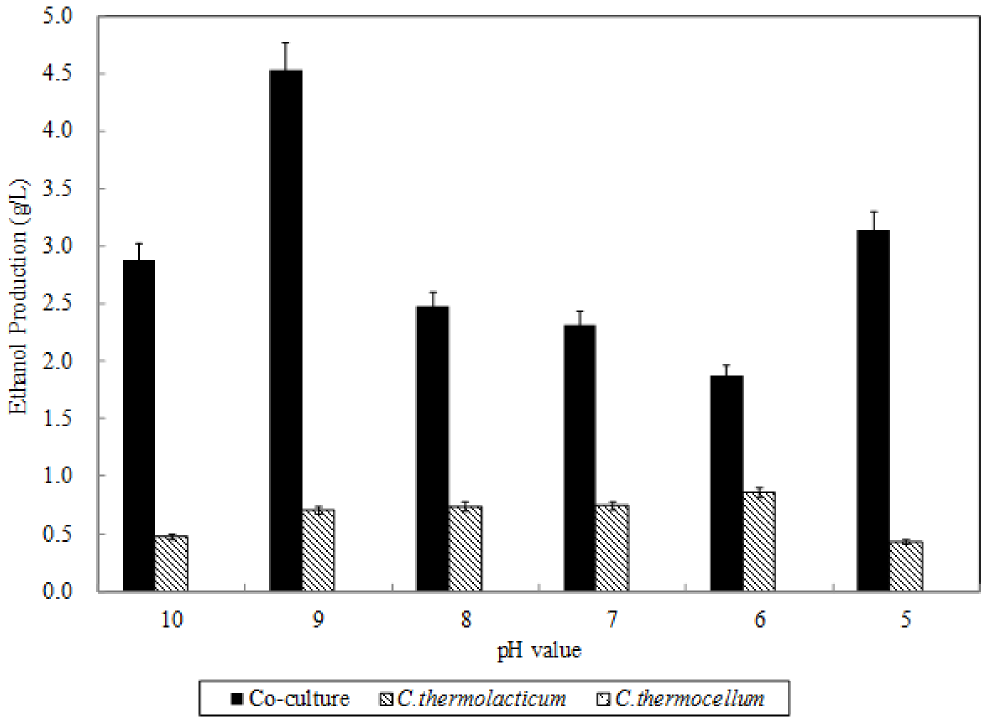


Figure 4.21 Effect of pH on ethanol production at 4 g/L initial ethanol level for xylose fermentation at 57ºC for 10 days

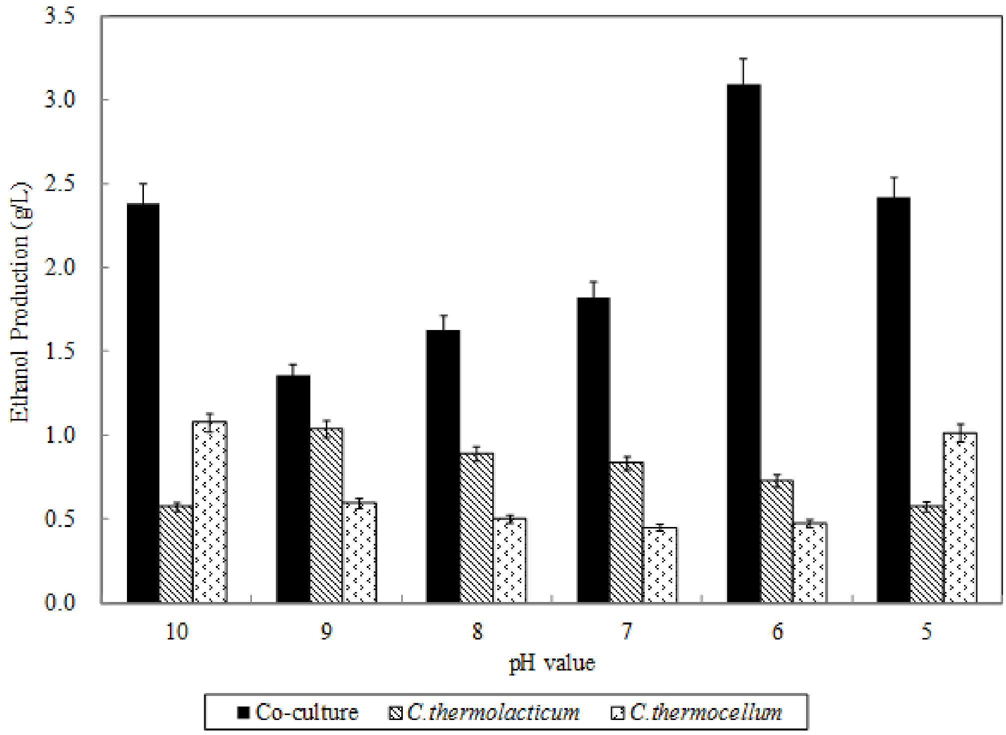


Figure 4.22 Effect of pH on ethanol production at 4 g/L initial ethanol level for glucose fermentation at 57ºC for 10 days

While the ethanol production by *C. thermolacticum* mono-culture and co-culture are significantly enhanced through exposure to initial ethanol levels up to 4 g/L on all substrates and at all pH values (Table 4.1, Figure 4.19), for *C. thermocellum* the positive effect of the initial ethanol appeared to be dependent on pH and substrate. If cellobiose is added as the sole carbon source, the positive correlation between initial ethanol addition and final ethanol concentration is terminated when the ethanol concentration approaches 2 g/L. As the ethanol concentration exceeds 2 g/L no additional positive effect on final ethanol yield is observed (Figure 4.23). For glucose and MCC fermentation, the ethanol production continues to increase as the initial ethanol concentration rises (Figure 4.24, 4.25). On the other hand, the production decline of acetate stopped at 2 g/L of initial ethanol level, and bounces back up slightly at 4 g/L initial ethanol level. In general, a higher initial ethanol level favors the ethanol production for both cultures and the co-culture and is accompanied by a lower acetate yield (Table 4.2). At the optimized condition, the ethanol production in MCC co-culture fermentation increases from 1.0 g/L at an initial ethanol level of 0 g/L to 3.8 g/L at an initial ethanol level of 4 g/L, an improvement of more than 3 g/L. Moreover, for ethanol yields in xylose co-culture fermentations, the increase reached over 4 g/L by increasing the initial ethanol concentration from 0 g/L to 4 g/L (Figure 4.26). The initial ethanol might contribute to changed fermentation in two ways: changing the metabolism pathway or changing the cell membrane fluidity (303).

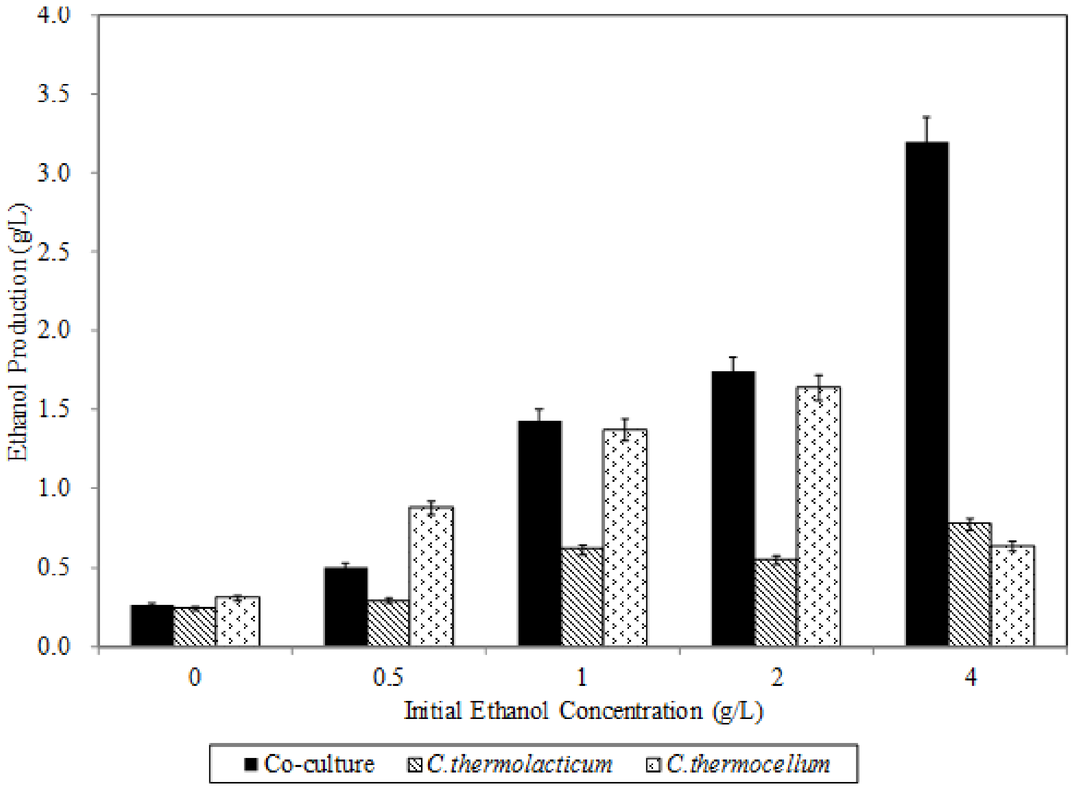


Figure 4.16 Effect initial ethanol concentrations on cellobiose fermentation at pH=9, 57ºC for 10 days

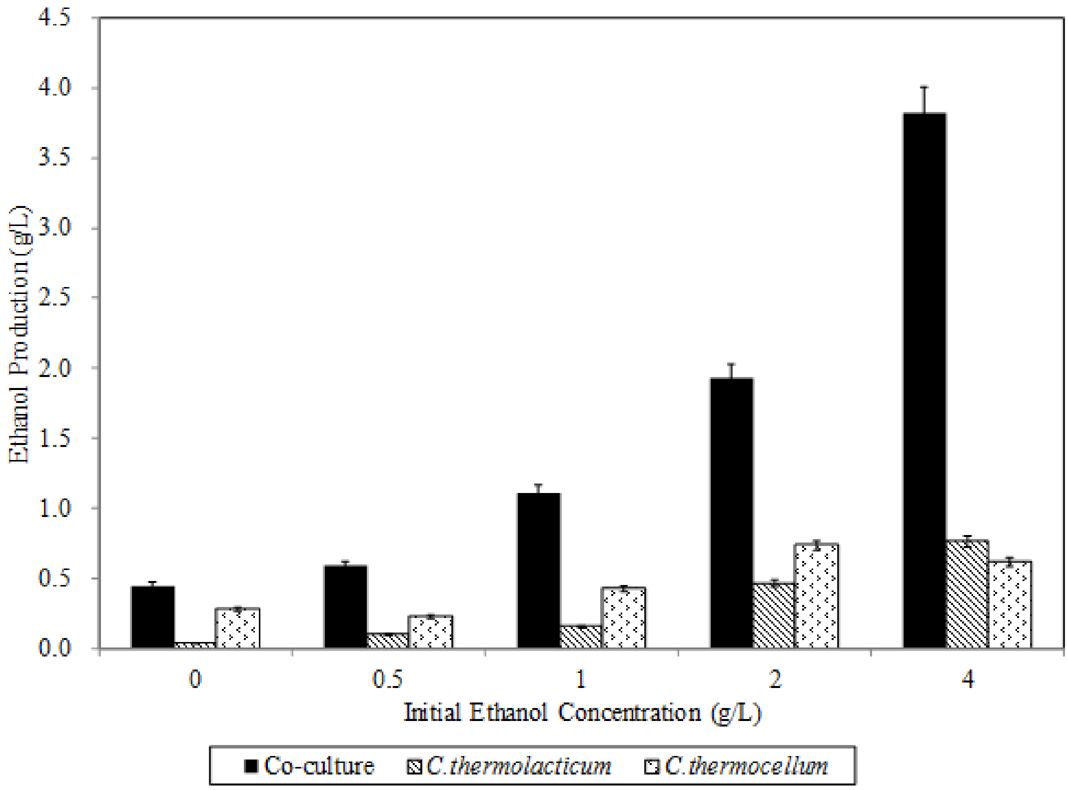


Figure 4.17 Effect initial ethanol concentrations on MCC fermentation at pH=9, 57ºC for 10 days

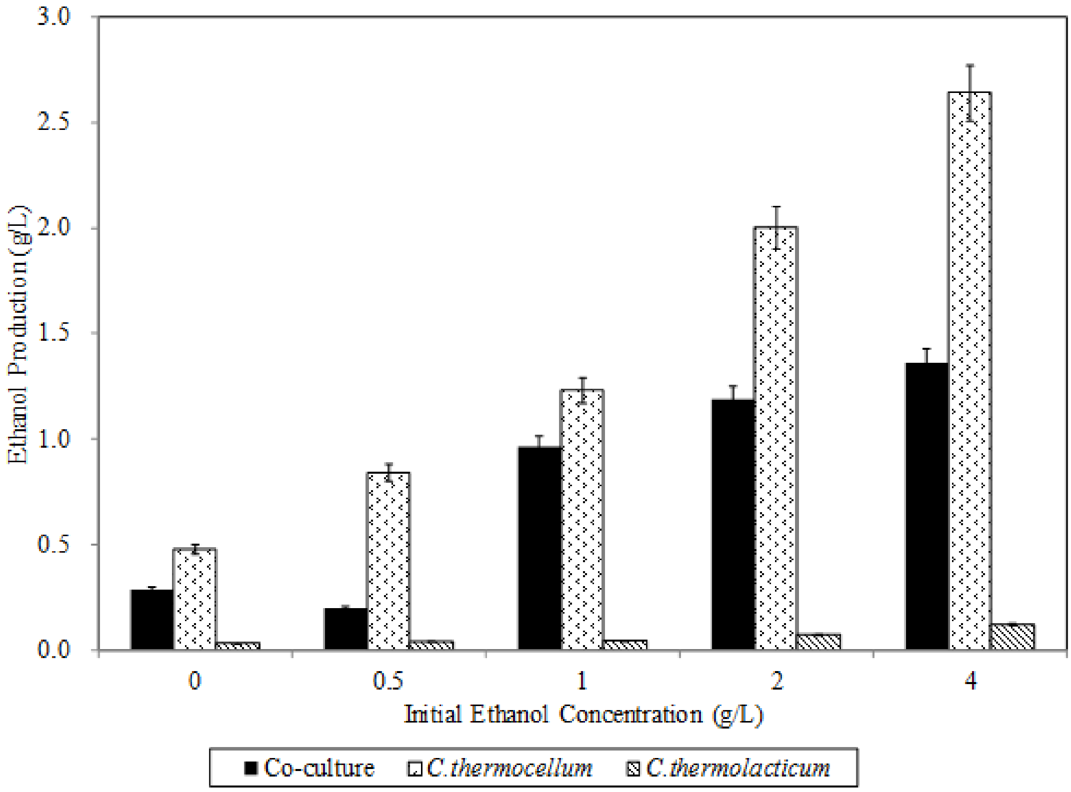


Figure 4.18 Effect initial ethanol concentrations on glucose fermentation at pH=9, 57ºC for 10 days

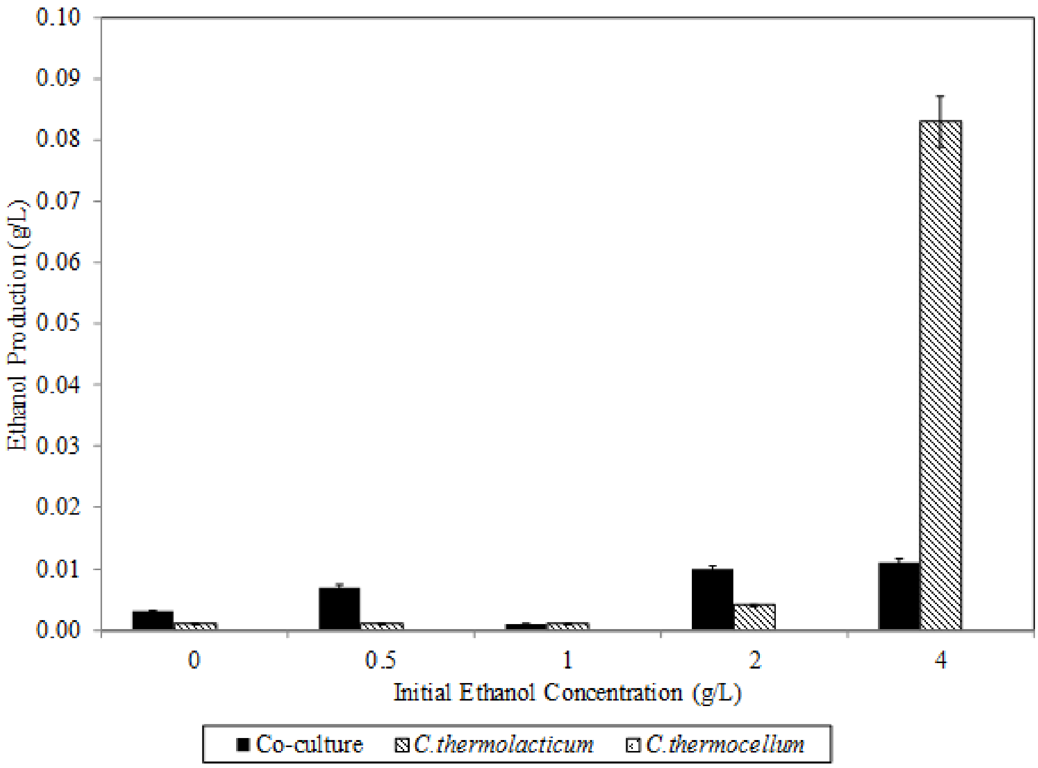


Figure 4.19 Effect initial ethanol concentrations on xylose fermentation at pH=9, 57ºC for 10 days

It is proven that the presence of ethanol will change the fatty acid composition of the cell membrane of *Clostridium* sp., which leads to higher cell membrane fluidity (303). Under extreme ethanol concentrations, which is over 8-16 g/L in this study, this fluidity change will lead to the blockage of the glycolysis, and results in low solvent production (304).

It is also conceivable that metabolic pathway regulation caused by the raising of the initial ethanol level can lead to higher ethanol production. In the co-culture fermentations, the enhancement of ethanol yield is accompanied with a decreased acetate yield. This observation suggests that the initial existence of ethanol might contribute to the shift of the co-culture fermentation pathway, shifting the hetero-fermentation towards the ethanol production, while reducing acetate production.

The other possible explanation is the interaction of the ethanol concentration and optimum culture temperature. In this study, the *C. thermocellum* is the major ethanol producer, the growth of *C. thermocellum* is extremely important. Previous studies suggest that the optimum temperature for the *C. thermocellum cultivation* will decrease with increasing ethanol or acetate concentrations within the medium (298). In order to test the effect of initial ethanol concentration on optimal temperature for this co-culture, the ethanol productions with the initial ethanol level at 0% and 4% were examined for the co-culture fermentation at several temperatures (55 ^{o}C – 65 ^{o}C). As shown in Figure 4.20-4.23, there was significant ($p< 0.05$) difference in ethanol production under the different fermentation temperatures at each initial ethanol level, indicating the temperature of fermentation strongly affects carbohydrate metabolisms including glucose, xylose, cellobiose and cellulose fermentations. A culture with 4 g/L ethanol was proven to have an optimum temperature at 57 ºC, instead of 60 ºC at 0 g/L ethanol level. This result agrees with the experiment performed by other researchers (298). Increasing the initial ethanol level from 0 g/L to 4 g/L, will lower the optimum cultivation temperature from 60 ºC to around 57 ºC which is close to the temperature chosen in this study. As a result, the lower the initial ethanol, the more the cultivation temperature (57 ºC) deviated from the optimum conditions, resulting in lower solvent productions.

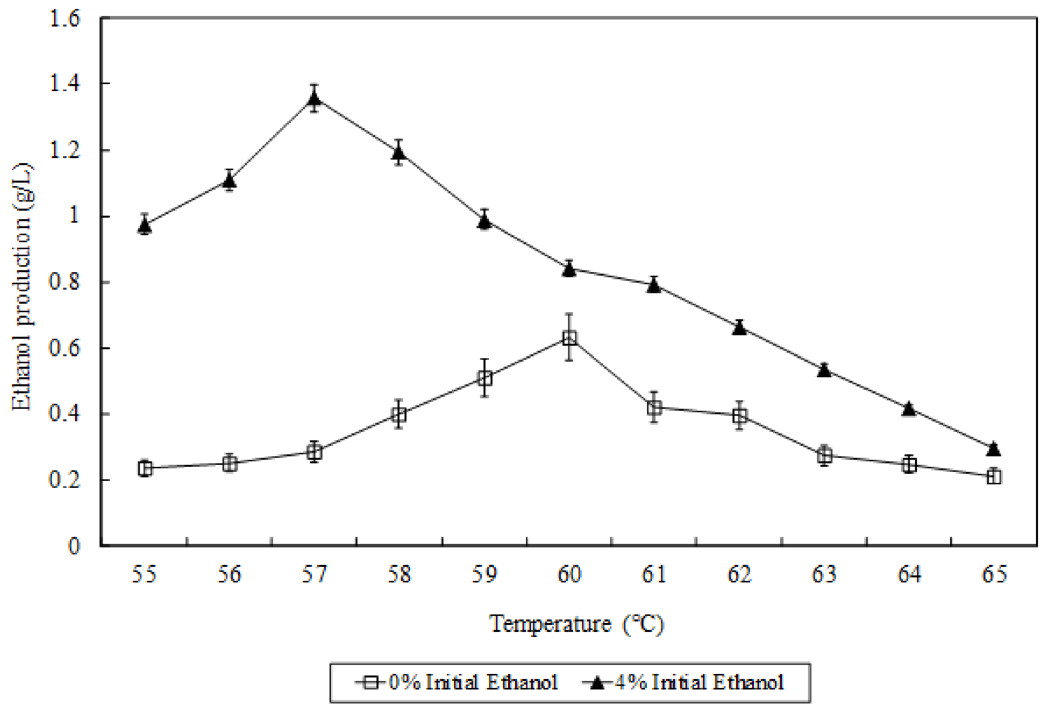


Figure 4.27 Relationship between optimum growth temperature and initial ethanol concentration for glucose fermentation at pH=9 for 10 days

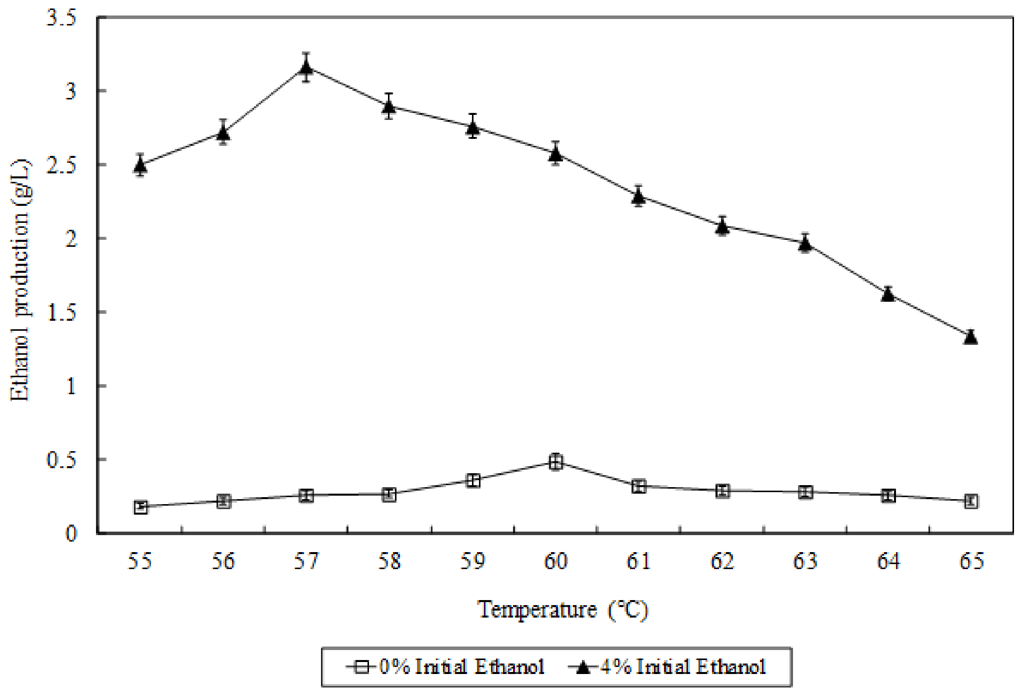


Figure 4.28 Relationship between optimum growth temperature and initial ethanol concentration for cellobiose fermentation at pH=9 for 10 days

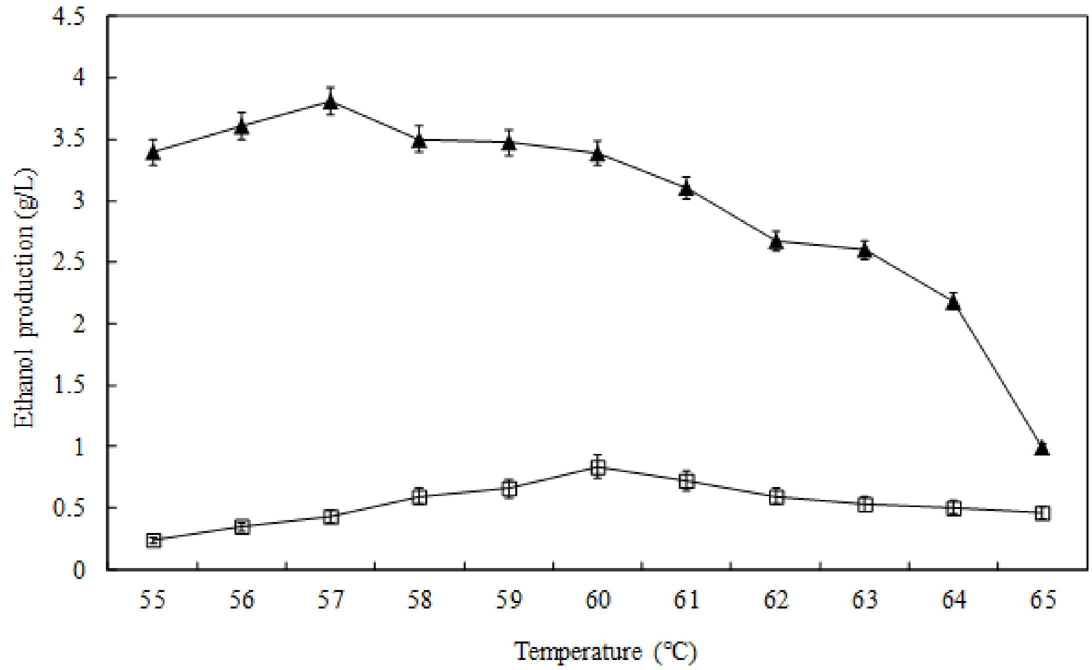


Figure 4.29 Relationship between optimum growth temperature and initial ethanol concentration for MCC fermentation at pH=9 for 10 days

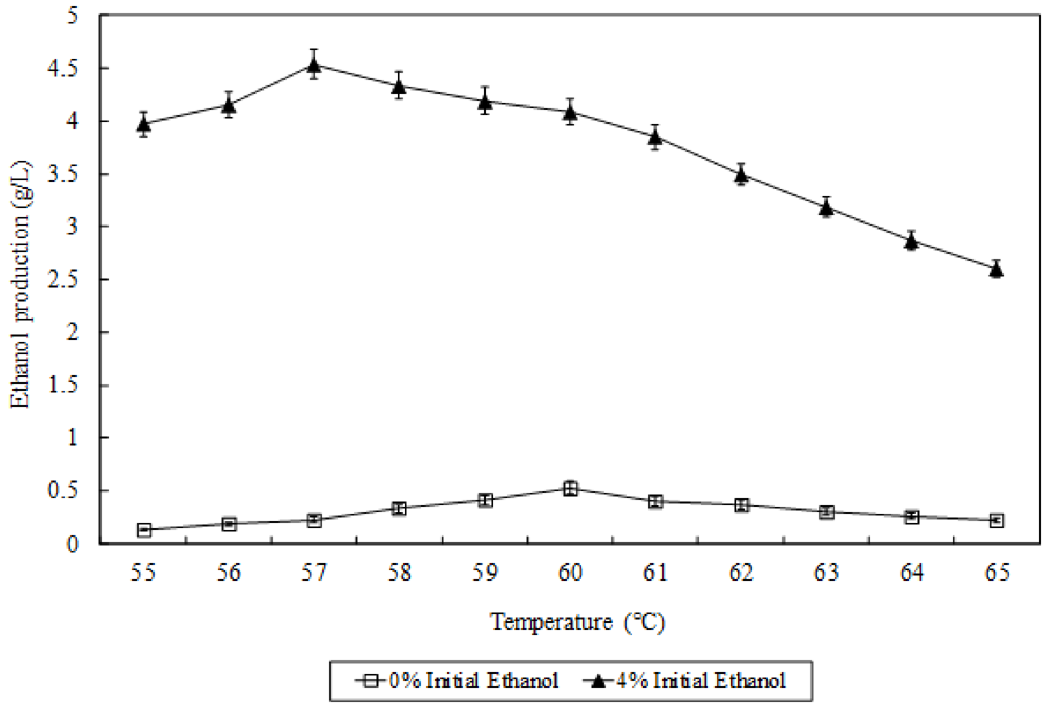


Figure 4.30 Relationship between optimum growth temperature and initial ethanol concentration for xylose fermentation at pH=9 for 10 days

No obvious effect was detected beyond this initial ethanol level (4 g/L) within the tolerance range. Further rising of the original ethanol concentration from 4 g/L to 8 g/L does not show additional improvement in solvent production for neither mono-cultures nor co-culture. Higher ethanol concentrations will lead to the termination of the fermentation. Increase of the initial ethanol concentration over 8 g/L stopped the fermentation activity completely for *C. thermocellum*, and the co-culture. Initial ethanol concentrations over 16 g/L will terminate the activity of *C. thermolacticum*.

