INITIAL INVESTIGATION ON XYLOSE FERMENTATION FOR

LIGNOCELLULOSIC BIOETHANOL PRODUCTION

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INITIAL INVESTIGATION ON XYLOSE FERMENTATION FOR LIGNOCELLULOSIC BIOETHANOL PRODUCTION

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INITIAL INVESTIGATION ON XYLOSE FERMENTATION FOR

LIGNOCELLULOSIC BIOETHANOL PRODUCTION

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VITA

Yanli Chen, daughter of Peiwen Chen and Ailan Song, was born on March 20, 1978 in Weihui, Henan Province of the People's Republic of China. She received her high school diploma from the First High School of Weihui in July, 1996. Ms. Chen entered Northwest University at Xi'an of the People's Republic of China in the fall of 1996, and graduated with a Bachelor of Chemical Engineering (distinction) in July 2000. As a research assistant, she has worked at the Institute of Process Engineering, Chinese of Academy of Sciences for almost four years. In the fall of 2004, she entered the Graduate School of University of Houston at Houston, Texas, United States. With a Master of Science in Chemical Engineering, she graduated from University of Houston in 2006. Then, she entered the Graduate School at Auburn University in the Department of Chemical Engineering in August 2006 and began working in the field of systems biology.

THESIS ABSTRACT

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Bioethanol production from lignocellulosic biomass has drawn tremendous attention, not only because bioethanol has a number of attractive traits as an alternative fuel to fossil fuel, but also due to attractive characteristics of lignocellulosic biomass as a feedstock. However, there is lack of a cost-competitive bioethanol production process since significant challenges exist, such as pretreatment of biomass feedstock, optimization of hydrolysis process, xylose fermentation and so on. In this work, we concentrate on the issue of xylose fermentation. Xylose is the second most abundant fermentable sugar in biomass hydrolysate, so its fermentation is essential for economic conversion of lignocellulose to bioethanol. However, the native strains of ethanol-producing microorganisms, such as *S. cerevisiae*, *P. stipitis*, *E. coli* and *Z. mobilis*, can not achieve effective cofermentation of glucose and xylose due to their intrinsic

limitations. This thesis presents two potential routes for solving the xylose fermentation issue, modified single strain and co-culture. The basic idea of modified single strain is to engineer native strains to selectively produce bioethanol from glucose and xylose by the use of recombinant DNA techniques. The basic idea of co-culture on ethanol production is to utilize two different microbial strains in a system and enable one to ferment glucose, the other to ferment xylose stably and effectively. Current research status, difficulties and possible strategies about the two routes are depicted in details. Furthermore, valuable future research directions of the two routes are recommended. Through a broad and complete review, some initial investigation results are provided.

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CHAPTER 1

INTRODUCTION

Pressing economic and environmental factors, such as soaring crude oil prices, global warming and diminishing oil reserves, have been driving global interest in searching for renewable energy to replace fossil fuels (Stephanopoulos, 2007; Lin and Tanaka, 2006). As one of the renewable fuels, biofuels have been considered as potential alternatives to fossil fuel for the future. Biofuels are defined as liquid, solid, or gaseous fuels derived from renewable biological sources. Bioethanol has been regarded as a clean energy alternative due to its more complete combustion and lower carbon monoxide emissions.

Currently, most commercial bioethanol is produced by the fermentation of starch or simple sugar from crops such as corn grain, sugar cane, and beets. Therefore, production costs are high due to higher source fees and operations fees. Moreover, the increasing and competing demand for bioethanol can result in serious supply scarcity and undesirable price increases of these agronomic crops. On the other hand, lignocellulosic biomass (such as agricultural residues, forestry wastes, waste paper, and energy crops) represents a non-food biomass resource and has been recognized as a potential sustainable source of mixed sugars to produce bioethanol due to vast quantities, low price and environmental benefits (DOE, 2006). In recent years, more and more attention has been focused on the conversion of lignocellulosic biomass to bioethanol production.

Lignocellulosic biomass mainly consists of carbohydrate polymers (cellulose and hemicellulose) and phenolic polymers (lignin). Lower concentrations of various other compounds are also present. Cellulose and hemicellulose, which typically make up two-thirds of lignocellulosic biomass by weight, are polysaccharides that can be hydrolyzed to sugars and then fermented to bioethanol. Lignin, accounting for 15-25% by weight due to different biomass sources, can not be used in fermentation processes.

Generally, the conversion of lignocellulosic biomass to bioethanol involves three basic steps: 1) pretreatment to liberate cellulose and hemicellulose from lignin; 2) hydrolysis of complex polysaccharides to simple sugars with acids or special enzymes. Upon hydrolysis, cellulose and hemicellulose can be broken down to a mixture of monomeric hexose (glucose, mannose and galactose) and pentose (xylose and arabinose). Among these sugars, glucose is normally the most abundant followed by xylose; 3) Fermentation of mixed hexose and pentose sugars to produce ethanol by ethanologenic microbes (Stephanopoulos, 2007).

Currently, converting lignocellulosic biomass to bioethanol is too expensive to be used on a commercial scale. Therefore, researchers are working to improve the efficiency and economics of the lignocellulosic biomass to bioethanol conversion process by focusing their efforts on the two most challenging steps: cellulose hydrolysis and sugar fermentation.

Since glucose and xylose are the two principal components of lignocellulosic biomass hydrolysates, the issue of sugar fermentation mainly refers to glucose and xylose fermentation. Glucose fermentation has been well studied. However, xylose fermentation is still a problem. Currently, most research efforts of solving xylose fermentation in the

process of bioethanol production from lignocellulosic biomass are mainly concentrated on two aspects: 1) genetic modification of glucose-fermenting microbial strains to ferment xylose; and 2) co-culture of glucose-fermenting and xylose-fermenting strains. This thesis presents the status of current research, difficulties and possible strategies, and future directions concerning these two methods. Moreover, some initial results regarding these two methods are given in the final chapter.

CHAPTER 2

BACKGROUND

This chapter provides background information about biomass, bioethanol, and conversion process of biomass to bioethanol. Also, current issues and strategies of producing bioethanol are described.

2.1 Biomass

Biomass is defined as consisting of all plant and plant-derived materials including livestock manures (DOE, 2006). Through photosynthesis, plants use light energy from the sun to convert water and carbon dioxide to sugars that can be stored. Some plants, like sugar cane and sugar beets, store the energy as simple sugars. Other plants, like corn, potatoes and root crops, store the energy as more complex sugars, called starches. Currently, industrial ethanol production is carried out by using starchy materials such as corn, wheat starch and potatoes. However, bioethanol from starchy materials has put the effort into direct competition with the food industry. Lignocellulosic biomass is the non-starch, fibrous part of plant materials. It is an attractive resource because it is renewable, abundant and low cost (Perlack et al., 2005). In recent years, more and more attention is being focused on the use of lignocellulosic biomass for the production of bioethanol via fermentation. Lignocellulosic biomass that can be used as feedstocks to produce bioethanol includes: 1) agricultural residues (leftover material from crops, such as corn

stover and wheat straw); 2) forestry wastes (chips and sawdust from lumber mills, dead trees, and tree branches); 3) municipal solid wastes (household garbage and paper products); 4) food processing and other industrial wastes (black liquor, a paper manufacturing by-product); and 5) energy crops (fast-growing trees and grasses, such as switchgrass, poplar and willow) (DOE, 2006).

For this discussion, the word "biomass" will refer to "lignocellulosic biomass." The primary components of biomass are carbohydrate polymers (cellulose, hemicellulose) and phenolic polymers (lignin). Low concentration of various other compounds, such as proteins, acids, salts, and minerals, are also present. The general composition of biomass is shown in Figure 2.1.

Cellulose is the most common form of carbon in biomass, accounting for 30%-50% by weight. It is a glucose polymer linked by β-1, 4 glycosidic bonds. The basic building block of this linear polymer is cellubiose, a glucose-glucose dimer. Hydrolysis of cellulose results in individual glucose monomers, which can be fermented to ethanol directly. Hemicellulose is a short, highly branched polymer containing five-carbon sugars (usually xylose and arabinose) and six-carbon sugars (glucose, galactose and mannose). It is at levels of between 20% and 40% by weight depending on the biomass types. Hemicellulose is more easily hydrolyzed than cellulose because of its branched, amorphous nature. When hydrolyzed, the hemicellulose from hardwoods releases products high in xylose (a five-carbon sugar). Lignin which provides structural integrity in plants is the largest non-carbohydrate fraction of lignocellulose. It makes up 15% to 25% by weight of biomass. Unlike cellulose and hemicellulose, lignin can not be utilized in the fermentation process. However, it contains a lot of energy and can be burned to

produce steam and electricity for the biomass-to-bioethanol process. The composition of cellulose, hemicellulose and lignin varies with the sources of biomass. Table 2.1 shows the composition of several selected agricultural residues, forestry wastes and energy crops.

2.2 Bioethanol

The principle fuel used as a gasoline substitute for road transport vehicles is bioethanol. Bioethanol has a number of advantages over fossil fuels. Firstly, it comes from a renewable resource. Secondly, it is biodegradable, low in toxicity and causes little environmental pollution. Bioethanol is a high octane fuel and can be added into gasoline as an octane enhancer. In the United States, "ethanol is blended with gasoline at a 10:90 ethanol-to-gasoline ratio to boost the fuel's octane rating, which allows it to burn more cleanly, reducing urban smog" (Service, 2007). Thirdly, the use of bioethanol can reduce the greenhouse gas emissions. Relative to fossil fuels, greenhouse gas emissions are reduced about 18% by the use of corn-based ethanol, but it can be up to 88% if using cellulosic ethanol (Service, 2007). A closed carbon dioxide cycle can be formed by using bioethanol as fuels. After combustion of bioethanol, the released carbon dioxide is recycled back into crops because crops use carbon dioxide to synthesize cellulose during photosynthesis (Chandel et al., 2007). In addition, blending bioethanol with gasoline will help extend the life of the diminishing fossil oil supplies and ensure greater fuel security, avoiding heavy reliance on oil producing nations that have not always been very stable. Another advantage of encouraging bioethanol use is that the rural economy would receive a boost from growing the necessary crops and creating new employment opportunities

(DOE, 2006). In addition, using agricultural and industrial residues to produce bioethanol can solve the waste disposal problem and provide environmental benefits. Currently in the United States, bioethanol is mainly produced from corn starch by the sugar fermentation process. However, the increased demand for bioethanol will result in serious problems, such as supply scarcity and dramatic increases in the cost of ~~the~~ food. Moreover, even converting all the starch to bioethanol, it can only reduce 10% of the gasoline demand (Service, 2007). Therefore, lignocellulosic bioethanol is thought to be the answer for solving these problems. We will introduce the conversion process of biomass to bioethanol in the following section.

2.3 Conversion Process of Biomass to Bioethanol

Basically, the overall process for converting lignocellulose to bioethanol is comprised of four major unit operations: pretreatment, hydrolysis, fermentation and product separation/distillation. Figure 2.2 shows in a block diagram the basic features of this process.

Pretreatment is an important first step in the conversion process of biomass to bioethanol. This step reduces the biomass size and opens up the plant structure since native lignocellulosic biomass is extremely recalcitrant to hydrolysis. There are several pretreatment methods such as mechanical combination, steam explosion, ammonia fiber explosion, acid or alkaline pretreatment and biological treatment (Chandel et al., 2007). Each of these is suitable for different types of biomass. Currently, pretreatment is still one of the most expensive processing steps with the cost as high as 30 cents per gallon produced (Moiser et al., 2005). Therefore, lowering the cost of the pretreatment process

is necessary in order to achieve the production of bioethanol from lignocellulosic biomass on commercial scale.

After pretreatment, the cellulose and hemicellulose portions need to be broken down further by enzymes or acids into monomeric sugars for the fermentation into ethanol. There are three principle methods of extracting sugars from biomass: dilute acid hydrolysis, concentrated acid hydrolysis and enzymatic hydrolysis.

2.3.1 Dilute acid hydrolysis

The dilute acid hydrolysis process is one of the oldest and simplest methods of extracting fermentable sugars from biomass. This process is carried out in two stages. In the first stage, the feedstock is mixed with 0.75% sulfuric acid and heated to approximately 50 ºC followed by transferring the material to the first stage acid impregnator where the temperature is increased to 190 ºC. Roughly, 80% of the hemicellulose and 29% of the cellulose are hydrolyzed in the first stage (Chandel et al., 2007). The second stage is optimized to yield the more resistant cellulose fraction. Normally, this is achieved by using 0.4% sulfuric acid at 230 ºC. Then, the liquid hydrolysate is neutralized and recovered from the process. Dilute acid hydrolysis has some limitations. If higher temperatures or longer residence time are applied, the monomeric sugars derived from hemicellulose will degrade to form some fermentation inhibitors, such as furan compounds and weak carboxylic acids (Olsson and Hägerdal, 1996). In order to remove these fermentation inhibitors, several chemical and biological methods could be used, such as ion exchange, charcoal adsorption and biological

detoxification (Chandel et al., 2007). However, the detoxification method will increase operating cost.

