Conversion of sugarcane bagasse to ethanol by the use of *Zymomonas mobilis* and *Pichia stipitis*

by

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Declaration

I hereby declare that this submission is my own work and to the best of my knowledge it contains no materials previously published or written by another person, nor material which to a substantial extent has been accepted for the award of any other degree or diploma at UWS or any other educational institution, except where due acknowledgement is made in the thesis. Any contribution made to the research by others, with whom I have worked at UWS or elsewhere, is explicitly acknowledged in the thesis.

I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the project’s design and conception or in style, presentation and linguistic expression is acknowledged.

_________________________
Nan Fu
Dedicated to my parents,
Kangping, Fu & Xiaolan, Wang
The year 2008 will stand out as a very special year in my life. Looking back sometimes I wonder how I finally made it. This thesis is a result of all the encouragement and support from those people mentioned below. I wish to express my sincere gratitude to my three supervisors. Dr. Paul Peiris who gave me invaluable suggestions on both the experimental work and the report; the academic attitude and research skills I have learned from him will be life-time treasures for me. Professor John Bavor who made me the first sieve plate; his insightful advice and kind help got me through many difficulties. I specially appreciate the scientific experimental design Ms. Julie Markham taught me; her careful logic and details showed me the value of being a good scientist.

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I am grateful for everything.
Abstract

The rapid development of the bioethanol industry globally demonstrates the importance of bioethanol as an alternate energy source to the depleting fossil fuels. To decrease costs and avoid undue pressure on the global food supply, the renewable lignocelluloses appear to be a better substrate for bioethanol production compared to others being investigated. This study investigated the conversion of lignocellulosic material, sugarcane bagasse, to ethanol by the use of *Zymomonas mobilis* and *Pichia stipitis*.

The investigation of fermentation characteristics of the two strains revealed that their performance on the ethanol production was closely related to the viable cell concentration in the medium. The increase of inoculum size to five fold resulted in an increase in the system co-efficiency to 2.2 fold and 5.2 fold respectively for *Z. mobilis* and *P. stipitis*. A theoretical value *de* (the cell instantaneous ethanol production rate) was introduced to describe the ethanol productivity based on biomass. System co-efficiency proved to be only affected by the viable cell concentration (*x_c*) and *de*, regardless of ethanol re-assimilation.

Immobilized culture of *Z. mobilis* and *P. stipitis* showed distinct differences in their characteristics. The bacterium acclimatized to the interior of gel beads; the biomass concentration within the beads increased greater than 10 fold during the reuse of the beads, resulting in an improved fermentation performance. The immobilized *P. stipitis* gave a similar system co-efficiency level of approximately 0.5 g/l/h under different culture conditions; cell growth in the medium was considerably more vigorous compared to that within the beads.

*P. stipitis* sole-culture on the glucose/xylose medium with a high inoculum size showed a comparable fermentation efficiency with the best result of the co-culture processes. Fermentation of 50.0 g/l of sugar mixture (30.0 g/l glucose and 20.0 g/l
xylose) was completed in 20 h with an ethanol yield of 0.44 g/g. No catabolite repression due to glucose was observed for the xylose assimilation.

Co-culture of immobilized Z. mobilis and free cells of P. stipitis proved to be the best fermentation scheme on the glucose/xylose sugar mixture co-fermentation. The removal of Z. mobilis after the utilization of glucose improved the stability of the performance. The best result showed that 50.0 g/l sugars were fully converted to ethanol within 19 h, giving an ethanol yield of 0.49 g/g, which is 96% of the theoretical rate. When co-cultured, viable cells of Z. mobilis inhibited the cell activity of P. stipitis, and were capable of growing to high concentration levels without an appropriate carbon source.

Acid and enzymatic hydrolysates of sugarcane bagasse showed similar fermentability, but the hydrolysate without overliming significantly inhibited both cell growth and ethanol production of P. stipitis. The co-culture process on the hydrolysate medium successfully utilized 53.56 g/l sugars (32.14 g/l glucose and 21.42 g/l xylose) in 26 h with a yield of 0.43 g/g; this value further increased to 0.49 g/g when ethanol peaked at 40 h.

A high cell density proved to be an effective method to improve the system co-efficiency for ethanol production. For the fermentation processes on the sugar medium, results achieved in this study, 10.54 g/l/h for Z. mobilis free cell culture on glucose, 0.755 g/l/h for P. stipitis free cell culture on xylose, 1.092 g/l/h for P. stipitis free cell culture on the glucose/xylose mixture and 1.277 g/l/h for glucose/xylose co-fermentation using co-culture, are higher than the best values reported in the literature in batch culture. In the fermentation of the hydrolysate, the system co-efficiency of 0.879 g/l/h achieved with co-culture is comparable to the best values reported for the fermentation of lignocellulosic hydrolysates.
Publications

Manuscripts

Fu, N., Peiris, P., Markham, J., and Bavor, J., (2008) “Improving ethanol productivity of *Zymomonas mobilis* and *Pichia stipitis* using a high cell density system”. In preparation for publication in *Applied Microbiology and Biotechnology*.

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Fu, N., Peiris, P., Markham, J., and Bavor, J., (2008) “Conversion of Sugarcane Bagasse to Ethanol by Co-culture of *Zymomonas mobilis* and *Pichia stipitis*”, *Proceeding of the Research Futures Postgraduate Conference 2008*, University of Western Sydney, Sydney, N.S.W., Australia, 3rd – 5th, June.

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Nomenclature

Definitions

**Co-fermentation**: Used to describe a fermentation process involving two sugars, which are converted to ethanol in a single process. It can be achieved by either the co-culture of two strains or the utilization of one strain only, as far as it works on the simultaneous conversion of mixed sugar substrate.

**Co-culture**: Used only when two microorganisms are cultured together and simultaneously existing in the medium; it is different from “sequential culture”, where microorganisms are cultured successively.

General Abbreviations

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>μg/mcg</td>
<td>microgram</td>
</tr>
<tr>
<td>atm</td>
<td>atmosphere</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>cfu</td>
<td>colonies forming unit</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
</tr>
<tr>
<td>DNS</td>
<td>dinitrosalicylic acid</td>
</tr>
<tr>
<td>ED</td>
<td>Entner-Doudoroff pathway</td>
</tr>
<tr>
<td>EMP</td>
<td>Embden-Meyerhof Pathway</td>
</tr>
</tbody>
</table>
FS fermentation scheme

gram

GC gas chromatography

GMO genetically modified organisms

GO glucose oxidase

hour

HPLC High Performance Liquid Chromatography

litre

molar (moles/l)

milligram

minute

millilitre

millimole

oxygen transfer rate

Pentose Phosphate Pathway

respiratory deficient

revolutions per volume

reducing sugar

second

separate hydrolysis and fermentation

simultaneous saccharification and co-fermentation

simultaneous saccharification and fermentation

time course

unit of enzyme activity

volume of inoculum medium per volume of fermentation medium

volume of enzyme per dry weight of substrate biomass

airflow rate (volume per min) per working volume of bioreactor

weight of solute per volume of solution
w/w  Weight per weight
XR  xylose reductase
XDH  xylitol dehydrogenase
YEIS  solution only containing yeast extract and inorganic salts

**Subscripts**

0  initial value
f  final value
1  start of time period
2  end of time period
yie  yield
con  consumed
p  time of product reaching peak
t  value at a specific time

**Kinetic Symbols**

$P$: the absolute amount of ethanol (g)
$N$: the total number of biomass (cells)
$p$: product (ethanol) concentration (g/l)
$s$: substrate concentration (g/l)
$v$: volume (l)
$t$: time (h)
$Q_p$: system co-efficiency / volumetric ethanol productivity (g/l/h)
$Q_s$: sugar uptake rate (g/l/h)
$Y_{p/s}$: ethanol yield / product conversion rate per substrate (g/g)
$q_p$: specific ethanol productivity (g/g/h)
de: cell instantaneous ethanol production rate (g/cells/h).
x_A: Biomass concentration – average dry weight (g/l)
x_C: Biomass concentration – viable cell concentration (cfu/ml)
Chapter 1

Introduction

1.1. Bioethanol production

Bioethanol production from renewable resources continues to attract considerable interest as an alternative to fossil fuel (Rortrup-Nielsen, 2005; van Maris et al., 2006; Lynd et al., 2008). The research and exploitation of bioenergy, such as bioethanol and biodiesel, are not recent topics. In order to cope with the predicted depletion of fossil fuel in the near future, a number of countries have taken steps to reduce their dependence on gas and oil imports, by developing and industrializing new energy forms (de Vries et al., 2007; Groom et al., 2007). Potential energy forms include nuclear energy and some renewable and clean forms, such as solar, hydro, biomass and wind. Amongst these, ethanol production from biomass is of particular interest due to following advantages: (1) low emission levels of carbon dioxide, and therefore contributing less to green house effects (Ignaciuk et al., 2006); (2) readiness to be blended with gasoline and used in the transportation industry without much modification to the vehicles (Katz, 2008); (3) mature production techniques. Ethanol has been produced from fruit or starch crops for alcoholic beverages for over thousands of years. Considering these advantages, the potential of fuel ethanol has been thoroughly explored for further development and industrialization.

The research and application of bioethanol for energy purposes were pioneered by the United States and Brazil. In the United States, bioethanol is produced mainly
from traditional starch crops such as corn (Katz, 2008); whereas in Brazil, bioethanol production is derived from and is closely related to the sugarcane industry, where both sugarcane and waste material from the sugar manufacture process serve as substrates for ethanol production (Goldemberg, 2007). Both countries have brought out a series of strategies to promote and popularize the utilization of ethanol for fuel purposes, e.g. the price reduction of ethanol-blended gasoline. Other strategies adopted in the United States include the enticement for growing plants for ethanol production and the additional duty levied for imported ethanol to encourage the domestic ethanol production industry (Katz, 2008). As a result, corn plantations have been greatly extended in the United States, replacing other crops such as soy for greater profit, e.g. 15.5% increase in acreage in 2007 (Kennedy, 2007; Katz, 2008). Even the harvested corn in the market has been increasingly diverted to ethanol production instead of being used as a traditional agricultural commodity. The biofuel industry consumed 25% of overall crops produced in 2007 in the United States and is projected to consume more than 30% in 2008 (Robertson et al., 2008; Tenenbaum, 2008). Due to all these measures, the bioethanol industry has rapidly developed and prospered. The production of renewable fuels in the United States is projected to achieve 36 billion gallons by 2022, which will mostly include ethanol from corn and cellulose (Potera, 2008). Cars and other vehicles driven by ethanol (either 100% or blended gasoline with ethanol up to 25%) are being commonly used in Brazil (Goldemberg, 2007). In Australia a program entitled Ethanol Production Grants was set up as early as in 2002 to encourage the use of biofuels for vehicles.

At the beginning of 2008, a global food crisis was observed worldwide. As pointed out by numerous reports, bioethanol production was considered one of the major factors responsible for this crisis (Kennedy, 2007; Runge and Senauer, 2007; Katz, 2008; Tenenbaum, 2008). Corn and other starch crops are basic food and feed material; the mass production of ethanol from these substrates in the United States substantially decreases the proportion of products used for food consumption, reduces the plantation area of other edible crops, and indirectly causes the shortage of both primary products and livestock. Bioethanol, however, remains a promising alternative to fossil fuels; its rapid development and spread in Brazil confirms this viewpoint. The utilization of ethanol is one of the safest methods to counteract the depletion of non-renewable resources. In order to retain bioethanol for fuel purpose
as well as to avoid the unnecessary pressure on the food supply, new substrates for ethanol production need to be set up, ensuring that all the starch crops are returned back to the agricultural commodity market. The sugarcane program in Brazil is considered acceptable because unlike starch, the sucrose is not a necessary food material. Nevertheless, for long term consideration, material which cannot be consumed by humans should be the ultimate choice for bioethanol production, e.g. lignocellulose.

Lignocellulose is basically the woody part of plants, constituting the most abundant raw material in nature (Kuhad and Singh, 1993; Chandrakant and Bisaria, 1998; Zhang, 2008). Each year approximately 200 billion tons of lignocellulosic waste is produced from agriculture and industry worldwide. These mainly include various agricultural residues, deciduous and coniferous woods, municipal solid wastes, pulp and paper industrial waste, and herbaceous energy crops (Saha, 2003; Das and Singh, 2004). Ethanol production from this kind of substrate can serve as a waste treatment and convert this apparently useless material into value-added products. Because the carbohydrate in the lignocellulose is originally produced by photosynthesis, the oxidation of these carbon atoms does not increase the level of carbon dioxide in the atmosphere, only simply recycles them (Zaldivar et al., 2001; Zhang, 2008). Consequently, lignocellulose is an ideal substrate for ethanol production, on account of its cheap cost and clean energy yield.

1.2. Lignocellulosic fuel

The concept of the conversion of lignocellulose to ethanol dates back as early as 1917 (Brooks and Ingram, 1995). However, currently ethanol production from lignocellulose substrates remains at a laboratory stage, while ethanol production from sugarcane and corn has already been commercialized. The major drawback in the utilization of lignocellulose is its natural structure. This will be discussed in detail in Chapter 2. Briefly, the intimate association of several macromolecules in the lignocellulose makes it recalcitrant to breakdown to single fermentable sugars. Even after the breakdown, the fact that it comprises a variety of monosugars makes the ethanol production process considerably difficult. The conversion efficiency of lignocellulose to ethanol is thus greatly limited.
As a potential fuel, the final price of ethanol products needs to be moderate and acceptable compared to the market price of traditional gasoline. On the other hand, it should bring in profit to the organization producing it. Both factors affect the commercial feasibility of producing ethanol from lignocellulose. A standard proposed in earlier research (du Preez, 1994) requires an ethanol-producing capability of 50-60 g/l within 36 h, with a yield no less than 0.40 g/g. Research on the lignocellulosic fuel production has continuously worked on the improvement of efficiency of both the hydrolysis process to depolymerize the macromolecular complex to fermentable sugars and the microbial fermentation process to convert the sugar mixture to ethanol.

The current research was therefore undertaken with the aim of assisting the bioenergy industry. Sugarcane bagasse was selected as the lignocellulosic substrate for the conversion to ethanol using two microbial strains.

1.3. Aim and objectives

Aim:
Maximization of ethanol production from sugarcane bagasse hydrolysate by the use of \textit{Zymomonas mobilis} and \textit{Pichia stipitis}.

Objectives:
- Determination of fermentation characteristics of \textit{Z. mobilis} and \textit{P. stipitis} on glucose and xylose medium respectively.
- Determination of effects of immobilization on the fermentation characteristics of \textit{Z. mobilis} and \textit{P. stipitis}.
- Evaluation of co-fermentation processes of glucose/xylose sugar mixture with different fermentation schemes, employing \textit{Z. mobilis} and \textit{P. stipitis}.
- Optimization of the hydrolysis of sugarcane bagasse to maximize fermentable sugars.
- Conversion of the sugarcane bagasse hydrolysate to ethanol by the use of \textit{Z. mobilis} and \textit{P. stipitis}. 
2.1. Lignocellulose for ethanol production

The conversion process from lignocellulose to ethanol usually involves four stages (Lee, 1997; Chandrakant and Bisaria, 1998). Starting with lignocellulosic raw material, firstly a pretreatment is carried out to remove the lignin portion and make the hemicellulose and cellulose structure more vulnerable to breakdown in the second hydrolysis stage. The released monosaccharides from saccharified carbohydrate polymers can be directly assimilated by microorganisms and converted to ethanol through metabolic processes. Finally the produced ethanol needs to be extracted from fermentation broth and purified into final products, which usually requires further energy input. Each step has its own difficulties; however, the major challenges are the hydrolysis process and the conversion of sugar mixture to ethanol as mentioned in Section 1.2.

Lignocellulosic biomass has a complicated structure; the composition of three basic components, lignin, hemicellulose and cellulose, is different from plant to plant (Kuhad and Singh, 1993; Das and Singh, 2004). The tight structural combination between three compounds in association with the crystalloid structure of cellulose all add up to the difficulties of hydrolysis (Martin et al., 2007b). Consequently, the hydrolysis of lignocellulose is difficult to achieve, requires a long time and expensive enzyme systems, and sometimes yields inhibitors of the microbial fermentation.
The cellulose portion in lignocellulose can be hydrolyzed to glucose, while the xylan in the hemicellulose can be hydrolyzed to xylose; the simultaneous fermentation of both sugars represents another technical difficulty (Chandrakant and Bisaria, 1998). Though glucose fermentation to ethanol can be efficiently accomplished by a number of microorganisms, including the yeast genus *Saccharomyces* and bacterium *Zymomonas mobilis*, the xylose fermentation is considerably more difficult. It is even harder when both sugars are required to be converted simultaneously. Nevertheless, for economical considerations, the conversion of xylose, which constitutes 15~30% of total sugars in the hydrolysate (Lee, 1997; Lawford and Rousseau, 2003), is crucial to achieve maximum ethanol production from lignocellulose.

Research has extensively focused on the hydrolysis and fermentation processes to optimize the overall process for the conversion of lignocellulose to ethanol. This can be achieved by both process engineering and microbial improvement, including the development of new processing schemes, optimization of environmental parameters, as well as the construction of desirable microorganisms through either mutation or recombinant DNA technology.

When it comes to the process considerations, it should be pointed out that though the four stages mentioned above constitute a complete process for the lignocellulosic ethanol production, in practice they are not strictly separated. There are a number of schemes combining steps. For example, in a simultaneous saccharification and fermentation (SSF) process (Ballesteros et al., 2004; Panagiotou et al., 2005), the pretreated lignocellulosic substrate is hydrolyzed to monosaccharides and then directly converted to ethanol in the same vessel while the saccharification is still in process. An advanced version of SSF process is called simultaneous saccharification and co-fermentation (SSCF) (Patel et al., 2005). In addition to the common SSF process, there are at least two different kinds of monosaccharides continuously released from carbohydrate polymers, and both are immediately converted to ethanol in this single process. Lynd’s group proposed a concept named the “Consolidated Process”, integrating all together four biotransformation processes into one (Lynd et al., 2005; van Zyl et al., 2007). Besides those three involved in the SSCF, they suggested that the production of cellulase enzymes should also be included in order
to minimize the cost of the enzyme system required for saccharification. Such a process would consist of the production of cellulolytic enzymes, the hydrolysis of lignocellulose and the co-fermentation of glucose and xylose, which make up four biotransformation steps.

In comparison with these integrated fermentation schemes, the separate hydrolysis and fermentation (SHF) process is more commonly adopted, since it allows the maximum optimization of each process individually. Because saccharification and fermentation usually require different optimal conditions for highest efficiency, the simultaneous process tends to result in inferior system performance.

In summary, SHF provides a higher efficiency in each stage respectively, but the integrated process has the potential to increase the whole system efficiency by combining more stages into a single step. The establishment of an optimized system thus depends on the evaluation and consideration of each factor that affects the efficiency. This literature review commences with basic stages involved in the conversion of lignocellulose to ethanol—the respective fermentation of glucose and xylose, and then moves to the co-fermentation and the hydrolysis. Finally some typical studies involving the SSF process and genetically modified microorganisms (GMO) are briefly reviewed.

2.2. Glucose fermentation by *Z. mobilis*

*Zymomonas mobilis* is highly recognized for its performance in ethanol production from glucose, fructose and sucrose. It is reported to show higher ethanol productivity and tolerance compared to *Saccharomyces cerevisiae* (Davis et al., 2006; Kalnenieks, 2006). However, for the co-fermentation process of a glucose/xylose mixture, *S. cerevisiae* is preferably used for the co-culture with another xylose fermenting strain [See Section 2.4.2]. In order to understand its metabolic mechanism and evaluate the possibility of *Z. mobilis* for co-culture, Section 2.2 reviews literature relating to the glucose fermentation by *Z. mobilis*.

2.2.1. Biochemistry of ethanol production by *Z. mobilis*

*Zymomonas* is Gram-negative and consists of large rods. Phylogenetically it belongs
to the class of Alphaproteobacteria and is affiliated to the pseudomonads, though the pseudomonads usually show respiratory metabolism whereas *Zymomonas* is strictly fermentative (Madigan and Martinko, 2006b). A similar trait of *Pseudomonas* and *Zymomonas* in the metabolism is that they are both able to carry out the Entner-Doudoroff (ED) pathway.

The ED pathway is different from the Embden-Meyerhof Pathway (EMP)—the glycolysis pathway, in that it only takes four steps to produce the first pyruvate and no ATP is produced during these four steps. As illustrated in Figure 2.1, the second pyruvate comes from the glyceraldehyde-3-phosphate and 2 ATP are yielded during the reactions. The pyruvates are decarboxylated into acetaldehyde, which is further reduced to ethanol as the end product. In other words, the ED pathway is not as energy efficient as the EMP pathway, since only half amount of ATP is produced [2 ATP in ED and 4 ATP in EMP].

*Zymomonas* is capable of carrying out glucose fermentation more rapidly than yeast with the EMP pathway probably because of the use of the ED pathway. Firstly, the ED pathway requires less reaction steps to convert glucose to ethanol; secondly, a larger amount of ethanol needs to be produced to match the ATP requirements of the bacterium. As pointed out by Kalnenieks (2006), the fast glucose catabolism rate of *Z. mobilis* decidedly exceeds the needs for its cellular biosynthesis, which is thus named as the “uncoupled growth” phenomenon. Nevertheless, the high ethanol productivity is beneficial to the industrialization of commercial ethanol production. Based on the stoichiometric reactions shown in Figure 2.1, the maximum ethanol yield of *Z. mobilis* from glucose follows the overall equation below (Madigan and Martinko, 2006a):

\[
\text{Glucose (C}_6\text{H}_{12}\text{O}_6) = 2 \text{ ethanol (C}_2\text{H}_5\text{OH}) + 2 \text{ CO}_2 \quad (1)
\]

A full conversion of 1.0 mole glucose yields 2.0 moles of ethanol, rendering a theoretical ethanol yield of 0.51 g/g glucose.
2.2.2. Culture of *Z. mobilis* on glucose

Amongst studies investigating the fermentation characteristics of *Z. mobilis* on glucose, early reports tended to suggest that it only gave best ethanol yield under strict anaerobic conditions, achieved by a continuous flush of nitrogen to the fermentation medium (Laplace et al., 1991a). Also, its high performance of the ethanol production was not affected by the increase in the initial glucose concentration; both ethanol yield and productivity of *Z. mobilis* were reported to be
superior to that of *S. cerevisiae* (Laplace et al., 1991b). Similar observations were made by Davis et al. (2006), who compared the ethanol production efficiency of the two strains on a hydrolyzed waste starch substrate. With an initial glucose concentration up to 80-110 g/l, *Z. mobilis* gave higher performance on both ethanol productivity and substrate consumption.

Moreover, the ethanol-producing ability of *Z. mobilis* can be further improved through the optimization of the fermentation scheme and environmental conditions. An ethanol production rate of 0.47-0.48 g/l/h was reported in a continuous culture of *Z. mobilis* using high cell density system (Laplace et al., 1993c). It was also found that *Z. mobilis* gives steady performance even in the presence of xylose or acetic acid, an inhibitor, in the medium; a conversion efficiency of 96% was achieved from glucose by a wild type strain of *Z. mobilis* cultured in a glucose/xylose medium (Lawford and Rousseau, 2003). Ethanol production models of *Z. mobilis* were established by Lee and Huang (2000) on both glucose and fructose substrates. The specific growth rate of the bacterium was claimed to correlate to the dependence of assimilated carbon fraction in both batch and continuous culture environments.

Fermentation processes employing wild type *Z. mobilis* on lignocellulosic hydrolysate have also been studied; however, sometimes results were comparably poor. The biomass growth rate in such a medium was low, consequently limiting the ethanol production rate (Delgenes et al., 1996b; Yu and Zhang, 2002). *Z. mobilis* is believed to be sensitive to some toxic components formed during the hydrolysis process, e.g. hydroxybenzaldehyde. Therefore detoxification of the hydrolysate is essential, and adaptation of the strain in the hydrolysate would also be helpful for eliminating the inhibition. A study culturing *Z. mobilis* on a hardwood derived cellulosic hydrolysate reported an ethanol yield of 0.35 g/g sugars (Delgenes et al., 1996a). The value is relatively low compared to a result of 0.50 g/g when the bacterium was cultured on the hydrolyzed starch medium (Davis et al., 2006). Other substrates used for *Z. mobilis* culture included agricultural wastes of a grain and oil seed mixture (Dey, 2001). Ethanol production of 205 g was obtained from 600 g of waste seeds using a two stage semi-continuous fluidized bed bioreactor, which equalled a conversion rate of 0.34 g/g.
In general, *Z. mobilis* shows excellent ethanol-producing performance on the sugar media. To further investigate its fermentation characteristics, immobilized cultures have been studied.

### 2.2.3. Immobilized *Z. mobilis*

Immobilization is a commonly used technique in biotechnology for fermentation and enzymatic studies. It has several advantages in comparison with free cells: (1) ability to concentrate a large amount of cells into a small volume, (2) easier management of cells, (3) possibility to provide semi-different environmental parameters within the immobilizing material (Grootjen et al., 1991b). Some studies involving immobilized *Z. mobilis* are discussed below.

Rebros et al. (2005) reported a remarkably high volumetric ethanol productivity of 43.6 g/l/h on glucose, by entrapping *Z. mobilis* in commercially available polyvinylalcohol beads Lentikat®. The result was achieved in batch culture with complete fermentation of 150 g/l glucose within 2 hours after repeated culture of the same batch of beads 72 times. The effects of bioreactor were also investigated for immobilized *Z. mobilis* (Kesava and Panda, 1996; Kesava et al., 1996). Gel beads cultured in an expanded bed bioreactor achieved higher kinetic parameters compared to those cultured in a regular mechanically stirred tank bioreactor; peak value of volumetric ethanol productivity was 21 g/l/h attained in a continuous culture. Bajpai and Margatitis (1985) studied the influence of initial glucose concentration on the ethanol productivity of immobilized *Z. mobilis* entrapped in calcium alginate gels. It was found that the yield coefficient was almost unaffected by the increase of substrate concentration. With a cell concentration of 58 g dry weight/l of bead volume, ethanol yield was more than 95% of the theoretical. Continuous culture for more than 72 days with the immobilized *Z. mobilis* was also reported with a volumetric ethanol productivity of 63 g/l/h in a vertical rotating immobilized cell reactor (Amin and Doelle, 1990).

It can be thus concluded that immobilization is an effective method for improving the performance of ethanol production by *Z. mobilis*. In addition, the co-immobilization system of *Z. mobilis* with other microorganisms or enzymes has also been set up to
extend the substrate utilization range of \( Z. \) mobilis. The relevant literature will be discussed together with other co-culture processes in Section 2.4.1.

2.3. Xylose fermentation

In contrast to the well-established glucose fermentation process, xylose fermentation has a much shorter history. Strains able to assimilate xylose were reported only from the early 1980s (Jeffries and Kurtzman, 1994). Since then extensive studies have been carried out on strain screening and selection to obtain the best ethanol-fermenting microorganisms on xylose (Roberto et al., 1991; Jeffries and Kurtzman, 1994). More than 40 kinds of yeasts are considered to be capable of converting xylose to ethanol (du Preez, 1994). Xylose-utilizing bacteria and molds have been reported; however, yeasts are of most interest because of their relatively high ethanol yield. In this section, three common xylose fermenting yeasts are discussed, and literature involving their fermentation studies is reviewed.

2.3.1 Biochemistry of ethanol production from xylose

There are three strains that have shown significant ethanol-producing capability from xylose, viz. *Pichia stipitis*, *Candida shehatae* and *Pachysolen tannophilus*. Among these three, *Pichia stipitis* is the most efficient xylose fermenter with the highest ethanol productivity (Chandrakant and Bisaria, 1998), but the average ethanol yields of all three are similar (Sanchez et al., 2002).

The metabolic pathways from xylose to ethanol are still being studied by some researchers. Figure 2.2 shows a general route that is most widely accepted. After assimilation, xylose is firstly converted to xylulose, by either the catalysis of xylose isomerase or the dehydrogenation of xylitol. Then xylulose enters the Pentose Phosphate Pathway (PPP) and transforms to glyceraldehyde-3-phosphate through a series of enzyme-mediated reactions. From here it connects to the glycolytic pathway, converting the glyceraldehyde-3-phosphate to pyruvate and then reducing the pyruvate to ethanol. Thus, the conversion of xylose to ethanol requires many more steps than that of glucose, resulting in a decreased efficiency of ethanol production. Moreover, some of the carbon atoms from xylose can be distributed to biomass synthesis via the PPP pathway, which further reduces the potential ethanol yield. As a
result, it is not surprising that ethanol production from xylose is less efficient and more time-consuming compared to that from glucose by *Z. mobilis*.

![Diagram of xylose metabolism to ethanol in yeasts](image)

For the calculation of the theoretical ethanol yield from xylose, it is assumed that all the xylose is converted to xylulose followed by a complete conversion of xylulose to ethanol without the formation of byproducts and consumption of carbon atoms by biosynthesis. Then the conversion process can be expressed by the equation below.

**Figure 2.2: Main pathway of xylose metabolism to ethanol in yeasts (Adapted from Chandrakant and Bisaria, 1998; Kruse and Schugerl, 1996).**
(Chandrakant and Bisaria, 1998):

\[
3 \text{ Xylose (C}_5\text{H}_{10}\text{O}_5) = 5 \text{ ethanol (C}_2\text{H}_5\text{OH}) + 5 \text{ CO}_2
\]

(2)

Overall, 1.67 moles of ethanol is produced from 1.0 mole xylose, which is less than the molar conversion rate of ethanol from glucose [2.0 mole/mole]; however, the mass conversion rate is the same, 0.51 g/g xylose.

A different interpretation, however, is proposed by other groups. In the whole process from xylose to ethanol, the first rate-limiting step is the conversion of xylose to xylulose, which can be achieved in two different ways. In the bacterial cells which metabolize xylose but not produce ethanol, e.g. *Escherichia coli*, xylulose is directly produced under the catalysis of xylose isomerase. By contrast, for all three yeasts, xylitol is firstly formed and then oxidized to xylulose. As shown in Figure 2.2, the reaction from xylose to xylitol is catalyzed by xylose reductase (XR); and then xylitol dehydrogenase (XDH) functions to oxidize xylitol to xylulose. Both steps need the participation of certain enzyme cofactors, which are different from yeast to yeast. In some cases the cofactors are reduced or oxidized in large amount and cannot be re-oxidized or re-reduced again, e.g. in *Candida utilis*. This directly causes a redox imbalance and therefore ceases further reactions, leading to xylitol excretion instead. Oxygen as an electron accepter can relieve this redox imbalance, but consumes some carbon atoms from the original xylose. In this case, the overall reaction from xylose to ethanol follows another stoichiometric equation (Jin and Jeffries, 2004):

\[
\text{Xylose (C}_5\text{H}_{10}\text{O}_5) + 0.5 \text{ O}_2 = 1.5 \text{ ethanol (C}_2\text{H}_5\text{OH}) + 2 \text{ CO}_2
\]

(3)

Only 1.5 moles of ethanol are produced from 1.0 mole of xylose, equalling a mass conversion rate of 0.46 g/g xylose. This value is less than that achieved under anaerobic conditions, where 0.51 g ethanol/g xylose is produced theoretically.

### 2.3.2. Fermentation of xylose to ethanol

Since the discovery of strains that are able to produce ethanol from xylose, there have been many efforts to investigate fermentation characteristics of these microorganisms. Some of these studies are discussed below.

#### Medium effects

Watson et al. (1984) studied the factors inhibiting ethanol production from xylose by
Chapter 2. Literature review

*P. tannophilus*. The study pointed out that ethanol itself was a kind of inhibitor and other possible factors included iron, chromium, copper, nickel, acetic acid and furfural. Most of these could be produced from the pretreatment and saccharification process of lignocellulose. The effects of fructose in the medium were also investigated (Bicho et al., 1989). Xylose metabolism of *P. tannophilus* was repressed by the presence of fructose in the medium, which was indicated as a repression in the induction of XR and XDH. By contrast, the presence of fructose did not affect the xylose utilization of *P. stipitis*.

Initial xylose concentration was another important factor accounting for ethanol productivity. Laplace et al. (1991b) suggested that sugar concentrations higher than 25 g/l tended to inhibit the cell growth of both *P. stipitis* and *C. shehatae*. Similar results were obtained by other research groups (Kruse and Schugerl, 1996, Bravo et al., 1995). According to Bravo et al. (1995), the optimum medium composition was 25 g/l xylose and 4 g/l yeast extract together with other inorganic salts. Culture of xylose fermenting yeasts on the glucose/xylose medium resulted in a sequential consumption of two sugars, firstly glucose and then xylose (Sanchez et al., 2002). Glucose metabolism apparently inhibited the assimilation of xylose and this was observed for all three yeast strains, viz. *P. stipitis* (Taniguchi et al., 1997b), *C. shehatae* and *P. tannophilus* (Sanchez et al., 1999).

**Temperature effects**

Barbosa et al. (1990) reported that ethanol production from xylose by *P. tannophilus* remained on a similar level when the temperature increased from 30°C to 37°C, though in the meantime xylitol yield decreased and CO₂ production increased. Converti et al. (2001) claimed that there were three metabolic pathways for the assimilated xylose in *P. tannophilus*. At a low temperature of 20°C respiration was the main process; between 25-30°C a large amount of xylitol was accumulated, and ethanol fermentation occurred only at higher temperatures such as 40°C. Other research, however, suggested that optimum temperature for ethanol production by *P. tannophilus* was 30°C, though the fastest cell growth was observed at 33°C (Sanchez et al., 2004).
**Oxygenation effects**

In contrast to *S. cerevisiae* whose metabolism is predominantly respiro-fermentative, the metabolism of xylose utilizing yeasts is considered to be fully respirative (Fiaux et al., 2003). However, this characteristic negatively affects the ethanol accumulation. The major disadvantages are: (1) difficulties in controlling the oxygenation at optimum values during fermentation, (2) ethanol re-assimilation under aerobic conditions, and (3) more complex techniques required for industrialization in comparison with anaerobic fermentation processes.

Currently it is accepted that the conversion of xylose to ethanol by yeasts requires strict micro-aerobic conditions. The optimum value of oxygenation, however, varies amongst reports. Kruse and Schugerl (1996) investigated the fermentative performance of *Pachysolen tannophilus* under different environmental conditions. Ethanol production only occurred in micro-aerobic conditions with an air flow of 0.009 vvm (volume/volume/minute). Under fully aerobic conditions the cells grew vigorously with rare ethanol accumulation, and under anaerobic conditions the cell growth was almost totally inhibited. Furlan et al. (1994) studied the effect of oxygenation on all three xylose-fermenters, viz. *P. stipitis*, *C. shehatae* and *P. tannophilus*. It was reported that for all three strains ethanol productivity significantly relied on oxygenation and the highest ethanol production was obtained under an air flow of 0.08 vvm and a stirring speed of 250 rpm. The optimum aeration level reported by Laplace et al. (1991a) was between 0.005 vvm and 0.001 vvm with a stirring speed of 800 rpm for both *P. stipitis* and *C. shehatae*. Other oxygenation levels used for ethanol production from xylose include an air flow level of 0.1 vvm with a stirring speed of 160 rpm (Taniguchi et al., 1997b), 400ml medium in a 500 ml conical flask on a rotary shaker at 150 rpm (Laplace et al., 1991b) and 100 ml medium in a 150 ml conical flask with 100 rpm shaking speed (Yu and Zhang, 2002). Skoog and Hahn-Hagerdal (1990) reported an ethanol yield up to 0.48 g/g by *P. stipitis* under an oxygen transfer rate (OTR) below 1 mmol/l/h.

The effect of oxygenation on the yeast cell activity was also demonstrated by the investigation on the enzyme XR (VanCauwenberge et al., 1989). It was found that under aerobic and anaerobic conditions there were two different enzyme forms in the *P. tannophilus* cells.
To summarize, research on both fermentation performance and metabolic mechanisms demonstrated that the oxygen plays a crucial part for the ethanol production from xylose by the three xylose fermenting yeasts. In order to achieve a maximum ethanol yield, oxygenation should be carefully controlled at an optimum level during the fermentation. Since the oxygenation level is affected not only by the air flow and stirring speed but also by the cell concentration and medium viscosity, the different values applied in the literature are confusing. The optimum aeration condition is best determined practically as done in the current study.

**Ethanol tolerance and re-assimilation**

Ethanol is a typical primary metabolite and is involved in energy metabolism; consequently, it is reasonable to presume that the ethanol produced by microorganisms could be re-assimilated under certain circumstances. On the other hand, ethanol is also an inhibitor of cell growth. The produced ethanol and external ethanol are suggested to have different toxicity to the microbial cells (du Preez, 1994). Thus, the effects of ethanol also need to be considered for the establishment of a successful fermentation process.

In contrast to traditional ethanol-producing strains, viz. *S. cerevisiae* and *Z. mobilis*, xylose fermenting yeasts tend to have low tolerance to ethanol (Lee, 1997). An increased ethanol concentration is inhibitory to both cell growth and ethanol production, but cell growth was inhibited at lower ethanol concentration compared to the ethanol production (du Preez et al., 1989). Ethanol tolerance of these yeasts was not improved by culturing on the glucose substrate, suggesting that their low ethanol tolerance was not a result of xylose catabolism. The ethanol re-assimilation rate of *P. stipitis* was found to be greater than that of *C. shehatae* (du Preez et al., 1989). The phenomenon of ethanol re-assimilation was observed in a number of studies (Maleszka and Schneider, 1982; Passoth et al., 2003; Gorgens et al., 2005). It occurred even in the presence of residual sugars in the medium, with a higher rate on xylose than on glucose. Skoog et al. (1992) reported that the increased oxygenation helped with the re-assimilation of ethanol by *P. tannophilus*; as a consequence, acetate formed and the production of CO₂ decreased.
Overall, the conversion of xylose to ethanol is considerably less efficient compared to the conversion of glucose to ethanol by *Z. mobilis*. It requires strict control on the environmental parameters to achieve satisfactory ethanol production, and an optimized oxygenation level is extremely crucial. Consequently, these yeasts appear to be incapable of individually carrying out the conversion of sugar mixture to ethanol.

2.4. Co-fermentation of glucose/xylose sugar mixture

The employment of two microorganisms in a single process, particularly the co-culture of strains simultaneously, is very different from processes with pure cultures. Strains may have different interactions with each other, e.g. killing effects, synergy effects, or no effects. In this study, two strains were employed for the production of ethanol from a mixed substrate; it was thus important to provide optimum environmental conditions for both strains in order to achieve the maximum product yield. In the case of *P. stipitis* and *Z. mobilis*, they both grow vigorously at 30°C (Laplace et al., 1991a), and are acclimatized to a pH range of 4 to 7 (Chandrakant and Bisaria, 1998). In general, the only conflict in the growing conditions of these two strains is the oxygen requirement. *P. stipitis* needs a strictly controlled micro-aerobic condition to ferment xylose to ethanol, while in earlier reports *Z. mobilis* has been reported to give maximum ethanol production only under absolute anaerobic conditions (Laplace et al., 1991a). This might account for the fact that *Z. mobilis* was rarely employed for co-culture processes with a xylose fermenting yeast. However, in other fermentation systems the bacterium has been widely used for the co-culture with other strains or with enzymes to extend its substrate utilization range. In this section, co-culture systems involving *Z. mobilis* are briefly reviewed at first, followed by the studies relating to the co-fermentation of glucose/xylose sugar mixtures with two strains. Finally the selection of *Z. mobilis* and *P. stipitis* for the co-fermentation will be justified.

2.4.1. Co-culture process involving *Z. mobilis*

Co-culture of two microorganisms is commonly used in fermentations with unfavorable substrates. One of the microorganisms degrades the substrate and converts it to a favorable material for the fermentation by the other microorganism,
e.g. converting starch to glucose, which can be directly utilized by *Z. mobilis*. Lee et al. (1993) co-immobilized *Z. mobilis* with both *Aspergillus awamori* and *Rhizopus japonicus* on raw starch material; an ethanol yield of 96% of the theoretical was achieved under anaerobic conditions. Gunasekaran and Kamini (1991) used the combination of *Kluyveromyces fragilis* and *Z. mobilis* on a lactose medium. Compared to fermentation schemes including free cells of both strains and immobilized *K. fragilis* with free cells of *Z. mobilis*, the co-immobilized system gave higher ethanol production.