4.2.3 Substrate selectivity

As shown in Table 4.1, 4.2, the substrate selection has significant impact on ethanol and acetate yield for co-culture fermentation as well as for mono-culture fermentations. Ethanol yield for *C. thermocellum* fermentation was non-detectible for hemicellulose and xylose conversions. This finding agrees with previous results demonstrating that *C. thermocellum* is unable to use xylose or xylan as sole carbon sources (305). Cellobiose and glucose were metabolized preferentially for both ethanol and acetate production. The efficiency of fermenting MCC is lower than fermenting simple sugars, including cellobiose and especially glucose under alkaline conditions (Figure 4.31, 4.32). Different from *C. thermocellum, C. thermolacticum* can directly convert all the selected substrates (crystalline cellulose, glucose, xylose and cellobiose) except xylan to ethanol and acetate. However, the proportions of these two major products are dramatically different between these two strains. For the conditions where the co-culture did not show any advantage, for example for glucose conversion at pH=9, the end-products obtained from *C. thermolacticum* fermentations were lower than product concentrations from *C. thermocellum* fermentation. Only exception was the fermentation of xylose. Starting ethanol concentration equal to 0 g/L at pH=6 is the only condition at which *C. thermolacticum* produces more product than *C. thermocellum* (Figure 4.33). These lower yields in *C. thermolacticum* fermentation can be explained by the media used in this study, which is optimized for *C. thermocellum* growth. The solvent production capacity of *C. thermolacticum* fermentation might not be fully utilized.

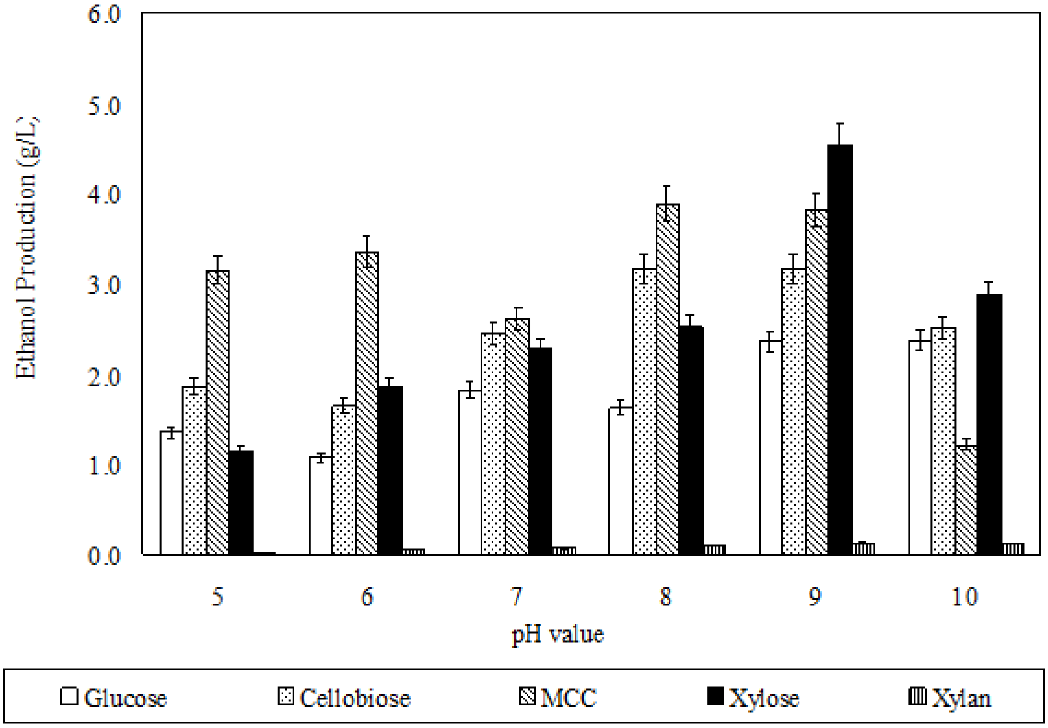


Figure 4.31 Effect of pH on ethanol production at 4 g/L initial ethanol level for co-culture fermentation on various substrates at 57 °C for 10 days

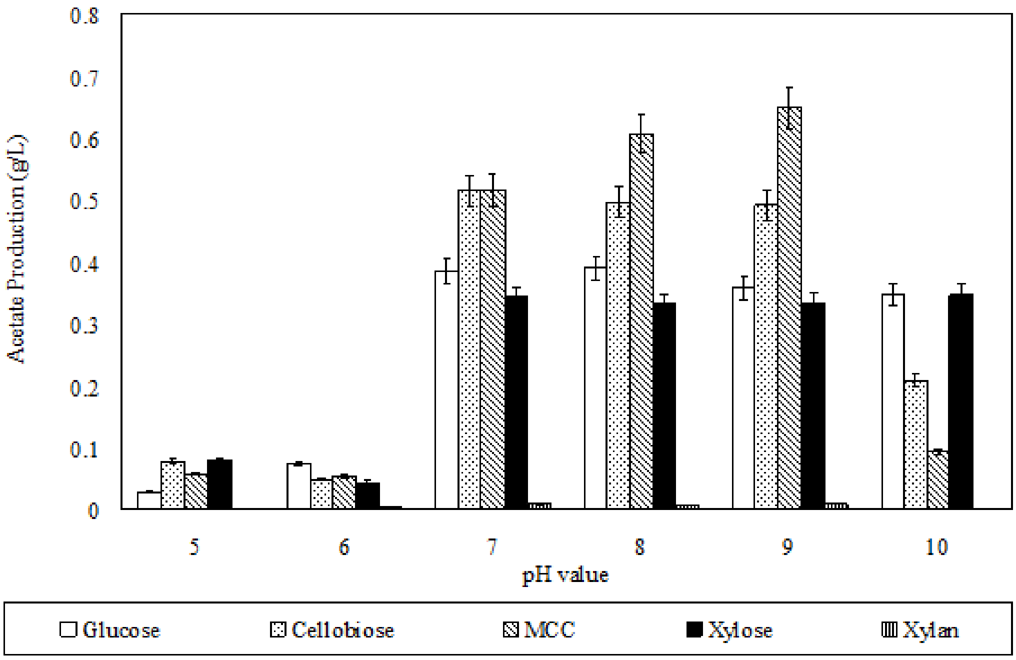


Figure 4.32 Effect of pH on acetate production at 4 g/L initial ethanol level for co-culture fermentation on various substrates at 57 °C for 10 days

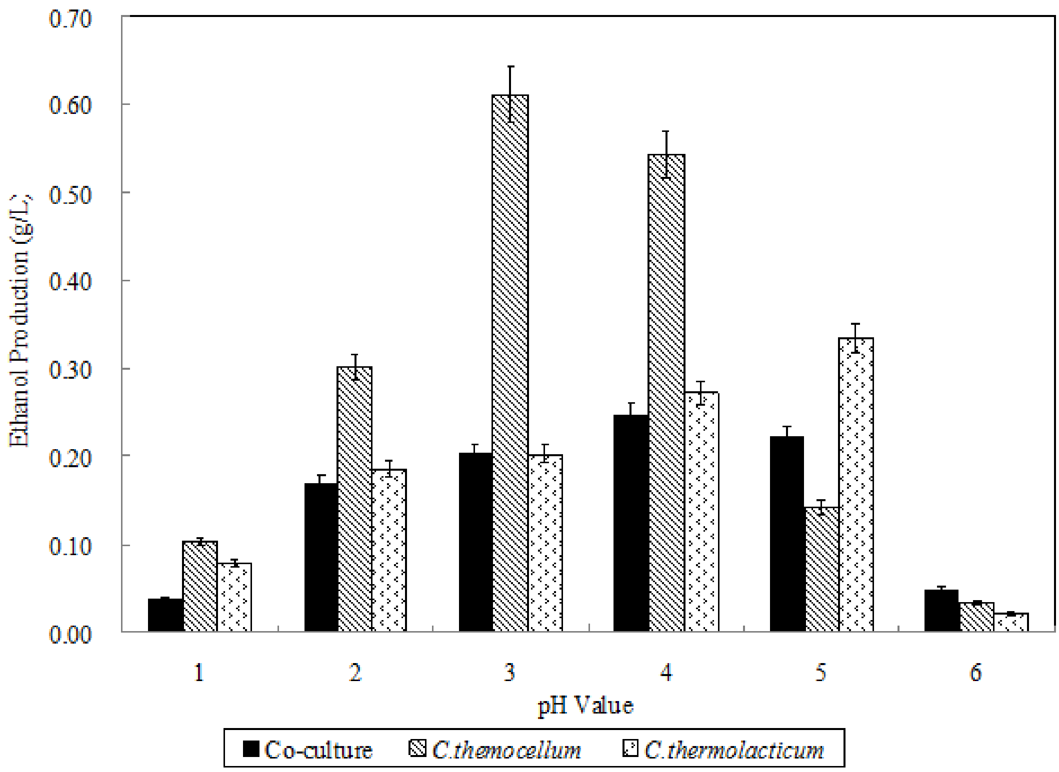


Figure 4.33 Effect of pH on acetate production at 0 g/L initial ethanol level for xylose fermentation at 57 °C for 10 days

The innovative co-culture is extremely efficiently in fermenting separate components of lignocellulosics for ethanol production. The substrates studied include cellulose, cellobiose, and xylose, which can be explained by selective substrate utilization ability observed in both strains. This improvement was most significant for ethanol yield at pH=9 with high initial ethanol level (4 g/L). Under these conditions, the ethanol yield from xylose was increased from 0.1 g/L for *C. thermolacticum* and 0 g/L for *C. thermocellum* to 4.5 g/L using the co-culture, which approaches 90% of the theoretical xylose fermentation efficiency (Table 4.1). This agrees with former studies showing the existence of cellulosic enzymes and xylanase in both strains (301, 302). The co-culture is extremely efficient in converting MCC to ethanol. Ethanol yields from MCC fermentation were dramatically improved through the use of a co-culture, from 1.4 g/L for *C. thermocellum* and 0.1 g/L for *C. thermolacticum* to a maximum of 3.8 g/L fro the co-culture. This represents 1.5 mol of ethanol per mol of sugar utilized for co-culture fermentation (around 75% of the theoretical efficiency) (306), almost three times the ethanol yield of the combined mono-cultures. For xylan and it fragments, an increase in the production of ethanol and acetate was observed with the application of the co-culture. No effective enzymes capable of fermenting high molecule weight xylan were reported in pervious studies for these two strains, only xylanase that can utilize degraded or partially degraded xylan was found (301, 302). In our case, the co-culture should only be able to utilize di- or mono-saccharides, including xylose. Nevertheless, tiny amounts of polymeric xylan are also consumed. Xylan fermentation appeared to be slower and less effective than conversion of other fermentable sugars. This small portion of xylan that was fermented is most possibly the modified xylan. The modifications like acetyl group and other side chain removal, took place during xylan preparation made part of the xylan more vulnerable to the enzymes. Thus, in this study, only small portion of xylan has found digested. In this case, by applying the co-culture of *C. thermolacticum* and *C. thermocellum* to lignocellulosic fermentation, optimization of the conditions should focus on the degradation conditions for cellulosic material and fragmentation of hemicellulose, mainly into xylose.

The polymerized hexoses appear to improve the fermentation end-product yields for the co-culture, whereas it depresses the fermentation for the mono-cultures. For fermentation applying a single strain, the substrate with simpler structure will be preferred. The cellobiose and glucose, which have simpler molecular structures, are more accessible than MCC since they don't have access restricting crystalline areas. This accessibility led to a higher microbial conversion which resulted in higher solvent productions compared to MCC. This result is consistent with earlier research suggesting that solubility can stimulate the fermentation (14).

It is clear that higher polymerization or lower solubility is able to initiate higher ethanol production during co-culture fermentation as compared to monocultures. For the co-culture increased utilization was observed for both cellobiose and MCC. MCC is proven to be the best substrate for ethanol production in the co-culture application in this study. However, decreased utilization of glucose was detected in co-culture as compared to mono-culture, and this observation is consistent under all chosen conditions. This is probably because for poly- or even di-saccharides, two strains are able to utilize different substrate instead of competing for one specific substrate as is the case with glucose in the system. While *C. thermocellum* is degrading di- or poly-saccharides into mono-saccharides (glucose) (307), *C. thermolacticum* and to some extent *C. thermocellum* ferment the glucose into end-products (31, 307). However, for the fermentation with only glucose, since both strains are able to utilize glucose, enzyme substrate competition is present between these strains, which means the total final yield in glucose fermentation is not improved.

4.2.4 Impact of pH value on co-culture fermentation

Determining the optimum pH range for both mono-culture fermentation systems is critical because of the interaction between pH value and substrate influence on microbial performance. However, the reported capability to digest the five chosen substrates for *C. thermocellum* and *C. thermolacticum* varies in previous reports due to the lack of uniform cultivation conditions (13, 216). In this work, the wild type

C. thermocellum strains ATCC 27405 and *C. thermolacticum* strain ATCC 43739 were grown in a standard medium as discussed by He and Sanford (300) on chosen substrates for 10 d. The influence of pH value on end product yield is shown in Table 4.1, 4.2. Fermentation yields for both strains were sensitive to pH changes. The sensitivity towards pH change was enhanced with the increase of initial ethanol concentration. When the starting ethanol concentration reaches 4 g/L, pH influence has the maximum input on ethanol production from all substrates and for all strains. No growth was found at a pH below 5 or above 10.

For the mono-cultures, pH=8 and pH=9 are the pH values providing the shortest lag period during fermentation for both strains, which is usually around 72 h. The optimum pH values for both strains dependent on the substrate. For cellulose and its fragments, including glucose, cellobiose and MCC, the largest ethanol production yields are found at pH=8 or 9. In contrast to this, for pentose fermentation, the biggest ethanol productions were seen at pH=6 to pH=9.

Alkaline conditions, especially pH equal to 9, is preferred for *C. thermocellum*/*C. thermolacticum* co-culture fermentation if the goal is high solvents production (Figure 4.24), confirming results obtained in our mono-culture fermentations (Figure 4.12, 4.34). When the pH was adjusted to 9, the co-culture fermentation was the most efficient ethanol producer, except for glucose. Almost all of the ethanol yields obtained from co-culture fermentation at this pH were higher than the sum of their mono-culture fermentations. At higher pH values (pH>9) the overall ethanol production is reduced and the advantage of a co-culture process disappeared for the mono sugars substrates. For MCC conversion, the co-culture was observed to be more effective than mono-cultures, even at less than optimum pH. No significant yield change was detected in xylan fermentation under high pH co-culture condition. A decline of ethanol formation was observed as pH decreased below 9. At lower pH, ethanol produced on selected substrates was significantly reduced.

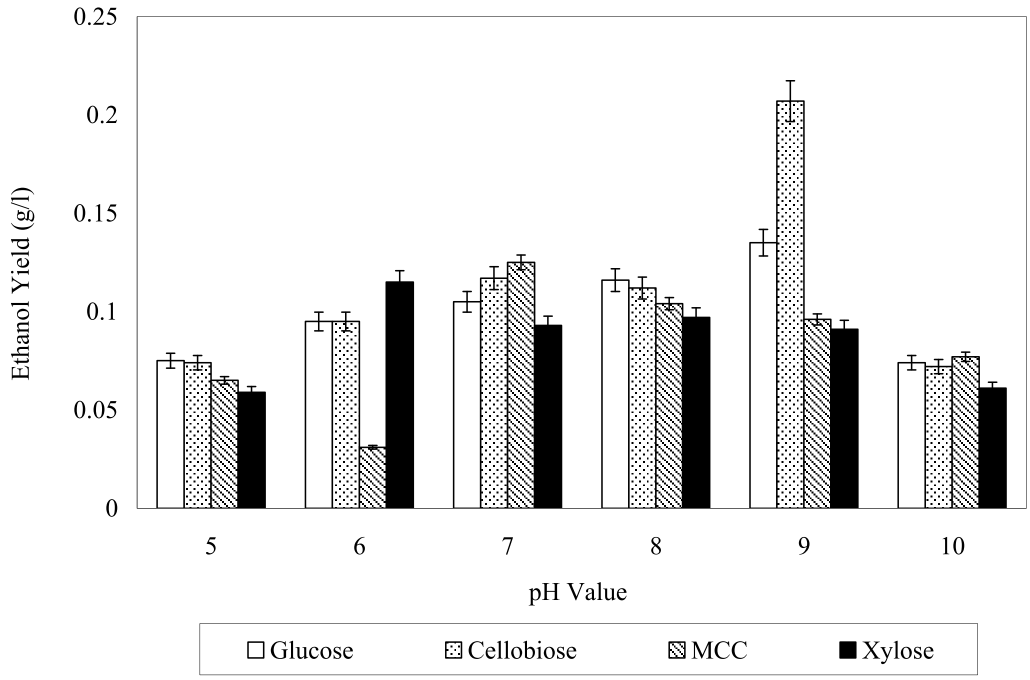


Figure 4.34 Effect of pH on ethanol production at 4 g/L initial ethanol level for C.thermolacticum fermentation on various substrates at 57ºC for 10 days

Similarly, the acetate production during fermentation by co-culture is higher when the strains were grown on cellulose, cellobiose, glucose, and xylose under neutral or alkaline conditions (Figure 4.34). Both pH=8 and pH=9 were considered as the optimum pH for acetate production since no significant yield difference was detected within this pH range. PH=7 resulted in a 0.1 g/L decrease in acetate production for MCC fermentation as compared to pH=9. Acetate production on none of the other substrates was observed to be affected by pH drop. However, once the pH dropped below 7, the acetate formed by co-culture fermentation dropped sharply from an average of 0.5 g/L to less than 0.1 g/L. In addition, under acidic conditions, co-culture application did not show significant advantages over mono-culture fermentations on cellobiose, glucose, hemicellulose and xylose (Figure 4.35, 4.36). The only exception is the MCC fermentation. A higher acetate yield always existed in co-culture fermentation of MCC, regardless of the pH. For acetate production, instead of *C. thermocellum* as observed for ethanol production, *C. thermolacticum* usually showed a better yield whenever co-culture did not provide any advantage.

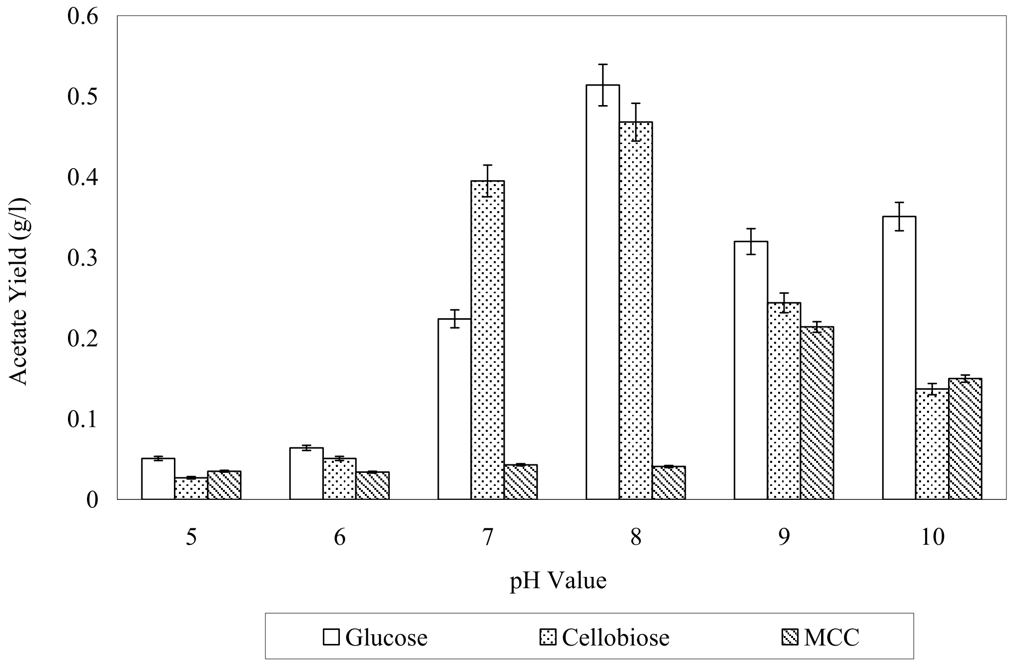


Figure 4.35 Effect of pH on acetate production at 4 g/L initial ethanol level for C. thermocellum fermentation on various substrates at 57ºC for 10 days

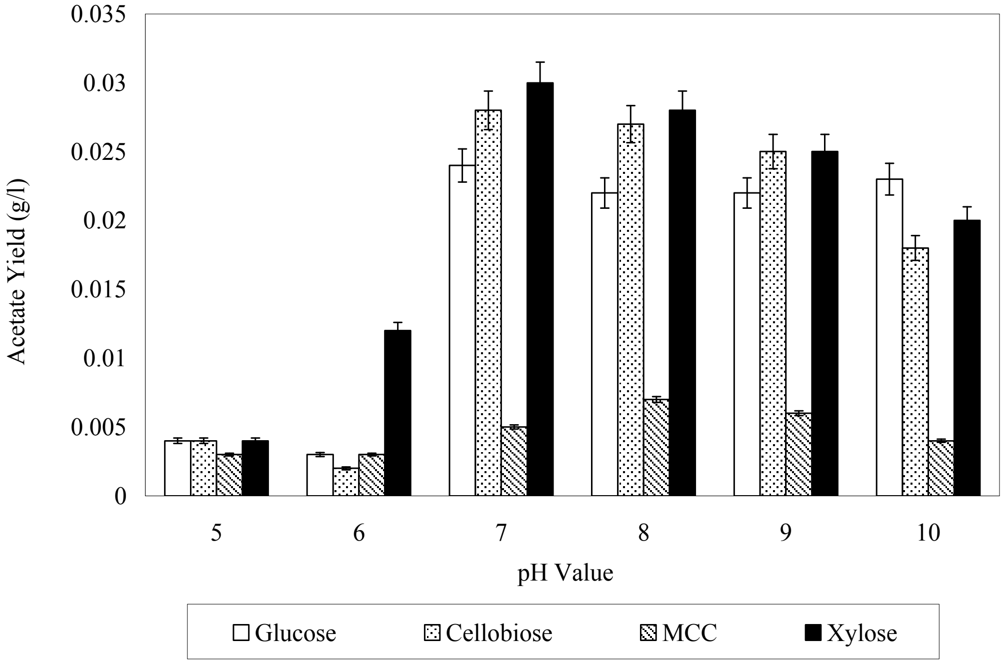


Figure 4.36 Effect of pH on acetate production at 4 g/L initial ethanol level for C. thermolacticum fermentation on various substrates at 57ºC for 10 days

In the relationship between pH and solvent production, mass action effects play the most important role. Both *C. thermocellum* and *C. thermolacticum* have a similar metabolic pathway. During hexose fermentation, both strains convert the substrates into pyruvate, and then into acetyl-coA and lactate. The acetyl-coA is fermented to a reductive product (ethanol) or an oxidative product (acetate) through the Embden-Meyerhof pathway (EMP) (31, 308). For xylose conversion, the pentose phosphate pathway and EMP are also important (309). In the Embden-Meyerhof pathway, during the formation of acetyl-CoA from pyruvate, pyruvate:ferredoxin oxidoreductase (E.C. 1.2.7.1) is the key enzyme to catalyzes the pyruvate oxidative decaboxylation (31). The optimum pH for this enzyme is reported to be around 7 (310). An activity decline of pyruvate:ferredoxin oxidoreductase will occur as the pH decreases below neutral condition (308). Instead of producing acetyl-CoA, glycolytic intermediates will form. Thus the yield of ethanol and acetate converted from acetyl-CoA dropped sharply under acidic condition. No obvious change in yield was detected by increasing pH from 7 to 9 for *C. thermolacticum* and from 8 to 9 for *C. thermocellum* in this research.

However, although acidic conditions reduced the solvent production, neutral condition (pH=7) are not the optimum pH for *C. thermocellum* and this co-culture, which indicates that other factors are involved in improving the co-culture activity. Higher acetate production compared to *C. thermolacticum* would be a possible reason leading to a higher optimum pH range. According to our observations, the accumulation of acetate within the medium will bring the pH down to 6 within the fermentation period which leads to lower yields. Thus, by applying higher initial pH, it will take longer for the system to drop below pH 7.

In summary, the co-culture produced the largest amount of acetate during fermentation under high initial ethanol levels, and the optimum pH of 9. For *C. thermocellum*, which had slightly lower acetate production compared to co-culture, pH=8 is efficient to provide a high pyruvate:ferredoxin oxidoreductase activity. This result suggests that in order to archive higher solvent productions, initial mild alkaline conditions, prompt removal of acetate from the system, or pH-control is essential.

4.2.5 Interaction among factors on formation of end-products

Based on ethanol and acetate yield after 10 d fermentation, statistical analysis indicated that the solvent yields were significantly related to the growth conditions, including the interactions among application of co-culture, initial ethanol level, pH value and substrate type. Interactions between each two factors, and among these four factors were found to be statistically significant (P<0.0001) at the 95% confidence level (Table 4.3). These results demonstrated that adjustment of pH, initial ethanol concentrations, substrates type and application of co-culture are all interrelated. Thus, the optimization of the co-culture fermentation should not only take the three main effects into account, the interactions among them cannot be ignored.

Source	DF	SS	MS	F Value	Pr > F 95% confidence level
Substrate	4	113.76	28.44	19238.3	< 0.0001
pH	5	13.67	2.73	185.04	< 0.0001
Initial Ethanol	4	200.67	50.17	33936.1	< 0.0001
Microbe	2	62.55	31.27	21155.4	< 0.0001
Substrate x pH	20	13.21	0.66	446.67	< 0.0001
Substrate x Initial Ethanol	16	52.67	3.29	2226.61	< 0.0001
Substrate x Microbe	8	62.75	7.84	5306.01	< 0.0001
pH x Initial Ethanol	20	3.97	0.20	134.37	< 0.0001
Microbe x pH	10	1.67	0.17	112.70	< 0.0001
Total Interaction	353	181.76	0.51	348.32	< 0.0001

Table 4.3 ANOVA table for ethanol production during free cell fermentation

4.2.6 Free cell fermentation of untreated aspen powder

The feasibility of fermenting untreated biomass by applying this co-culture was examined. Aspen powder, due to its complicated lignocellulosic structure, was expected to require longer fermentation times. At the optimized conditions, the highest ethanol production of 1.28 g/L was observed. This is significantly lower than ethanol yields observed with the pure sugar media (Figure 4.37). In addition, this maximum ethanol production was detected at pH=10 instead of pH=9 in pure saccharide fermentations. Higher wood loading rate did not have significantly positive effect on ethanol production. By changing the substrate amount from 1% to 1.5%, only a slight increase of ethanol production was observed. Further increasing the aspen concentration from 1.5% to 2% only changed the ethanol yield from 1.1 g/L to 1.2 g/L. Further increasing the wood loading rate from 2% to 10%, however, led to a decrease of ethanol production for co-culture fermentations.