2.3.2 Concentrated acid hydrolysis

The concentrated acid hydrolysis process can provide complete and rapid conversion of cellulose to glucose and hemicellulose to xylose with little degradation. In this process, 70% sulfuric acid is added to the biomass with 10% moisture content (in a ratio of 1.25 acid: 1 biomass) in a reactor. The temperature is controlled at 50 ºC for two to hours (Chandel et al., 2007). Water is then added to dilute the acid to 20-30% and the mixture is again heated to 100 ºC for one hour. The gel produced from this mixture is then pressed to release an acid sugar mixture and a chromatographic column is used to separate the acid and sugar mixture. The obtained acid can be reconcentrated through multiple effect evaporators. Approximately, 90% of both cellulose and hemicellulose can be depolymerized into their monomeric sugars with concentrated hydrolysis, so this process has the advantage of high sugar recovery efficiency (Chandel et al., 2007). In addition, monomeric sugar degradation can be avoided with low temperature and pressure in this process.

2.3.3 Enzymatic hydrolysis

Besides acid, cellulolytic enzymes which are highly specific can be used to hydrolyze the pretreated biomass into fermentable sugars. These enzymes can be produced from some bacterial and fungal sources or obtained from some commercial companies, such as Novozymes. However, the process is very expensive compared with

acid hydrolysis due to the high enzyme cost. Although the cost of cellulolytic enzyme has come down to 20 to 30 cents per gallon of ethanol produced, this conversion process cannot be competitive with the process of ethanol production from starch in corn kernels at a cost of 3 to 4 cents per gallon of ethanol (Stephanopoulos, 2007).

After hydrolysis, the primary fermentable sugars in hydrolyzate are pentose and hexose, such as glucose and xylose. Different microorganisms are used to ferment these sugars to produce bioethanol, such as *Saccharomyces cerevisiae*, *Pichia stipitis*, *Kluyveromyces marxianus*, *Candida shehatate*, *Zymomonas mobilis* and *Escherichia coli.* Currently, the fermentation of a mixture of hexose and pentose is inefficient because no wild organism has been found that can convert all sugars into ethanol at a high yield (Ragauskas et al., 2006). For example, the most widely used glucose-fermenting yeast *S. cerevisiae* is able to effectively ferment glucose, but it can not take xylose as a carbon source. Although *E. coli* does metabolize xylose, it naturally produces a variety of acetic and lactic acids as fermentation products, so the bioethanol yield is comparatively low (Service, 2007). The lack of industrially suitable microbes for converting biomass into bioethanol has been a major technical problem.

Fermentation can be preformed as a batch, fed batch and continuous process. Generally, batch fermentation is the most widely used mode by the fermentation industry. The kinetic properties of microorganisms, type of lignocellulosic hydrolysate and process economics aspects are the determinative factors for the choice of most suitable process mode. After fermentation, dilute bioethanol can be purified through distillation and/or filtration to produce the desired fuel-grade quality ethanol.

During the biomass-to-bioethanol process, there are two significant steps: hydrolysis and fermentation. In order to improve the efficiency and economics of the ethanol production process and use it at commercial scale, intensive research has been conducted to improve biomass hydrolysis and fermentation technologies.

Enzymatic hydrolysis performed separately from fermentation step is known as separate hydrolysis and fermentation (SHF). This process offers various processing advantages. It enables enzymes to operate at higher temperature (45-50 ºC) for increased performance and fermenting microbes to operate at moderate temperature (28-35 ºC) for optimizing of the sugars utilization (Lin and Tanaka, 2006). However, it has some shortcomings. For instance, the hydrolytic enzymes can be inhibited by hydrolysis products: glucose and short cellulose chains. In addition, the operating cost is higher since separated reactors for saccharification and fermentation are needed.

In order to prevent inhibition of the hydrolytic enzymes by the reaction products, enzymatic hydrolysis and fermentation can be combined in one unit, termed as simultaneous saccharification and fermentation (SSF). This is a one-step process in which enzymatic hydrolysis and sugar fermentation are carried out in one reactor. Except keeping the glucose concentration low and prevent inhibition of the hydrolytic enzymes, SSF has other advantages, such as increasing the overall rate of biomass to bioethanol conversion, shortening fermentation time and reducing operating cost. However, it also has some drawbacks due to the low rate of cellulose hydrolysis and most ethanol producing microorganisms can not utilize all of the sugars derived after hydrolysis. By using promising ethanol bacteria recombinant *E. coli* K011, *Klebsiella oxytoca* and *Z. mobilis*, SSF process has been improved after including the co-fermentation of multiple

sugar substrates present in the hydrolysate (Chandel et al., 2007). The new variant of SSF is known as simultaneous saccharification and co-fermentation (SSCF). SSCF also has the advantages of lower cost, higher ethanol yield and shorter processing time. The most promising conversion method is consolidated bioprocess (CBP), featuring cellulase production, cellulose hydrolysis and fermentation in one step. In this process, ethanol and all required cellulolytic enzymes are produced by a single microorganism. This can be achieved by expression of cellulases in fermenting organisms or transfer of the bioethanol-synthesizing pathway into a cellulase-producing organism. CBP will have the benefit of completely eliminating the cost of purifying cellulase (Stephanopoulos, 2007). Moreover, it has the potential to provide the lowest cost route for bioconversion from lignocellulosic biomass to bioethanol and other products (Lynd et al., 2005). However, this process can not become the leading process at present due to the relative lag in development of molecular biological methods to manipulate organisms.

2.4 Current Issues and Strategies

Lignocellulosic bioethanol is proposed as having such benefits as: reduction of greenhouse gas emissions, reduction of fossil fuel use, increased national energy security, increased rural development, a sustainable fuel supply for the future. Although significant advances have been made at bench scale toward the bioethanol generation from lignocellulose, there are still technical and economical barriers, which make the bioethanol program unsuccessful on a commercial scale. Currently, the challenges include 1) low bulk density feedstock; 2) high viscosity substrate; 3) optimization of

hydrolysis and fermentation; 4) fermentability of substrate; 5) xylose fermentation; 6) cost challenges.

"Xylose is a five-carbon sugar abundant in hardwoods and agriculture residues, so its fermentation is essential for economic conversion of lignocellulose to ethanol" (Jeffries et al., 2007). The widely used microorganism in crop-based ethanol production, *S.* cerevisiae cannot ferment xylose, therefore, it has limited use for conversion of lignocellulose to ethanol. The ethanologenic bacterium *Z. mobilis* has the same limitation. Some xylose-fermenting microorganisms, such as *P. stipitis*, *C. shehatate*, and *E. coli*, have other drawbacks including low ethanol yield and low ethanol tolerance to hamper their large-scale utilization in biomass to bioethanol process (Zaldivar et al., 2001). In this thesis, we focused on the problem of xylose fermentation to do some initial investigation. To date, there are two viable strategies for solving xylose fermentation issue: modified single strain and co-culture. By introducing pathways for the xylose fermentation, some microorganisms have been engineered to coferment glucose and xylose effectively. The successful examples are recombinant *E. coli*, *Z. mobilis* and *S. cerevisiae* (Alterthum and Ingram, 1989; Amore et al., 1991; Takuma et al., 1991; Zhang et al., 1995; Eliasson et al., 2000; Sedlak and Ho, 2004). Besides the methods of engineered microbes, co-culture conversion strategy has been suggested as a breakthrough, high-payoff opportunity in bioethanol production (DOE, 2006). In co-culture systems, there are two different microbial species which can ferment glucose and xylose respectively. We will explain the two routes for solving xylose fermentation problem in the following chapters in more details.

Table 2.1 Cellulose, hemicellulose, and lignin content in various sources of biomass

Feedstock	Cellulose	Hemicellulose	Lignin	Reference
Corn stover	36.4	22.6	16.6	DOE, 2006
Corn cob	42.0	39.0	14.0	Kuhad and Singh, 1993
Rice straw	32.0	24.0	13.0	Kuhad and Singh, 1993
Wheat straw	30.0	24.0	18.0	Kuhad and Singh, 1993
Rice hulls	36.0	15.0	19.0	Kuhad and Singh, 1993
Saw dust	55.0	14.0	21.0	Olsson and Hägerdal, 1996
Willow	37.0	23.0	21.0	Olsson and Hägerdal, 1996
Switchgrass	31.0	24.4	17.6	DOE, 2006
Poplar	49.9	20.4	18.1	DOE, 2006

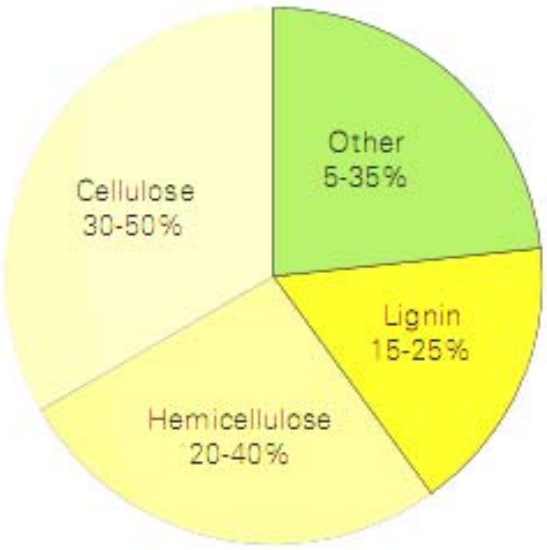


Figure 2.1 General composition of lignocellulosic biomass (Lee et al., 2007)

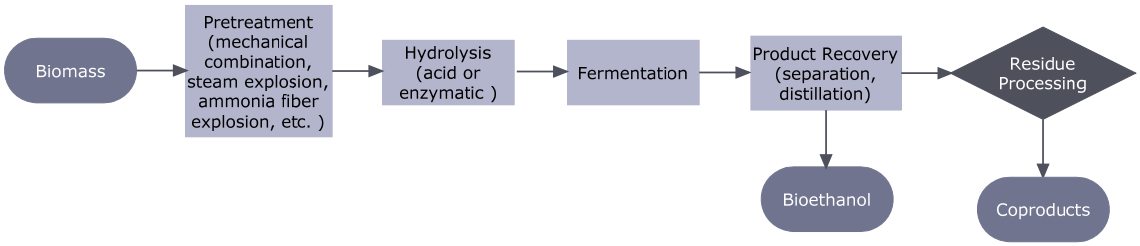


Figure 2.2 Schematic diagram of the conversion process of biomass to bioethanol

CHAPTER 3

MODIFIED SINGLE STRAIN

FOR GLUCOSE AND XYLOSE COFERMENTATION

The efficient fermentation of xylose is critical to attaining economically feasible processes for bioethanol production from lignocellulosic biomass since xylose is so abundant in biomass. However, there are no robust industrial microorganism strains that can ferment glucose and xylose simultaneously and efficiently. One detour to solve this problem is to genetically modify or adjust some potential strains by introducing pathways for xylose fermentation. The greatest successes have been made in the engineering of *S. cerevisiae*, *E. coli* and *Z. mobilis*. In this chapter, we will introduce several examples of successful recombinant strains, then address the current difficulties of modified single strain research. Finally, metabolic network modeling will be suggested to help solve these difficulties.

3.1 Successful Examples of Modified Single Strain

The characteristics required for an industrially suitable microorganism are summarized in Table 3.1 (Dien et al., 2003). However, no naturally occurring microorganisms can satisfy all of these features. The lack of industrially suitable microbes for converting biomass into ethanol has been a major technical barrier. Among all of the traits, ethanol yield is the most important one. In order to obtain a high ethanol

yield, the stains should produce ethanol with few side productions, and metabolize all major sugars. The primary fermentable sugars in hydrolysate from cellulose and hemicellulose are pentose and hexose (e.g., glucose and xylose). However, the well-known fermentative yeast *S. cerevisiae* naturally converts glucose to ethanol, but does not ferment xylose. Although *E. coli* metabolize pentose sugars, it naturally produces a variety of acetic and lactic acids as fermentation products, but ethanol as a minor fermentation product (Service, 2007). By introducing pathways for the xylose fermentation, some microorganisms have been engineered to ferment both glucose and xylose and selectively produce ethanol. The successful examples are recombinant *S. cerevisiae*, recombinant *E. coli*, and recombinant *Z. mobilis* (Ingram et al., 1997; Bothast et al., 1999; Dien et al., 2003; Jeffries and Jin, 2004; Potera, 2006; Chu and Lee, 2007). It has been shown that metabolic engineering of microorganisms is a very efficient tool for increasing ethanol yield. Table 3.2 compared the fermentation performance of several genetically engineered microorganisms on pretreated corn fiber hydrolyzates or glucose/xylose mixtures. From this table, we can see that utilization of these recombinant strains in the fermentation with biomass hydrolyzates or glucose/xylose mixture can keep high ethanol yield (from 0.41~0.50 g g^{-1}) which is very close to the theoretical ethanol yield from glucose or xylose (0.51g g^{-1}) (Zhang et al., 1995) . In addition, high ethanol volumetric productivity can also be obtained by using these recombinant strains.