The immobilization of enzyme directly with *Z. mobilis* instead of an enzyme-producing microorganism also proved to be an effective system on starch. Bandaru et al. (2006) investigated optimum conditions for the co-immobilization of amyloglucosidase and *Z. mobilis* cells on sago starch. Kannan et al. (1998) successfully prevented the formation of byproducts and improved ethanol yield on sucrose with the use of a sucrase deficient mutant of *Z. mobilis* co-cultured with the invertase. A co-immobilization system of *Saccharomyces diastaticus* and *Zymomonas mobilis* reported an improved ethanol production in comparison with both systems involving only the sole *S. diastaticus* and involving free cell co-culture of *S. diastaticus* and *Z. mobilis* (Amutha and Gunasekaran, 2001).

In conclusion, though *Z. mobilis* has rarely been used for co-culture with a xylose fermenting strain, it shows excellent performance when co-cultured with other microorganisms or even enzymes. The current study using *Z. mobilis* and *P. stipitis* to co-ferment the glucose/xylose mixture therefore represents a new challenge for *Z. mobilis* studies.

2.4.2. Employment of a glucose fermenter and a xylose fermenter

Extensive research has been carried out on the co-employment of a glucose fermenting strain and a xylose fermenting strain, for its potential to significantly improve the fermentation efficiency on the sugar mixture substrate. Based on different combinations of strain selection, fermentation mode and inoculation sequence, numerous fermentation schemes can be created. This section discusses the previously reported fermentation processes to exemplify the application and effect of each factor, followed by the evaluation of some successful fermentation schemes.
The first factor to be considered is the strains used to carry out the respective glucose and xylose fermentations. Common strains for glucose fermentation are either *S. cerevisiae* or *Z. mobilis*. Though *Z. mobilis* have rarely been employed in such co-culture processes, a number of *Saccharomyces* strains and their mutants have been studied. For xylose fermenters, all three yeasts, viz. *P. stipitis*, *C. shehatae* and *P. tannophilus*, have been attempted in order to set up an optimized strain combination. Reported combinations include *S. cerevisiae* and *P. stipitis* (Grootjen et al., 1991a; Taniguchi et al., 1997a), *S. cerevisiae* and *C. shehatae* (Lebeau et al., 1997), *S. cerevisiae* and *P. tannophilus* (Qian et al., 2006), a respiratory deficient (RD) mutant of *S. cerevisiae* and *Pichia stipitis* (Laplace et al., 1993a; Taniguchi et al., 1997b), and a RD mutant of *S. cerevisiae* with a mutant of *P. stipitis* showing restricted glucose catabolite repression (Kordowska-Wiater and Targonski, 2002). *Saccharomyces diastaticus* and its RD mutants have also been studied (Laplace et al., 1993b; Delgenes et al., 1996a). The glucose fermenter has shown significant effects on xylose fermentation in a co-culture process. Therefore an appropriate selection of a strain combination is the first decisive factor for a successful co-fermentation process.

The next factor affecting the system performance is the culture mode. There are options of immobilized culture (Grootjen et al., 1991b; de Bari et al., 2004) and free cell culture (Taniguchi et al., 1997b; Qian et al., 2006), as well as batch culture (Taniguchi et al., 1997b) and continuous culture (Laplace et al., 1993c). Continuous culture consists of a prolonged exponential phase of cell growth (Stanbury et al., 2000) and therefore allows a continuous ethanol production at maximum rate. Whole cell immobilization has also been applied in various ways. Firstly, it is able to provide a modified environment within the immobilizing material which is different from the medium environment, and thus is particularly suitable for culturing microorganisms with different environmental requirements (Grootjen et al., 1991b). On other occasions, the co-immobilization of cell mixture was suggested to be an effective method to control the cell number of two strains at a certain ratio (de Bari et al., 2004).

The inoculation sequence of the two microorganisms also has a significant effect on
the fermentation scheme, by affecting the potential interactions of the two strains and the fermentation sequence of the sugar mixture. When two strains are inoculated at the same time, they both exist in the fermentation medium, and hence the strain interaction needs to be considered. In the case of a glucose fermenter and a xylose fermenter, the direct exposure to each other often resulted in low ethanol yield and low productivity. The major interferences were either the oxygen competition between two strains, which led to the poor growth of xylose fermenter (Laplace et al., 1993a; Taniguchi et al., 1997b), or the repressed xylose metabolism resulting from the glucose catabolism (Kordowska-Wiater and Targonski, 2002).

When two strains are inoculated successively, the progress of fermentation generally depends on which strain is first inoculated. This scheme has the advantage of providing each microorganism an optimum environment individually. However, if inoculated second, the low ethanol tolerance of the xylose fermenter is a problem on account of the ethanol produced by the first-inoculated glucose fermenter. If the xylose fermenter is inoculated first, it tends to consume glucose before xylose in the mixture (Kruse and Schugerl, 1996; Sanchez et al., 2002), which often leads to a decreased system performance due to its low efficiency of glucose fermentation. Moreover, after the completion of the first substrate, the corresponding microorganism can be either kept in the medium (Taniguchi et al., 1997b) or inactivated (Fu and Peiris, 2008). The inoculation manner therefore significantly affects the fermentation process and needs to be carefully selected along with the strain combination and the culture mode, in order to establish a rational fermentation scheme.

A commonly reported problem in the co-culture process is the conflict on the oxygen requirement. Laplace et al. (1993a) co-cultured *S. cerevisiae* and *C. shehatae* on the sugar mixture medium. Though the micro-aerobic condition was provided, ethanol was only produced from glucose and *C. shehatae* showed a slow cell growth rate and poor fermentation performance. The cause was considered to be the entire consumption of oxygen by *S. cerevisiae*. In order to eliminate the oxygen competition, a RD mutant of *Saccharomyces diastaticus* was applied for the co-culture with *P. stipitis* in another study in continuous fermentation (Laplace et al., 1993b). Again xylose could not be fully consumed except for an initial concentration
of 50 g/l and a dilution rate no more than 0.006 h\(^{-1}\). Similar results were obtained by other research groups. The strain combination of \textit{P. stipitis} and \textit{S. cerevisiae} was attempted either through immobilization (Grootjen et al., 1991b) or in two sequential bioreactors (Grootjen et al., 1991a). In both processes the xylose uptake was relatively slow, resulting from the insufficient oxygen supply within the gel beads and the oxygen competition respectively.

Repression of xylose assimilation by glucose catabolism was observed in a co-culture process of \textit{C. shehatae} and a RD mutant of \textit{S. cerevisiae} (Lebeau et al., 1997). A mutant of \textit{P. stipitis} showing restricted glucose catabolism was used to counteract this problem (Kordowska-Wiater and Targonski, 2002); however, xylose fermentation was not improved.

Currently only three co-fermentation processes employing two strains have reported impressive results. Taniguchi et al. (1997b) co-cultured a RD mutant of \textit{S. cerevisiae} together with \textit{P. stipitis} on 50 g/l glucose and 25 g/l xylose, achieving an ethanol yield of 0.50 g/g sugars in 40 h. de Bari et al. (2004) co-immobilized \textit{S. cerevisiae} and \textit{P. stipitis} using different biomass proportions to convert 42 g/l glucose and 12 g/l xylose. Under optimum conditions both sugars were utilized by 24 h, but the ethanol only peaked at 40 h, giving a yield of 0.38 g/g sugars. Qian et al. (2006) introduced a recombinant \textit{E. coli} strain and co-cultured it with \textit{S. cerevisiae} on a softwood hydrolysate. The medium contained 37.5 g/l sugar mixture, of which 75% was glucose. An ethanol yield of 0.45 g/g was reached within 24 h with the full consumption of sugars. The ethanol yield was even higher, up to 0.49 g/g, when \textit{P. tannophilus} was used to replace the recombinant \textit{E. coli}; however, a longer time of 48 h was required in this instance.

It is notable that amongst these three reports, two of them employed an engineered strain for the co-culture process [a RD mutant of \textit{S. cerevisiae}, and a recombinant strain of \textit{E. coli}], and the other one adopted the whole cell immobilization technique. This implies that it is difficult to achieve high levels of ethanol production by a basic co-culture process, where free cells of the two strains are simply inoculated into the same bioreactor. Consequently, a successful co-fermentation of sugar mixture needs a careful design of fermentation scheme and suitable engineering of the bioprocess.
and/or the strains. Another significant feature observed in these three reports is the high glucose proportion in the sugar mixture [67%, 78% and 75% of the total sugars respectively]. The fermentation of xylose is the actual bottleneck in the co-fermentation, and thus a high content of glucose makes the process easier and helps to increase the overall kinetic parameters.

In general, co-culture processes in earlier years gave poorer results compared to the pure culture fermentation. This resulted from a number of difficulties in the process, e.g. oxygen competition and catabolism repression due to glucose. Recently some efficient conversion processes have been reported, by optimizing environmental parameters and exploring novel fermentation schemes. For the establishment of an efficient co-fermentation process of the glucose/xylose sugar mixture, critically analyzing the previous study, selecting reasonable fermentation schemes and further optimizing them will be the most rational approach. In next section, the justification of using \textit{Z. mobilis} as a glucose fermenter and \textit{P. stipitis} as a xylose fermenter will be discussed.

\subsection*{2.4.3. Justification for the strain combination of \textit{Z. mobilis} and \textit{P. stipitis}}

As mentioned above, previous studies generally employed \textit{Saccharomyces} and its mutants as the glucose fermenting strain in co-culture; processes involving \textit{Z. mobilis} was rarely reported. However, although the ability of \textit{S. cerevisiae} to grow under both aerobic and anaerobic conditions seemed to be superior to \textit{Z. mobilis}, the resultant oxygen competition between \textit{S. cerevisiae} and the xylose fermenter only led to reduced fermentation performance (Laplace et al., 1993a; Taniguchi et al., 1997b). Therefore, in later research the RD mutant of \textit{Saccharomyces} has been widely employed. On the other hand, recently \textit{Z. mobilis} has been reported to tolerate limited oxygen (Kalmenieks, 2006; Madigan and Martinko, 2006b). Its fermentation performance under semi-anaerobic conditions is substantially high and steady, achieved under conditions without airflow rather than a continuous flush of nitrogen (Davis et al., 2006). Considering that best results for \textit{Saccharomyces} in co-culture were obtained with RD mutants, \textit{Z. mobilis} as an anaerobic strain with limited oxygen tolerance has the potential to give high ethanol productivity. The fermentation efficiency could even be improved, since \textit{Z. mobilis} is known to show better performance on the ethanol production from glucose. Consequently, the
co-culture process employing *Z. mobilis* has a need to be investigated.

In a previous study, sequentially culturing *Z. mobilis* and *Pachysolen tannophilus* on the glucose/xylose sugar mixture indicated that the efficiency of the co-fermentation can be further optimized with an improved performance of the xylose fermentation (Fu and Peiris, 2008). One approach to achieve this is to use a better xylose fermenter. Amongst the three xylose fermenting strains reported, *P. stipitis* gave the highest ethanol productivity and the least byproducts when cultured on xylose (Sanchez et al., 2002). Therefore *P. stipitis* was selected for this study together with *Z. mobilis*.

The composition of the sugar mixture selected for this study was a glucose to xylose ratio of 60:40 to simulate the composition of these two sugars in the sugarcane bagasse (Chandrakant and Bisaria, 1998). It was recognized that the high concentration of xylose represented a challenge; accordingly, a number of fermentation schemes were designed to overcome this difficulty.

### 2.5. Hydrolysis of lignocellulose

The aim of the current research is to convert the lignocellulosic material to ethanol. Sugarcane bagasse as a typical lignocellulose was chosen as a starting material. Bagasse, an industrial waste from the sugar industry, is the residual fibrous material after juice has been extracted from the sugarcane. Typically bagasse contains approximately 50% cellulose, 24% hemicellulose, and the rest is lignin (Chandrakant and Bisaria, 1998; Marton et al., 2006). In nature, there is no single wild type microorganism that can efficiently convert raw bagasse directly to ethanol. Pretreatment and saccharification processes need to be carried out to hydrolyze the bagasse to its component sugars prior to fermentation. To be commercially viable, this process should be fast, with a high yield of sugars and low levels of inhibitors.

#### 2.5.1. Physicochemical methods of hydrolysis

Physicochemical methods of hydrolysis are relatively cheaper and quicker in contrast to enzymatic hydrolysis. However, they require harsh conditions and yield less sugar and higher concentration of inhibitors. Common techniques include steam explosion (Kaar et al., 1998; Martin et al., 2001), acid hydrolysis (Gong et al., 1993; Aguilar et
al., 2002; Carvalho et al., 2004; Fogel et al., 2005; Silva et al., 2005) and other hydrolysis methods such as alkaline hydrolysis (Qu et al., 2007) and organic solvent processes (Rossell et al., 2006). Among these, acid hydrolysis is the most widely used treatment for a single hydrolysis process, whereas steam explosion is often employed as a pretreatment method for enzymatic hydrolysis.

Acid hydrolysis in general is to treat the lignocellulosic material with dilute acid at a temperature ranging from 80°C to 250°C for a period of time, after which the depolymerized sugars are harvested in the liquid phase. The monosaccharides released into the medium are usually from the hemicellulose part of the lignocellulose. Since the crystalloid structure of cellulose is relatively hard to degrade by dilute acid, the glucose yield is comparably low. Consequently, acid hydrolysis usually yields xylose-rich hydrolysates.

There are various kinds of acid that have been used, including sulfuric acid, hydrochloric acid, phosphoric acid and other uncommon acids. Amongst these, dilute sulfuric acid hydrolysis has been investigated most. Gong et al. (1993) used 3% (w/v) sulfuric acid to hydrolyze the sugarcane bagasse, which resulted in a hydrolysate with 11% fermentable sugars containing xylose as the dominant sugar. When this hydrolysate was used as the substrate for ethanol production, prior detoxification was necessary to alleviate the inhibition of cell growth caused by inhibitors in the hydrolysate. Silva et al. (2005) investigated the effect of sulfuric acid loading and residence time on the bagasse hydrolysis. It was reported that at a temperature of 121°C and bagasse loading of 10% (w/v), a sulfuric acid loading of 130 mg/g for 30 min resulted in the maximum xylose concentration. Other research groups investigating the optimum conditions for sulfuric acid hydrolysis reported a high xylose concentration up to 57.25 g/l from bagasse, achieved with a solid-liquid ratio of 1:4, a sulfuric acid loading of 2%, at 1.0 atm for 40 min in an autoclave (Fogel et al., 2005). This is the highest xylose concentration reported. However, the low solid-liquid ratio greatly increased the difficulty of separating the liquid phase out of the bagasse fiber. Moreover, more raw material bagasse is required for producing a given volume of hydrolysate.

Lavarack et al. (2002) evaluated the kinetic models of acid hydrolysis of bagasse and
suggested that sulfuric acid was more efficient and yielded higher xylose concentrations in comparison with hydrochloric acid. Another investigation on the kinetic models of acid hydrolysis with sulfuric acid reported the following optimum conditions, i.e. 2% sulfuric acid at 122°C for 24 min, which resulted in a xylose concentration of 21.6 g/l in the hydrolysate (Aguilar et al., 2002). A notable trend observed in studies investigating the conversion of bagasse into value-added products, including both ethanol and xylitol, was that the produced acid hydrolysate needed to be concentrated to increase the xylose concentration to a fermentable level (Roberto et al., 1991; Sene et al., 1998; Sene, 2001; Baudel et al., 2005a; Santos et al., 2005).

For acid hydrolysis processes carried out with phosphoric acid, a longer reaction time is usually required to achieve a similar level of sugar in comparison with processes carried out with sulfuric acid (Carvalho et al., 2004; Gamez et al., 2004; Gamez et al., 2006). Others investigating nitric acid hydrolysis claimed that it was the most efficient catalyst amongst a variety of acids (Rodriguez-Chong et al., 2004). However, the reported concentration of xylose in the hydrolysate [18.6 g/l] was lower than that achieved with sulfuric acid as discussed above.

Some physicochemical hydrolysis processes comprised two stages, pretreatment and saccharification. Yu et al. (2002) investigated the hydrolysis of waste cotton cellulose pretreated by pyrolysis. For both acid and alkaline hydrolysis, removal of inhibitors was reported to be crucial in achieving a high ethanol yield. Other methods of pretreatment prior to acid hydrolysis included γ-irradiation (Pereira and Lancas, 1996) and the Organosolv Process (Rossell et al., 2006). The latter has been attempted on a pilot scale up to 5000 l/day, using an ethanol-water mixture as solvent for pretreatment and dilute sulfuric acid as catalyst for acid hydrolysis.

In summary, amongst various physicochemical hydrolysis approaches, dilute sulfuric acid method is most commonly used for lignocellulose hydrolysis. Its major disadvantages include the low sugar yields and the requirement of harsh conditions, i.e. temperature up to 220°C and low pH. Consequently, techniques based on enzymatic hydrolysis have been developed.
2.5.2. Enzymatic hydrolysis

Disadvantages associated with enzymatic hydrolysis are the cost of the enzyme system and the comparatively low efficiency of these enzymes (Chandrakant and Bisaria, 1998). As a result, the reaction is often carried out with a prior physicochemical treatment to remove the lignin part and loosen the cellulose structure, making it more accessible to cellulolytic enzymes (Martin et al., 2002). A number of pretreatment methods have been reported. Martin et al. (2001) studied the enzymatic hydrolysis of sugarcane bagasse pretreated by steam explosion at a temperature range between 205°C and 210°C. The obtained hydrolysate contained approximately 22 g/l of glucose and 9.0 g/l of xylose, with the use of enzyme combination of Celluclast 1.5 L and Novozyme 188. In other studies sugarcane bagasse was exposed to a hydrothermal pretreatment at 200°C (Walch et al., 1992); the resulting hydrolysate showed improved sugar yields during the subsequent enzymatic saccharification.

Dilute acid treatment has also been attempted as a pretreatment for the enzymatic saccharification. Using wheat straw pretreated by sulfuric acid as substrate, Saha et al. (2005) reported a yield of monomeric sugars of 565 mg/g after the enzymatic hydrolysis and a maximum ethanol yield of 0.24 g/g dry solid during the fermentation. Palmarola-Adrados et al. (2005) achieved a sugar yield of 80% of the theoretical on the wheat bran; enzymatic hydrolysis with prior treatment by a small amount of acid was suggested to give higher sugar yields compared to that without the pretreatment. Other pretreatment approaches included peracetic acid treatment (Teixeira et al., 1999) and microwave treatment (Kitchaiya et al., 2003); both studies claimed that the pretreatment increased the sugar yield during the enzymatic hydrolysis.

Adsul et al. (2005) evaluated enzymatic hydrolysis with different delignified bagasses; a maximum hydrolysis rate of 95% was reported. Jeffries and Schartman (1999) working with different hydrolysis schemes reported a highest saccharification rate of 65%, with the use of 5 FPU/g of cellulase plus 10 IU/g glucosidase. Sequential addition of enzymes was reported to give a better yield compared to the addition of both enzymes as a single dose.
In general, enzymatic hydrolysis is considered to be an efficient method of obtaining high sugar content from lignocellulosic material. On account of the compact structure of cellulose, a prior pretreatment process helps to improve the sugar yield. Though the cellulolytic enzyme systems are costly, enzymatic hydrolysis is a promising method to obtain hydrolysate with higher sugar levels. However, for a commercial ethanol production process, the production of cellulolytic enzymes must be taken into consideration in the overall cost.

2.5.3. Fermentation of hydrolysate

Although enzymatic hydrolysis produces sufficiently high sugar levels for direct fermentation, there are other factors limiting the fermentability of the lignocellulosic hydrolysate, i.e. the presence of inhibitors in the hydrolysate. These inhibitors are usually byproducts formed during the acid hydrolysis, including acetic acid, phenolic compounds, furfural, 5-hydroxymethylfurfural, etc (Takahashi et al., 2000). They are capable of inhibiting cell growth and metabolism of the microorganism responsible for ethanol production (Watson et al., 1984; du Preez, 1994; Klinke et al., 2004). Martin (2007a) summarized three major approaches to eliminate the effect of these inhibitors on the fermentation process:

1. Minimizing the inhibitor formation during the hydrolysis,
2. Detoxifying the hydrolysate and removing inhibitors prior to fermentation,
3. Selecting and adapting the fermenting strains to the hydrolysate.

Harsh conditions in the physicochemical hydrolysis are reported to promote the formation of undesirable compounds (Delgenes et al., 1996b). Enzymatic hydrolysis with milder conditions along with an appropriate pretreatment method can effectively limit the inhibitor formation. Steam explosion and wet oxidation, which is an advanced method derived from steam explosion, are widely accepted as the best pretreatment methods for this purpose (Kaar et al., 1998; Martin et al., 2006a; Martin et al., 2007b). Both methods subject the bagasse raw material to a high temperature of 170-220°C under humid conditions to delignify, and then expose the delignified slurry to cellulolytic enzymes.

Detoxification is the most commonly used method of improving the fermentability of the hydrolysate, due to its effectiveness on the inhibitors removal. There are various
detoxification methods, including overliming (Amartey and Jeffries, 1996), pH neutralization (Yu and Zhang, 2002), ion exchange (Watson et al., 1984; Qu et al., 2007), alkaline extraction (Baudel et al., 2005b), activated charcoal adsorption (Marton et al., 2006; Qu et al., 2007) and solvent extraction (Griffin and Shu, 2004). Amongst these, overliming as a simple process without specific equipment requirements has often been employed as a general detoxification method (Sene et al., 1998; Takahashi et al., 2000). By adjusting the pH of the hydrolysate to 10-10.5 with either CaO or Ca(OH)$_2$, followed by neutralization back to 7, a majority of undesirable components, such as acid, phenolic components and furfural, can be precipitated and easily removed by centrifugation (Amartey and Jeffries, 1996; Olsson and Hahn-Hagerdal, 1996). It was reported that the overliming of sugarcane bagasse hydrolysate doubled the ethanol productivity compared to the hydrolysate without treatment (Martin et al., 2002).

For strain selection and adaptation, most research chose to repeatedly culture the same batch of cells in the renewed hydrolysate medium, which permitted a natural adaptation of cells to the hydrolysate environment (Parekh et al., 1986; Yu et al., 1987; Kwon et al., 2006). Yu et al. (1987) collected cells of *Candida shehatae* from the fermentation process by centrifugation and used them as the inoculum for the next fermentation process. The resultant strain was capable of completely fermenting the substrate of spent sulphite liquor at pH 1. Parekh et al. (1986) recycled *Candida shehatae* 72 times and *Pichia stipitis* 12 times; both strains showed improved ethanol productivity and ethanol tolerance. However, sometimes it is difficult to distinguish whether the improvement of kinetic parameters was a consequence of the strain adaptation or only due to the increase of biomass from the recycling. For instance, Kwon et al. (2006) cultured *Candida tropicalis* in a fermentor with a membrane filter system; the accumulation of viable cells in the medium would dramatically increase the whole system efficiency. Other studies forced the strain to adapt to the hydrolysate by a series of subcultures to media containing increased substrate concentrations (Amartey and Jeffries, 1996; Sene et al., 1998; Sene, 2001). This principle was also applied to the adaptation of the microorganism to an increased inhibitor concentration (Martin et al., 2007a).

The hydrolysis process produces the fermentable sugars for ethanol production, and
its fermentability directly determines the overall efficiency of the conversion of lignocellulose to ethanol. The improvement on the convertibility of hydrolysate greatly benefits the subsequent fermentation. In order to obtain a hydrolysate with a high sugar yield and lower inhibitor concentration, the hydrolysis process of sugarcane bagasse should comprise three stages: (1) the pretreatment of bagasse raw material to delignify and increase the accessibility of the cellulose, (2) enzymatic saccharification of the pretreated bagasse to achieve a high level of sugar concentration which could be directly fermented by microorganisms, (3) detoxification to minimize the inhibitory components in the hydrolysate.

2.6. Conversion of lignocellulose using other fermentation schemes

For the conversion of lignocellulose to ethanol, besides the separate hydrolysis and fermentation (SHF) processes, there are other fermentation schemes that combine two or more processes together into an integrated process. An ultimate fermentation scheme should include a genetically constructed microorganism which is able to produce cellulolytic enzymes, hydrolyze the lignocellulose to sugars and co-ferment the sugar mixture to ethanol in a single step reaction. However, at present this scheme remains conceptual; preliminary research focuses on either the simultaneous saccharification and fermentation (SSF) process or the use of genetically modified organisms (GMO) to co-ferment glucose/xylose sugar mixture. Some typical studies regarding these two aspects are reviewed below.

2.6.1. Simultaneous saccharification and fermentation

SSF processes can be further divided into two sub-categories. The first one employs only a single strain, either a wild type or genetically modified, to directly produce ethanol from macromolecular carbohydrates such as cellulose. The other process co-cultures a common ethanol producing strain with strains or enzymes that can degrade those macromolecular carbohydrates into single sugars. A major advantage of SSF process is that, by continuous removal of the released monosugars, SSF can break the equilibrium of enzymatic reactions and propel the reaction towards the production of sugars, consequently improving the efficiency of saccharification (Chandrakant and Bisaria, 1998).
Kadar et al. (2004) used a glucose-fermenting yeast co-cultured with enzymes, achieving an ethanol yield up to 17 g/g on both substrates of old corrugated cardboard and paper sludge. Two glucose-fermenters, *S. cerevisiae* and *Kluyveromyces marxianus*, showed comparable ethanol productivity. Similar ethanol yields were obtained by Ballesteros et al. (2004) culturing *K. marxianus* on a number of lignocellulosic substrates. Within 160 h, maximum ethanol production was around 16-19 g/l, which was 50-72% of the theoretical. Golias et al. (2002) investigated SSF processes carried out by three strains. The highest ethanol productivity was achieved with a recombinant strain of *Klebsiella oxytoca* P2, but both strains of *K. marxianus* and *Z. mobilis* gave higher ethanol yield than *K. oxytoca* P2. The application of recombinant *Z. mobilis* with xylose fermenting ability on the pretreated hardwood substrate resulted in an ethanol yield over 23 g/l (McMillan et al., 1999); however, the sugar conversion rate was considerably low [54% in 7 days]. Latif and Rajoka (2001) attempted an SSF process with two microorganisms, *Saccharomyces cerevisiae* and *Candida tropicalis*, either individually or co-cultured on the substrate of dry corn cobs. The highest product yield was obtained by *S. cerevisiae* for ethanol and *C. tropicalis* for xylitol; the co-culture of two microorganisms with cellulase enzymes showed hardly any advantage.

For the co-culture of a cellulolytic strain and an ethanol producing strain, the combination of *Trichoderma viride* and *Pachysolen tannophilus* has been studied on the natural untreated straw (Zayed and Meyer, 1996). *T. viride* was inoculated at first, inactivated after the completion of the saccharification process, and then *P. tannophilus* was inoculated to the same medium to convert the released sugars to ethanol. Shama (1991) carried out the co-culture process with *Bacillus stearothermophilus* and *Clostridium thermocellum*. With the use of a lactate dehydrogenase deficient mutant of *B. stearothermophilus*, the process produced 50% more ethanol in comparison with *C. thermocellum* sole culture. Direct fermentation of cellulose to ethanol was studied by Balusu et al. (2005). Using response surface methodology, ethanol production up to 0.41 g/g was reported with optimized medium components. In other studies, Panagiotou et al. (2005) achieved an ethanol yield of 0.35 g/g by employing *Fusarium oxysporum* to directly produce ethanol from cellulose.
Though the SSF process has the potential to achieve higher system efficiency, currently there are no reports of SSF giving competitive results to the SHF processes. The low saccharification efficiency and the consequently slow ethanol production greatly limit the performance of SSF. An SSF process for the commercial application still needs more studies.

2.6.2. Application of genetically modified microorganisms

Genetic engineering makes the construction of strains with all desirable characteristics theoretically possible. With time and the development of new techniques, it may be possible to construct a recombinant strain that is able to carry out all the reactions from lignocellulose to ethanol. Up to date some GMO with excellent performance on the glucose/xylose co-fermentation have already been reported. These artificially engineered strains are often referred as “biocatalysts”, for they are only produced to transform certain substrates into desirable products (Fujita et al., 2004; Ishige et al., 2005). In terms of co-fermentation of glucose/xylose sugar mixture, recombinant biocatalysts are usually constructed in three ways: (1) extending the substrate utilization range of traditional ethanol producing strains, such as *S. cerevisiae* or *Z. mobilis*, (2) modifying the metabolic system of strains with wide substrate-assimilating range to make them produce ethanol, typically *E. coli*; (3) improving the ethanol productivity of the common xylose fermenting strains, e.g. *P. stipitis*, by inserting gene fractions from *S. cerevisiae* or *Z. mobilis*.

One of first successful reports on the genetic modification of glucose fermenters were achieved by Zhang et al. (1995). They modified the genome of *Z. mobilis* to produce a pentose metabolism pathway using genes derived from *Xanthomonas campestris* or *Klebsiella oxytoca*. Davis et al. (2005) cultured a xylose-utilizing strain of *Z. mobilis* on a wheat stillage hydrolysate. However, the xylose fermentation was considerably less efficient than the glucose fermentation. Hahn-Hagerdal et al. (1994) pointed out that the low performance of recombinant glucose fermenters on xylose fermentation was mainly because of the unfavorable kinetic properties and an inadequate pentose phosphate pathway in the cells. To solve this problem and improve the xylose conversion rate, Jin and Jeffries (2003) manipulated the expression of *P. stipitis* genes coding for XR and XDH in the *S. cerevisiae* cells, and reported an increased enzymatic activity. An efficient conversion of glucose/xylose
sugar mixture was achieved by Krishnan et al. (2000) using a recombinant \textit{Z. mobilis} CP4 (pZB5). An ethanol concentration up to 44.3 g/l was produced from 76/l glucose and 33.8 g/l xylose within 24 h, with a xylose consumption more than 80%.

\textit{E. coli}, which is one of the most studied and best understood strains, has been widely used for genetic modification work, for it can efficiently metabolize a number of sugars including both glucose and xylose. Takahashi et al. (2000) investigated the ethanol production performance of a recombinant strain of \textit{E. coli} with the ethanol pathway genes from \textit{Z. mobilis} on the glucose/xylose sugar mixture. The research compared the fermentation efficiency of \textit{E. coli} KO11 in the synthetic medium and hemicellulosic hydrolysate, and reported a maximum ethanol yield of 96.4% of the theoretical from the synthetic medium. Continuous culture employing a recombinant \textit{E. coli} has also been attempted (Martin et al., 2006b). With a 26 day culture, a stable ethanol yield of 80-85% of the theoretical rate was achieved. The plasmid retention rate was 96-97% without the addition of antibiotics. Qureshi et al. (2006) reported a salt tolerance up to 10 g/l and an initial xylose tolerance up to 250 g/l of a recombinant strain of \textit{E. coli}. Maximum ethanol yield of 0.50 g/g was achieved, which was 98% of the theoretical value.

The yeast \textit{P. stipitis} has also been used for genetic engineering as the most promising xylose fermenter in nature. A recombinant \textit{P. stipitis} strain carrying genes from \textit{S. cerevisiae} was constructed as early as in 1994 (Yang et al., 1994). Another study investigating a \textit{P. stipitis} strain bearing a \textit{URA1} gene from \textit{S. cerevisiae} reported an ethanol yield of 0.41 g/g from glucose (Shi and Jeffries, 1998). The recombinant strain proved to be viable under anaerobic conditions on glucose medium but failed to grow on xylose medium.

In conclusion, some GMO have been reported to give comparably high ethanol productivity on the co-fermentation of glucose/xylose sugar mixture, and new strains carrying better fermentative characteristics will be continually constructed. However, Kim et al. (2007) suggested that recombinant organisms are still not very favorable for a large scale production in industry. Therefore, the research on the fermentation engineering to solve the problems on the glucose/xylose co-fermentation remains necessary.
Chapter 3

Materials and Methods

3.1. Overview of experimental design
The experimental design of the current study is presented in Figure 3.1.

3.2. General Procedures
Chemicals and reagents were obtained from Sigma-Aldrich Inc. (U.S.A.), Oxoid Ltd. (U.K.) and Alfa Aesar (U.K.). The enzymes were kindly provided by Novozymes Australia Pty. Ltd. Distilled water was used in all solutions and media preparations unless otherwise stated.

Accurate weight measurements for chemical compounds and dry cell pellets were made using a Sartorius BP110S analytical balance (four decimal accuracy) [Selby-Biolab, Germany]. Measurements with less precision requirements were made on an A&D electronic balance FX-3200 [A&D company Ltd., Japan].

The pH of solutions was determined using a WTW pH meter [pH 315i/SET, WTW, Germany]. All pH probes were calibrated using color-coded buffers for pH 7 and pH 4 [Labchem, APS Finechem, Australia]. Solutions used for pH adjustment will be stated wherever relevant.
Figure 3.1: Schematic diagram of the overall experimental design in this study
Thorough mixing of substances was achieved by Chiltern MT19 auto vortex mixer [Selby, Australia], or a Gallenkamp magnetic stirrer [A. Gallenkamp & Co. Ltd., U.K., Catalogue No. SWT 310 010 U]. The magnets used were approximately 3 cm in length by 0.5 cm in diameter.

A bench top Jouan B3.11 centrifuge [Thermoline Scientific Equipment Pty. Ltd., Australia] was used for routine centrifugation. Larger volumes of liquid were centrifuged using a refrigerated Sorvall RC 5B Plus centrifuge [Sorvall Products L.P., U.S.A.].

Spectrophotometric measurements were carried out with a Jenway 6300 Spectrophotometer [Barloworld Scientific Ltd., U.K.]. Cuvettes used were 2.5 ml with a path length of 1 cm [Greiner Bio-one, Germany, Catalogue No. Bestell-Nr. 614101].

Immobilization and other operations requiring a strict aseptic environment were carried out in a Class 2 Biological Safety Cabinet BH120 [Gelman Sciences, U.S.A.]. Operations involving volatile chemicals were carried out in a Clyde-Apac LFC series fume cabinet [Clyde-Apac, Australia].

Sterilization of glassware, media and equipment was effected by autoclaving at 121°C for 15 min by either a pressure cooker [Namco, Australia], or an Atherton 110A series sterilizer [Atherton Pty. Ltd., Australia]. Fermentors were sterilized under the same conditions using an Atherton GEC612 sterilizer [Atherton Pty. Ltd., Australia]. Malt Extract Agar (Oxoid CM0059) was prepared following the company instruction and sterilized at 115°C for 10 min. Heat labile solutions were filter sterilized using vacuum filtration with a 0.45μm membrane filter. Where appropriate, equipment was dried in a 60°C oven.

Julabo SW22 Water bath [Julabo Labortechnik GMBH, Germany] was used for enzyme assays.

Shaking incubation was carried out in orbital shakers. Based on the number and the
size of flasks, three shakers were employed: the Orbital Shaking Refrigerated Incubator TFSIR-383-1, TLM 2-590 and the Orbital Shaking Incubator TU-400, all from Thermoline Scientific Equipment Pty. Ltd. (Australia).

Bioreactors used in this study were a continuous stirred tank bioreactor with 1 l working volume [Biostat B, B. Braun Biotech International, Germany]. The pH probe used for the bioreactor was from Mettler Toledo GMBH [Switzerland, Catalogue No. 59904068].

Figure 3.2: A 1 l bioreactor used in this study
3.3. Microorganisms

*Zymomonas mobilis* UWS HAWK 203 was obtained from the Microbiology Culture Collection in the School of Natural Sciences, University of Western Sydney, Sydney, Australia; originally from the American Type Culture Collection (ATCC) No. 10988.

*Pichia stipitis* WM 810 was kindly provided by the Westmead Clinical School, University of Sydney, Sydney, Australia; originally from Centraalbureau voor Schimmelcultures (CBS) No. 5773.

Both strains were cultured on agar plates at 30°C, *Z. mobilis* on Glucose Agar and *P. stipitis* on Xylose Agar; the medium composition of the two media is listed in Section 3.4. Culture plates were stored in a 4°C refrigerator and subcultured to fresh media once every fortnight.

3.4. Media

Unless otherwise stated, the composition of solid and liquid media used in this study is as shown in Tables 3.1 and 3.2.

<table>
<thead>
<tr>
<th>Table 3.1: Solid media composition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solid media</strong></td>
</tr>
<tr>
<td>Glucose Agar</td>
</tr>
<tr>
<td>Xylose Agar</td>
</tr>
<tr>
<td>Malt Extract Agar (Oxoid, CM0059)</td>
</tr>
</tbody>
</table>
### Table 3.2: Liquid media composition

<table>
<thead>
<tr>
<th>Liquid media</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose Medium</td>
<td>50.0 g/l glucose, 10.0 g/l yeast extract, 1.0 g/l MgCl₂, 1.0 g/l (NH₄)₂SO₄ and 1.0 g/l KH₂PO₄.</td>
</tr>
<tr>
<td>Xylose Medium</td>
<td>50.0 g/l xylose, 10.0 g/l yeast extract, 1.0 g/l MgCl₂, 1.0 g/l (NH₄)₂SO₄ and 1.0 g/l KH₂PO₄.</td>
</tr>
<tr>
<td>Co-fermentation Medium</td>
<td>30.0 g/l glucose, 20.0 g/l xylose, 10.0 g/l yeast extract, 1.0 g/l MgCl₂, 1.0 g/l (NH₄)₂SO₄ and 1.0 g/l KH₂PO₄.</td>
</tr>
<tr>
<td>Glucose Storage Medium</td>
<td>10.0 g/l glucose, 2.5 g/l yeast extract and 5.55 g/l CaCl₂</td>
</tr>
<tr>
<td>Xylose Storage Medium</td>
<td>10.0 g/l xylose, 5.0 g/l yeast extract and 5.55 g/l CaCl₂</td>
</tr>
</tbody>
</table>

### 3.5. Inoculum preparation

All inocula were prepared in 250 ml conical flasks with 50 ml medium at 30°C. *Z. mobilis* inocula were cultured on Glucose Medium in a stationary incubator for 24 h, and *P. stipitis* inocula were cultured on Xylose Medium in an orbital shaker with 150 rpm shaking speed for 36-48 h.

In this study, three levels of inoculum size were commonly used, viz. 10%, 50% and 100% (v<sub>cell</sub>/v<sub>ferm</sub>). For the inoculum size of 10%, the traditional volume to volume inoculum was used, i.e. inoculum medium with a volume that is 10% of volume of the fermentation medium was transferred directly into the fermentation medium. For the inoculum size of 50% and any size larger than this, cells from the corresponding volume of inoculum medium were firstly concentrated and then inoculated into the fermentation medium; the procedure is shown in Figure 3.3.
In order to attain the desirable volume of inoculum, multiple flasks containing 50 ml inoculum medium were simultaneously incubated. Subculture up to three times was carried out to ensure that cells in all flasks were initially from the same colony on the culture maintenance agar plate. For example, for 800 ml fermentation medium with an inoculum size of 100%, all together 800 ml inoculum medium was required, equalling 16 inoculum flasks. To prepare 16 flasks of inoculum, firstly a single inoculum flask was inoculated by transferring a single colony from the culture maintenance plate; after incubation, 5 ml of culture was transferred into another two inoculum flasks containing fresh medium, and then these two flasks were used to inoculate 16 inoculum flasks, ensuring that each flask had the identical culture.

A comparison between the traditional volume to volume inoculation and the concentrated cell inoculation is shown in Table 3.3.
Table 3.3: Comparison of some features between two inoculation modes

<table>
<thead>
<tr>
<th></th>
<th>Conventional (v/v)</th>
<th>Concentrated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduce inoculum medium to fermentation</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Permit high initial cell concentration</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Dilute the initial sugar concentration in fermentation medium</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

3.6. Whole cell immobilization

Calcium alginate gel encapsulation was used to immobilize both *Z. mobilis* and *P. stipitis* cells (Becerra et al., 2001). An outline of the procedure is shown in Figure 3.4.