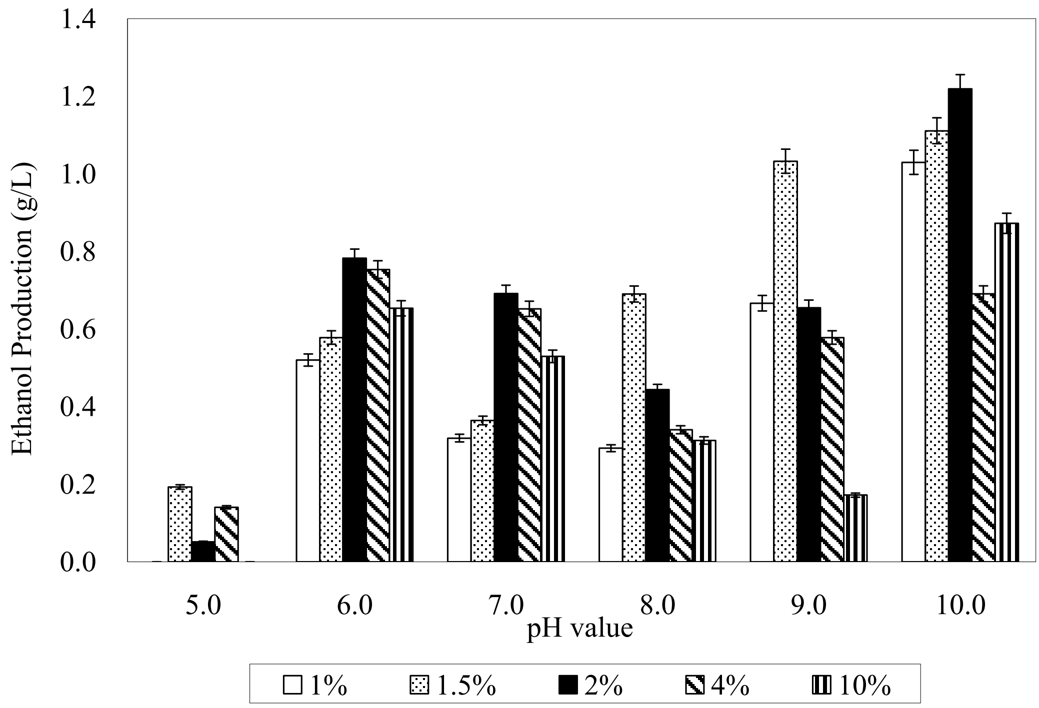


Figure 4.37 pH effects on ethanol yield for aspen co-culture fermentation at 57ºC for 10 days with wood loading rate from 1% to 10%

4.3 Immobilized cell fermentation

The *Clostridium* co-culture used in this study can effectively produce enzymes to degrade chosen substrates. But they were proved to be sensitive to pH changes as well as extreme ethanol concentrations. Therefore, as a potential way to modify the the microbes responses towards ethanol and pH changes, alginate immobilization was performed using the *Clostridium* co-culture. All attributes (immobilization, initial pH, substrates and initial ethanol concentration) as main effect variables as well as interactions among these factors were investigated. Regression analysis was conducted to characterize the association between immobilization, and the averaged ethanol productions or the average acetate production within the co-cultures. Comparisons fermentation efficiency between free cell fermentation and immobilized cell fermentation were also investigated under given initial ethanol concentration and given initial pH values. The outcome illustrated the benefit of using immobilization technique on this co-culture fermentation.

4.3.1 Benefit of immobilization

The benefit of applying the immobilization on chosen the *Clostridium* co-culture is clearly demonstrated in CBP fermentations in improving the ethanol production for selected substrates. This approach showed the potential of using both co-cultures and immobilization techniques for direct ethanol production from lignocellulosics biomass under alkali condition and up to 4 g/L of initial ethanol. Ethanol production is dramatically improved in immobilized cell fermentation as compared to free cell fermentations under almost all chosen conditions. Almost all the ethanol yields obtained from immobilized cell fermentation are above any of the free cell fermentation yields, approaching over 80% of the theoretical conversion efficiency under identical conditions on all the substrate. The application of immobilization is shifting the metabolism of ethanol production into acetate production. The acetate generation, which is considered to have a positive contribution to final ethanol production, turns out to lie in the same narrow range irrespective of the substrate or pH value used (300). However, the correlation between ethanol productivity improvement and increased E/A ratio is missing, suggesting that mass action effects are not the only factors that influencing the final ethanol production during immobilized cell fermentations. The other potential influences might be caused by the existence of Ca^{2+}, or the property changes caused by cells' growth state change.

4.3.2 Impact of immobilization on ethanol tolerance

The productions of ethanol and acetate as measured in the liquid samples after completion of the fermentations were depicted in Figure 4.38. According to the ANOVA (table 4.4), even by applying immobilization, the initial ethanol concentration still plays a critical role in adjusting the final solvent productions ($p<0.005$). However, the effect of immobilization and the interaction between immobilization and initial ethanol concentration appeared to be not statistically significant by holding pH values and substrates constant. The only expectations are the glucose fermentation at pH=10, xyolse fermentation at pH=8, and MCC fermentation at pH=9. These results suggest that the immobilization method applied is not able to change the cells responds towards ethanol concentration changes for this co-culture. This might be caused by the pore size of the alginate gel produced. Existence of flavin, nicotinamide, and cytochrome in the medium is proved to be able to diffuse 1% (w/v) of gel out of the pellets (295). Thus, the pore sizes of the pellets were enlarged when the encapsulation was performed within the cultural medium. The ethanol molecule was able to permeate the alginate gel structure freely, indicating that the gel was not able to set up a protection for the *Clostridium* sp. Extensive polymerization of the alginate gel would be a possible way to provide extra protection for *Clostridium* co-culture against ethanol. However, over-protection of the co-culture would cause the reduction of the positive relationship between initial ethanol concentration and final ethanol production under 0-4 g/L initial ethanol levels. High degree of polymerization would also make the transition of nutrients and wastes difficult. Thus, further augmentation of the gel polymerization by increasing alginate concentration is not recommended for final ethanol production enhancement. Although the micro-organisms behavior changes with the phase of medium, the ethanol production alteration due to the ethanol concentration change remained the same. Similar to the free cell fermentations, as the initial ethanol concentration increases from 0 g/L to 4 g/L, the final ethanol production is dramatically improved for all substrate under all chosen pH values. Continuously increasing the initial ethanol concentration to 8g/L still led to the decline of final ethanol production. Over 40% decrease in ethanol productions were observed under all conditions when the initial ethanol concentration was increase from 4 g/L to 8 g/L for immobilized cell fermentations. Except for the fermentations performed under pH=7, and glucose fermentation performed under pH=10, the ethanol production were considerably low (<1.0g/L) at 8g/L initial ethanol level. Therefore, the application of immobilization is not considered to be an efficient way to avoid ethanol inhibition at extreme levels.

		PH=7	PH=8	PH=9	PH=10
Glucose	Initial Ethanol	<.0001	0.0003	0.0007	<.0001
	Immobilization	0.4944	0.3216	0.5346	0.0238
	Interaction	0.0098	0.5734	0.0194	0.2586
Xylose	Initial Ethanol	<.0001	<.0001	0.0010	<.0001
	Immobilization	0.7125	0.0258	0.3060	0.1764
	Interaction	0.6684	0.9239	0.4982	0.4587
Cellobiose	Initial Ethanol	<.0001	<.0001	<.0001	0.0011
	Immobilization	0.2977	0.5233	0.2214	0.4381
	Interaction	0.3941	0.2425	0.2430	0.6542
MCC	Initial Ethanol	<.0001	<.0001	<.0001	0.0004
	Immobilization	0.0083	0.6586	0.0011	0.5906
	Interaction	0.0690	0.7619	0.6655	0.3081

Table 4.4 P value for comparison of ethanol production for immobilized cell fermentation and free cell fermentation. Variables include initial ethanol, immobilization and the interaction between these two.

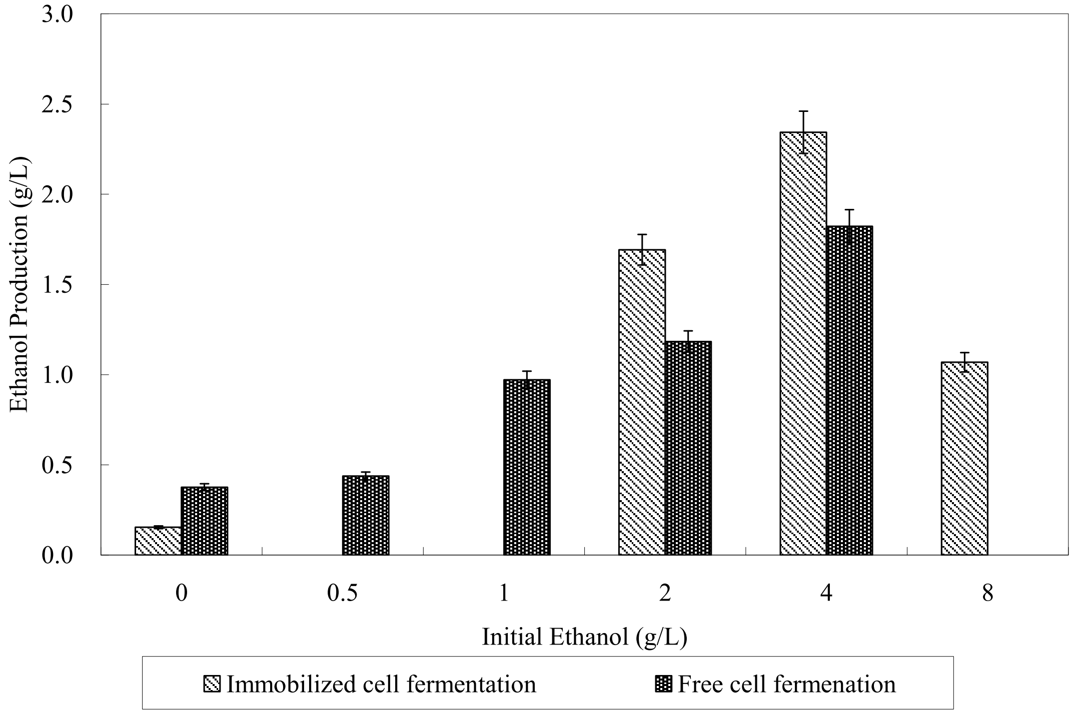


Figure 4.38a Comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for glucose fermentation at pH = 7, 57ºC for 10 days

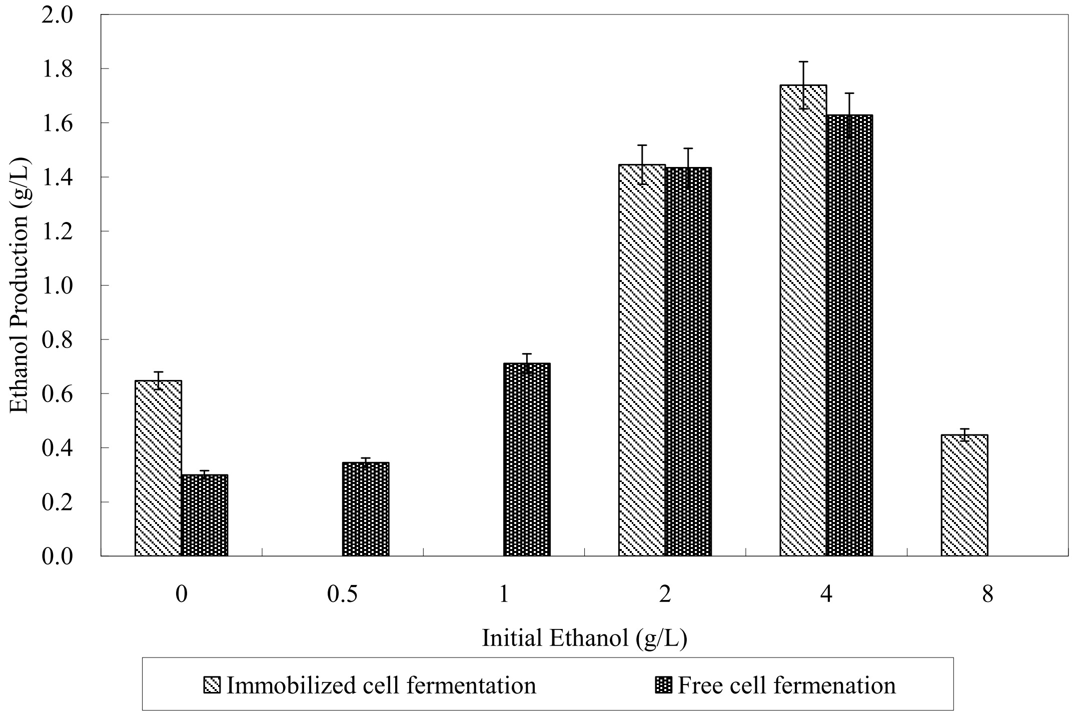


Figure4.38b Comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for glucose fermentation at pH = 8, 57ºC for 10 days

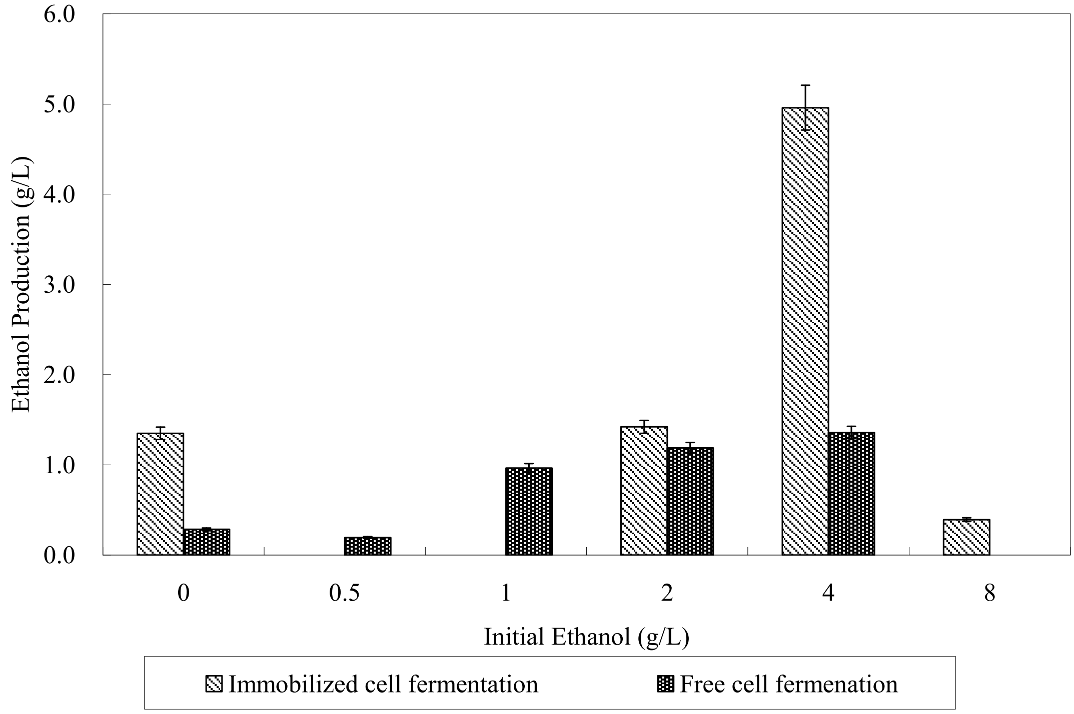


Figure 4.38c Comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for glucose fermentation at pH = 9, 57ºC for 10 days

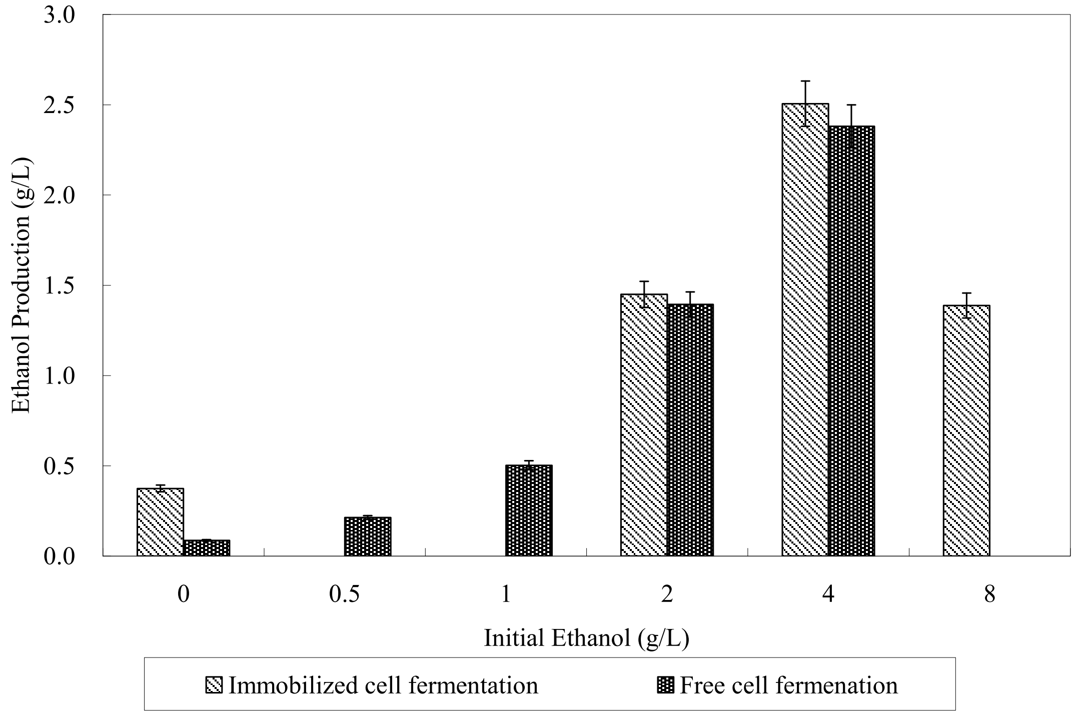


Figure 4.38d Comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for glucose fermentation at pH = 10, 57ºC for 10 days

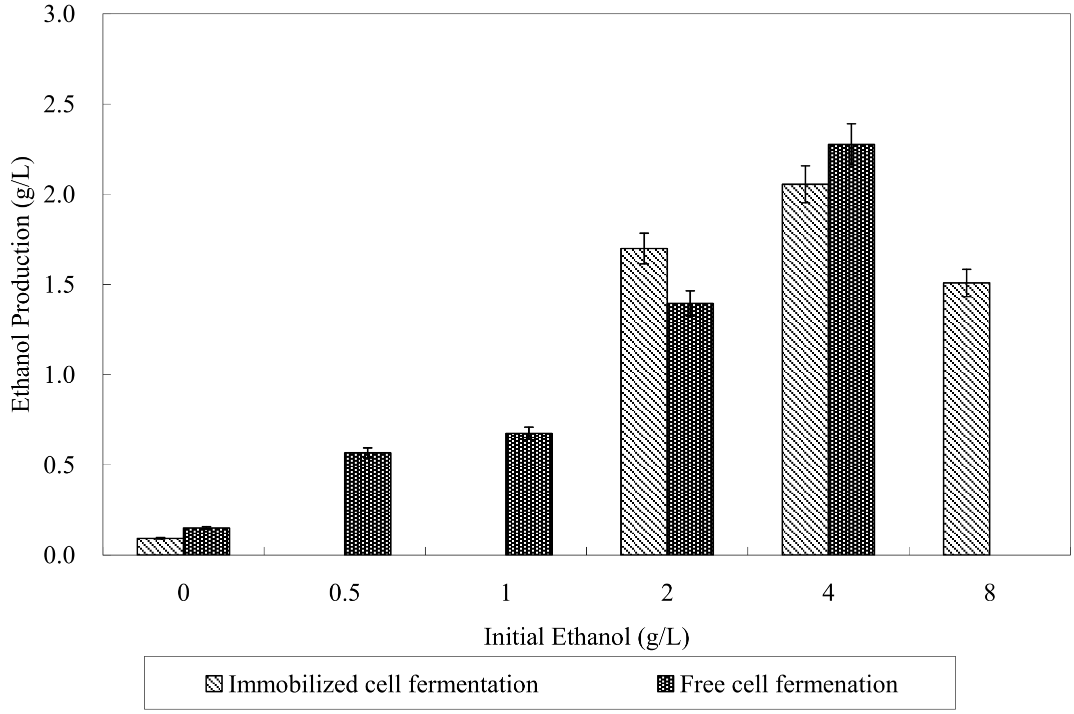


Figure 4.38e Comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for xylose at pH = 7, 57ºC for 10 days

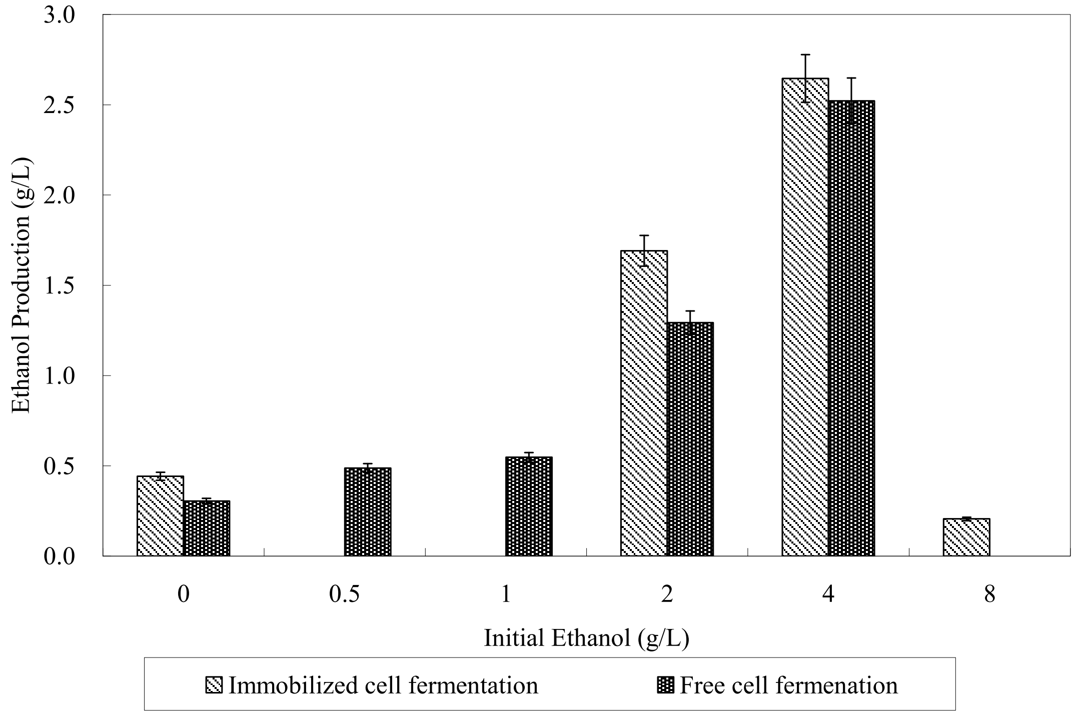


Figure 4.38f Comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for xylose fermentation at pH = 8, 57ºC for 10 days

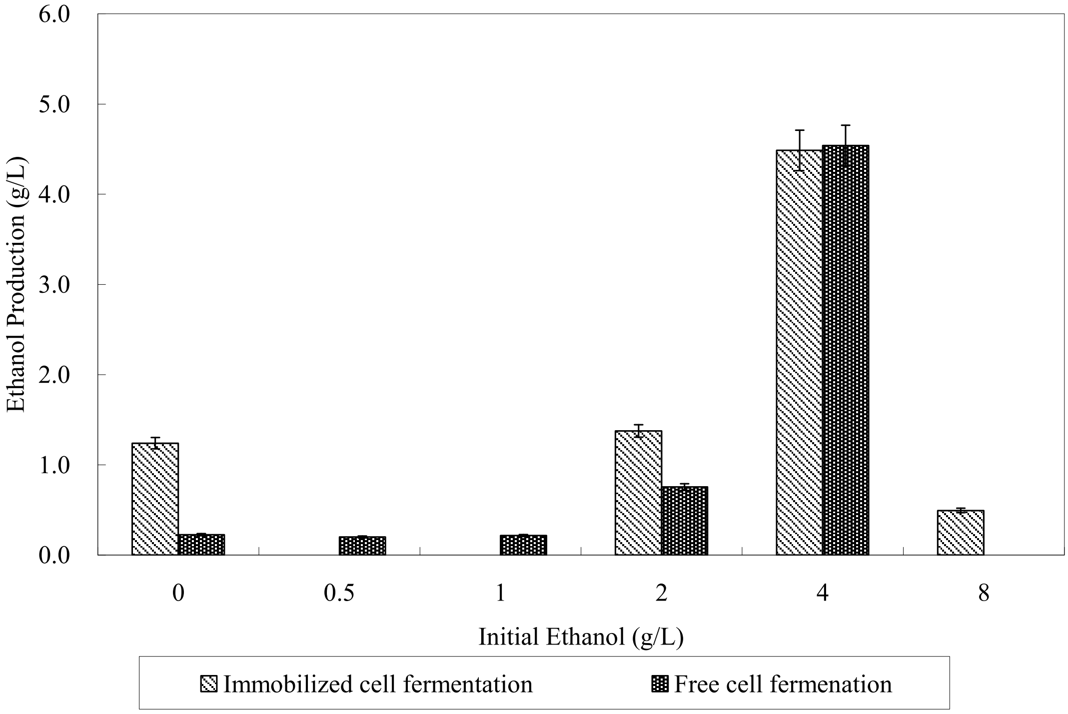


Figure 4.38g Comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for xylose at pH = 9, 57ºC for 10 days

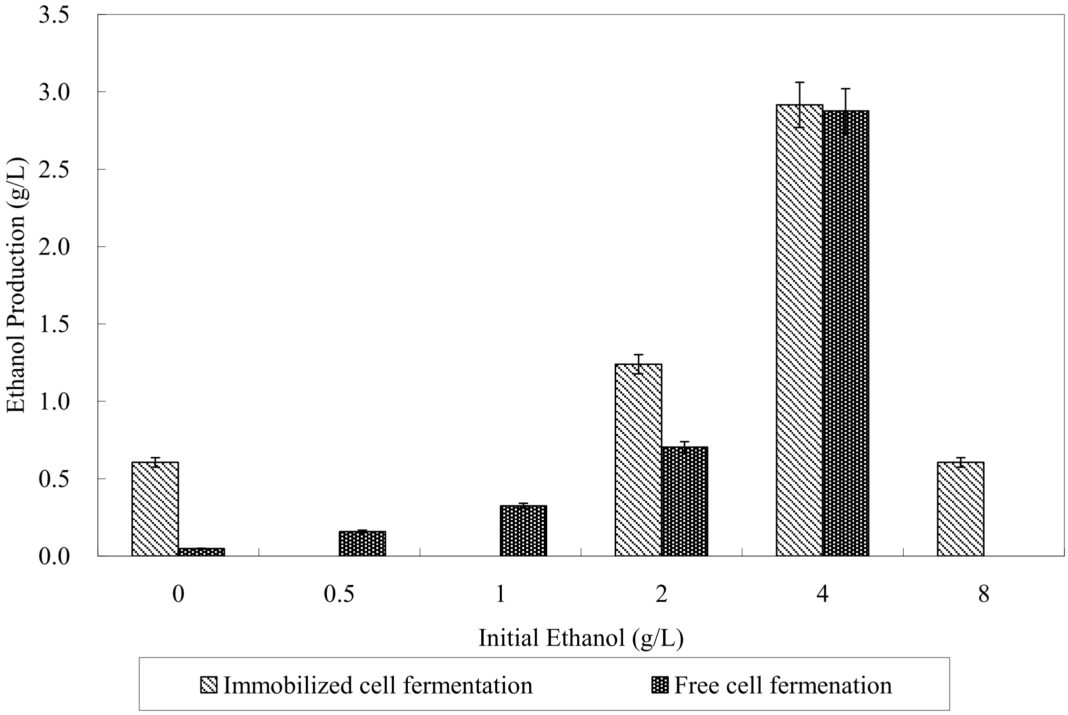


Figure 4.38h Comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for xylose fermentation at pH = 10, 57ºC for 10 days

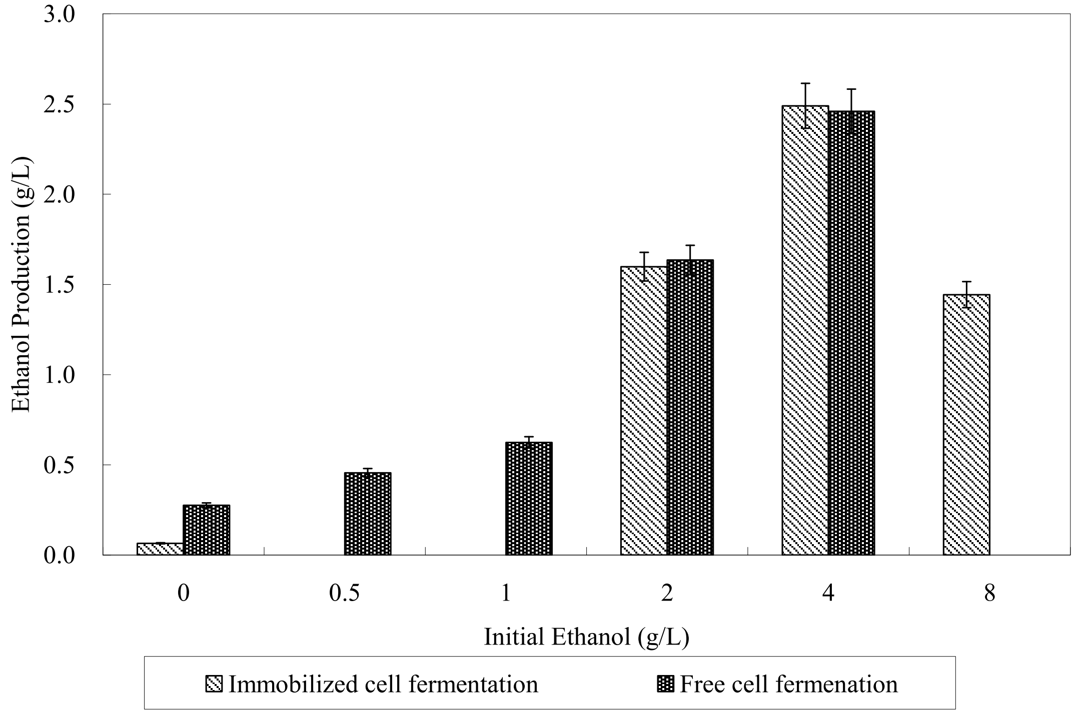


Figure 4.38i Comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for cellobiose fermentation at pH = 7, 57ºC for 10 days