3.1.1 Recombinant *S. cerevisiae*

As a promising candidate for industrial bioethanol production, *S. cerevisiae* has attractive strengths, such as robustness, public acceptance, inhibitor tolerance and high

ethanol productivity (Karhummaa et al., 2007). However, the native strain cannot ferment xylose. Xylose is a five-carbon monosaccharide that exists in the hydrolysate of biomass with large amount. Naturally, there are some bacteria, yeasts and fungi which can metabolize xylose. Bacteria generally accomplish conversion of xylose to xylulose in one step catalyzed by xylose isomerase (XI) encoded by the *XYLA* gene, while yeasts and fungi use different pathway to degrade xylose to xylulose (Bothast et al., 1999). Xylose reductase (XR) and xylitol dehydrogenase (XDH) are the two main enzymes used in this method of xylose degradation. XR, encoded by the *XYL1* gene, is responsible for the reduction of xylose to xylitol and is aided by cofactors NADH or NADPH. Xylitol is then oxidized to xylulose by XDH, which is expressed through the *XYL2* gene, and accomplished exclusively with the cofactor NAD^+ (Chu and Lee, 2007). Therefore, there are two main pathways of xylose metabolism, "Xylose Reductase-Xylitol Dehydrogenase" or XR-XDH pathway and "Xylose Isomerase" (XI) pathway. After producing xylulose, both of XR-XDH and XI pathways proceed through enzyme xylulokinase (XK), encoded on gene *XKS1*, to further modify xylulose into xylulose-5-P where it then enters the pentose phosphate pathway for further catabolism. Figure 3.1 clearly describes the two metabolic pathways for xylose utilization in bacteria, yeasts and fungi. The two different pathways have been applied to construct recombinant xylose-fermenting *S. cerevisiae*.

In 1991, researchers successfully obtained xylose-fermenting recombinant *S. cerevisiae* strains transformed with *P. stipitis* native genes *XYL1* encoding xylose reductase (XR) (Amore et al., 1991; Takuma et al., 1991). The success of obtaining *S. cerevisiae XYL1* transformants gave the first indication that the metabolic engineering

approach could be used to improve xylose fermentation. Later, Kötter and Ciriacy (1993) isolated the *XYL1* and *XYL2* genes coding for XR and XDH from *P. stipitis* and expressed them in *S. cerevisiae*. Although this recombinant strain can metabolize xylose, the primary product is xylitol not ethanol. Moreover, the growth and fermentation rate is very slow. These limitations for this recombinant strain are probably caused by cofactor imbalance and an insufficient capacity of xylulose conversion by the pentose phosphate shunt (Kötter and Ciriacy, 1993). Since the native *S. cerevisiae* xylulokinase (XK) activity is insufficient for xylose or xylulose fermentation, over-expression is required to obtain high ethanol yields. In 1998, Ho et al. firstly constructed a recombinant *S. cerevisiae* strain 1400 (pLNH32) expressing the XR and XDH from *P. stipitis* and overexpressing XK from a high copy number yeast-*E. coli* shuttle plasmid. The ethanol yield can reach 0.46 g g^{-1} and the ethanol productivity is 1.15 g l^{-1} h^{-1} by using this strain to ferment a mixture of glucose (90 g l^{-1}) and xylose (40 g l^{-1}) (Ho et al. 1998). Another important recombinant strain, *S. cerevisiae* TMB3001, was metabolically engineered with a xylose-utilizing pathway by integrating the *P. stipitis* genes *XYL1* and *XYL2* and the endogenous *XKS1* (Eliasson et al., 2000). This strain can convert xylose to ethanol under anaerobic condition as well as aerobic condition (Eliasson et al., 2000; Zaldivar et al., 2002). Ethanol yields of 0.30 g g^{-1} was obtained from xylose-glucose mixtures in anaerobic chemostat cultures, with a dilution rate of 0.06 h^{-1} (Jeppsson et al., 2002). Although the recombinant *S. cerevisiae* TMB3001 can grow on xylose, the xylose utilization rate is low due to the imbalance of redox co-factors. Metabolic engineering of the ammonium assimilation in xylose fermenting *S. cerevisiae* has been used as a strategy to improve ethanol yield by modulating the redox metabolism (Roca et al., 2003;

Grotkjaer et al., 2005). The stable recombinant strain TMB3001, carrying XR and XDH from *P. stipitis* and over-expressed endogenous *XKS1* was modified by deleting the *GDH1* gene encoding an NADPH-dependent glutamate dehydrogenase, and by over-expressing either the *GDH2* gene encoding an NADH-dependent glutamate dehydrogenase or the glutamate synthase (*GLT1*) and glutamine synthase (*GLN1*) genes encoding the GS-GOGAT complex. These modifications in the ammonia assimilation pathway resulted in beneficial alterations of the redox metabolism and hereby of the xylose metabolism. The over expression of *GDH2* strains increased ethanol yield from 0.43 to 0.51 mol of carbon (Cmol) $Cmol^{-1}$ mainly due to a 44% reduction of xylitol excretion, and the over expression of GS-GOGAT complex strains increased ethanol yield by 16% in carbon-limited continuous cultivation at a low dilution rate (Roca et al., 2003). Grotkjaer et al. (2005) constructed a metabolic flux model for two recombinant *S. cerevisiae* trains TMB30001 and CPB. CR4. "Metabolic flux analysis suggested that the ratio of the specific XR activity utilizing different co-factors (NADH/NADPH) was shifted from being primarily NADPH dependent for the TMB3001 strain to be partly NADH dependent for the CPB.CR4 strain. A higher NADH/NADPH ratio for co-factor usage by the XR is clearly beneficial for solving the redox imbalance for xylose fermenting *S. cerevisiae* (Grotkjaer et al., 2005)".

The redox co-factors imbalance problem associated with XR- and XDH- coupled reactions that are particularly harmful to efficient anaerobic xylose fermentation can also be circumvented by introducing XI pathway into *S. cerevisiae*. Heterologous expression of several xylose isomerases (XI) from different microorganisms in *S. cerevisiae* has been attempted (Sarthy et al., 1987; Amore et al., 1989; Moes et al., 1996; Walfridsson et

al., 1996; Gárdonyi and Hahn-Hägerdal, 2003; Kuyper et al., 2003). However, the activity of XI when expressed in *S. cerevisiae* is generally reduced or lost due to some reasons, such as differences in internal pH between bacteria and yeasts, protein misfolding and unsuitable post-translational modifications (Bothast et al., 1999; Chu and Lee, 2007). The overall ethanol yields by using recombinant *S. cerevisiae* expressing XI have been low, and further development to improve XI activity and heterologous expression in *S. cerevisiae* through adaptation and metabolic manipulation will be required to achieve high ethanol yield.

3.1.2 Recombinant *E. coli*

Although E. coli has the natural ability to metabolize glucose, xylose, galactose, mannose and other sugars, the fermentation products are a mixture of acidic and neutral products. More important, the amount of ethanol is very small. Since 1986, the microbiologist Lonnie Ingram of the University of Florida and his research group have been working on the genetic engineering of different *E. coli* strains for ethanol production (Ingram et al., 1987; Ingram and Conway, 1988; Alterthum and Ingram, 1989; Ingram et al., 1991; Ohta et al., 1991; Ingram et al., 1997). They developed ethanologenic derivatives of *E. coli* B (ATCC11303) in which pyruvate metabolism was redirected to ethanol and carbon dioxide by the integration and functional expression of *Z. mobilis* genes encoding pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adhB*). A deletion was also introduced into the fumarate reductase gene (*frd*) to minimize succinate production and increase ethanol yield (Ingram et al., 1997; Tao et al., 2001). The resulting strains, K011, can efficiently ferment high concentrations of glucose and xylose

to ethanol at yields 103% to 106% of theoretical. “The extra ethanol was thought to arise from fermentation of carbohydrates present in rich medium that were not account for in the sugar balance ”(Dien et al., 2003). Furthermore, *E. coli* K011 appears to grow more rapidly than the parent on plates and in broth (Tao et al., 2001). The major shortcomings of using recombinant *E. coli* in industrial fermentation are a narrow and neutral pH growth range (pH 6.0-8.0), less hardy cultures compared to yeast, and public perceptions regarding the danger of *E. coli* strains (Dien et al., 2003). Also, it has low ethanol tolerance (6.4% ethanol in the final fermenting solution) (Service, 2007). Regarding the stability of the recombinant *E. coli* K011, some researchers (Dumsday et al., 1999) compared it in batch and chemostat culture. They found that chemostat culture on glucose was remarkably stable, but on mannose, xylose and a xylose/glucose mixture, this recombinant strain progressively lost their hyperethanologenicity. Their results indicate the genetic instability of the recombinant *E. coli* K011 on xylose in the chemostat culture.

3.1.3 Recombinant *Z. mobilis*

As one of potential biocatalysts for bioethanol production, *Z. mobilis* has some attractive properties, such as high ethanol tolerance (up to 120 g/L ethanol), high ethanol yield, high specific ethanol productivity and public safety (Dien et al., 2003). However, wild-type *Z. mobilis* has an extremely limited substrate range, such as glucose, fructose and sucrose (Lawford and Rousseau, 2002), therefore, it can not ferment xylose. Much effort has been put into attempts to give *Z. mobilis* a xylose-fermenting capacity by the use of recombinant DNA techniques. Researchers at the National Renewable Resources Laboratory (NREL) were the first to construct a recombinant strain CP4 (pZB5) capable

of fermenting xylose (Zhang et al., 1995). This recombinant strain was engineered by transforming four *E. coli* genes (xylose isomerase (*xylA*), xylulokinase (*xylB*), transketolase (*tktA*), and transaldolase (*talB*)) into *Z. mobilis*. The recombinant *Z. mobilis* CP4 (pZB5) was shown to grow on 25 g/L xylose as the sole carbon source at a specific growth rate of 0.057 h^{-1} and to produce ethanol as the principal fermentation product at 86% of the theoretical yield. In the presence of a mixture of glucose and xylose (25 g/L each), the transformed strain CP4 (pZB5) can ferment both sugars to ethanol at 95% of theoretical yield within 30 hours. However, xylose was utilized much more slowly than glucose. The anaerobic fermentation of xylose and glucose to ethanol was achieved through a combination of the pentose phosphate and Entner-Doudoroff pathways. By using the same general strategy, they also constructed a recombinant strain *Z. mobilis*(ATCC 39676 (pZB186)) that can ferment arabinose (Deanda et al., 1996). Five genes, encoding L-arabinose isomerase (*araA*), L-ribulokinase (*araB*), L-ribulose-5-phosphate-4-epimerase (*araD*), transaldolase (*talB*), and transketolase (*tktA*), were isolated from *E. coli* and introduced into *Z. mobilis* under the control of constitutive promoters. The engineered *Z. mobilis* strain grew on medium containing L-arabinose 25 g/L as the sole carbon source, and produced ethanol at 98% of the maximum theoretical ethanol yield based on arabinose consumed. However, the yield based on arabinose supplied was lower (84%) due to residual unfermented arabinose. In the presence of glucose and arabinose (both 25 g/L), the overall ethanol yield (based on sugars supplied) was 84% of theoretical, due to incomplete utilization of arabinose. Arabinose was used at a much slower rate than glucose, and only after glucose was nearly depleted. The strain was stable in the presence of tetracycline or arabinose as the sole carbon source. However,

only 40% of the cells retained their ability to ferment arabinose when grown on complex medium at 30 ºC and they completely lost their ability to ferment arabinose within seven generations at 37 ºC. Later, Chou et al. (1997) reported the construction of a single strain of *Z. mobilis* 206C (pZB301) capable of fermenting both xylose and arabinose. The strain contains seven plasmid-borne genes encoding xylose- and arabinose-metabolising and pentose phosphate pathway enzymes. This recombinant strains was capable of fermenting a mixture of 30 g/L glucose, 30 g/L xylose and 20 g/L arabinose to ethanol at an overall yield of ~82-84% of the theoretical (based on sugar supplied). Uptake of arabinose was slow compared to that of glucose and xylose, accounting for the long fermentation time (80-100 h at 30 ºC). Very recently, researchers in Rogers laboratory constructed a better recombinant strain ZM4 (pZB5) by transforming pZB5 into their best *Z. mobilis* ethanol producing strain ZM4 (ATCC 31821) (Joachimsthal and Rogers, 2000). This strain demonstrated much higher ethanol tolerance than the CP4 derivatives on glucose/xylose mixtures. Furthermore, in order to enhance its genetic stability, researchers developed several genomic DNA–integrated strains of *Z. mobilis* 206C (pZB301) through the insertion of all seven genes necessary for xylose and arabinose fermentation into the *Zymomonas* genome (Mohagheghi et al., 2002). Four strains were selected from all the integrants developed and tested for stability. Based on the stability test, one of the integrants (AX101) was selected further to evaluate the cofermentation of glucose, xylose, and L-arabinose in batch and continuous modes (Mohagheghi et al., 2002). Lawford and Rousseau (2002) assessed the fermentation performance characteristics of strain AX101, in batch and continuous pH-controlled fermentations using different pure sugar synthetic biomass hydrolysate media (Lawford and Rousseau, 2002). They found that only 50% of

the xylose (initial concentration 30 g/L) was consumed when both acetic acid (2.5 g/L, pH 5.5) and ethanol (30 g/L) were added to the medium (Lawford and Rousseau, 2002). The results indicated that acetic acid inhibition was exacerbated in the presence of ethanol. Although the recombinant *Z. mobilis* AX101 has several appealing properties as biocatalyst for ethanol production, such as higher ethanol process yield, low pH performance and minimal by-product formations, the main obstacle for its application in industrial process is the inhibition by acetic acid in continuous for ethanol productivity (Mohagheghi et al., 2002; Dien et al., 2003). Acetic acid is commonly found in hydrolysates and originates from acetyl side-chain groups of hemicellulose. To solve this problem, there are two possible routes: 1) adapting the strain AX101 to acetic acid or 2) removing acetic acid from the hydrolysate prior to the fermentation (Dien et al., 2003).