The harvested beads had a final concentration of 2% alginate. *Z. mobilis* beads were approximately 2-3 mm in diameter, with an off white color; the original beads contained cells from 400 ml culture concentrated into 100 ml of beads. *P. stipitis* beads were approximately 3-4 mm in diameter, with a creamy/yellow color; cells from 1000 ml culture were concentrated into 250 ml of beads.

For beads that were used after storage, a reconditioning process was carried out prior to fermentation, by incubating beads in a small volume of fermentation medium to restore the biomass level inside the beads. The reconditioning of *Z. mobilis* was carried out with 100 ml Glucose Medium in 250 ml conical flasks, cultured stationary for 4-10 h based on the beads condition. The reconditioning of *P. stipitis* was performed by separating the 250 ml beads into 2 flasks each containing 100 ml Xylose Medium, and incubating in an orbital shaker with 100 rpm shaking speed for 12 h.

The biomass concentration within the beads was determined by dissolving 5 beads in 10 ml of 1% sodium citrate solution. Incubation was carried out in a 30°C shaker with 150 rpm shaking speed, for approximately 30 min to 1 h, till all the beads completely dissolved. Then 1 ml of cell suspension was used for plating to determine the viable cell number, and the remaining 9 ml was centrifuged and dried in a 104°C
oven to monitor the dry biomass weight [See details of both methods in Section 3.10.2].

![Diagram of immobilized cells preparation](image)

**Figure 3.4:** Outline for the preparation of immobilized cells.

### 3.7. Fermentations

Fermentation processes were carried out as time courses experiments. General conditions were as follows.
3.7.1. Shake flask fermentations
All time courses with shake flasks were carried out in 250 ml conical flasks in a 30°C orbital shaker. Two levels of fermentation medium volume were used for different experiments. For experiments with 50 ml medium in each flask, the shaking speed of 100 rpm was employed and the inoculum size was 10% (v/v). For experiments with 100 ml medium in each flask, various shaking speeds and inoculum sizes were used, which will be stated wherever relevant.

3.7.2. Glucose fermentation with Z. mobilis
For both free cell culture and immobilized culture, the fermentations were carried out in a 1 l bioreactor with 800 ml Glucose Medium. For immobilized culture, no CaCl\textsubscript{2} was added to the medium. The temperature was controlled at 30°C and the stirring speed was 100 rpm. No aeration was provided during the fermentation and pH was uncontrolled.

3.7.3. Xylose fermentation with P. stipitis
Similar to Z. mobilis, fermentations with both free cell and immobilized P. stipitis were carried out in a 1 l bioreactor, at 30°C with 100 rpm stirring speed. The fermentation medium was 800 ml of Xylose Medium, with no CaCl\textsubscript{2} addition to the immobilized culture. Various aeration levels were used for different fermentation time courses, and pH was uncontrolled. Some commonly used aeration levels and the corresponding oxygen transfer rates are listed in Table 3.4.

<table>
<thead>
<tr>
<th>Air flow (cm\textsuperscript{3}/min)</th>
<th>None</th>
<th>20</th>
<th>50</th>
<th>80</th>
<th>100</th>
<th>150</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTR (mmol/l/h)</td>
<td>0</td>
<td>66.96</td>
<td>167.41</td>
<td>267.86</td>
<td>334.82</td>
<td>502.23</td>
</tr>
</tbody>
</table>

3.7.4. Co-fermentations
Co-fermentation processes were carried out with 800 ml Co-fermentation Medium in a 1 l bioreactor. The temperature was 30°C and the stirring speed was 100 rpm, with uncontrolled pH. Unless otherwise stated, in all co-fermentation experiments, the inoculum size was 50% for both strains.
In all processes of glucose/xylose co-fermentation, the glucose fermentation by *Z. mobilis* was much more efficient compared to the xylose fermentation by *P. stipitis*. The completion of glucose fermentation was determined by observing the pH change. Both glucose and xylose fermentations produce carbon dioxide during the ethanol fermentation process. The produced CO\textsubscript{2} enters the fermentation medium and forms carbonic acid, which decreases the pH of the medium (Fu and Peiris, 2008). During the glucose fermentation, the decrease of pH was considerably rapid; then it would remain constant for a period of time, which indicated the complete utilization of glucose. After that, the pH would again decrease slowly, indicating the utilization of xylose.

### 3.7.4.1. Sequential culture
Sequential culture of *Z. mobilis* and *P. stipitis* was achieved by inoculating *Z. mobilis* at first to convert all the glucose in the medium. After glucose was utilized, the bioreactor was autoclaved at 115°C for 5 min to inactivate all the bacteria in the medium. *P. stipitis* was then inoculated to the medium after the temperature of bioreactor decreased to room temperature, which usually took 12 h. For inoculation, the harvested *P. stipitis* cell pellet after centrifugation was re-suspended in 200 ml of 20 g/l xylose and inoculated to the bioreactor. The addition of the xylose in the inoculum also compensated for the loss of xylose in the fermentation medium due to the sampling during the glucose fermentation. The resulting fermentation medium after the inoculation of *P. stipitis* contained 20 g/l xylose and had a volume of approximate 800 ml. No aeration was provided for the glucose fermentation, while during the xylose fermentation the air flow was controlled at 80 cm\textsuperscript{3}/min.

### 3.7.4.2. Co-culture with free cells
Both strains were simultaneously inoculated into the bioreactor to start the fermentation. After the completion of glucose fermentation, xylose fermentation continued under the same condition without any adjustment. Fermentation ended when the pH of the medium remained constant. Various aeration levels and inoculum sizes were tested in order to optimize the system.
3.7.4.3. The use of immobilized Z. mobilis in the co-culture

This fermentation scheme co-cultured the immobilized Z. mobilis and free cells of P. stipitis simultaneously on the glucose/xylose medium. Again after the glucose fermentation was completed, no adjustment to conditions was made; the beads remained in the fermentation medium till the end of the process. Time courses were repeated with same batch of Z. mobilis beads in order to examine the effect of the reuse of the immobilized cells. In all the time courses, air flow was controlled at 80 cm³/min.

3.7.4.4. Co-culture processes in a modified fermentor

This fermentation scheme was an improved version of the previous one with immobilized Z. mobilis. A specially designed sieve plate was introduced to the bioreactor, together with a movable device to lift the sieve plate up and down [see Figure 3.5]. When both immobilized Z. mobilis and free cells of P. stipitis were inoculated, the sieve plate would prevent the immobilized beads from passing through and permit the yeast cells to pass. Then after all the glucose was used up, the immobilized beads could be conveniently removed from the fermentation medium, simply by lifting the sieve plate up to the upper part of the bioreactor where they had no contact with the medium. Therefore this scheme enabled the removal of the majority of Z. mobilis cells from the medium without disturbing the ongoing xylose fermentation, consequently minimizing the potential effects of Z. mobilis on the xylose fermentation by P. stipitis. Furthermore, this would also prevent undue damage to the gel beads from the prolonged exposure to the fermentation medium.

For this fermentation scheme, time courses with different inoculum levels were carried out to investigate the effect when different ratios of two strains were employed as inoculum. All experiments were carried out in duplicate. Air flow was controlled at 80 cm³/min for all time courses.

3.7.4.5. Culture of P. stipitis on glucose/xylose mixture

Experimental conditions were similar to those described for sole xylose fermentation in Section 3.7.3, except 30 g/l glucose and 20 g/l xylose were used as carbon sources instead of 50 g/l xylose. Time courses were carried out with several aeration levels and inoculum sizes in order to achieve an optimized performance.
3.8. Sampling techniques

In order to ensure consistency, the sampling procedure used in this study is as shown in Figure 3.6.

Figure 3.5: Modified bioreactor with the added sieve plate.

Figure 3.6: Sampling procedure used in this study.
3.9. Hydrolysis of sugarcane bagasse

In order to investigate the effect of hydrolysis conditions on the hydrolysate, three batches of sugarcane bagasse were prepared and simultaneously subjected to the treatments of acid hydrolysis and enzymatic hydrolysis under different conditions. Parameters including acid concentration, enzyme loading and recovery method were examined to set up an optimal hydrolysis process.

3.9.1. Acid hydrolysis

Acid hydrolysis process generally followed the description of Takahashi et al. (2000). The basic procedure is shown in Figure 3.7.

![Figure 3.7: Procedure of acid hydrolysis.](image-url)
3.9.2. Enzymatic hydrolysis

Enzymatic hydrolysis was carried out as described by Martin et al. (2006a) and Davis et al. (2005). Basically, the bagasse material was mixed with 0.04 M acetate buffer at pH 5.0 [prepared by dissolving 3.28 g sodium acetate anhydrous in 1 l distilled water and adjusting the pH to 5.0 using acetate acid glacial]. Enzymes were added at several loadings with different enzyme combinations; the hydrolysis was carried out in an orbital shaker at 60°C, 200 rpm. The details of enzymes employed in this study are shown in Table 3.5.

Table 3.5: Enzymes used for hydrolysis

<table>
<thead>
<tr>
<th>Enzyme name</th>
<th>Class</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellclast 1.5 L</td>
<td>Cellulase</td>
<td>700 EGU/g</td>
</tr>
<tr>
<td>Viscozyme L</td>
<td>β-glucanase (endo-1,3(4)-)</td>
<td>100 FBG/g</td>
</tr>
<tr>
<td>Novozyme 188</td>
<td>β-glucosidase</td>
<td>250 CBU/g</td>
</tr>
<tr>
<td>Shearzyme 500 L</td>
<td>Xylanase (endo-1,4-)</td>
<td>500 FXU-S/g</td>
</tr>
<tr>
<td>Biofeed Wheat L</td>
<td>Xylanase (endo-1,4-)</td>
<td>650 FXUW/ml</td>
</tr>
</tbody>
</table>

3.9.3. Preparation of hydrolysates for fermentation

Hydrolysate was supplemented with sugars and other components to increase the initial sugar level and improve the fermentability. The composition of Concentrated Sugar solution and Concentrated YEIS (yeast extract and inorganic salts) solution is listed in Table 3.6.

Table 3.6: Composition of supplements added to bagasse hydrolysate

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentrated Sugars</td>
<td>200.0 g/l glucose and 120.0 g/l xylose</td>
</tr>
<tr>
<td>Concentrated YEIS</td>
<td>80.0 g/l yeast extract, 8.0 g/l MgCl₂, 8.0 g/l (NH₄)₂SO₄ and 8.0 g/l KH₂PO₄.</td>
</tr>
</tbody>
</table>

For the preparation of adaptation medium, 80 ml filter-sterilized hydrolysate was
mixed with 10 ml Concentrated Sugar and 10 ml Concentrated YEIS solution, made up to 100 ml for each adaptation flask. The fermentation medium comprised 600 ml of hydrolysate medium together with 100 ml of Concentrated Sugars and 100 ml Concentrated YEIS solution, totaling 800 ml.

3.9.4. Strain adaptation to the hydrolysate
Both strains were adapted to the hydrolysate medium prior to fermentation. For immobilized *Z. mobilis*, beads were firstly reconditioned as described in 3.5, and then transferred into a 250 ml conical flask containing 100 ml supplemented hydrolysate medium. Incubation was carried out for 8-12 h in a 30°C stationary incubator. *P. stipitis* cells were firstly harvested from 400 ml Xylose Medium as described in Section 3.5. After centrifugation, cells were re-suspended into 4 conical flasks, each containing 100 ml supplemented hydrolysate medium. Incubation lasted for 24 h in a 30°C orbital shaker with a shaking speed of 200 rpm.

Inoculation and fermentation conditions were identical to those reported for glucose/xylose co-fermentation in the previous section. Fermentations were carried out with the co-culture in a modified fermentor and also with *P. stipitis* sole culture [see fermentation conditions in Sections 3.7.4.4 and 3.7.4.5 respectively].

3.10. Analytical methods
3.10.1. Determination of sugar concentration
Three methods were employed for the assay of sugar concentration, viz. Reducing sugar (RS) assay, Glucose oxidase (GO) assay, and HPLC. RS assay was generally used for all sugar media to estimate the reducing sugar content. GO assay was used to measure the glucose concentration in the glucose/xylose mixture. HPLC was employed to determine the glucose and xylose concentration in the sugarcane bagasse hydrolysate.

3.10.1.1. Reducing sugar assay
Both glucose and xylose are reducing sugars and can be assayed by the Dinitrosalicylic Acid (DNS) method (Miller et al., 1960) using the corresponding sugar standards.
For samples from time courses using only glucose or xylose as the sole carbon source, sugar concentration was determined as follows. The supernatant of the centrifuged sample was diluted 1 in 10, 1 in 50 and 1 in 100. Then standard sugar solutions were prepared using either glucose or xylose at concentration levels of 200, 400, 600, 800 and 1000 µg/ml. For the assay, reagents were added into test tubes as shown in Table 3.7.

**Table 3.7: Reagents added in the RS assay**

<table>
<thead>
<tr>
<th>Standard solution</th>
<th>Test sample</th>
<th>Distilled water</th>
<th>DNS reagent *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>3 ml</td>
<td>2 ml</td>
<td></td>
</tr>
<tr>
<td>Standards</td>
<td>1 ml</td>
<td>2 ml</td>
<td>2 ml</td>
</tr>
<tr>
<td>Test samples</td>
<td>1 ml</td>
<td>2 ml</td>
<td>2 ml</td>
</tr>
</tbody>
</table>

* DNS reagent comprised 10.0 g/l 3, 5 dinitrosalicylic acid, 182.0 g/l Rochele salt (potassium sodium tartrate), 10.0 g/l sodium hydroxide, 2.0 g/l phenol and 0.5 g/l sodium bisulphite, made up in distilled water.

The tubes were then vortexed and capped, placed in a boiling water bath for exactly ten minutes. All samples and standards were boiled at the same time. Tubes taken from the water bath were cooled to room temperature for approximately 1 h. The absorbances were measured at 640 nm. Reducing sugar content was determined from the standard curve with correction made for dilution.

For co-fermentations with glucose/xylose mixture, RS content in the samples was determined as xylose equivalents using the xylose standard. This was because in a co-fermentation process, two sugars were consumed at a different rate; hence it was impossible to give an accurate estimation for the glucose: xylose ratio in each sample. In this case, the use of xylose standard had two advantages: (1) after all the glucose was used up, the residual xylose could be accurately measured using xylose standard; (2) compared to a sugar mixture standard curve with random ratio of two sugars, the error caused by xylose standard was predictable and consequently could be minimized.
As shown in Figure 3.8, with the same concentration, the xylose standard always
gives higher absorbance readings compared to the glucose standard. In this case,
when a glucose/xylose sugar mixture was expressed as xylose equivalent, the
resulting value would be smaller than the actual sugar concentration. This would
affect the RS results for all the samples in the earlier stage of a co-fermentation,
when there was still glucose in the medium. Nevertheless, the use of xylose standard
still gave an accurate measurement after all the glucose had been completely
consumed. Moreover, it could still reflect the actual trend of sugar uptake, even
though the values were slightly lower. In association with the GO method, which was
used exclusively to assay the glucose concentration, the combination of two methods
enabled a reliable monitor for the sugar uptake trend in the co-fermentation process.

![Figure 3.8: Standard curves of glucose and xylose for reducing sugar assay](image)

3.10.1.2. Glucose oxidase assay
Glucose oxidase assay was used to determine the glucose concentration from the
sugar mixture. The procedure used in this study was a modified method based on
both methods of AOAC Official Method 969.39 (AOAC-International, 2006) and
Glucose Oxidase Method described in the “Methods of Enzymatic Analysis” (Kunst
et al., 1984). The principle of this method is as follows.
Glucose is oxidized under the catalysis of glucose oxidase, generating $\text{H}_2\text{O}_2$ which can react with the chromogen $\text{o-Dianisidine} \cdot 2\text{HCl}$ under the catalysis of peroxidase and result in the formation of orange color. The color intensity is directly proportional to the amount of $\text{H}_2\text{O}_2$ and therefore directly proportional to the glucose concentration in the sample. By quantifying the color intensity with a spectrophotometer, the glucose concentration could be estimated from suitable glucose standards.

For the assay, reagents were firstly prepared following the quantities below, and stored at 4°C refrigerator.

**Reagents:**

1. **Acetate buffer**: 0.1 M, pH 5.5. Dissolved 13.608 g sodium acetate in 1 l distilled H$_2$O, added 2.7 ml acetate acid, adjusted pH using sodium acetate or acetate acid glacial when necessary.

2. **Peroxidase solution**: 7,748 units/l. Dissolved 1.3 mg peroxidase (Sigma P-8375, Type VI from Horseradish, 298 units/mg) in 50 ml acetate buffer.

3. **Glucose oxidase solution**: 60,995 units/l. Dissolved 9.8 mg glucose oxidase (Sigma G7141, Type X-S from *Aspergillus niger*, 155,600 units/g) in 25 ml acetate buffer.

4. **Chromogen solution**: 0.5 mmol/l. Dissolved 46.40 mg o-Dianisidine•2HCl (Sigma D3252) into 250 ml buffer.

Reaction mixture was prepared freshly for every assay by taking 1 ml of Solution (2), 1 ml of Solution (3), 4 ml of Solution (4) and 4 ml of distilled $\text{H}_2\text{O}$ to make up 10 ml. Glucose standards were prepared at concentration levels of 50, 100, 150, 200 and 250 $\mu$g/ml. Samples were diluted 1 in 10, 1 in 50 and 1 in 250 respectively. Tubes were added with 0.1 ml of samples and standards, and 0.1 ml of distilled $\text{H}_2\text{O}$ for the blank. The prepared reaction mixture and all tubes containing reaction substrate were
incubated separately in a 30°C water bath for 5 min before commencing the assay.

The assay commenced with the addition of 1.0 ml of reaction mixture to each of the tubes. The addition was processed at 30 sec interval so that each tube could be incubated for exactly 30 min. The reaction was stopped by the addition of 2 ml prechilled distilled H₂O. The absorbance of each tube was read immediately against the blank at 525 nm. The glucose concentration of each sample was determined from the standard curve with correction for dilution.

The method described above was optimized. It is accurate for glucose concentration up to 60 g/l and sensitive to 0.05 g/l [see the Appendix section for the details of method optimization].

3.10.1.3. HPLC

HPLC used in this study was a software-based Perkin Elmer system for carbohydrate assay [Perkin Elmer Ltd, U.K.]. Turbochrom 4 workstation was used for system control and data integration. The detector was a Series 200 Refractive Index detector and the column was a Biorad Aminex HPX-87P column [Biorad, U.S.A.]. The mobile phase was milli-Q water [Milli-RO 12plus, Millipore, Australia] filtered with 0.45 μm filter and degassed with Helium. The column temperature was 85°C and the flow rate was 0.6 ml/min.

3.10.2. Determination of biomass growth

3.10.2.1. Viable cell concentration

The samples were serially diluted up to 10⁷ in 5% (w/v) peptone solutions. 0.1 ml was spread plated onto the appropriate agar medium and incubated for 48 h. Plates containing 25 – 250 colonies were used to calculate the amount of viable cells (cfu) per ml.

Glucose Agar plates were used for Z. mobilis, while Malt Extract Agar plates were used for P. stipitis. For the co-culture process involving both strains, Glucose Agar was used. The colonies of the yeast and bacterium can be easily distinguished from each other after 48 h incubation. As shown in Figure 3.9, P. stipitis colonies were larger and more opaque, whereas Z. mobilis colonies were smaller with a slightly
translucent edge.

This method only worked when the viable cell concentration of *P. stipitis* and *Z. mobilis* in the medium was approximately on a similar level. If the difference in the cell concentration between two strains was greater than 100 times, e.g. in a co-culture process of immobilized *Z. mobilis* and free cells of *P. stipitis*, the large amount of yeast colonies would obscure the few colonies of bacteria. Under this circumstance, the viable cell concentration of *Z. mobilis* was reported as $1 \times 10^5$ cfu/ml, referring to “undetectable on the plate of $10^4$ dilution”.

![Image of Glucose Agar plate showing colonies of *P. stipitis* (big) and *Z. mobilis* (small).](image)

**Figure 3.9:** Glucose Agar plate showing colonies of *P. stipitis* (big) and *Z. mobilis* (small).

### 3.9.2.2. Dry biomass weight

Dry weight assay is commonly employed to monitor the biomass growth and calculate kinetic parameters for a fermentation process. To estimate the dry weight concentration of cells, an aliquot of culture was centrifuged and washed by distilled water. The obtained cell pellet was dried in a 104°C oven for approximately 24 h till
the weight was constant. The dry weight value was determined by measuring the weight difference of the empty centrifuge tubes and tubes with dry cell pellets in them.

3.10.3. Determination of ethanol
Ethanol concentration was measured by a gas chromatography (GC) system [GC-3BT, Shimadzu, Seisakusho Ltd, Japan], except for the co-fermentation processes carried out in the modified fermentor, with \textit{P. stipitis} sole culture, and the fermentation of bagasse hydrolysates. The column was a packed Poropak-Q column, approximately 176 cm in length by 0.5 mm in diameter. The oven temperature was 165°C and a thermal conductivity detector was used. The carrier gas was Hydrogen. The internal standard used for this GC system was 2-propanol.

For those co-fermentation processes mentioned above, another GC system with a flame ionization detector [Hewlett Packard 5890A, Hewlett Packard, U.S.A.] was used. It consisted of a capillary HP column, approximately 125 cm in length by 0.2 mm in diameter. The injector temperature was 190°C and the oven temperature was 40°C. The carrier gas was Hydrogen with internal standard 1-propanol.

3.11. Calculation of kinetic parameters
Theoretical conversion rates for ethanol yield were as follows: 0.51 g/g from glucose, 0.51 g/g from xylose, and thus 0.51 g/g from sugar mixture [60\% of glucose and 40\% of xylose].

Kinetic parameters are calculated as follows. The general nomenclature and definitions of terms below are given in the Nomenclature section.

\textbf{Ethanol yield} \((Y_{p/s}, \text{ g/g})\): highest ethanol concentration \((p_f, \text{ g/l})\) / sugar consumed \((\Delta s, \text{ g/l})\). Expressed by equation:

\[ Y_{p/s} = \frac{p_f}{s_0 - s_f} (\text{g} / \text{g}) \quad (4) \]

where the \(\Delta s\) was determined by the initial sugar concentration \((s_0, \text{ g/l})\) minus the residual sugar concentration in the fermentation medium \((s_f, \text{ g/l})\).
Volumetric ethanol productivity/Overall system co-efficiency ($Q_p$, g/l/h): highest ethanol concentration ($p_f$, g/l) / time used to achieve ($t_p$, h). Expressed by equation:

$$Q_p = \frac{p_f}{t_p} (g/l/h)$$

Specific ethanol productivity ($q_p$, g/g/h): highest ethanol concentration ($p_f$, g/l) / average dry cell concentration ($x_A$, g/l) / time used to achieve the highest ethanol ($t_p$, h). Expressed by equation:

$$q_p = \frac{1}{x_A} \frac{p_f}{t_p} (g/g/h)$$  \hspace{1cm} (6)

where the $x_A$ was the average of dry biomass weight up to Time $t_p$.

Sugar uptake rate ($Q_s$, g/l/h): sugar consumed ($\Delta s$, g/l) / time ($t_f$, h). Expressed by equation:

$$Q_s = \frac{s_0 - s_f}{t_f} (g/l/h)$$  \hspace{1cm} (7)

where the $t_f$ was the time when all sugar was used up. In a process where the sugar utilization was finished at the same time when ethanol reached peak, $t_f$ was equal to $t_p$; in a process where sugar consumption was not complete, $t_f$ referred to the overall fermentation time.
Chapter 4

Free cell culture of Z. mobilis and P. stipitis on sugar medium

In order to investigate fermentation characteristics of Z. mobilis and P. stipitis and establish optimum environmental conditions for co-culture experiments, the two microorganisms were cultured on the corresponding sugar media respectively. Both Z. mobilis and P. stipitis have been extensively studied for their fermentative capability, optimum culture conditions, and their effects on other strains in a co-culture process. There are a number of studies reporting an increased ethanol productivity in an immobilized culture (Amin and Doelle, 1990; Rebros et al., 2005) or through a cell recycling process (Parekh et al., 1986; Yu et al., 1987). Both processes caused cell accumulation and consequently increased the level of viable cells. However, there is little information directly describing the relationship between the viable cell concentration and the ethanol production. In a previous study it has been shown that the ethanol production rate by Z. mobilis increased with an increase in viable cell concentration (Fu and Peiris, 2008). To confirm this relationship and obtain more detailed information, experiments investigating the fermentation performance with different inoculum sizes were carried out for both Z. mobilis and P. stipitis. For P. stipitis, the effect of oxygenation was also examined.
4.1. Effect of inoculum size on the performance of *Z. mobilis*

In this experiment, three time courses culturing *Z. mobilis* on glucose were carried out under conditions described in Section 3.7.2. Inoculum sizes of 10%, 50% and 100% were used for each time course respectively. The resulting biomass levels of the three time courses are shown in Figure 4.1, and the kinetic parameters are compared in Figure 4.2.

![Figure 4.1](image)

**Figure 4.1**: Comparison of the biomass concentration in time courses with different inoculum sizes for *Z. mobilis*: (a) viable cell concentration, (b) dry cell weight.
As shown in Figure 4.1, the increase of inoculum size directly increased the level of biomass. Results for viable cell concentration and dry weight showed a similar trend. The highest biomass level was achieved in the time course with the largest inoculum size [100%]. The initial viable cell level [1.2x10^8 cfu/ml] in time course with 100% inoculum size was approximately 2 fold and 10 fold higher than the one with 50% [6.1x10^7 cfu/ml] and 10% [1.7x10^7 cfu/ml] respectively, which was consistent with the increase in the inoculum size. In all three time courses, the biomass concentration showed a steady increase and peaked when all the glucose was used up [2.5 h for time course with 100% inoculum size, 4 h for the one with 50% and 8 h for the one with 10%; see Figure 4.2]. The peak values of viable cell concentration in all three time courses were at a similar level [~1.5x10^9 cfu/ml]; however, highest dry weight value was achieved in the time course with largest inoculum size 100% [2.95 g/l]. Because of the lower levels of initial biomass amount, the peak dry weight values in both time courses with smaller inoculum size were lower. The lowest dry weight peak was in the time course with lowest inoculum size, 10% [1.63 g/l compared to 2.54 g/l in the time course with 50% inoculum size].

![Figure 4.2: Comparison of kinetic parameters of time courses with different inoculum sizes of Z. mobilis.](image)

In association with the different levels of biomass concentration achieved in three
time courses, the kinetic parameters also showed some significant differences. In all three time courses, ethanol concentration reached a peak when all the glucose was completely utilized. The ethanol peak time \( t_f \) decreased significantly from 8 h to 4 h to 2.5 h along with an increase in the inoculum size from 10% to 50% to 100%. The decrease on the fermentation time led to a corresponding increase in the system co-efficiency \( Q_p \), from 2.93 g/l/h to 6.39 g/l/h to 10.54 g/l/h. This was approximately a linear correlation to the inoculum size. When the amount of inoculated cells increased by 40-50% of the medium volume [4-5 fold increase based on the traditional 10% inoculum size], the system co-efficiency doubled, and the fermentation time halved. Both values had the potential to be further improved if a higher inoculum size was provided.

Apart from the system co-efficiency \( Q_p \), an increase was also observed in the specific ethanol productivity \( q_p \), which measures the ethanol production capability based on biomass. With the increase in the inoculum size from 10% to 50% to 100%, the value increased from 3.26 g/g/l to 4.00 g/g/l to 5.02 g/g/l. Ethanol yields in all three time courses were similar, around 0.50-0.51 g/g, which was more than 98% of the theoretical yield.

In summary, the increase in the viable cell concentration in the fermentation medium significantly contributed to the improvement of the overall performance of \( Z. \) mobilis on the ethanol production from glucose.

### 4.2. Optimization of ethanol production by \( P. \) stipitis from xylose

As discussed in Section 2.3, although a large number of studies have been conducted on the xylose fermentation, the reported optimum conditions were sometimes inconsistent. Hence there was a need to experimentally determine and establish optimal conditions of \( P. \) stipitis culture on xylose. Firstly, the effect of medium composition was investigated; then experiments were carried out to decide the optimum oxygenation level when different inoculum sizes were employed. Similar to the glucose fermentation by \( Z. \) mobilis, three levels of inoculum size, 10%, 50% and 100% were investigated.
4.2.1. Effect of medium composition on the performance of \textit{P. stipitis}

This experiment was carried out in shake flasks, with conditions as described in Section 3.7.1. All together three different medium compositions were investigated; the details are shown in Table 4.1.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
Compositions & Sources & \\
\hline
Medium 1 & 50.0 g/l xylose, 10.0 g/l yeast extract, 1.0 g/l MgCl\textsubscript{2}, 1.0 g/l (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 1.0 g/l KH\textsubscript{2}PO\textsubscript{4}. & (Yu and Zhang, 2002) \\
 & Medium 2 & 50.0 g/l xylose, 4.0 g/l yeast extract, 3.6 g/l peptone, 1.0 g/l MgSO\textsubscript{4}, 3.0 g/l (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 2.0 g/l KH\textsubscript{2}PO\textsubscript{4}. & (Sanchez et al., 2004) \\
 & Medium 3 & 50.0 g/l xylose, 3.0 g/l yeast extract, 5.0 g/l peptone, 3.0 g/l malt extract. & (Parekh et al., 1986) \\
\hline
\end{tabular}
\caption{Composition of the three media for \textit{P. stipitis} culture on xylose}
\end{table}

For each medium, three culture flasks were prepared, with 50 ml medium in 250 ml conical flasks. The experiment commenced by inoculating the first flask of each medium with a single colony from \textit{P. stipitis} culture maintenance plate respectively, and then serially subcultured to the other two flasks after 24 h incubation. Samples were taken at 24 h for the first two subculture flasks for viable cell assay, while for the third subculture flask, samples were taken at both 24 h and 48 h and assayed for sugar consumption and ethanol production as well.

Figure 4.3 presents the result for biomass growth on the three media. \textit{P. stipitis} showed best growth in Medium 3 during all three subcultures. However, after two subculture processes and 48 h incubation, the difference in the values of viable cell concentration between the three media markedly decreased in the third subculture. The vigorous growth of \textit{P. stipitis} on Medium 3 was considered to be a result of the abundant nutrition supply. As shown in Table 4.1, Medium 3 was more nutritive compared to the other two, containing higher levels of rich components such as yeast extract, peptone and malt extract.
Chapter 4. Free cell culture of *Z. mobilis* and *P. stipitis* on sugar medium

The sugar consumption and ethanol production of the third subculture are shown in Figure 4.4. Again Medium 3 proved to be the best for the conversion of xylose to ethanol by *P. stipitis*. Resulting from the high level of viable cell concentration, the xylose in Medium 3 was consumed much more rapidly compared to the other two. It was the only medium that used up all the xylose in 48 h. For the ethanol production, at 24 h the ethanol concentration in Medium 3 reached a level of 11.89 g/l, whereas for the other two media the ethanol concentration remained too low to be detected by GC. Nevertheless, both Medium 1 and Medium 2 showed an increased sugar utilizing rate after 24 h, leading to a rapid accumulation of ethanol.

In summary, Medium 3 showed superior performance for both biomass growth and ethanol production when *P. stipitis* was cultured on xylose. This could again be attributed to the abundant rich components in Medium 3. However, the high cost associated with these rich components makes it economically unviable to be used on a large scale industrial production. In order to decrease the dependence of *P. stipitis* on rich media components, further study was undertaken with Medium 1, which gave better results on ethanol production compared to Medium 2. Medium 1 is referred as the Xylose Medium in Table 3.2 [see Section 3.4].

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**Figure 4.3: Comparison of biomass growth of *P. stipitis* on three media during three subcultures.**
4.2.2. Time courses with 10% inoculum size

According to reported literature, oxygenation is of great importance for the conversion of xylose to ethanol [see section 2.3.2]. Therefore, the effect of oxygenation was investigated on three inoculum sizes respectively. The fermentations were carried out in a 1 l bioreactor, with conditions as described in Section 3.7.3. For the inoculum size of 10%, three time courses were carried out, at air flow levels of 0, 20 and 100 cm³/min respectively, corresponding to an OTR of 0, 66.96 and 167.41 mmol/l/h. The kinetic parameters of three time courses are shown in Figure 4.4.

Figure 4.4: Comparison of sugar consumption and ethanol production of *P. stipitis* on three media after three subcultures: (a) reducing sugar, (b) ethanol.
Chapter 4. Free cell culture of *Z. mobilis* and *P. stipitis* on sugar medium

in Table 4.2, and the trends in biomass growth are shown in Figure 4.5.

**Table 4.2: Kinetic parameters for xylose fermentation with an inoculum size of 10%**

<table>
<thead>
<tr>
<th>Kinetic parameters</th>
<th>Air flow level (cm³/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Fermentation time (h)</td>
<td>86</td>
</tr>
<tr>
<td>Time when ethanol peaked (h)</td>
<td>N/A</td>
</tr>
<tr>
<td>Sugar consumption (%) *</td>
<td>17%</td>
</tr>
<tr>
<td>Ethanol yield (g/g)</td>
<td>0</td>
</tr>
<tr>
<td>System co-efficiency (g/l/h)</td>
<td>0</td>
</tr>
<tr>
<td>Specific ethanol productivity (g/g/h)</td>
<td>0</td>
</tr>
</tbody>
</table>

* Sugar consumption (%) refers to the percentage of the consumed sugar out of the total initial sugar in the medium.

![Figure 4.5: Viable cell concentration for xylose fermentations with an inoculum size of 10%](image)

The fermentation process carried out without an air flow, which provided the yeast a semi-anaerobic condition, showed a considerably low rate of sugar uptake [only 17% of xylose was consumed in 86 h]. This was associated with a low level of biomass
Chapter 4. Free cell culture of Z. mobilis and P. stipitis on sugar medium

4.2.3. Time courses with 50% inoculum size

When the inoculum size was increased to 50%, the increased cell density affected the oxygenation level in the medium; consequently, the optimum OTR value would be different to that using a 10% inoculum size. In this experiment, four aeration levels of 20, 50, 80 and 100 cm$^3$/min were investigated respectively, which was equivalent to an OTR of 66.96, 167.41, 267.86 and 334.82 mmol/l/h. Kinetic parameters and the biomass growth trends are shown in Table 4.3 and Figure 4.6 respectively.

As a result of the increased inoculum size, in all four time courses the xylose was used up within 50 h. The fermentation time $t_p$ decreased from 48 h to 28 h when the air flow increased from 20 to 100 cm$^3$/min, and the system co-efficiency $Q_p$ increased from 0.533 g/l/h to 0.734 g/l/h accordingly. As expected, with the increase in the inoculum size from 10% to 50%, the optimum air flow also increased in order to meet the oxygenation requirement for the increased cell number [20 cm$^3$/min for inoculum size of 10% and 100 cm$^3$/min for the size 50%]. The highest system co-efficiency of 0.734 g/l/h achieved with 100 cm$^3$/min was 5.2 fold as high as the best achieved using the 10% inoculum size [0.142 g/l/h]. The highest ethanol yield [$Y_{p/s}$] and the highest specific ethanol productivity [$q_p$], however, were attained in the time course with slightly lower air flow level at 80 cm$^3$/min [0.45 g/g and of 0.247 concentration in the fermentation medium [$\approx 10^7$ cfu/ml]; no significant cell growth was observed during the whole process. The fermentation with an air flow level of 20 cm$^3$/min gave the highest ethanol yield of 0.43 g/g; however, up to 8% of sugar was un-utilized by the end of the fermentation at 140 h. Ethanol peaked at 130 h with a concentration of 18.52 g/l and decreased slightly to 18.19 g/l at 140 h, indicating possible ethanol re-assimilation even when there was still xylose available in the medium. When the air flow increased to 100 cm$^3$/min, all xylose was fully utilized at 140 h, but ethanol peaked at 120 h. The decreased ethanol concentration after the peak again demonstrated possible ethanol re-assimilation. The peak ethanol concentration in this time course was 14.98 g/l, giving an ethanol yield of 0.34 g/g, 20% lower compared to the value achieved with 20 cm$^3$/min aeration [0.43 g/g]. Therefore, for the inoculum size of 10%, an air flow of 20 cm$^3$/min gave the best fermentation performance, although the overall system co-efficiency remained low at 0.142 g/l/h.
g/g/h respectively. It is possible that the increased aeration at 100 cm³/min accelerated the ethanol re-assimilation and led to a slightly reduced ethanol yield [0.44 g/g]. A similar observation was made for the time courses with an inoculum size of 10%. Biomass concentration in all four time courses was at a similar level. There was a slight drop of viable cell concentration at the later stage of all processes, which could result from the foaming caused by the air flow.

Table 4.3: Kinetic parameters for xylose fermentation with an inoculum size of 50%

<table>
<thead>
<tr>
<th>Kinetic parameters</th>
<th>Air flow level (cm³/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Ethanol peak time (h)</td>
<td>48</td>
</tr>
<tr>
<td>Sugar consumption (%)</td>
<td>100%</td>
</tr>
<tr>
<td>Ethanol yield (g/g)</td>
<td>0.41</td>
</tr>
<tr>
<td>System co-efficiency (g/l/h)</td>
<td>0.533</td>
</tr>
<tr>
<td>Specific ethanol productivity (g/g/h)</td>
<td>0.194</td>
</tr>
</tbody>
</table>

Figure 4.6: Viable cell concentration for xylose fermentations with an inoculum size of 50%
4.2.4. Time courses with 100% inoculum size

When the inoculum size was increased to 100%, foaming became a major problem affecting the overall fermentation performance. Amongst the four air flow levels tested in this experiment, viz. 0, 50, 100 and 150 cm$^3$/min, serious foaming occurred on both levels of 100 and 150 cm$^3$/min. As an illustration, the biomass results of the four time courses are shown in Figure 4.7.

![Graph](image-url)

**Figure 4.7:** Biomass concentration for xylose fermentations with an inoculum size of 100%: (a) viable cell concentration, (b) dry cell weight.

As shown in Figure 4.7, though the same inoculum level was employed for these
four time courses, the biomass concentration showed a big variation. Time course with 50 cm\(^3\)/min gave the highest cell concentration overall, although a gradual decrease of dry biomass weight was observed after 12 h. Under semi-anaerobic conditions without airflow, the biomass concentration showed a slight increase during the 66 h fermentation time. By contrast, in the time courses with higher aeration levels, viz. 100 and 150 cm\(^3\)/min, the cell concentration showed a dramatic decrease. The viable cells in the medium dropped to approximately 1/5 of the peak value, whereas the dry weight of cells started with a level around 4.5 g/l and fell to 0.4~0.5 g/l at the lowest. In both processes, the foaming caused by the high levels of cell concentration and aeration could not be suppressed by the manual addition of antifoam. The foam forced the cells out of the medium and made them stick to the wall of the vessel, consequently causing a significant loss of viable cells. As a result, the high level of initial cell concentration was maintained throughout the fermentation only in the two time courses with lower aeration levels.

**Table 4.4: Kinetic parameters for xylose fermentation with an inoculum size of 100%**

<table>
<thead>
<tr>
<th>Kinetic parameters</th>
<th>Air flow level (cm(^3)/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Ethanol peak time (h)</td>
<td>66</td>
</tr>
<tr>
<td>Sugar consumption (%)</td>
<td>93%</td>
</tr>
<tr>
<td>Ethanol yield (g/g)</td>
<td>0.42</td>
</tr>
<tr>
<td>System co-efficiency (g/l/h)</td>
<td>0.270</td>
</tr>
<tr>
<td>Specific ethanol productivity (g/g/h)</td>
<td>0.043</td>
</tr>
</tbody>
</table>

Table 4.4 summarizes the kinetic parameters for these four time courses. Resulting from the erratic biomass concentrations, the fermentation performance also showed significant variations. Both the highest system co-efficiency of 0.755 g/l/h and the highest ethanol yield of 0.45 g/g were achieved in the time course with an air flow level of 50 cm\(^3\)/min. For the time course without aeration, despite the high level of yeast concentration, the xylose failed to be used up within 66 h [7% sugar un-utilized]. In other two time courses showing significant cell loss, the system
performance was also poor. A sugar residue up to 21% was observed for the one with an air flow of 150 cm$^3$/min. Though the time course with an air flow of 100 cm$^3$/min completely utilized all the xylose in 49 h, the system co-efficiency was only 1/2 of that achieved with air flow of 50 cm$^3$/min [0.359 compared to 0.755 g/l/h].