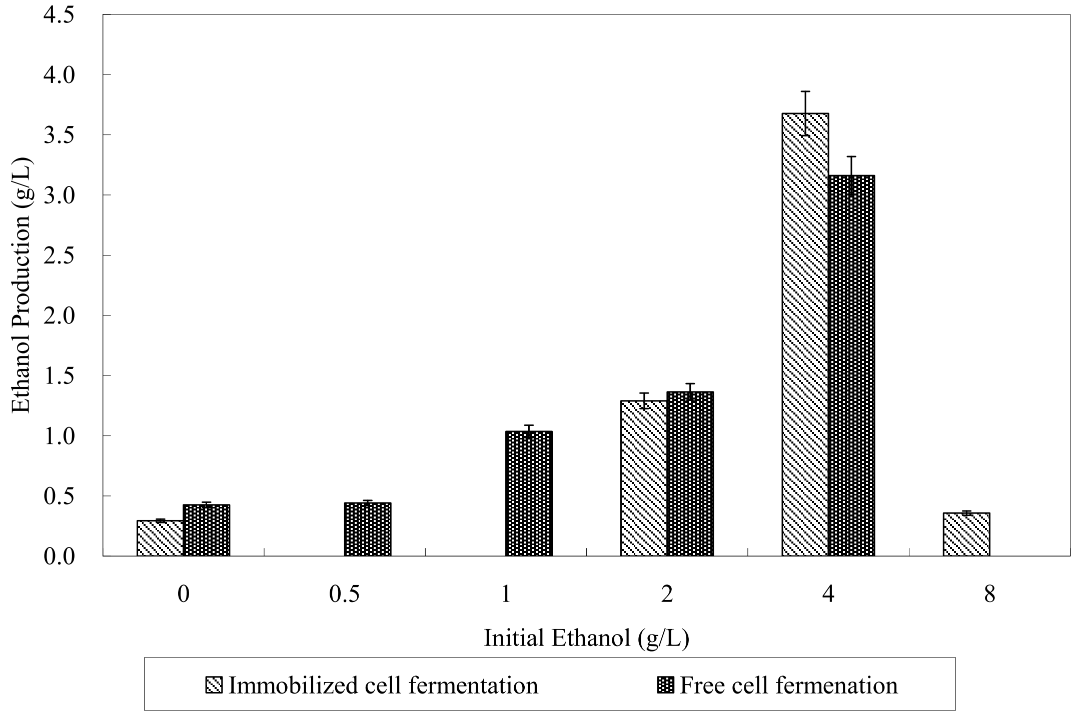


Figure 4.38j Comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for cellobiose fermentation at pH = 8, 57ºC for 10 days

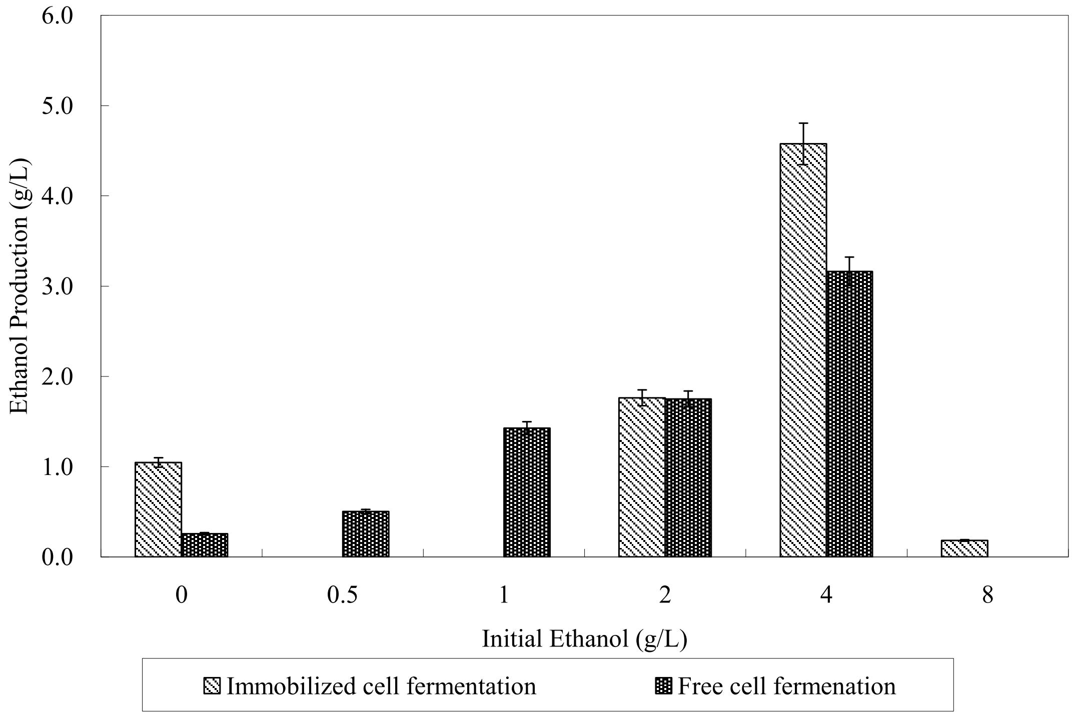


Figure 4.38k Comparison between immobilized cell fermentation and free cell fermentation at various ethanol levels for cellobiose fermentation at pH = 9, 57ºC for 10 days

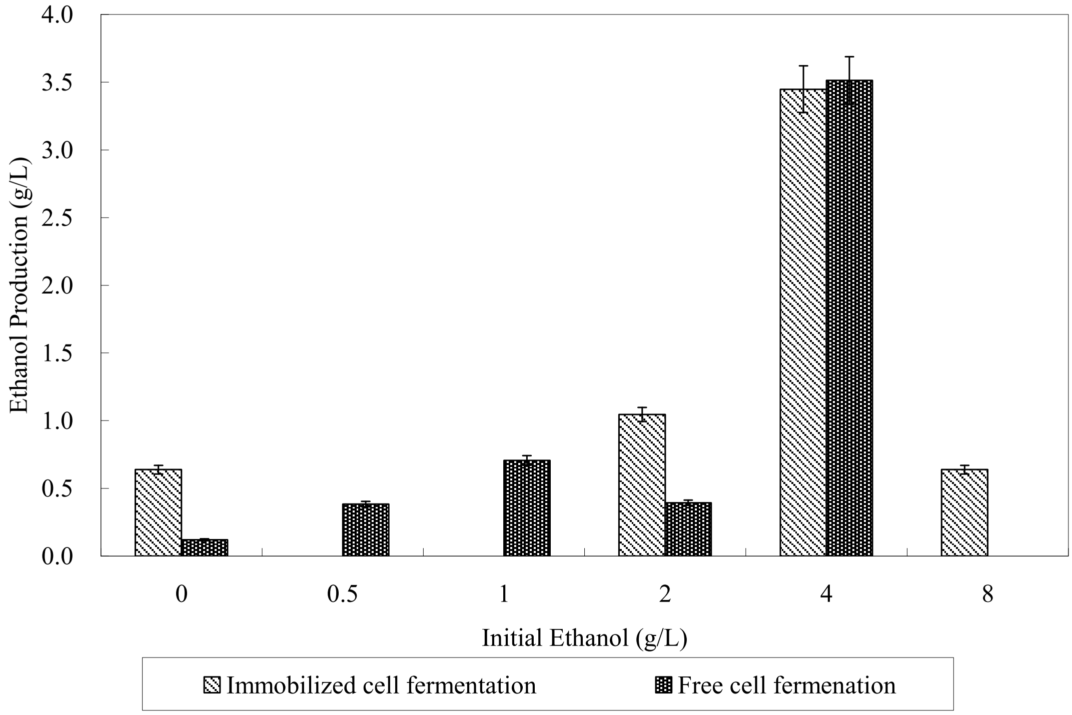


Figure 4.38l Comparison between immobilized cell fermentation and free cell fermentation at various ethanol levels for cellobiose fermentation at pH = 10, 57ºC for 10 days

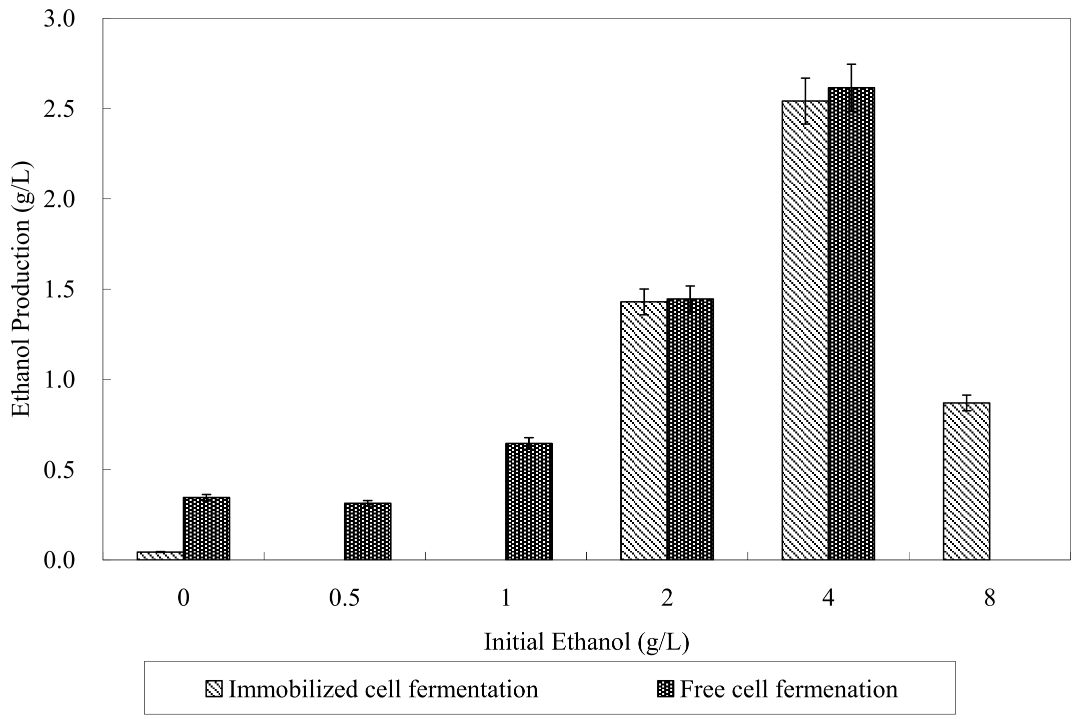


Figure 4.38m Comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for MCC fermentation at pH = 7, 57ºC for 10 days

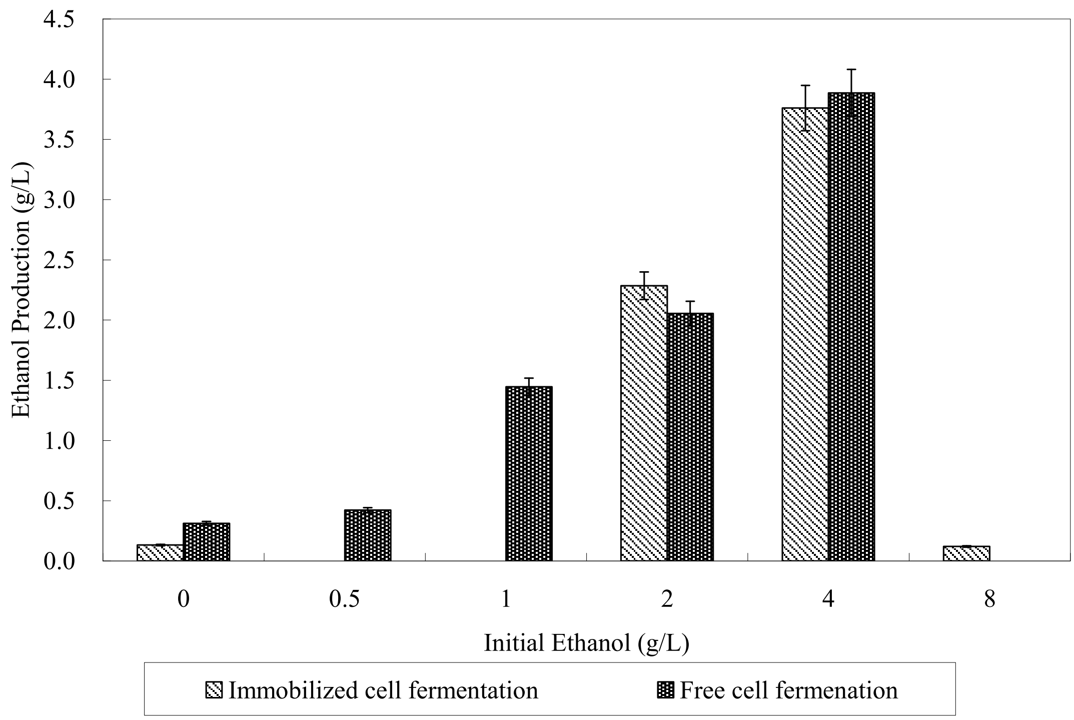


Figure 4.38n Comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for MCC fermentation at pH = 8, 57ºC for 10 days

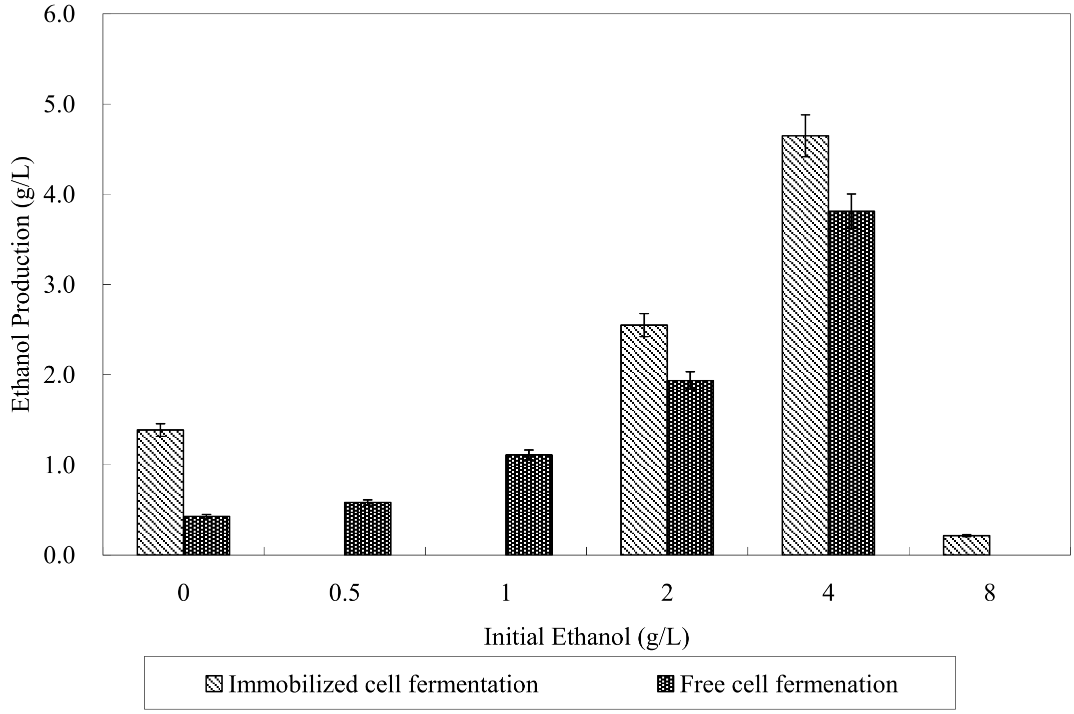


Figure 4.38o Comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for MCC fermentation at pH = 9, 57ºC for 10 days

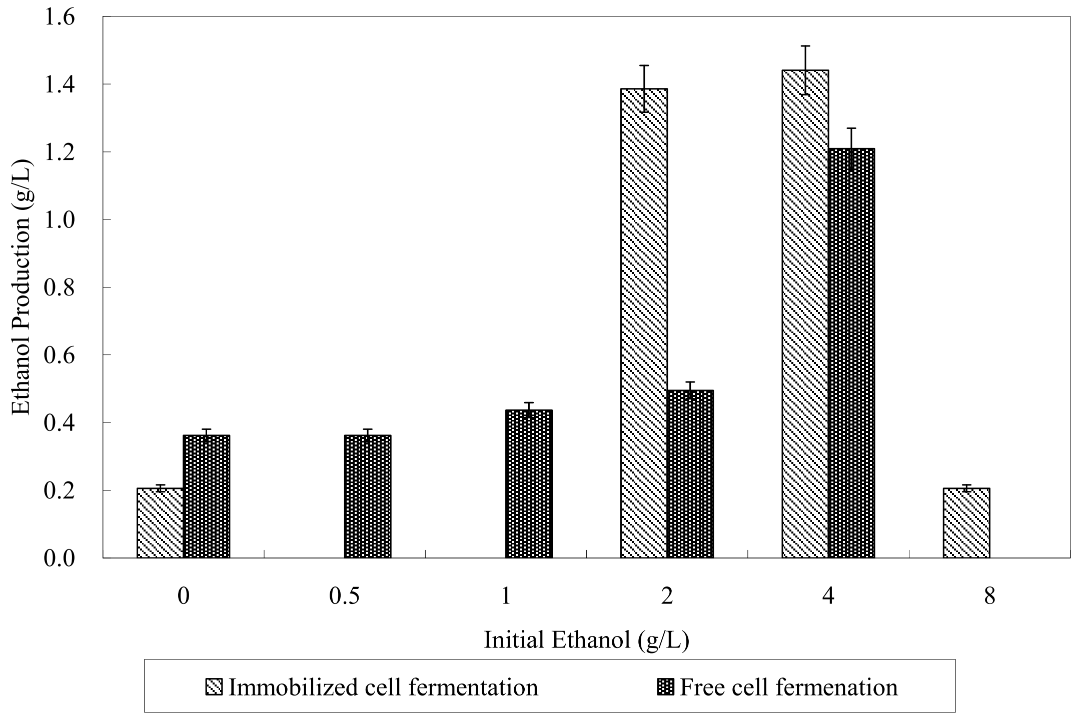


Figure 4.38p Comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for MCC fermentation at pH = 10, 57ºC for 10 days

Although the ethanol production change was reported insignificant, the immobilization technique was shown to be able to reduce acetate production, the major by-product during fermentation. As shown in Table 4.5, immobilization modified the co-culture's ability to produce acetate. Noticeably, less amount of acetate were produced as compared to free cell fermentation under all conditions, except for xylose fermentation at pH=7 ($p<0.05$) (Figure 4.39). Especially at pH=8, the production of acetate dropped to less than 0.1g/L for all substrates, indicating this immobilization technique is able shift the hetero-fermentative pathway towards the homo-fermentative pathway by blocking the acetate production mechanisms or shifting the acetate production to other by-products production like carbon dioxide, since the increase of ethanol production is not related to the decrease of acetate production. This production reduction is caused by the property changes of *Clostridium* spp. after immobilization. It is possible that the immobilization change *Clostridium* cell cultivation environment from liquid phase into solid phase.

		PH=7	PH=8	PH=9	PH=10
Glucose	Initial Ethanol	0.4389	0.0027	0.0007	0.3548
	Immobilization	0.0096	<.0001	0.0276	0.0174
	Interaction	0.0123	0.0095	0.5583	0.0996
Xylose	Initial Ethanol	0.1333	0.0025	0.0038	0.6624
	Immobilization	0.0003	<.0001	0.0113	0.8874
	Interaction	<.0001	0.0083	0.0177	0.4899
Cellobiose	Initial Ethanol	0.0200	0.0013	0.0483	0.2834
	Immobilization	<.0001	<.0001	0.1717	0.2533
	Interaction	<.0001	0.0028	0.8717	0.0201
MCC	Initial Ethanol	0.7493	0.0006	0.0039	0.3467
	Immobilization	<.0001	<.0001	0.0375	0.0782
	Interaction	0.0004	0.0132	0.9760	0.0759

Table 4.5 P value for comparison of acetate production between free cell fermentation and immobilized cell fermentation. Variables include initial ethanol, immobilization and the interaction between these two.

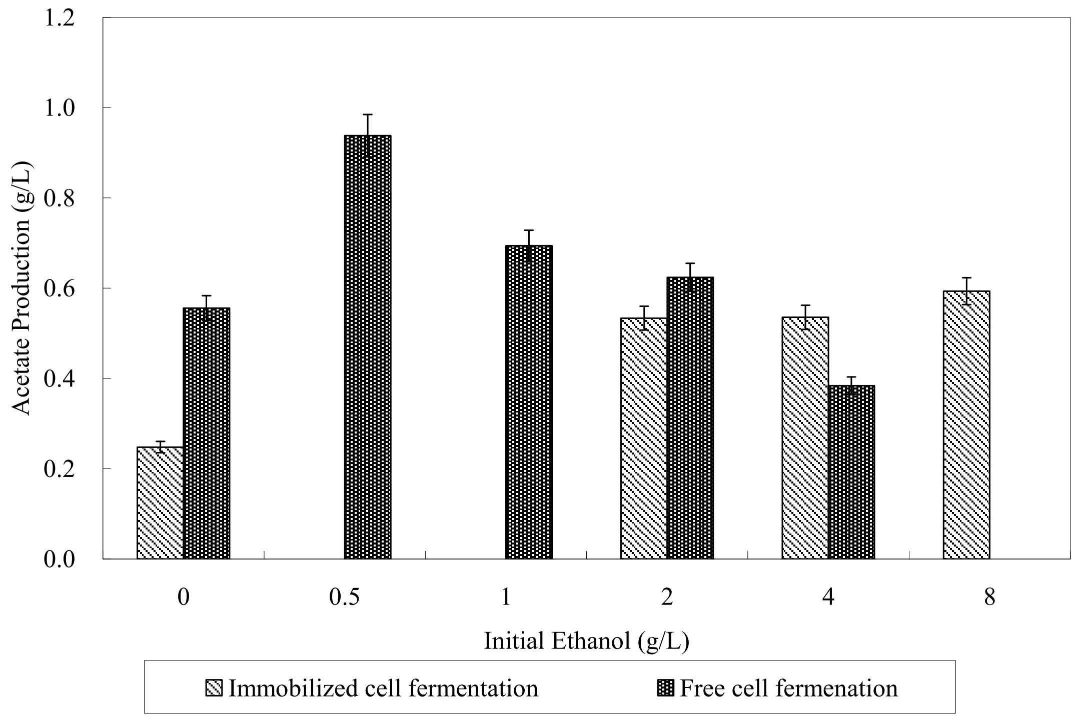


Figure 4.39a Acetate production comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for glucose fermentation at pH = 7, 57ºC for 10 days

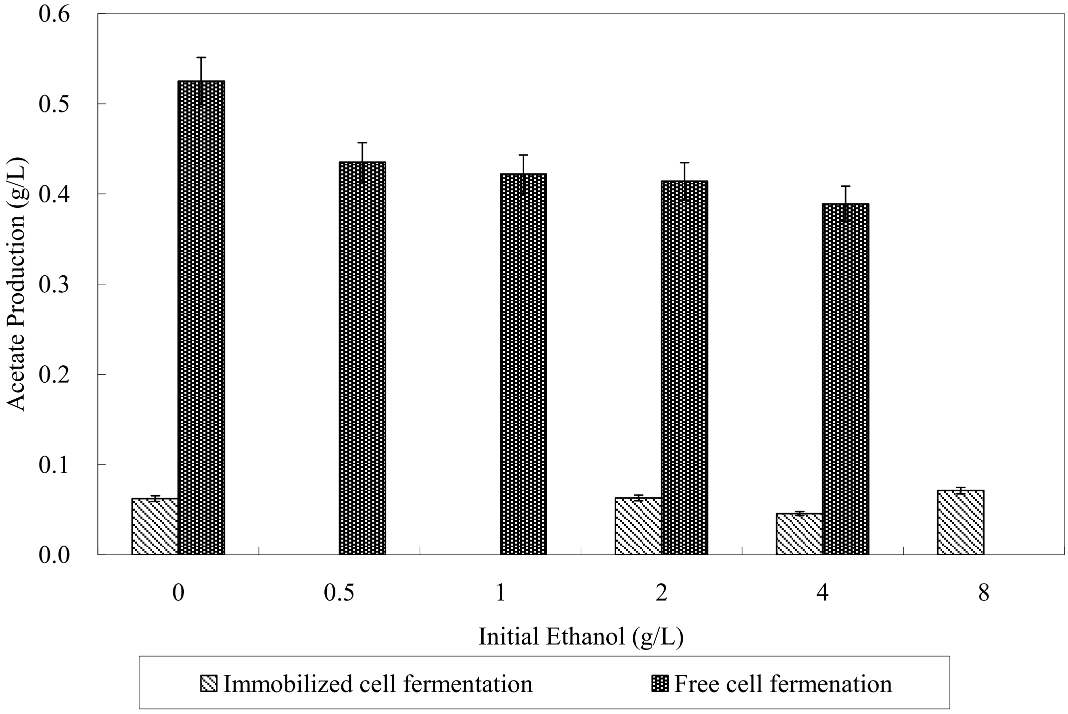


Figure 4.39b Acetate production comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for glucose fermentation at pH = 8, 57ºC for 10 days

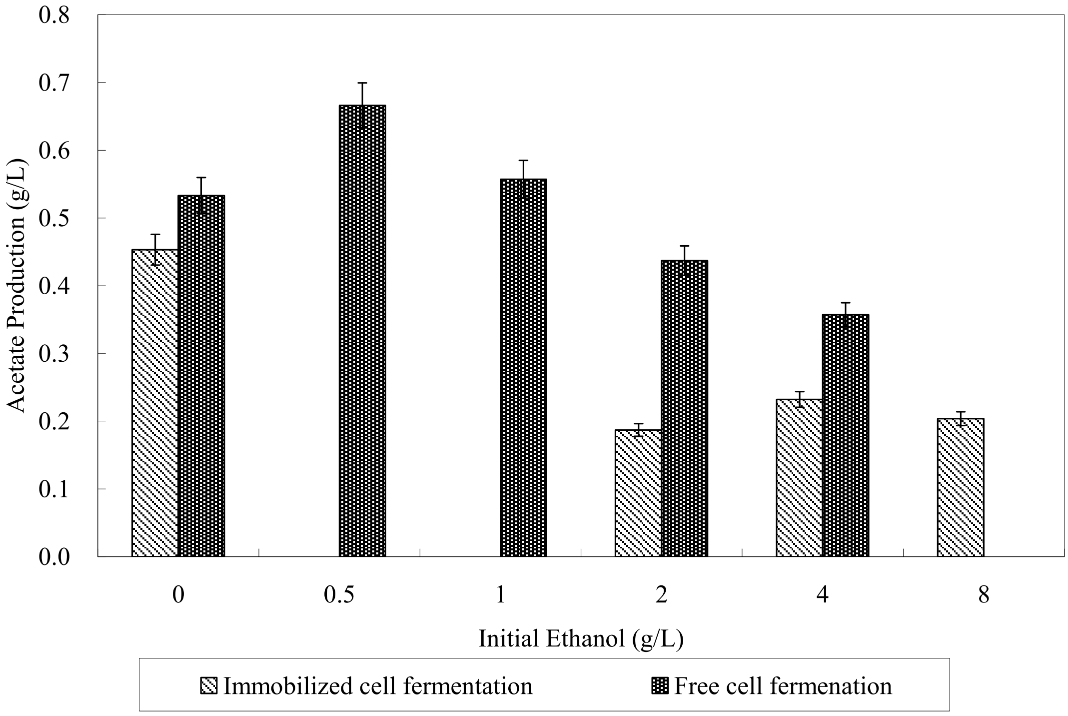


Figure 4.39c Acetate production comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for glucose fermentation at pH = 9, 57ºC for 10 days