3.2 The Current Difficulties of Modified Single Strain Research

The successful construction of recombinant bacterial and yeast strains through genetic engineering exhibited an improved ability to ferment xylose to ethanol, which arguably represents the most significant development in xylose fermentation research in the past few decades. However, there are some limitations for the genetic manipulation strategy when attempting to develop recombinant strains for industrial bioethanol production process. These limitations include 1) gene insertion may cause pleiotropic effects in the host organism that are difficult to predict (McMillan, 1993); 2) recombinant constructs are often unstable in long-term culture; 3) fermentation performance on real hydrolyzates has seldom been evaluated; 4) formation of undesirable co-products that can inhibit the prolong fermentation.

Actually, these limitations are result from one significant difficulty involving in lack of system-level understanding of cell metabolism. Mathematical models play an important role in the analysis of metabolism from a systems engineering perspective. The mathematical models widely used in metabolic engineering fall into two classes: kinetic models and stoichiometric models (Patil et al., 2004). Kinetic models describe the dynamic properties of metabolic network by combining kinetics information about specific cellular process with known stoichiometry. Due to the lack of kinetic data and the difference between *in vivo* and *in vitro* kinetic parameters for numerous biochemical reactions in metabolic network, application of kinetic models in metabolic engineering is in the preliminary stage. Different from kinetic models, stoichiometric models quantitatively describe steady-state flux distribution in metabolic network. Based on well-known stoichiometric coefficients, it does not require determination of parameters like kinetic constants. Due to this advantage, stoichiometric models have been well utilized in metabolic engineering. We will introduce its application and give a successful example in the following part.

3.3 Metabolic Network Modeling

3.3.1 Introduction

Researchers have long desired the ability to quantitatively describe metabolic fluxes through metabolic networks. So far this endeavor has been hampered by the need for extensive kinetic information describing enzyme catalysis within a living cell. Detailed information about all of the enzymes in a specific metabolic network has not

always been available. Recently, this dilemma has been partially resolved by the development of mass balance-based stoichiometric models.

Generally, the system of equations that described mass balances for the metabolites is:

$$\frac{dX}{dt} = S \bullet v - b \tag{1}$$

Where X is the vector of metabolite concentrations, where S is the $m \times n$ stoichiometric matrix (m is the number of metabolites and n is the number of biochemical reactions in the metabolic network), where v is the vector of reaction fluxes, and where b is the vector of known substrate consumption rates, production formation rates and cell growth rates. The element S_{ij} is the stoichiometric coefficient of the *ith* metabolite in the *jth* reaction. Since the rates of substrate consumption, product formation and cell growth are much slower than that of metabolic reactions, metabolites concentrations in the cell can be assumed to be at pseudo-steady state. This assumption reduces Eq. (1) to

$$S \bullet v = b \tag{2}$$

Typically, the number of metabolic fluxes is greater than the number of mass balance equations (i.e. $n > m$). Therefore, the system is underdetermined, which means more than one solution could exist. Technically, the stoichiometry of the metabolic network does not uniquely specify the fluxes through the cell's pathways, so that the number of possible flux distributions allowed by the stoichiometry is infinite. The cell's choice of flux distribution is determined by a complex interplay of enzymatic and genetic regulatory events (Varma and Palsson, 1994). However, in the absence of detailed knowledge of these events, the metabolic flux distribution can be estimated by defining

an "objective" which underlies the cell's behavior according to the evolutionary selection process. Linear programming provides an expedient method for finding a feasible steady-state flux vector that maximizes an objective function. This technique is defined as Flux Balance Analysis (FBA).

FBA has been successfully applied to describe metabolism in *E. coli* (Edwards and Palsson, 2000), *Haemophilus influenzae* (Edwards and Palsson, 1999) and *S. cerevisiae* (Förster et al., 2003) and its recombinant strain (Jin and Jeffries, 2004). We will provide more details about the genome-scale reconstruction of the *S. cerevisiae* metabolic network model and its application for improved bioethanol production in the next section.

The determination of a particular metabolic flux distribution was formulated as a linear programming problem, in which the solution that maximizes an objective function was identified. The linear programming problem can be formulated as the following system of equations:

$$\text{Max} \qquad Z = \sum c_i v_i$$

$$\text{Subject to} \qquad S \bullet v = b$$

$$\alpha_i < v_i < \beta_i$$

Where Z is the objective function, and where c is the weight vector that determines the contribution of each flux to the objective function. The maximization of Z is subject to both equality and inequality constraints. The equality constraints are referenced as stoichiometric constraints (mass balance), and the inequality constraints are referenced as enzymatic capacity constraints (using an appropriate $v_{\max}$ values). Each row of the stoichiometric constraints $S \bullet v = b$ represents the steady state balance equation for a

particular metabolite. If the i th row of the equation is associated with an internal metabolite, b_i should be zero to reflect the pseudo-steady state assumption. On the other hand, if the i th row is associated with an external metabolite, which is transferred to/from the surrounding environment or is otherwise accumulated or depleted over time, the corresponding element of b should equal to the net exchange rate of accumulation rate of this metabolite as determined from experimental measurements. Inequality constraints are necessary to confine the feasible flux vectors between suitable lower and upper bounds, given by the vector α and β. Generally, the flux vector is bounded in the interval $[0, \infty)$ if the i th reaction is irreversible or $(-\infty, \infty)$ if it is reversible. Nevertheless, setting tighter bounds is beneficial in order to eliminate unrealistic or undesirable flux distributions from the feasible set. This should depend on the availability of experimental data for individual fluxes. As to objective functions, there are three types 1) to represent exploration of the metabolic capabilities of a network; 2) to represent physiologically meaningful objectives (such as maximum cellular growth rate); and 3) to represent bioengineering design objectives. Different objective functions have been used to analyze metabolic networks, including minimization of ATP production, minimization of total nutrient uptake, maximization of metabolite production and maximization of growth rate (Savinell and Palsson, 1992).

3.3.2 Reconstruction and Application of the *S. cerevisiae* Metabolic Network Model

Along with its industrial importance, *S. cerevisiae* was the first eukaryotic organism whose genome was fully sequenced and annotated (Goffeau, 1997). With the availability of *S. cerevisiae* genome sequence database and other information, such as

biochemical and physiological data, genome-scale metabolic network model for *S. cerevisiae* was first reconstructed by Förster and his colleagues in 2003 (Förster et al., 2003). This genome-scale reconstructed metabolic network contains 584 metabolites and 1175 metabolic reactions that were compartmentalized between the cytosol and the mitochondria, and included transport steps between the compartments and the environment. The reconstruction process consisted of collecting a set of biochemical reactions, constructing stoichiometric model of metabolism using mass balance, and gathering other necessary information, such as knowledge on the biomass composition, the growth-associated and nongrowth-associated ATP requirements.

However, the process is iterative. The general process of reconstructing metabolic network model is depicted in Figure 3.2 (Covert et al., 2001). When the initial model is designed, it could be applied to some quantitative analytical methods to simulate cellular behavior under certain genetic and physiological conditions. Then computed results would be compared with experimental results. If no agreement was found, adjustments and corrections on the initial reaction list would need to be done until simulated results were in agreement with experimental results. Once an accurate metabolic network is reconstructed, then mathematical methods, such as convex analysis and linear programming, could be used to analyze structural properties, calculate metabolic capabilities, and examine the integrated functions of the reconstructed metabolic network. The resulting simulated results could be applied to provide valuable information for the development of metabolic engineering strategies for the construction of strains with desired and improved properties.

Metabolic engineering is most effective when guided by systematic biochemical models to integrate the intrinsic variables and extrinsic changes (Jeffries, 2006). Bro et al. (2006) used the genome-scale metabolic network model of *S. cerevisiae* to simulate the effect of genome modification (gene insertion, gene overexpression and/or gene deletion) on cellular behavior (biomass, ethanol and glycerol yield). By testing different alternative strategies for engineering of the redox metabolism in *S. cerevisiae*, they identified the most optimal strategies in terms of reducing glycerol formation and increasing the ethanol yield on glucose or mixture of glucose and xylose and the best-scored strategies were predicted to completely eliminate formation of glycerol and increase ethanol yield with 10%. In addition, they also performed experiments to verify their prediction from their simulation results. By expressing the gene *gapN* which encodes non-phosphorylating $NADP^+$-dependent glyceraldehyde-3-phophate dehydrogenase (GAPN) in glucose-fermenting *S. cerevisiae*, they obtained a transformant that had a 40% lower glycerol yield while the ethanol yield increased with 3% without affecting the maximum specific growth rate by anaerobic batch cultivation on glucose. By expressing GAPN in a xylose-fermenting *S. cerevisiae* strain harbouring xylose reductase (XR) and xylitol dehydrogenase (XDH), they obtained a recombinant strain that could lead to an improvement in ethanol yield by up to 25% on xylose/glucose mixtures. Both the model predictions and the experimental results showed that heterologous expression of GAPN in *S. cerevisiae* seems to be an attractive strategy to redirect flux that is going to glycerol toward ethanol and that overexpression of GAPN may lead to a further reduction of glycerol yield and increase of ethanol yield either on glucose or on glucose/xylose mixture.

In conclusion, their results showed that the genome-scale metabolic model represents a powerful tool for directing the construction of recombinant strains without doing numerous of biological experiments for trial. Therefore, it is necessary to build metabolic network models for other ethanol-producing strains, such as *P. stipitis* and *Z. mobilis*. .

Table 3.1 Important traits for ethanol production (Dien et al., 2003)

Trait	Requirement
Ethanol yield	>90% of theoretical
Ethanol tolerance	>40 g l^{-1}
Ethanol productivity	>1 g l^{-1} h^{-1}
Robust grower and simple growth requirements	Inexpensive medium formulation
Able to grow in undiluted hydrolysates	Resistance to inhibitors
Culture growth conditions retard contaminants	Acidic pH or higher temperature

Table 3.2 Comparison of recombinant strains for fermentation with pretreated corn fiber hydrolyzates or glucose/xylose mixtures (adapted from Bothast et al., 1999)

Strain	Maximum ethanol (g/L)	Ethanol yield[a] (g/g)	Maximum ethanol volumetric productivity (g $L^{-1}h^{-1}$)	Reference
E. coli K011	34.7	0.41	1.16	Dien et al., 1997
E. coli SL40	31.7	0.42	1.12	Dien et al., 1997
Z. mobilis CP4(pZB5)	22.6	0.45	1.04	Dien (unpublished data, 1998)
Z.mobilis ZM4(pZB5)[c]	62	0.46	1.29[b]	Bothast et al., 1999
Saccharomyces 1400 (pLNH32)	21.0	0.50	1.60	Moniruzzaman et al., 1997

[a] Grams of ethanol per gram of sugar consumed
[b] Calculated from Bothast et al.1999 (p 871)
[c] Fermentation with a mixture of 65g/L each of glucose and xylose