In summary, it was difficult to control the fermentation conditions at an optimum level for the *P. stipitis* culture with an inoculum size of 100%. The foaming that occurred under high aeration levels significantly reduced the biomass concentration and resulted in decreased fermentation performance. The time course without aeration failed to achieve high ethanol productivity either. It was thus confirmed that oxygen supply was essential for an efficient xylose fermentation.

### 4.2.5. Comparison of best performance on each inoculum level

In order to establish the correlation between the amount of biomass and ethanol production for *P. stipitis* culture on xylose, the time courses with best results from each inoculum level are compared and discussed. The three selected time courses are: inoculum size 10% with air flow level of 20 cm$^3$/min, inoculum size 50% with air flow level of 100 cm$^3$/min, and inoculum size 100% with air flow level of 50 cm$^3$/min. The ethanol peak time \([t_f]\) and average dry cell weight \([x_A]\) of these time courses are shown in Table 4.5, and the kinetic parameters are presented in Figure 4.8.

As shown in Table 4.5, an increase in the inoculum size from 10% to 50% resulted in an increase in the biomass from an average of 1.94 g/l to 3.36 g/l. The fermentation time decreased from 130 h to 28 h, and the system co-efficiency increased from 0.142 g/l/h to 0.734 g/l/h, which is the highest reported value for free cells of *P. stipitis* in batch culture. In the fermentation with an inoculum size of 100%, the average dry weight further increased to 6.21 g/l; however, the system co-efficiency remained at a similar level to the one with 50% inoculum size [0.755 g/l/h compared to 0.734 g/l/h]. Both fermentations with an increased inoculum size needed 28 h to completely utilize the sugar, but the specific ethanol productivity \([q_p]\) with 100% inoculum level was only half of that of the 50% inoculum level [0.122 g/g/h compared to 0.218 g/g/h].
Table 4.5: Fermentations with best results from three inoculum levels for *P. stipitis* culture on xylose

<table>
<thead>
<tr>
<th>Inoculum size</th>
<th>Air flow (cm$^3$/min)</th>
<th>Ethanol peak time (h)</th>
<th>Average dry cell weight (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%</td>
<td>20</td>
<td>130</td>
<td>1.94</td>
</tr>
<tr>
<td>50%</td>
<td>100</td>
<td>28</td>
<td>3.36</td>
</tr>
<tr>
<td>100%</td>
<td>50</td>
<td>28</td>
<td>6.21</td>
</tr>
</tbody>
</table>

The reason for the decreased specific ethanol productivity was considered to be the oxygenation level. It can be seen in Table 4.5 that, although the amount of biomass in the time course with the 100% inoculum size was approximately twice as high as that in the time course with 50% [6.21 g/l compared to 3.36 g/l], the air flow level was only half [50 cm$^3$/min compared to 100 cm$^3$/min]. This mismatch of the cell concentration and air flow level could lead to insufficient redox activity and therefore limit the efficiency of the xylose metabolism by *P. stipitis*. It was thus expected that...
with an increase in the air flow level, the system co-efficiency of time course with 100% inoculum size could be further improved. Due to the severe foaming it was not possible to carry out this experiment in a 1 l bioreactor. Therefore, a shake flask experiment was resorted to.

4.2.6. Correlation between the biomass concentration and ethanol production for *P. stipitis*

This experiment was carried out using shake flasks under conditions described in Section 3.7.1. Six flasks of xylose medium were prepared, each containing 100 ml of Xylose Medium. Three were cultured on an orbital shaker with a shaking speed of 100 rpm, whereas the other three were cultured at 200 rpm to test the effect of oxygenation. For each oxygenation level, three flasks were inoculated with cells from 50 ml, 100 ml and 200 ml of a 48 h *P. stipitis* culture respectively, giving an inoculum size of 50%, 100% and 200% for each flask. Inoculation was carried out by centrifuging to harvest the cells and then re-suspending them with the fermentation medium. The results of the six flasks are shown in Figure 4.9.

As shown in Figure 4.9 (a), the biomass concentration of *P. stipitis* on xylose increased with the increase in the inoculum size as well as the aeration level. Since the first sample was taken at 12 h, the initial biomass concentration was not determined. However, flasks with same inoculum size were expected to have the same amount of cells initially. During 36 h culture, for the inoculum size of 50% and 100%, both flasks cultured under 200 rpm gave higher biomass concentrations compared to those under 100 rpm. For flasks with 200% inoculum size, no more cell accumulation beyond 10.5 g/l was observed, which could be a limitation for the *P. stipitis* cell growth on the xylose medium in the shake flask environment. Nevertheless, at 12 h before cell concentration started to decrease, the biomass in the flask at 200 rpm was on a higher level than that at 100 rpm. It can be thus said that at a higher air flow level, the biomass growth improved.
Chapter 4. Free cell culture of *Z. mobilis* and *P. stipitis* on sugar medium

Figure 4.9: Results of shake flask experiments of *P. stipitis* on Xylose Medium: (a) dry cell weight, (b) reducing sugar, (c) ethanol production.
In accordance with the trend for biomass, in Figure 4.9 (b), it can be seen that flasks with higher biomass concentration showed faster sugar utilization. As for the effect of oxygenation, all three flasks cultured under 200 rpm gave lower level of residual xylose concentration at 36 h in comparison with those cultured under 100 rpm with the same inoculum size. On the other hand, when aeration level stayed the same, higher sugar utilization was always achieved in the flasks with higher biomass concentration [200%>100%>50%]. Furthermore, an interesting observation was that, in the flask with less biomass but higher aeration level [50% inoculum with 200 rpm], the sugar was consumed at a higher efficiency compared to the flask with higher biomass concentration but lower aeration level [100% inoculum with 100 rpm]; the residual xylose concentration of these two flasks at 36 h was 9.68 g/l and 12.86 g/l respectively. This further demonstrated that both aeration level and the biomass concentration significantly affected the xylose fermentation process, though the effect of oxygenation appeared to be more crucial.

Results for ethanol production are shown in Figure 4.9 (c). At 100 rpm, the ethanol production increased with the increase in the inoculum size; three inoculum sizes of 50%, 100% and 200% respectively produced ethanol of 9.37 g/l, 12.38 g/l and 16.43 g/l at 36 h. However, these values were lower than those obtained at 200 rpm [14.04 g/l, 15.23 g/l and 17.48 g/l respectively]. The three flasks cultured at 200 rpm showed an increase in ethanol concentration from 24 h to 36 h, and the higher ethanol concentration was always obtained with higher levels of biomass.

This experiment proved that both increase in the biomass concentration and oxygenation level benefited the ethanol production by P. stipitis on xylose. The significance of this phenomenon will be discussed in later sections.

4.3. Discussion

4.3.1. The correlation between the amount of biomass and ethanol production for Z. mobilis culture on glucose

Previous literature has reported a range of values for the volumetric ethanol productivity [system co-efficiency] of Z. mobilis culture on glucose. For free cell
culture, values of 1.19 g/l/h [23.9 g/l of ethanol in 20 h] and 4.33 g/l/h [36 g/l of ethanol in 9 h] have been reported from 50 g/l glucose medium (Laplace et al., 1991b) and 80 g/l of glucose in the starch hydrolysate medium (Davis et al., 2006) respectively. For immobilized Z. mobilis culture, values ranging from 2.44 g/l/h [39 g/l of ethanol in 16 h] (Kesava et al., 1996) to 43.6 g/l/h (Rebros et al., 2005) have been reported. Although the system co-efficiency is definitely affected by the medium environment and the initial sugar level, these two factors are insufficient to account for such a wide variation. It is also observed that a high system co-efficiency [> 30 g/l/h] is usually associated with a high cell concentration (Amin and Doelle, 1990; Rebros et al., 2005). In a previous study by us, ethanol production was suggested to be closely related to the viable cell concentration in the medium (Fu and Peiris, 2008). From all these trends an inference could be made that the ethanol production of Z. mobilis on glucose is directly proportional to the total amount of biomass in the medium.

The findings in this study support the above stated deduction. Figure 4.10 presents the relationship of fermentation time [$t_f$] and system co-efficiency [$Q_p$] to the average biomass concentration [$x_A$] in three time courses with different inoculum sizes. Associated with an increase in biomass from 0.899 to 1.597 to 2.099 g/l, the fermentation time significantly reduced from 8 h to 4 h to 2.5 h, resulting in a corresponding increase in the system co-efficiency.

![Figure 4.10: The relationship of fermentation time and volumetric productivity to biomass concentration for Z. mobilis culture on glucose](image-url)
It has been pointed out by a number of researchers that a sufficient level of biomass in the medium is a prerequisite for an efficient fermentation process. Underwood et al. (2004) stated that limited cell growth directly resulted in a low system co-efficiency for the ethanol production process. The use of a large inoculum is a common method employed by the dairy industry to ensure a sufficient biomass level for the dairy fermentation (Montville and Matthews, 2008). In contrast to the specific ethanol productivity \( q_p \) which represents the ethanol production rate based on biomass, system co-efficiency \( Q_p \) represents the ethanol production rate based on the substrate. Assuming that cells in the medium do not assimilate the substrate for its own growth, but only convert all of the substrate [glucose] to ethanol through enzymatic reactions in metabolism, the whole process of the conversion of glucose to ethanol can be treated as a large, complicated reaction. The overall reaction can be shown by Formula (1) in Section 2.2.1. Ignoring the intermediate reactions, each cell of \( Z. \) mobilis could be treated as a reaction unit, a biocatalyst to carry out the reaction from glucose to ethanol. Each cell has a certain production efficiency, i.e. a single cell can only process a certain amount of substrate within a certain time. Consequently, with more cells in the medium, more such reactions will be simultaneously carried out, resulting in an increase of the overall ethanol production rate.

The concept of “whole cell biocatalyst” has been widely accepted in the field of bioenergy (Aristidou and Penttila, 2000; Fujita et al., 2004; Kim et al., 2007). Microorganisms involved in the biotransformation process, either saccharification or fermentation, are considered as catalysts, which means that in an ideal state, the microorganisms should not participate in the reactions and not utilize the substrate for its own growth, but only transform the substrate into the desirable products. \( Z. \) mobilis thus has the quality of an ideal biocatalyst for ethanol production. It converts the substrate to product efficiently and consumes negligible amount of glucose for its own growth [the overall ethanol yield was more than 98% of the theoretical]. At present no study has been reported for tracing the substance transfer during the cell division of \( Z. \) mobilis. However, Kalnenieks (2006) has suggested that the amount of ATP produced through the ED pathway far exceeds the requirement of the cell-biomass synthesis of the bacterium. It is claimed that there are futile extraneous
metabolic reactions consuming ATP, resulting in the high requirement of ATP production by *Z. mobilis*. An inference could thus be made that the biomass synthesis needed by cell growth of *Z. mobilis* is greatly dependent on the ATP produced during the ED pathway; there is little need for consumption of the carbon atoms from the glucose substrate, especially in a process with a large inoculum size which gives less cell growth. In other words, it could be expected that all glucose is theoretically used for ethanol production under optimal conditions.

Therefore, it can be proposed that: “in a given volume of medium (/l), the amount of ethanol produced (g) within a given time (/h) is only related to the total amount of biomass (cells) in the medium under optimal conditions. In other words, “under optimal conditions, the system co-efficiency $Q_p$, g/l/h] is only related to the viable cell concentration [x$_C$, cells/l] in the medium”. Conceptually, the amount of ethanol produced [$P_{yie}$, g] within a given time period [$t_1$ to $t_2$] equals the integral of the ethanol produced by each cell within this time period; this could be expressed by a formula as follows:

$$P_{yie} = \int_{t_1}^{t_2} N \times de \,(g)$$

where $N$ represents the biomass – number of viable cells in the medium (cells), and $de$ is defined as cell instantaneous ethanol production rate (g/h/cells). Consequently:

$$P_{yie} = N \times de \times (t_2 - t_1)(g) \quad (8.1)$$

When the medium volume [$v$, l] is known, both $P_{yie}$ and $N$ can be converted to their corresponding concentration in the medium:

$$p_f = \frac{P_{yie}}{v} \text{ (g/l)}; \quad x_C = \frac{N}{v} \text{ (cells/l)}$$

where $p_f$ represents the final ethanol concentration, and $x_C$ represents the viable cell concentration. In this case, Formula (8.1) can be expressed as:

$$p_f = x_C \times de \times (t_2 - t_1)(\text{g/l}) \quad (8.2)$$

$$\frac{p_f}{t_2 - t_1} = x_C \times de \,(\text{g/l/h}) \quad (8.3)$$

As shown by Formula (5) in Section 3.11, the system co-efficiency $Q_p$] can be expressed as:
\[ Q_p = \frac{P_f}{t_p} = \frac{P_f}{t_{2-1}} \text{ (g/l/h)} \]  \hspace{1cm} (5.1)

Integrating Formulae (8.2) and (5.1):

\[ Q_p = x_C \times de = \frac{N}{v} \times de \text{ (g/l/h)} \]  \hspace{1cm} (9)

Therefore, under optimal conditions, when the value of \( de \) stays the same, the overall system co-efficiency \([Q_p]\) is only determined by the viable cell concentration \([x_C]\).

4.3.2. The correlation between the amount of biomass and ethanol production for \( P. stipitis \) culture on xylose

Irrespective of strains and substrates, an ethanol production process could be described by the three formulae in general, viz. Formulae (5), (8) and (9). For ethanol production from xylose by \( P. stipitis \), wherever a high system co-efficiency has been reported in literature, a high biomass concentration was always associated. This was achieved by either the recycling of cells (Parekh et al., 1986; Yu et al., 1987), or the utilization of a large inoculum size (Nigam, 2001a; b; Agbogbo et al., 2007). In contrast, when low cell concentrations were observed, the system co-efficiency remained low (Sanchez et al., 2002; Sanchez et al., 2004; Agbogbo et al., 2006).

A similar trend was observed in this study. In the experiment testing the effect of medium composition [see Section 4.2.1], the efficient conversion of xylose to ethanol on Medium 3 was associated with the highest biomass concentration among three tested media. For Medium 1 and Medium 2, the ethanol production rate between 24 h and 48 h also showed a significant increase in comparison with that between 12 h and 24 h, resulting from the increased number of cells due to the multiplication of the yeast.

When three levels of inoculum size were used for the xylose fermentation [see Section 4.2.5], the increase in the average biomass concentration \([x_A]\) from 1.94 g/l to 3.36 g/l resulted in a 5.2 fold increase in the system co-efficiency \([0.142 \text{ compared to 0.755 g/l/h}]\). This relationship was further demonstrated by the shake flask experiments with different inoculum sizes under two oxygenation levels [see Section 4.2.6]. On each level of oxygenation, higher cell concentrations always gave more
efficent ethanol production.

All of the above results support the viewpoint that the system co-efficiency $Q_p$ is directly proportional to the viable cell concentration in the medium $x_c$ for *P. stipitis* culture on xylose. The only exception occurred in the fermentation with the inoculum size of 100% [see Section 4.2.4]. Amongst the four time courses carried out, the best one was obtained under an air flow level of 50 cm$^3$/min, giving a system co-efficiency of 0.755 g/l/h, which was almost at a similar level to that achieved with the inoculum size of 50% [0.734 g/l/h]. In order to explain this discrepancy and further clarify the relationship between viable cell concentration and system co-efficiency, the effect of oxygenation needs to be considered.

### 4.3.3. The role of oxygenation in the xylose fermentation

As discussed in Section 2.3.2, oxygenation is considered to be one of the most crucial factors affecting the efficiency of ethanol production from xylose. Overly high oxygenation levels help ethanol re-assimilation and thus decrease the ethanol yield (Passoth et al., 2003; Gorgens et al., 2005). Overly low oxygenation levels inhibit the cell growth and also result in low efficiency of ethanol production (du Preez, 1994; Fiaux et al., 2003; Agbogbo and Coward-Kelly, 2008). The latter trend was confirmed by the two time courses carried out under semi-anaerobic conditions in this study: one with an inoculum size of 10%, and the other one with an inoculum size of 100% [see Sections 4.2.2 and 4.2.4 respectively]. In the first time course, the lack of oxygenation and the low cell concentration led to an extremely low sugar consumption rate; in the second instance, though high cell concentration was maintained through the whole process [$x_A$: 6.28 g/l], the sugars failed to be utilized within 66 h of fermentation.

The reduced efficiency of ethanol production was further proved by the shake flask experiment in Section 4.2.6. In all three flasks with lower aeration level of 100 rpm, the ethanol production was considerably less compared to the corresponding flasks cultured at 200 rpm [9.37 compared to 14.04 g/l, 12.38 g/l compared to 15.23 g/l and 16.43 g/l compared to 17.48 g/l]. It can be thus deduced that when the actual oxygenation level was below the optimum, a further reduction of oxygenation level led to a decreased system co-efficiency.
Based on this deduction, the system co-efficiency achieved in the time course with an inoculum size of 100% can be explained. The air flow level used in this time course was 50 cm$^3$/min, only half of the level used for the time course with 50% inoculum size [air flow 100 cm$^3$/min]. An increased biomass concentration accompanied with a reduced air flow resulted in the insufficient oxygenation for each cell, and the overall ethanol production efficiency decreased as a consequence.

Relating this trend to Formula (9),

$$Q_p = xc \times de (g/l/h)$$

obviously the oxygenation level affects the overall system co-efficiency $Q_p$ by altering the cell instantaneous ethanol production rate $de$. Consequently, the statement proposed for the glucose fermentation by Z. mobilis, which states that “under optimal conditions, the system co-efficiency $Q_p$, g/l/h] is only related to the viable cell concentration [$xc$, cells/l] in the medium”, could be further extended as follows: “in an ethanol production process, when the value of $de$ is constant, system co-efficiency $Q_p$ is only related to the viable cell concentration [$xc$] in the medium; likewise, when viable cell concentration [$xc$] is constant, system co-efficiency $Q_p$ is only related to the value of $de$.” Therefore, when the viable cell concentration in the medium remains constant, the optimization of oxygenation level could always improve the overall system co-efficiency. The results of the shake flask experiment in Section 4.2.6 support this statement.

Apart from the effect of decreasing the fermentation efficiency when its level is overly low, oxygenation also affects the ethanol production by promoting the ethanol re-assimilation when it is overly high. The increase of oxygenation enables the microorganisms to further oxidize the produced ethanol, thus causing the reduction of ethanol yield. In this study, this was demonstrated by several fermentation time courses. Firstly, at the inoculum level of 10%, the time course with 100 cm$^3$/min air flow gave an ethanol yield 20% lower than the one with 20 cm$^3$/min [0.34 g/g compared to 0.43 g/g]. Secondly, at the inoculum level of 50%, the ethanol yield in the time course with 80 cm$^3$/min [0.45 g/g] was slightly higher than that with 100 cm$^3$/min which gave the highest system co-efficiency [0.44 g/g]. Thirdly, at the
inoculum level of 100%, with the increase in the air flow from 50 to 100 to 150 cm³/min, the ethanol yield decreased from 0.45 to 0.37 to 0.30 g/g respectively.

The final level of ethanol concentration \( p_f \) achieved in the fermentation medium can be expressed as follows:

\[
p_f = \frac{P_{\text{yie}} - P_{\text{con}}}{v} \text{ (g/l)}
\]  (10)

where \( P_{\text{yie}} \) and \( P_{\text{con}} \) respectively represent the total ethanol produced and consumed by the microorganism, and \( v \) is the volume of the fermentation medium. Based on Formula (8):

\[
P_{\text{yie}} = \int_{t_1}^{t_2} N \times de \text{ (g)}
\]  (8)

the oxygenation affects the final ethanol concentration for both values of \( P_{\text{con}} \) and \( de \). Therefore, an optimized oxygenation value would be essential to achieve a maximum ethanol production from xylose by \( P. stipitis \).

4.3.4. Significance and application of the cell instantaneous ethanol production rate \([de]\)

As far as \( de \), the cell instantaneous ethanol production rate, is concerned, it is only a theoretical value and cannot be experimentally determined. However, based on Formula (9), \( de \) can be expressed as:

\[
de = \frac{1}{x_c} Q_p = \frac{1}{x_c} \frac{p_f}{t_p} \text{ (g/cells/h)}
\]  (9.1)

According to Formula (6) in Section 3.11, the specific ethanol productivity \([q_p]\) equals:

\[
q_p = \frac{1}{x_A} \frac{p_f}{t_p} \text{ (g/g/h)}
\]  (6)

Comparing Formula (9.1) and Formula (6), both values of \( de \) and \( q_p \) represent the ethanol productivity based on biomass. For \( de \), it evaluates the fermentation capability of each cell \( Q_p \) divided by viable cell concentration \( x_c \), whereas \( q_p \) is a commonly used kinetic parameter to evaluate the efficiency of a fermentation process \([Q_p \text{ divided by average dry biomass } x_A]\). However, as viable cell
concentration \([x_c]\) and average dry biomass \([x_d]\) are both used to describe the biomass concentration in the medium, the value of \(de\) can be partly reflected by the value of \(q_p\) in the fermentation process. For instance, the xylose fermentation with 100\% inoculum size at 50 cm\(^3\)/min air flow gave similar system co-efficiency to that with 50\% inoculum size at 100 cm\(^3\)/min air flow. In Section 4.3.3, it was suggested that this was due to the reduced level of \(de\) under insufficient oxygenation conditions. As an evidence, the specific ethanol productivity\([q_p]\) in the time course with larger inoculum size was only half of that in the time course with smaller inoculum size but higher oxygenation level [0.122 g/l/h compared to 0.218 g/l/h; see Figure 4.8]. Thus, the value of \(q_p\) could be used to examine the value of \(de\) for the same strain cultured under different conditions.

In Section 4.3.3, oxygenation was used as an example for demonstration of its effects on the value of \(de\); there are however a number of other factors that may affect \(de\) and thus alter the fermentation performance. These include the medium composition, temperature and other environmental parameters. Furthermore, \(de\) as a value to describe the fermentation capability could also be used to describe the different fermentation efficiency between processes, or even between strains. It is an absolute value for the ethanol amount produced by a single cell within a given time; therefore it eliminates the potential error in the value of \(q_p\), which is caused by the weight difference of a single cell from different species.

For instance, supposing both \(Z. \text{mobilis}\) and \(P. \text{stipitis}\) are cultured under optimum conditions on glucose and xylose respectively and there are exactly the same number of viable cells for each strain, after a given culture time, the ethanol production of \(Z. \text{mobilis}\) will be substantially higher than that of \(P. \text{stipitis}\). This is because the glucose metabolism in the cells of \(Z. \text{mobilis}\) is much simpler and more efficient than the xylose metabolism in the cells of \(P. \text{stipitis}\). Therefore, even though both strains are cultured under optimum conditions, the \(de\) of \(Z. \text{mobilis}\) is higher than that of \(P. \text{stipitis}\), resulting in better performance of ethanol production.

4.3.5. Improvement of ethanol production by \(Z. \text{mobilis}\) and \(P. \text{stipitis}\)

In this chapter, \(Z. \text{mobilis}\) and \(P. \text{stipitis}\) were cultured on the glucose and xylose medium respectively to investigate their fermentation characteristics for the
co-fermentation. Viable cell concentration proved to be a key factor to improve the ethanol productivity for both strains, though in the case of *P. stipitis*, the oxygenation also played a crucial part.

The best result of *Z. mobilis* was achieved with an inoculum size of 100%, giving a system co-efficiency of 10.54 g/l/h and a specific ethanol productivity of 5.02 g/l/h. Both values are highest reported in the literature for *Z. mobilis* free cell in batch culture using a low initial sugar level of 50 g/l. Compared to the traditional 10% volume to volume inoculum, using a large inoculum size prepared as described in Section 3.5 significantly improved the system co-efficiency by 3.6 fold, and specific ethanol productivity by 1.5 fold. The system co-efficiency could be further improved if more cells were provided or a higher initial sugar concentration was used. Higher values of system co-efficiency have been reported in the literature using immobilized culture (Rebros et al., 2005) or continuous culture (Amin and Doelle, 1990).

The improvement of the specific ethanol productivity, however, was unexpected. The increased inoculum size was expected to increase the efficiency of ethanol production by increasing the viable cell concentration \[x_C\], but have no effects on the de, because it did not alter any environmental parameters. However, a 1.5 fold increase in the specific ethanol productivity was observed. It was suggested that the sufficient initial cells in the medium enabled a significant ethanol accumulation as soon as the fermentation commenced; therefore, the time required for cells to reach this concentration level by multiplication was reduced. The ethanol production capability based on biomass was thus improved.

For *P. stipitis*, the highest volumetric productivity of 0.755 g/l/h was achieved with an inoculum size of 100% at an air flow level of 50 cm³/min; however, when an inoculum size of 50% was supplied with an air flow level of 100 cm³/min, a similar volumetric productivity was obtained [0.734 g/l/h], and the specific ethanol productivity was double that of the former [0.218 g/g/h compared to 0.122 g/g/h]. This demonstrated that the time course with inoculum size of 50% would be economically more viable as it achieved a similar system co-efficiency using a considerably lower number of cells.
Both values of 0.734 g/l/h and 0.755 g/l/h were the highest system co-efficiency known for xylose fermentation in free cell batch culture. For fermentation with 50% inoculum size, it improved the system co-efficiency 5.2 fold and the specific ethanol productivity 2.9 fold compared to the best result obtained with a traditional inoculum size of 10% [0.142 g/l/h and 0.074 g/g/h respectively]. By providing cells an optimum oxygenation level, the system co-efficiency in the time course with 100% inoculum size had the potential to be further improved. For instance, if the specific ethanol productivity could be increased to a similar level to the time course with 50% inoculum size, the system co-efficiency would increase proportionally. However, this would require an increased aeration level for the high cell concentration in the medium. In order to find a solution for the concomitant foaming problem, other fermentation schemes could be tried out.

There was another reason for using the large inoculum size for *P. stipitis* culture on xylose. In this study for all fermentation processes culturing *P. stipitis* in a 1 l bioreactor with both inoculum sizes of 10% and 50%, it was observed that the efficiency of biomass growth was relatively low [see Figures 4.5 and 4.6]. Highest biomass accumulation occurred in the time course with 10% inoculum size at 100 cm$^3$/min air flow; cell concentration started with 2.2x10$^7$ cfu/ml and increased to 6.0x10$^8$ cfu/ml in 120 h. This rate of growth was comparatively low for xylose-utilizing yeast. In a previous study by us,*Pachysolen tannophilus* was able to achieve a cell concentration of 2.0x10$^9$ cfu/ml within 48 h using a traditional 10% inoculum size (data unpublished). In addition, considering the cell loss caused by the foaming problem, the only strategy to ensure a sufficient cell level of *P. stipitis* during the fermentation process was to inoculate a large amount of cells at the beginning.

The effect of oxygenation on the xylose fermentation observed in this study was in accordance with previous reports (Kruse and Schugerl, 1996; Taniguchi et al., 1997b; Chandrakant and Bisaria, 1998). Under anaerobic conditions, when the inoculum size was low, neither ethanol accumulation nor cell reproduction was observed. Even with a high inoculum size [see Table 4.4], the increased viable cell concentration in the medium could not compensate for the extremely low *de* value [$q_p$: 0.043 g/g/h]; the overall system co-efficiency remained low [0.270 g/l/h]. Ethanol re-assimilation
occurred in almost every time course. The trend was intensified when higher oxygenation levels were used. Ethanol loss up to 10% of the peak value was observed in the time course with 50% inoculum size and 100 cm$^3$/min air flow [20.56 g/l at 28 h to 18.15 g/l at 48 h].

For the co-fermentation process, inoculum size of 50% was chosen as a standard for both Z. mobilis and P. stipitis. Amongst the three inoculum levels investigated, size of 50% was the most moderate, giving a reasonably high performance of ethanol production. The inoculum size of 100% was unfavorable for the co-fermentation mainly for two reasons: (1) the overly high cell concentration made it considerably more difficult to control, especially in the case of P. stipitis; (2) the preparation of large inoculum requires additional inputs, e.g. time, media and equipment; compared to the 100% inoculum size, the 50% gave a better balance between production outcome and cost effectiveness.
Chapter 5

Immobilized cultures of *Z. mobilis* and *P. stipitis*

Immobilized culture of *Z. mobilis* and *P. stipitis* was investigated on glucose and xylose respectively for their feasibility to be employed in a co-fermentation process. In previous literature, gel encapsulation of *Z. mobilis* whole cells was reported to give a high ethanol productivity up to 43.6 g/l/h (Rebros et al., 2005). Immobilized *P. stipitis* was developed for the xylose fermentation with the objective of carrying out the process with a high cell density under aerobic conditions, eliminating the interference due to foaming.

### 5.1 Fermentations employing immobilized *Z. mobilis*

The techniques used for whole cell immobilization were described in Section 3.6. Cells from 400 ml inoculum medium were concentrated into 100 ml calcium alginate beads. This batch of beads was repeatedly cultured for all time course experiments with immobilized *Z. mobilis* culture till the beads were damaged. Then another batch of beads was made for further use.

#### 5.1.1 Effect of immobilization on the fermentation performance of *Z. mobilis*

Experiments were carried out for immobilized *Z. mobilis* using the same conditions as for free cell culture to determine the effect of immobilization on fermentation
performance. Beads used for this time course were freshly made, without a prior reconditioning process to allow cell accumulation. Thus the inoculum size of this time course was at a similar level to that of time courses with 50% inoculum size [also using cells from 400 ml inoculum medium to inoculate]. The fermentation conditions are as described in Section 3.7.2.

Figure 5.1 presents the results of sugar utilization and ethanol production of two time courses, employing free cells of Z. mobilis and immobilized Z. mobilis respectively. Both time courses showed similar trends on the substrate consumption and ethanol yield; however, results of immobilized culture were slightly inferior. It took an additional hour for immobilized Z. mobilis to use up all the sugars in the medium [5 h compared to 4 h in the free cell culture]. Ethanol production in the free cell culture peaked at the same time when all the glucose was completely consumed [4h]. By contrast, for immobilized culture, a perceptible increase in the ethanol concentration was observed after the full consumption of sugars at 5 h [22.92 g/l at 5 h and 23.53 g/l at 6 h]. Moreover, it was also observed that the peak value of ethanol concentration in the immobilized culture was slightly less than that in the free cell culture [23.53 g/l compared to 25.28 g/l].

![Figure 5.1: Comparison of sugar utilization and ethanol production between a free cell Z. mobilis culture and an immobilized culture.](image)

Figure 5.1: Comparison of sugar utilization and ethanol production between a free cell Z. mobilis culture and an immobilized culture.
Biomass of the immobilized culture was carried out both within the beads and in the fermentation medium. Dry weight biomass inside the beads showed a relative increase up to 6 fold during the fermentation process [0.8 mg before and 4.8 mg after the time course]. On the other hand, cell growth in the fermentation medium was considerably low. The medium remained clear during 11 h culture time. Cell growth in the culture fluid was undetectable by the current method of dry weight assay; viable cells in the medium slowly increased from an initial level of $10^4$ cfu/ml to $10^6$ cfu/ml at the end. The value was only 1/1000 of the highest viable cell concentration achieved in the free cell culture [$\sim 1.5 \times 10^9$ cfu/ml].

5.1.2. Effect of recycling of the immobilized \textit{Z. mobilis} beads

During the above culture process of the immobilized \textit{Z. mobilis}, the biomass accumulation within the beads was notably significant. According to the discussion for the free cell culture in Chapter 4, the performance of \textit{Z. mobilis} was definitively affected by the viable cell concentration in the medium. It was therefore considered important to determine the effect of the increased biomass within the beads on the fermentation performance. For this purpose, a batch of \textit{Z. mobilis} beads was repeatedly cultured under the same conditions. For this experiment only, fermentation medium of 1000 ml was used for two processes, instead of the usually employed 800 ml.

Table 5.1 summarizes the kinetic parameters of the two time courses using repeatedly cultured immobilized \textit{Z. mobilis} beads. The fermentation performance in the second time course was largely improved through the recycling of the beads. In association with an increase of the biomass within the beads from 6.8 to 11.5 mg, the fermentation time decreased from 7 h to 4 h, resulting in an increase in the system co-efficiency from 3.42 to 5.93 g/l/h. In both processes, ethanol yield was steady at 0.49 g/g, which was approximately 96% of the theoretical value, slightly lower than those obtained in the free cell culture with a large inoculum size [0.50–0.51 g/g].

Given that the biomass dry weight inside the beads was only 0.8 mg when the beads were initially made, the biomass level of 11.5 mg demonstrated a significant increase in the biomass concentration within the beads. However, after reusing the same batch
of beads for three time courses [including the one reported in Section 5.1.1] and two reconditioning processes prior to fermentation, the beads were severely damaged. At this stage, most of the beads split into half, resulting from the carbon dioxide produced during the ethanol producing process. Furthermore, due to the enormous cell propagation within, the physical characteristics of beads altered. The gel surface became increasingly rough and adherent to the glassware, which greatly increased the difficulty of inoculating these beads into the bioreactor. Concomitant with the destruction of beads, more cell leakage occurred during the fermentation process. In the time course reported in Section 5.1.1, it took 8 h for free cell concentration in the fermentation medium to reach a level of $1 \times 10^6 \text{ cfu/ml}$. When the same beads were cultured again in the 1000 ml of fermentation medium, by 6 h the free cell concentration in the medium increased to a level of $1 \times 10^6 \text{ cfu/ml}$; the repetition of this time course further reduced this time to 2 h.

**Table 5.1: Kinetic parameters of immobilized Z. mobilis on repeated culture**

<table>
<thead>
<tr>
<th>Fermentation time (h)</th>
<th>Biomass in the beads (mg) *</th>
<th>System co-efficiency (g/l/h)</th>
<th>Ethanol yield (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st time course</td>
<td>7</td>
<td>6.8</td>
<td>3.42</td>
</tr>
<tr>
<td>2nd time course</td>
<td>4</td>
<td>11.5</td>
<td>5.93</td>
</tr>
</tbody>
</table>

*Biomass concentration within the beads was assayed before and after each time course finished, using the method described in Section 3.6. Data was expressed as the absolute value from the dry weight assay by dissolving five beads in 10 ml of sodium citrate solution.

Another observation during the repeated culture process concerned the increased volume of fermentation medium. In the experiment reported in Section 5.1.1, it took 5 h for the beads to utilize 800 ml of Glucose Medium; however, with the same batch of beads and the same initial glucose concentration, another extra 2 h was required to use up all the glucose in the 1000 ml medium.

To summarize, the recycling of the same batch of beads greatly increased the fermentation performance of Z. mobilis culture on glucose. However, the calcium
alginate gel beads tended to be damaged easily during repeated usage.

**5.1.3. Effect on glucose fermentation of a sieve plate in the modified bioreactor**

As described in Section 3.7.4.4, one of the fermentation schemes used for the co-fermentation process employed a modified bioreactor, which is an innovative design in this study [see Figures 3.5 and 6.4]. Through the addition of a sieve plate to the bioreactor, this fermentation scheme enabled an easy removal of all immobilized beads from the fermentation medium, without altering other conditions within the bioreactor. However, under this circumstance *Z. mobilis* beads were not able to pass through the sieve plate and therefore were only exposed to the fermentation medium above the sieve plate. The distribution of material in the medium only relied on the continuous stirring by the stirrer underneath the sieve plate. Furthermore, the addition of the sieve plate also changed the medium rheology. Therefore, it was necessary to identify the potential changes of fermentation characteristics when the glucose fermentation was restricted to above the sieve plate.

Two experiments were carried out for this purpose. In the first experiment, immobilized *Z. mobilis* beads that was freshly made was cultured in the bioreactor with the sieve plate under standard conditions, and the obtained results are compared to those reported in Section 5.1.1. In the second experiment, the same batch of beads was repeatedly cultured with and without the sieve plate.

Figure 5.2 displays the results of sugar utilization and ethanol production for time courses carried out in a normal bioreactor and in a modified bioreactor. In Figure 5.2 (a), both time courses employed *Z. mobilis* beads that were freshly made from two batches; as an identical procedure was used, the biomass concentration for the two batches were similar. Because of this similarity in biomass, both trends of sugar utilization and ethanol consumption in these two time courses were remarkably similar. The sugar consumption in the time course with the sieve plate was slightly slower [complete at 6 h, with 1.92 g/l left at 5 h]. However, the ethanol production in both time courses peaked at 6 h, giving a similar level of peak ethanol concentration [23.40 g/l compared to 23.11 g/l].
Chapter 5. Immobilized cultures of \textit{Z. mobilis} and \textit{P. stipitis}

![Figure 5.2](image.png)

**Figure 5.2:** Comparison of sugar utilization and ethanol production between immobilized \textit{Z. mobilis} cultures with and without the sieve plate: (a) different batches with similar biomass; (b) same batch repeatedly cultured.

In Figure 5.2 (b), again similar results were obtained for time courses with and without the sieve plate. However, since this time the same batch of \textit{Z. mobilis} beads was repeatedly used, the time course with the sieve plate gave a higher performance compared to that without the sieve plate. Ethanol peaked at 5 h together with a complete utilization of all the glucose, which was 1 h earlier than the one without the sieve plate. Nevertheless, the final ethanol concentration of two time courses was at a
similar level [22.99 g/l and 22.77 g/l respectively].

It can be thus concluded that the addition of the sieve plate had little effect on the performance of glucose fermentation by *Z. mobilis*. The addition of the sieve plate could have decreased the liquid fluidity of the fermentation medium; however, this disadvantage is relatively minor and could be easily compensated by the vigorous metabolism of the bacterium resulting from the high density of viable cells within the beads.

### 5.1.4. Biomass accumulation of immobilized *Z. mobilis* during the reconditioning process

It was shown that the biomass concentration within the beads had a significant impact on the fermentation characteristics. As the reconditioning process of the immobilized beads was used to ensure a sufficient biomass level for the fermentation process, studies were carried out to determine the best conditions for the reconditioning process. The effect of the following three factors was investigated, viz. the reconditioning times, the addition of CaCl₂, and the glucose concentration in the reconditioning medium.