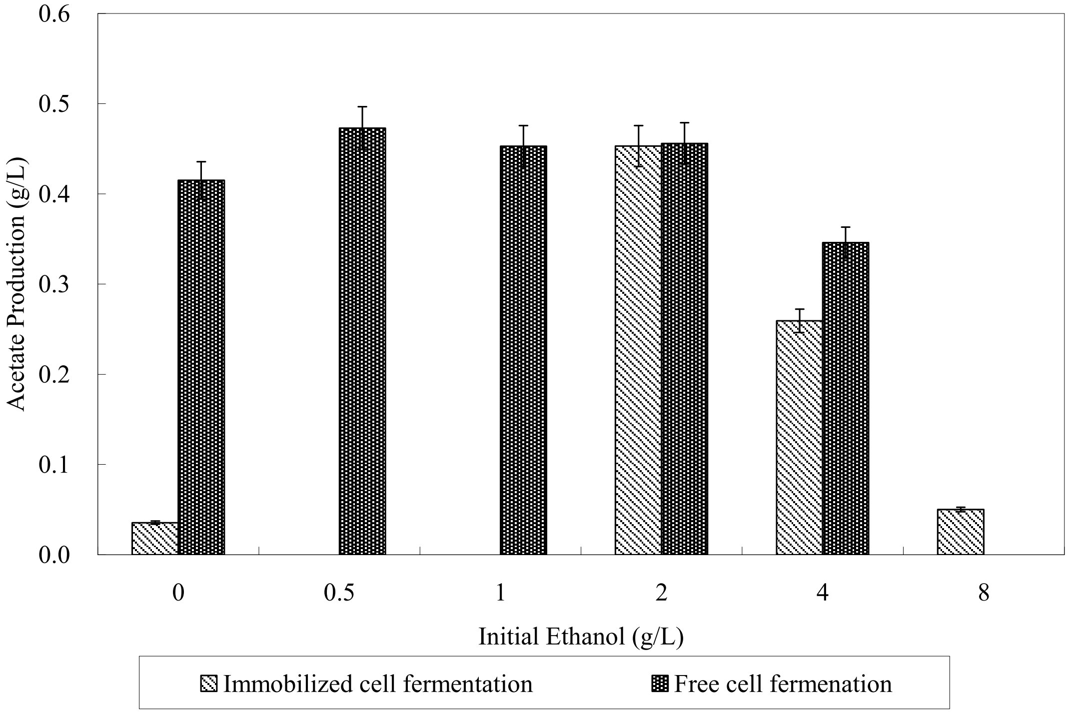


Figure 4.39d Acetate production comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for glucose fermentation at pH = 10, 57ºC for 10 days

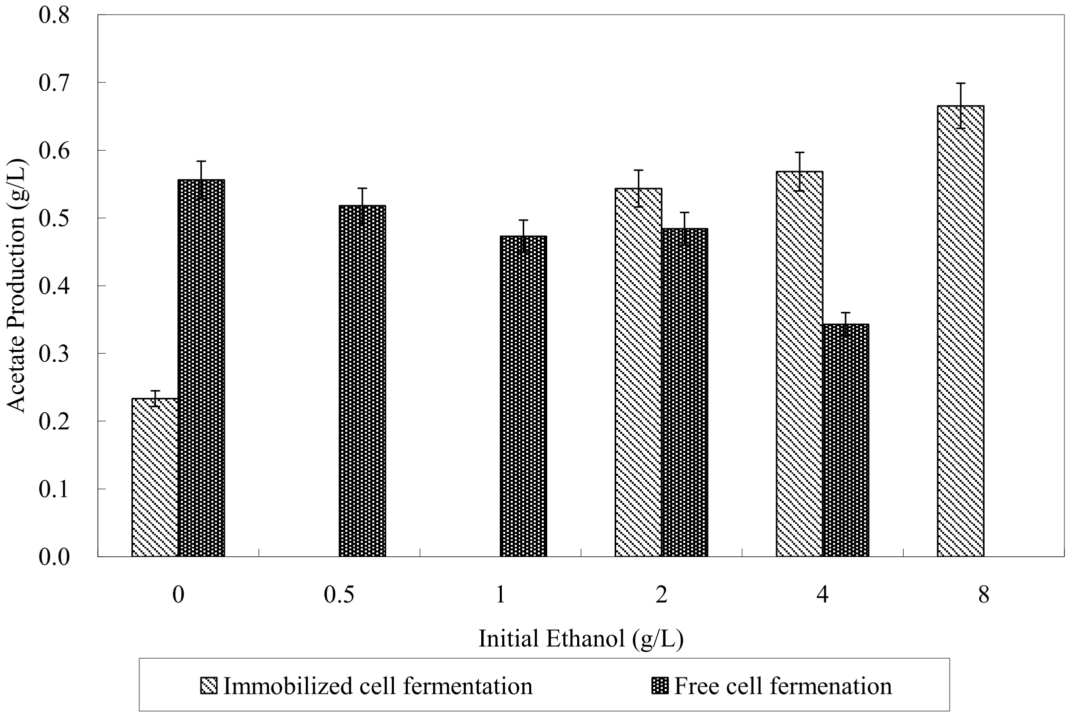


Figure 4.39e Acetate production comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for xylose fermentation at pH = 7, 57ºC for 10 days

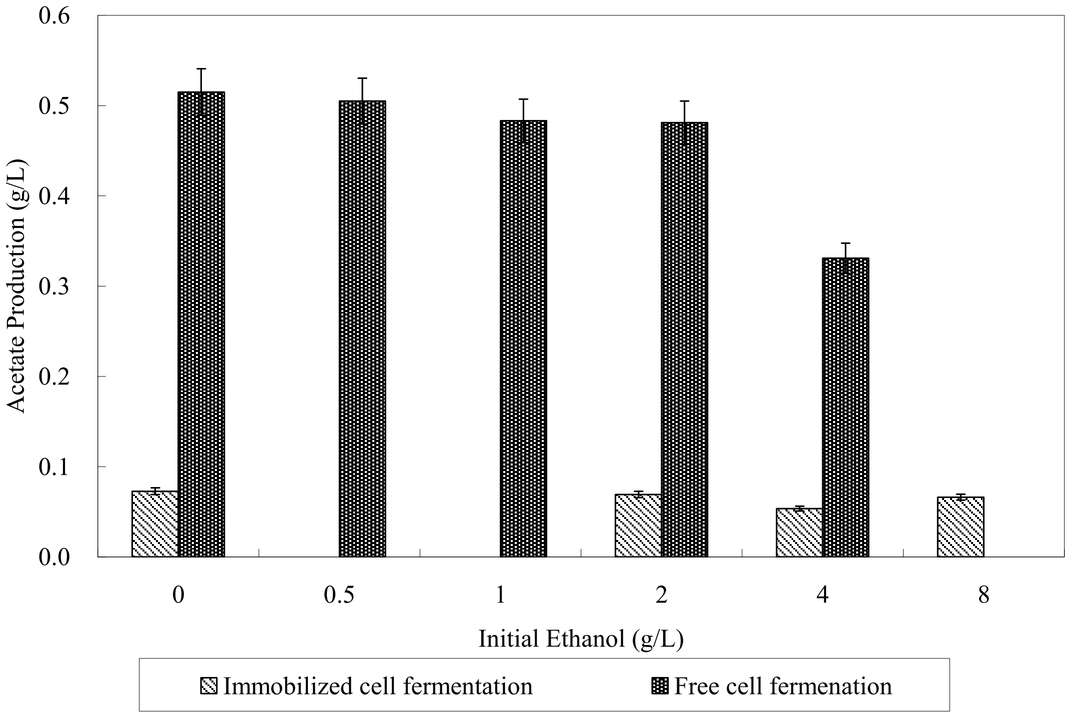


Figure 4.39f Acetate production comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for xylose fermentation at pH = 8, 57ºC for 10 days

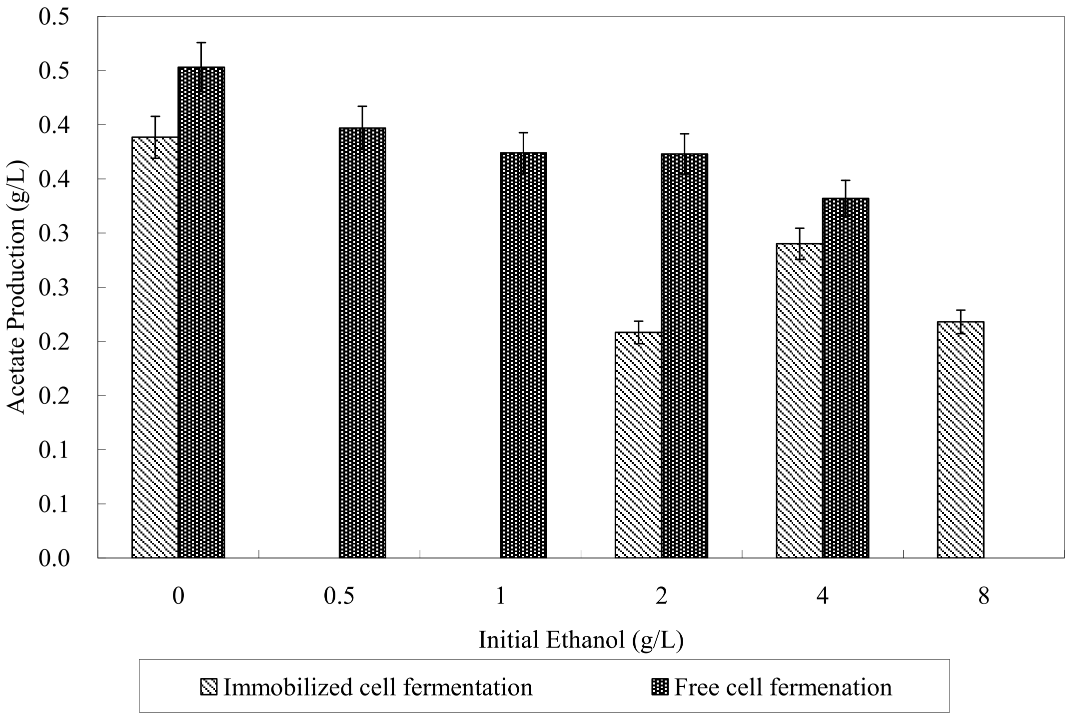


Figure 4.39g Acetate production comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for xylose fermentation at pH = 9, 57ºC for 10 days

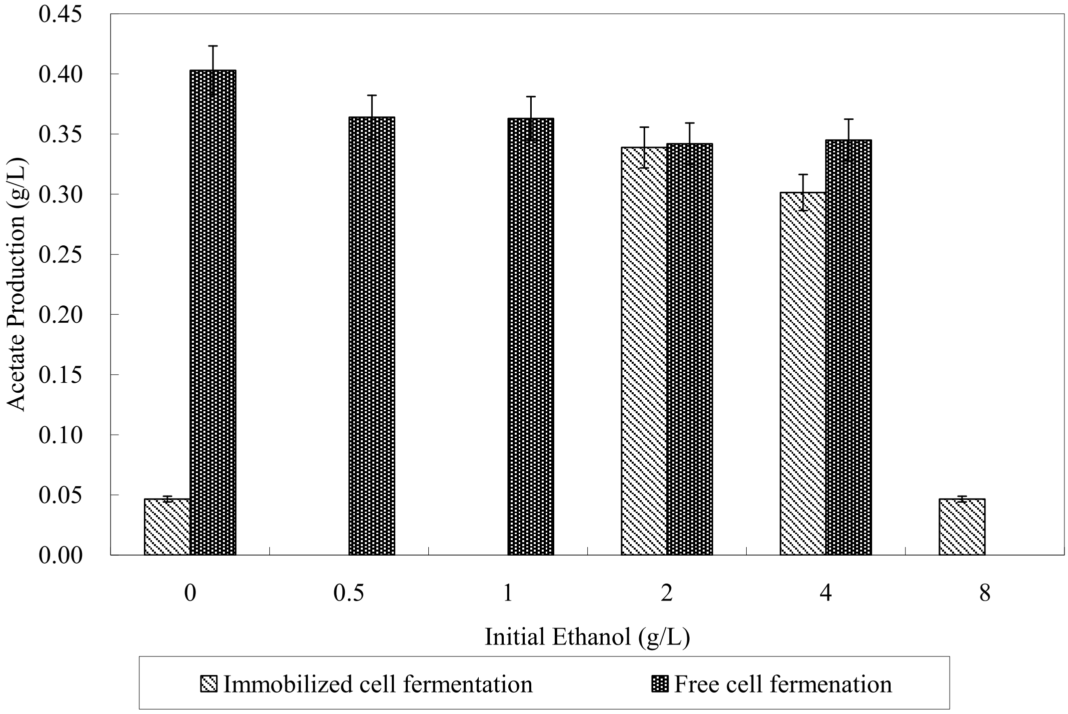


Figure 4.39h Acetate production comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for xylose fermentation at pH = 10, 57ºC for 10 days

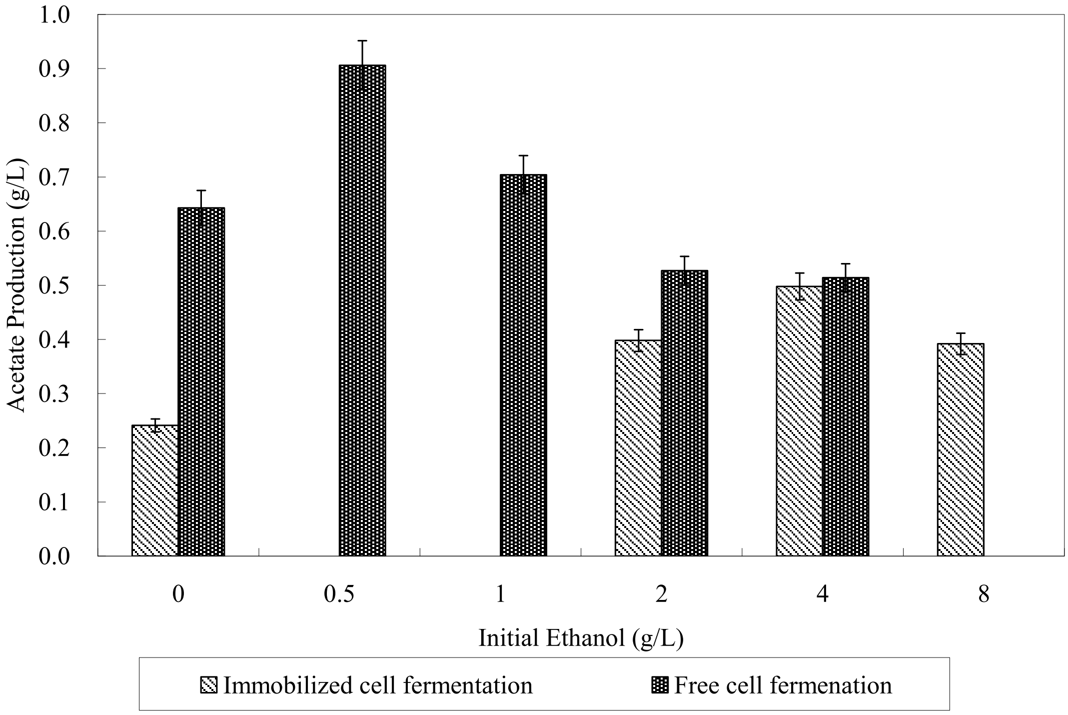


Figure 4.39i Acetate production comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for cellobiose fermentation at pH = 7, 57ºC for 10 days

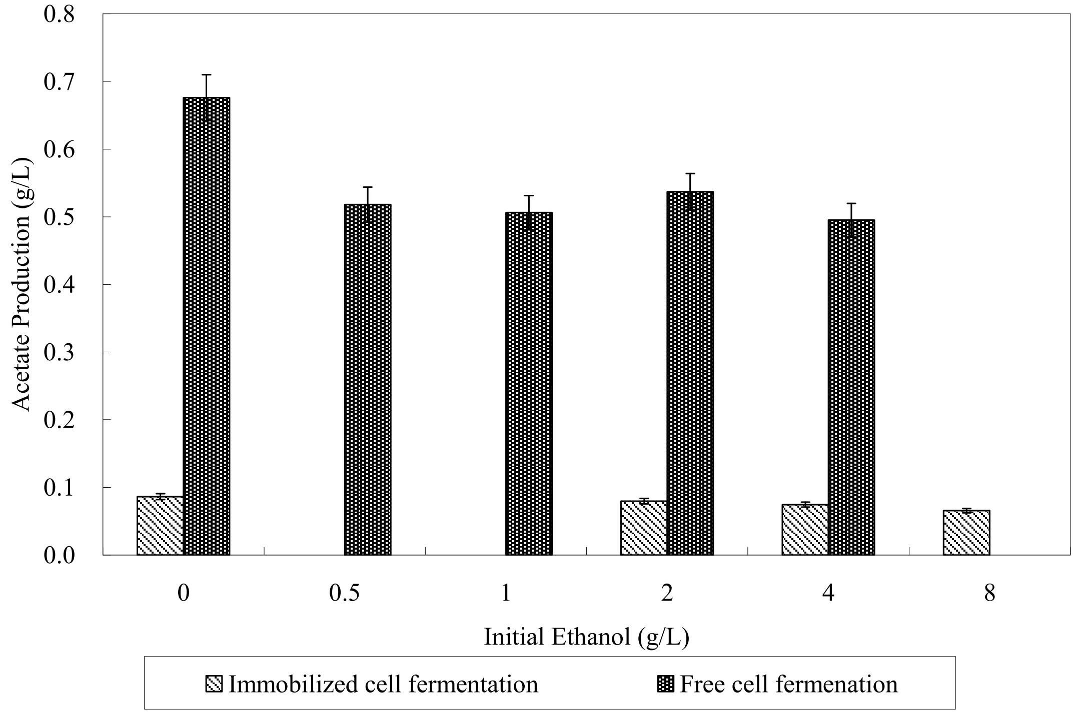


Figure 4.39j Acetate production comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for cellobiose fermentation at pH = 8, 57ºC for 10 days

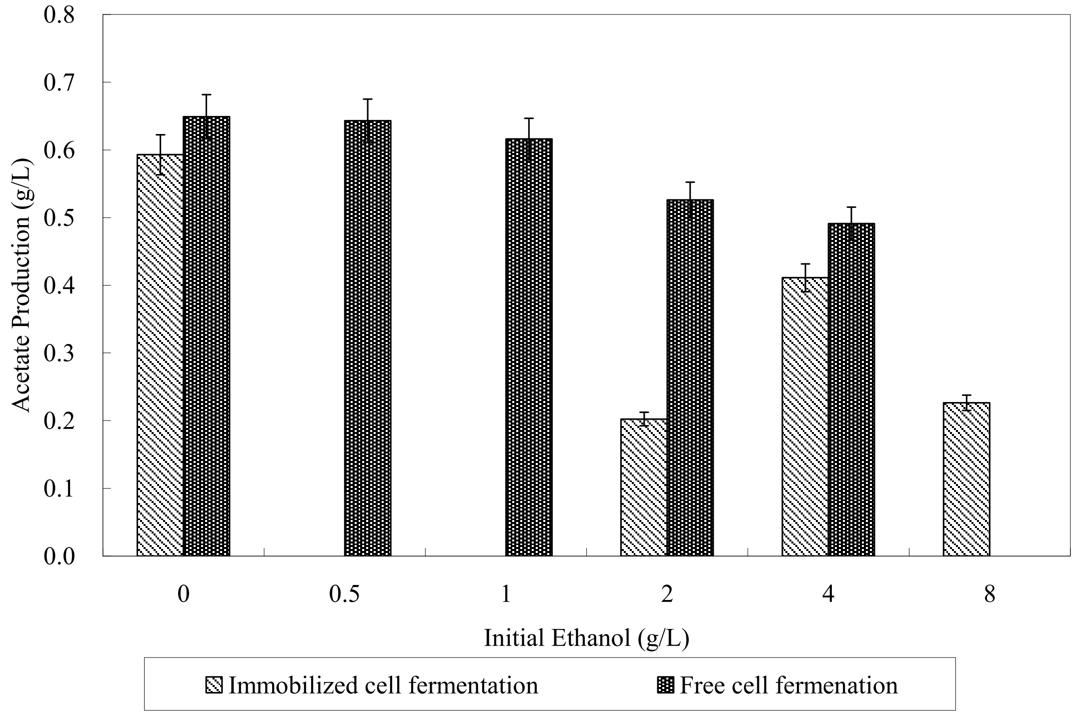


Figure 4.39k Acetate production comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for cellobiose fermentation at pH = 9, 57ºC for 10 days

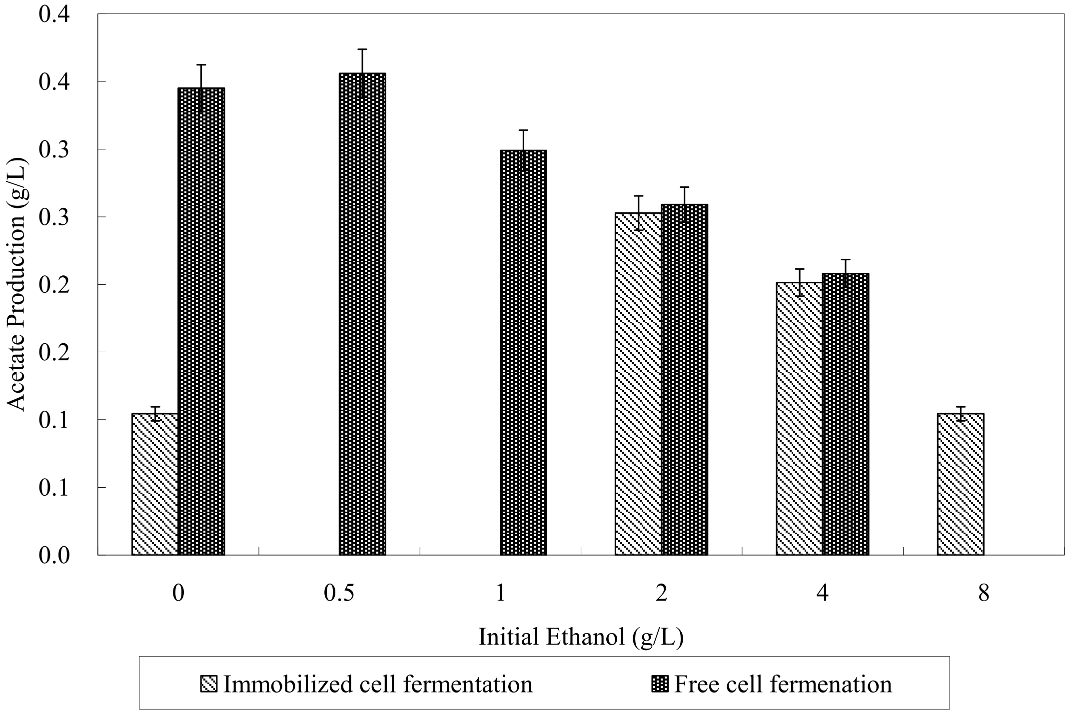


Figure 4.39l Acetate production comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for cellobiose fermentation at pH = 10, 57ºC for 10 days

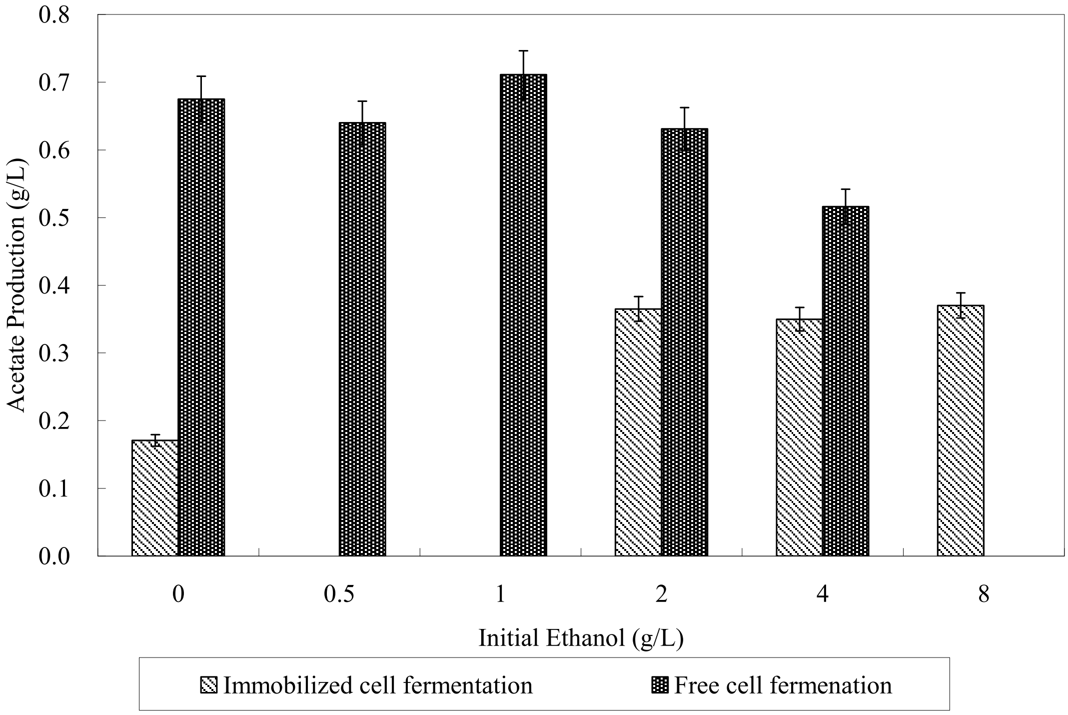


Figure 4.39m Acetate production comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for MCC fermentation at pH = 7, 57ºC for 10 days

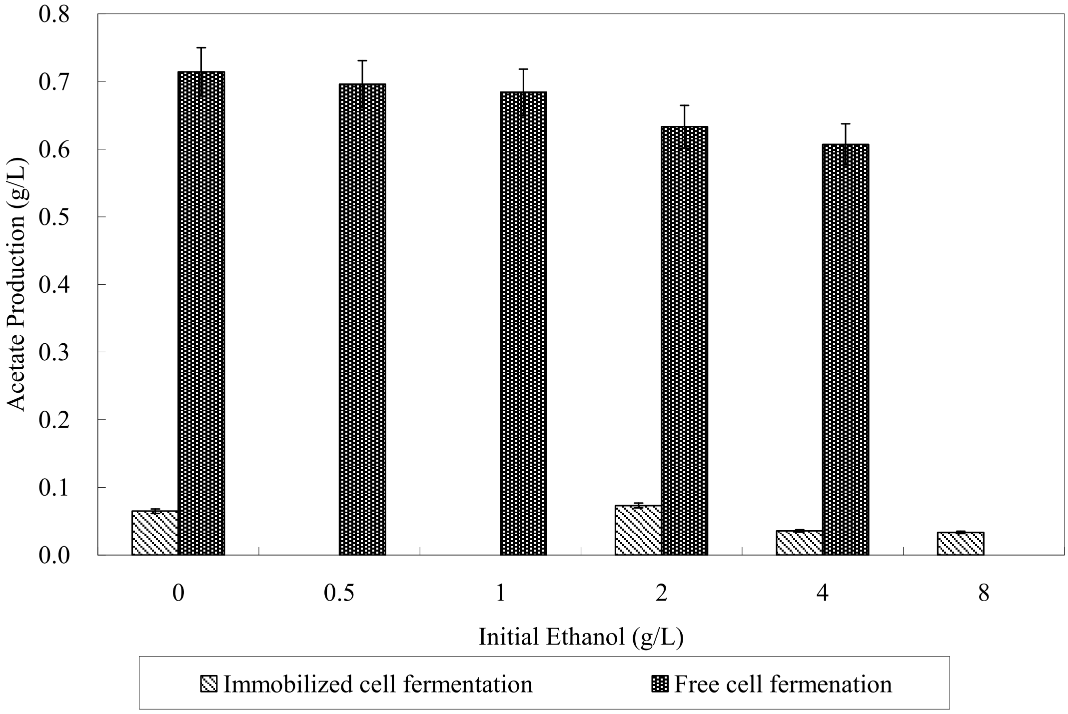


Figure 4.39n Acetate production comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for MCC fermentation at pH = 8, 57ºC for 10 days

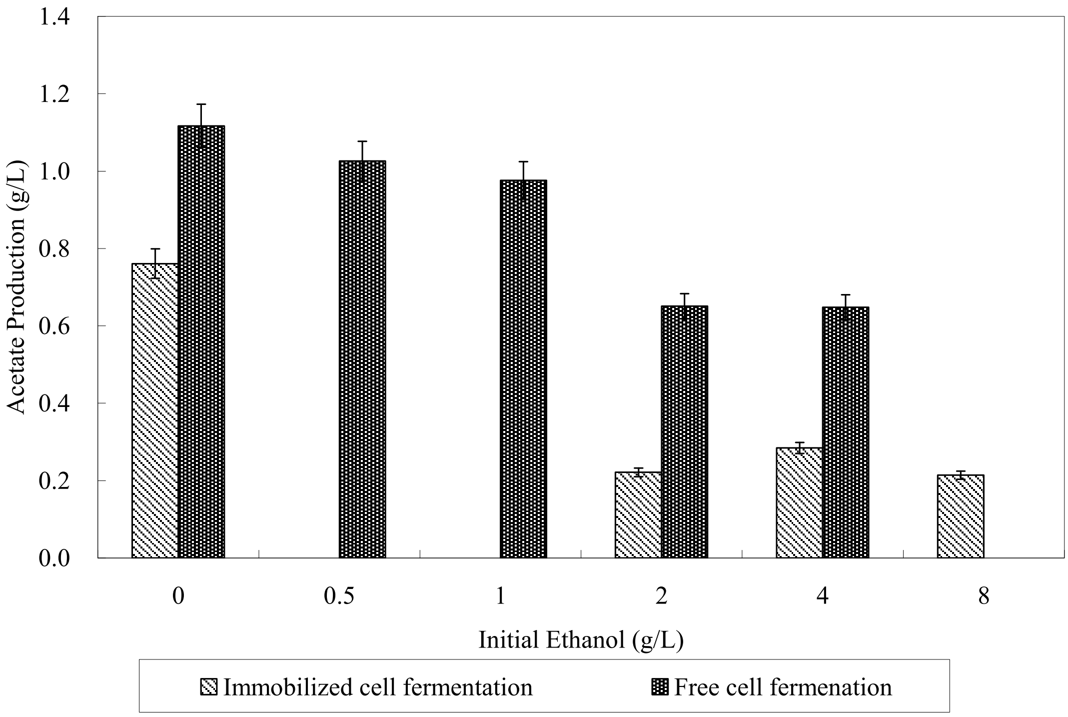


Figure 4.39o Acetate production comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for MCC fermenation at pH = 9, 57ºC for 10 days

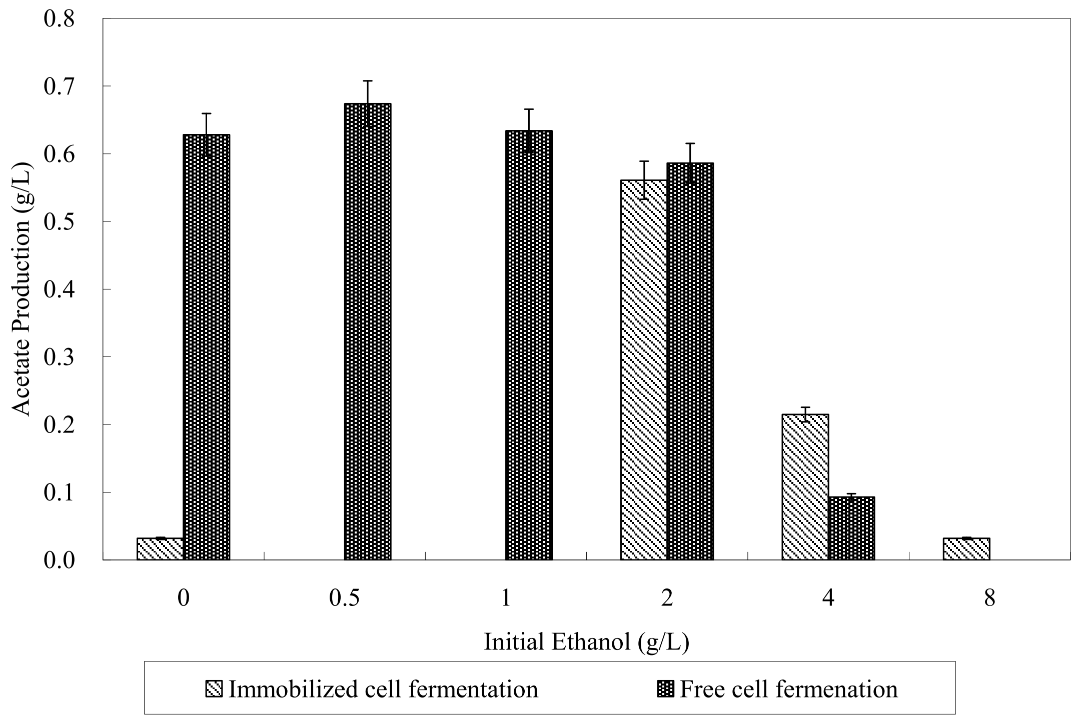


Figure 4.39p Acetate production comparison between immobilized cell fermentation and free cell fermentation at various initial ethanol levels for MCC fermenation at pH = 10, 57ºC for 10 days

4.3.3 Impact of immobilization on pH tolerance

To examine the relationship between immobilization and pH changes, regression models were fit for each treatment at fixed initial ethanol levels for certain substrates in this study. As shown in the results (Figure 4.33), ethanol and acetate remain to be the major end-products in immobilized *C. thermocellum/C. thermolacticum* co-culture fermentation. The sensitivity towards pH change was enhanced with the increase of initial ethanol concentration.