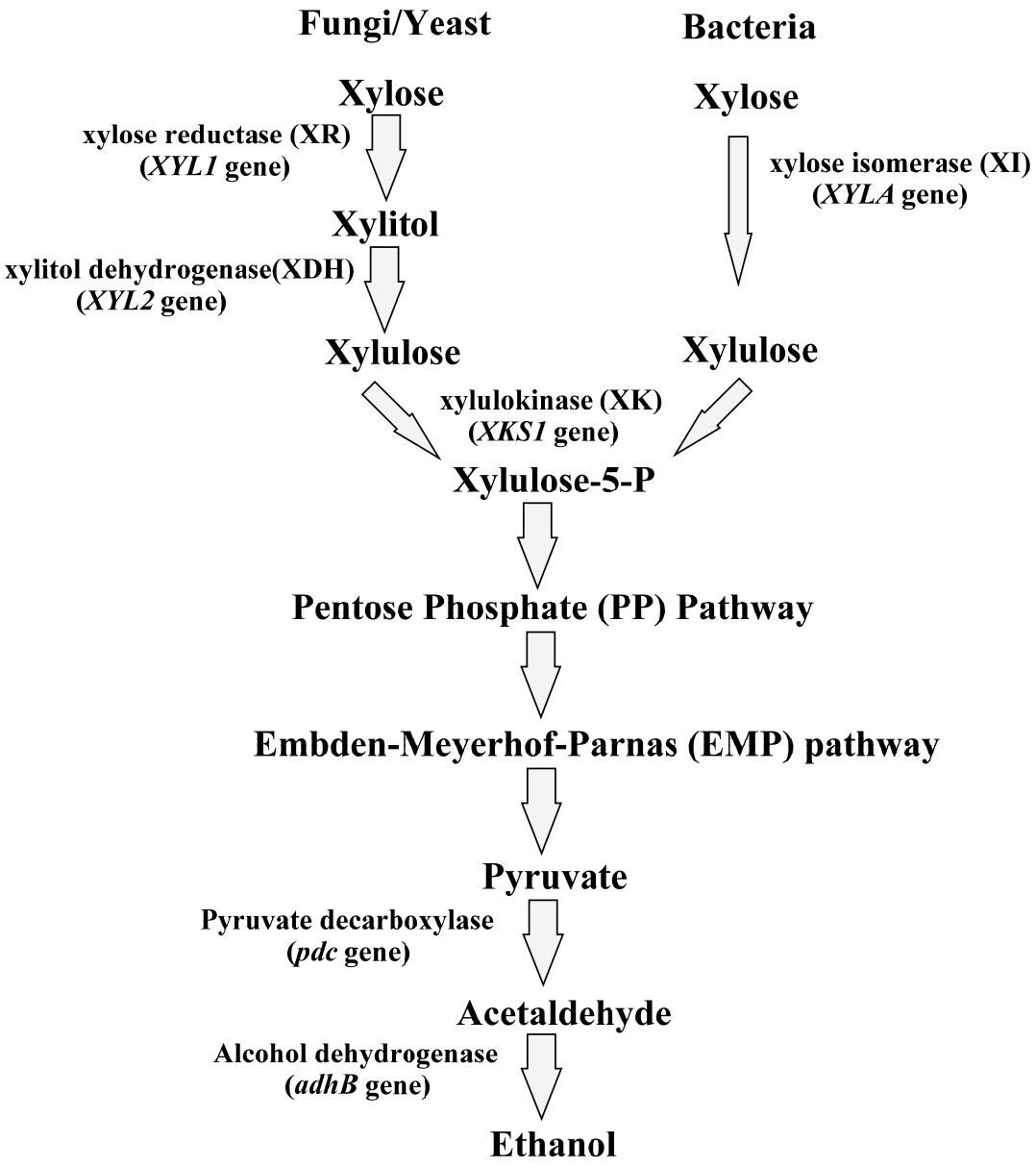


Figure 3.1 Metabolic pathways for xylose utilization in fungi, yeast and bacteria

(edited from Bothast et al., 1999)

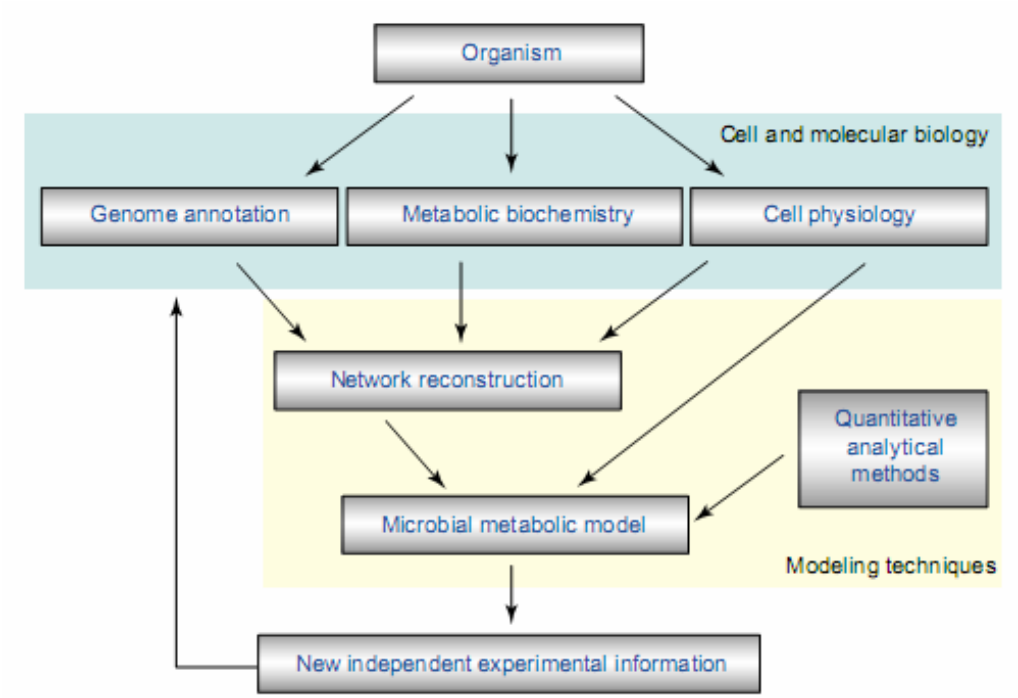


Figure 3.2 Integrated process of microbial metabolic network model construction (Covert et al., 2001)

CHAPTER 4

CO-CULTURE FOR GLUCOSE AND XYLOSE COFERMENTATION

Development in glucose and xylose cofermentation is progressing rapidly. Although recombinant strains can achieve glucose and xylose cofermentation, there is no robust modified strain which can be use in the bioethanol production from biomass at industrial scale due to their limitations. Some research on co-culture indicates that it has great potential to solve the problem of glucose and xylose cofermentation. In this chapter, we will give a broad overview of the current status of co-culture study on bioethanol production. Strains, fermentation mode, condition and performance for current co-cultures systems will be compared and discussed. Finally, the issues about co-culture systems will be addressed.

4.1 Description and Feature of Co-Culture Systems

Currently most industrial bioconversions rely on pure microbial cultures, such as production of amino acids, nucleotides, antibiotics and enzymes; however, some other commercial processes, for instance, alcohol brewing, biological waste-water treatment and manufacture of dairy and conventional fermenting products (such as soy sauce, pickles) are accomplished by multiple microbial species (Taniguchi et al., 2004). Moreover, many environmental bioconversions are catalyzed by mixed microbial cultures

with specialists “working” together with an apparently stable fashion in natural systems. Based on some insights from commercial processes and natural systems, the main idea for the co-culture of ethanol production from cofermentation of glucose and xylose is to utilize two different microbial strains and enable each of them to carry out their own tasks stably and effectively. In particular, one microbe could ferment glucose and the other microbe could ferment xylose in the same environment, and there is no any negative effect between them, such as competition of substrate, inhibition of products and so on. In order to achieve this goal, the pivotal part is to find two appropriate microbial strains that can fully finish their special tasks in a co-culture system. In addition, the co-culture system should be resistant and stable for long periods of time despite being contaminated by “outside” microbes or other potentially toxic materials.

4.2 Strains Used in Co-Culture Systems and Their Interactions

The natural ethanologenic microorganisms are categorized as yeasts, fungi and bacteria. Examples of these yeasts are *S. cerevisiae*, *Kluyveromyces marxianus*, *P. stipitis*, *C. shehatate* and *Pachysolen tannophilius*. Examples of these bacteria are Z. mobilis, *Zymobacter palmae*, *Clostridium cellulolyticum*, *E. coli* and *Bacillus stearothermophilus*. Table 4.1 summarized all of the known strain combinations currently used in co-culture systems. In these co-culture systems, *P. stipitis*, *P. tannophilu*, *C. shehatae* and recombinant *E. coli* are usually responsible of for xylose fermentation; *K. marxianus* and *S. cerevisiae* or its respiratory deficient mutant are normally used as the glucose-fermenting microbe.

To gain greater insights, we performed a statistic analysis on 18 strain combinations and listed the result in Table 4.2. As shown in Table 4.2, the combination of *P. stipitis* and *S. cerevisiae* appeared eight times, and the combination of *P. stipitis* and respiratory deficient mutant *S. cerevisiae* appeared three times; the combination of *C. shehatae* and *S. cerevisiae* appeared two times in the total of 18 strain combinations. In addition, the combination of *C. shehatae* and respiratory deficient mutant *S. cerevisiae* appeared once. In respect to a bacteria and yeast combination, *S. cerevisiae* with *E. coli*. and *P. tannophilis* with *Z. Mobilis* appeared once each. As to the combination of bacteria with bacteria, there appears to be no research in this area. Perhaps this is due to the lack of bacterial strains that can effectively ferment xylose. From Table 4.2, we can conclude that the most commonly used combination in current co-culture systems is the combination of *P. stipitis* and *S. cerevisiae* since both of them are yeasts, therefore they have much better compatibility as well as similar fermentation conditions, such as temperature and pH. Furthermore, *S. cerevisiae* has been regarded as one of the most efficient glucose-fermenting yeast with a number of attractive properties (safe, high ethanol tolerance etc.). And as one of the best xylose-fermenting yeasts, *P. stipitis* can convert xylose at a relatively high conversion rate and with high yields (Grootjen et al., 1991b). Therefore, their combination should be considered as the natural choice. However, some researchers found that a limited oxygen supply was optimal for *P. stipitis* to ferment xylose (Grootjen et al., 1990b and 1991b). In order to solve the problem of limited oxygen condition for P. stipitis to ferment xylose lowering the S. cerevisiae fermentation yield, respiratory deficient (RD) mutants of S. cerevisiae have be used in some co-culture systems in stead of normal S. cerevisiae (Laplace et al., 1993b;

Taniguchi et al., 1997a; Kordowska-Wiater and Targoński, 2001). Considering the function of xylose fermenting, *P. stipitis* probably could be combined with recombinant *E. coli* and *Z. mobilis* in co-culture systems. Also, *C. shehatae* could be associated with *K. Marxianus*, recombinant *E. coli* and *Z. Mobilis* for simultaneous glucose and xylose fermenting. Moreover, it is meaningful to test the combination of *P. tannophilis* with *K. Marxianus*, *S. cerevisiae* and/or its RD mutants, recombinant *E. coli*. These can be a potential direction for the future co-culture research.

When selecting strains for a co-culture system, it is very important to understand the interactions and investigate the compatibility of these associated strains. Generally speaking, possible interactions between two microbial species can be categorized as neutralism, positive interactions (commensalisms, protocooperation and mutualism), and negative interactions (amensalism, competition, parasitism, and predation) (Taniguchi et al., 2004). Neutralism means that there is no ill or harmful effect between two different populations in the system. In commensalisms, one population benefits while the other remains unaffected. Protocooperation (synergism) benefits both populations, but the association is not obligatory. Mutualism is an obligatory relationship between two microbial populations that benefits both populations. Close mutualistic relationships are known as "symbiosis." Different from positive interactions, amensalism and competition represent a negative relationship between two microbial populations. When one microbial population produces a substance that is inhibitory to the other population, the relationship is called amensalism. Competition occurs when two populations use the same resource, either space or a limiting nutrient. Parasitism exerts a negative influence on susceptible

host populations and benefits the parasite. Predation typically occurs when one organism, the predator, engulfs and digests another, the prey.

For an effective co-culture system, utilization of positive interactions and avoidance of negative interactions between two microorganisms should be the criterion for selecting microorganisms. Although Laplace and his coworker have presented the compatibility tests and used resistance to mitochondrial inhibitors assays to type and quantify both strains in the co-culture process (Laplace et al., 1992), there is no research on developing impactful experimental methods for studying the interactions or distinguishing different microbial species in co-culture systems. Obviously, this can be one of future directions for co-culture research as well.

4.3 Fermentation Mode and Condition of Co-Culture Systems

All of the co-culture systems that have been studied so far are summarized in Table 4.3. As shown in this table, most of these co-culture systems have good fermentation performances, such as higher overall ethanol yield, shorter fermentation time, and higher substrate utilization. It turned out that co-culture could be a very appealing strategy for increasing ethanol yield and reducing the whole processing cost of bioethanol production. In this part, we will discuss the fermentation modes and conditions used in these co-culture systems.

4.3.1 Fermentation Mode

Normally, there are two fermentation modes utilized in co-culture systems: batch and continuous. An obvious difference between these two modes is that there is no feed

and outflow for a batch fermentor, but a continuous fermentor (chemostat) has its feed and outflow. A simple schematic of a batch fermentor and a typical chemostat is shown in Figure 4.1.

From Table 4.3, we can see that most current co-culture systems are operated in a batch mode, because it is simple and the process is easy to control. However, the batch mode has some drawbacks. For instance, the existing glucose can suppress the xylose fermentation especially at the initial stage, because the xylose conversion is inhibited completely at glucose concentration of 2.3 g/L and higher (Grootjen et al., 1991b). On the other hand, when the glucose is close to depletion, the high ethanol concentration (around 30 g/L) can inhibit xylose fermentation (Rouhollah et al., 2007).

Different from batch mode, continuous fermentation can avoid the accumulation of ethanol and other inhibiting metabolites in the system due to the existence of outflow (Lebeau et al., 1997). Moreover, by adjusting the volumetric feed rate (dilution rate), the glucose concentration in the chemostat can be controlled below 2.3 g/L. Therefore, fast and simultaneous conversion of glucose and xylose with co-cultured systems can be easily achieved by continuous fermentation. If *S. cerevisiae* is used in a co-cultivated system, its high fermentative potential may also generate a glucose concentration low enough to allow fast xylose conversion (Laplace et al., 1993b). However, there are no good methods to get the two ethanologenic microorganisms in the outflow back into the fermentor or to separate the ethanologenic microorganisms within the solution in the outflow when using continuous mode. This is a big issue that limits the utilization of continuous fermentation in co-culture systems. We expect that this problem should be solved in the future co-culture research.

4.3.2 Fermentation Condition

The selection of fermentation conditions, such as temperature, pH, initial sugar concentration and composition, aeration or anaerobic environment and so on, should mainly depend on the two selected microorganism strains in a co-culture system. Normally, the fermentation temperature of a current co-culture system is at the range of 28~30 °C no matter which combination is used, because the optimal fermentation temperature for most monoculture, including *S. cerevisiae*, *P. stipitis*, *E. coli*, *Z. mobilis*, *C. shehatae* and *P. tannophilis* is around 30°C. The pH of fermentation medium is controlled at 4.5~7.0 by adding sodium hydrate or hydrogen chloride, since the optimal pH for the monoculture of *S. cerevisiae*, *P. stipitis* and *Z. mobilis* is 5.0, but for *E. coli* is about 7.0 (Zaldivar et al., 2001). Figure 4.2 shows the distribution of pH selection in current co-culture systems. From this figure, a conclusion can be drawn that most current co-culture systems choose pH values at 5.0, since most current co-cultures are the combination of *S. cerevisiae* and *P. stipitis*, and pH of 5.0 is the optimal pH for these two yeast strains. For cofermentation of glucose and xylose, the initial total sugar concentration and composition play an important role in affecting the fermentation performance. However, very little research has been performed to determine the factor's effect on the fermentation performance. As shown in Figure 4.3, different researchers have chosen different initial total sugar concentration and different glucose/xylose ratio in their co-culture systems according to their conventionality. Generally, the xylose composition changes from 20%~50% and most cases are at about 30% which is close to the xylose composition in the corn stove hydrolysate and other biomass hydrolysate. Some research indicates that *S. cerevisiae* regulates fermentation by sensing the presence

of glucose, however, *P. stipitis* induces fermentative activity in response to oxygen limitation (Jeffries et al., 2007). Moreover, Skoog and Hahn-Hägerdal (1990) reported the maximum specific productivity and ethanol yield was achieved when the oxygen transfer rate below 1mmol/L/h on xylose fermentation by *P. stipitis* CBS6054. Normally, the oxygen transfer rate (OTR) or the average specific oxygen uptake rate was maintained at a low level by changing the airflow and/or agitation rate in some current co-culture systems (Laplace et al., 1993a,b; Taniguchi et al., 1997a, b).

In addition to temperature, pH, initial total sugar concentration and composition, oxygen transfer rate, inoculum volume is another important factor which influences the fermentation performance. However, there is no related research that has been conducted so far. Generally, the inoculum volume in current co-culture systems is in the range of 1%~3% (v/v). As a result, a study on how the inoculum volume impacts the other fermentation conditions and how it impacts performance needs to be conducted in the future.

4.4 Fermentation Performance

Generally, there are several parameters to evaluate fermentation results, such as ethanol yield ($Y_{p/s}$), volumetric ethanol productivity (Q_p), specific ethanol production rate (q_p), efficiency of substrate utilization (E) and so on. Of these parameters, the ethanol yield is the most vital parameter. It is calculated as grams of ethanol produced per gram of total sugars consumed (g ethanol/g substrate). The theoretical ethanol yield for glucose fermentation or xylose fermentation is 0.51 g ethanol/ g sugar (Krishnan et al., 1999). So far the highest ethanol yield for mono-culture has been achieved by using recombinant *E.*

coli K011 and *Z. mobilis* ZM4. The recombinant strain *E. coli* K011 can achieve the yields of 0.48 and 0.52 g/g for glucose and xylose, respectively. They represent 94% of the theoretical yield for glucose, and more than 100% for xylose due to extra ethanol produced from the catabolism of other complex nutrient components in the medium (McMillan, 1993). When using the recombinant strain of *Z. mobilis* ZM4 (pZB5), sugar concentrations of about 50 g/L glucose and 50 g/L xylose can result in an ethanol productivity of approximately 5 gL−1h−1, and a yield of 0.50 g ethanol/g substrate, which is 98% of the theoretical yield (Joachimsthal and Rogers, 2000). From Table 4.3, we can see that the overall ethanol yield of different co-culture systems changes from 0.29~0.50 g ethanol/ g substrate. The highest overall ethanol yield was obtained at 0.5 g ethanol/g substrate which is about 98% of the theoretical yield (Taniguchi et al., 1997a). By employing the strain combination of *P. stipitis* CBS5773 and respiratory deficient mutant *S. cerevisiae* no. 7 in batch fermentation, Taniguchi et al. obtained greater volumetric ethanol productivity with 0.94 $gL^{-1}h^{-1}$ and the maximum ethanol concentration with 37.5 g/L which is the highest value in all of the current co-cultures systems. Consequently, under some optimal conditions, excellent fermentation performance (high ethanol yield, high average volumetric productivity and final ethanol concentration) can be realized through co-culture.

4.5 The Current Issues and Related Strategies in the Co-Culture Research

Jeffries (1985) suggested the minimum values (Table 4.4) of some important performance parameters for a commercially attractive xylose conversion process. In order to compare the performance parameters of different single strains (wild type and

recombinant strains) and co-culture systems, we extracted some experimental results for each case from literatures and listed them in Table 4.5. Comparing the results shown in Table 4.5 with the minimum requirements for commercial xylose conversion process, we can conclude that co-culture is a very appealing strategy to be used in the ethanol production at industrial scale. However, there are some shortcomings that exist for the current culture systems. We will address the issues of using the *P. stipitis* and *S. cerevisiae* co-culture as an example, since this co-culture is a well-studied case with the best fermentation performance so far.

First of all, there is diauxic growth behavior for most microbes, such as *P. stipitis*, *E. coli* and other strains. This growth pattern was originally discovered by Monod (1942). When growing in multiple substrates, microbes with the diauxic behavior actively control substrate uptake by regulating their internal repertoire of enzymes. Normally, there are two growth phases. The first growth phase involves exclusive consumption of the preferred substrate. After the preferred substrate has been completely exhausted, the microorganisms undergo a lag period during which they switch to synthesizing the enzymes needed to metabolize the less preferred substrate. For example, when both of glucose and xylose simultaneously present in the environment, *P. stipitis* ferments glucose first, then ferments xylose after the glucose concentration is reduced to 2.3 g/L (Grootjen et al., 1991c). In this case, it is hard to achieve cofermentation of glucose and xylose simultaneously, especially at the beginning of batch fermentation. Continuous fermentation probably can solve this problem with proper dilute rate to keep the concentration of glucose in the fermentor is lower than 2.3 g/L. Unfortunately, there is little research about this strategy.

Secondly, there is different fermentation mechanisms between two different strains. For instance, *S. cerevisiae* regulates fermentation by sensing the presence of fermentable sugars such as glucose, while *P. stipitis* induces fermentative activity in response to oxygen limitation. However, when oxygen exists in the medium, pyruvate is metabolized by cellular respiration but fermentation for *S. cerevisiae* and ethanol is not produced, therefore, the total ethanol yield will be reduced. In order to avoid the reduction of ethanol yield with limited oxygen condition for the co-culture system, it is better to use RD mutant *S. cerevisiae* cultivated with *P. stipitis* in the system. Another big issue is that there is lack of understanding on interspecies interactions (synergic effect, competitive effect and so on). In addition, the possible underlying metabolic changes caused by genetic or environmental perturbations (such as the metabolic products of another strain in the co-culture system) for each strain need to be studied. One possible way for solving this issue is the integration of experimental and computational methods. The approach includes developing a robust mathematical model that can describe the dynamic characteristics of the model system and predict the changes upon environmental conditions, devising effective measurement methods to measure some important intracellular metabolites concentrations *in vivo* by using nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry combined with gas chromatography (GC-MS).

Table 4.1 The combinations of two strains used in different co-culture systems for ethanol production

Strains	References
P. stipitis + *K. marxianus*	Rouhollah et al., 2007
P. stipitis + *S. cerevisiae*	Rouhollah et al., 2007
Z. mobilis + *P. tannophilus* (Successive inoculation)	Fu and Peiris, 2008
S. cerevisiae + *P. tannophilus*	Qian et al., 2006
S. cerevisiae + recombinant *E. coli*	Qian et al., 2006
coimmobilized *P. stipitis* + *S. cerevisiae*	Bari et al., 2004
Restricted catabolite repressed mutants of *P. stipitis* + *S. cerevisiae*	Kordowska-Wiater and Targoński, 2002
Restricted catabolite repressed mutants of *P. stipitis* + RD mutant of *S. cerevisiae*	Kordowska-Wiater and Targoński, 2002
P. stipitis + RD mutant *S. cerevisiae*	Kordowska-Wiater and Targoński, 2001
coimmobilized *S. cerevisiae* + *C. shehatae*	Lebeau et al., 1997
P. stipitis + *S. cerevisiae*	Taniguchi et al., 1997a
P. stipitis + RD mutant *S. cerevisiae*	Taniguchi et al., 1997a
P. stipitis + RD mutant *S. diastaticus*	Laplace et al., 1993a
C. shehatae + *S. cerevisiae*	Laplace et al., 1993b
C. shehatae+ respiratory deficient mutant *S. cerevisiae*	Laplace et al., 1993b
coimmobilized *P. stipitis* + *S. cerevisiae*	Grootjen et al., 1991a and 1990a
immobilized *P. stipitis* + suspended *S. cerevisiae*	Grootjen et al., 1991a

Table 4.2 Statistics of the strain combinations in different co-culture systems

Strain Combination	Appearance Times/Total Times
P. stipitis + *K. marxianus*	1/18
P. stipitis + *S. cerevisiae*	8/18
P. stipitis + RD mutant *S. cerevisiae*	3/18
P. stipitis + RD mutant *S. diastaticus*	1/18
C. shehatae + *S. cerevisiae*	2/18
C. shehatae + RD mutant *S. cerevisiae*	1/18
E. coli + *S. cerevisiae*	1/18
Z. mobilis + *P. tannophilis*	1/18

Table 4.3 Summary of different co-culture systems

Co-culture Systems	Fermentation Mode	Fermentation Condition	Performance	Reference
P. stipitis CCUG18492+*K. marxianus*	Batch	Initial substrate concentration:30g/L glucose, 30g/L xylose, 12g/L mannose, 8g/L galactose pH: 4.5 Working volume:100mL	$Y_{p/s}$: 0.36 $Y_{x/s}$: 0.08 Q_{pmax}: 1.08 $C_{E,max}$: 31.87 T_F: 72 h E: 99%	Rouhollah et al., 2007
P. stipitis CCUG18492 + *S. cerevisiae*	Batch	Initial substrate concentration: 30g/L glucose, 30g/L xylose, 12g/L mannose, 8g/L galactose pH: 4.5 Working volume:100mL	$Y_{p/s}$: 0.41 $Y_{x/s}$: 0.08 Q_{pmax}: 0.77 $C_{E,max}$: 29.45 T_F: 60 h E: 94%	Rouhollah et al., 2007
Z. mobilis + *P. tannophilus* (Successive inoculation)	Batch	Initial substrate concentration:60g/L glucose and 40g/L xylose Temperature: 30°C Inoculations were carried out successively with firstly Z. mobilis, and after all the glucose had been converted to ethanol. P. tannophilus we then inoculated. Cofermentation with no aeration at glucose fermentation stage and full aeration at xylose fermentation stage	$Y_{p/s}$: 0.29 X_{max}: 2.41×10^7 (Z. mobilis) X_{max}: 9.4×10^7 (P. tannophilus) Q_P: 2.38 T_F: 12 h (Z. mobilis) T_F: 120 h (P. tannophilus)	Fu and Peiris, 2008
Z. mobilis + *P. tannophilus* (Successive inoculation)	Batch	Initial substrate concentration:60g/L glucose and 40g/L xylose Temperature: 30°C Inoculations were carried out successively with firstly Z. mobilis, and after all the glucose had been converted to ethanol. P. tannophilus we then inoculated. Cofermentation with no aeration at glucose fermentation stage and a aeration level <1 mmol/L/h at xylose fermentation stage	$Y_{p/s}$: 0.33 X_{max}: 5.1×10^7 (Z. mobilis) X_{max}: 5.7×10^7 (P. tannophilus) Q_P: 2.32 T_F: 12 h (Z. mobilis) T_F: 144 h (P. tannophilus)	Fu and Peiris, 2008
S. cerevisiae 2.535 + *P. tannophilis* ATCC 2.1662	Batch	Treated or untreated Softwood hydrolysate was used as the substrate in the fermentation. Temperature: 30°C pH: 5.5 150mL hydrolysate and 50mL inoculum of co-cultures	For treated softwood hydrolysate fermentation by adapted co-culture $Y_{p/s}$: 0.49 Q_P: 0.38 Sugar consumed (%):>99 Ethanol(g/L): 18.2	Qian et al., 2006
S. cerevisiae + recombinant *E. coli*	Batch	Treated or untreated Softwood hydrolysate was used as the substrate in the fermentation. Temperature: 30°C pH: 7.0 150mL hydrolysate and 50mL inoculum of co-cultures	For treated softwood hydrolysate fermentation by adapted co-culture $Y_{p/s}$: 0.45 Q_P: 0.71 Sugar consumed (%):>99 Ethanol(g/L): 17.1	Qian et al., 2006
P. stipitis (NRRL Y-11544) + *S. cerevisiae* coimmobilized in Ca-alginate gel beads	Batch	Mixed sugar syrups containing 45 g/L glucose and 12 g/L xylose was used as substrate. Temperature: 30°C pH: 5.5	Best conditions for the cofermentation of 40g/L glucose and 10 g/L xylose $Y_{p/s}$: 0.396 Conversion: 0.995 $_{\text{g consumed/g initial}}$	Bari et al., 2004

Co-culture Systems	Fermentation Mode	Fermentation Condition	Performance	Reference
Restricted catabolite repressed mutant *P. stipitis* CCY39501 + respiratory deficient mutant *S. cerevisiae* Ja(a)	Batch	Initial substrate concentration:35g/L glucose and 15g/L xylose Temperature: 28°C pH: 5.5 Fermentation time: 120 h 1% v/v of respiratory deficient mutant *S. cerevisiae* V_{30} and 1% v.v of *P. stipitis* were inoculated in 150 mL F_3 medium.	S_G: 100.00% S_K: 68.00% $C_{E,max}$: 20.30 $Y_{p/s}$: 0.45 $Y_{x/s}$: 0.20 Q_P: 0.169	Kordowska-wiater and Targoński, 2002
P. stipitis CCY39501+ *S. cerevisiae* V_{30}	Batch	Initial substrate concentration:35g/L glucose and 15g/L xylose Temperature: 28°C pH: 5.5 Fermentation time: 96 h 2% v/v of respiratory deficient mutant *S. cerevisiae* V_{30} and 1% v.v of *P. stipitis* were inoculated in 150 mL medium.	S_G: 99.71% S_K: 26.67% $C_{E,max}$:15.00 $Y_{p/s}$: 0.39 $Y_{x/s}$: 0.14 Q_P: 0.318	Kordowska-Wiater and Targoński, 2001
P. stipitis CCY39501+ respiratory deficient mutant *S. cerevisiae* V_{30}	Batch	Initial substrate concentration:35g/L glucose and 15g/L xylose Temperature: 28°C pH: 5.5 Fermentation time: 96 h 2% v/v of respiratory deficient mutant *S. cerevisiae* V_{30} and 1% v/v of *P. stipitis* were inoculated in 150 mL medium.	S_G: 100.00 % S_K: 99.67 % $C_{E,max}$: 18.80 $Y_{p/s}$: 0.38 $Y_{x/s}$: 0.12 Q_P: 0.264	Kordowska-Wiater and Targoński, 2001
S. cerevisiae CBS 1200 + *C. shehatae* ATCC 22984 coimmobilized in a two-chambered bioreactor	Batch	Initial substrate concentration: 10g/L glucose and 4.5g/L xylose Initial cell loading: *C. shehatae* 0.65 mg dry wt/mL *S. cerevisiae* 5.00mg dry wt/mL	Best conditions: T_m: 230 h Total Ethanol produced:5.15g $Y_{p/s}$: 0.47 $Y_{x/s}$: 0.088 Q_P: 7.5	Lebeau et al., 1997
P. stipitis CBS5773 + *S. cerevisiae* no. 7	Batch	Initial substrate concentration: 50g/L glucose and 25g/L xylose The initial concentration of *P. stipitis* and *S. cerevisiae* were tentatively 7.1 g/L and 1.5g/L, respectively. pH: 5.0 Working volume: 1L Fermentation time: 40h qO_2 was controlled at 66.7 mg/g cell/h for glucose consumption and then controlled at 14.3 mg/g cell/h for xylose consumption .	$Y_{p/s}$: 0.39 $C_{E,max}$: 29.4 Q_P: 0.74	Taniguchi et al., 1997a
P. stipitis CBS5773 + respiratory deficient mutant *S. cerevisiae* no. 7	Batch	Initial substrate concentration: 50g/L glucose and 25g/L xylose The initial concentration of *P. stipitis* and *S. cerevisiae* were tentatively 7.1 g/L and 1.5g/L, respectively. pH: 5.0 (controlled by 2N NaOH and 2N HCl) Working volume: 1L Fermentation time: 40h	$Y_{p/s}$: 0.50 $C_{E,max}$: 37.5 Q_P: 0.94	Taniguchi et al., 1997a

Co-culture Systems	Fermentation Mode	Fermentation Condition	Performance	Reference
P. stipitis CBS5773 + *S. cerevisiae* no. 7	Batch	Initial substrate concentration: 50g/L glucose and 25g/L xylose The initial concentration of *P. stipitis* and *S. cerevisiae* were tentatively 3.5 g/L and 0.75 g/L, respectively. Fermentation time: 68h qO_2 was controlled at 66.7 mg/g cell/h for glucose fermentation stage and 14.3 mg/g cell/h for xylose fermentation stage.	$Y_{p/s}$: 0.35 $C_{E,max}$: 26.2 Q_P: 0.39	Taniguchi et al., 1997b
P. stipitis CBS5773 (fermentor A) + *S. cerevisiae* no. 7 (fermentor B)	Batch	Initial substrate concentration: 50g/L glucose and 25g/L xylose The initial concentration of P. stipitis and S. cerevisiae were 7.0 g/L and 0.75g/L, respectively. Filtered air was sparged at constant flow rate (0.2vvm) into fermentor A. Meanwhile, nitrogen gas was sparged at 0.2 vvm into fermentor B. Fermentation time: 56h Working volume in Fermentor A: 1L Working volume in Fermentor B: 1L Total working volume:2 L	For *P. stipitis* in Fermentor A $Y_{p/s}$: 0.44 $C_{E,max}$: 33.1 Q_P: 0.59 For *S. cerevisiae* in Fermentor B $Y_{p/s}$: 0.45 $C_{E,max}$: 33.7 Q_P: 0.60	Taniguchi et al., 1997b Taniguchi and Tanaka, 2004
P. stipitis + respiratory deficient mutant *S. diastaticus*	Continuous	3% (v/v) inoculum was used. Temperature: 30°C pH: 5.0 Working volume: 1.5L O_2 transfer rate:1.75 mmol/h Sugar mixture in fermentation medium: 70% glucose and 30% xylose	When the initial substrate concentration did not exceed 20 g/L, the substrate was entirely consumed. When the initial substrate concentration equaled to 80g/L, the residual xylose concentration reached 20.5 g/L. With the initial substrate concentration at 50 g/L, the xylose was entirely consumed when dilution rate did not exceed 0.006 h^{-1} whereas the glucose was entirely consumed whatever the D is.	Laplace et al., 1993a
C. shehatae ATCC 22984 + *S. cerevisiae* CBS 1200	Batch	Initial substrate concentration: 14g/L glucose and 6g/L xylose (a mixture of 70% glucose and 30% xylose) Inoculums of each yeast strain were 1.5%. Temperature: 30°C pH: 5.0 Working volume: 1.5L Stirring speed:800rpm Aeration rate: 0.005vvw Oxygen transfer rate:1.75mmol/l/h	T_F: 14 h $C_{E,max}$: 14.5 g/L $Y_{p/s}$: 0.39 S_G: 100 % S_K: 8 %	Laplace et al., 1993b
C. shehatae ATCC 22984 + respiratory deficient mutant *S. cerevisiae* CBS1200	Batch	Initial substrate concentration: 14g/L glucose and 6g/L xylose (a mixture of 70% glucose and 30% xylose) Inoculums of each yeast strain were 1.5%. Temperature: 30°C pH: 5.0 Working volume: 1.5L Stirring speed:800rpm Aeration rate: 0.005vvw Oxygen transfer rate:1.75mmol/l/h	T_F: 14 h $C_{E,max}$: 14.7 g/L $Y_{p/s}$: 0.40 S_G: 100 % S_K: 6 %	Laplace et al., 1993b

Co-culture Systems	Fermentation Mode	Fermentation Condition	Performance	Reference
C. shehatae ATCC 22984 + respiratory deficient mutant *S. cerevisiae* CBS1200	Continuous	Initial substrate concentration: 14g/L glucose and 6g/L xylose (a mixture of 70% glucose and 30% xylose) Inoculums of each yeast strain were 1.5%. Temperature: 30°C pH: 5.0 Working volume: 1.5L Stirring speed:800rpm Aeration rate: 0.005vvw Oxygen transfer rate:1.75mmol/l/h	T_F: 14 h $C_{E,max}$: 14.5 g/L $Y_{p/s}$: 0.39 S_G: 100 % S_K: 8 %	Laplace et al., 1993b
coimmobilized *P. stipitis* CBS 5773 + *S. cerevisiae* CBS 8066	Continuous	Temperature: 30°C pH: 5.0 Working volume: 400mL	Best condition: D: 0.07 Influent concentration: Glucose: 40.9 g/L Xylose: 10.5 g/L S_G: 100 % S_K: 37 %	Grootjen et al., 1991a
immobilized *P. stipitis* CBS 5773 + suspended *S. cerevisiae* CBS 8066	Continuous	Temperature: 30°C pH: 5.0 Working volume: 400mL	Best condition: D: 0.11 Influent concentration: Glucose: 40.5 g/L Xylose: 11.2 g/L S_G: 100 % S_K: 11 %	Grootjen et al., 1991a
coimmobilized *P. stipitis* CBS 5773 + *S. cerevisiae* CBS 8066	Continuous	The medium contained a mixture of glucose 40 g/L and xylose 10 g/L Temperature: 30°C pH: 5.0 Working volume: 200mL	Best condition: D: 0.375 $Y_{p/s}$: 0.4 $C_{E,max}$: 20	Grootjen et al., 1990a

$Y_{p/s}$: Overall Ethanol yield (g ethanol/g substrate); X_{max}: maximum biomass yield (CFU/ml); Q_P: volumetric productivity of ethanol (g/L/h); Q_{pmax}: maximum volumetric ethanol productivity (g/L/h); T_F: time of maximum ethanol concentration to be reached (h); T_m: time at which maximum ethanol produced reached (h); $C_{E,max}$: maximum ethanol concentration (g/L); $Y_{x/s}$: cell biomass yield (g cell biomass/g substrate); E: efficiency of substrate utilization (%); S_G: glucose used (%); S_K: xylose used (%); qO_2: specific oxygen uptake rate, mg/g cell/h; D: dilution rate (h^{-1})

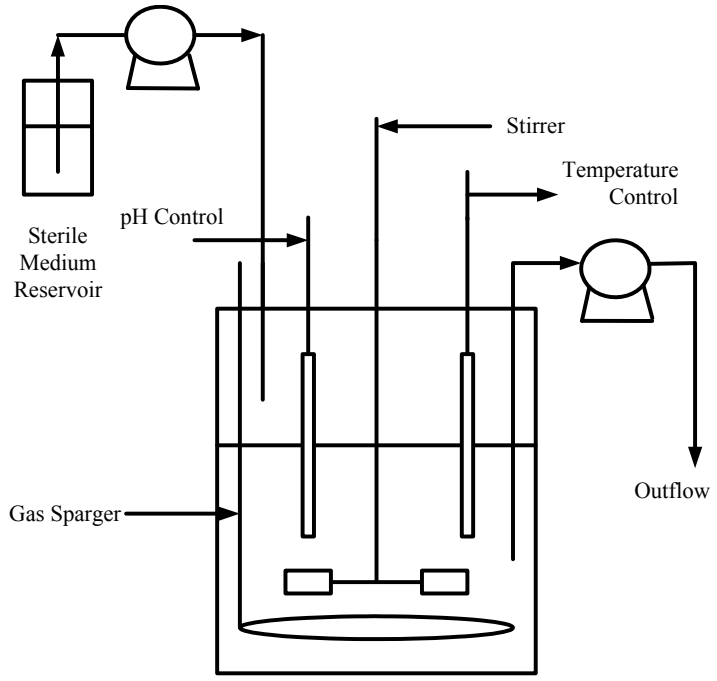
Table 4.4 Minimum requirements of performance parameters for commercial xylose conversion process

Performance parameter	Minimum level to achieve for commercialization
Ethanol yield	0.40 g/g
Final ethanol concentration	5% (w/v)
Average volumetric productivity	1.4 g L^{-1} h^{-1}

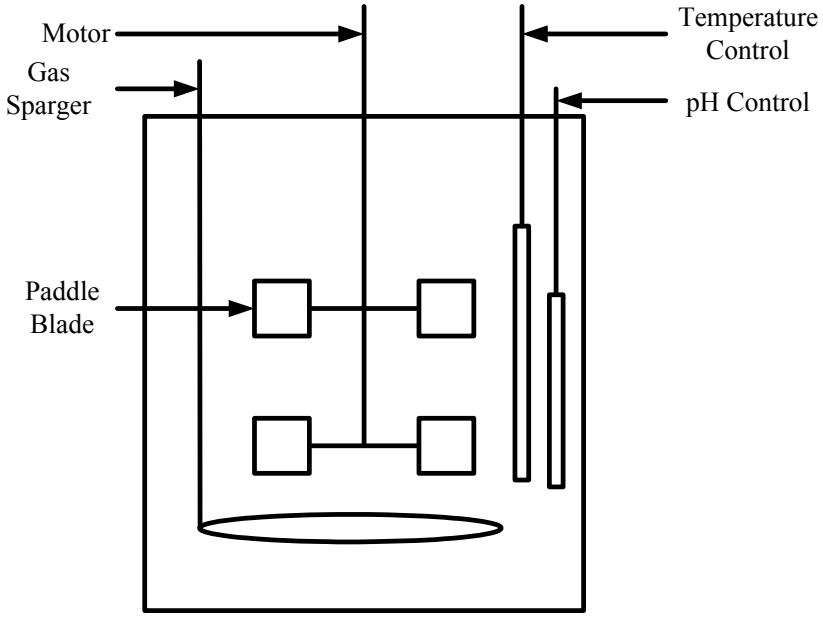
Table 4.5 Comparison of fermentation performance of different single strain (wild type, recombinant strains) and co-culture systems

	Strains	Carbon source (g/L)	Ethanol Concentration (g/L)	Ethanol yield ($g\ g^{-1}$)	Ethanol volumetric productivity ($g\ L^{-1}\ h^{-1}$)	Reference
Single strain	*P. stipitis* Wild type	X50	-	0.43	0.9	Du Preez et al., 1986
	Recombinant *S. cerevisiae* 1400 (*XYL1*, *XYL2*) integrated	X50	2.7	0.05	0.02	Tantirungkij et al., 1993
	Recombinant *S. cerevisiae* (*XYL1*, *XYL2*, *XKS1*) integrated	G90, X40	60.0	0.46	1.15	Ho et al., 1998
	Recombinant *S. cerevisiae* TMB3001 (*XYL1*, *XYL2*, *XKS1*) integrated	X50	-	0.31	0.20	Jeppson et al., 2002
	Recombinant *Z.mobilis* CP4 (pZB5)	G25, X25	24.2	0.48	0.81	Zhang et al., 1995
	Recombinant *Z.mobilis* CM4 (pZB5)	G65, X65	62.0	0.46	1.29	Joachimsthal et al., 1999
	Recombinant *E. coli* K011(*pdc*, *adhB*, *frd*) integrated	X80	41.6	0.52[a]	0.87	Ohta et al., 1991
Co-culture	*P. stipitis* + *S. cerevisiae*	G50, X25	26.2	0.35	0.39	Taniguchi et al., 1997b
	P. stipitis + RD mutant *S. cerevisiae*	G50, X25	37.5	0.50	0.94	Taniguchi et al., 1997a

[a] Attributed to nutrient supplement; G stands for glucose; X stands for xylose



A. Continuous fermentor



B. Batch fermentor

Figure 4.1 Schematic of batch and continuous fermentors

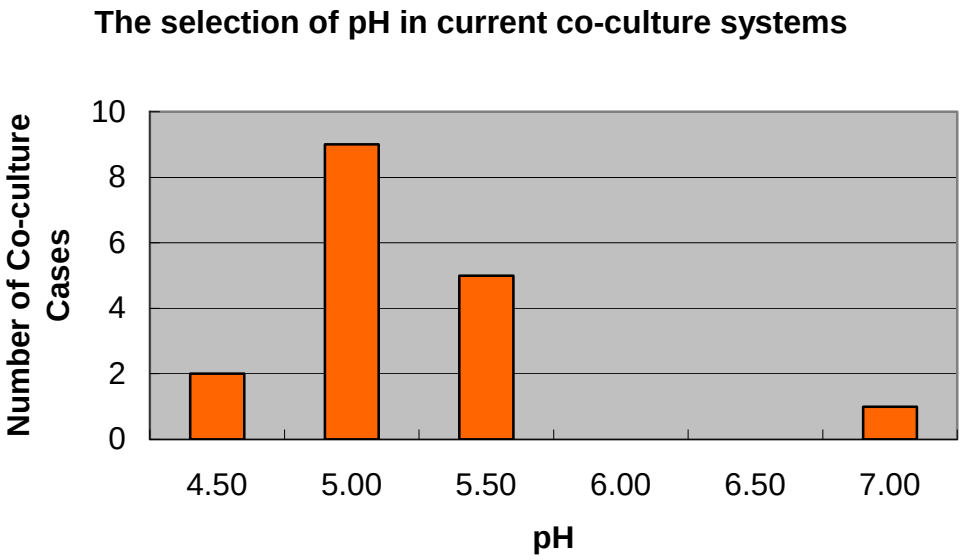


Figure 4.2 Distribution of pH values in current co-culture systems

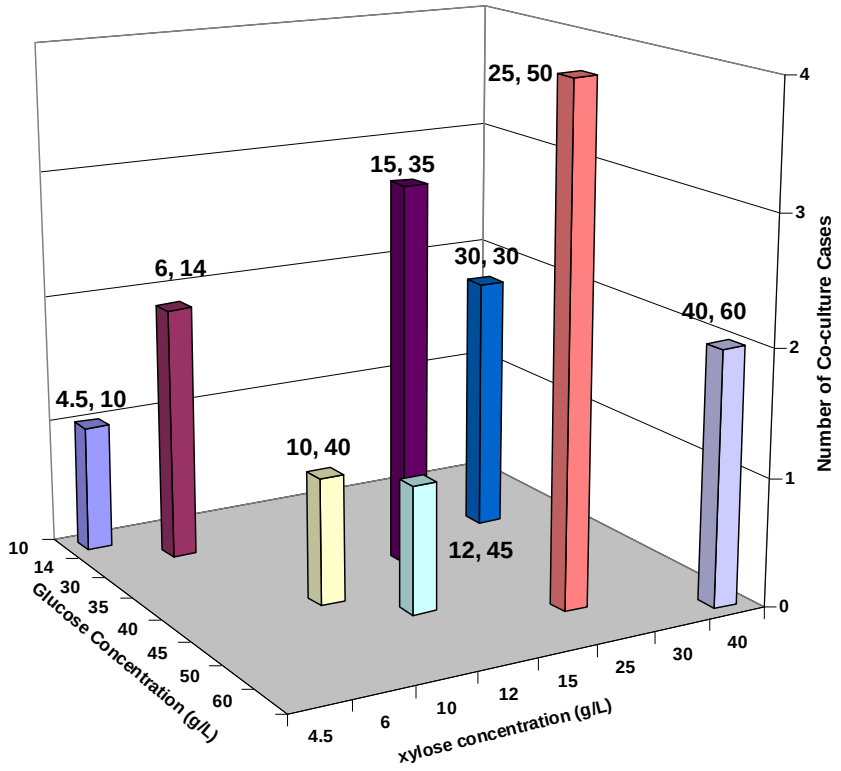


Figure 4.3 The distribution of initial glucose/xylose concentration in current co-culture systems

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

Currently, research efforts of unraveling xylose fermentation in the process of bioethanol production from biomass are mainly focusing on two aspects: 1) genetic modification of glucose-fermenting microbial strains to ferment xylose; and 2) co-culture of glucose-fermenting and xylose-fermenting strains. This research presented some initial results regarding ~~to~~ these two routes. A comprehensive overview of each method has been given. In this chapter, we will draw some conclusions based on the investigated work. In addition, future research directions associated with these two routes will be presented.

5.1 Conclusions

The conclusions we can draw based on the initial investigation are listed below:

1) Cofermentation of glucose and xylose can be achieved by genetic modification of glucose-fermenting microbial strains or co-culture of glucose-fermenting and xylose-fermenting microbial strains.

2) lthough successful construction of recombinant strains through genetic engineering makes it feasible for single strain to coferment glucose and xylose, there are some drawbacks that hinder application of recombinant strains on bioethanol production at industrial scale, such as pleiotropic effects caused by

gene insertion, non-stability in long-term culture, inhibition of undesirable co-products

3) The above drawbacks of recombinant strains may result from a lacking of understanding of cell metabolism on a system level. It was shown that stoichiometric models of genome-scale metabolic network are powerful tools that can help study cells' metabolic functions from the perspective of systems engineering. Primary application of genome-scale metabolic network models includes prediction of outcomes of genetic manipulation, identification of novel metabolic functions and design of microbial strains for desirable products.

4) Existing experimental results of current co-culture research show that it is a potential strategy to accomplish simultaneous cofermentation of glucose and xylose by selecting a proper combination of glucose-fermenting strains and xylose-fermenting strains.

5) The most widely used strain combination during the current co-culture systems is the combination of *P. stipitis* and *S.* cerevisiae or its RD mutants. The best fermentation performance is obtained by *P. stipitis* CBS5773 and RD mutant *S. cerevisiae* no. 7 in batch fermentation. The overall ethanol yield is 0.50 g ethanol/ g substrate, ethanol volumetric productivity reaches 0.94 $gL^{-1}h^{-1}$ and the maximum ethanol concentration is 37.5 g/L.

6) Although co-culture exhibits some appealing traits to improve the simultaneous cofermentation of glucose and xylose, there are some shortcomings of this strategy. For example, the diauxic effect exists for most xylose-fermenting strain candidates (*P. stipitis* and *E. coli*). Another issue is the difference of

fermentation conditions for different strains in the co-culture system. Solving these problems can hasten the process of co-culture utilization in bioethanol production from biomass on a commercial scale.

5.2 Recommendations for Future Work

We conclude with a summary of related research directions that deserve future investigation.

5.2.1. Stability of recombinant strains in continuous fermentation

When attempting to develop recombinant strains for industrial bioethanol production process, genetic stability and environmental acceptability should be considered. There is no research that has been conducted to test and improve the stability of recombinant strains in continuous fermentation so far. This needs further investigation.

5.2.2. Genome-scale metabolic network model for P. stipitis

Due to its importance on xylose fermentation, it is necessary to build a genome-scale metabolic network model for *P. stipitis*. Since the complete genome of *P. stipitis* has been sequenced and annotated in 2007 (Jeffries, 2007), it is possible to set up a genome-scale metabolic model for it. The principles that have been developed and the experiences that have been gained from modeling *E. coli* (Varma and Palsson, 1993a, b; Varma and Palsson, 1994; Pramanik and Keasling, 1997) and *S. cerevisiae* (Förster et al., 2003; Jin and Jeffries, 2004) could be directly applied to modeling *P. stipitis*. With the availability of genome sequences for more and more ethanologenic microorganisms, such

as *Z. mobilis*, *C. shehatae* and *P. tannophilis*, building genome-scale metabolic network model for each of them will be possible in the future.

5.2.3. Different strain combinations for co-culture systems

Based on our investigation, we can draw a conclusion that the most used combination in current co-culture systems is *P. stipitis* and *S. cerevisiae* or its respiratory deficient mutant. Some research work has been done on this combination due to the better compatibility and good match for co-culture purpose. In respect to the combination of yeast and bacteria, *S. cerevisiae* with *E. coli*. and *P. tannophilis* with *Z. Mobilis*, has appeared only one time, respectively. As to the combination of bacteria and bacteria, there has been no research performed so far, it is probably due to unavailable bacterial strains which can effectively ferment xylose. Considering the function of xylose fermenting, *P. stipitis* could be cultured with recombinant *E. coli* or *Z. mobilis* simultaneously in the fermentation system. Since *C. shehatae* is also among the best yeasts for fermenting xylose, *C. shehatae* could be associated with *K. Marxianus*, recombinant *E. coli* or *Z. mobilis* for simultaneous glucose and xylose fermenting. Another promising area would be to test the combination of *P. tannophilis* with *K. Marxianus*, *S. cerevisiae* or its respiratory deficient mutants, recombinant *E. coli* respectively. All of these would be good potential directions for the future co-culture research.

5.2.4. Effect of different fermentation parameters on fermentation performance in different co-culture systems

Fermentation parameters, such as pH, temperature, initial sugar concentrations and compositions, initial inoculum volume, and initial inoculum composition of the two strains, have significant influence on the fermentation performance. In addition, different co-culture systems require different optimal fermentation parameters. To date, there is very little research being done concerning these aspects. These aspects deserve future investigation for improving the potential application of co-culture in bioethanol production process.

5.2.5. Kinetic model for co-culture systems

In principle, kinetic models capture the dynamic properties of systems, and can be a powerful tool to help obtain optimum operating conditions for achieving sufficient profitability and reduce tests for eliminating extreme possibilities. Diverse kinetic models about mono-culture have been proposed in the literature (Thatipamala et al., 1992; Lee et al., 1995; Olsson and Hahn-Hägerdal, 1995; Birol et al., 1998; Lee and Huang, 2000; Leksawasdi et al., 2001; Hodge and Karim, 2002). However, none of these proposed models has put forth a kinetic model that can predict the cell, substrate, and ethanol concentrations for co-culture systems in either batch or continuous operation. Probably, a major problem associated with setting up this model is the lack of kinetic data and the difference between in *vivo* and in *vitro* kinetic parameters for co-culture systems. Therefore, building kinetic models for potential co-culture systems is also a good future research direction.

5.2.6. Metabolic network model for co-culture systems

By using the genome-scale reconstructed metabolic network models for each ethanol-producing microorganism, a combined metabolic network model for any potential co-culture system that captures the dominant metabolic interactions between two strains can be readily built. Elementary mode and extreme pathway analyses for the combined metabolic network model can give us valuable information, such as which genes are essential for producing ethanol, which enzymes would most likely be regulated for changing growth conditions, what kind of metabolic interactions would appear between two strains in the co-culture system. This research work could be one of the interesting directions for co-culture systems in the future as well.

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