For the first experiment, six reconditioning processes were carried out on a batch of freshly-made beads. Incubation was carried out in 250 ml conical flasks with 100 ml Glucose Medium. In the first three flasks, CaCl₂ was added at a concentration of 5.55 g/l (50 mM), a level used for the Glucose Storage Medium [see Table 3.2]; the other three flasks were free of CaCl₂. In the second experiment, another batch of freshly-made beads was reconditioned three times, in the Glucose Medium with increased glucose concentration of 20g/l, 50 g/l and 80 g/l respectively. Both batches of beads were then subjected to the fermentation time course after reconditioning. The results of biomass growth during the reconditioning experiments are shown in Table 5.2 and Table 5.3.
Table 5.2: The effect of CaCl$_2$ on the biomass of immobilized Z. mobilis during reconditioning

<table>
<thead>
<tr>
<th></th>
<th>1$^{\text{st}}$ batch of Z. mobilis beads</th>
<th>Dry biomass weight of 5 beads (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original beads</td>
<td>Freshly-made beads</td>
<td>0.9</td>
</tr>
<tr>
<td>With CaCl$_2$</td>
<td>After 1$^{\text{st}}$ culture</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>After 2$^{\text{nd}}$ culture</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>After 3$^{\text{rd}}$ culture</td>
<td>2.2</td>
</tr>
<tr>
<td>Without CaCl$_2$</td>
<td>After 4$^{\text{th}}$ culture</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>After 5$^{\text{th}}$ culture</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>After 6$^{\text{th}}$ culture</td>
<td>4.8</td>
</tr>
<tr>
<td>Time course</td>
<td>After Time course</td>
<td>9.2</td>
</tr>
</tbody>
</table>

Table 5.3: The effect of glucose concentration on the biomass growth of immobilized Z. mobilis during reconditioning

<table>
<thead>
<tr>
<th></th>
<th>2$^{\text{nd}}$ batch of Z. mobilis beads</th>
<th>Dry biomass weight of 5 beads (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original beads</td>
<td>Freshly-made beads</td>
<td>0.8</td>
</tr>
<tr>
<td>Reconditioning</td>
<td>20 g/l glucose</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>50 g/l glucose</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>80 g/l glucose</td>
<td>4.3</td>
</tr>
<tr>
<td>Time course</td>
<td>After Time course</td>
<td>7.8</td>
</tr>
</tbody>
</table>

As shown in both tables, the dry cell weight of both batches of beads showed a steady increase with serial reconditioning. Biomass accumulation in the reconditioning processes with CaCl$_2$ supplement was inferior compared to that achieved in the processes without CaCl$_2$. Biomass increase in the three processes with CaCl$_2$ was respectively 0.1 [original to 1$^{\text{st}}$ reconditioning], 0.6 [1$^{\text{st}}$ to 2$^{\text{nd}}$] and 0.6 mg [2$^{\text{nd}}$ to 3$^{\text{rd}}$]. In other processes, much higher increase was achieved [1.1 mg from 3$^{\text{rd}}$ to 4$^{\text{th}}$ and 1.8 mg from 4$^{\text{th}}$ to 5$^{\text{th}}$ for the 1$^{\text{st}}$ batch of beads, and 0.6, 1.0 and 1.9 mg respectively in the three reconditioning processes for the 2$^{\text{nd}}$ batch]. Therefore it appears that the addition of CaCl$_2$ into the medium was not beneficial to the biomass growth within the beads.
In the reconditioning processes for the first batch of beads, despite the steady biomass increase during the first five times, the biomass concentration slightly decreased at the sixth process [5.1 to 4.8 mg], but again showed dramatic increase after the beads were cultured in the fermentation time course [4.8 mg into 9.2 mg]. A similar trend was observed for the reconditioning processes of the second batch of beads. As can be seen from Table 5.3, after increasing gradually during the three reconditioning processes, the biomass within the beads showed a large rise after the beads were used for fermentation time course [4.3 mg to 7.8 mg]. In addition, though both batches of beads had been reconditioned several times under various conditions prior to the time course experiments, their performance was at a similar level to the batch that had not been reconditioned but directly employed for fermentation [results reported in Section 5.1.1]. Consequently, no matter what the medium composition, reconditioning times, or initial sugar level was used, the reconditioning processes did not improve the biomass concentration within the beads to a very significant level, especially in comparison with the fermentation time courses.

Physical bead damage was observed for both batches of beads during the repeated usage in the reconditioning process. It started with the split of the beads and occurred even with the lowest glucose concentration of 20 g/l. The damage became increasingly severe with the progress of the experiment. This suggested that the bead damage was mainly attributed to the metabolic activity of the cells within the beads. The continuously stirring impeller in the bioreactor was not a significant factor compared to that.

5.2. Fermentations employing immobilized P. stipitis

As described in Section 3.6, a batch of P. stipitis beads concentrated P. stipitis cells from 1000 ml inoculum medium into 250 ml beads. Consequently, when the full batch was inoculated into 800 ml fermentation medium, the inoculum size was 125% [cells from 1000 ml inoculum medium into 800 ml fermentation medium]. The utilization of a large amount of yeast cells was mainly to ensure a high biomass concentration for the xylose fermentation.
5.2.1. Fermentation characteristics of immobilized *P. stipitis* culture

In order to investigate the fermentation characteristics of immobilized *P. stipitis* culture, the first two time courses were carried out under standard xylose fermentation conditions as described in Section 3.7.2. The air flow of these two time courses was controlled at 80 cm$^3$/min, which was a level higher than the best air flow of 50 cm$^3$/min for the free cell culture with 100% inoculum size. The result of the first time course employing the freshly prepared beads of immobilized *P. stipitis* was compared to that of the free cell culture with the most efficient fermentation, i.e. the time course with 50% inoculum size and 100 cm$^3$/min air flow. The immobilized culture had an inoculum size 2.5 fold higher than the free cell culture. The comparison of the kinetic parameters is made in Table 5.4. As the majority of biomass in the immobilized culture was entrapped in the calcium alginate beads, the specific ethanol productivity was not calculated. Despite a much higher inoculum size, the immobilized *P. stipitis* gave considerably lower kinetic parameters. Both time courses achieved an ethanol yield of 0.44 g/g; however, it took immobilized yeast 36 h to utilize all the xylose in the medium, 8 h longer than the free cell culture [28 h]. This led to an inferior system co-efficiency of 0.553 g/l/h, in contrast to the 0.734 g/l/h in the free cell culture.

<table>
<thead>
<tr>
<th>Time courses</th>
<th>Inoculum size (%)</th>
<th>Air flow (cm$^3$/min)</th>
<th>Ethanol peak time (h)</th>
<th>System co-efficiency (g/l/h)</th>
<th>Ethanol yield (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free cell</td>
<td>50%</td>
<td>100</td>
<td>28</td>
<td>0.734</td>
<td>0.44</td>
</tr>
<tr>
<td>Immobilized</td>
<td>125%</td>
<td>80</td>
<td>36</td>
<td>0.553</td>
<td>0.44</td>
</tr>
</tbody>
</table>

The second time course employing immobilized *P. stipitis* was a repeat of the first time course, in order to investigate the fermentation characteristics when the same batch of *P. stipitis* beads was reused. As shown in Table 5.5, the kinetic parameters of these two time courses were almost identical to each other, except that in the second culture process, ethanol peaked 2 h earlier [34 h compared to 36 h in the first
process], resulting in a slightly higher system co-efficiency [0.581 compared to 0.553 g/l/h].

**Table 5.5: Kinetic parameters of immobilized *P. stipitis* in repeated culture**

<table>
<thead>
<tr>
<th>Time courses</th>
<th>Inoculum Size (%)</th>
<th>Air flow (cm³/min)</th>
<th>Ethanol peak time (h)</th>
<th>System co-efficiency (g/l/h)</th>
<th>Ethanol yield (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st TC *</td>
<td>125%</td>
<td>80</td>
<td>36</td>
<td>0.553</td>
<td>0.44</td>
</tr>
<tr>
<td>2nd TC *</td>
<td>125%</td>
<td>80</td>
<td>34</td>
<td>0.581</td>
<td>0.44</td>
</tr>
</tbody>
</table>

* TC: time course

During the repeated culture of immobilized *P. stipitis*, there was little biomass increase within the beads. Table 5.6 summarizes the results of the biomass assay for the two time courses employing immobilized *P. stipitis*. It can be seen that the increase of dry weight was relatively low [3.0 mg after the 1st culture, and 2.5 mg after the 2nd culture] and the viable cell concentration even showed a slight decrease [1.8x10⁸ to 9.8x10⁷ to 8.4x10⁷ cfu/ml].

**Table 5.6: Biomass concentration within the immobilized *P. stipitis* beads during two time courses**

<table>
<thead>
<tr>
<th>Sampling time</th>
<th>5 beads in 10 ml sodium citrate solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dry weight (mg/9 ml)</td>
</tr>
<tr>
<td>Fresh beads</td>
<td>9.1</td>
</tr>
<tr>
<td>After 1st time course</td>
<td>12.1</td>
</tr>
<tr>
<td>After 2nd time course</td>
<td>14.6</td>
</tr>
</tbody>
</table>

It was also found that cell leakage was significant for the immobilized *P. stipitis* beads. During the culture processes, vigorous cell growth was observed in the fermentation medium, making the initially clear medium turbid and cloudy as cells multiplied. Along with the increased cell concentration in the medium, foaming occurred and it was necessary to add antifoam to control the foaming at the later
stage of the fermentation. The trends of biomass concentration in the fermentation medium in these two time courses are shown in Figure 5.3. Viable cells in both time courses started at approximately $1 \times 10^6$ cfu/ml, gradually increased to a level of $1 \times 10^8$ cfu/ml by 20 h and then leveled out.

![Figure 5.3: Cell growth in the fermentation medium of immobilized P. stipitis culture](image)

Bead damage for immobilized *P. stipitis* was not as severe as that for immobilized *Z. mobilis*. Though both beads of *P. stipitis* and *Z. mobilis* were made following the same procedure, most *P. stipitis* beads remained intact after two fermentation time courses.

Results so far indicated that the fermentation characteristics of immobilized *P. stipitis* were not promising. The overall system co-efficiency was lower compared to free cell cultures, although the biomass concentration in the immobilized culture was considerably higher. In addition, biomass accumulation within the beads was relatively low, and yeast cells tended to reproduce in the fermentation medium rather than within the beads. All these factors were considered unfavorable for a co-culture process on the glucose/xylose sugar mixture. The beads were then cultured under different environmental conditions to determine whether the fermentation performance could be improved by optimizing the conditions.
5.2.2. Immobilized \textit{P. stipitis} culture under different oxygenation levels

The two time courses reported in the previous section employing immobilized \textit{P. stipitis} were carried out with an air flow of 80 cm\textsuperscript{3}/min. As shown in the experiments with free cell culture, the oxygenation level was extremely crucial for xylose fermentation by \textit{P. stipitis}. With an increased biomass level in the medium, an increased level of oxygenation needed to be provided to meet the oxygen requirement of each cell [see Sections 4.2.2 and 4.2.3]. Given that the biomass entrapped in the immobilized \textit{P. stipitis} was exceptionally high [cells from 1000 ml into 250 ml beads], time courses with increased aeration level were carried out as an effort to improve the overall system co-efficiency.

Table 5.7: Kinetic parameters of immobilized \textit{P. stipitis} culture under different oxygenation levels

<table>
<thead>
<tr>
<th>Time courses</th>
<th>Kinetic Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air flow (cm\textsuperscript{3}/min)</td>
<td>Ethanol peak time (h)</td>
</tr>
<tr>
<td>80</td>
<td>34</td>
</tr>
<tr>
<td>100</td>
<td>32</td>
</tr>
<tr>
<td>150</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 5.7 summarizes kinetic parameters of two time courses with increased air flow levels for immobilized \textit{P. stipitis}, and compares them with the time course reported in Section 5.2.1. With an increase of air flow from 80 to 100 to 150 cm\textsuperscript{3}/min, the fermentation time required for a complete consumption of the xylose in the medium decreased from 34 h to 32 to 30 h respectively. However, this was associated with a decrease in the ethanol yield, from 0.44 g/g to 0.41 to 0.36 g/g. As a result, the system co-efficiency did not increase along with the increase in the air flow. On the contrary, it decreased slightly, from 0.581 g/l/h to 0.577 to 0.524 g/l/h for the time courses with air flow levels of 80, 100 and 150 cm\textsuperscript{3}/min respectively. The decrease in the ethanol yield was probably due to the accelerated ethanol re-assimilation under higher oxygenation conditions. It could hence be said that for the immobilized \textit{P.}
Chapter 5. Immobilized cultures of *Z. mobilis* and *P. stipitis*

*stipitis*, the increase of oxygenation level was not advantageous for the improvement of fermentation performance.

### 5.2.3. Immobilized *P. stipitis* culture with different initial sugar concentrations

The immobilized *P. stipitis* culture was also employed to investigate the effect of initial sugar concentration on xylose fermentation. The immobilized culture was employed for this experiment because the foaming problems in the free cell culture made it difficult to maintain the high cell concentration for a prolonged period of fermentation time. In this experiment, two time courses were carried out, with an initial xylose concentration of 20 g/l and 80 g/l respectively. Results of these two time courses are shown in Table 5.8, with the results from time course of 50 g/l xylose as a comparison.

**Table 5.8: Kinetic parameters of immobilized *P. stipitis* culture with different initial xylose concentrations**

<table>
<thead>
<tr>
<th>Time courses</th>
<th>Kinetic Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial xylose concentration (g/l)</td>
<td>Air flow (cm$^3$/min)</td>
</tr>
<tr>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>50</td>
<td>80</td>
</tr>
<tr>
<td>80</td>
<td>150</td>
</tr>
</tbody>
</table>

As shown in Table 5.8, along with the increased sugar concentration, the time used to finish all the sugars increased from 15 h to 34 h to 52 h. Time courses carried out with 20 and 50 g/l xylose showed similar results on the system co-efficiency [0.536 and 0.581 g/l/h] and ethanol yield [0.43 and 0.44 g/g]. Though the time course with 80 g/l gave slightly inferior system co-efficiency of 0.489 g/l/h, it could be mainly a result of the reduced ethanol yield of 0.37 g/g, which indicated an increased ethanol re-assimilation in this time course due to the increased air level [150 cm$^3$/min compared to 80 cm$^3$/min in other two time courses]. Considering this fact, the different initial sugar levels used in these three time courses appeared to have little effect on the fermentation performance of the immobilized *P. stipitis*. 

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5.3. Discussion

5.3.1. Immobilized *Z. mobilis* culture

5.3.1.1. Fermentation characteristics of immobilized *Z. mobilis*

Based on the results reported in Section 5.1.1, when the amount of biomass in the fermentation medium was at a similar level, the immobilized culture tended to show slightly inferior results compared to the free cell culture. This was further confirmed by all time course experiments carried out with freshly prepared *Z. mobilis* beads, which contained similar amount of cells to the inoculum size of 50% [see Sections 5.1.3 and 5.1.4]. It is considered that the time required for material to diffuse in and out of the beads could account for the extended time for ethanol to peak and the slow sugar utilization. This problem, however, had only minimal effect on the fermentation performance.

Another problem observed for the immobilized *Z. mobilis* was the decreased peak ethanol concentration. As reported in Section 5.1.2, ethanol yield in both time courses was 0.49 g/g, which was slightly less than those obtained in the free cell culture [0.50–0.51 g/g]. Again this could be attributed to the diffusion; some ethanol produced could be retained within the beads and slowly released to the medium. The second cause might be the undesirable dilution of the initial sugar concentration from the inoculation of immobilized *Z. mobilis*. Gel beads could carry over some liquid from their previous environment into the bioreactor, and the diluted initial sugar concentration could result in a reduced final ethanol concentration.

In spite of these problems, the whole cell immobilization has some distinct advantages in the fermentation process. Firstly, it can be conveniently recycled and reused, therefore lowering the costs involved. This is of particular significance when a large inoculum size is required. Secondly, the biomass within the beads would continuously accumulate during the culture processes when recycled, and the increased biomass concentration greatly benefits the ethanol production [see Section 4.3.1].
5.3.1.2. Biomass accumulation within the beads

As reported in Section 5.1.2, when the same batch of beads was repeatedly cultured, the fermentation time significantly decreased from 7 h to 4 h. This was associated with a one fold increase of biomass within the beads [6.8 mg after 1st time course, and 11.5 mg after 2nd time course]. Considering that the dry biomass weight was only 0.8 mg when originally made [see Section 5.1.1], the cell concentration increased more than 10 fold for the immobilized Z. mobilis after only a few fermentation processes. The efficient biomass accumulation further indicated that Z. mobilis cells were acclimatized to the gel environment and grew well inside the beads.

The increased cell amount within the beads also led to the improvement of system co-efficiency [3.42 g/l/h to 5.93 g/l/h]. This trend was in accordance with that observed for the free cell culture [see Section 4.1]. An advantage of immobilized Z. mobilis culture is that through a number of repeated culture processes, the biomass within the beads could naturally accumulate to an extremely high level, and result in exceptionally high system co-efficiency. According to the report by Rebros et al. (2005), a system co-efficiency of 43.5 g/l/h was achieved after 72 times recycling of the same batch of immobilized Z. mobilis.

5.3.1.3. Reconditioning of the beads

Results reported in Section 5.1.4 showed that the biomass accumulation in the reconditioning was of relatively low efficiency compared to that in a fermentation time course. Therefore, the reconditioning process was considered as an inefficient way for improving the biomass concentration within the beads, especially when high initial cell concentration was entrapped inside [cells from 400 ml medium into 100 ml beads]. Nevertheless, it remained an effective method to revive the metabolic activity of the cells and allow cells to adapt to the medium when beads were returned from storage. Furthermore, these experiments provided important information regarding the fermentation characteristics of immobilized Z. mobilis.

As far as the effect of the CaCl₂ added into medium is concerned, the purpose of adding Ca²⁺ was to reduce the potential bead damage caused by the dissolution of calcium alginate gel. However, as shown in Section 5.1.4, the addition of CaCl₂ appeared to be a disadvantage for the cell growth. Moreover, the actual presence of
Ca$^{2+}$ in the medium is uncertain. During the media preparation, a small amount of a white precipitate was formed after the addition of CaCl$_2$, which could be CaSO$_4$ formation resulting from the reaction between CaCl$_2$ and (NH$_4$)$_2$SO$_4$. Therefore, there was no positive outcome by the addition of CaCl$_2$ and it was no longer added for experiments that followed.

It is, however, necessary to minimize bead damage, since it considerably reduces the number of times that the beads could be recycled and increases the costs involved. During the reconditioning processes [see Section 5.1.4], it was observed that the bead damage occurred even in media with glucose concentration as low as 20 g/l. To overcome this problem and increase the beads durability, new immobilization material able to withstand multiple recycling should be developed.

The amount of glucose in the medium is considered a major reason for the inability of the reconditioning process to significantly improve the biomass concentration within the beads. As shown in Tables 5.2 and 5.3, the biomass level within the beads did show some increase after reconditioning, but it was too slight to have any notable effect on the fermentation performance. Significant cell accumulation occurred only in the time course experiment with 800 ml fermentation medium. Beads that were repeatedly cultured in the time courses showed markedly improved fermentation performance, while those that had been reconditioned six times made hardly any difference to the ethanol productivity [see Sections 5.1.2 and 5.1.4 respectively]. This indicated that the amount of glucose in the medium was crucial in cell accumulation. The reconditioning medium and fermentation medium contained 5 g and 40 g glucose respectively [50 g/l with respective volume of 100 ml and 800 ml]. As Z. mobilis cells need the released ATP from the glucose metabolism process for cell growth, the increased amount of glucose could provide the bacterium more ATP for reproduction, leading to a higher level of cell number.

A similar trend was also observed for the fermentation time required to convert an increased volume of fermentation medium. As reported in Sections 5.1.1 and 5.1.2, when the fermentation medium increased from 800 ml to 1000 ml, an extra two hours were required to convert all the glucose to ethanol by Z. mobilis. Both observations indicated that for a glucose fermentation process by Z. mobilis, the total
amount of substrate available is the most decisive factor in terms of both biomass growth and fermentation performance, rather than the concentration of the substrate in the medium. When the same amount of cells was employed, in the medium with a larger volume which contained a larger amount of substrate, cell growth could achieve a higher level which was unattainable with less substrate; likewise, longer fermentation time would be required to transform the larger amount of substrate.

Specific to the fermentation performance, it is suggested that the fermentation time is only determined by the ratio of substrate to the biomass, which could further affect the system co-efficiency and other kinetic parameters. According to Formula (8.1) in Section 4.3.1,

\[ P_{\text{yie}} = N \times de \times (t_2 - t_1)(\text{g}) \]  \hspace{1cm} (8.1)

the fermentation time can be expressed as:

\[ t_2 - t_1 = \frac{1}{de} \frac{P_{\text{yie}}}{N} (\text{h}) \]  \hspace{1cm} (8.4)

Consequently, in a fermentation process where \( de \) stays constant, the fermentation time required to utilize all the substrate is only determined by the absolute amount of the produced ethanol \([P_{\text{yie}}]\) and the number of viable cells \(N\) in the medium. Since the amount of ethanol produced is directly proportional to the amount of substrate in the medium, it is the ratio of the substrate to the biomass that determines the fermentation time and the corresponding process efficiency. This explains why in a scaled-up process, with a large volume of medium, a large inoculum is always required to ensure a reasonable fermentation efficiency.

To summarize, significant characteristics shown by immobilized \( Z. \text{ mobilis} \) include the efficient conversion of glucose to ethanol, ability to be easily recycled, low cell leakage during the fermentation process and the potential to accumulate cells to a high concentration within the beads. All these characteristics made the immobilized \( Z. \text{ mobilis} \) an ideal form to be used in the co-culture process.

### 5.3.2. Immobilized \( P. \text{ stipitis} \) culture

As mentioned at the beginning of Chapter 5, the primary motive of immobilizing \( P. \text{ stipitis} \) was to control the foaming problem occurred during the free cell culture.
Moreover, immobilization was also a good method in terms of the employment of a large inoculum size and the repeated use of the same batch of beads. During the experiments, *P. stipitis* showed several unique characteristics when immobilized; these were neither similar to the immobilized *Z. mobilis*, nor like the free cell culture of *P. stipitis*.

### 5.3.2.1. Differences between the immobilized *P. stipitis* and immobilized *Z. mobilis*

Major differences between immobilized *P. stipitis* and immobilized *Z. mobilis* are as follows.

**No improvement in the fermentation efficiency when repeatedly cultured**

When same batch of *P. stipitis* beads was repeatedly used in two time courses, similar results was obtained [See Table 5.5; Section 5.2.1]. This observation was very dissimilar to the immobilized *Z. mobilis* culture, where the fermentation performance significantly improved during the repeated use of the same batch of beads [see Sections 5.1.2 and 5.1.3].

**Insignificant increase in biomass within the beads**

The repeated culture of the same batch of immobilized *P. stipitis* beads had no effect on the improvement of the fermentation efficiency; neither could it increase the biomass concentration within the beads. For immobilized *Z. mobilis*, an increase greater than 10 fold [0.8 mg to 11.5 mg] in biomass was observed after three time course experiments [see Section 5.3.1.2]. In contrast, the increase in biomass for immobilized *P. stipitis* was only 60% [9.1 mg to 14.6 mg] after two time course experiments [see Section 5.2.1].

**Cell leakage during the fermentation process**

Time courses employing immobilized *Z. mobilis* showed a low level of free cells [maximum level 1x10⁶ cfu/ml] in the fermentation medium [see Sections 5.1.1 and 5.1.2]. However, in the time courses with immobilized *P. stipitis*, cell multiplication in the medium was vigorous; the free cell concentration increased from a level of 1x10⁶ cfu/ml to 1x10⁸ cfu/ml within 20 h. This number of viable cells was significant for *P. stipitis* culture on xylose; it was almost half the value of the average viable cell
Chapter 5. Immobilized cultures of *Z. mobilis* and *P. stipitis*

concentration \( \sim 2.3 \times 10^8 \) cfu/ml] in a time course with a regular inoculum size of 10% [see Figure 4.5; Section 4.2.2], and even higher than the lowest value of viable cell concentration \( \sim 8.0 \times 10^7 \) cfu/ml] in the time course with 100% inoculum size after cell loss [See Figure 4.7; Section 4.2.4].

There are probably two reasons contributing to this difference in the cell leakage between immobilized *Z. mobilis* and *P. stipitis* cultures. Firstly, *Z. mobilis* is an anaerobic bacterium with little oxygen tolerance; it is able to grow well under anaerobic conditions and therefore prefers to reproduce within the beads. In contrast, *P. stipitis* is an aerobic yeast. Reported literature indicated that the yeast hardly grows under anaerobic conditions. Therefore a medium environment with limited oxygen supply is more favorable for the cell growth compared to the interior of gel beads. This also explains its inability to multiply significantly within the beads.

Secondly, the different fermentation time to utilize all the sugars could also lead to the different final levels of free cell concentration in the medium. Fermentations using immobilized *P. stipitis* usually took more than 30 h to consume all the xylose, which gave cells sufficient time to reproduce and reach a high cell concentration. By contrast, all sugars in glucose fermentation with immobilized *Z. mobilis* usually could be completely consumed within 8 h.

**Low level of bead damage**

In contrast to the easily damaged *Z. mobilis* beads, immobilized *P. stipitis* beads were considerably more durable, able to withstand six fermentation time courses, while most beads remained intact and maintained their shape. The damage caused to *Z. mobilis* beads was considered to result from the large amount of carbon dioxide produced during the metabolism process of ethanol production; consequently, the low level of bead damage in the immobilized *P. stipitis* only indicated that the carbon dioxide production within the beads was at such a slow rate that was unable to cause any damage to the beads. It can be thus inferred that the simultaneous ethanol production was also at a low efficiency.

All these characteristics suggested that *P. stipitis* cells within the gel beads had a low activity. Because of the low cell activity, the efficiency of both biomass growth and
cell metabolism was low. This conclusion is also supported by the relatively low system co-efficiency [~0.5 g/l/h] achieved with the large amount of cells entrapped in the beads [equal to an inoculum size of 125%]. As shown in Table 5.6 [see Section 5.2.1], although the biomass within the beads did not show a significant increase, a stable level of viable cells was maintained during the culture processes. In association with the high level of free cells in the fermentation medium [see Section 5.2.1], the overall amount of biomass in the time courses with immobilized \( P. stipitis \) was at a substantially high level. In Section 4.3.3 it is suggested that the system co-efficiency of xylose fermentation by \( P. stipitis \) only relates to two values, viz. viable cell concentration \( [x_c] \) and the cell instantaneous ethanol production rate \([de]\). In this instance, the value of \( x_c \) was sufficiently high; thus the only factor that could cause the low system co-efficiency was the \( de \). According to Table 5.4 [see Section 5.2.1], comparing the kinetic parameters between a free cell \( P. stipitis \) culture and an immobilized culture, the immobilized culture with a much higher inoculum size gave an inferior system co-efficiency [0.553 g/l/h compared to 0.734 g/l/h in free cell culture]. This indicated that the value of \( de \) of \( P. stipitis \) cells entrapped in the beads was exceptionally low, which further supports the low cell activity.

Major factors affecting the value of \( de \) include the intrinsic fermentation capability of the fermenting strain and the environmental conditions. The decreased \( de \) value in the immobilized \( P. stipitis \) culture suggested that the environment within the gel beads was unfavorable for the yeast cells. The aeration level inside the gel beads was significantly limited. In addition with the large amount of cells entrapped inside, it can be treated as a semi-anaerobic condition within the beads. The insufficient oxygen level benefits the cell activity of \( Z. mobilis \), but was inhibitory to the metabolic system of \( P. stipitis \). The cell activity was consequently limited. Previous literature studying the immobilized \( P. stipitis \) culture also reported a poor fermentation performance on xylose (Grootjen et al., 1991b).

### 5.3.2.2. Effects of oxygenation level on the immobilized \( P. stipitis \) culture

Time courses with increased aeration levels were carried out for the immobilized \( P. stipitis \) culture [see Section 5.2.2]. With a higher air flow provided, it was expected that this would increase the cell activity within the beads and therefore improve the overall fermentation performance. However, as shown in Table 5.7 [see Section
5.2.2, the increased oxygenation only reduced the ethanol yield, having no positive effect on the system co-efficiency.

According to the discussion in Section 4.3.3, oxygenation affects the xylose fermentation on two counts: (1) accelerating ethanol re-assimilation, (2) affecting the value of $de$. Under the conditions of these time courses, the ethanol re-assimilation was accelerated by the increased aeration, but the $de$ hardly improved. It is considered that the increased air flow to the bioreactor hardly changed the aeration levels inside the gel beads; but the excessive oxygen definitely helped the further oxidation of the excreted ethanol in the fermentation medium, and thus decreased the final ethanol yield.

The reason for the unimproved aeration within the beads might be the compactness of the gel beads, which made it difficult for air to diffuse into the centre of beads. As the biomass concentration in the immobilized $P. stipitis$ beads tended to remain at a constant level and the aeration within the beads was hardly affected by the conditions outside, both values of $x_C$ and $de$ remained approximately the same. Therefore, according to Formula (9) [see Section 4.3.1], during the repeated culture of the same batch of $P. stipitis$ beads, the system co-efficiencies of all these fermentation processes were expected to be similar. Experimental results support this theory. All together six time courses were carried out using immobilized $P. stipitis$. Two were carried out to investigate the fermentation characteristics [see Section 5.2.1]; two were carried out at different aeration levels [see Section 5.2.2], and two were carried out with different initial xylose concentrations [see Section 5.2.3]. Similar system co-efficiencies were obtained in all six time courses [0.553, 0.581, 0.577, 0.524, 0.536 and 0.489 g/l/h respectively]. This evidence further confirms the viewpoint that the system co-efficiency only relates to the viable cell concentration $x_C$ and cell instantaneous ethanol production rate $[de]$.  

The similar system co-efficiencies also indicated that in contrast to free cell culture of $P. stipitis$, the system co-efficiency of immobilized culture was hardly affected by external environmental conditions. It is more an intrinsic characteristic for each batch of immobilized beads, determined by the original biomass entrapped within, and the diameter and compactness of the gel beads. Consequently, the system co-efficiency
reported here \(-0.5 \text{ g/l/h}\) was only a characteristic of the beads used in this study; the variation of the value was mainly from the different ethanol yield caused by ethanol re-assimilation.

5.3.2.3. Effects of initial xylose concentration on the immobilized \(P. \textit{stipitis}\) culture

According to Formula (9) [see Section 4.3.1], the initial sugar concentration does not have a direct effect on the system co-efficiency \(Q_p\). However, higher initial sugar concentration is always desirable for an industrial production process, because it leads to a higher final ethanol concentration, which will decrease the equipment cost and make the subsequent distillation process more cost efficient. In some early studies investigating the optimum initial sugar concentration for xylose fermentation (Laplace et al., 1991b; Bravo et al., 1995; Kruse and Schugerl, 1996), 25 g/l was reported as the optimum value; any higher level of xylose including 50 g/l was claimed to inhibit the fermentation.

The results obtained in this study were contrary to these reports. In the free cell culture of \(P. \textit{stipitis}\) on xylose, efficient ethanol production was achieved with 50 g/l initial xylose [system co-efficiency of 0.755 g/l/h with ethanol yield of 0.45 g/g]. In the immobilized culture with an increased xylose concentration of 80 g/l, inferior system co-efficiency was achieved [0.489 g/l/h], but it was mainly due to the reduced ethanol yield [0.37 g/g]. Moreover, the value of 0.489 g/l/h still compared favorably with other recent studies [0.44 g/l/h as reported by Nigma’s (2001a) and 0.20 g/l/h as reported by Agbogbo et al. (2006)]. The increased initial xylose level did not show inhibitory effects on the xylose metabolism by \(P. \textit{stipitis}\) in this study.

Another interesting feature from the experiment involving different initial sugar levels was the increased fermentation time associated with the increased xylose concentration. The fermentation time for time courses with 20 g/l, 50 g/l and 80 g/l initial xylose was 15 h, 34 h and 52 h respectively [see Section 5.2.3]. As discussed in Section 5.3.2.2, for the immobilized \(P. \textit{stipitis}\) culture, the viable cell concentration \([x_v]\) within the beads and the cell production efficiency \(\xi_{le}\) were generally at a similar level when the same batch of beads was recycled. Therefore with larger amount of sugars in the medium, longer fermentation time was required to utilize all
the sugars. The situation was similar to that discussed for the immobilized \( Z. \) \( \textit{mobilis} \) culture using Formula (8.4) in Section 5.3.1.3.

\[
T_2 - T_1 = \frac{1}{d_e} \frac{P_{\text{yie}}}{N} (h)
\]

(8.4)

The fermentation time is determined by the total amount of ethanol produced, which is a result of the total sugar amount. The only difference in this instance is that the increase in sugar was from the increase of initial sugar concentration, rather than the increase of medium volume.

At the beginning of this section, it was said that the system co-efficiency \( Q_p \) was not directly determined by the initial sugar concentration; this was also explained by Formula (8.4). By definition,

\[
Q_p = \frac{P_f}{t_p} (g/l/h)
\]

(5)

where the final ethanol concentration \( [p_f] \) is directly related to the initial sugar concentration. However, as shown by Formula (8.4), if both values of \( N \) and \( d_e \) remain constant, the increase of initial sugar concentration only results in an increase of fermentation time \( [t_p] \), which would counter the increase in \( p_f \), and the system co-efficiency \( Q_p \) would remain at the same level.

In this case, obviously an effective method to increase the system co-efficiency is to decrease the ratio of substrate \( [P_{\text{yie}}] \) to biomass \( [N] \). With a small amount of sugar in the medium and a relatively high biomass level, an efficient conversion could be easily achieved. For instance, a number of previous studies reporting a high \( Q_p \) value were achieved in shake flasks with small volumes of fermentation medium (Parekh et al., 1986; Qian et al., 2006).

In conclusion, immobilized \( P. \) \( \textit{stipitis} \) showed some significant differences in the fermentation characteristics in comparison with the immobilized \( Z. \) \( \textit{mobilis} \). The yeast preferred to grow in the fermentation medium rather than inside the beads, with significant cell leakage occurring in all time courses. The low rate of biomass accumulation within the beads and the similar fermentation performance when recycled suggested that the yeast cells had a low activity inside the beads. The
system co-efficiency of immobilized *P. stipitis* culture was hardly affected by the different aeration levels and different initial xylose levels in the fermentation process. Therefore it could be said that the system co-efficiency is more of an intrinsic characteristic for each batch of *P. stipitis* beads, determined by the amount of biomass inside the beads and the properties of the gel material. Given all of the above factors, *P. stipitis* was not considered suitable for the micro-encapsulation. Fermentation schemes involving immobilized *P. stipitis* were therefore considered to be inappropriate for the co-fermentation process of a glucose/xylose sugar mixture.
Prior to the fermentation of the bagasse hydrolysate to ethanol, a simulated sugar mixture was fermented using *Z. mobilis* and *P. stipitis* to establish an optimized fermentation scheme. The process design of the sugar mixture co-fermentation was based on the results from Chapters 4 and 5. Four fermentation schemes were investigated, viz. sequential culture, free cell co-culture, co-culture involving immobilized *Z. mobilis* and co-culture in a modified bioreactor. Conditions for each fermentation scheme were as described in Section 3.7.4. In all co-fermentation processes, unless otherwise stated, the inoculum size used for the two strains was 50% respectively, and the air flow level was controlled at 80 cm$^3$/min. According to results of these experiments, the potential interactions between *Z. mobilis* and *P. stipitis* during a co-culture process were discussed and validated using shake flask experiments. Finally, *P. stipitis* was also individually cultured on the glucose/xylose sugar mixture, as a contrast to the co-culture process.
6.1. Comparison of four fermentation schemes involving both Z. mobilis and P. stipitis

6.1.1. Time course with sequential culture

This fermentation scheme has been reported before, using the strain combination of Z. mobilis and P. tannophilus (Fu and Peiris, 2008). According to the description in Section 3.7.4.1, this fermentation process could be clearly divided into two separate stages, viz. the glucose fermentation stage by Z. mobilis and the xylose fermentation stage by P. stipitis. The results of the time course employing this fermentation scheme are shown in Figure 6.1. Divided by the Autoclave Point, the left hand side of the graph shows the results of the glucose fermentation stage, and the right hand side shows the results of the xylose fermentation stage.

Figure 6.1: Co-fermentation process employing a sequential culture of Z. mobilis and P. stipitis: ◆ reducing sugars (g/l); □ glucose concentration (g/l); ¶ ethanol production (g/l); ○ viable cell concentration of Z. mobilis (cfu/ml); ▲ viable cell concentration of P. stipitis (cfu/ml).

As shown in Figure 6.1, in this time course the overall fermentation time was 28.5 h, with the glucose fermentation taking 2.5 h and xylose fermentation taking 26 h to completely utilize each sugar respectively. During the glucose fermentation stage, the viable cell concentration of Z. mobilis steadily increased from 4x10^7 cfu/ml at the
beginning to $5.9 \times 10^8$ cfu/l in 2 h, giving an ethanol yield of approximately 0.51 g/g [$15.31$ g/l ethanol from 30 g/l glucose]. The concentration of ethanol however decreased to 7.61 g/l because of the evaporation at the autoclaving stage, and then re-increased to 16.04 g/l at the end of the xylose fermentation process. During the 30 h of *P. stipitis* culture, only a two fold increase in the viable cell concentration was observed [from $2.1 \times 10^8$ cfu/ml to $4.0 \times 10^8$ cfu/ml].

### Table 6.1: Kinetic parameters of the time course employing a sequential culture

<table>
<thead>
<tr>
<th>Fermentation process</th>
<th>Glucose stage</th>
<th>Xylose stage</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol peak time (h)</td>
<td>2.5</td>
<td>26</td>
<td>28.5</td>
</tr>
<tr>
<td>System co-efficiency (g/l/h)</td>
<td>6.13</td>
<td>0.32</td>
<td>0.83</td>
</tr>
<tr>
<td>Ethanol yield (g/g)</td>
<td>0.51</td>
<td>0.42</td>
<td>0.47</td>
</tr>
</tbody>
</table>

Table 6.1 summarizes the kinetic parameters of the sequential culture process. The overall fermentation performance was high, achieving a system co-efficiency of 0.83 g/l/h and an ethanol yield of 0.47 g/g. Glucose fermentation by *Z. mobilis* kept its usual high efficiency even in the presence of 20 g/l xylose in the medium; the system co-efficiency was 6.13 g/l/h, similar to that achieved in other sole glucose fermentation processes using an inoculum size of 50% [6.39 g/l/h; see Section 4.1]. The system co-efficiency of the xylose fermentation stage [0.32 g/l/h] showed an inferior result compared to the sole xylose fermentation [0.734 g/l/h] reported in Section 4.2.3. However, the low efficiency of the xylose fermentation stage was compensated by the efficient conversion of glucose. The overall system efficiency of the sequential culture [0.83 g/l/h] remained higher than the best result obtained in the sole xylose fermentation [0.755 g/l/h].

#### 6.1.2. Time courses with co-culture of free cells

Theoretically the simultaneous culture of *Z. mobilis* and *P. stipitis* was expected to result in the simultaneous utilization of glucose and xylose, and consequently reduce the total fermentation time in comparison with the sequential culture. However, previous studies (Taniguchi et al., 1997b; Sanchez et al., 2002) pointed out that the
xylose assimilation of *P. stipitis* was significantly affected by catabolite repression due to glucose. Furthermore, interactions between the two co-cultured strains could also affect the fermentation process. For the purpose of maximizing the ethanol production, four time courses were carried out employing the co-culture of free cells of *Z. mobilis* and *P. stipitis* under various conditions. As shown in Table 6.2, three of them employed an inoculum size of 50% for both strains with different aeration levels, and the last one increased the inoculum size of *P. stipitis* to 100%, with *Z. mobilis* inoculum staying at 50% and air flow level controlled at 80 cm$^3$/min.

**Table 6.2: Kinetic parameters of four time courses using free cell co-culture**

<table>
<thead>
<tr>
<th>Kinetic parameters</th>
<th>Co-culture processes with free cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Air flow level (cm$^3$/min)</td>
</tr>
<tr>
<td></td>
<td>50</td>
</tr>
<tr>
<td>Fermentation time (h)</td>
<td>47</td>
</tr>
<tr>
<td>Sugar consumption (%)</td>
<td>87%</td>
</tr>
<tr>
<td>Sugar uptake rate (g/l/h)</td>
<td>0.92</td>
</tr>
<tr>
<td>Ethanol yield (g/g)</td>
<td>0.38</td>
</tr>
<tr>
<td>System co-efficiency (g/l/h)</td>
<td>0.349</td>
</tr>
</tbody>
</table>

Table 6.2 presents the kinetic parameters of four time courses employing the free cell co-culture of *Z. mobilis* and *P. stipitis*. Although different environmental conditions were used, in all four processes the sugars could not be completely utilized by the end of the fermentation. In general the four time courses gave a similar performance. Highest system co-efficiency [0.518 g/l/h] was obtained in the process with the standard inoculum size at 80 cm$^3$/min air flow; however, this was mainly due to the shorter fermentation time in this time course [35 h compared to 44-47 h in other time courses]. Though not shown in the table, glucose fermentation in all time courses was efficient and not affected by the presence of the xylose substrate and yeast cells. All the glucose was completely converted into ethanol within 3 h after the fermentation began. By contrast, the efficiency of the xylose fermentation was considerably low. A sole xylose fermentation process with an initial concentration of
20 g/l [carried out with immobilized *P. stipitis* culture; see Section 5.2.3] could successfully convert all the xylose to ethanol within 15 h. However, with the same concentration of xylose in the co-culture, all four processes failed to use up the xylose by the end of the time course [35-47 h]. The xylose fermentation after 20 h was almost stagnant. The adjustment of both aeration level and viable cell concentration of *P. stipitis* had hardly any effect on the improvement of xylose fermentation efficiency.