PH=9 was still considered to be the optimum pH for ethanol production under all conditions, which means as the initial ethanol level increases from pH=7 to pH=9, the final ethanol production for most substrates continuously rises and hits the maximum at pH=9. This can be explained by the high oxidation / reduction potential provide at low pH. The dehydrogenase complexes in *Clostridium* spp, such as NAD^+, will lose their activity under high oxidization/reduction potential. In this case, higher pH is more favorable. Further increase of pH value of the medium to pH=10 led to dramatic ethanol production decline for all substrates at all initial ethanol levels probably due to enzyme denature (Figure 4.40). The only exceptions to the optimum being at pH 9 are the glucose fermentation at 2% initial ethanol level, and xylose fermentation at 2% initial ethanol level (Figure 4.40). Neither free cell fermentation nor immobilized fermentation were observed being optimized at pH=9 possibly due to the interaction between pH and initial ethanol concentration.

Statistical analysis showed that both the main factors (pH value and immobilization) and interactions are significant by holding substrate and initial ethanol constant. Considering pH variations, the ethanol productions by immobilized cell fermentation are significantly enhanced through exposure to most fermentation conditions. At a given initial ethanol concentration (0-4 g/L) and a given substrate, the fermentation using immobilized cells results in higher ethanol production than the fermentation using free cells on average. Effect of applying immobilization technology is significant at most conditions ($p<0.01$). Considerable enhancement in ethanol productions were reported for immobilized cell fermentation as compared to free cell fermentation. These observations proved that applying entrapment technology can increase the cells' ability to adapt to pH changes. PH changes within medium lead to less effect on cells after being immobilized. The protection provided by the gel, existence of calcium chloride, or cell characteristic changes caused by the immobilization process are the potential explanations for this production improvement.

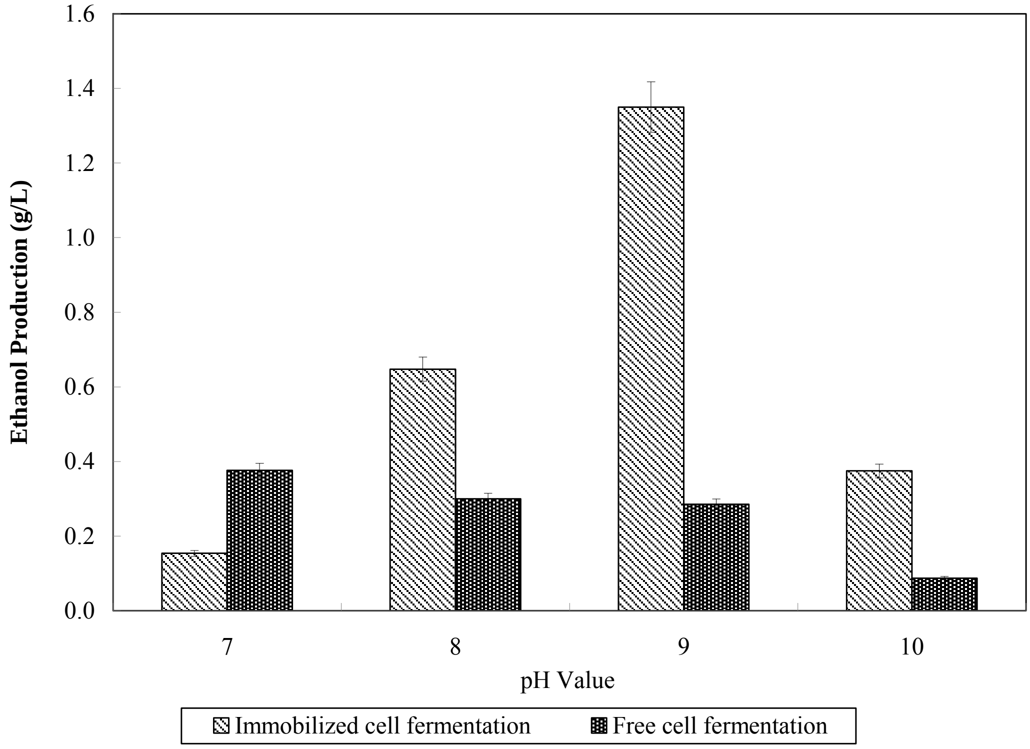


Figure 4.40a Ethanol production comparison between immobilized cell fermentation and free cell fermentation at various pH for glucose fermentation at 0g/L initial ethanol level, 57ºC for 10 days

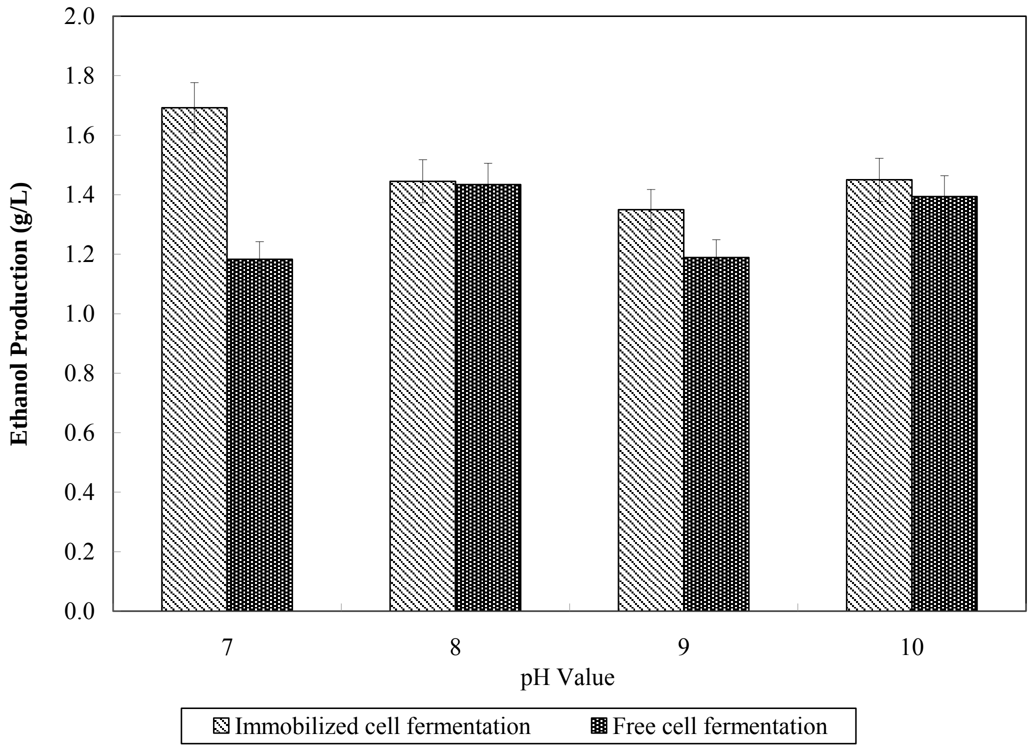


Figure 4.40b Ethanol production comparison between immobilized cell fermentation and free cell fermentation at various pH for glucose fermentation at 2g/L initial ethanol level, 57ºC for 10 days

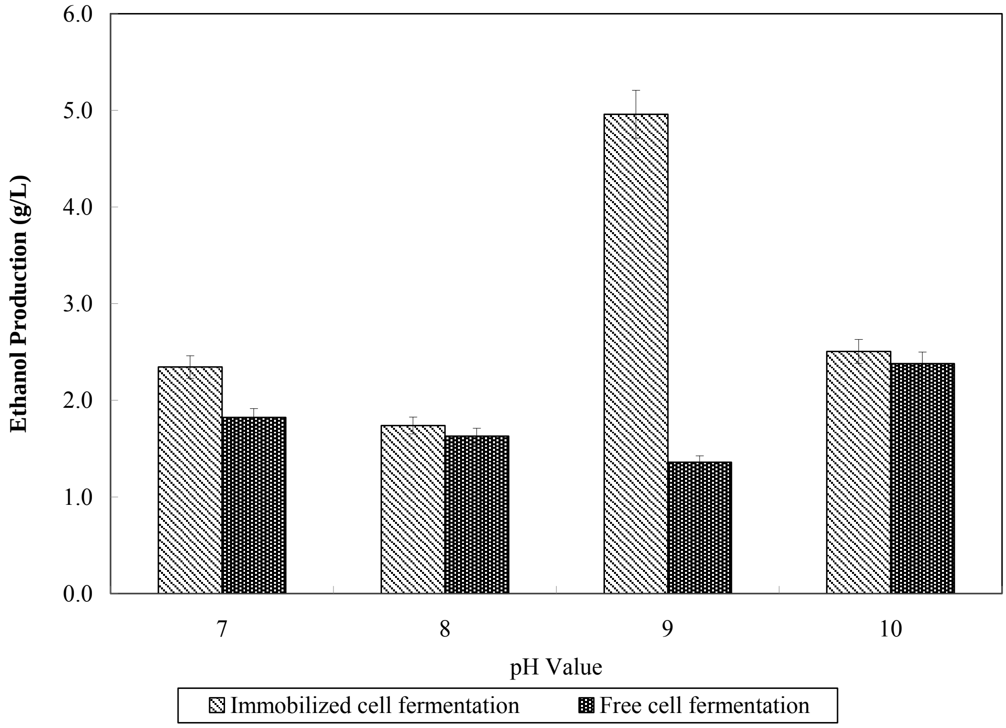


Figure 4.40c Ethanol production comparison between immobilized cell fermentation and free cell fermentation at various pH for glucose fermentation at 4g/L initial ethanol level, 57ºC for 10 days

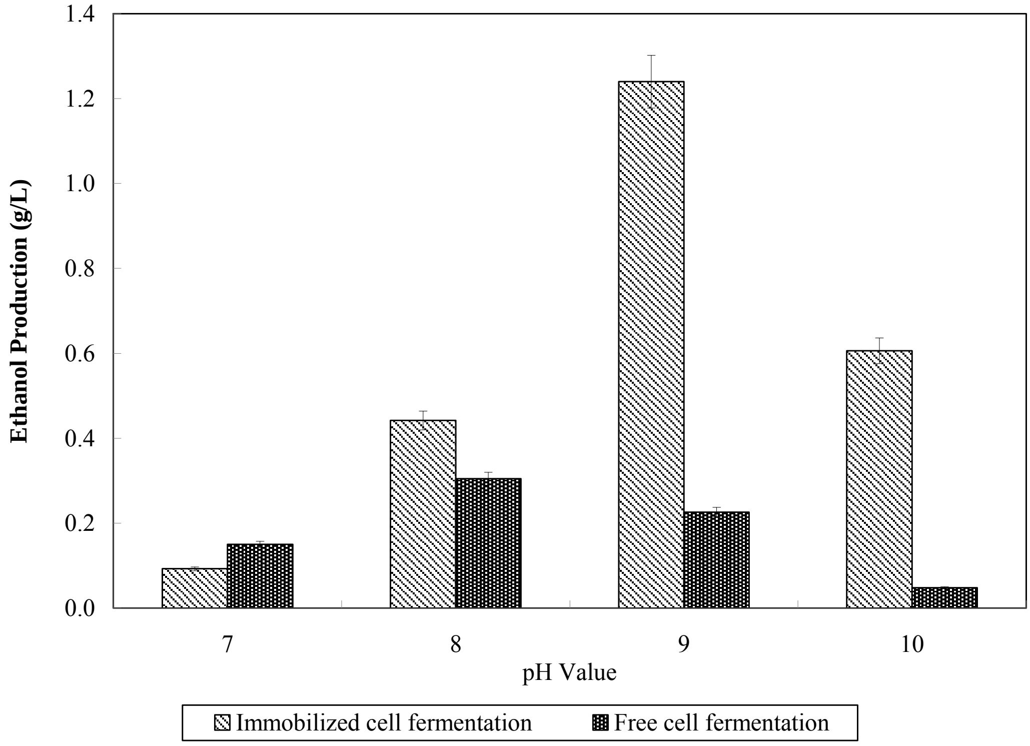


Figure 4.40d Ethanol production comparison between immobilized cell fermentation and free cell fermentation at various pH for xylose fermentation at 0g/L initial ethanol level, 57ºC for 10 days

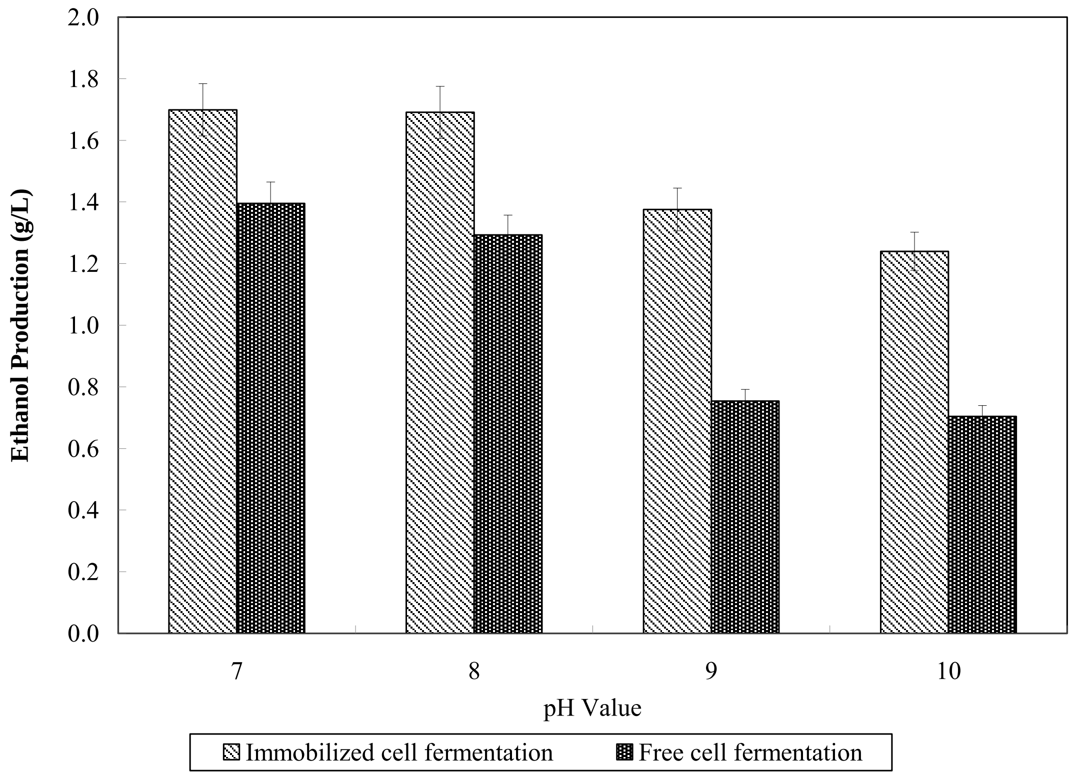


Figure 4.40e Ethanol production comparison between immobilized cell fermentation and free cell fermentation at various pH for xylose fermentation at 2g/L initial ethanol level, 57ºC for 10 days

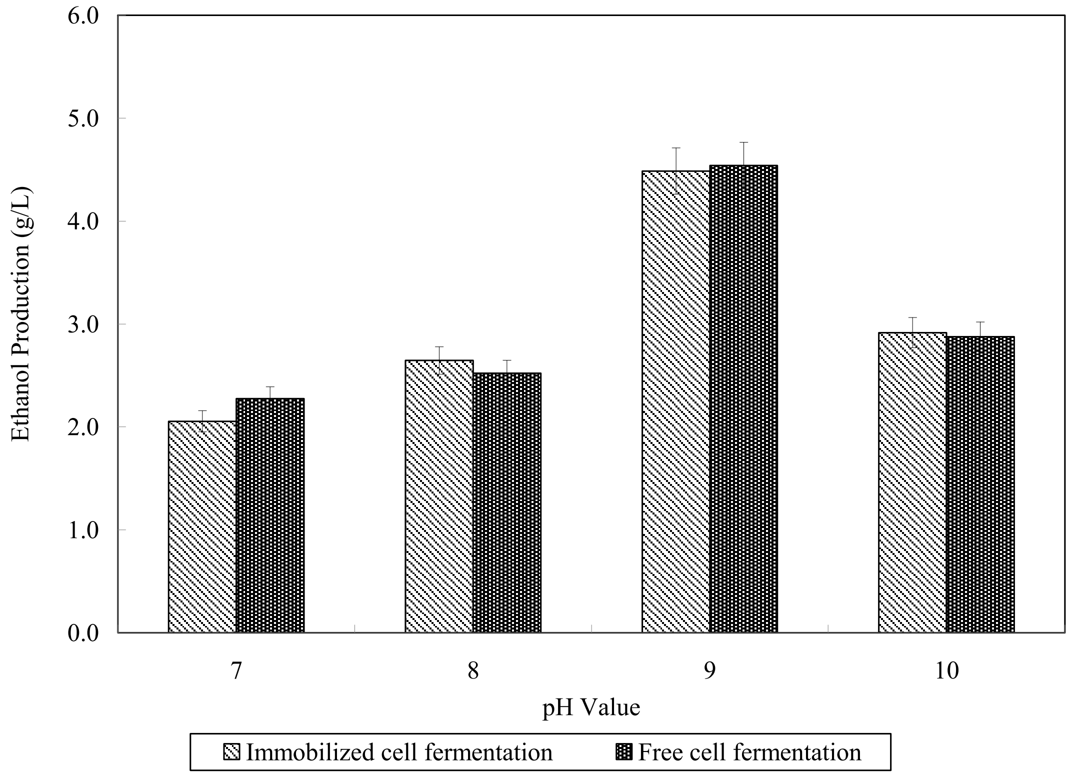


Figure 4.40f Ethanol production comparison between immobilized cell fermentation and free cell fermentation at various pH for xylose fermentation at 4g/L initial ethanol level, 57ºC for 10 days

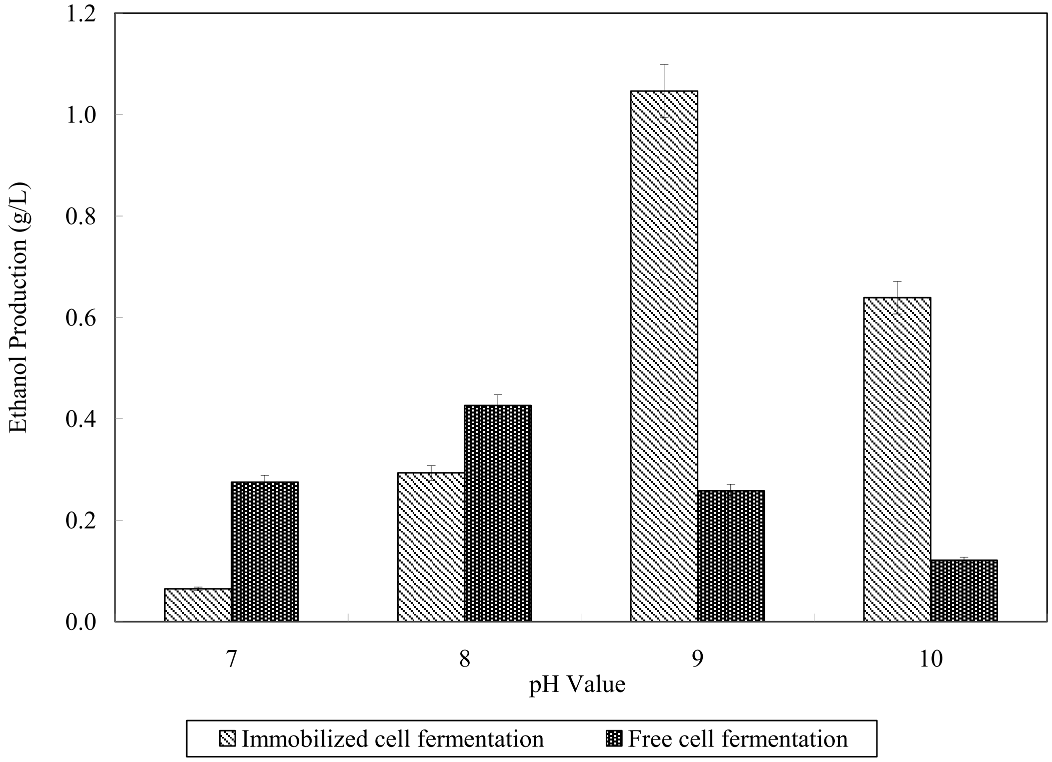


Figure 4.40g Ethanol production comparison between immobilized cell fermentation and free cell fermentation at various pH for cellobiose fermentation at 0g/L initial ethanol level, 57ºC for 10 days

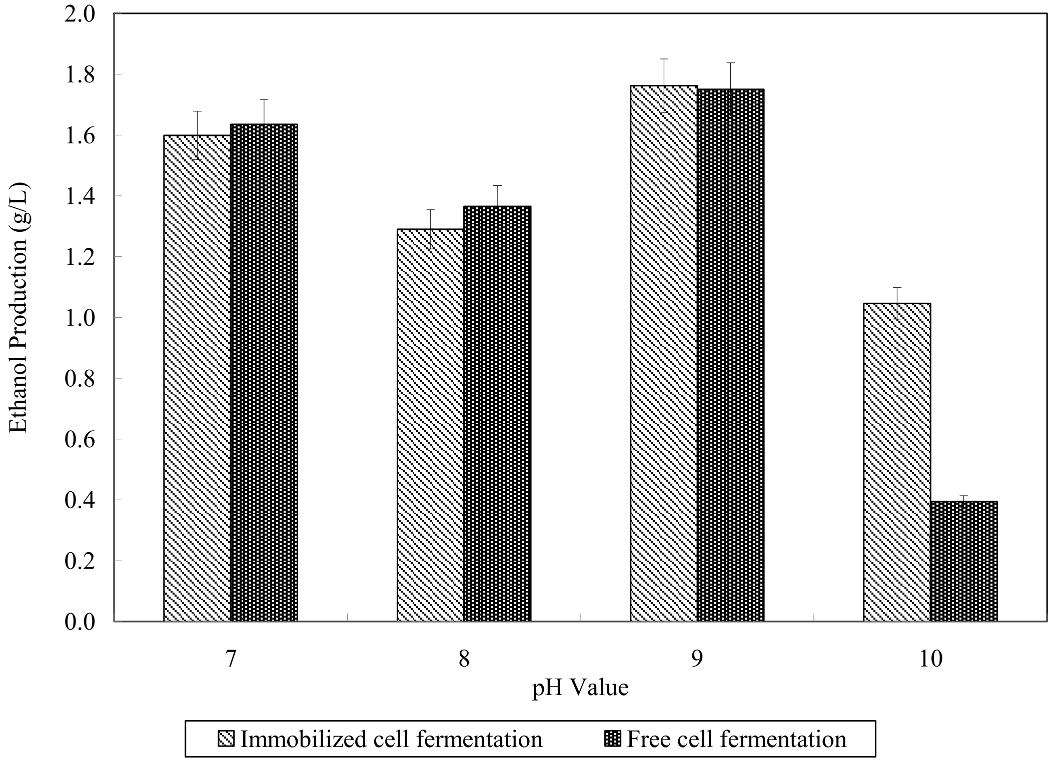


Figure 4.40h Ethanol production comparison between immobilized cell fermentation and free cell fermentation at various pH for cellobiose fermentation at 2g/L initial ethanol level, 57ºC for 10 days

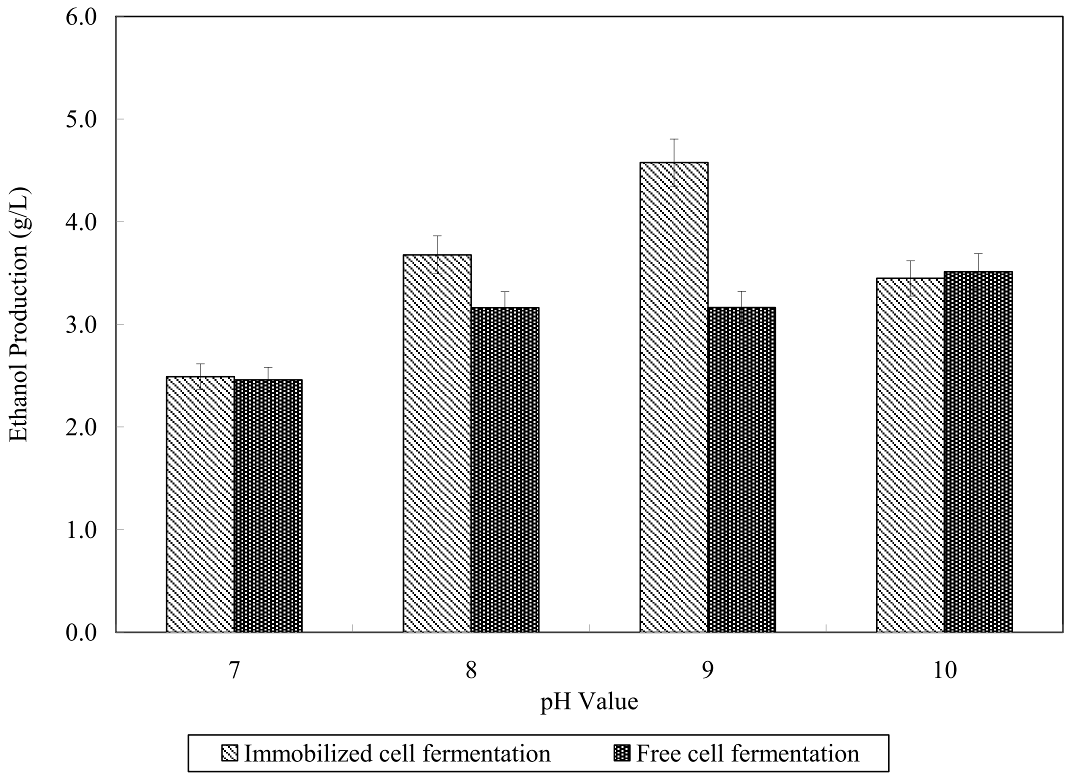


Figure 4.40i Ethanol production comparison between immobilized cell fermentation and free cell fermentation at various pH for cellobiose fermentation at 4g/L initial ethanol level, 57ºC for 10 days

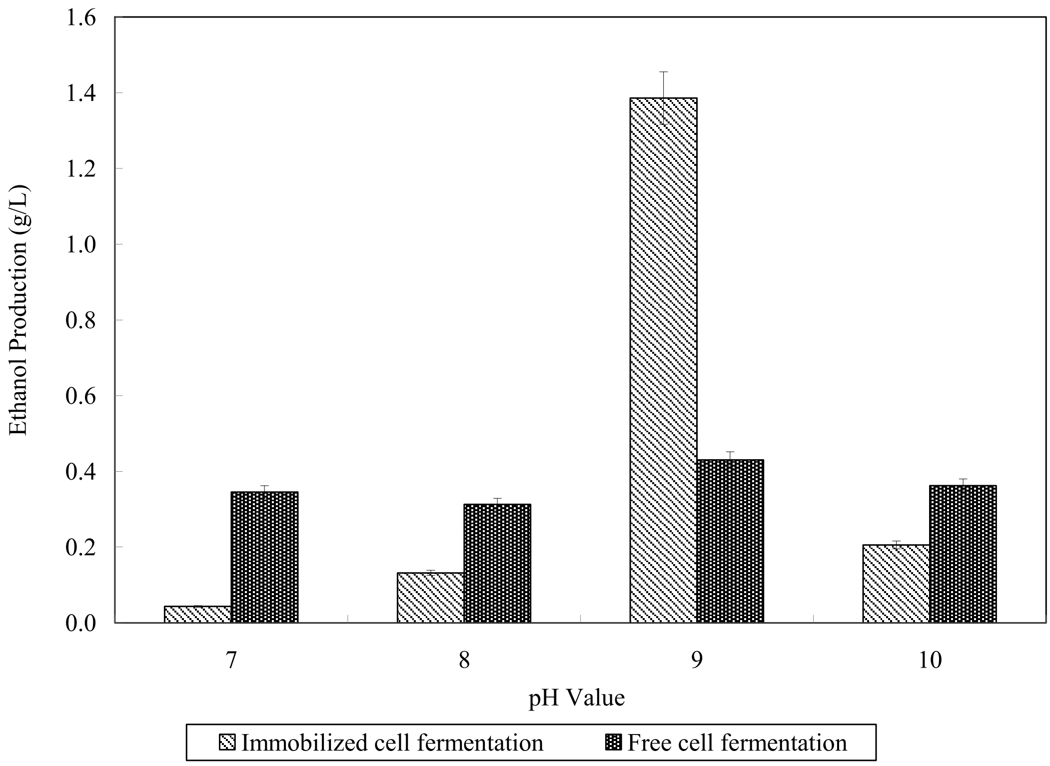


Figure 4.40j Ethanol production comparison between immobilized cell fermentation and free cell fermentation at various pH for MCC fermentation at 0g/L initial ethanol level, 57ºC for 10 days

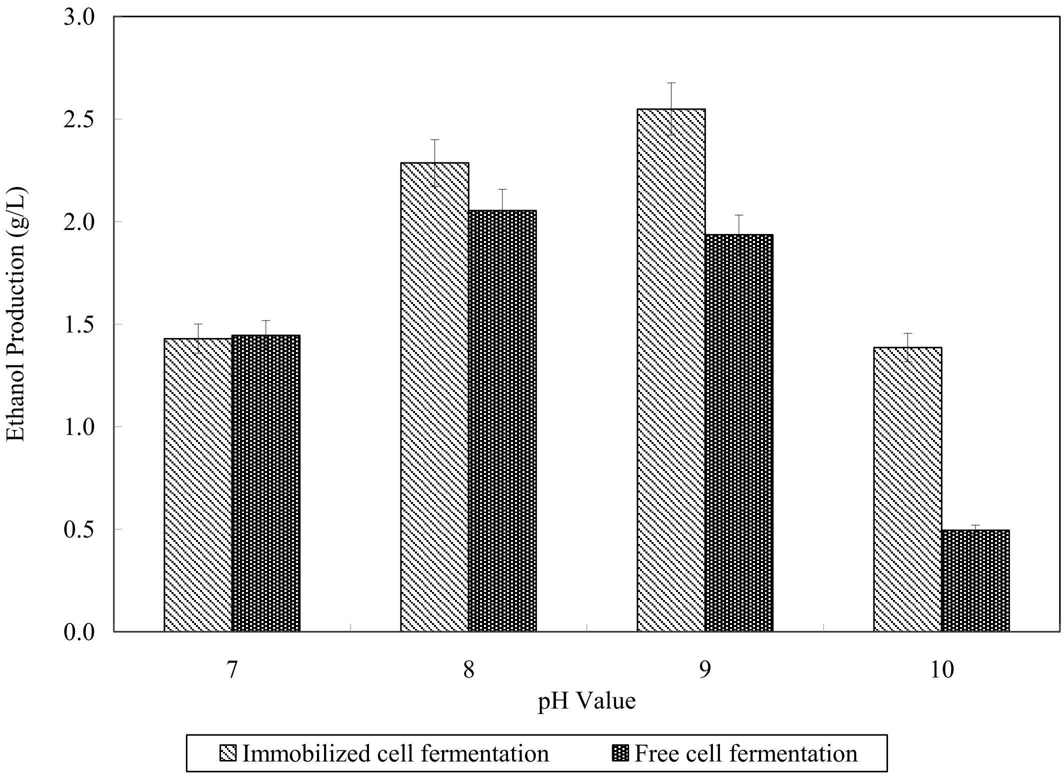


Figure 4.40k Ethanol production comparison between immobilized cell fermentation and free cell fermentation at various pH for MCC fermentation at 2g/L initial ethanol level, 57ºC for 10 days

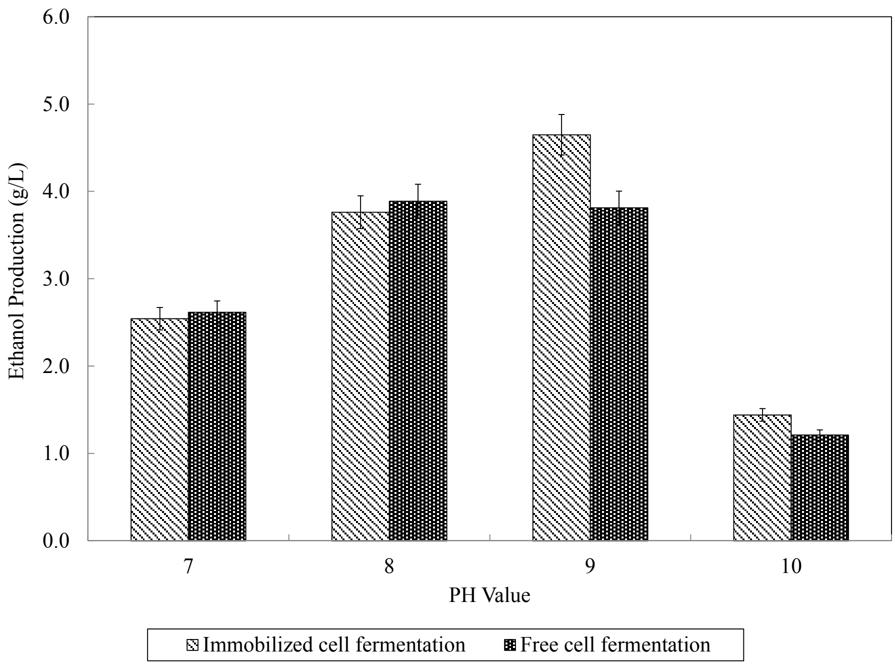


Figure 4.40l Ethanol production comparison between immobilized cell fermentation and free cell fermentation at various pH for MCC fermentation at 4g/L initial ethanol level, 57ºC for 10 days

When the alginate is considered as protection for co-culture cells, the acetate production is supposed to increase proportionally with the ethanol production improvement since this improvement is caused by higher cell density achieved during immobilized cell fermentation. With higher concentration of the cells, higher solvent production will be reached. However, the increase of ethanol yield caused by applying an immobilization was observed not to correlate to ethanol/acetate ratio. Acetate productions are significantly lower in immobilized cell fermentation as compared to free cell fermentation on cellulose, cellobiose, glucose, and xylose under neutral or alkaline conditions (Figure 4.41). PH=8 is the condition that leads to the sharpest acetate formed decrease by immobilized cell fermentation as compared to free cell fermentations. For mono-sacchrides, glucose and xylose, the drop is about 0.3 g/L. This decrease is even more dramatic for cellobiose and MCC, an acetate production decline over 0.5 g/L was observed. A higher acetate yield always existed in co-culture fermentation of MCC in immobilized cell fermentation, regardless of the pH.

For the free cell fermentation, low pH leads to high oxidation/ reduction potential, which is unfavorable for anaerobic micro-organisms' metabolism. In this case, lower pH will lead to the decline of the cell activity. Without protections, the cell activity decrease will become more significant as pH is lowered. Thus, compare to the immobilized cells, which are less effected by their environment, the ethanol production differences between free cell fermentation and immobilized cell fermentation will become larger as the pH value become much extreme for cell growth. However, according to our results, no clear relationship between the pH changes and ethanol production differences were observed between immobilized cell fermentation. Thus, other changes might be involved in this fermentation process, besides gel protection and cell population increase.

Based on some preliminary tests, the existence of Ca^{2+} might be the potential reason for ethanol production improvement in the immobilization experiments. In order to examine the influence on the addition of calcium chloride, a set of comparison studies has been performed. The amount of calcium chloride to be used for the immobilization process was added during free cell fermentation at pH=9 at 4 g/L initial ethanol level for all chose substrate. The immobilized cell fermentations performed with 3% calcium chloride appeared to be significantly better than free cell fermentation performed with 3% calcium chloride added. Furthermore, increase of ethanol productions is proportional with pH value increase. However, the decline of acetate productions and disproportional increase of ethanol productions are both found in this study, indicating that other factors, like cell characteristics changes, are involved in the ethanol production improvement during immobilization processes.

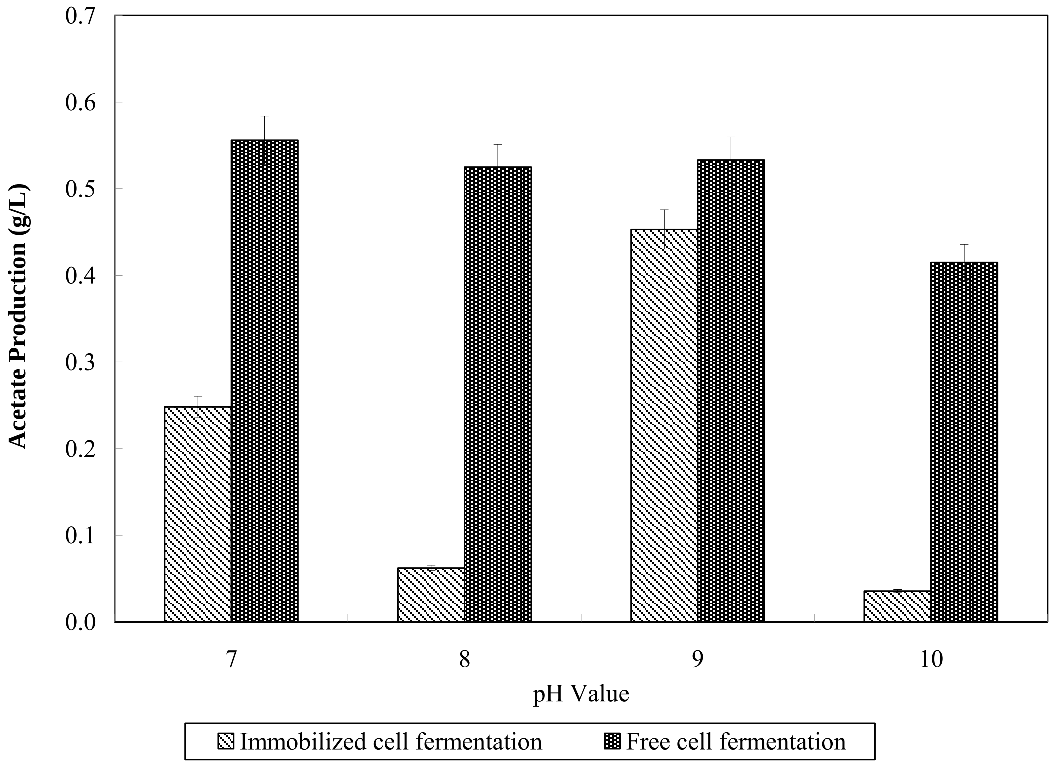


Figure 4.41a Acetate production comparison between immobilized cell fermentation and free cell fermentation at various pH for glucose at 0g/L initial ethanol level, 57ºC for 10 days

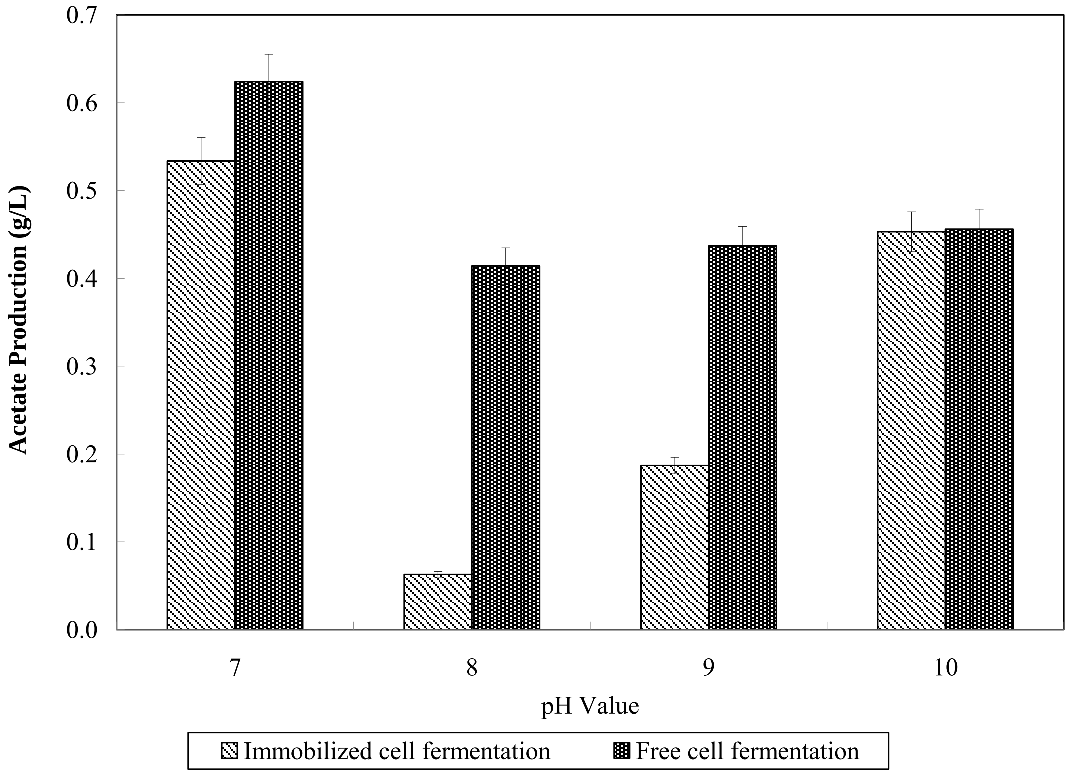


Figure 4.41b Acetate production comparison between immobilized cell fermentation and free cell fermentation at various pH for glucose fermentation at 2g/L initial ethanol level, 57ºC for 10 days

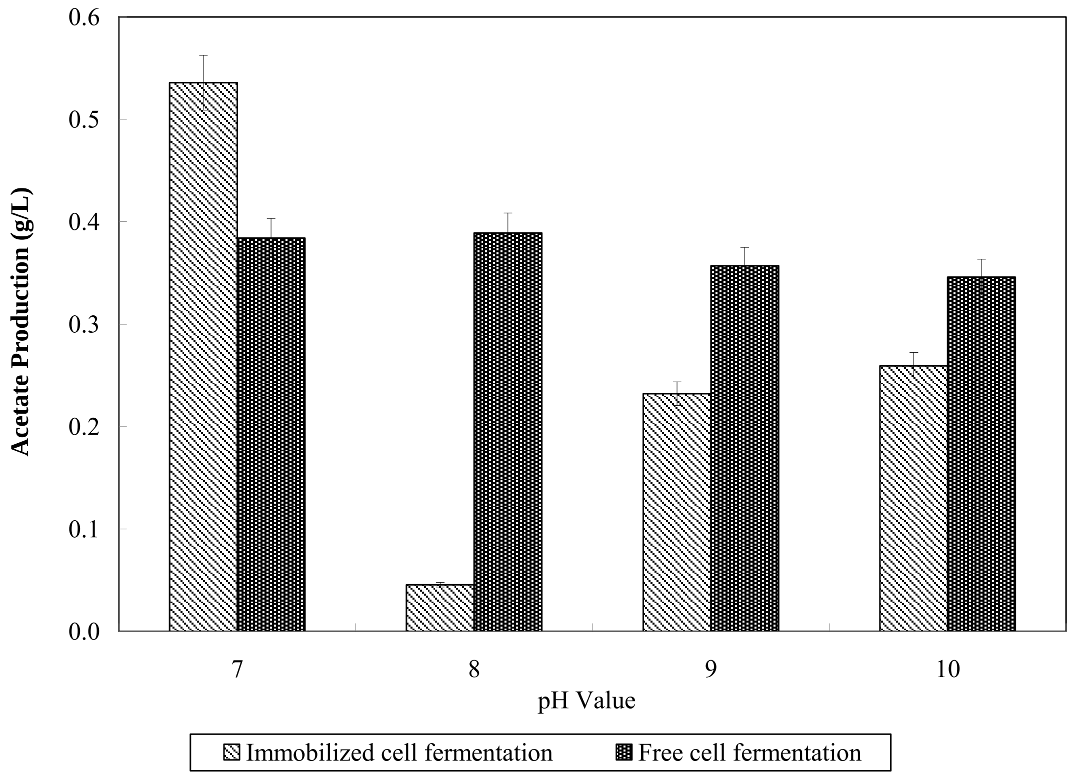


Figure 4.41c Acetate production comparison between immobilized cell fermentation and free cell fermentation at various pH for glucose fermentation at 4g/L initial ethanol level, 57ºC for 10 days

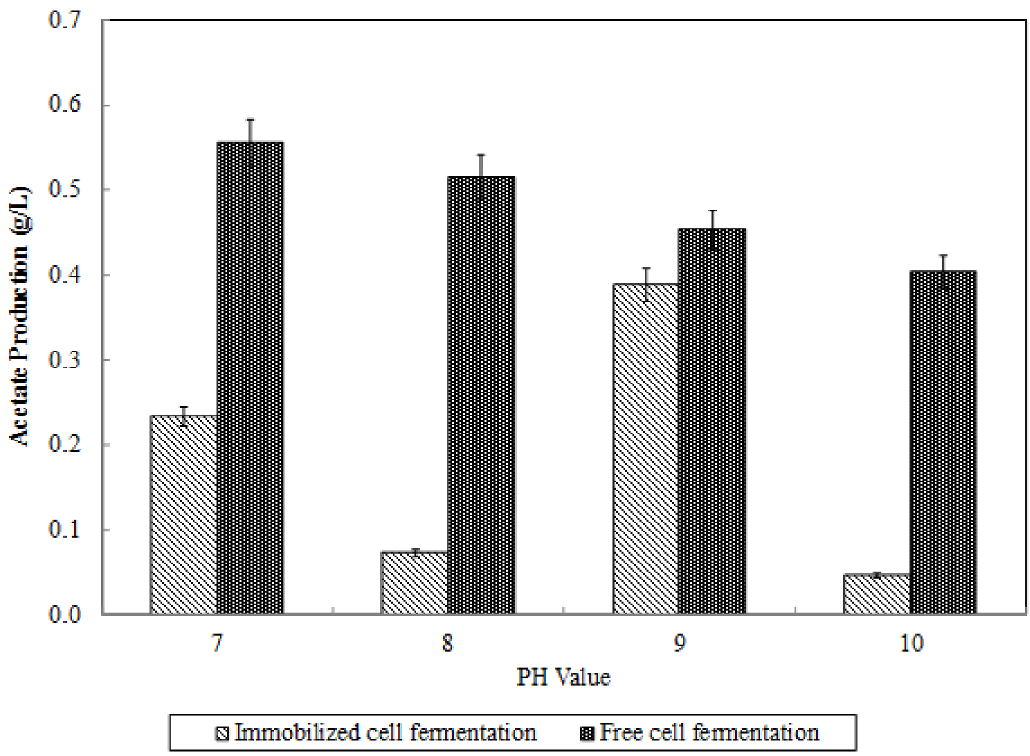


Figure 4.41d Acetate production comparison between immobilized cell fermentation and free cell fermentation for xylose at 0g/L initial ethanol level, 57ºC for 10 days

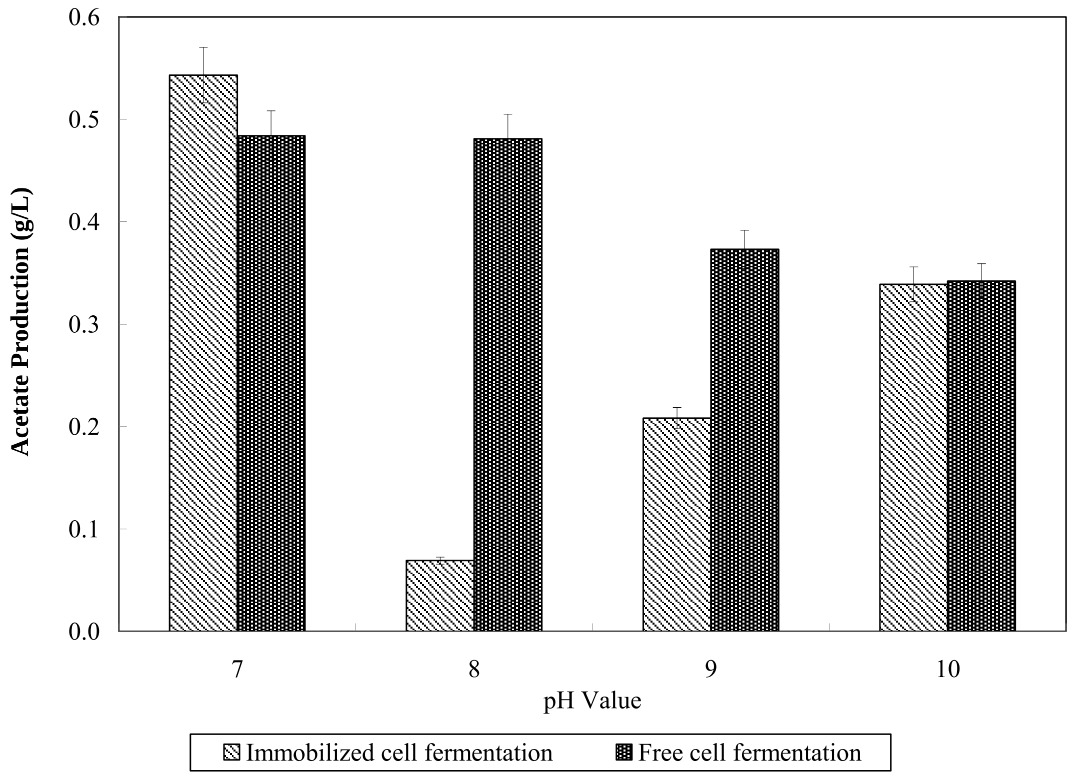


Figure 4.41e Acetate production comparison between immobilized cell fermentation and free cell fermentation at various pH for xylose fermentation at 2g/L initial ethanol level, 57ºC for 10 days

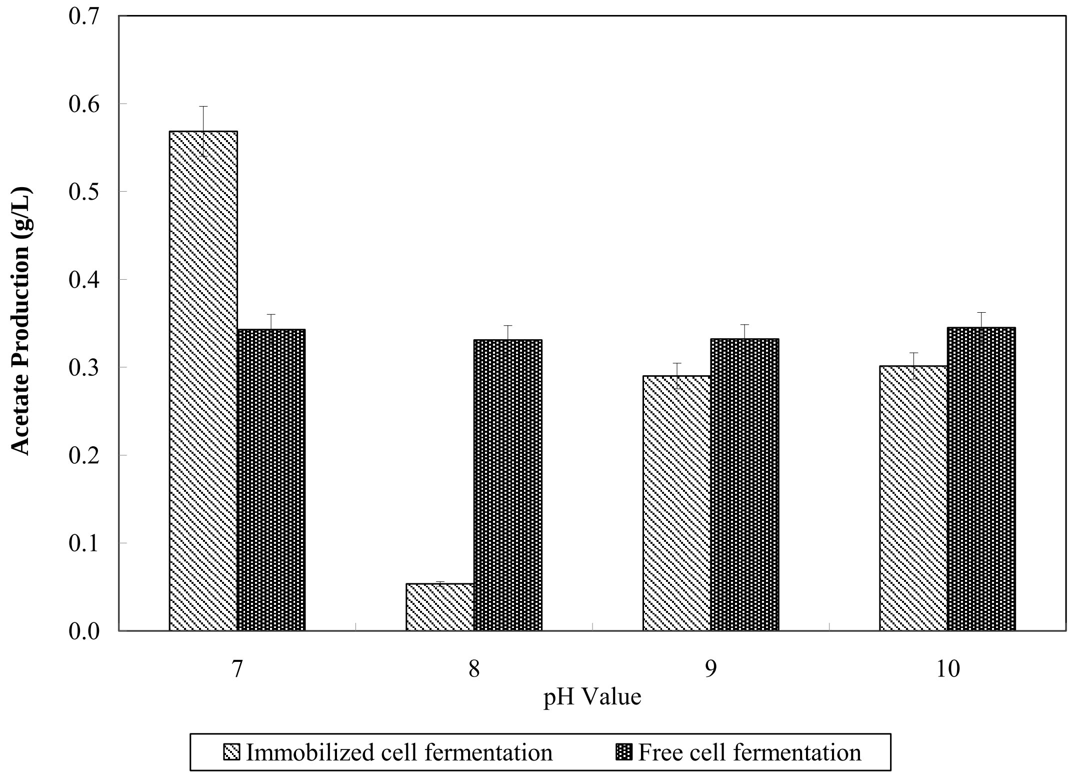


Figure 4.41f Acetate production comparison between immobilized cell fermentation and free cell fermentation at various pH for xylose fermentation at 4g/L initial ethanol level, 57ºC for 10 days

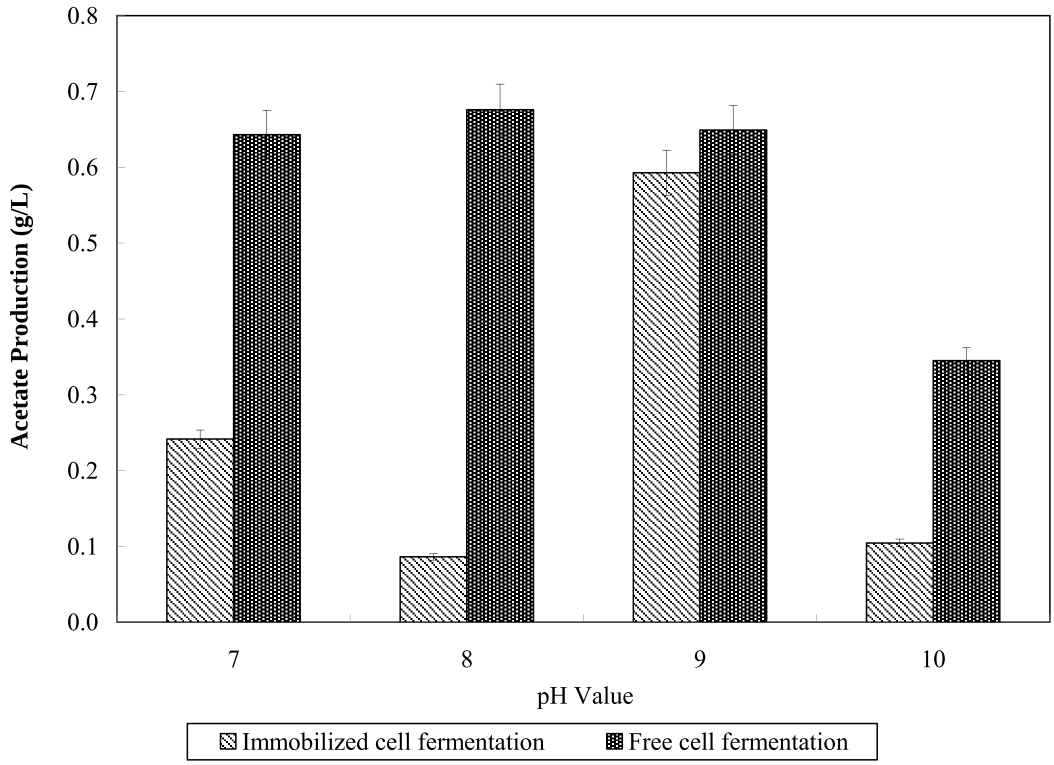


Figure 4.41g Acetate production comparison between immobilized cell fermentation and free cell fermentation at various pH for cellobiose fermentation at 0g/L initial ethanol level, 57ºC for 10 days

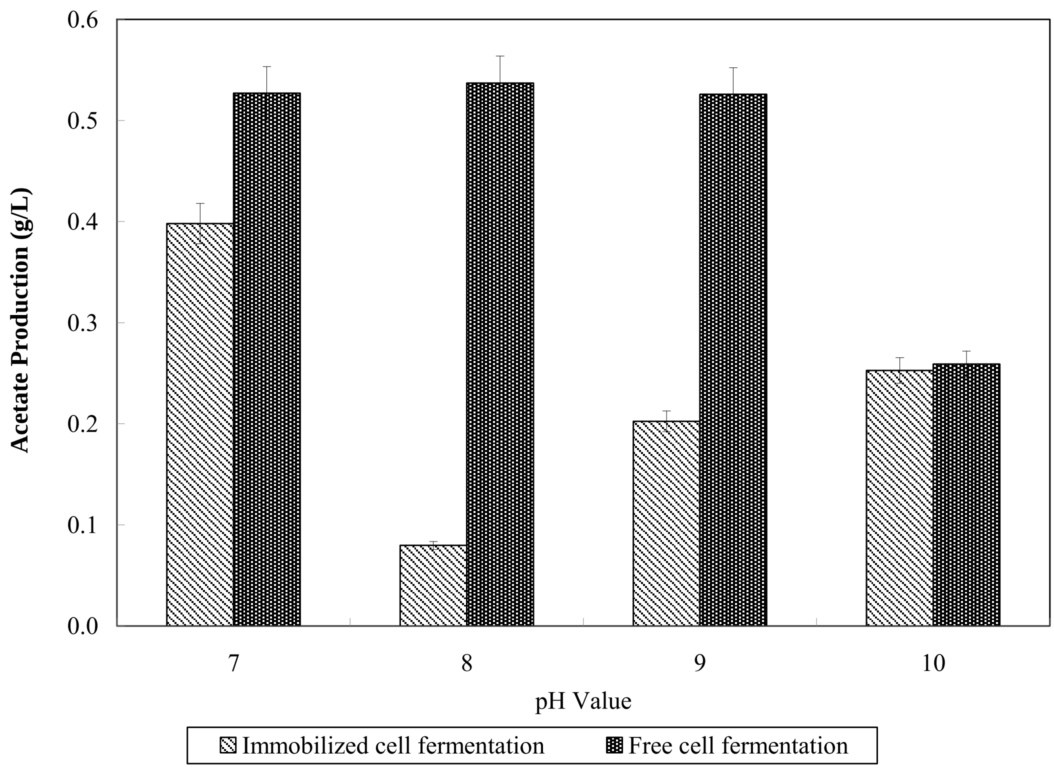


Figure 4.41h Acetate production comparison between immobilized cell fermentation and free cell fermentation at various pH for cellobiose fermentation at 2g/L initial ethanol level, 57ºC for 10 days

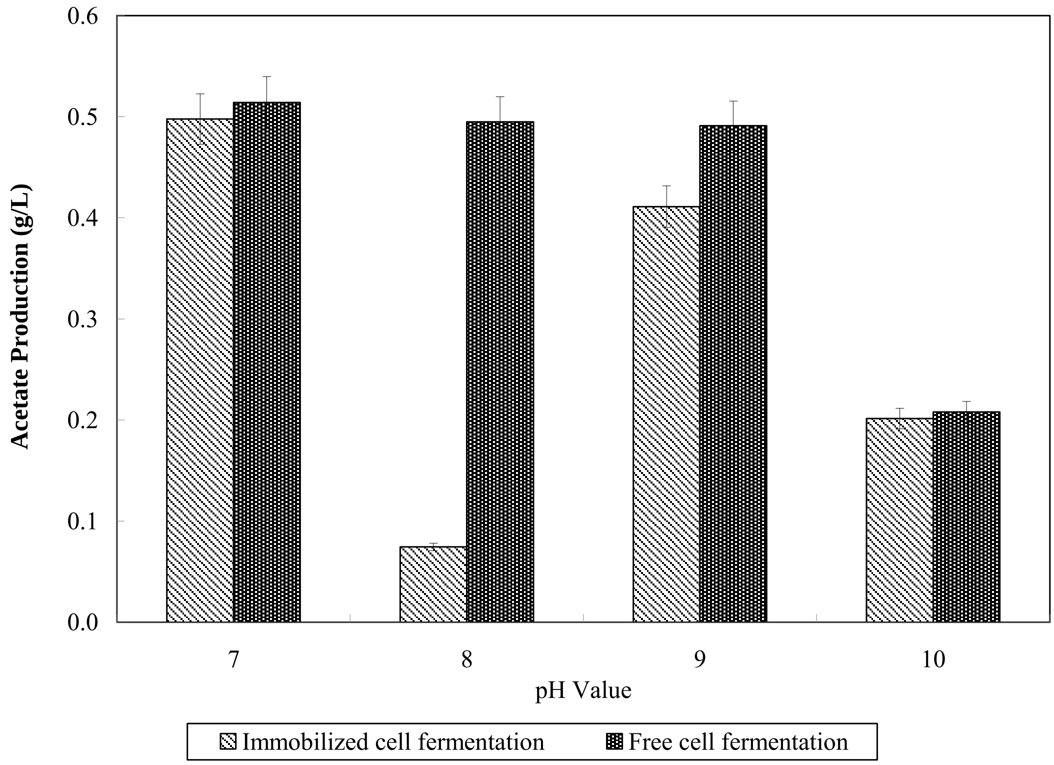


Figure 4.41j Acetate production comparison between immobilized cell fermentation and free cell fermentation at various pH for cellobiose fermentation at 4g/L initial ethanol level, 57ºC for 10 days

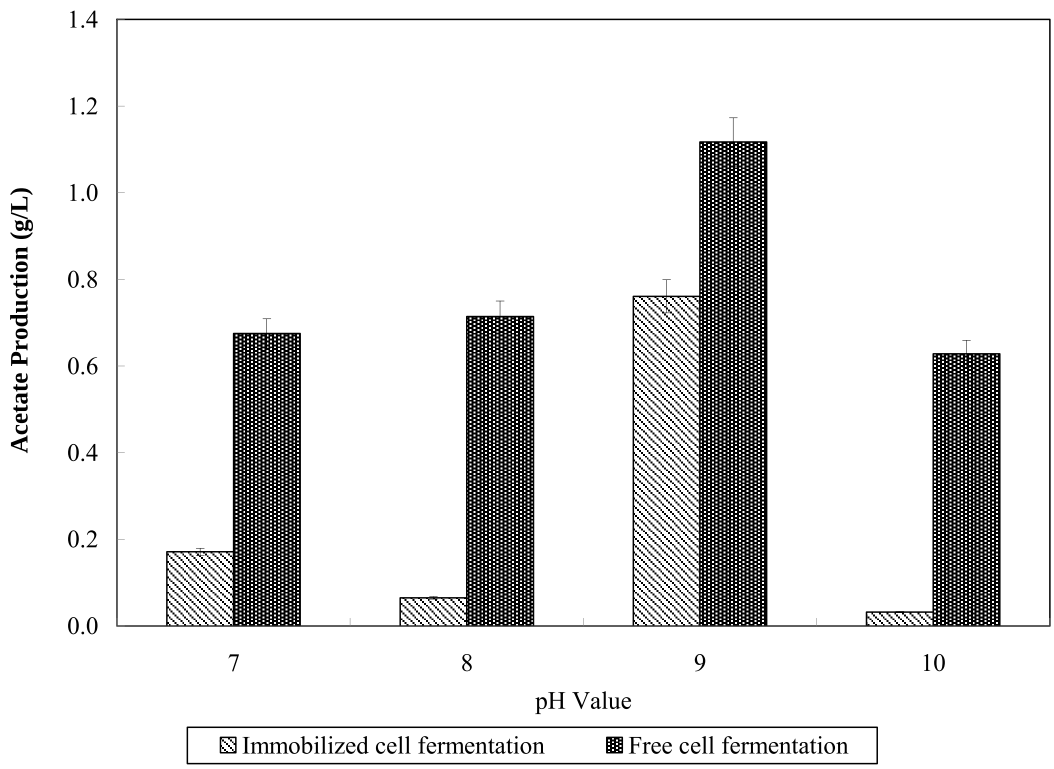


Figure 4.41k Acetate production comparison between immobilized cell fermentation and free cell fermentation at various pH for MCC fermentation at 0g/L initial ethanol level, 57ºC for 10 days

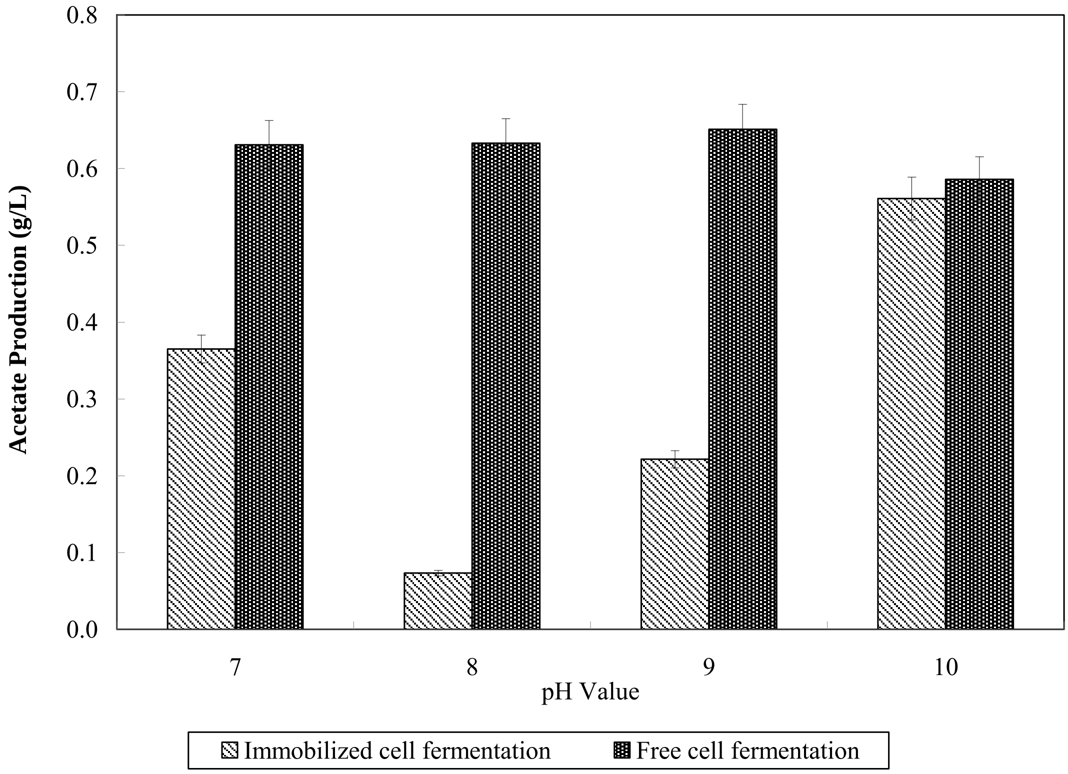


Figure 4.41l Acetate production comparison between immobilized cell fermentation and free cell fermentation at various pH for MCC fermentation at 2g/L initial ethanol level, 57ºC for 10 days

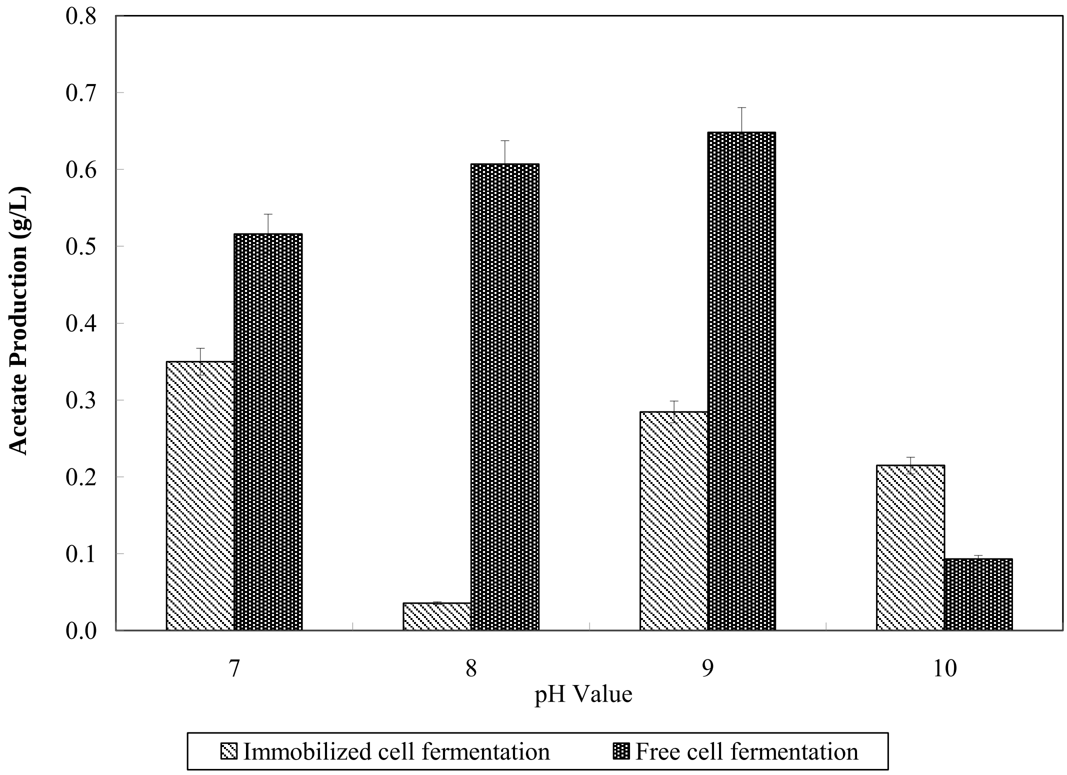


Figure 4.41m Acetate production comparison between immobilized cell fermentation and free cell fermentation at various pH for MCC fermentation at 4g/L initial ethanol level, 57ºC for 10 days

4.3.4 Impact of immobilization on substrate selectivity

The application of the immobilization technique on the *Clostridium* spp. co-culture dramatically enhances the efficiency in converting separate components of lignocellulosics for ethanol production. Ethanol productions from all chosen substrates including glucose, cellulose, cellobiose, and xylose were significantly improved under most conditions. Selectivity of the substrates remains unchanged as compared to free cell fermentation. The improvement resulted in the best ethanol production at pH=9 with high initial ethanol level (4 g/L) especially for hexose fermentation. Under this condition, the ethanol yield through the use of immobilized technique increased from 1.4 g/L to 4.9 g/L for glucose (97% of the theoretical efficiency), from 3.2 g/L to 4.8 g/L for cellobiose (85% of the theoretical efficiency), and from 3.8 g/L to 4.7 g/L for MCC (83% of the theoretical efficiency) (306) (Figure 4.42). This represents almost 20% of efficiency improvement for ethanol production compared to the maximum efficiency approached for free cell fermentation on hexoses. Xylose fermentation was dramatically improved when comparing immobilized cell fermentation to free cell fermentation at lower initial ethanol level. However, at pH=9 with 4 g/L initial ethanol level, improvement of ethanol production was not significant. Similar to free cell fermentations, the efficiency also approached around 90% of theoretical yield in immobilized fermentation. The MCC utilization lost its significant advantage over immobilized cell fermentation. The MCC was not observed as the most favorable substrate for co-culture as compared to free cell fermentation. This can be explained by the properties of the alginate gel used for immobilization. With the entrapment of alginate gel, the 18nm cellulosome degrading the MCC were retained within the gel (218). However, due to the large MCC molecule diameter ranges, some of them might not be able to diffuse into the immobilized cells and react with the cellulosome. Although the immobilization was able to increase the MCC utilization efficiency, limited molecules reacting with the cellulosome and other enzymes balanced out the improvement. This made the MCC lose its significant advantage over cellobiose and glucose utilization. For cellobiose and glucose, the diffusions within alginate gel are performing well during fermentation due to their small molecule size. Thus, the improvements of ethanol production for these substrates were more significant than MCC fermentation.

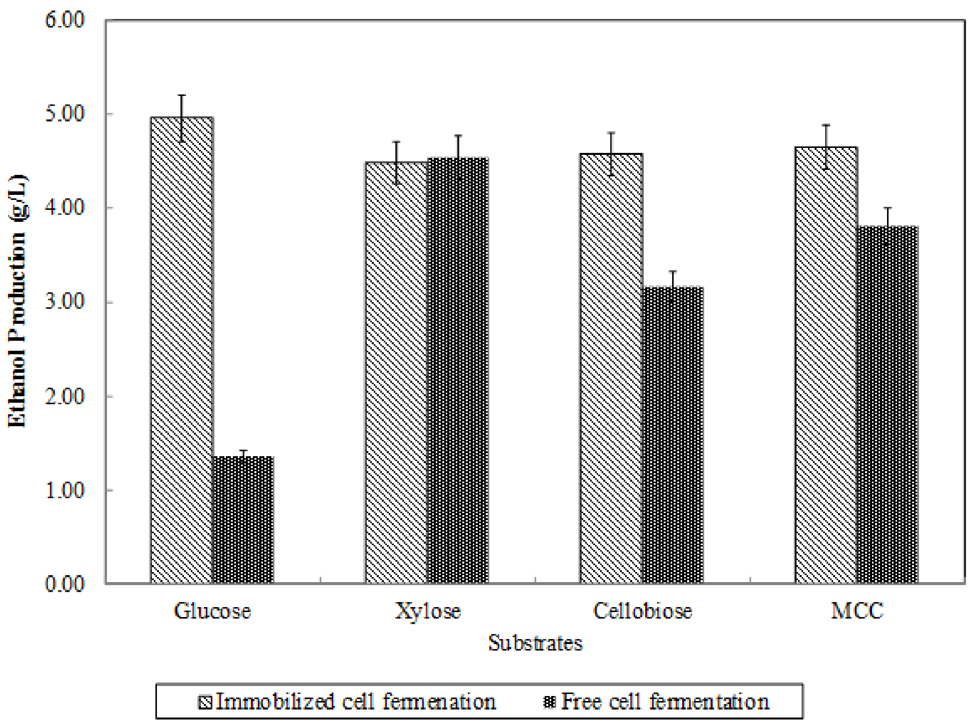


Figure 4.42 Ethanol production comparison between immobilized cell fermentation and free cell fermentation on various substrates under pH=9, at 4g/L initial ethanol level, 57ºC for 10 days

4.3.5 Immobilized cell co-culture fermentation of untreated and alkali treated aspen powder

The feasibility of fermenting lignocellulosic biomass by applying this immobilized co-culture was examined by applying immobilized co-culture on untreated aspen powder and alkali pretreated aspen powder at 2% loading rate. The alkaline pretreatment was performed by addition of 9% NaOH, heat at 100ºC for 3 hours in a 1L bioreactor. Upon complete of the pretreatment, no washing step was involved. The treated wood and liquid was neutralized with 0.5N HCl to optimum pH (pH=9). Composition changes as well as structural changes were investigated. At the optimized condition, the highest ethanol production of 1.37 g/L was observed for untreated wood fermentation and 2.78 g/L for alkaline pretreated wood.

4.3.5.1 Composition change and ethanol production

The significantly enhancement in ethanol production observed in our immobilized cell co-culture fermentation are mainly due to the compositional modifications during pretreatment and fermentation (Table 4.6). NaOH is strong agent, capable of dissolving some hemicellulose while opening up the lignocellulosic structure. Alkaline pretreatment significantly dissolved considerable amounts of xylan and galactan although cellulose did not show much loss during pretreatment. About 27% of the original hemicellulose is dissolved in the NaOH pretreatment (311). It also is known that alkaline treatment effectively removes the acetyl groups from hemicelluloses, allowing easier access. In addition, some lignin was also dissolved or modified during pretreatment. Since the co-culture is unable to utilize hemicellulose, and hemicellulose is considered to be a barrier for access to cellulose, alkaline pretreated biomass showed a clear advantage over fermentation of not pretreated biomass. By applying the alkaline pretreatment, the ethanol production was almost tripled. Through application of the immobilization technique to the pretreated material, the fermentation efficiency of aspen wood approached about 79% of the maximum theoretical yield based on glucan and xylan for CBP. Even with only the immobilization technique without pretreatment, 41.88% of the maximum theoretical yield was observed, showing the application of immobilized co-culture is an efficient way to convert lignocellulosic biomass into ethanol. These results clearly demonstrate the feasibility for using immobilized *Clostridium* co-culture for bio-ethanol production in a one-pot process. Even with the help of our pretreatment process, no detoxification is required before fermentation, only neutralization will be performed. However, for industrialization, the low biomass loading restriction will need to be overcome.

Immobilization	Original Cells		Free Cells		Immobilized Cells	
Sample / Content, %	Untreated Aspen	Alkaline Treated Aspen	Fermented Untreated Aspen	Fermented Alkaline Treated Aspen	Fermented Untreated Aspen	Fermented Alkaline Treated Aspen
Glucan	44.3	41.3	29.9	15.2	20.7	6.5
Xylan	17.9	12.3	14.8	10.1	12.5	4.7
Galactan	1.2	0.3	0.7	0.2	0.5	0.1
Arabinan	0.6	0.4	0.3	0.2	0.2	0.1
Mannan	1.7	0.8	0.9	0.5	0.5	0.2
Klason Lignin	21.2	19.4	21.1	19.3	21.1	19.2
Ethanol Production (g/L)	N/A	N/A	0.58	1.47	1.37	2.78

Table 4.6 Composition analysis for aspen wood before and after alkine pretreatment (9% NaOH,100ºC for 3 hours) and fermentation (at 4% initial ethanol level, pH=10, 57ºC for 10 days)

4.3.5.2 Preliminary test for increasing biomass loading rate for immobilized cell fermentation

In order to further increase the biomass loading rates for ethanol production, series of experiments stimulate the continuous fermentation process was done at pH=9 with 4 g/L initial ethanol concentration. Cellobiose was chosen as the substrate for this process. After the 10 days fermentation, fermentation medium containing unused substrates and final products were extracted out from reactor. Newly made medium containing 1% cellobiose and required nutrients was added to react with the remaining immobilized cells. The final production of ethanol for the fermentation process was performed. Figure 4.36 illustrates the amount of ethanol produced during this continuous fermentation process. The ethanol accumulated during this 10-day process. For the first three days, ethanol production was not noticeable due to the growth disturbance of the immobilization process. On the fourth day, the microbes start to enter the exponential growth period. Ethanol accumulation increased dramatically. After the eighth day of fermentation, most of the existing substrate was exhausted, and the cell growth started to decline. The maximum ethanol production (4.8 g/L) was approached on the ninth day of the fermentation and the production remained unchanged. If the old medium was replaced by fresh medium, no noticeable ethanol production was detected, even after 10 days cultivation. The immobilized cells were considered to be dead after the initial 10-day fermentation. In this case, the time of replacing the medium needs to be optimized so that for each cycle of fermentation, the ethanol production was optimized and the immobilized cell can be reused longer. Further investigations are required for the continuous process optimization.

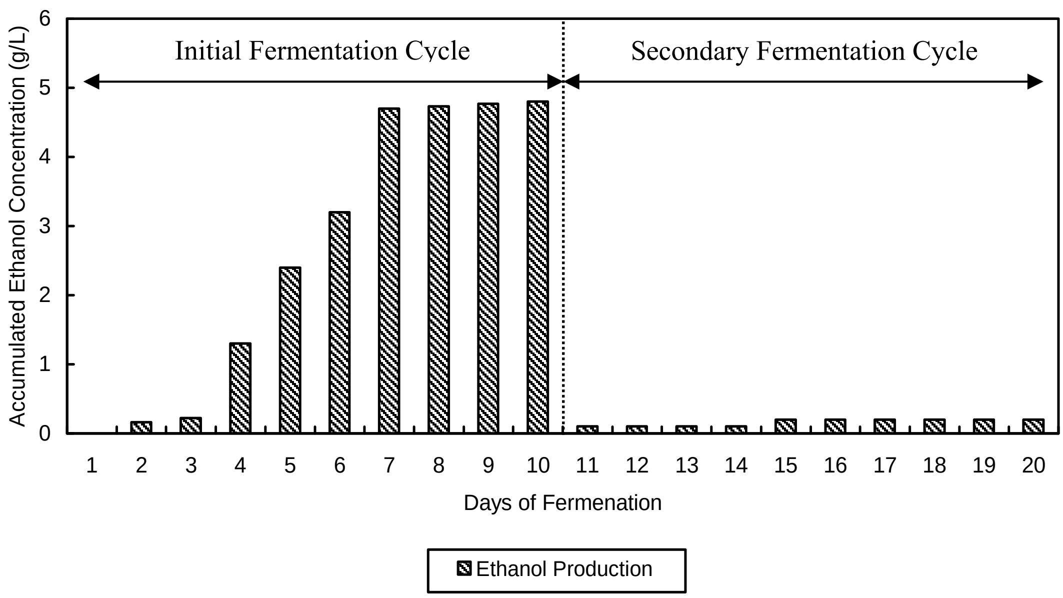


Figure 4.43 Accumulated ethanol production of continuous cellobiose fermentation (1% loading rate) process at pH=9, 57ºC for 10 days with 4 g/L initial ethanol concentration

Chapter 5

CONCLUSION

This study clearly shows the technical feasibility of using lignocellulosic biomass for ethanol production with our innovative co-culture, especially advantageous it the use of the co-culture together with an immobilization technique. The co-culture results shorter lag time and higher solvent production. Applying the chosen simultaneous *C. thermocellum/C. thermolacticum* co-culture in CBP fermentations dramatically improve the ethanol production for selected substrates, especially for MCC and xylose. Furthermore, the lag time for the strains has been shortened into 48 hours. These enhancements of MCC and xylose degradation clearly demonstrate synergistic effect between these two strains. The co-culture is able to tolerate up to 4 g/L initial ethanol level. Higher initial ethanol level led to improvement of ethanol production due to the relationship between existing ethanol concentration and optimum bacterium growth temperature. Alkaline condition, especially, pH = 9 was observed as the most favorable condition for bio-ethanol production.

For the ethanol production, it is even further increased through the application of the immobilization technique. Alkali condition with 4 g/L of initial ethanol is still considered to be the optimum fermentation condition. Ethanol production is dramatically improved in immobilized cell fermentation as compared to free cell fermentations, approaching over 80% of the theoretical conversion efficiency under identical conditions. Metabolism pathway shifting, existence of Ca^{2+}, and the property changes caused by cells' growth state alteration are considered the three possible explanations for this improvement. When comes to the influence of immobilization on the pH sensitivity, ethanol sensitivity and substrate selectivity, the immobilization is not able to protect the strains from extreme pH, however, ethanol sensitivity is found to be improved. The substrate selectivity was also changed due to the prosperities of the alginate gel. MCC is not considered to be the best substrate that has clear advantage over glucose and cellobiose for immobilized cell fermentations.

Comparatively higher starting pH is necessary to obtain maximum ethanol yield due to the acetyl groups found in native aspen which, as they are released reduce the pH. The ethanol yield obtained from untreated aspen fermentation is relatively low compared to pure cellulosic due to the hindered accessibility of wood or lack of fermentable substrates at the early fermentation stages. Therefore, adding sugars at the beginning of the fermentation to initiate the microbial growth, modifying the substrate or adding hemicellulose degraders to provide higher accessibility might be necessary. In our study 9% NaOH was added as pretreatment agent before immobilized co-culture are introduced. During the alkaline pretreatment, considerable amount of hemicellulose is dissolved or modified. Thus, the carbohydrate-lignin complex structure is disrupted. The lignocellulosic structure provides higher accessibility to the enzymes secreted by immobilized co-culture.

Approximately 80% of the maximum theoretical efficiency is approached for ethanol production, which is twice the amount of un-pretreated aspen fermentation. No expensive detoxification process or extra piece of equipment is requires. Only a simple neutralization within the same reactor would be necessary. This study optimized an immobilized *C. themocellum*/*C. thermolacticum* co-culture, by overcoming the potential inhibitor effect of high pH and high ethanol concentrations, allowing for an effective merging of pretreatment and fermentation processes. Immobilized co-culture CBP fermentation will result in reduced equipment and processing cost, bringing the technology closer to economic feasibility

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