Biomass growth in these four time courses, however, showed some unusual trends. The trends in the three time courses employing the 50% inoculum size for *P. stipitis* were comparatively similar. The results of viable cell concentration in two time courses using different inoculum sizes of *P. stipitis*, viz. 100% inoculum size and 50% inoculum size with 50 cm$^3$/min, are shown in Figure 6.2. As can be seen from Figure 6.2 (a), in both time courses there was no biomass growth observed for the yeast cells. Time course with an inoculum size of 100% for *P. stipitis* had a higher level of viable cells compared to the one with 50% inoculum size [3~4x10$^8$ cfu/ml compared to 1~2x10$^8$ cfu/ml]; but the cell concentration did not show any significant increase in either process.

On the other hand, the *Z. mobilis* cell concentration showed a steady decrease in both time courses after the glucose was completely utilized [~3 h], which was a rational trend considering that glucose was the only carbon source for the bacterium in the medium. However, a large increase of *Z. mobilis* viable cells occurred in both time courses around 11 h, when there was no glucose but only xylose left in the fermentation medium. This observation of re-increase of *Z. mobilis* during the xylose fermentation stage was also observed for the time course employing 50% inoculum size of *P. stipitis* at 80 cm$^3$/min. In the time course with 100% inoculum size, it even occurred twice [11 h & 33 h]; each time the bacterial cells showed a more than 10 fold increase [~1x10$^7$ cfu/ml to ~3x10$^8$ cfu/ml]. In this study, it has been proved that *Z. mobilis* is unable to utilize xylose for it is unable to grow on the Xylose Agar plate. Therefore, this re-increase of bacterial cells in an environment lacking necessary carbon sources is very unusual. It indicates some interactions between *P. stipitis* and *Z. mobilis* cells which have the potential to stimulate the bacterial cell growth.
In general, all four time courses co-culturing the free cells of *P. stipitis* and *Z. mobilis* gave a poor fermentation performance. The low efficiency of the xylose fermentation suggested that this fermentation scheme is not suitable for the co-fermentation of glucose/xylose sugar mixture. In order to improve the fermentation performance of co-culture as well as to investigate the potential interactions between the two strains, immobilized *Z. mobilis* was used to replace the free cells for the co-culture with *P. stipitis*.

**Figure 6.2:** Viable cell results of two time courses with free cell co-culture: (a) *P. stipitis*, (b) *Z. mobilis*. 

![Graphs showing viable cell concentration over time for *P. stipitis* and *Z. mobilis*.](image-url)
6.1.3. Time courses with co-culture involving immobilized *Z. mobilis*

This fermentation scheme co-cultured immobilized *Z. mobilis* in gel beads together with free cells of *P. stipitis*. For all three time courses carried out, the inoculum size for *Z. mobilis* culture was a full batch of beads, which was originally made of cells from 400 ml medium into 100 ml beads; for *P. stipitis*, the inoculum size of 50% was used. Similar to the experiments carried out for both immobilized *Z. mobilis* and immobilized *P. stipitis* culture, the same batch of beads were repeatedly cultured to investigate the effect of recycling the beads on the fermentation performance. All three time courses were carried out under same environmental conditions with an air flow level of 80 cm$^3$/min.

<table>
<thead>
<tr>
<th>Table 6.3: Kinetic parameters of three time courses employing co-culture with immobilized <em>Z. mobilis</em></th>
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</thead>
<tbody>
<tr>
<td>Kinetic parameters</td>
</tr>
<tr>
<td>--------------------</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Fermentation time (h)</td>
</tr>
<tr>
<td>Sugar consumption (%)</td>
</tr>
<tr>
<td>Ethanol yield (g/g)</td>
</tr>
<tr>
<td>System co-efficiency (g/l/h)</td>
</tr>
</tbody>
</table>

Table 6.3 presents the kinetic parameters of three time courses repeatedly culturing the same batch of *Z. mobilis* beads together with free cells of *P. stipitis* on the glucose/xylose sugar mixture medium. Although environmental parameters employed for the three time courses were identical, the fermentation performance showed some significant differences with the repeated usage of the same beads. In the first time course, all sugars [30 g/l glucose & 20 g/l xylose] were completely consumed within 24 h, resulting in a system co-efficiency of 0.868 g/l/h and an ethanol yield of 0.44 g/g. This system co-efficiency was higher than that achieved with sequential culture [0.83 g/l/h], which provided an optimized fermentation environment for each strain respectively. However, with the repeated usage of the
same batch of immobilized beads, the performance deteriorated. Fermentation time increased to 29 h to consume all the sugars, and the system co-efficiency and ethanol yield decreased to 0.687 g/l/h and 0.42 g/g respectively. In the third time course with the second reuse of the beads, sugars failed to be consumed within 37 h, and the system co-efficiency further decreased to 0.501 g/l/h.

The lowered fermentation performance during the bead recycling process was unexpected, given that the same process led to an increase of fermentation performance for the sole glucose fermentation [see Section 5.1.2]. It was also observed that the bead damage became increasingly severe while recycling. The trends of viable cell growth in the fermentation medium of these three processes are shown in Figure 6.3.

Similar to the free cell co-culture processes [see Section 6.1.2], no increase in the viable cell concentration was observed for \textit{P. stipitis}, suggesting a poor biomass growth. In the first two time courses, the yeast cells leveled off at a level of $4 \sim 5 \times 10^8$ cfu/ml through the whole process. In the third time course, viable cells of \textit{P. stipitis} in the medium decreased gradually from $1.8 \times 10^8$ cfu/ml to $7.0 \times 10^7$ cfu/ml during the fermentation. The low cell concentration could result in the low efficiency of xylose fermentation in this process.

For all three time courses, the free cell concentration of \textit{Z. mobilis} in the fermentation medium was generally at a low level [maximum $3.5 \times 10^7$ cfu/ml], as a result of the immobilization. Viable cell concentration of the bacterial cells in the medium increased during the first 4 h of the fermentation, when there was still glucose left in the medium. The highest level was achieved in the third time course [$3.5 \times 10^7$ cfu/ml], which gave the poorest fermentation performance. By contrast, the first time course showing the highest ethanol production efficiency had the lowest cell leakage between 0 h and 4 h [free cell concentration: $1 \sim 3 \times 10^6$ cfu/ml]. During the later xylose fermentation, the concentration of viable \textit{Z. mobilis} cells was low, but still detectable by the viable cell assay [$3 \sim 7 \times 10^6$ cfu/ml]. The large re-increase of \textit{Z. mobilis} cells during the xylose fermentation which occurred in the free cell co-culture was not observed in any of the three time courses.
As for the reduced fermentation performance associated with the repeated culture, it was suggested that the reuse of the same batch of *Z. mobilis* beads resulted in increasing bead damage, which released more free cells of *Z. mobilis* to the fermentation medium and the xylose fermentation by *P. stipitis* was thus affected. This difference in the viable cell concentration of *Z. mobilis* could hardly be seen from Figure 6.3 (b), because the high concentration of *P. stipitis* cells greatly suppressed the growth of the *Z. mobilis* under the co-culture conditions.
interfered with the plate reading of *Z. mobilis* colonies when there was a 100 fold difference in the cell concentration of the two strains. However, the bead damage could be observed visually. The fermentation process of a co-culture usually lasted for approximately 30 h, while the glucose fermentation stage generally finished within 4 h. After that, all the immobilized *Z. mobilis* beads remained in the fermentation medium, playing no part in the ethanol production but interfering with the xylose fermentation. The exposure for a long time to the fermentation medium at pH around 4 with a rotor and impellor significantly decreased the bead life. As a consequence, with recycling of the beads in the co-culture time courses, the bead damage became increasingly severe.

In summary, the efficiency of xylose fermentation in the sugar mixture decreased with the continuous bead damage during the recycling of the beads and the subsequent increased cell leakage of *Z. mobilis*. In addition with the results obtained from the free cell co-culture processes, where all four processes failed to fully utilize the xylose [see Section 6.1.2], it appears that the existence of *Z. mobilis* cells in the fermentation medium inhibited the xylose fermentation by *P. stipitis*. The bead damage of immobilized *Z. mobilis* due to the prolonged exposure to the fermentation medium also affected the bead life and increased the cost. For both reasons, a fermentation scheme employing a modified bioreactor was developed to remove all the immobilized beads after the completion of glucose fermentation.

### 6.1.4. Time courses with co-culture in a modified bioreactor

As described in Section 3.7.4.4, this fermentation scheme was a novel design used in this study. It enabled the removal of immobilized *Z. mobilis* from the fermentation medium during a co-fermentation process without disturbing the ongoing xylose fermentation. The use of the modified fermentor in a time course is shown in Figure 6.4. As can be seen in Figure 6.4 (a), the added sieve plate in the modified bioreactor had a mesh size that was smaller than the diameter of the gel beads; therefore after inoculation, the immobilized *Z. mobilis* beads could only stay on top of the sieve plate, while the free cells of *P. stipitis* were distributed throughout the medium, making the medium turbid and cloudy because of the high cell concentration. Stirring of the fermentation medium was carried out with the rotor underneath the sieve plate. The results reported in Section 5.1.3 showed that the efficiency of the glucose
fermentation was not much affected in this system. After the glucose fermentation was completed, the sieve plate was moved up by a movable device [see Figure 3.5]. As shown in Figures 6.4 (b) and 6.4 (c), the immobilized beads were consequently separated from the fermentation medium. Xylose fermentation continued without being interrupted by the removal process. Furthermore, the potential interactions between \textit{Z. mobilis} and \textit{P. stipitis} were minimized.

In this experiment, three inoculum levels with different ratios of the two strains were investigated for the effect on the fermentation characteristics, with duplicate time courses at each level. The details are shown in Table 6.4. Same batch of immobilized \textit{Z. mobilis} beads were repeatedly used and replaced with fresh beads when the damage was severe. In each time course, the sieve plate was moved up to remove all the immobilized \textit{Z. mobilis} after glucose fermentation was completed.

\textbf{Table 6.4: Three inoculum levels used for co-culture processes in modified bioreactor}

<table>
<thead>
<tr>
<th>Level</th>
<th>Immobilized \textit{Z. mobilis}</th>
<th>Free cells of \textit{P. stipitis}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level 1</td>
<td>Full batch *</td>
<td>50%</td>
</tr>
<tr>
<td>Level 2</td>
<td>1/4 batch</td>
<td>50%</td>
</tr>
<tr>
<td>Level 3</td>
<td>1/4 batch</td>
<td>100%</td>
</tr>
</tbody>
</table>

* For the immobilized \textit{Z. mobilis} inoculum, “full batch” refers to the use of all beads made from 400 ml \textit{Z. mobilis} cells into 100 ml gel beads, while “1/4 beads” refers to the use of 1/4 beads (w/w) out of a full batch.
Figure 6.4: Modified bioreactor showing the removal of immobilized *Z. mobilis* beads by the sieve plate: (a) immobilized *Z. mobilis* co-cultured with free cells of *P. stipitis* in the bioreactor; (b) & (c) sieve plate was raised, separating the immobilized beads containing *Z. mobilis* and xylose fermentation continuing with the beads away from the fermentation medium.
Kinetic parameters of time courses with different inoculum levels are reported in Table 6.5. With the addition of the sieve plate, the results of co-fermentation processes were remarkably more stable compared to that achieved without a sieve plate [see Section 6.1.3]. All six time courses [duplicate of three inoculum levels] gave steady and comparable fermentation efficiencies. For time courses with inoculum level 1, the inoculum size was similar to that employed for the co-culture process without a sieve plate [full batch of \(Z.\ mobilis\) beads and 50\% of \(P.\ stipitis\)], and a similar result was obtained. The fermentation took 25 h to utilize both sugars in the medium; the overall system efficiency was 0.868 g/l/h and the ethanol yield was 0.45 g/g. The fermentation performance was further improved for time courses using the level 2 inoculum. With a decrease of \(Z.\ mobilis\) inoculum size to 1/4 of the inoculum level 1, the fermentation was completed within 22 h, giving an exceptionally high ethanol yield of 0.50 g/g, which was 98\% of the theoretical conversion rate. The system co-efficiency reached 1.126 g/l/h, which was the highest value achieved in a co-fermentation process so far. The inoculum level 3 employing a 100\% inoculum size of \(P.\ stipitis\) was an attempt to further increase the efficiency of the xylose conversion. It was expected that the double inoculum size of \(P.\ stipitis\) could improve the system co-efficiency proportionally; however, the result was not as high as expected. Fermentation time decreased to 19 h, together with an increase in the system co-efficiency to 1.277 g/l/h and an ethanol yield of 0.49 g/g.

Table 6.5: Kinetic parameters of time courses with co-culture in modified bioreactor

<table>
<thead>
<tr>
<th>Kinetic parameters</th>
<th>Co-culture in modified bioreactor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inoculum level *</td>
</tr>
<tr>
<td></td>
<td>Level 1</td>
</tr>
<tr>
<td>Fermentation time (h)</td>
<td>25</td>
</tr>
<tr>
<td>Ethanol yield (g/g)</td>
<td>0.45</td>
</tr>
<tr>
<td>System co-efficiency (g/l/h)</td>
<td>0.867</td>
</tr>
</tbody>
</table>

* See details in Table 6.4.
Viable cell results of three time courses at each inoculum level are presented in Figure 6.5. The trends of biomass in these time courses were steady as expected, which consequently ensured a stable fermentation efficiency. *P. stipitis* cell concentration in time courses using an inoculum size of 50% generally leveled off at 4~5x10^8 cfu/ml, with a slight decrease observed while the fermentation was progressing. In the time courses with higher inoculum size of 100%, a higher level of yeast cell concentration was attained [7~9x10^8 cfu/ml]. On the other hand, the free cell concentration of *Z. mobilis* was controlled at a minimum level as a result of the removal of immobilized *Z. mobilis* beads after the completion of glucose fermentation. As stated in Section 3.10.2.1, in Figure 6.5 (b) all values of 1x10^6 cfu/ml indicate that at this sampling point no *Z. mobilis* cells could be discerned on the plates of viable cell assay. It can be clearly seen that in all the time courses the free cell concentration of *Z. mobilis* was generally low, indicating a low level of cell leakage from the immobilized beads. Even in the time courses with inoculum level 2, where higher bacterial cell levels were observed, the maximum value remained lower than 1x10^8 cfu/ml.

In summary, the results of this experiment suggested that the fermentation scheme with the sieve plate gave a much more stable fermentation performance compared to the one without the sieve plate. When the same inoculum size was used for two time courses with and without sieve plate, similar results were obtained. A decrease of inoculum size for immobilized *Z. mobilis* beads resulted in significant increases in both the system co-efficiency and ethanol yield. A conversion rate of 96-98% of the theoretical was achieved. Further increase of inoculum size of *P. stipitis* also helped to improve the fermentation performance. The optimized ethanol production resulting from the increased ratio of *P. stipitis* cells to *Z. mobilis* cells further suggests that the *Z. mobilis* cells might have some inhibitory effects on the xylose fermentation by *P. stipitis*. 
6.1.5. Comparison of three co-culture processes with different fermentation schemes

Amongst the four fermentation schemes involving both *Z. mobilis* and *P. stipitis* for the co-fermentation of glucose/xylose mixture in this study, three of them were co-culture processes simultaneously inoculating two strains to start the fermentation process, viz. the co-culture with free cells, the co-culture with immobilized *Z. mobilis*, and the co-culture carried out in the modified bioreactor. A comparison is
made here on the sugar utilization and ethanol production of three time courses from each fermentation scheme. The time courses selected are: free cell co-culture with an air flow level of 80 cm$^3$/min, the first time course employing the co-culture with immobilized $Z.\ mobilis$, and a time course carried out in the modified bioreactor at inoculum level 2. The selection was based on the inoculum size of $P.\ stipitis$; all three time courses employed an inoculum size of 50%. Therefore, the contribution of $P.\ stipitis$ on the fermentation performance is presumably the same; the effects of the fermentation scheme and the inoculum size of $Z.\ mobilis$ are the major factors to be compared. For convenience of identification, these three fermentations are named as “FS 1”, “FS 2” and “FS 3” (FS: fermentation scheme) in this section.

The results of sugar utilization and ethanol production of these three time courses are shown in Figure 6.6. As can be seen in Figure 6.6 (a), glucose in the fermentation medium was consumed efficiently in all three time courses. Both Time course FS 1 employing an inoculum size of 50% for $Z.\ mobilis$ and Time course FS 2 employing a full batch of immobilized $Z.\ mobilis$ beads completely consumed all the glucose [30 g/l] within 3 h, whereas Time course FS 3 with only 1/4 batch of immobilized $Z.\ mobilis$ beads took 6 h to complete the glucose fermentation.

In all three time courses, xylose was hardly consumed when glucose was used up [remained at a level of 20 g/l]. It can be seen from Figure 6.6 (b) that the trends of reducing sugar utilization in both time courses of FS 1 and FS 2 could be clearly distinguished as the glucose fermentation stage and the xylose fermentation stage. Between 0 h and 3 h, i.e. the glucose fermentation stage, the reducing sugar concentration showed a steep decrease; after that, the sugar utilization rate markedly slowed down. By contrast, the reducing sugar curve of time course FS 3 is much smoother. Though the glucose utilization in this time course was not as efficient as that in the other two, the xylose consumption was faster, demonstrating the best balanced glucose and xylose fermentation amongst the three time courses.
Figure 6.6: Comparison of co-culture processes in different fermentation schemes: (a) glucose, (b) reducing sugar, (c) ethanol (FS 1: free cell co-culture; FS 2: co-culture with immobilized *Z. mobilis* and free cells of *P. stipitis*; FS 3: co-culture in a modified bioreactor).
The trends of ethanol production in the three time courses were in accordance with the trends of sugar utilization. As shown in Figure 6.6 (c), in time course FS 1 and FS 2, the ethanol production increased steeply during the first 3 h of glucose fermentation, indicating a high efficiency of ethanol production by *Z. mobilis* from glucose. This rate significantly slowed down on the commencement of the xylose fermentation, which corresponded to the slow sugar utilization rate in Figure 6.6 (b). In contrast, the ethanol production in FS 3 was less steep in the first 3 h, but increased steadily during the xylose fermentation stage, and finally peaked at 22 h.

The corresponding trends between sugar utilization and ethanol production indicate that the ethanol production is directly related to the sugar consumption. For time courses with free cell co-culture and co-culture with immobilized *Z. mobilis*, the fermentation process can be clearly divided into the glucose fermentation stage and xylose fermentation stage, based on the different rate of sugar uptake and ethanol yield. The efficient conversion of glucose to ethanol was completed within 3 h because of the large inoculum size used for *Z. mobilis*; however, the efficiency of xylose fermentation was low. In contrast, the time course carried out in the modified bioreactor showed a lower efficiency of the glucose fermentation as a result of the reduced inoculum size of *Z. mobilis*. However, the subsequent xylose fermentation gave a comparably high performance, resulting in the most balanced glucose and xylose fermentation and the best results for the glucose/xylose co-fermentation amongst the three fermentation schemes.

### 6.2. Effect of increased initial sugar level in the co-culture process

As pointed out in Section 5.3.2.3, for industrial ethanol production it is always desirable to ferment with a high initial sugar level for economic viability. This experiment investigated the co-culture process with increased sugar levels. The initial sugar concentration was increased to 45 g/l glucose and 30 g/l xylose, which was 1.5 fold of the standard medium. The other medium components were as listed in Table 3.2. The experiment employed the fermentation scheme with the best results for the glucose/xylose co-fermentation, i.e. the co-culture carried out in a modified bioreactor. The inoculum sizes used for two time courses were a full batch and a 1/2
batch of immobilized \textit{Z. mobilis} beads respectively, co-cultured with 50\% \textit{P. stipitis}.

Table 6.6 presents the kinetic parameters of these two time courses. It took 45 h and 42 h for two time courses to completely utilize the sugars respectively. The resulting system co-efficiency was 0.679 g/l/h and 0.878 g/l/h, which was lower than that achieved in the time courses with standard initial sugar level of 50 g/l [0.867, 1.126 and 1.277 g/l/h for each inoculum level; see Table 6.5]. The time course with a decreased inoculum size of \textit{Z. mobilis} gave a relatively higher system co-efficiency [0.878 g/l/h]. However, the ethanol concentration reached a peak [31.61 g/l] at 36 h when there was 3.14 g/l xylose left in the medium, and then decreased to 27.22 g/l at 42 h when all sugar was completely utilized.

\textbf{Table 6.6: Kinetic parameters of two time courses with co-culture on 45 g/l glucose and 30 g/l xylose}

<table>
<thead>
<tr>
<th>Inoculum size</th>
<th>Kinetic parameters</th>
<th>1 batch \textit{Z. mobilis} &amp; 50% \textit{P. stipitis}</th>
<th>1/2 batch \textit{Z. mobilis} &amp; 50% \textit{P. stipitis}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermentation time (h)</td>
<td>45</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Time when ethanol peaked (h)</td>
<td>45</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Ethanol yield (g/g)</td>
<td>0.42</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>System co-efficiency (g/l/h)</td>
<td>0.679</td>
<td>0.878</td>
<td></td>
</tr>
</tbody>
</table>

Comparing the fermentation performance of the two time courses carried out with 75 g/l initial sugar, the one with the larger inoculum size of \textit{Z. mobilis} [a full batch compared to 1/2 batch of beads] showed an inferior fermentation performance; both the system co-efficiency [0.679 g/l/h] and ethanol yield [0.42 g/g] was lower than those in the time course with fewer cells of \textit{Z. mobilis} [0.878 g/l/h and 0.46 g/g respectively]. This trend was in accordance with that observed for fermentation processes with lower initial sugar level of 50 g/l reported in previous sections. The efficiency of xylose fermentation improved when there were fewer free cells of \textit{Z. mobilis} released into the fermentation medium from the immobilized beads.
The viable cell results of these two time courses supported this observation. Figure 6.7 shows the biomass growth of *P. stipitis* and *Z. mobilis* in the fermentation medium. The cell concentration of *P. stipitis* in two time courses was at a similar level because of the same inoculum size employed; it leveled out at $4\times10^8$ cfu/ml with slight decrease towards the end. By contrast, the free cell level of *Z. mobilis* was considerably different in the two processes. During the whole process of the time course with a full batch of *Z. mobilis*, the bacterial cells in the medium were generally around $10^6-10^7$ cfu/ml, while for the one with lower inoculum level, the viable cells of *Z. mobilis* were barely detectable for most of the fermentation process.

Figure 6.7: Viable cell results of the two time courses with co-culture on 45 g/l glucose and 30 g/l xylose: (a) *P. stipitis*, (b) *Z. mobilis* in the medium.
6.3. Cell interaction between *Z. mobilis* and *P. stipitis* in the co-culture process

Results of the co-culture processes reported in above sections indicated that the viable cells of *Z. mobilis* may have been inhibitory to the conversion of xylose to ethanol by *P. stipitis*. In order to confirm this observation and further investigate the strain interaction, semi-quantitative experiments were carried out using shake flasks. Incubation conditions were as described in Section 3.7.1. For each experiment, flasks containing 100 ml standard fermentation medium were prepared, the first group with the Xylose Medium and the second group with the Co-fermentation Medium [see medium composition in Table 3.2]. Each flask in the respective group was inoculated with *P. stipitis* cells at an inoculum size of 50% [cells from 50 ml medium into 100 ml fermentation medium], together with an increasing number of *Z. mobilis* cells. The five inoculum levels used for each experimental group are shown in Table 6.7.

**Table 6.7: Five inoculum levels employed for the investigation of the interactions between *P. stipitis* and *Z. mobilis*.**

<table>
<thead>
<tr>
<th>Inoculum level</th>
<th>Test 1</th>
<th>Test 2</th>
<th>Test 3</th>
<th>Test 4</th>
<th>Test 5</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. stipitis</em></td>
<td>50%</td>
<td>50%</td>
<td>50%</td>
<td>50%</td>
<td>50%</td>
</tr>
<tr>
<td><em>Z. mobilis</em></td>
<td>10%</td>
<td>20%</td>
<td>30%</td>
<td>40%</td>
<td>50%</td>
</tr>
</tbody>
</table>

For inoculation, *Z. mobilis* culture of given volume and 50 ml *P. stipitis* culture [both were harvested after appropriate incubation; see incubation conditions in Section 3.5] were transferred into a sterilized centrifuge bottle and centrifuged at 10,000 rpm for 10 min. The resulting cells pellets were re-suspended using the corresponding fermentation medium. Samples were taken at 12 h time interval for assay. The group with the Xylose Medium as substrate had a “Control” flask inoculated with only 50 ml *P. stipitis* without *Z. mobilis* cells. The group with the Co-fermentation Medium had no “Control” flask, because *P. stipitis* was not expected to consume glucose in the co-culture process. For convenience of identification, flasks with varying amounts of *Z. mobilis* were referred as Test 1, 2, 3, 4 and 5 respectively; “Test 1.x”
referred to those with the Xylose Medium, and “Test 2.x” referred to those with the Co-fermentation Medium.

6.3.1. Effect of Z. mobilis cells on the P. stipitis fermentation on the Xylose Medium

Samples were taken at 12 h, 24 h and 36 h; results of biomass concentration of P. stipitis and Z. mobilis and ethanol production in each flask are shown in Figure 6.8. The viable cell concentration of P. stipitis was not significantly affected by the presence of Z. mobilis cells in the medium. In all five “Test” flasks, P. stipitis maintained a substantial biomass level between $10^8$ and $10^9$ cfu/ml, due to the same inoculum size used for each flask. However, after 36 h culture, the biomass concentration in all “Test” flasks was generally less than that in the “Control” flask, implying less cell growth of P. stipitis in the presence of Z. mobilis cells.

The biomass growth of Z. mobilis in these five flasks showed some unusual trends. As the Xylose Medium was used as substrate for the two strains, there was no available carbon source for Z. mobilis because of its inability to assimilate xylose. As a consequence, the bacterium in all five “Test” flasks managed to survive the first 12 h, and decreased to a level that was undetectable on all plates in the viable cell assay at 24 h. However, a re-increase of bacterial cells occurred in all five “Test” flasks at 36 h; viable cells of Z. mobilis generally increased to approximately $10^7$–$10^8$ cfu/ml, with higher concentrations observed in the flask with higher inoculum size.

The overall ethanol production at 36 h of these flasks is shown in Figure 6.8 (c). It can be clearly seen that, compared to the “Control” flask without Z. mobilis, all five “Test” flasks containing bacterial cells gave much less ethanol production [only 1/2–2/3 of the concentration in the “Control” flask]. Moreover, the ethanol concentration tended to decrease along with the increase of Z. mobilis cell concentration in the medium [Flask “Test 1.5” gave the lowest ethanol yield of all, 4.48 g/l].
Figure 6.8: Strain interactions between *Z. mobilis* and *P. stipitis* – Xylose

Medium: (a) viable cells of *P. stipitis*, (b) viable cells of *Z. mobilis*, (c) overall ethanol production.
6.3.2. Effect of *Z. mobilis* cells on the *P. stipitis* fermentation on the Co-fermentation Medium

For the experiment carried out on the Co-fermentation Medium, samples were taken at 12 h and 24 h and assayed for sugar utilization, biomass and ethanol production. Figure 6.9 presents the results of *P. stipitis* growth, *Z. mobilis* growth and the ethanol yield. Results obtained in this experiment on the Co-fermentation Medium were considerably different to those obtained on the Xylose Medium. Although the inoculum size of *P. stipitis* was the same for all flasks, the viable cell concentrations of *P. stipitis* achieved in this experimental group [2–4x10⁸ cfu/ml] were generally lower than those achieved in the first experimental group [4–5x10⁸ cfu/ml]. A notable trend was that at 12 h, when the glucose fermentation by *Z. mobilis* in each flask was just completed, the flasks with a higher initial number of *Z. mobilis* clearly showed fewer *P. stipitis* cells. However, with the progress of the fermentation and the exhaustion of glucose, this difference was reduced. *P. stipitis* cells at 24 h in all five flasks were at a similar level, which was still less than those achieved in the five flasks reported above without glucose as a substrate.

By contrast, the biomass growth of *Z. mobilis* in these five flasks was remarkably vigorous. Though different inoculum levels were employed, at 12 h all five flasks gave a similar cell concentration of the bacterium around 1.0x10⁹ cfu/ml. From 12 h to 24 h, although its only carbon source, glucose, had been completely utilized, in the first three flasks *Z. mobilis* managed to maintain this high level of viable cells. In “Test 2.4” & “Test 2.5” which were inoculated with higher amount of bacterial cells, the cell concentration decreased slightly. This trend could explain the difference in growth of *P. stipitis* in these five flasks. As shown in Figure 6.9 (a), between 12 h and 24 h, in “Test 2.1” & “Test 2.2” where *Z. mobilis* concentration stayed constant, the viable cell concentration of *P. stipitis* decreased, whereas in “Test 2.4” & “Test 2.5” where *Z. mobilis* concentration decreased, the viable cell concentration of *P. stipitis* increased.
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Figure 6.9: Strain interactions between *Z. mobilis* and *P. stipitis* – Co-fermentation Medium: (a) viable cells of *P. stipitis*, (b) viable cells of *Z. mobilis*, (c) overall ethanol production.
Ethanol production in these five flasks containing a glucose/xylose sugar mixture showed some significant differences. It can be seen from Figure 6.9 (c) that, at 12 h all five flasks yielded a high ethanol concentration, as a result of the complete glucose fermentation by \textit{Z. mobilis}. Flasks containing higher initial levels of \textit{Z. mobilis} cells tended to give slightly higher ethanol concentration [ethanol concentration in “Test 4.4” & “Test 4.5” was slightly higher compared to that in other flasks], because the earlier the glucose fermentation was completed, the sooner the xylose fermentation commenced. However, from 12 h to 24 h, the situation changed. There was little ethanol production in all five flasks during these 12 h, even when \textit{P. stipitis} cells and the residual xylose in the medium were both at a high concentration. The highest ethanol accumulation occurred in “Test 2.1” which was inoculated with the lowest amount of \textit{Z. mobilis} cells; however, the increase in the ethanol production was only 1.74 g/l between 12 h and 24 h [16.60 g/l to 18.34 g/l]. The lowest overall ethanol production by the end of the time course was in the flask containing the highest initial concentration of \textit{Z. mobilis} cells [“Test 2.5”]; ethanol concentration showed a decrease from 17.23 g/l to 16.86 g/l between 12 h and 24 h. The addition of glucose to the medium, which resulted in an increased level of \textit{Z. mobilis} cells, was accompanied by a poor performance of xylose fermentation by \textit{P. stipitis}. The xylose fermentation efficiency in this experimental group was considerably lower compared to that in the group using only xylose as carbon source.

Based on these results, the interactions between \textit{Z. mobilis} and \textit{P. stipitis} in a co-culture process could be as follows. The presence of \textit{Z. mobilis} cells in the medium, whether with or without glucose, affected the xylose fermentation by \textit{P. stipitis} in an adverse manner. Firstly, flasks containing a higher initial level of \textit{Z. mobilis} cells gave a lower ethanol production; secondly, the efficiency of xylose fermentation considerably decreased when \textit{P. stipitis} was co-cultured with \textit{Z. mobilis} in the Co-fermentation Medium, where the bacterium was able to grow to a high cell concentration. The second interaction is that \textit{Z. mobilis} appeared to be able to grow on the xylose metabolic process of \textit{P. stipitis}. This enabled \textit{Z. mobilis} to multiply to a high cell concentration even in a medium completely devoid of an appropriate external carbon source.
6.4. Sole culture of *P. stipitis* on glucose/xylose sugar mixture medium

All three xylose fermenters, viz. *P. stipitis*, *C. shehatae* and *P. tannophilus*, are capable of assimilating both glucose and xylose, and producing ethanol under suitable conditions (Kruse and Schugerl, 1996; Taniguchi et al., 1997b; Sanchez et al., 1999; Sanchez et al., 2002; Agbogbo et al., 2006). The reported fermentation efficiencies were low, due to the yeast's low fermentation capability of glucose and the catabolite repression caused by the glucose on the xylose fermentation. These results were, however, achieved with the normal inoculum size. In this study it was proved that an increased inoculum size of *P. stipitis* significantly improves its fermentation performance; consequently there was a need to investigate the effect of increased inoculum size of *P. stipitis* on the glucose/xylose co-fermentation. The experiment was first carried out in shake flasks and then scaled up into a 1 l bioreactor.

6.4.1. Investigation of fermentation performance in shake flasks

Experimental conditions were as described in Section 3.7.1. Flasks with 100 ml Co-fermentation Medium were inoculated with *P. stipitis* cells at an inoculum size of 100%. Incubation was carried out at both 100 and 200 rpm shaking speeds, and samples were taken at 12 h and 24 h for sugar, biomass and ethanol assays.

![Figure 6.10: Results of shake flask experiments of *P. stipitis* on the Co-fermentation Medium.](image-url)
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Results for reducing sugar and ethanol are shown in Figure 6.10. The sugar utilization at both shaking speeds was considerably efficient. At 12 h there was only 11.68 g/l and 7.63 g/l residual sugar at 100 and 200 rpm respectively, and at 24 h all the sugars was completely utilized. An ethanol yield of 0.49 g/g was achieved within 24 h in both flasks [24.1 g/l for Flask “100 rpm” and 24.47 g/l for Flask “200 rpm”]. The flask with the higher shaking speed [200 rpm] performed the fermentation process more efficiently, giving faster sugar utilization and ethanol production. This again indicated that with higher biomass concentration, a higher aeration level was required to match the oxygen requirement for each cell. The increased efficiency of ethanol production in the flask cultured at 200 rpm was associated with a higher biomass concentration in the medium. As can be seen in Figure 6.11, at both 12 h and 24 h, the viable cell concentration of \textit{P. stipitis} in the Flask “200 rpm” was approximately 30% higher than that in the Flask “100 rpm” [~2.0x10^{9} cfu/ml compared to ~1.5x10^{9} cfu/ml].

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.11.png}
\caption{Comparison of biomass growth of \textit{P. stipitis} on the Co-fermentation Medium and the Xylose Medium}
\end{figure}

It was also observed that the biomass growth of \textit{P. stipitis} was much more vigorous on the glucose/xylose sugar mixture substrate compared to that on the sole xylose substrate. Figure 6.11 compares the viable cell concentration of \textit{P. stipitis} cultured on
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The Co-fermentation Medium [results of this experiment] and on the Xylose Medium [see Section 4.2.6]. Under the same culture conditions, the biomass concentration in the flasks with glucose was generally higher than that in the flasks containing only xylose.

The sole culture of *P. stipitis* on the glucose/xylose sugar mixture medium gave a remarkably high fermentation performance, which was at a similar level to the co-culture process involving immobilized *Z. mobilis* and free cells of *P. stipitis* in a modified 1 l bioreactor. A comparison of kinetic parameters of the two processes is made in Table 6.8; the result achieved in the shake flask at 200 rpm shaking speed is compared to that from the co-culture process in a modified bioreactor using “inoculum level 3” [see Section 6.1.4]. Both processes employed an inoculum size of 100% for *P. stipitis*. Since the sample point at “24 h” may not be the ethanol peak time for the shake flask experiment, values at “12 h” are compared to those obtained at “11 h” in the co-culture process for the calculation of kinetic parameters.

**Table 6.8: Comparison of two co-fermentation processes: *P. stipitis* sole culture in the shake flask and two strains co-culture in the bioreactor**

<table>
<thead>
<tr>
<th>Process characteristics</th>
<th>Fermentation scale</th>
<th><em>P. stipitis</em> sole culture</th>
<th>Co-culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sampling time [t, h]</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Ethanol yield [Y_{p/s}, g/g]</td>
<td>0.49</td>
<td>0.50</td>
</tr>
<tr>
<td>Kinetic parameters</td>
<td>Sugar uptake rate [Q_s, g/l/h]</td>
<td>3.531</td>
<td>3.594</td>
</tr>
<tr>
<td></td>
<td>System co-efficiency [Q_p, g/l/h]</td>
<td>1.752</td>
<td>1.819</td>
</tr>
<tr>
<td></td>
<td>Average viable cell concentration of <em>P. stipitis</em> [x_C, cfu/ml]</td>
<td>2.0x10^9</td>
<td>4.9x10^8</td>
</tr>
</tbody>
</table>

As can be seen in Table 6.8, although these two processes of glucose/xylose co-fermentation were carried out in different bioreactors with different strains, the kinetic parameters of two processes were exceptionally similar. This similarity of the
fermentation performance could be directly attributed to the high biomass concentration of *P. stipitis* in the flask. The viable cell concentration of *P. stipitis* in the flask was four times as high as that in the 1 l fermentor [$2.0 \times 10^9$ cfu/ml compared to $4.9 \times 10^8$ cfu/ml]. This high cell concentration probably compensated for the inferior glucose fermentation efficiency of *P. stipitis* compared to that of *Z. mobilis*, resulting in a similar overall fermentation performance. These results suggested that *P. stipitis* under suitable conditions had the potential to give as good a result as the co-culture on the glucose/xylose co-fermentation. Therefore, the *P. stipitis* sole culture on the sugar mixture medium was scaled up to 1 l bioreactor to further investigate its fermentation performance.

### 6.4.2. Co-fermentation of glucose/xylose sugar mixture by sole culture of *P. stipitis* in a 1 l bioreactor

This fermentation scheme would be more suitable than a co-culture process for industrial applications if it could give a better or similar fermentation performance. The culture process of one strain is simpler, easier and more controllable, without the need to consider strain interactions when two strains are co-cultured. In order to investigate further this possibility, three time courses were carried out, culturing *P. stipitis* on 800 ml Co-fermentation Medium in a 1 l bioreactor. The main experimental conditions are as described in Section 3.7.4.5. The inoculum size and aeration level employed for each time course are shown in Table 6.9, together with the kinetic parameters achieved in each process.

| Table 6.9: Process conditions and kinetic parameters for *P. stipitis* sole culture on the Co-fermentation Medium |
| --- | --- | --- | --- |
| Process conditions | Inoculum size | 50% | 100% | 100% |
| Air flow (cm$^3$/min) | 50 | 80 | 300 |
| Fermentation time (h) | 22 | 24 | 20 |
| Ethanol yield (g/g) | 0.42 | 0.44 | 0.44 |
| System co-efficiency (g/l/h) | 0.952 | 0.874 | 1.092 |
| Specific ethanol productivity (g/g/h) | 0.184 | 0.102 | 0.124 |
As shown in Table 6.9, the adopted inoculum and aeration conditions are significantly different amongst these three time courses. The air flow level of 300 cm$^3$/min employed in Time Course 3 was the highest level used in this study. However, the fermentation performance of the three processes was found to be similar. The fermentation time of three time courses was 22 h, 24 h and 20 h, resulting in a system co-efficiency of 0.952 g/l/h, 0.874 g/l/h and 1.092 g/l/h respectively. Time Course 3 using a 100% inoculum size of *P. stipitis* with 300 cm$^3$/min air flow gave the highest fermentation efficiency. The system co-efficiency of 1.092 g/l/h was comparable to the best results achieved with the co-culture process in a modified fermentor [1.126~1.277 g/l/h, see Section 6.1.4], but the ethanol yield was considerably lower [0.44 g/g compared to 0.50 & 0.49 g/g]. Ethanol re-assimilation could be an explanation of the reduced ethanol yields, which was observed in all three time courses after the ethanol peaked.

The differences in the kinetic parameters between these three time courses are not so significant when the different process conditions are taken into consideration. It can be seen from Figure 6.12, the trends of biomass concentration in these three time courses were generally similar, although different inoculum sizes and aeration levels were employed. The viable cell concentration in Time course 1 with 50% inoculum size increased to a similar level to that in the other two time courses with 100% inoculum size after only 12 h growth. Moreover, Time courses 2 and 3 had a 3.75 fold difference on the aeration level [80 cm$^3$/min for TC 2 and 300 cm$^3$/min for TC 3], which could lead to a significant difference in the fermentation performance when *P. stipitis* is cultured on xylose [see Section 4.2.4], but it had no apparent effect when the glucose/xylose sugar mixture was used as substrate.

The fermentation details of Time Course 3 are shown in Figure 6.13. Glucose in the medium [30 g/l] was completely utilized in 9 h, and xylose [20 g/l] was fully consumed by 20 h. The reducing sugar curve was smooth during the whole process, without a clear distinction of the glucose and xylose fermentation stages. Ethanol production showed a steady increase during the 20 h of fermentation time, and perceptibly decreased after peak, indicating the ethanol re-assimilation by the yeast cells. All these trends suggested a sequential utilization of the glucose and xylose;
however, the reported catabolite repression caused by glucose on xylose assimilation was not observed.

Figure 6.12: Viable cell results of three time courses culturing *P. stipitis* on the Co-fermentation Medium.

In conclusion, with the use of a large inoculum size, the co-fermentation process of glucose/xylose sugar mixture by *P. stipitis* gave an exceptionally high performance.
The achieved system co-efficiency was similar to the best results from the co-culture process, but the ethanol yield was lower.

6.5. Discussion

6.5.1. Comparison of co-fermentations with four fermentation schemes employing two strains

6.5.1.1. Sequential culture

As shown in Table 6.1, the overall system co-efficiency and the ethanol yield achieved using this scheme were 0.83 g/l/h and 0.47 g/g respectively. Both values demonstrated a high ethanol production efficiency in comparison with previous reports (Lebeau et al., 1997; Kordowska-Wiater and Targonski, 2002; de Bari et al., 2004; Agbogbo et al., 2006). Details of these studies have been reviewed in Section 2.4.2. The main factor ensuring this high fermentation performance was the separate cultivation processes for two strains respectively. Each strain was provided with optimum conditions; thus both fermentations of glucose and xylose could be carried out efficiently.

However, the system co-efficiency of the xylose fermentation stage in the sequential culture was 0.32 g/l/h, which was only half the value obtained in the optimized sole xylose fermentation process [0.734 g/l/h; see Section 4.2.3]. The possible causes could be the initial ethanol concentration from the preceding glucose fermentation [7.61 g/l left after autoclaving], or the un-optimized oxygenation level during this stage. Nevertheless, because of the remarkably high efficiency of the glucose fermentation by Z. mobilis, the overall system co-efficiency remained high [0.83 g/l/h]. This indicated that the separate culture of Z. mobilis and P. stipitis sequentially was a simple and convenient approach for the glucose/xylose sugar mixture co-fermentation.

This process overcame several difficulties reported in other processes. Firstly, it successfully converted the glucose/xylose sugar mixture to ethanol at a high efficiency. The process is considered difficult, and unsuccessful processes of glucose/xylose co-fermentation often lead to inferior system co-efficiency compared to the sole culture processes of two strains individually (Grootjen et al., 1991a).
Previous processes reporting high system co-efficiency were often established at the expense of incomplete consumption of xylose in the mixture (Laplace et al., 1993a). Compared to a reported process using the same fermentation scheme, the use of *P. stipitis* instead of *P. tannophilus* improved the overall system co-efficiency 4 fold [0.83g/l/h compared to 0.23 g/l/h] (Fu and Peiris, 2008). It was also found that the fermentation performance of *Z. mobilis* was not affected by the presence of xylose, which was in accordance with a previous study (Lawford and Rousseau, 2003).

The fermentation scheme of sequential culture, however, had some disadvantages. The separation of sugar mixture into two parts for fermentation increased the time required for the utilization of sugars. Furthermore, as *Z. mobilis* cells needed to be inactivated by autoclaving, extra energy input was required, resulting in an increase in costs.

### 6.5.1.2. Co-culture process of free cells

Simultaneous culture of two strains on the glucose/xylose sugar mixture medium is expected to combine both fermentations of glucose and xylose into a single stage and therefore reduce the fermentation time. However, as shown in Table 6.2, in none of four time courses were all the sugars in the medium completely utilized, regardless of inoculum size and air flow level. This again proved that the co-fermentation of a glucose/xylose sugar mixture represents a technical difficulty, due to the interference of the two fermentation processes. The efficient glucose fermentation in association with the stagnant xylose fermentation indicated that this simple co-culture of *Z. mobilis* and *P. stipitis* was not a good fermentation scheme for glucose/xylose co-fermentation. This conclusion was in accordance with the observations in the literature. All three reports of successful co-culture of a glucose fermenter and a xylose fermenter needed some engineering to either the strains or the process (Taniguchi et al., 1997b; de Bari et al., 2004; Qian et al., 2006) [see Section 2.4.2].

A possible reason for the poor fermentation performance of free cell co-culture could be the unfavorable biomass growth. In Chapters 4 and 5 it was found that the viable cell concentration is very crucial to the overall fermentation efficiency. An interesting observation on the biomass growth in the free cell co-culture was that *Z. mobilis* was able to multiply to a high cell concentration during the xylose fermentation stage.
when there was no external carbon source available [see Figure 6.2]. It would be interesting to know the source from which the bacterial cells obtained the energy required for multiplication. The observation of the re-increase of *Z. mobilis* cells was made in three time courses out of four, and also occurred in the shake flask experiment investigating the two strains' interactions [see Section 6.3.1]. Therefore, it appears that some factors from *P. stipitis* metabolism on xylose stimulated the bacterial growth.

### 6.5.1.3. Co-culture process with immobilized *Z. mobilis* and free cells of *P. stipitis*

The co-culture of immobilized *Z. mobilis* and free cells of *P. stipitis* has some prominent advantages for the conversion of the glucose/xylose sugar mixture to ethanol. Firstly, it is able to provide each strain with optimum environmental conditions, i.e. semi-anaerobic conditions for *Z. mobilis* inside the beads and optimum level of oxygenation for *P. stipitis* in the fermentation medium. Secondly, the simultaneous fermentation of two sugars saves the overall fermentation time compared to sequential culture. Thirdly, it has all the advantages of the immobilized culture for *Z. mobilis*, e.g. the easier recovery and multiple reuses by recycling. In addition, it also allows the separation of *Z. mobilis* cells from the fermentation medium whenever necessary, without disturbing the ongoing xylose fermentation.

As shown in Table 6.3, the utilization of immobilized *Z. mobilis* instead of free cell *Z. mobilis* in the co-culture with *P. stipitis* resulted in a successful conversion of 30 g/l glucose and 20 g/l xylose to ethanol within 24 h. This is one of the best results reported for the glucose/xylose co-fermentation to date. The resulting system co-efficiency of 0.868 g/l/h was even higher than that of the sequential culture [0.83 g/l/h]. Previous studies on the co-culture process reporting a comparable result to this included a system co-efficiency of 0.94 g/l/h within 40 h (Taniguchi et al., 1997b), 0.53 g/l/h within 40 h (de Bari et al., 2004), and 0.70 g/l/h within 24 h (Qian et al., 2006); all of these have been discussed in detail in Section 2.4.2. The result reported here also compared favorably with some of the best results using recombinant strains, e.g. system co-efficiency of 0.76 g/l/h within 24 h using a recombinant *Z. mobilis* strain (Krishnan et al., 2000), and a system co-efficiency of 0.66 g/l/h within 48 h using a recombinant *E. coli* strain (Takahashi et al., 2000). Moreover, in this study a high xylose proportion up to 40% [60% of glucose and 40% of xylose] was used as
the substrate, which further increased the difficulty of the co-fermentation process. It is thus demonstrated that the fermentation scheme of immobilized \textit{Z. mobilis} and free cells of \textit{P. stipitis} is an excellent scheme for the co-fermentation of glucose/xylose sugar mixture.

Compared to the fermentation scheme with free cell co-culture, the result achieved in this time course indicated that the strain combination of \textit{Z. mobilis} and \textit{P. stipitis} is able to carry out the glucose/xylose co-fermentation efficiently, with the optimum environmental conditions provided for each strain. With \textit{Z. mobilis} immobilized within the gel beads, and \textit{P. stipitis} exposed to micro-aerobic conditions in the fermentation medium, both fermentations of glucose and xylose could be processed without the interference from each other, and the overall fermentation efficiency was thus improved.

However, when the same batch of beads was repeatedly used, the fermentation efficiency decreased significantly. In Section 6.1.3, this was suggested to result from the increased cell leakage of \textit{Z. mobilis} due to the reuse of beads and the prolonged exposure of beads to the fermentation medium. It was also suggested that the released \textit{Z. mobilis} cells from beads are inhibitory to the xylose fermentation by \textit{P. stipitis}. In support of this theory, the removal of the immobilized \textit{Z. mobilis} beads from the fermentation medium greatly improved the stability of the fermentation performance.

\textbf{6.5.1.4. The removal of \textit{Z. mobilis} cells from the co-culture process after the completion of glucose fermentation}

The only difference between the co-culture processes carried out in the modified bioreactor and the co-culture processes involving immobilized \textit{Z. mobilis} and free cells of \textit{P. stipitis} is that it allows the removal of \textit{Z. mobilis} beads from the fermentation medium after the completion of glucose fermentation. Through this procedure, it was possible to decrease the damage to the gel beads and maintain them in good condition for longer periods. Moreover, the potential effect of \textit{Z. mobilis} cell leakage on the xylose fermentation was minimized. Fermentations carried out in the modified bioreactor demonstrated these advantages. The results of repeated culture processes were steadier and more reliable, with similar kinetic parameters obtained
for duplicate time courses.

When the same inoculum size was used for the co-culture processes with and without the sieve plate [full batch of \textit{Z. mobilis} beads & 50\% of \textit{P. stipitis}], the resulting fermentation performance was similar. The processes carried out in the modified bioreactor took 25 h to completely utilize all 50 g/l of the sugar mixture, giving a system co-efficiency of 0.867 g/l/h, in contrast to 24 h of sugar utilization and 0.868 g/l/h of system co-efficiency for the co-culture process in the unmodified bioreactor. The slightly longer fermentation time in the modified bioreactor could be attributed to the addition of the sieve plate, which might increase the difficulty of material distribution. However, the results achieved in two processes could be considered at a similar level. This was in accordance with the results reported in Section 5.1.3, which suggested that the adverse effect of the sieve plate, if any, was negligible. The improved xylose fermentation performance, which was considerably steadier in the modified bioreactor, was more significant.

Results also indicated that the free cells of \textit{Z. mobilis} were inhibitory to the xylose fermentation process. As shown in Table 6.5, a decreased inoculum size of \textit{Z. mobilis} reduced the overall fermentation time from 25 h to 22 h, and significantly improved the ethanol yield from 0.45 g/g to 0.50 g/g, which was 98\% of the theoretical rate. This was the highest conversion rate achieved in all fermentations involving \textit{P. stipitis} in this study. This high ethanol yield remained the same when the inoculum size of \textit{P. stipitis} increased to 100\% [0.49 g/g], implying that the increased sugar-ethanol conversion rate was probably from the reduced number of \textit{Z. mobilis} cells, rather than any change in the yeast concentration.

This observation is interesting as it relates to how the bacterial cells could possibly affect the final ethanol yield. While ethanol re-assimilation by \textit{P. stipitis} has been reported many times in the literature (Maleszka and Schneider, 1982; Skoog et al., 1992; Passoth et al., 2003; Gorgens et al., 2005), the same cannot be said for re-assimilation by \textit{Z. mobilis}. However, a previous research study by Fu and Peiris (2008) reported that the ethanol produced by \textit{Z. mobilis} on glucose was completely consumed within 36 h under aerobic conditions. Other studies have pointed out that the wild type \textit{Z. mobilis} is strictly fermentative, but possesses a complete redox
enzyme system on its cell membrane (Kalnenieks, 2006). Therefore, it might be possible that the cells of Z. mobilis in the fermentation medium could oxidize the produced ethanol in a co-culture process. If this is true, then the improved ethanol yield arising from the reduced inoculum size of Z. mobilis could be explained; the reduced number of Z. mobilis free cells released to the medium resulted in a lower ethanol re-assimilation rate during xylose fermentation by P. stipitis. This theory could also explain the apparent inhibition caused by viable cells of Z. mobilis on the P. stipitis cell activity. If Z. mobilis oxidizes ethanol under aerobic conditions, then it would consume oxygen and less oxygen would be available for the P. stipitis metabolism. Therefore, higher the concentration of Z. mobilis cells in the fermentation medium, the slower would be the xylose utilization.

Finally, when it comes to the effect of P. stipitis inoculum size, an increase from 50% to 100% further decreased the overall fermentation time from 22 h to 19 h, giving the highest system co-efficiency of 1.277 g/l/h amongst all the co-fermentation processes. However, considering that a double number of P. stipitis cells were in the medium, the improvement on the fermentation performance was not as high as expected. Similar to the discussion in Section 4.3.3, this could result from insufficient oxygenation when the increase in the oxygenation level could not match the increase in the viable cell concentration.

Given the high levels of both system co-efficiency and ethanol yield as well as the steady performance during repeated culture, this fermentation scheme of co-culturing immobilized Z. mobilis and free cells of P. stipitis is considered to be the best fermentation scheme for the glucose/xylose co-fermentation process using Z. mobilis as the glucose fermenter and P. stipitis as the xylose fermenter.

### 6.5.2. Possible interactions between Z. mobilis and P. stipitis in a co-culture process

Based on the fermentations involving co-culture, there are at least two possible interactions between Z. mobilis and P. stipitis. Firstly, Z. mobilis viable cells appeared to inhibit the cell activity of P. stipitis, and consequently both efficiencies of the yeast cell growth and the xylose fermentation were reduced. Secondly, Z. mobilis showed an abnormal re-growth during xylose fermentation stage when there
was no appropriate carbon source for utilization. It was also suggested that *Z. mobilis* might be able to oxidize the ethanol produced by *P. stipitis* and further reduce the overall ethanol yield. This is however more an effect on the fermentation efficiency rather than an interaction between cells. These observations were confirmed with the experiments reported in Section 6.3.

As can be seen in both Figures 6.8 and 6.9, the decreased final ethanol concentration along with the increase in the initial cell number of *Z. mobilis* indicated the inhibitory effect of *Z. mobilis* cells on xylose fermentation by *P. stipitis*. As discussed in Section 6.5.1.4, this could be caused by either the ethanol re-assimilation, or a reduced ethanol production resulting from oxygen competition. As far as the biomass growth of *P. stipitis* is concerned, in both figures there was a similar trend in that the highest final concentration of the yeast was achieved in the “Test 3” flask containing an initial biomass ratio of *P. stipitis* to *Z. mobilis* of 5:3, while the lowest concentration was achieved in the “Test 2” flask containing an initial biomass ratio of 5:2. The reason responsible for this observation was still unclear. However, comparing the viable cell levels of *P. stipitis* between the culture on the Xylose Medium and on the Co-fermentation Medium, in the Co-fermentation Medium where *Z. mobilis* maintained a high cell concentration, *P. stipitis* cell growth was considerably lower, and the corresponding xylose fermentation was approximately at a standstill. Both observations indicate an intense inhibitory effect on the yeast cell activity when there were more *Z. mobilis* cells in the medium.

The re-increase of *Z. mobilis* cells in an environment free of an appropriate carbon source was shown in Figure 6.8. Both the temporary reduction of cells at 24 h and the re-increase at 36 h implied that *Z. mobilis* cells had access to some external nutrient in the later phase of the process which could stimulate its growth. A possibility could be that *Z. mobilis* is able to multiply based on the metabolism process of xylose by *P. stipitis*, and this might be achieved by the utilization of some intermediate metabolites from the xylose metabolism. In Figure 2.2 showing the metabolism of *P. stipitis* [see Section 2.3.1], xylose is converted to glyceraldehydes-3-phosphate through PPP pathway and then enters the glycolytic pathway, from where the same reactions are used to convert the glyceraldehydes-3-phosphate to pyruvate and then to ethanol as in the ED pathway.
of glucose metabolism in *Z. mobilis* [see Figure 2.1; Section 2.2.1]. Though the specific mechanism is still not clear, it is considered to be possible for the bacterium to utilize some metabolites from *P. stipitis* for its own growth, since some parts of the metabolic pathways are the same.

A bolder speculation would be that *Z. mobilis* directly utilizes the ATP produced during the glycolytic pathway of xylose metabolism in *P. stipitis*. This is from the inference stated in Section 4.3.1 that the cell division of *Z. mobilis* greatly depends on the energy supply from ATP. As the metabolism process from glyceraldehydes-3-phosphate to pyruvate generates 2 ATP in both the glucose metabolism of *Z. mobilis* and the xylose metabolism of *P. stipitis*, it leads to an interesting question whether it could be possible for the bacterium to directly utilize the ATP produced by the yeast. Currently there is no literature elaborating the detailed reproduction mechanism of *Z. mobilis*. Prior to attempting further explanations, details of the cell division of *Z. mobilis* and substance transport between cells need to be known.

### 6.5.3. Process considerations for the co-culture process

#### 6.5.3.1. Effects of initial sugar level

A high initial sugar level is a desirable requisite for industrial ethanol production. However, the co-culture processes with 75 g/l of initial sugars showed inferior results compared to those with 50 g/l of initial sugars [see Section 6.2]. The reduced fermentation performance was probably a result of ethanol re-assimilation during the prolonged fermentation time. As discussed in Section 5.3.2.3, the system co-efficiency \(Q_p\) is only related to the viable cell concentration \(x_C\) and cell instantaneous ethanol production rate \(de\), and the increased initial sugar level has no direct effect on \(Q_p\). In these two fermentation processes with 75 g/l initial sugars, since the inoculum size and environmental conditions were similar to those processes containing 50 g/l sugars, both values of \(x_C\) and \(de\) remained constant; consequently a longer fermentation time was required to utilize the larger amount of sugar in the medium. During the prolonged fermentation, significant ethanol re-assimilation was observed for both processes, which directly led to a decreased ethanol yield [0.42 and 0.46 g/g respectively]. The system co-efficiency thus reduced accordingly, on account of the decreased final ethanol concentration.
Z. mobilis cells in the fermentation medium are considered to be the major reason for the increased ethanol re-assimilation along with the increased fermentation time. There were several facts supporting this statement. Firstly, ethanol re-assimilation was a common phenomenon observed in almost every time course with aeration, resulting in an ethanol yield around 0.44–0.46 g/g in this study. The further oxidation of ethanol by P. stipitis was observed and reported in a number of time courses with sole xylose fermentation [both free cell culture and immobilized culture]. However, in a co-culture process with oxygen supply, it was difficult to determine which strain was responsible for ethanol re-assimilation. The only indirect evidence was that in those time courses achieving high ethanol yields of 0.49–0.50 g/g, it was the reduced amount of Z. mobilis cells that led to the increased ethanol accumulation [see Section 6.1.4]. Secondly, as Z. mobilis proved to be inhibitory for P. stipitis cell activity, the yeast’s capability for ethanol re-assimilation under these conditions was doubtful. Finally, Z. mobilis was able to multiply during the xylose fermentation by P. stipitis. During a short fermentation time, it is difficult for the bacterium to reproduce to high levels which could affect the ethanol concentration. However, with longer fermentation time, the increase of Z. mobilis viable cell concentration to a significant level is very probable and could cause notable ethanol re-assimilation. The results of biomass assay for these two processes with 75 g/l sugars provide evidence for this theory. As shown in Figure 6.7 (b), even after the majority of the bacterial cells were removed from the fermentation medium at 6 h, the free cells of Z. mobilis in the fermentation medium showed an increase in the later phase of the fermentation process.

Though the two fermentation processes with a higher initial sugar level showed a slightly lower system co-efficiency compared to that with 50 g/l initial sugars, the inferior results are thought to be mainly from the increased ethanol assimilation during the longer fermentation time to utilize all the sugars in the medium. Apart from this trend, there was no apparent inhibitory phenomenon observed for both the strains’ performance, either by the increased sugar level or by the increased ethanol level. Consequently, with further process optimization, it is believed that fermentation with higher initial sugar levels has the potential to achieve a high performance similar to that with 50 g/l initial sugars.
6.5.3.2. Inoculum size and economic considerations

For an economically feasible process, both the input cost and output benefit need to be considered. The large inoculum size employed in this study is a cost-increasing factor, as it requires more medium and a longer time to prepare. Moreover, when *P. stipitis* was utilizing xylose as its main carbon source, in both the sole xylose fermentation process and the co-culture process with *Z. mobilis*, no significant biomass growth was observed with an inoculum size larger than 50%. As can be seen in all figures presenting the biomass trends of the yeast on xylose substrate [see Figures 4.6 & 4.7 for sole xylose fermentation and 6.2, 6.3 & 6.5 for co-culture], when the inoculum size was larger than 50%, the overall cell concentration leveled out, with a slight decrease at the later phase of the process. This biomass loss was mainly due to the foaming problem, resulting from both the high cell concentration and the introduced air flow. However, the lack of biomass reproduction indicated a weak growth capability of *P. stipitis* on xylose. As reported in Section 4.3.5, the biomass increase of *P. stipitis* was considerably lower than that of *P. tannophilus* when cultured under similar conditions. Moreover, the yeast's performance on the glucose/xylose sugar mixture medium further demonstrated its poor capability of biomass growth when xylose was the main carbon source. As shown in Figure 6.11 [see Section 6.4.1], with similar inoculum size and incubation conditions, the achieved biomass concentration of *P. stipitis* growing on the xylose medium was only 3/4 of that on the glucose/xylose mixture medium in shake flasks. A similar trend was also observed in the fermentor [see Figure 6.12, Section 6.4.2]. *P. stipitis* showed a steady growth with an inoculum size of 50% when cultured on the glucose/xylose sugar mixture medium. The peak cell concentration in all three time courses was around $1.3\sim1.7 \times 10^9$ cfu/ml, a level that was never achieved by *P. stipitis* culture on the xylose medium in this study. Even in the shake flask experiment with an inoculum size of 200%, the highest biomass concentration was only $1.2\sim1.4 \times 10^8$ cfu/ml [see Figure 4.9, Section 4.2.6].

All of above data indicate that xylose was not a favorable substrate for *P. stipitis* cell reproduction. If this is the case, there are two methods that might be useful to decrease the cost of a large inoculum size. The first is to use a smaller inoculum size wherever possible. According to the results for both co-culture process [see Table 6.5,
Section 6.1.4] and \( P. \) stipitis sole culture on the xylose medium [see Figure 4.8, Section 4.2.5], the use of a 100\% inoculum size did not increase the fermentation performance proportionally. The results achieved with a 50\% inoculum size were only slightly inferior. Therefore, unless the fermentation efficiency with a 100\% inoculum size could be further optimized, it is not worth using a larger inoculum size just for a small gain in performance.

The second approach concerns cell recycling. In the literature review [see Section 2.5.3], it was pointed out that recycling cells was a common method for adapting the strain to the hydrolysate medium and thus improving fermentation efficiency. It was, however, hard to determine whether the improvement of performance was as a result of strain adaptation or simply because of the increased cell concentration during recycling. Nevertheless, collecting cells from the fermentation process, and reusing them as an inoculum for the next fermentation process is a very economical method as it saves on the inoculum cost and improves the cells' fermentation capability. There are a number of approaches for achieving this purpose, and each of them has the potential for industrial application. These include immobilization [not micro-encapsulation], natural cell sedimentation and membrane recycling system. The ultimate method of continuously employing the same batch of cells would be continuous fermentation.

6.5.4. The potential of \( P. \) stipitis sole culture for the glucose/xylose co-fermentation

This experiment was originally carried out as a basic investigation for the performance of \( P. \) stipitis sole culture on the glucose/xylose mixture medium. However, as pointed out in Section 6.4.1, the results obtained were comparable to the best results achieved with the co-culture process. The fermentation was consequently scaled up to a 1 l bioreactor to investigate the fermentation characteristics in detail.

6.5.4.1. Fermentation characteristics of \( P. \) stipitis sole culture on the glucose/xylose mixture medium

Processes carried out in the shake flask experiments and in the 1 l bioreactor [see Sections 6.4.1 and 6.4.2] gave similar fermentation performance, indicating a successful scale-up process. In all five processes [two flasks & three time courses in...
fermentor], all the sugars in the medium were completely utilized within 24 h, giving a final ethanol concentration of 24 g/l for the flasks, and 21~22 g/l for the fermentor. This result was the highest reported in studies investigating the conversion of glucose/xylose sugar mixture by *P. stipitis*. The highest system co-efficiency of 1.092 g/l/h was achieved with 100% inoculum size at an air flow of 300 cm$^3$/min [see Table 6.9; Section 6.4.2]. By contrast, it has been reported recently that a system co-efficiency of 0.19 g/l/h was obtained for *P. stipitis* on a substrate comprising 75% glucose and 25% xylose (Agbogbo et al., 2006).

As analyzed in Sections 6.4.3 and 6.5.3.2, the efficient conversion of sugar mixture was mainly a result of the vigorous *P. stipitis* cell growth on the glucose substrate. The peak viable cell level of *P. stipitis* in these three time courses was almost twice as high as that on the sole xylose substrate with an inoculum size of 100%, and 3-4 times as high as that with an inoculum size of 50%. The increased cell concentration greatly accelerated the fermentation speed, and compensated for the difference on the glucose fermentation efficiency between *Z. mobilis* and *P. stipitis*.

As shown in Figure 6.13, it took 9 h for *P. stipitis* to consume all 30 g/l glucose in the medium, whereas for *Z. mobilis*, this value was only 3 h for a full batch of immobilized beads and 6 h for a 1/4 batch. However, at 9 h when all the glucose was used up, the residual xylose in the medium had already decreased to a level between 15~16 g/l, indicating a 3/4 consumption of all the xylose in the medium. This trend was different from that observed in a co-culture process, where the xylose consumption hardly occurred during the glucose fermentation by *Z. mobilis*. In all three time courses culturing only *P. stipitis* on the glucose/xylose mixture, glucose was preferentially consumed; however, xylose utilization also started even when there was still residual glucose in the medium. Catabolite repression by glucose on the xylose assimilation was not observed. The change of utilizing the substrate from glucose to xylose was smooth. This is possibly because of the high cell concentration used for the fermentation; *P. stipitis* cells were forced to utilize every possible carbon source to avoid starvation.

Finally, it was observed that the adjustment of both the aeration level and the viable cell concentration had little impact on the overall fermentation efficiency. This
indicated that the effect of aeration level on the cell instantaneous ethanol production rate \( [de] \) was not as significant as it was when \( P. stipitis \) was cultured on the medium only containing xylose. A possible reason might be that the metabolism process of \( P. stipitis \) cells on the glucose/xylose mixture substrate was complex, and thus \( de \) was affected by multiple factors rather than simply an aeration level. Nevertheless, the relationship between system co-efficiency \( [Q_p] \) and viable cell concentration \( [x_C] \) remained true for a co-fermentation process like this, since the high viable cell level in these three time courses directly led to a high fermentation efficiency comparable with the co-culture process involving \( Z. mobilis \). However, the optimization of the value \( de \) for \( P. stipitis \) culture on glucose/xylose mixture still needs more experimental investigation.

6.5.4.2. Comparison between \( P. stipitis \) sole culture and co-culture for glucose/xylose co-fermentation

The two processes of \( P. stipitis \) sole culture and co-culture gave comparable results on the glucose/xylose co-fermentation, and each scheme had its own advantages and disadvantages.

For \( P. stipitis \) sole culture, the most notable advantage was the high biomass level that could be achieved. As shown in Figure 6.12, even with an inoculation size of 50%, the cell concentration in the medium was able to increase and reach a level similar to that inoculated with 100% cells. This solved a major problem which occurred in the co-culture process with a large inoculum size that no biomass reproduction was observed for \( P. stipitis \) [see Section 6.5.3.2]. Secondly, the culture of a single strain is much simpler, especially given the inhibitory effect of \( Z. mobilis \) cells on the \( P. stipitis \) cell activity. Finally, yeast is always preferable for an industrial process, because bacteria can be infected by the bacteriophage and die out quickly, leading to the termination of the fermentation process (Montville and Matthews, 2008).

On the other hand, the first advantage of the co-culture process is that it employs \( Z. mobilis \) for the glucose fermentation, whose \( de \) level is significantly higher than that of \( P. stipitis \). Even though both fermentation schemes gave a similar performance, further optimization of the co-culture process has the potential to improve the
fermentation performance. In contrast, for the *P. stipitis* sole culture, the inferior ethanol productivity per cell was unlikely to be improved only by process engineering. Secondly, ethanol re-assimilation remained a problem for the *P. stipitis* sole culture [see Section 6.4.2]. It decreased the final ethanol yield and thus the exact time control of the harvest would be required to obtain a maximum ethanol level. However, in the co-culture process, this was not a problem; with a reduced amount of *Z. mobilis* cells, a high ethanol yield up to 0.49-0.50 g/g was achieved, which was 96-98% of the theoretical value.

Finally, as far as the process control was concerned, both processes showed similar difficulties, with no apparent advantages over each other. For *P. stipitis* sole culture, though the growth and incubation of one strain was much easier, the fermentation of mixed substrate still needs more work to set up optimized de values for glucose fermentation and xylose fermentation respectively, as suggested in Section 6.5.4.1. In contrast, co-culture process of *Z. mobilis* and *P. stipitis* gave best fermentation performance when the xylose fermentation was not interfered by *Z. mobilis* viable cells; this can be realized by several simple process engineering techniques, which will be discussed in “Recommendations for future work” in Chapter 8. Both schemes of *P. stipitis* sole culture and co-culture were used for the fermentation of sugarcane bagasse hydrolysate to further compare their fermentation characteristics.
Chapter 7

Conversion of sugarcane bagasse hydrolysate to ethanol

Sugarcane bagasse has been commonly used as a starting material in research investigating ethanol production from lignocelluloses. It contains a high proportion of cellulose and a relatively low content of impurities, both of which are beneficial for the fermentation process (Marton et al., 2006; Qu et al., 2007). Moreover, because the bagasse has already been transported to the sugar factory for the purpose of sugar production, there is no additional transport cost involved, which is another advantage over other lignocellulosic material, such as wheat straw or corn stalk (Martin et al., 2006a; Rossell et al., 2006). This study investigated three kinds of hydrolysates prepared from bagasse raw material, viz. the acid hydrolysate, the enzymatic hydrolysate, and the enzymatic hydrolysate without pretreatment and overliming, for the production of ethanol. Each medium was used as substrate for ethanol production either by co-culture of \textit{Z. mobilis} and \textit{P. stipitis} or by sole culture of \textit{P. stipitis}.

7.1. Optimization of conditions for bagasse hydrolysis

7.1.1. Acid hydrolysis

Three batches of milled bagasse of particle size 1.0 mm were hydrolyzed following the acid hydrolysis procedure as described in Section 3.9.1. For each batch, 50 g raw
bagasse was mixed with 300 ml of dilute sulfuric acid, and hydrolyzed under different conditions. After collecting the hydrolysate in the liquid phase, the residual bagasse was mixed with dilute sulfuric acid again and re-hydrolyzed for the second time, in order to determine the effect of the repeated hydrolysis on the sugar yield. For convenience of identification, the three batches of bagasse were named as Bagasse 1, 2 and 3 respectively. The detailed conditions used for each batch of bagasse are shown in Table 7.1, and the resulting sugar yields are shown in Figure 7.1.

Table 7.1: Conditions for acid hydrolysis of the three batches of bagasse

<table>
<thead>
<tr>
<th>Hydrolysis conditions</th>
<th>Bagasse 1</th>
<th>Bagasse 2</th>
<th>Bagasse 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st acid hydrolysis</td>
<td>H₂SO₄ concentration (v/v)</td>
<td>1%</td>
<td>1%</td>
</tr>
<tr>
<td></td>
<td>Water bath at 80°C</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>2nd acid hydrolysis</td>
<td>H₂SO₄ concentration (v/v)</td>
<td>2%</td>
<td>2%</td>
</tr>
<tr>
<td></td>
<td>Water bath at 80°C</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Figure 7.1: Comparison of sugar yields under various acid hydrolysis conditions.

As can be seen in Figure 7.1, the first hydrolysis yielded a higher level of sugar concentration compared to the second hydrolysis. A notable trend was the increased
xylose concentration with the repetition of the acid hydrolysis process; by contrast, the glucose yield decreased significantly. This might be related to the increased acid concentration used for the hydrolysis [2% H$_2$SO$_4$ for all three batches of bagasse in the second hydrolysis]. The first batch of bagasse gave the lowest sugar yield in both hydrolysis processes, indicating that subjecting the hydrolysate to 80°C for 30 min also increased the sugar yield. Besides the reported glucose and xylose, cellobiose and arabinose were also observed in low concentrations in the hydrolysates. All hydrolysates were assayed for sugar concentration after the overliming process; there were no significant impurities detected by HPLC.

### 7.1.2. Enzymatic hydrolysis

The same three batches of bagasse were then repeatedly employed for the investigation of the enzymatic hydrolysis. The first experiment was carried out to investigate the effect of enzyme combination and incubation time. Each batch of bagasse was mixed with 300 ml acetate buffer, and incubated under conditions as described in Section 3.9.2. The enzyme combination employed for each batch is shown in Table 7.2, and details of each enzyme are recorded in Table 3.5 [see Section 3.9.2]. An enzyme loading of 1% (v/w) was used for each enzyme. Incubation continued for 96 h and samples were taken at 24 h interval.

<table>
<thead>
<tr>
<th>Batch of bagasse</th>
<th>Enzyme combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bagasse 1</td>
<td>Cellclast 1.5 L + Shearzyme 500 L + Biofeed Wheat L</td>
</tr>
<tr>
<td>Bagasse 2</td>
<td>Cellclast 1.5 L + Novozyme 188</td>
</tr>
<tr>
<td>Bagasse 3</td>
<td>Cellclast 1.5 L + Viscozyme L</td>
</tr>
</tbody>
</table>

Results of this experiment are shown in Figure 7.2. Most of the hydrolysis in all three batches was completed within 24 h; the extended incubation time made little improvement to the sugar yield. Enzyme combination of “Cellclast 1.5 L + Shearzyme 500 L + Biofeed Wheat L” used in the first batch of bagasse gave the lowest yield of glucose, which led to the lowest level of total sugar. In the other two
batches the overall sugar yields were at a similar level [10~11 g/l], but the enzyme combination of “Cellclast 1.5 L + Novozyme 188” yielded a higher glucose concentration compared to the combination of “Cellclast 1.5 L + Viscozyme L”.

![Figure 7.2: Sugar yields with different enzyme combination after 96 h incubation.](image)

The second experiment of enzymatic hydrolysis investigated the effect of an increased enzymatic loading and a sequential addition of enzyme combinations. Two batches of bagasse were employed, viz. Bagasse 1 & 3, and mixed with 300 ml acetate buffer followed by the addition of a combination of Cellclast 1.5 L and Novozyme 188 to start hydrolysis. After 24 h incubation, enzyme Shearzyme 500 L and Biofeed Wheat L were added. All enzymes were used at a loading of 2% (v/w). The results of this experiment are shown in Figure 7.3. Comparing the sugar yields between the first and the second experiments, it was found that the increased enzyme loading increased the final sugar concentration in the hydrolysate. After 24 h, the sugar concentration in the “Bagasse 1” with an enzyme loading of 2% was 5.92 g/l glucose and 5.92 g/l xylose respectively, whereas with a loading of 1%, these values were 2.1 g/l and 4.67 g/l respectively [see Figure 7.2] The overall sugar yields in the “Bagasse 3” was also higher in this experiment than in the first experiment [11.99 g/l compared to 10.12 g/l]. The addition of enzyme combination of Shearzyme 500 L
and Biofeed Wheat L at 24 h only slightly increased the overall sugar yield in both batches of bagasses.

![Figure 7.3: Sugar yields with sequential enzyme addition and increased enzyme loading.](image)

In the third experiment, all these three batches of bagasse were hydrolyzed using the enzyme combination of Cellclast 1.5 L and Novozyme 188 again at an enzyme loading of 2% (v/w), with 200 ml of acetate buffer. After centrifuging to collect the liquid phase, the residual liquid in the bagasse fibre was manually squeezed for recovery. Sugar was assayed both before and after the squeeze to test the change on sugar composition. Figure 7.4 shows the results of this experiment. Since all three batches of hydrolysis were carried out under similar conditions, the resulting sugar yield was similar. The overall sugar level was around 10–11 g/l, slightly lower compared to that achieved in the second experiment [11–12 g/l]; this was thought to be a result of the depleting bagasse material during the repeated experiments. Squeezing the liquid hydrolysate out of bagasse fibre had no apparent effect on the sugar concentration; however, the available hydrolysate volume increased significantly. It was thus kept as the recovery method; together with the enzyme combination of Cellclast 1.5 L and Novozyme 188, enzyme loading of 2% and incubation time 24 h, these conditions were chosen as the standard condition for the enzymatic hydrolysis in this study.
7.2. Preparation of hydrolysate for fermentation

Based on the results reported above, the procedure of hydrolysis used in this study is shown in Figure 7.5. For the preparation of fermentation substrate of “Acid hydrolysate” and “Enzymatic hydrolysate”, 100 g of fresh bagasse material was used. In addition, residual bagasses from the experiment reported in Section 7.1, “Bagasse 1, 2 & 3”, were combined together and subjected to the same procedure, to prepare the adaptation medium for the microbial strains. The third batch of raw bagasse was mixed with 600 ml of acetate buffer and directly subjected to the same procedure of enzymatic hydrolysis, without the prior acid hydrolysis. The resulting liquid, without overliming for detoxification, was used as the third fermentation substrate. This time, the adaptation medium was prepared by enzymatically hydrolyzing the solid phase of bagasse residue in Figure 7.5. A comparison of the sugar yields in the three processes of hydrolysis, viz. the acid hydrolysis, the enzymatic hydrolysis and the enzymatic hydrolysis without prior acid treatment, is shown in Table 7.3.
As shown in Table 7.3, the final sugar concentration in all six hydrolysate media was around 8–12 g/l, similar to that achieved in the experiments reported in Sections 7.1.1 and 7.1.2. Comparing the effect of acid hydrolysis and enzymatic hydrolysis, the overall sugar yield was higher in the enzymatic hydrolysate compared to that in the acid hydrolysate [12.35 compared to 11.03 g/l for fresh bagasse, and 8.84 compared to 8.10 g/l for residual bagasse]. However, acid hydrolysis in general yielded higher concentration of xylose [6.16 compared to 3.37 g/l for fresh bagasse, and 4.96 compared to 3.14 g/l for residual bagasse]. For all three hydrolysates, the
sugar yield in the bagasse material that had been repeatedly hydrolyzed was lower than that in the fresh bagasse material.

### Table 7.3: Sugar yields in the hydrolysates prepared for fermentation

<table>
<thead>
<tr>
<th></th>
<th>Acid hydrolysate</th>
<th>Enzymatic hydrolysate</th>
<th>Hydrolysate without acid treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh</td>
<td>Residual(^a)</td>
<td>Fresh</td>
</tr>
<tr>
<td>Glucose (g/l)</td>
<td>4.87</td>
<td>3.14</td>
<td>8.98</td>
</tr>
<tr>
<td>Xylose (g/l)</td>
<td>6.16</td>
<td>4.96</td>
<td>3.37</td>
</tr>
<tr>
<td>Glucose+Xylose (g/l)</td>
<td>11.03</td>
<td>8.10</td>
<td>12.35</td>
</tr>
</tbody>
</table>

\(^a\): Bagasse residue from the experiment in Section 7.1.2.

\(^b\): Bagasse residue from the solid phase after enzymatic hydrolysis in Figure 7.5.

Considering the effect of acid treatment on the following enzymatic hydrolysis, both glucose and xylose yields were slightly lower in the enzymatic hydrolysate without the acid treatment [7.63 g/l and 2.54 g/l compared to 8.98 g/l and 3.37 g/l]. A further comparison is shown in Table 7.4. The “Bagasse hydrolysis rate” for the process with prior acid hydrolysis included the bagasse consumption during the acid hydrolysis; thus the higher hydrolysis rate of 35.4% did not reflect the actual performance of the sole enzymatic hydrolysis. However, it indicated a higher overall efficiency of bagasse conversion when both acid hydrolysis and enzymatic hydrolysis were involved compared to a single enzymatic hydrolysis process. The higher level of sugar recovery further confirmed this trend [79.4% compared to 59.4%]. The enzymatic hydrolysate without prior acid treatment was directly used for the fermentation time course without overliming and HPLC analysis showed no significant impurities in its contents.

Overall, the final sugar concentration of 10–12 g/l in all three hydrolysates was too low to be used in a fermentation process for ethanol production. Therefore both glucose and xylose were supplemented as described in Section 3.9.3. Moreover, yeast extract and inorganic salts were also added to the hydrolysate medium to improve the fermentability.
Table 7.4: Comparison of hydrolysis efficiency of enzymatic hydrolysis with and without prior acid treatment

<table>
<thead>
<tr>
<th></th>
<th>Sugar level in hydrolysate (g/l)</th>
<th>Bagasse hydrolysis rate (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sugar recovery efficiency (%)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>With acid treatment</td>
<td>12.35</td>
<td>35.4%&lt;sup&gt;c&lt;/sup&gt;</td>
<td>79.4%&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Without acid treatment</td>
<td>10.17</td>
<td>17.1%</td>
<td>59.4%</td>
</tr>
</tbody>
</table>

<sup>a</sup>: Calculated as the consumed dry biomass weight / original weight used for hydrolysate.

<sup>b</sup>: Calculated as overall concentration of sugar yield in the hydrolysate / consumed biomass concentration.

<sup>c</sup>: Included sugar yield during the acid hydrolysis process.

7.3. Fermentation of bagasse hydrolysate

Table 7.5 shows the conditions used for the three time courses carried out with bagasse hydrolysate. Three hydrolysate media were randomly selected for the fermentation processes with different fermentation schemes.

Table 7.5: Conditions for three fermentation processes using bagasse hydrolysate

<table>
<thead>
<tr>
<th></th>
<th>Time course 1</th>
<th>Time course 2</th>
<th>Time course 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>Acid hydrolysate</td>
<td>Enzymatic hydrolysate</td>
<td>Hydrolysate without acid treatment</td>
</tr>
<tr>
<td>Fermentation scheme</td>
<td>Co-culture in the modified bioreactor</td>
<td>Co-culture in the modified bioreactor</td>
<td>P. stipitis sole culture</td>
</tr>
<tr>
<td>Inoculum size</td>
<td>1/4 batch of Z. mobilis &amp; 50% of P. stipitis</td>
<td>1/2 batch of Z. mobilis &amp; 50% of P. stipitis</td>
<td>50% of P. stipitis</td>
</tr>
<tr>
<td>Times of strain adaptation</td>
<td>Twice for both strains</td>
<td>Once for both strains</td>
<td>Once</td>
</tr>
<tr>
<td>Air flow (cm³/min)</td>
<td>80</td>
<td>80</td>
<td>250</td>
</tr>
</tbody>
</table>
7.3.1. Time course on the enzymatic hydrolysate

The adaptation media of *Z. mobilis* and *P. stipitis* were analyzed for residual sugars at the end of each adaptation period. *Z. mobilis* successfully utilized all the glucose in the hydrolysate medium, with all the xylose left remained after 12 h of adaptation. *P. stipitis* completely utilized both the glucose and xylose within 24 h of adaptation. The corresponding ethanol accumulation was observed for both strains. The results of the time course carried out on the enzymatic hydrolysate of bagasse are shown in Figure 7.6.

![Graph showing sugar utilization and ethanol production](a)

![Graph showing biomass growth](b)

**Figure 7.6: Time course using enzymatic hydrolysate of bagasse as substrate**: (a) sugar utilization and ethanol production; (b) biomass growth.
This time course, using the co-culture of immobilized \textit{Z. mobilis} and free cells of \textit{P. stipitis}, gave a poor fermentation performance. As can be seen in Figure 7.6 (a), it took 9.5 h to utilize all the glucose in the medium, and the xylose was consumed at a low rate. The utilization of xylose terminated at 18.5 h when there still was 7.51 g/l of xylose left in the medium. Ethanol peaked 6.5 h later [25 h] after the termination of xylose utilization, at a concentration of 17.59 g/l. After that, ethanol re-assimilation was observed, i.e. ethanol concentration decreased to 15.31 g/l at 31 h.

The performance was much inferior compared to that of the time course using same fermentation scheme on the sugar medium. As shown in Figure 6.6, with the same inoculum size [1/4 batch of \textit{Z. mobilis} and 50\% of \textit{P. stipitis}], glucose and xylose were completely utilized at 6 h and 22 h respectively, and the peak ethanol concentration was 24.78 g/l.

The poor fermentation performance was considered as a result from the unfavorable biomass growth during the fermentation process. As can be seen from Figure 7.6 (b), \textit{P. stipitis} cell concentration decreased steadily throughout the whole time course, from $1 \times 10^9$ cfu/ml to only 1/5 by the time that xylose utilization terminated [$2.3 \times 10^8$ cfu/ml at 18.5 h]. This trend continued to the end of the process, giving a final \textit{P. stipitis} level of $3.0 \times 10^7$ cfu/ml. The large decrease in the biomass concentration of the yeast could be the explanation for the low efficiency of the xylose fermentation.

By contrast, though the majority of \textit{Z. mobilis} cells entrapped in the immobilized beads was removed from fermentation medium as early as at 6 h, the bacterial cells in the medium kept a steady increase during the process, reaching a peak level of $2.5 \times 10^9$ cfu/ml at 28.5 h. This abnormal increase of \textit{Z. mobilis} cells in a medium lacking an appropriate carbon source was in accordance with the earlier observations on the sugar medium [see Sections 6.1.2 & 6.3.1].

Some of the unique fermentation characteristics observed for the time course are as follows. Firstly, the inability of estimating the progress of fermentation by monitoring the change of pH. The glucose fermentation in this time course was complete at 9.5 h, but the immobilized \textit{Z. mobilis} beads were removed from the fermentation medium at 6 h. The misjudging of the completion of glucose fermentation was due to the observation of a re-increase of the pH around 6 h. As described in Section 3.7.4, on the sugar medium this would represent the full
consumption of the glucose in the sugar mixture substrate. However, at the time when the pH of hydrolysate medium showed a slight re-increase, there was still 7.59 g/l of glucose left in the medium. This irregular pH profile was probably because of the fact that the bagasse hydrolysate was repeatedly treated at extreme pH conditions [pH 1~2 during the acid hydrolysis and pH 10.5 for overliming] during the hydrolysis process.

Secondly, the effect of the adaptation process prior to fermentation. After adapting the immobilized Z. mobilis beads twice in the hydrolysate medium, the surface of gel beads became rough and sticky. This directly affected the difficulty of inoculating the beads into the bioreactor because they tended to stick onto the glass funnel used for inoculation. This change of the bead surface was different to that caused by the accumulation of bacterial cells inside the beads [see Section 5.1.2]. The texture of gel surface changed after the adaptation on the hydrolysate. P. stipitis cell growth also showed some significant trends. After being twice adapted in the hydrolysate medium, the initial cell concentration of P. stipitis in this time course was around $1.0 \times 10^9$ cfu/ml, which was approximately twice as high as that achieved with a normal 50% inoculum size [$4.0 \sim 6.0 \times 10^8$ cfu/ml; see Figures 4.6 and 6.3]. During the fermentation, the yeast cells showed an unusual flocculant nature. The cells aggregated into tiny grains and sedimented quickly if not stirred; the appearance looked very different to the smooth and turbid nature in the sugar medium [see Figure 6.4]. This could be caused by both the composition in the hydrolysate and the cell aging during the prolonged incubation time of repeated subculture [48 h in the sugar medium for the preparation of 50% inoculum size, and 24 h twice in the adaptation medium]. It is considered that this abnormal nature of P. stipitis cells could be responsible for the significant cell decrease during the fermentation process.

In general, the main reasons for the poor fermentation performance on the enzymatic hydrolysate were thought to be the unfavorable biomass levels in the medium. The early removal of Z. mobilis from the fermentation medium decreased the efficiency of glucose fermentation, and the reduction in the cell concentration of P. stipitis resulted in a poor performance on the conversion of xylose to ethanol. Consequently, in the second time course using the acid hydrolysate, the inoculum size of Z. mobilis was increased to 1/2 batch, and the adaptation times of both strains on the
Chapter 7. Conversion of sugarcane bagasse hydrolysate to ethanol

hydrolysate medium was reduced to a single adaptation.

7.3.2. Time course on the acid hydrolysate

The results of the time course on the acid hydrolysate of bagasse are shown in Figure 7.7. Resulting from the adjustment of the inoculum, the fermentation efficiency was notably improved. As can be seen in Figure 7.7 (a), the glucose utilization in the first 4 h by \textit{Z. mobilis} was rapid and efficient, similar to the trend shown in Figure 6.6 (a) with the sugar medium. The decreased glucose utilization rate observed from 4 h to 13.5 h was mainly from the inappropriate sampling time. The majority of \textit{Z. mobilis} was removed from the fermentation medium at 4.5 h; however, the residual glucose in the medium [3.68 g/l] showed no interference with the xylose fermentation. All xylose was used up at 26 h, resulting in a fermentation time comparable with those achieved with the sugar medium [19-25 h]. However, ethanol production only peaked at 40 h, giving an exceptionally high concentration of 27.98 g/l. The overall conversion rate was 0.49 g/g sugar mixture, 96% of the theoretical value [0.51 g/g].

Biomass growth in this time course was in accordance with the excellent fermentation performance. \textit{Z. mobilis} cells were not observed on any plates of the viable cell assay, except for a low concentration at 4 h [1.0x10^6 cfu/ml], which means that the potential adverse effect of \textit{Z. mobilis} cells on the xylose fermentation was minimized [see Section 6.5.2]. \textit{P. stipitis} with a high level of cell concentration [5~7x10^8 cfu/ml] throughout the whole process also guaranteed an efficient conversion of xylose to ethanol.

Similar to the first time course, the pH again showed abnormal variation and led to the early removal of the \textit{Z. mobilis} beads from the fermentation medium before the completion of glucose fermentation. A freshly-made batch of immobilize\textit{Z. mobilis} was employed for this fermentation; however, after adaptation, the texture of bead surface showed similar changes to earlier observations [see Section 7.3.1].
Figure 7.7: Time course using acid hydrolysate of bagasse as substrate: (a) sugar utilization and ethanol production; (b) biomass growth.

7.3.3. Time course on the hydrolysate without pretreatment and detoxification
In Section 6.4 the sole culture of P. stipitis on the glucose/xylose mixture gave comparable results with the co-culture; therefore there was a need to investigate the performance of P. stipitis sole culture on the hydrolysate medium. Figure 7.8 summarizes the results of this time course. A notable trend in this fermentation was the lag phase of sugar utilization and ethanol production. Although the viable cells of P. stipitis leveled off at a high concentration level around 5~7x10⁸ cfu/ml, the rapid glucose utilization and ethanol production only started at 13.5 h, and the overall fermentation efficiency was low. It took 21.5 h [13.5 h to 35 h] to utilize all the
glucose in the medium [32.75 g/l], whereas on the sugar medium, 30 g/l of glucose could be fully assimilated within 9 h by *P. stipitis* sole culture [see Figure 6.13, Section 6.4.2]. Xylose was almost un-consumed during the whole process, except for a sharp decrease between 35 h to 44 h, which corresponded to a slight decrease in ethanol concentration in the medium. Ethanol peaked at 35 h, giving a low concentration of 12.54 g/l. In the processes reported earlier with *P. stipitis* sole culture on the glucose/xylose sugar mixture, it was observed that the yeast grew vigorously on both the sugar medium [see Section 6.4] and the hydrolysate medium [during the adaptation process; see Section 7.3.1]. However, in this fermentation, there was no significant cell growth observed.

This time course employed similar conditions to the third time course reported in Section 6.4.2 culturing sole *P. stipitis* on the glucose/xylose mixture. Both the viable cell concentration and the air flow were at a sufficiently high level [4~7x10^8 cfu/ml and 250 cm^3/min respectively], which excluded the possibility that the poor fermentation performance was caused by an unfavorable biomass level. Therefore, the most possible reason for the long lag phase and the low fermentation efficiency would be the unfavorable substrate, i.e. the hydrolysate medium of bagasse without the pretreatment and detoxification process.

Figure 7.8: Time course using hydrolysate without pretreatment and detoxification as substrate.
7.3.4. **Kinetic parameters of three time courses on the hydrolysate medium**

Kinetic parameters of three time courses carried out on three different hydrolysate media are shown in Table 7.6. In both Time courses 1 and 3 where the sugars were not completely utilized, the ethanol peaked before the fermentation ended, indicating an ethanol re-assimilation even when there was still xylose left in the medium. In Time course 2, however, the peak of ethanol occurred 14 h later than the complete utilization of both glucose and xylose in the medium. Time course 1 gave lower system co-efficiency $[Q_p]$ compared to Time course 2 [0.703 g/l/h and 0.879 g/l/h respectively], and its average viable cell concentration of *P. stipitis* [$x_c$] was accordingly lower [4.4x10^8 compared to 5.1x10^8 cfu/ml]. This suggested that in both time courses the overall value of $de [Q_p/x_c]$ was at a similar level, and therefore the poor fermentation performance in Time course 1 was mainly due to the depletion of *P. stipitis* viable cells at the later phase of fermentation [see Figure 7.6]. By contrast, Time course 3 with highest $x_c$ level of *P. stipitis* showed the lowest system co-efficiency [0.358 g/l/h] amongst three time courses. Considering that on the sugar medium the sole culture of *P. stipitis* could give comparable results to the co-culture of two strains, it could be thus inferred that the fermentation efficiency of *P. stipitis* cells in this time course was inhibited, which was indicated as a decrease in the value of cell instantaneous ethanol production rate [$de= Q_p/x_c$].

<table>
<thead>
<tr>
<th>Table 7.6: Kinetic parameters of three time courses on the hydrolysate medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kinetic parameters</td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Sugar utilization time [$t_f$, h]</td>
</tr>
<tr>
<td>Ethanol peak hour [$t_p$, h]</td>
</tr>
<tr>
<td>Sugar consumption [%]</td>
</tr>
<tr>
<td>Ethanol yield [$Y_{p/s}$, g/g]</td>
</tr>
<tr>
<td>System co-efficiency [$Q_p$, g/l/h]</td>
</tr>
<tr>
<td>Average viable cell concentration of <em>P. stipitis</em> [$x_c$, cfu/ml]</td>
</tr>
</tbody>
</table>
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7.4. Discussion

7.4.1. Hydrolysis process

Some important observations are as follows.

**Low concentration of sugar yield**

In all experiments, the final sugar yields in the hydrolysate from both acid hydrolysis and enzymatic hydrolysis were below 15 g/l [see Sections 7.1.1 and 7.1.2]. This result was in accordance with previous reports, in which the acid hydrolysate of bagasse needed to be concentrated to increase the sugar level and therefore make it more fermentable (Roberto et al., 1991; Sene et al., 1998; Sene, 2001; Baudel et al., 2005a; Santos et al., 2005). Takahashi et al. (2000) however reported a total sugar level up to 68 g/l without any concentration procedure. The acid hydrolysis method used in this study was adopted from Takahashi et al. (2003), but the achieved sugar yield level was only 11–12 g/l. Even after multiple experiments of repeating the procedure with different batches of bagasse under different conditions, the sugar yield could not be improved. Therefore, enzymatic hydrolysis was resorted to. Other reports employing similar acid hydrolysis techniques also have achieved higher sugar levels. For instance, xylose concentrations of 57.25 g/l and 21.6 g/l were reported by Fogel et al. (2005) and Aguilar et al. (2002) respectively. These suggested that the acid hydrolysis process carried out in this study has the potential for further optimization.

Reports on the enzymatic hydrolysis stated that the final sugar concentration in the medium was low and additional glucose was needed as a supplement to improve the fermentability of the hydrolysate medium (Davis et al., 2005). In another study, Martin et al. (2006a) employed a loading ratio of acetate buffer to bagasse at 50:1 (v/w) and also used an SSF process to improve the conversion performance. These techniques were, however, impractical for the current study. Adsul et al. (2005) investigating the characteristics of enzymatic hydrolysis used a higher liquid:solid ratio of 100:1 (v/w). Jeffries and Schartman (1999) suggested that a sequential addition of cellulase and glucosidase improved the saccharification rate of cellulosic material, but no values were given for the final sugar concentration. In this study, the
sequential addition of enzymes failed to increase the sugar levels significantly. In the second experiment of enzymatic hydrolysis [see Figure 7.3], the addition of two xylanases after 24 h of the saccharification process only slightly increased the overall sugar yield.

**Similar sugar yield in different enzymatic hydrolysis processes**

Section 7.1.2 reported enzymatic hydrolysis processes carried out under different environmental conditions; however, the resulting sugar yields in these processes were very similar. The enzyme combination of cellulase and xylanase gave an inferior yield of glucose (~2 g/l). All other hydrolysates contained approximately similar levels of total sugars, with the only difference being the proportion of glucose and xylose. Neither the increase of the enzyme dose nor the supplement of additional enzymes markedly affected final sugar levels in the hydrolysate. In contrast, when the same batch of bagasse was repeatedly hydrolyzed, it continued to yield sugars of an approximately similar level. All these observations showed a possible equilibrium in the enzymatic catalysis reactions. As suggested by Chandrakant and Bisaria (1998), the released sugars tended to inhibit the activity of cellulolytic enzymes. Without the removal of these sugars from the medium, the saccharification process somehow was suspended, consequently limiting the achievable final sugar level.

**Higher xylose yield from the acid hydrolysis process**

The sugar yield pattern was also in accordance with previous reports. As discussed in Section 2.5.1, a number of reports have pointed out that the hemicellulose in the lignocellulose was relatively easier to hydrolyze during an acid hydrolysis process compared to the cellulose, resulting in xylose-rich hydrolysates (Taniguchi et al., 1997b; Chandrakant and Bisaria, 1998). The results of this study demonstrated this pattern [see Section 7.2]. During the complete hydrolysis process including both acid treatment and enzymatic saccharification, the sugar yield in the acid hydrolysate contained a higher level of xylose. By contrast, the glucose concentration in the enzymatic hydrolysate was substantially higher, leading to a higher total sugar yield in the enzymatic hydrolysate than in the acid hydrolysate.

**Improvement of hydrolysate fermentability by overliming**

Comparing the fermentations on the hydrolysates, only the time course carried out in
the hydrolysate medium without pretreatment and overliming showed a lag phase of sugar utilization and ethanol production [see Section 7.3.3]. The inhibition of \textit{P. stipitis} could be a result from the un-detoxified hydrolysate, although no inhibitors were detected in any significant levels by the HPLC analysis of the hydrolysate. Overliming is considered as an effective detoxification method for the lignocellulosic hydrolysates. It removes inhibitors such as acid, phenolic components and furfural by precipitating them with calcium cations (Amartey and Jeffries, 1996; Takahashi et al., 2000). It was reported that \textit{Z. mobilis} is sensitive to the inhibitors in the hydrolysate medium (Delgenes et al., 1996b; Yu and Zhang, 2002). The successful glucose fermentation without a lag phase in the first two time courses suggested that both media of acid hydrolysate and enzymatic hydrolysate were free of inhibitors, as a result of the overliming process carried out. In contrast, though no inhibitor was observed by HPLC analysis, the poor fermentation performance in Time course 3 indicated the low fermentability of the hydrolysate without overliming. A detoxification process was therefore considered to be a crucial step for an efficient conversion of lignocellulose to ethanol. Both the acid and enzymatic hydrolysates with proper detoxification appeared not to have a significant effect on the fermentation performance, except for the effect on the gel beads texture. With appropriate biomass levels and optimum conditions, the hydrolysate could be expected to show similar results to the sugar medium.

Despite the failure to achieve a high sugar concentration, the major features observed for the hydrolysis process of sugarcane bagasse in this study were in accordance with the previous literature. The process still needs extensive research to make it economically viable on an industrial scale. Future plans for the improvement of hydrolysis efficiency could include using microorganisms as whole cell catalysts instead of cellulosytic enzymes. This has the potential to reduce the cost of using expensive enzymes, and efficient processes could be established with constructed strains.

\textbf{7.4.2. Fermentation processes using the hydrolysates}

\textbf{7.4.2.1. Process considerations for the co-culture and the \textit{P. stipitis} sole culture on the hydrolysate medium}

For co-culture processes in both Time course 1 and 2, the ethanol concentration
peaked hours later than the time when xylose was fully consumed or stopped being utilized. In Time course 1, the sugar consumption terminated at approximately 18.5 h and ethanol peaked 6.5 h later at 25 h. In Time course 2, it took an additional 14 h for ethanol to reach the peak [40 h compared to 26 h when xylose was completely utilized]. A similar trend was reported in previous research. Bari et al. (2004) co-immobilized *S. cerevisiae* and *P. stipitis* to produce ethanol from the aspen hydrolysate; while both glucose and xylose were fully consumed by 24 h, the ethanol concentration reached the peak at 40 h. However, in his study this trend was also observed when a sugar mixture medium was used as the substrate. By contrast, in the current study, the time of the complete sugar consumption and the time of the ethanol peaking were approximately the same in the fermentations with the sugar mixture medium [see Figure 6.6]. The lag in the ethanol peak time on the hydrolysate medium is considered to result from the time needed to convert all the assimilated xylose to ethanol in the *P. stipitis* cells. The increased time difference between the complete sugar consumption and the ethanol peaking implied a reduction in the *P. stipitis* metabolism efficiency. This reduced metabolism efficiency was not reflected in the sugar uptake rate, since in Time course 2 the 26 h taken for sugar utilization was approximately the same as the time required in the time courses carried out on the sugar medium [with an inoculum size of 50% for *P. stipitis*, the sugar was completely utilized in 25 h when a full batch was employed, and in 22 h when 1/4 batch of *Z. mobilis* was employed; see Table 6.5]. However, the delayed ethanol peak time indicated a requirement for a longer time to convert all of the assimilated xylose to ethanol.

Considering the interactions between two strains, the cell growth of *Z. mobilis* without an appropriate carbon source was observed once again in Time course 1, and it was associated with a decline of *P. stipitis* viable cells in the medium. From 25 h to 31 h when *Z. mobilis* reached a high cell concentration of $2.5 \times 10^9$ cfu/ml and *P. stipitis* decreased to less than $1 \times 10^8$ cfu/ml, a reduction of ethanol concentration was observed, from 17.59 g/l to 15.31 g/l. By contrast, in Time course 2 where the free cells of *Z. mobilis* were controlled to a minimum level, such an ethanol decrease was not observed and the ethanol concentration peaked 14 h after all the xylose had been consumed. Both observations support the viewpoint that *Z. mobilis* is able to re-assimilate ethanol under aerobic conditions.
As far as the *P. stipitis* growth on the hydrolysate medium is concerned, firstly it had the potential to multiply to a high concentration when cultured in this environment. As reported in Section 7.3.1, an inoculum size of 50% was employed for *P. stipitis* in Time course 1 [cells from 400 ml inoculum medium into 800 ml fermentation medium]. However, after twice adaptation to the hydrolysate prior to fermentation, the initial cell concentration of *P. stipitis* in the fermentation medium reached a level of $1 \times 10^9$ cfu/ml, which was approximately at a concentration level of the 100% inoculum size. This indicated that after the adaptation process, yeast cells multiplied to twice the level of before the adaptation. Given that the initial cells in the 400 ml adaptation medium were from 400 ml of inoculum medium after 48 h incubation [see Section 3.9.3], which equalled an inoculum size of 100%, the doubling of the cell concentration indicated a vigorous cell growth during the adaptation process. This high level of cell concentration achieved in the adaptation medium was in accordance with the observation reported in Section 6.4, where it is suggested that *P. stipitis* gave higher biomass yield on the glucose/xylose mixture medium than on the xylose medium.

Moreover, if considering each adaptation flask as a mini fermentation process inoculated with 100% *P. stipitis* cells, the yeast showed reasonable performance on the conversion of both sugars to ethanol in the hydrolysate medium with prior detoxification. Therefore, the inhibition of both the cell growth and the ethanol production of *P. stipitis* in Time course 3 suggested that the yeast was sensitive to some inhibitors in the hydrolysate, which could be removed by overliming. Nevertheless, the longer metabolism time needed for converting sugars to ethanol as discussed earlier would be a disadvantage for the improvement of fermentation efficiency. Once the mechanism of metabolism responsible for this problem is unraveled, improvements could be attempted.

The co-culture process also showed some shortcomings when used on the hydrolysate medium. For example, the pH change could no longer be associated with the sugar consumption [see Sections 7.3.1 and 7.3.2], which made the determination of fermentation status more difficult. The removal of *Z. mobilis* beads from the medium could be impractical on an industrial scale. Some ideas for overcoming this
problem are suggested in Chapter 8.

7.4.2.2. General discussion

Similar to the fermentations on the sugar medium, the biomass proved to be the most decisive factor for the overall fermentation performance. Low level of biomass only resulted in reduced fermentation efficiency [see Section 7.3.1]. A sufficient viable cell level in the medium is a prerequisite for an efficient ethanol production [see Section 7.3.2], and a vigorous metabolic activity is also very crucial [see Section 7.3.3]. Both strains of *Z. mobilis* and *P. stipitis* grew well on the detoxified hydrolysate, showing comparable fermentation performance to the cultures on the sugar medium.

In Time course 2, the successful co-culture of immobilized *Z. mobilis* and free cells of *P. stipitis* converted 32.14 g/l glucose and 21.42 g/l xylose to ethanol within 26 h; the ethanol peaked at 40 h, giving a yield rate of 0.49 g/g. The fermentation performance compared favorably with previous reports. Qian et al. (2006) co-cultured a recombinant *E. coli* with *S. cerevisiae* on softwood hydrolysates, and a full conversion was achieved within 24 h with an ethanol yield of 0.45 g/g. Their study, however, used a low initial sugar level of 37.5 g/l and was carried out in shake flasks, which would help to improve the fermentation efficiency because of the smaller ratio of substrate to biomass [see Section 5.3.2.3]. The research of de Bari et al. (2004) showed a pattern of delayed ethanol peaking similar to this study, but with a much lower ethanol yield of 0.39 g/g. Other studies (Nigam, 2001b; Saha et al., 2005) investigating the conversion of wheat straw hydrolysate to ethanol required considerably longer fermentation times to completely utilize all the sugars in the medium [around 110~120 h]. Amongst studies with GMO, Krishnan et al. (2000) reported an ethanol concentration up to 44.3 g/l within 24 h by a recombinant *Z. mobilis*; however, only 80% xylose was consumed in this process. Takahashi et al. (2000) employed a recombinant *E. coli* strain on the acid hydrolysate of bagasse; sugars were fully consumed by 48 h, with an ethanol yield of 0.47 g/g.

It can thus be concluded that the fermentation scheme of immobilized *Z. mobilis* and free cells of *P. stipitis* designed in the current study gave an excellent performance on the conversion of bagasse hydrolysate to ethanol, and has the potential to be
employed for industrial production. However, for the establishment of an economically feasible process for the conversion of lignocellulose to ethanol, the increase of hydrolysis efficiency and the improvement of hydrolysate convertibility still need more work.
This study investigated fermentation processes for ethanol production from lignocellulosic material by \textit{Z. mobilis} and \textit{P. stipitis}; optimized fermentation schemes were first established on a sugar mixture medium, and then applied to the hydrolysate medium. The research covered fermentation process engineering, strain interactions in the co-culture, microbial metabolism on complex media containing two carbon sources and the hydrolysis process of sugarcane bagasse.

\subsection*{8.1. General conclusions}

\subsubsection*{8.1.1. The correlation between viable cell concentration and system co-efficiency}

It was proved that for both \textit{Z. mobilis} culture and \textit{P. stipitis} culture, the system co-efficiency \([Q_p]\) of ethanol production is directly proportional to the viable cell concentration \([x_c]\) in the medium. Based on Formula (8), an empirical formula describing ethanol production, and Formula (5), the definition of system co-efficiency, another formula was derived:

\begin{equation}
Q_p = x_c \times de (g/ll/h)
\end{equation}

where \(de\) is a theoretical value, defined as the cell instantaneous ethanol production rate (g/h/cells). Further derivatives of these formulae explained a number of
Chapter 8. Conclusions and recommendations for future work

observations during the ethanol production process.

Firstly, for *P. stipitis* culture, when the viable cell concentration \( [x_c] \) remains constant, the oxygenation level directly determines the system co-efficiency. This is because an optimized oxygenation level improves the metabolism efficiency of xylose in the *P. stipitis* cells, and thus the value of the cell instantaneous ethanol production rate \((de)\) increases.

Secondly, the fermentation time used to convert a certain amount of sugar is only decided by the ratio of substrate to the biomass.

\[
t_2 - t_1 = \frac{1}{de} \frac{P_{yie}}{N} (h)
\]  
(8.4)

When the sugar concentration in the medium remains the same, a smaller volume of medium results in lower amounts of sugars. In association with an increased total number of biomass \([N]\), the overall fermentation time decreases. This explains the observation that when a conical flask is used as a bioreactor, the fermentation efficiency tends to improve.

Finally, the system co-efficiency \( Q_p \) is not determined by the initial sugar concentration and the corresponding final ethanol concentration. By definition, \( Q_p \) is the quotient of peak ethanol concentration divided by the time. However, as shown in Formula (8.4), when both values of \( de \) and \( N \) stay constant, the change in the initial sugar concentration would only lead to the corresponding change in the fermentation time; as a result, no matter what the sugar concentration is, the system co-efficiency \([Q_p]\) remains unaffected. This accounts for the use of the absolute amount of \( P_{yie} \) and \( N \) in Formula (8.4), rather than their corresponding concentration values.

The cell instantaneous ethanol production rate \([de, g/h/cells]\) is partly reflected by the specific ethanol productivity \([q_{lp}, g/g/h]\) during a fermentation process, since both values describe the ethanol productivity based on biomass. However, \( de \) gives a better description of the fermentation capability of a strain by eliminating the potential error attributed to the weight variation of a single cell from different species.
8.1.2. Fermentation characteristics of sole strain culture

Free cell culture of \textit{Z. mobilis} requires simple techniques and gives an efficient ethanol production process. Because of the simple metabolism reactions from glucose to ethanol in \textit{Z. mobilis} cells, its profile of ethanol production fits best into Formula (9). When the initial cell concentration was increased to 5 fold and 10 fold, the system co-efficiency increased to 2.2 fold and 3.6 fold respectively.

Free cell culture of \textit{P. stipitis} shows more efficient cell growth on a complex medium than on a simple medium, and on glucose substrate than on xylose substrate. Its fermentation performance on xylose medium is mainly affected by both viable cell concentration and oxygenation level, which affects its cell instantaneous ethanol production rate \([de]\). The increase of initial cell concentration to 5 fold resulted in a 5.2 fold increase in the system co-efficiency. However, further increase in the biomass level required a concomitant increase in the oxygenation level.

Sole culture of \textit{P. stipitis} on the glucose/xylose mixture medium gave comparable results to the co-culture of \textit{Z. mobilis} and \textit{P. stipitis}. Glucose and xylose were consumed sequentially; however, there was no observation of catabolite repression due to glucose on the xylose metabolism. It was speculated that the high cell concentration used for fermentation led to the starvation of cells in an environment with limited substrate, consequently forcing them to utilize every potential carbon source.

\textit{Z. mobilis} showed its acclimatization to the interior of calcium alginate beads during microbial encapsulation. Biomass inside the beads accumulated to a high concentration during their repeated usage, resulting in an improved fermentation performance. The main disadvantage of immobilized \textit{Z. mobilis} was the bead damage caused by the intensive carbon dioxide production during the ethanol production process.

Immobilized \textit{P. stipitis} showed a constant fermentation efficiency when cultured under various conditions, which resulted from the steady levels of both biomass concentration and cell instantaneous ethanol production rate within the beads. The
yeast preferred to grow in the medium environment rather than inside the beads. Both the limited fermentation efficiency achieved with high biomass concentration entrapped in the beads and the minimal bead damage during repeated culture indicated a low cell activity in the interior of the gel beads.

8.1.3. Co-culture of Z. mobilis and P. stipitis
The utilization of immobilized Z. mobilis and free cells of P. stipitis achieved efficient conversion of the glucose/xylose sugar mixture to ethanol by providing each strain an optimum environment within the beads and in the medium respectively. The resulting ethanol production performance was approximately 25 g/l ethanol within 19-22 h, with a complete conversion of a high xylose proportion [40% in the sugar mixture, i.e. 30g/l of glucose and 20 g/l of xylose], giving an ethanol yield of 0.49-0.50 g/g sugar. Except for the low final ethanol concentration, all other values exceeded the standard suggested by du Preez (1994) for industrial production: 50-60 g/l ethanol within 36 h and an ethanol yield no less than 0.40 g/g.

During the co-culture process, Z. mobilis cells proved to be inhibitory to the cell activity of P. stipitis. Increased viable cells of Z. mobilis in the medium led to a significant decrease in both efficiencies of cell growth and xylose fermentation of the yeast. Moreover, the bacterium appeared to be able to grow on the xylose metabolic process of P. stipitis; cell reproduction to a level of $1.0 \times 10^9$ cfu/ml occurred in the medium environment lacking an appropriate carbon source for utilization. Finally Z. mobilis tended to assimilate the produced ethanol under aerobic conditions, which was not a strain interaction, but reduced the overall production efficiency.

Therefore, the removal of the immobilized Z. mobilis from the medium after the completion of glucose fermentation is recommended to achieve a reasonable fermentation efficiency. Moreover, this method increases the life of gel beads by reducing the extended period of exposure to the fermentation medium.

8.1.4. Fermentation of bagasse hydrolysate
The hydrolysis processes of sugar bagasse showed similar trends to previous literature. The sugar yield of both acid hydrolysis and enzymatic hydrolysis was low. Through all methods attempted in this study, there was no significant improvement in
the sugar yield achieved. With proper overliming, both the acid and enzymatic hydrolysates showed similar fermentability; the fermentation of both glucose and xylose proceeded without any apparent inhibition. In contrast, for the enzymatic hydrolysate without overliming, a lag phase of ethanol production up to 13.5 h was observed for the sole culture of \textit{P. stipitis}. The best results achieved with the acid hydrolysate gave a complete sugar utilization at 26 h, by which time the ethanol yield was already 0.43 g/g; at 40 h when ethanol peaked, the yield further increased to 0.49 g/g. These results still compare favorably with du Preez's standard (1994); however, for further improvement of the overall efficiency from bagasse to ethanol, the realization of an efficient and complete hydrolysis process could be the major problem to overcome.

8.2. Recommendations for future work

Based on the current study, the following areas are recommended.

Strain improvement. As discussed in Section 2.6.2, there are different possibilities of constructing recombinant strains with desired characteristics, and some strains have already been constructed and applied in research. However, here the potential of \textit{P. stipitis} is specially considered. In this study, \textit{P. stipitis} gave comparable results with co-culture on the glucose/xylose sugar medium; it is also believed that with appropriate optimization, a similar performance could be achieved on the hydrolysate medium as well. The major shortcoming of \textit{P. stipitis} is the relatively slow metabolic efficiency, i.e. \textit{theale}. The strain should be improved through mutation or genetic engineering to increase its metabolic rate, since it shows some unique advantages over other strains. It is a yeast, thus eliminating the possible attack by bacteriophage; it naturally ferments both glucose and xylose, without the need to artificially add new genes for the xylose assimilation. Moreover, recent research on the heterologous xylanase production by \textit{P. stipitis} (Gorgens et al., 2005) enables the strain to hydrolyze the xylan while fermenting. The further exploitation of \textit{P. stipitis} into a desirable biocatalyst for an integrated process from lignocellulose to ethanol looks exciting and worth attempting.
Improvement of the immobilization. The bead damage of immobilized \textit{Z. mobilis} during the fermentation reduces the bead life and increases the cost. A new immobilizing material will be necessary for longer usage and better performance. Furthermore, when the co-culture of \textit{Z. mobilis} and \textit{P. stipitis} is considered, an ideal immobilization mode should contain \textit{Z. mobilis} within the immobilization material [e.g. tiny beads or fine fibres], and \textit{P. stipitis} on the surface of the immobilization material for better aeration. In this manner, both strains are provided with optimum oxygenation conditions and can be easily recycled during fermentations.

Continuous culture. Results reported in this study are the highest values achieved in batch culture for the different processes. However, continuous culture has the potential to further increase the fermentation efficiency compared to batch culture, by maintaining a continuous exponential phase for all cells in the medium. In association with the utilization of high cell density, it is possible to set up a continuous culture using a column filled with both \textit{Z. mobilis} and \textit{P. stipitis}. With a sufficient amount of biomass and a proper length of column, all the sugars in the medium flowing through the column would be completely converted to ethanol before flowing out. For better manipulation, the co-culture of \textit{Z. mobilis} and \textit{P. stipitis} should be preferably immobilized. It can either be a co-immobilization as described in the earlier paragraph, or a sequential two-stage fermentation with \textit{Z. mobilis} first to convert all the glucose and then \textit{P. stipitis} later to convert all the xylose.

Metabolic study for a co-culture of \textit{Z. mobilis} and \textit{P. stipitis}. As suggested above, there were some special interactions between these two strains during the co-culture. A detailed, step by step investigation of each strain's metabolic reactions in this process would contribute to the knowledge of the biochemistry of interaction between two strains, as well as the nature of how cells from different genera interact with each other.

Improvement of hydrolysis and development of an integrated process. This study mainly focuses on the fermentation stage of the four stages required to convert the lignocellulose to ethanol, as mentioned in Section 2.1. An efficient
fermentation process has been established for the ethanol production from the glucose/xylose sugar mixture. However, industrial production of ethanol from lignocellulose also requires an economically feasible process to obtain fermentable single sugars from carbohydrate macromolecules. A cost-efficient hydrolysis process still needs to be worked out. Ultimately, an integrated, single stage process for the conversion of lignocellulose to ethanol would be preferable. It may be possible to achieve this aim either by the utilization of a genetically constructed biocatalyst, or simply through process integration and optimization. These are questions that need to be answered through further research.
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Appendix

A. Optimization of the Glucose oxidase method

In order to optimize the Glucose oxidase method and obtain a linear standard curve, a number of parameters were examined experimentally. These included the wavelength, the amount of chromogen and the reagent used to stop the enzymatic reaction. Results of these experiments are shown in both Figure A.1 and Figure A.2.

Figure A.1: Effects of reaction parameters on the Glucose oxidase method: (a) wavelength, (b) amount of chromogen.
Figure A.2: Effects of stopping reagent on the Glucose oxidase method: (a) 2 ml of concentrated sulfuric acid, (b) 5 ml of 25% sulfuric acid, (c) 2 ml of 25% sulfuric acid, (d) 2 ml of pre-chilled distilled water.
From Figure A.1 it can be seen that, under all six conditions, the standard curve of Glucose oxidase method was approximately linear. However, the wavelength of 525 nm gave the highest absorbance readings for all tested glucose concentrations. On the other hand, when 6 ml of chromogen solution and 2 ml distilled H$_2$O were used for the reaction mixture, the resulted absorbance readings were considerably higher than those obtained with 4 ml of chromogen and 4 ml water. Nevertheless, for economic considerations, the latter combination was chosen, together with 525 nm wavelength, as the optimal conditions.

In the Figure A.2 testing the effect of stopping reagent, Reagent (a) 2 ml of concentrated sulfuric acid and Reagent (b) 5 ml of 25% sulfuric acid were respectively suggested by previous literature (Chow and Landhausser, 2004) and AOAC Official Method (AOAC-International, 2006). However, as shown in the Figure A.2, both methods failed to completely stop the reaction; 30 min after the addition of the stopping reagent, the spectrophotometric reading of each standard altered significantly. Moreover, when the concentrated sulfuric acid was used, samples turned the originally orange color into pink, which may indicate some undesirable side reactions. Reagent (3) with 2 ml 25% sulfuric acid was not able to stop the reaction either; the divergence of two curves indicated the color change of the solutions as the enzymatic reaction continued. Finally, with Reagent (4) 2 ml distilled water prechilled in the ice bath for more than 30 min, superimposed standard curves were achieved, indicating no change of color during the 30 min after the addition of the stopping reagent. This suggested that the prechilled distilled water had stopped the reaction satisfactorily, probably because of the significant decrease in the temperature. Thus 2 ml of pre-chilled distilled water was selected as the stopping reagent, which gave repeatable results for the assay.

The Glucose oxidase assay carried out under these conditions provided a reliable standard curve and a high sensitivity; the resulting medium color stayed stable for 30 min after the assay, thus preventing the potential color change during the time of absorbance measurement. The procedure described in the Section 3.10.1.2 of this thesis is recommended as an optimized procedure for the glucose assay in a sugar mixture.
References:
