The development of a biological pretreatment strategy for the conversion of wheat straw to biofuels or platform chemicals

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Thesis submitted to the University of Nottingham
for the degree of Doctor of Philosophy

SEPTEMBER 2015

Abstract

The increasing concern of energy shortage and environmental pollution attracts worldwide exploration of using sustainable biomaterials for the production of biofuels and biochemicals. Utilising lignocellulosic raw materials for valuable bio-products production is generally considered as a preferred biosynthetic technology. Although various processes have already been proposed, lignocellulose hydrolysis is still remaining as one of the major challenges that prevents wide spread application of lignocellulosic raw materials in biofuel and biochemical production.

The aim of this study was to investigate the feasibility of applying soft-rot fungi as a biological pretreatment of wheat straw for the generation of cellulase enzymes and then use the freshly produced enzymes to hydrolyse the fermented wheat straw to a sugar rich hydrolysate. The wheat straw hydrolysate had also been examined for the production of bioethanol and biochemicals, such as succinic acid and itaconic acid.

Solid State Fermentations (SSF) of wheat straw were carried out using both *Aspergillus niger* and *Trichoderma reesei*. The fermentation conditions, such as moistures content, culture time, addition of nutrients, and modification of wheat straw were optimised for the production of cellulase. In a SSF using autoclaved wheat straw, an enzyme activity of 9.5 FPU/g was achieved. When 0.5% yeast extract and mineral solution were added, the enzyme activities increased to 24.0 FPU/g after 5 days of cultivation. In a SSF of an alkali

soaked wheat straw (wheat straw treated with 1% NaOH at 25˚C for 24 hours), 21.8 FPU/g was obtained after just 1-day culture.

Optimisation of hydrolysis process led to a hydrolysate containing 59.8 g/L glucose, which was achieved from the hydrolysis of biologically pretreated wheat straw at 18% solid loading, with an enzyme loading rate of 55 FPU/g at 50˚C. Fermentations using the wheat straw hydrolysate resulted in 28.6 g/L ethanol, which was equivalent to 93.4% of theoretic yield.

Utilisation of wheat straw hydrolysate for succinic acid production was investigated using recombinant yeast strains. For *Saccharomyces cerevisiae* D2, the deletion of SDH1 and SDH2 genes enhanced succinic acid production by 68%. Optimisation of fermentation conditions and fermentation scales led to a succinic acid production to around 12 g/L, which was nearly 100-folds of what succinic acid production using the wild *S. cerevisiae* D2 strain at initial fermentation conditions. Use wheat straw hydrolysate to replace commercial glucose based semi-defined medium resulted in the same succinic acid production yield, but lower concentration due to the low sugar concentration in the hydrolysate. Biosynthesis of itaconic acid using wheat straw hydrolysate was also explored, but no significant itaconic acid production was observed.

Acknowledgement

This thesis would not have been complete without the support of many people around me. First and foremost I would like to thank my supervisor Dr Chenyu Du, Professor Gregory A. Tucker and Dr Chris D. Powell for their guidance and supervision during my PhD.

I would like to thank Naresuan University, Thailand for providing the funding for this work.

I appreciated the X-Ray Diffraction training from Dr Bill MacNaughtan and Scanning Electron Microscopy training from Miss Christine Grainger-Boultby. It has been an honour to work in the Division of Food sciences at the University of Nottingham. I would like to thank everyone in Division of Food Sciences, especially Dr Abhishek Somani, Dr Roger Ibbett, Dr Darren Greetham and Dr Scott Davies for their valuable discussions.

Lastly, I would like to thank my family for all their love and encouragement. For my parents, Mr Nuttaporn Pensupa and Mrs Tassanee Pensupa who supported me in all my pursuits.

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Chapter 1 Introduction

1.1 Energy crisis and environmental concerns

World energy consumption has been increasing every year. In 2013, primary energy consumption reached 12,730.430 million tonnes oil equivalent, which is 2.3% higher than the previous year (BPstats, 2014). The increase in global population, life style changes and enlargement of industrial sector in many countries are the main causes of growing energy demand (IEA, 2014). Currently, fossil fuels (coal, petroleum and natural gas) are still the main primary energy sources in the world. With the current technology, the utilisation of fossil fuels generates various pollutants, such as carbon dioxide, sulphur dioxide and nitrous oxide. They may cause environmental problems to ecosystem. Moreover these pollutants have been identified as a cause of global warming. Carbon dioxide is one of the main components of greenhouse gases in the atmosphere. It has been reported that to produce 1 kWh of electricity from coal, around 1 kg of carbon dioxide was emitted (Zevenhoven and Beyene, 2011). The increase in energy consumption has, of course, led to increasing carbon dioxide concentration in the atmosphere (Figure 1.1). Yeh and Bai (1999) have reported that carbon dioxide caused 55% of global warming and they also suggested that the reduction of carbon dioxide emissions from the fossil fuel was a very urgent issue to reduce the global warming trend.

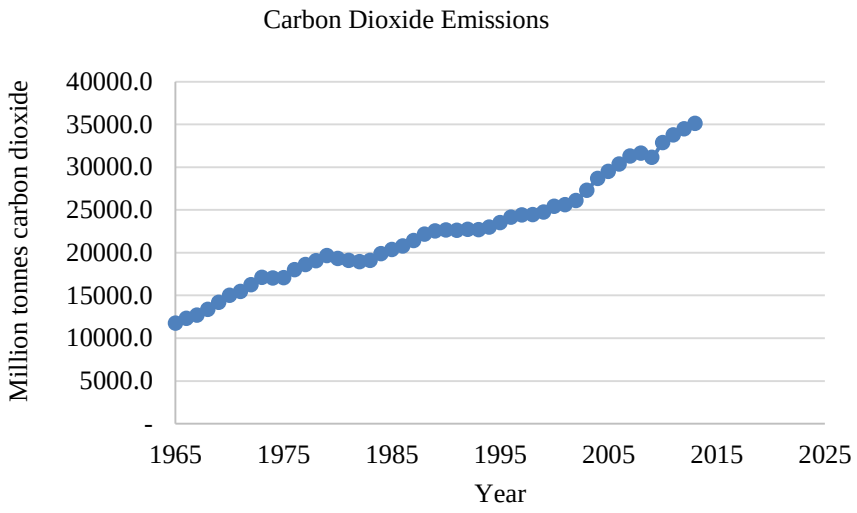


Figure 1. 1 Global carbon dioxide emission of year 1965 to 2013 (The-world-bank-group, 2015a)

Energy crisis is caused by a variety of factors, such as, overconsumption of energy, overpopulation, poor infrastructure and growth of the industrial sector. According to data from The World Bank, the average energy consumption per person increased 6.5% (from 1774.9 to 1890.1 kg of oil equivalent per capita) between 2005 to 2011, while world population increased 6%. The United States has been the biggest energy consumer since 1990, but the high growth rate of the industrial sector and population growth brought China to the top of the energy consumer list since 2009 (The-world-bank-group, 2015b). A year later, the amount of energy used in China was 2,492 million tonnes of oil equivalent (19.4% of world energy consumption) followed by the United States and India with 2,248.8 and 692 million tonnes of oil equivalent, respectively. This high growth in energy consumption has become a serious issue.

There are several possible solutions to this energy crisis and environmental pollution. One of them is to find alternative, renewable, sustainable and environmental benign energy sources, such as using bioethanol. The global ethanol production has increased from 10.7 billion gallons in 2004 to 20.4

billion gallons in 2008 (Demirbas, 2011). The main ethanol producers in the world are the United States and Brazil as shown in Table 1.1. The total world ethanol production contributed to 1% of world's transport fuel consumption in 2006 (Vincent, 2010a). The world ethanol production exceeded 94.5 million tonnes in 2013 (Levdikova, 2014).

Table 1. 1 Global ethanol production, in billion gallons per year (Demirbas, 2011).

Country	**2004**	**2008**	**Share 2008 (%)**
United States	3.40	8.93	43.8
Brazil	3.87	6.90	33.9
China	0.92	1.02	5.0
India	0.32	0.61	3.0
France	0.22	0.40	1.9
Canada	0.06	0.26	1.3
Germany	0.06	0.22	1.1
Thailand	0.65	0.15	0.7
Russia	0.20	0.15	0.7
Spain	0.09	0.13	0.6
South Africa	0.10	0.11	0.5
United Kingdom	0.08	0.11	0.5
Remaining countries	1.35	1.40	6.9
World	10.75	20.37	100.0

1.2 Current problem with biofuel production

Although the first generation of biofuel production has been widely commercialised recently, it still receives plenty of questions regarding its competing with food industry and its true sustainability. However, utilising lignocellulosic raw materials for the biofuel and biochemical production is generally considered as a preferred and an advanced technology for biosynthesis. Various life cycle assessment studies have demonstrated that lignocellulosic bioethanol production process has net CO_2 reduction in its life cycle. One of the main problems with lignocellulosic biosynthesis is financial concern. The current lignocellulosic biofuel technology could be divided into pretreatment, hydrolysis, fermentation and distillation processes. The pretreatment is designed to disrupt biomass structure, which enhances the

efficiency in the subsequent enzymatic hydrolysis step. It has been found that some pretreatment technologies generate unfavourable inhibitory compounds for the subsequent hydrolysis and fermentation (see Figure 1.2). The detoxification of these inhibitory compounds will, of course, influence the total production cost.

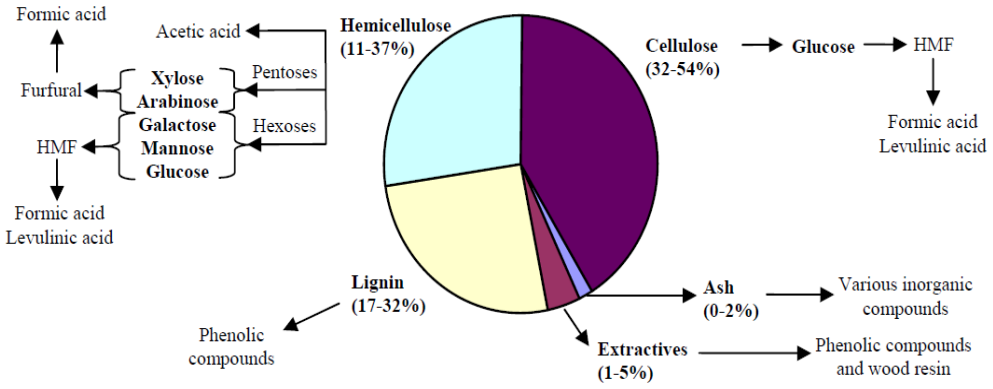


Figure 1. 2 Composition of lignocellulosic material and their potential hydrolysis products (Taherzadeh and Karimi, 2007)

Currently, there are several possible approaches to increase the economical competency of lignocellulosic bioethanol process. Firstly the feed stock supply cost could be diminished by using agricultural waste such as wheat straw. Secondly, the reduction of operating cost can be achieved by selecting a proper pretreatment technique. Thirdly, the cost of enzyme in the hydrolysis process can be trimmed down by producing on-site enzyme. Lastly, the lignocellulosic bioethanol production process could be adapted to produce other value added biochemical instead of bioethanol.

1.3 Objectives of this project

The objectives of this project are to investigate feasibility of the potential approaches that could improve the lignocellulosic biofuel production process as listed above.

The first aim was to investigate the possibility of applying soft-rot fungi as a biological pretreatment of wheat straw. The observations on the chemical and physical alterations of wheat straw using biological pretreatment on wheat straw can be found in chapter 4.

The second aim was to develop methods to reduce the production cost by generating on-site cellulolytic enzymes. The details of factors that influence enzyme production are reported in chapter 5.

The third aim of this project was to select a proper hydrolysis technique for the biomass and to optimise the hydrolysis step in order to obtain sugar-rich hydrolysate. The details of the optimisation analysis are provided in chapter 6.

The forth aim was to explore the application of wheat straw hydrolysate as a biorefinery platform. Besides the ethanol production, the production of value added chemicals such as succinic acid and itaconic acid were investigated. The details of fermentations are presented in chapter 7 and 8.

1.5 Thesis outline

The thesis is structured and presented as the following way:

Chapter 1 Introduction: in this chapter provides an overview of the project, explains the current problems and suggests some ways to solve them.

Chapter 2 Literature review: a review of the bioethanol and biochemical (succinic acid and itaconic acid) production was described.

Chapter 3 Material and methods: will describe all procedures carried out in this project including quantitative analysis.

Chapter 4 Biological pretreatment: will report the performance of biological pretreatment on wheat straw using *Aspergillus niger* and *Trichoderma reesei*. The chapter ends with a co-culture technique for cellulase production. I will demonstrate that *A. niger* is a promising strain to use in short term biological pretreatment.

Chapter 5 Optimisation for cellulase production: will explain relevant factors influencing cellulase production. The optimisation of cellulase production in solid state fermentation by *A. niger* was investigated.

Chapter 6 Hydrolysis: in this chapter, the sugar yields from pretreated wheat straw with acid hydrolysis or enzymatic hydrolysis are presented. The selected hydrolysis method has been optimised to improve sugar concentration in hydrolysate.

Chapter 7 Succinic acid production: the details of succinic acid production from synthetic medium using yeast strain will be discussed. In order to increase the production yield, the yeast strain will be manipulated. This chapter will end with discussing a development of fermentation condition to improve the succinic acid yield.

Chapter 8 Fermentation: in chapter 8, I will present the application of wheat straw hydrolysate to produce value added chemicals including succinic acid, ethanol and itaconic acid.

Chapter 9 Conclusion and future work: in this chapter, I will summarise all the findings of this study and suggest some potential future work that can be further explored.

Chapter 2 Literature Review

This chapter will provide broad information about general background of lignocellulosic bioethanol production, informative knowledge on each step of bioethanol production process and main challenges in this research field.

2.1 Lignocellulosic raw materials

Bioethanol has been considered as a potentially important transportation fuel. It can be produced from different kinds of raw materials such as sugar rich material, starchy material and cellulose-based material. The world biggest bioethanol producer is the United States together with Brazil which they use corn and sugarcane as their raw materials. Both sources contain high amounts of starch and sugar. However, to avoid food and fuel issues, for example, rising food prices and reduction of cereal production since the price of the fuels are, of course, higher than the price of cereal; this makes agricultural sector prefer to plant the starchy plant for fuels rather than food. Therefore lignocellulosic bioethanol has been developed. The lignocellulosic material includes agricultural residues, short rotation herbaceous crops and forestry residues, such residues have been identified as a cheap and effective raw material for production of bioethanol.

2.1.1 Lignocellulose structure

The lignocellulose is composed of 2 polymeric forms of carbohydrate, which are cellulose and hemicellulose and a phenolic polymer, which is lignin. The components of lignocellulose biomass vary by plant species, plant organ, age and stage of growth some examples are shown in Table 2.1.

Table 2. 1 Cellulose, hemicellulose and lignin contents in common agricultural residues and wastes (Kumar *et al.*, 2009)*,(Rodriguez Couto and Sanromán, 2005)** (Mosier *et al.*, 2005)***, (Conde-Mejía *et al.*, 2012)****

Lignocellulosic material	**Cellulose (%)**	**Hemicellulose (%)**	**Lignin (%)**
Aspen****	50.8–53.3	26.2–28.7	15.5–16.3
Barley bran **	23	32.7	21.4
Cane bagasses**	33	22	14
Coastal Bermuda grass*	25	35.7	6.4
Corn cobs*	45	35	15
Corn leaves**	37.6	34.5	12.6
Cotton seed hairs*	80-95	5-20	0
Eucalyptus viminalis****	41.7	14.1	31
Fresh bagasse*	33.4	30	18.9
Fir ****	43.9	26.5	28.4
Grape seeds**	7.10	31.13	43.54
Grape stalks**	29.95	35.33	22.94
Grasses*	25-40	35-50	10-30
Hardwood stems*	40-55	24-40	8-25
Leaves*	15-20	80-85	0
Newspaper*	40-55	25-40	18-30
Nut shells*	25-30	25-30	30-40
Softwood stems*	45-50	25-35	25-35
Oat straw**	49.3	25	18
Paper*	85-99	0	0-15
Pinus banksiana****	41.6	25.6	28.6
Pinus pinaster****	42.9	17.6	30.2
Popular***	49.9	17.4	18.1
Primary wastewater solid*	8-15	NA	24-29
Rice straw*	32.1	24	18
Solid cattle manure*	1.6-4.7	1.4-3.3	2.7-5.7
Sorted refuse*	60	20	20
Switch grass*	45	31.4	12.0
Swine waste*	6.0	28	NA
Waste paper from chemical pulp*	60-70	10-20	5-10
Wheat straw*	30	50	15
White birch****	41	36.2	18.9

The structure of the components listed in Table 2.1 can be explained in detail as:

Cellulose

Cellulose is normally found in both primary and secondary cell wall of plant cell. It is linear homopolymeric form of D-glucose that is linked together with 1,4-β glycosidic bonds. The repeating unit of cellulose forms a crystalline fibril which makes cellulose very rigid and sturdy.

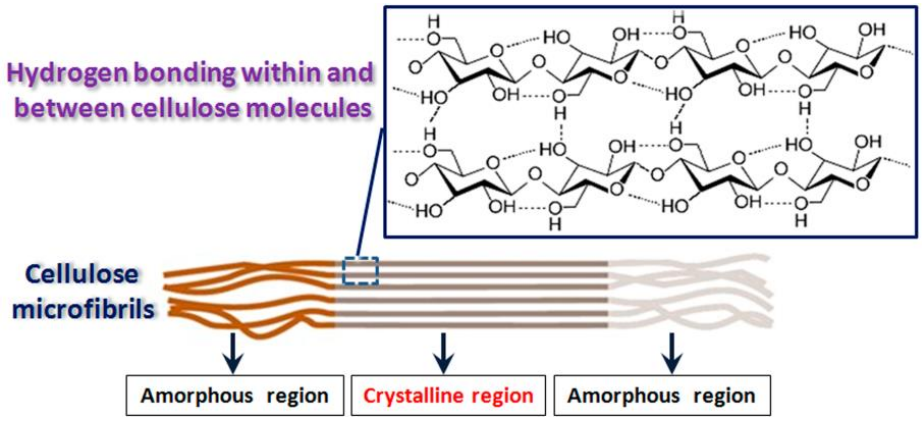
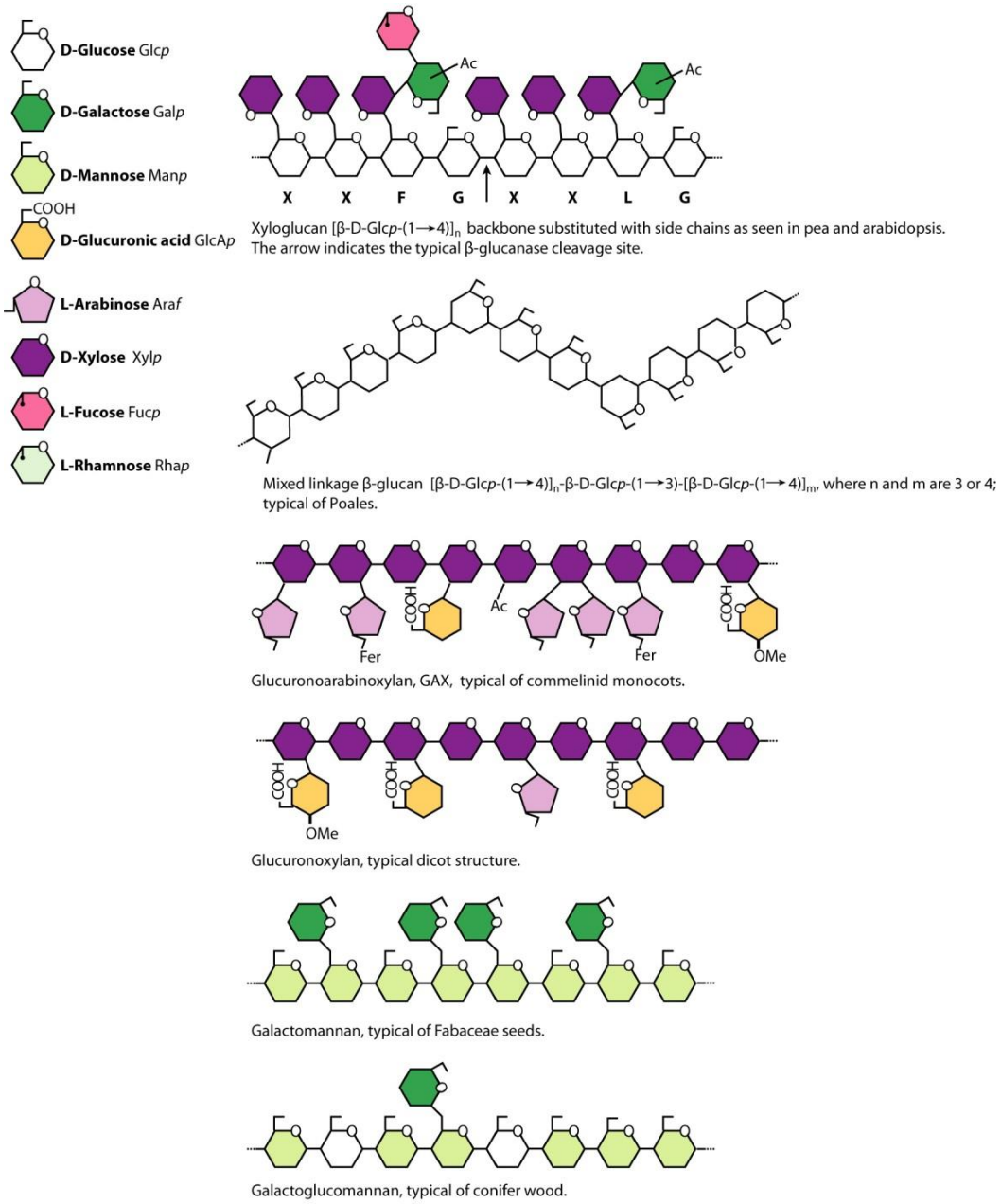


Figure 2. 1 Cellulose structure (Zhou and Wu, 2012)

Hemicellulose

Hemicellulose is a polymer that is mostly found in primary and secondary cell walls. Unlike cellulose, hemicellulose is heteropolymeric compound since it consists of D-xylose, D-glucose, D-galactose, D-mannose, L-arabinose, 4-O-methyl-glucuronic acid, D-galacturonic acid and D-glucuronic acid. These compounds are linked together by β-1, 4 linkage and occasionally β-1, 3 and β-1, 6 linkage (Saha, 2003). Different structures of hemicellulose polysaccharide can be found, for example, xyloglucan, galactomannan, glucomannan, glucuronoxylan and xylan. Their backbone structures are shown in Figure 2.2. Since hemicellulose is a mixture of carbohydrate components that link together

with ester bonds, it cannot form crystalline structures due to primarily to the side chains. Therefore it can be easily broken by using chemical agent or enzyme due to the extensively branched structure.



Scheller HV, Ulvskov P. 2010.
Annu. Rev. Plant. Biol. 61:263–89

Figure 2. 2 Basic structure of hemicellulose (Scheller and Ulvskov, 2010).

Lignin

Lignin is a polyaromatic compound and is usually found in secondary cell walls of the plant cell. Lignin consists of 3 aromatic alcohols monomers which are p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (see Figure 2.3). The monomers are linked together with covalent bond (carbon to carbon bond and ether bond) to form 3 types of subunit (hydroxyphenol- (H-type), guaiacyl- (G-type) and syringyl subunits (S-type) (Harmsen *et al*., 2010).

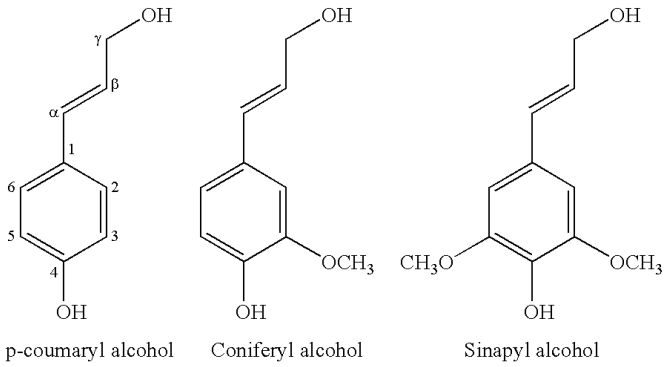


Figure 2. 3 p-coumaryl- , coniferyl- and sinapyl alcohol: building blocks of lignin polymer (Ekman, 2010)

Lignin is a very unique compound because it has none repeating bonds between the subunits (see Figure 2.4) (Lankinen, 2004). It is a non-soluble compound. In plant cell wall, lignin can be bound to cellulose with hydrogen bond and ether bond. (see Table 2.2). This crosslink strengths the cell wall. Then this compound is more difficult to degrade than cellulose and hemicellulose (Harmsen *et al*., 2010).

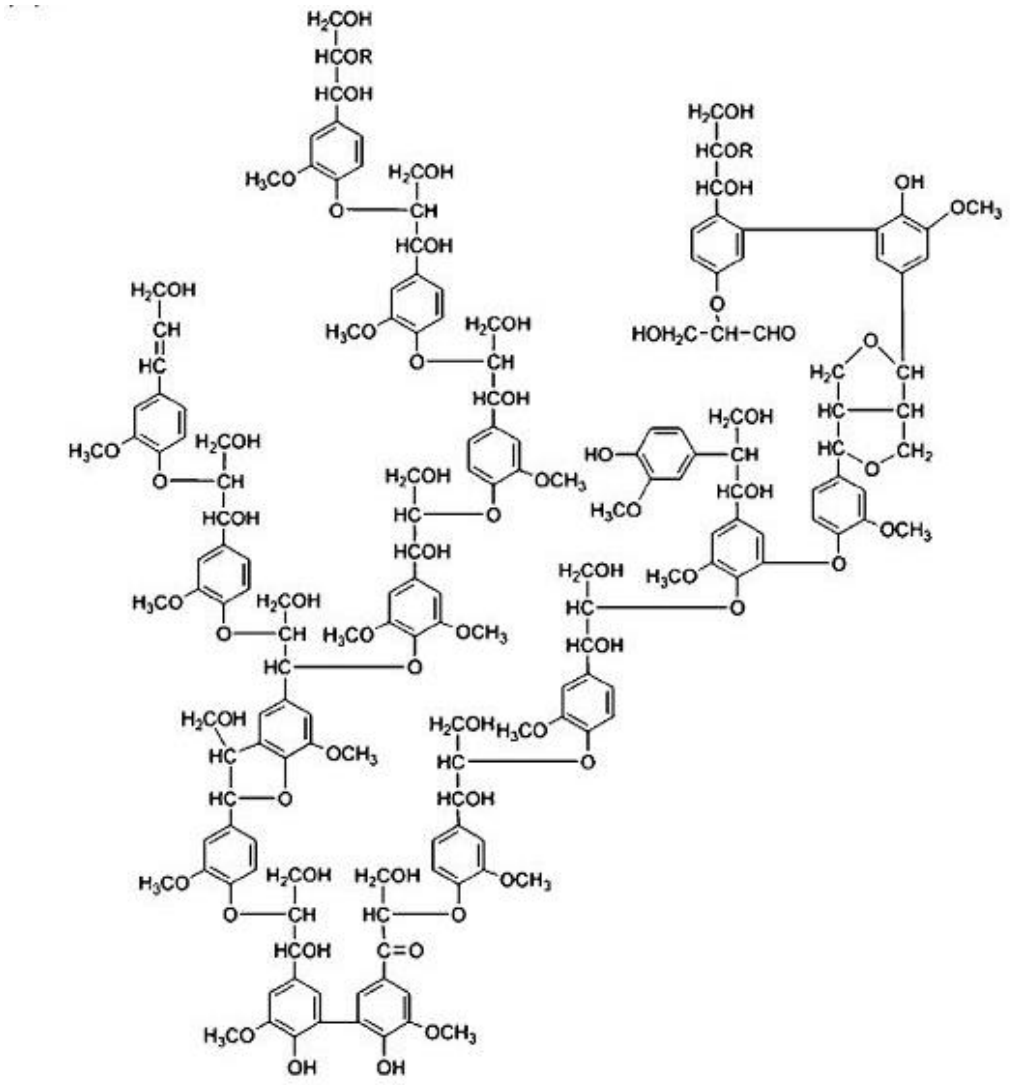


Figure 2. 4 Lignin structure (Vivekanandhan *et al*., 2013)

Table 2. 2 Chemical bonding of lignocellulosic component (Gupta and Tuohy, 2013)

Intrapolymer linkage	**Compound**
Ether	Lignin, hemicellulose
Carbon to carbon	Lignin
Hydrogen	Cellulose
Ester	Hemicellulose
Interpolymer linkage	**Compound**
Ether	Cellulose– lignin
Ester	Hemicellulose-lignin
Hydrogen	Cellulose-hemicellulose Hemicellulose-lignin Cellulose-lignin

Lignocellosic biomass is a promising candidate for a renewable energy resource because it contains complex polysaccharides (cellulose, hemicellulose) and it is an abundant material. Scientists have been trying to find a suitable technology to extract all sugars out from lignocellulosic materials. Conventional method being used nowadays is normally very specific to a particular substrate, such as, acid treatment in corn.

2.2 Lignocellulosic bioethanol production process

Bioethanol production process from lignocellulosic material consists of 4 steps, which are (i) pretreatment, (ii) hydrolysis, (iii) fermentation and (iv) distillation. Details of each step are:

2.2.1 Pretreatment

Pretreatment process has an important role in bioethanol production. It aims to break down the recalcitrance matrix in plant cell wall in order to increase accessibility of biomass. The exposed biomass structure is preferable for hydrolysis step because it can increase efficacy of biomass conversion into fermentable sugar. By improving biomass conversion efficacy, this definitely improves the ethanol yield. In the best possible way, the pretreatment process must enhance sugar releasing during hydrolysis step without generating by-products that can reduce the efficacy of subsequent hydrolysis and fermentation processes in the later stage. Furthermore, the process must be a cost-effective process (Kumar *et al*., 2009). Pretreatment method has been classified into 4 categories.

Physical pretreatment

Physical pretreatment or mechanical pretreatment requires physical forces to break the lignocellulose structure and increase surface area of biomass. For example, chipping, grinding and milling. There are many research groups that have been active on applying physical pretreatment on feedstock as shown in Table 2.3. Da Silva *et al* (2010) have studied the effect of milling as a pretreatment process on sugarcane bagasse. The result shows that sugar yield of pretreated bagasse was improved up to 70% compared with untreated bagasse. Similar result can be also found in applying wet grinding process on water hyacinth; Harun *et al* (2011) reported that after wet grinding the sugar yield increased from 24.69 mg sugar/g dry wt to 59.32 mg sugar/ g dry wt. This was equivalent to a 40.26% improvement compared to untreated water hyacinth.

Table 2. 3 Physical pretreatment on different lignocellulosic feedstock.

Substrate	Technique	Results	Reference
Corn slurry	Ultrasonic	330% than untreated	(Montalbo-Lomboy et al., 2010)
Sugarcane bagasse	Wet disk milling	49.3% glucose	(da Silva et al., 2010)
Rice straw	Wet disk milling	78.5% glucose	(Hideno et al., 2009)
Rice straw	Ball milling	89.4% glucose	(Hideno et al., 2009)
Water hyacinth	Milling	n.a.	(Harun et al., 2011)
Water hyacinth	Ultrasonic	n.a.	(Harun et al., 2011)
Switchgrass	Extruding	40.6% glucose	(Karunanithy and Muthukumarappan, 2011)

Some physical pretreatment have applied a freezing technique. This kind of pretreatment process requires extremely low temperatures. In the extremely low temperature conditions, embedded water inside biomass material is solidified into ice particles breaking down the biomass structure. Chang *et al*

(2011) investigated the effect of freezing as a pretreatment process on rice straw. The result shows that freeze-pretreated rice straw generated 4 times higher glucose yield than untreated rice straw.

Another physical pretreatment processes that have been studied are mechanical wave for example ultrasonic wave (Harun *et al*., 2011) and electromagnetic wave such as microwave (Zhu *et al*., 2006a, Saha *et al*., 2008), gamma ray (Yang *et al*., 2008, Wang *et al*., 2012). These techniques are energy intensive processes and require costly equipment, therefore manufacturers normally overlook these processes.

It can be concluded that size reduction enhanced the hydrolysis process because the surface area of biomass is increased and hydrolysing reagent can penetrate into the biomass easier than the untreated one.

Physical pretreatment is a simple process and can be applicable to different types of feedstock. However, it still lacks support from bioethanol producers due to the physical pretreatment processes requiring high energy to proceed. Hence, manufacturer have tried to minimise the energy usage in physical pretreatment by combined it with another pretreatment processes. Begum *et al* (1988) reported that 9.2 g/L of reducing sugar was obtained from rice straw pretreated by 500 kGy gamma ray. The reducing sugar yield increased to 33 g/L after combining the gamma ray pretreatment with 10% alkali solution.

Chemical pretreatment

Chemical pretreatments use chemical reagents to disrupt biomass structure. In this process, each chemical reagent has specificity on individual chemical bond within the biomass.

Acid pretreatment

Lignocellulosic biomass pretreatment by using acid solution has been studied since 1883 due to the acid has ability to depolymerise cellulose into oligosaccharide (Yoon *et al*., 2014, Shahbazi and Zhang, 2010). And the process was started to develop since then. The most common acids used in hydrolysis are sulphuric acid, hydrochloric acid and phosphoric acid. These acid are high destructive catalyst and cheap. Acid pretreatment process is commercially used nowadays due to the high efficacy of the process. Generally this process can be classified into 2 groups based on acid concentration and temperature used:

(i) Concentrated acid pretreatment and

(ii) Diluted acid pretreatment.

Concentrated acid pretreatment requires strong acid (30-70%) such as sulphuric acid, hydrochloric acid and phosphoric acid at low temperature (50-60˚C) for treating biomass (Khan, 2010, Janga *et al*., 2012, Goshadrou *et al*., 2011). The hydrogen bonds in the cellulose fraction are broken down by the acidic reagent. The amorphous cellulose is then degraded almost instantly to glucose. This degradation reaction would solubilise polysaccharide and leave lignin in the solid phase (Carvalheiro *et al.*, 2008). This process maybe applied as a simultaneous pretreatment and saccharification process for the biomass. Because of the strength of the acid, which can completely break down cellulose structure at ambient temperature this method does not require an enzymatic hydrolysis step. The effect of concentrated phosphoric acid on microcrystalline cellulose was investigated by Zhang *et al* (2010b). It has been found that the

microcrystalline cellulose is degraded to amorphous cellulose after being treated with strong acid at 50˚C for 10 hours. The sugar yield from treated microcrystalline substrate increased over 3 times compared with untreated microcrystalline cellulose. Goshadrou *et al* (2011) have reported that glucose yield from sweet sorghum bagasse increased by 26.4% after treatment with 85% (v/v) phosphoric acid at 50°C for 30 min compared to untreated sweet sorghum bagasse. The advantage of the concentrated acid pretreatment is that it can be applied to all types of feedstock, but it has major disadvantages as it requires (i) corrosion resistant equipment, (ii) by product formation and (iii) processes to recycle the acid reagent are needed (Tichagwa, 2012).

Dilute acid pretreatment is one of the most effective methods that have been applied for treating biomass. Low concentration of acid, up to 5%, in combination with heat has been used in this process. The temperature can normally vary from 160°C to 220ºC and is held for a short period around 10-30 minutes (Gupta and Tuohy, 2013). This method degrades hemicellulose and cellulose polymer into short oligomers by acid disruption on covalent bonds (hydrogen bonds, and van der Waals forces) (Li *et al*., 2010b). Then the oligomer continues to break down into monomeric sugars (Jacobsen and Wyman, 2000). Compared to the concentrated acid pretreatment, this dilute acid pretreatment has less corrosion problems and it can generate fewer by products such as hydroxymethylfurfural, levulinic acid, and formic acid (Carvalheiro *et al*., 2008, Lee *et al*., 2014). However these inhibitors could increase when higher temperatures and higher acid concentrations were applied in pretreatment process (Lenihan *et al*., 2011). This process has been applied to many type of biomass shown in Table 2.4. The dilute acid pretreatment on hard

wood (aspen) and soft wood (balsam) were investigated by Jensen *et al* (2010). Their results show that the highest total sugar yields of aspen was 88.3% obtained from pretreating aspen with 0.5 % (w/w) sulphuric acid at 175ºC for 30 minutes followed by enzymatic hydrolysis. For balsam, the sugar yield increases to 21.2% when pretreated balsam with 0.25 % (w/w) sulphuric acid at 160ºC for 71 minutes. Dilute acid pretreatment has been also studied in herbaceous crops. Guo *et al* (2008) have investigated the effect of dilute acid pretreatment on sugar yield from silvergrass. Their results show that xylose yield from dilute acid pretreated (2% sulphuric acid at 121ºC) silvergrass was 70% higher than untreated silvergrass. However, 4–5% of furfural was generated under these conditions. Chen *et al* (2011) have proposed that acid concentration in the pretreatment has an influence on the level of inhibitor formation (furfural and acetic acid). They have studied dilute acid pretreatment of rice straw by using different sulphuric acid concentrations in a range of 1-15% at 180˚C for 2 minutes. It has been found that less than 1 g/L of inhibitor compounds were generated using less than 3% (w/w) sulphuric acid in the pretreatment process and when the acid concentration was increased the level of furfural was increased to 1.5-3.5 g/L and acetic acid was increased to 2.9–3.5 g/L.

Table 2. 4 Diluted acid pretreatment on various lignocellulosic feedstock.

Substrate	Acid concentration (%)	Acid	Temperature (˚C)	Time (minutes)	Reference
Almond shell	0.8	H_2SO_4	160	10	Gong *et al*. (2011)
Bermuda grass	0.6-1.5	H_2SO_4	121	30-90	Sun and Cheng, (2005)
Bermuda grass	0.3-1.2	H_2SO_4	120-180	5-60	Redding *et al*. (2011)
Corn stover	85	H_3PO_4	50	30	Sathitsuksanoh *et al*. (2012)
Miscanthus	0.25-4	H_2SO_4	121	20	Xu *et al.* (2012)
Switch grass	1.2	H_2SO_4	160	20	Li *et al*. (2010b)
Rice straw	0.5-1	H_2SO_4	160-190	1-25	Hsu *et al*. (2010)
Rye straw	0.6-1.5	H_2SO_4	121	30-90	Sun and Cheng, (2005)
Wheat straw	0-1	H_2SO_4	121	60	Saha *et al*. (2005)

Alkali pretreatment

Alkaline pretreatment is a process that uses alkali reagent to break down chemical bonding in lignocellulosic biomass. Sodium hydroxide is normally employed as well as calcium hydroxide, ammonium hydroxide and potassium hydroxide (Nadeem *et al*., 2013, McIntosh and Vancov, 2011, Brodeur *et al*., 2011). These alkali reagents can cleave the bond in lignin structure and the bonds between lignin and other fractions and thus lead to lignin solubilisation (Mosier *et al*., 2005). The alkali reagent partially degrades glycosidic bonds in cellulose and hemicellulose which result in biomass swelling and reduced crystallinity of the biomass (Agbor *et al*., 2011). It also removes acetyl and uronic group from hemicellulose which may impact on enzyme accessibility due to loss of steric interference (Wang, 2009). The alkali pretreatment can improve the biomass conversion efficacy in the subsequent hydrolysis step (Bensah and Mensah, 2013).

Xu *et al*. (2010b) have studied the effect of sodium hydroxide pretreatment on switchgrass. Their results show that after pretreating the switchgrass with 1% (w/w) sodium hydroxide at 50˚C for 12 hours, the lignin content was reduced by 77.8% and sugar yield was increased 3.78 times compared to the untreated switchgrass. They have also proposed that delignification efficacy of the pretreatment process was strongly influenced by the temperature used. Cheng *et al* (2010) also support the theory that temperature plays an important role in delignification and more lignin is removed at higher temperatures.

Alkali pretreatment can be performed at a wide range of temperatures (50-135˚C) (Chang *et al*., 1998). Some alkali pretreatments can be carried out at ambient conditions but this might require a higher concentration of the alkali reagent and take longer time compared to the acid pretreatment (Taherzadeh and Karimi, 2008, Brodeur *et al*., 2011).

Kim and Lee (2005) have investigated the effect of soaking in aqueous ammonia on corn stover. It has been found that this pretreatment can be performed at room temperature without agitation. This can remove 55-74% of the lignin. It has minimal impact on cellulose with nearly 100% of glucan left in the solid phase. Since soaking in aqueous ammonia is operated in mild conditions, the sugar degradation is minimal (Sharma *et al*., 2013). Consequently the inhibitor compounds like furfural, hydroxymethylfurfural (HMF) and organic acids are not detected (Zhang *et al*., 2014). However this process also generates inhibitors during depolymerisation of lignin; the inhibitors are phenolic compound, formic acid, acetic acid (Jönsson et al., 2013b). These inhibitors have to be removed prior to the hydrolysis step (Chaturvedi and Verma, 2013).

The efficacy of alkali pretreatment depends on the amount of lignin in the biomass (Canilha *et al*., 2012). It has been found that biomass having low lignin content such as herbaceous crops (Chaturvedi and Verma, 2013) is a very effective biomass. Alkaline pretreatment has been studied in various types of lignocellulosic biomass as shown in Table 2.5. Nguyen *et al*. (2010) has studied the alkali pretreatment of rice straw by using ammonia for lignocellulose conversion to fermentable sugars. Their result show that after pretreating rice straw with 10% (v/v) ammonia solution at 100ºC for 6 hours, the lignin content in rice straw was reduced by 6% and cellulose content increased by 21% compared to untreated rice straw.

Table 2. 5 Alkali pretreatment on various lignocellulosic feedstocks.

Substrate	**Alkali concentration (%)**	**Alkali**	**Temperature (˚C)**	**Time**	**Reference**
Almond shells	0.9	$Ca(OH)_2$	130	60 min	Gong *et al*. (2011)
Barley hull	15	aqueous ammonia	75	48 h	Kim *et al*. (2008)
Barley straw	1	NaOH	70-90	20 min	Iroba *et al.* (2013)
Cattails	1-4	NaOH	n.a	24 h	Zhang *et al*. (2010a)
Cotton stalks	2	NaOH	121	90 min	Silverstein *et al*. (2007)
Corn stover	1.5	$Ca(OH)_2$	120	4 h	Kaar and Holtzapple (2000)
Miscanthus	0.5-4	NaOH	50	2 h	Xu *et al*. (2012)
Oil palm, empty fruit brunches	21	aqueous ammonia	60	12 h	Jung *et al*. (2011)
Sorghum straw	2	NaOH	121	60 min	McIntosh and Vancov (2010)
Wheat straw	2	NaOH	121	30 min	McIntosh and Vancov (2011)

Oxidative delignification pretreatment

Oxidative delignification pretreatment is a process that uses oxidizing agent to degrade the lignin fraction in biomass (Chaturvedi and Verma, 2013). Hydrogen peroxide (H_2O_2) is usually used in this process (Harmsen *et al*., 2010). Hydrogen peroxide is highly reactive with aromatic rings. Then aromatic structure in lignin fraction can be converted into carboxylic acid, carbon dioxide and water (Kaparaju and Felby, 2010). Silverstein *et al*. (2007) have studied the effect of oxidative delignification on cotton stalks. The cotton stalk was pretreated with 2% hydrogen peroxide at 30 °C. It was found that 50% of the lignin was removed which resulted in an improvement of biomass conversion efficacy.

In order to improve delignification efficacy of pretreatment on wheat straw Curreli *et al*. (1997) and Taherzadeh and Karimi (2008) have developed a method by adding an alkali reagent, such as, sodium hydroxide or calcium hydroxide as a catalyst to the pretreatment process. Wheat straw was immerged with 1% sodium hydroxide for 24 hours to solubilise the hemicellulose. This was mixed with solution containing 1% sodium hydroxide and 0.3% hydrogen peroxide for 24 hours. The aromatic rings in lignin complex were decomposed by the mixed reagent. The result shows that 81% of lignin was removed and cellulose increased by 90%.

Ozonolysis

Ozonolysis is a process that utilises ozone to degrade lignin in the biomass. Ozone is a very reactive oxidizing agent. it will break down the aromatic ring structure in lignin by incorporating a double bond in the aromatic structure

(Gupta, 2008). The low molecular weight organic acid compounds, such as, formic acid and acetic acid are then released causing a pH drop (Aresta *et al*., 2012). Although the ozone can partially solubilise the hemicellulose, it does not degrade the cellulose (Chaturvedi and Verma, 2013). Vidal and Molinier (1988) have reported the effect of ozonolysis on saw dust showing that the ozonolysis pretreated saw dust has enhanced enzyme accessibility. The sugar yield of ozonolysis pretreated saw dust improved from 15.3 to 32.1% compared to none pretreated saw dust. They have also proposed that a small amount of hemicellulose was removed from biomass because the linkage between hemicellulose and lignin was broken down.

The ozonolysis pretreatment is normally operated under a mild condition, so it does not cause sugar degradation (Canilha *et al*., 2012, Eqra *et al*., 2014). The ozonolysis has been studied on many feedstocks such as wheat straw (García-Cubero *et al*., 2009), rye straw (García-Cubero *et al*., 2009), sugarcane bagasse (Eqra *et al*., 2014), poplar saw dust (Vidal and Molinier, 1988). In all cases, the sugar yield of ozonolysis pretreated biomass after enzymatic hydrolysis increased. Although this pretreatment has high efficacy in delignification, it is not a preferred manufacturing process since the ozone is not stable and likely to transform into some other oxides under high temperature. This is a major disadvantage of this pretreatment, which means that it requires a large amount of ozone making it noneconomic due to the cost of ozone used (Aresta *et al*., 2012).

Organosolv process

Organosolv is a process that applies an organic solvent to remove the lignin fraction from biomass (Zhao *et al*., 2009). The organic solvent such as methanol, ethanol, acetone, ethylene glycol, tri-ethylene glycol and tetra-hydrofurfuryl alcohol are normally used to break the linkage between hemicellulose and lignin in this process (Kumar *et al*., 2009). The organosolv process has been studied on biomass residues, such as, olive tree (Diaz *et al*., 2011), switchgrass (Hu *et al*., 2012), sugarcane bagasse (Mesa *et al*., 2011), miscanthus (Brosse *et al*., 2009), almond shell (Gong *et al*., 2011). All the studies have reported that the organosolv pretreatment has removed lignin speeding up the cellulose conversion rate. The sugar yield after hydrolysis step was higher than none pretreated biomass. Bai *et al*. (2013) investigated the structural change during the organosolv pretreatment on bamboo. The results show that after treating bamboo with 70% ethanol at 180˚C for 2 hours, the lignin content was decreased from 23.8% to 17.3%.

This pretreatment can be performed at a wide range of temperatures (100-200˚C) depending on the type of solvent (Zhao *et al*., 2009). The efficacy of this pretreatment method depends on the solvent used, biomass structure and condition of the process. Gong *et al*. (2011) have investigated the efficacy of different organosolv solvents on almond shell. It was found that when 45% acetone was used to treat almond shell at 180˚C for 30 minutes, 55% of the lignin was removed. The organosolv pretreatment showed even better results when 45% ethanol was applied under the same conditions. This removed 60% of lignin and 45% of hemicellulose from the biomass and there was no evidence of glucose degradation.

In some cases, the inorganic acid catalysts (HCl or H_2SO_4) or alkali are applied to increase the delignification rate and enhance the degradation of hemicellulose (Alvira *et al*., 2010, Zhao *et al*., 2009). Furthermore, the acid combined with organosolv pretreatment can increase the rate of cellulose conversion in the hydrolysis step (Sannigrahi and Ragauskas, 2013).

The advantage of this pretreatment is that it can be employed with all kinds of feedstock. More importantly it separates the pure lignin fraction from biomass and this lignin by-product can be converted to a valuable product (Harmsen *et al*., 2010). However a major drawback of this pretreatment is the cost of solvent and it is necessary to remove the solvent from the system afterward since it might interfere with the hydrolysis step in the later stage (Alvira *et al*., 2010).

Hydrothermal pretreatment

This pretreatment method uses high temperature water (180-200˚C) without compressive condition to break the biomass structure down. Hydrothermal pretreatment process is similar to dilute acid pretreatment, but it does not require any acid catalyst. Although the hydrothermal pretreatment is usually carried out under high temperature conditions, it, however, produces fewer inhibitor compounds compared to the dilute acid pretreatment. In order to prevent inhibitor formation, Alvira *et al*. (2010) have suggested that the hydrothermal pretreatment should maintain its pH in the range of 4-7.

The hydrothermal process has been studied on a variety of feedstocks, including herbaceous crop (see Table 2.6).

Table 2. 6 Hydrothermal pretreatment of various lignocellulosic feedstocks.

Substrate	Temperature (°C)	Time (min)	Reference
Almond shells	210	10	Gong *et al*. (2011)
Bamboo	140–200	10–120	Xiao *et al*. (2014)
Corn stover	195	10-30	Xu *et al*. (2010c)
Rice straw	180	20	Chen *et al*. (2011)
Sorghum bagasse	210	20	Dogaris *et al*. (2009)
Sugarcane bagasse	170-230	1-46	Laser *et al*. (2002)
Rapeseed straw	210–220	30–50	Díaz *et al*. (2010)
Wheat straw	188	40	Pérez *et al*. (2008)
Wheat straw	195	6-12	Petersen *et al*. (2009)
Wheat straw	195	6	Kaparaju and Felby (2010)
Wheat straw	185	10	Hansen *et al.* (2011)
Wheat straw	210-220	2.5	Alvira *et al*. (2011)

Physicochemical pretreatment

Physicochemical pretreatment is a process that combines both chemical and physical pretreatment methods to treat the biomass material in one step. Examples of studies of physicochemical pretreatment on lignocellulosic residues are shown on Table 2.7. The physicochemical pretreatments, for example, steam explosion, ammonia fibre explosion and carbon dioxide explosion are mainly used to enhance the porosity of the biomass and to reduce the particle size and crystalline cellulose.

Steam explosion

Steam explosion is a pretreatment process that treats lignocellulosic biomass with high pressure steam. At the end of the process the pressure dramatically decreases. The sudden pressure change causes an explosion from inside of the biomass matrix. The consequences of the explosion are an increasing cellulose accessibility since the explosion has disrupted the biomass structure (Chen and Chen, 2011). The temperature of this pretreatment is in the range of 170-240˚C

and pressure is varied from 0.60-4.12 MPa (Zhang *et al*., 2008, Han *et al*., 2010, Wang *et al*., 2009, Sun *et al*., 2005, Bauer *et al*., 2014).

Han *et al*. (2010) have studied the effect of steam explosion on wheat straw. Their results show that the pretreating wheat straw at 200ºC for 3 minute resulted in a smaller particle size than the untreated wheat straw. The pretreated wheat straw also became more hydrophilic since the wax layer coating on the surface was removed. This improves the ability of bonding between the straw and hydrolytic enzymes. This group also suggested that steam explosion at high temperature with long retention time led to a high weight loss of biomass.

The factors that affect the efficacy of the steam explosion are retention time, temperature, size of biomass and moisture content. Zhang *et al*. (2008) have investigated the effect of pressure during steam explosion on lignin content in wheat straw. The results show that 5.75 % of lignin content in the wheat straw was removed after steam explosion at 0.6 MPa. It has been found that the lignin degradation increased to 14.48% at a pressure of 0.8MPa. The hemicellulose content in biomass was also affected by the steam explosion. Wang et al. (2009) have reported that increasing the temperature during pretreatment from 200˚C to 240˚C for Lespedeza stalk, resulted in a reduction in the hemicellulose, in the form of xylan, from 8.5% to 1.2%.in Cara *et al*. (2008) studied the effect of steam explosion on olive prunings. The results indicate that soaking with water before steam explosion at high temperature can increase both the sugar and ethanol yields.

In order to reduce the temperature used during steam explosion, catalyst agents, such as, sulphuric acid have been applied in the process. This acid-assisted steam explosion can be performed with a shorter retention time and at lower temperature. The technique can improve the efficacy of the hydrolysis step and reduce the production of inhibitor compounds (Zimbardi *et al*., 2007).

Ammonia fibre explosion

Ammonia Fibre Explosion (AFEX) is a similar process to steam explosion. Liquid ammonia is used as a catalyst in this pretreatment process. The ammonia fibre explosion is carried out at low temperature (60-100˚C) and high pressure condition (Alvira *et al*., 2010). The pretreatment condition is held for a desired time and then the pressure suddenly released. After ammonia fibre explosion the hemicellulose fraction in biomass is converted to smaller oligomers. The bonds between lignin and carbohydrate are broken down resulting in an increased cellulose accessibility (Kumar *et al*., 2009). Ammonia fibre explosion has been reported to be not very effective in biomass with a high lignin content. Studies on ammonia fibre explosion have only been done on herbaceous crops and some agricultural residues, for instance, coastal bermuda grass (Lee *et al*., 2010), corn stover (Teymouri *et al*., 2005, Chundawat *et al*., 2010, Zhao *et al*., 2014, Steele *et al*., 2005), sweet sorghum bagasse (Li *et al*., 2010a), rice straw (Vlasenko *et al*., 1997), miscanthus (Murnen *et al*., 2007) and switch grass (Alizadeh *et al*., 2005, Bals *et al*., 2010).

Teymouri *et al*. (2005) have worked on determining optimal conditions for ammonia fibre explosion on corn stover for maximising the sugar yield. The optimal pretreatment condition for corn stover is 90ºC for 5 minutes and this method leads to a higher yield of ethanol which increases up to 2.3 times over that of the untreated raw material.

Carbon dioxide explosion pretreatment

Carbon dioxide explosion pretreatment is a process that uses supercritical carbon dioxide which is carbon dioxide compressed at temperature to above its critical point and is again used to break down biomass structure. During the carbon dioxide explosion, the supercritical carbon dioxide penetrates through the porous biomass under high pressure then the pressure is drastically decreased. The biomass structure explodes after the rapid pressure change resulting in an increase in biomass surface area. This process is usually operated at a lower temperature compared to steam explosion (Kumar *et al*., 2009). Zheng *et al*. (1998) have studied the effect of varying temperature and pressure levels during carbon dioxide explosion on avicel. The pretreatment process was carried out at temperatures of 25, 35 and 80°C at a pressure of 3000 psi. They found that the glucose yields from the pretreated avicel at either 80˚C or 35˚C were not statistically different. On the other hand, when they varied the pressure, the results showed a positive response on glucose yield as well as the rate of hydrolysis after carbon dioxide explosion.

Table 2. 7 Physicochemical pretreatment on various lignocellulosic feedstocks.

Substrate	Pretreatment	Reference
Coastal Bermuda grass	Ammonia fibre explosion	Lee *et al*. (2010)
Corn stover	Ammonia fibre explosion	Teymouri *et al*. (2005)
Rice straw	Ammonia fibre explosion	Vlasenko *et al*. (1997)
Aspen	Carbon dioxide explosion	Kim and Hong (2001)
Corn stover	Carbon dioxide explosion	Narayanaswamy *et al.* (2011)
Pine	Carbon dioxide explosion	Kim and Hong (2001)
Switchgrass	Carbon dioxide explosion	Narayanaswamy *et al.* (2011)
Switchgrass	Steam explosion	Samuel *et al.* (2011)
Wheat straw	Steam explosion	Montané *et al*. (1998)
Wheat straw	Steam explosion	Ballesteros *et al*. (2006)

Biological pretreatment

Biological pretreatment is an alternative process that increases the surface area of biomass by using microorganisms to remove lignin and break down carbohydrate structures (Shi *et al*., 2008, Sun *et al*., 2011, Taniguchi *et al*., 2005). This process is becoming more attractive for pretreating biomass because it can be carried out under mild conditions. The biological pretreatment is a relatively safe and environmentally benign process compared to chemical or physicochemical pretreatments. Moreover, the biological pretreatment does not generate toxic compounds such as furfural, hydroxymethylfurfural (HMF). These compounds have an inhibitory effect on yeast fermentation process.

Many species of ligninolytic microorganisms have been investigated in biological pretreatment, such as, white-rot fungi (Yang *et al*., 2011), Brown-rot fungi (Ray *et al*., 2010), Soft-rot fungi (Brown *et al*., 2012) and Cellulolytic bacteria (Dashtban *et al*., 2009). Solid state fermentation is commonly used in biological pretreatment at a broad range of culture times ranging from 7 days to

150 days (Zeng *et al*., 2011, Xu *et al*., 2010a). Each of these microorganisms have their own specific mechanism to break down biomass structure.

White rot fungi

White rot fungi is a microorganism that has an ability to degrade the lignin fraction in biomass. *Phanerochaete chrysosporium* has been used as a standard model for white-rot fungi. *P. chrysosporium* can express the entire ligninolytic system, and can completely oxidize the lignin forming carbon dioxide as the product (Jäger *et al*., 1985). The hemicellulose and cellulose are then hydrolysed by hemicellulase and cellulase, respectively (Kumar *et al*., 2009). Zeng *et al*. (2011) have investigated the effect of biological pretreatment using *P. chrysosporium* on wheat straw. They found that after 7 days of pretreatment, the total lignin (which is around 25% of dry weight biomass) in the straw was almost completely removed whereas most of the cellulose and the hemicellulose components were not consumed. Shi *et al*. (2008) also studied the biological pretreatment using *P. chrysosporium* on cotton stalks. They reported that 27.6% of the lignin was degraded within 2 weeks and that carbohydrate consumption was not detected. On the other hand, Salvachúa *et al.* (2011) have reported that after using *P. chrysosporium* to treat wheat straw for 21 days that lignin degradation was not detected, while there was a 35% degradation of cellulose and 70% of degradation of hemicellulose.

Some of white-rot fungi, such as *Ceriporiopsis subvermispora*, *Trametes versicolor* and *Trametes hirsute*, can simultaneously degrade lignin, cellulose and hemicellulose. Biological pretreatment using *C.subvermispora* has been studied on many types of feedstock, for example, wheat straw (Wan and Li,

2011), corn stover (Wan and Li, 2010), sugarcane bagasse (Ramos *et al*., 2004) and wood (Tanaka *et al*., 2009). Salvachúa *et al.* (2011) have reported that there were no inhibitors found after pretreating wheat straw with *C. subvermispora* for 21 days; however 13% lignin, 36% cellulose and 30% hemicellulose were removed by this process. Wan and Li (2010) also support this statement. They have found that 31.6% lignin and 6% cellulose were removed from corn stover after pretreatment with *C. subvermispora* for 18 days. The component degradation, of course, enhances the subsequent enzymatic hydrolysis. It has been found that the biological pretreatment by *C. subvermispora* for 18 days on soybean straw, switchgrass, and hardwood can increase the glucose yield around 2-3 fold compared to the untreated biomass (Wan and Li, 2011).

Trametes sp. have been widely used to break down the lignocellulosic structure since this strain has an ability to oxidise lignin, however, the strain also degrades cellulose (Canam *et al*., 2011). In order to reduce cellulose consumption , Canam *et al*. (2011) have proposed a promising technique using a mutated *Trametes versicolor* strain in which cellobiose dehydrogenase has been repressed. They used this strain for the biological pretreatment of canola straw since it has ability to produce extra-cellular laccase (Schlosser *et al*., 1997). Although the mutant strain grew relatively slowly it did show high efficacy on delignification than the wild strain after 12 weeks of pretreatment. Moreover, the saccharification efficacy was improved in comparison with the response observed in the *T. versicolor* wild type.

Although white-rot fungi are very promising candidates for the lignocellulolytic enzyme system, this fungal group is still not a favoured choice for industrial scale production since the long residence time of pretreatment and the consumption of the cellulose by the fungus itself remain quite significant (Canam *et al*., 2011).

Brown-rot fungi

Brown-rot fungi have the ability to both degrade cellulose and modify lignin structure by demethylation (Nicholas, 1982). However, brown rot fungus has a limited ability to remove lignin because it lacks ligninolytic enzymes (Valásková and Baldrian, 2006).

The brown-rot fungi that are most commonly used for pretreatment are *Gloeophyllum sepiarium*, *Gloeophyllum trabeum*, *Fomitopsis palustris*, *Fomitopsis pinicola*, *Polyporus schweinitzii*, *Serpula incrassata*, *Piptoporus betulinus* and *Tyromyces palustris*. (Cohen *et al*., 2005, Valásková and Baldrian, 2006).

Biological pretreatment of hardwood aspen using *G. trabeum* has been investigated. It has been found that a glucose yield of 72% of was obtained after 2 weeks of pretreatment (Schilling *et al*., 2012). Biological pretreatment using brown rot fungi on soft wood has also been studied. *Coniophora puteana* was applied to pretreat Scots pine for 15 days and it was found that the glucose saccharification yield exceeded 70% and only 9% wood mass was lost (Ray *et al*., 2010).

Soft-rot fungi

Soft-rot fungi can degrade cellulose and lignin in a similar manner to the white rot fungi. The soft rot fungi can grow under extremely wet or dry conditions (Gupta *et al*., 2013). One of the most extensively studied soft rot fungus is *Trichoderma sp*. *Chaetomium globosum* is another stain that has been used as a pretreatment process. Ray *et al*. (2010) have studied the efficacy of biological pretreatment on pinus radiate sapwood using different fungal strains. They reported that biomass pretreated with *Chaetomium globosum* ATCC 6205 at 25˚C for 20 days, showed a 10% weight loss. However, the glucose yield did not improve.

The efficacy of the pretreatment step depends on the characteristics of the biomass, biomass composition and pretreatment conditions (Vincent, 2010b). It is difficult to evaluate and compare pretreatment technologies because they involve variable upstream and downstream processes (Agbor *et al*., 2011). Each method has their own unique advantages and disadvantages (see Table 2.8).

Table 2. 8 The advantages and disadvantages of each pretreatment technique.

Pretreatment	**Advantage**	**Disadvantage**
Physical pretreatment :		
Mechanical	Reduce cellulose crystallinity, increase surface area and pore size	High power and energy consumption
Physicochemical :		
Steam explosion	Solubilized lignin and hemicellulose, cost effective, higher yield of glucose	Generate inhibitor compounds, hemicellulose degradation
Ammonia fibre explosion	Increases accessible surface area, low formation of inhibitor, not require small particle size for efficiency, decrystalizes cellulose, lignin removal	Not efficient for raw material with high lignin content, high cost from amount of ammonia
Carbon dioxide explosion	Increases accessible surface area, cost effective, prevent decomposition of monosaccharide by acid, not generate toxic compounds	Does not affect lignin and hemicellulose, very high pressure requirement
Chemical pretreatment :	**Advantage**	**Disadvantage**
Ozonolysis	Reduces lignin content, not generate inhibitory components	High cost of large amount of ozone needed
Acid Hydrolysis		
-Concentrated acid	High glucose yield, ambient temperature, hemicellulose removal	High cost of chemicals and need to be recovered, reactor corrosive problem, formation of inhibitor
-Diluted acid	Less corrosive problem than concentrate acid, hemicellulose removal, less formation of inhibitor	Low sugar concentration in exit steam
Alkaline hydrolysis	Remove lignin, solubilize hemicellulose, increase surface area, need ambient condition to react, lignin removal	Takes a long time
Oxidative delignification	Efficient remove of lignin, low formation of inhibitors	High cost
Organosolv	Cause lignin and hemicellulose hydrolysis	High cost, solvent need to be drain and recycle
Biological pretreatment	**Advantage**	**Disadvantage**
Fungi	Degrade lignin and hemicellulose, low energy consumption	Takes a long time, hydrolysis rate is low

2.2.2 Hydrolysis

The hydrolysis step is required to convert the lignocellulosic biomass into fermentable sugar. There are 2 major methods that have been used these being (i) chemical hydrolysis and (ii) enzymatic hydrolysis.

Chemical hydrolysis

Acid is the main chemical reagent which is used in chemical hydrolysis predominantly sulphuric acid and hydrochloric acid. High concentration of acid (30-70% by vol.) without heat was applied to break the amorphous portion structure down by disrupting hydrogen bond in cellulose, ester and ether bond in hemicellulose and turn it into sugar (Khan, 2010).

Acid hydrolysis for producing sugar has been studied in many types of feedstock, such as, potato peel (Lenihan *et al*., 2010), olive tree pruning (Romero *et al*., 2007), sugarcane bagasse (Gámez *et al*., 2006, Aguilar *et al*., 2002) and rice straw (Kim *et al*., 2012, Wang *et al*., 2012).

Yield from acid hydrolysis is dependent on retention time, temperature, acid concentration, particle size and mixing rate. In order to improve yield and avoid sugar degradation, 2 acid hydrolysis stages have been introduced. Janga *et al.* (2012) have investigated a 2 stage acid hydrolysis on aspen and pine wood. In the first stage, high concentrations of acid (96-98%wt) is used to de-crystallise cellulose and degraded hemicellulose. After cellulose has been de-crystallised, a lower concentration of acid (20%wt) is then applied to cleave the glycosidic bond in cellulose fraction into monosaccharide sugar. The result shows 44% of glucose was deliberated after the 2 hydrolysis stages. The advantage of acid hydrolysis over enzymatic hydrolysis is the faster reaction rate. However this hydrolysis technique has huge disadvantages, which are corrosive problems on equipment and acid residues (Verardi and Calabrò, 2012).

Enzymatic hydrolysis

Enzymatic hydrolysis is another method that converts lignocellulose into fermentable sugar. Enzymatic hydrolysis is favoured for the degradation of biomass for many reasons. Firstly, the enzymatic hydrolysis technique is carried out under mild conditions. It does not require corrosion resistant equipment nor the downstream process required to recover the acid residue. More importantly, the enzymatic hydrolysis does not generate inhibitory compounds.

During enzymatic hydrolysis, lignocellulose material is degraded using lignocellulolytic enzymes. This is normally in the form of an enzyme-cocktail, comprised of cellulase, hemicellulase and ligninolytic enzymes.

Cellulase

Cellulolytic enzymes are a group of enzymes that are specific for hydrolysing cellulose. Cellulases can be divided by their mechanism of action as described below:

1. Endo-1,4-β-D-glucanase or endoglucanase, EG (EC 3.2.1.4)

This enzyme randomly hydrolyses the β-1,4-D-glycosidic linkages in the cellulose chain. The endoglucanase prefers to degrade the amorphous regions in cellulose rather than the crystalline cellulose.

2. Exoglucanase also known as cellobiohydrolase, CBH (EC 3.2.1.91)

This enzyme hydrolyses β-1,4-D- glycosidic linkage in the crystalline cellulose and amorphous cellulose. It cleaves the cellulose polymer at the ends of the

linear chain resulting in the disaccharide cellobiose unit. Cellobiohydrolases can be classified into 2 types based on their substrate specificity. Cellobiohydrolase I (CBH I) acts on the reducing end and cellobiohydrolase II (CBH II) on the non-reducing end of the cellulose chain. The cellobiohydrolase is a very effective enzyme for crystalline cellulose degradation. However, the cellobiose molecule can suppress the activity of cellobiohydrolase.

3. β-glucosidase or cellobiases (BG) (EC 3.2.1.21)

This enzyme can convert oligosaccharides, such as, cellobiose into glucose. It has been reported that β-glucosidase can increase the reaction rate of cellulose hydrolysis (Sørensen *et al*., 2013).

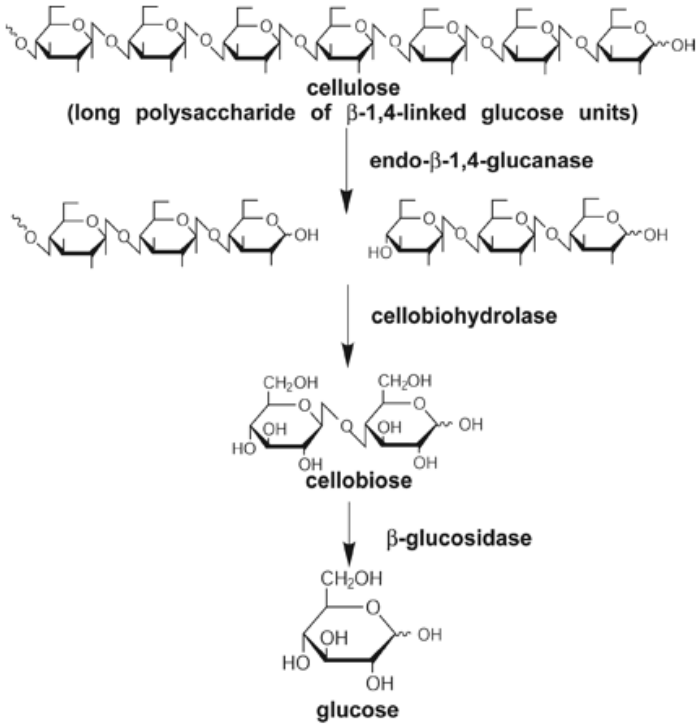


Figure 2. 5 Cellulose degradation mechanism (Xie *et al*., 2007)

The combined mechanism of these cellulolytic enzymes is shown in Figure 2.5. Firstly, the cellulose fibre is hydrolysed by endoglucanase and generates

smaller chain polymers. Then cellobiohydrolase grasps the short chain polymer and hydrolyses it producing cellobiose. After that two molecules of glucose are deliberated from a single cellobiose by β-glucosidase.

Hemicellulase

Hemicellulose is a heteropolymeric polysaccharide. The structure of this compound is a mixture of branched and linear polysaccharides. They are bound together with ether and ester bonds. Moreover the hemicellulose can be linked to other compounds, such as, lignin and cellulose. Hemicellulose degradation requires concerted action of multiple enzymes as shown in Figure 2.6.

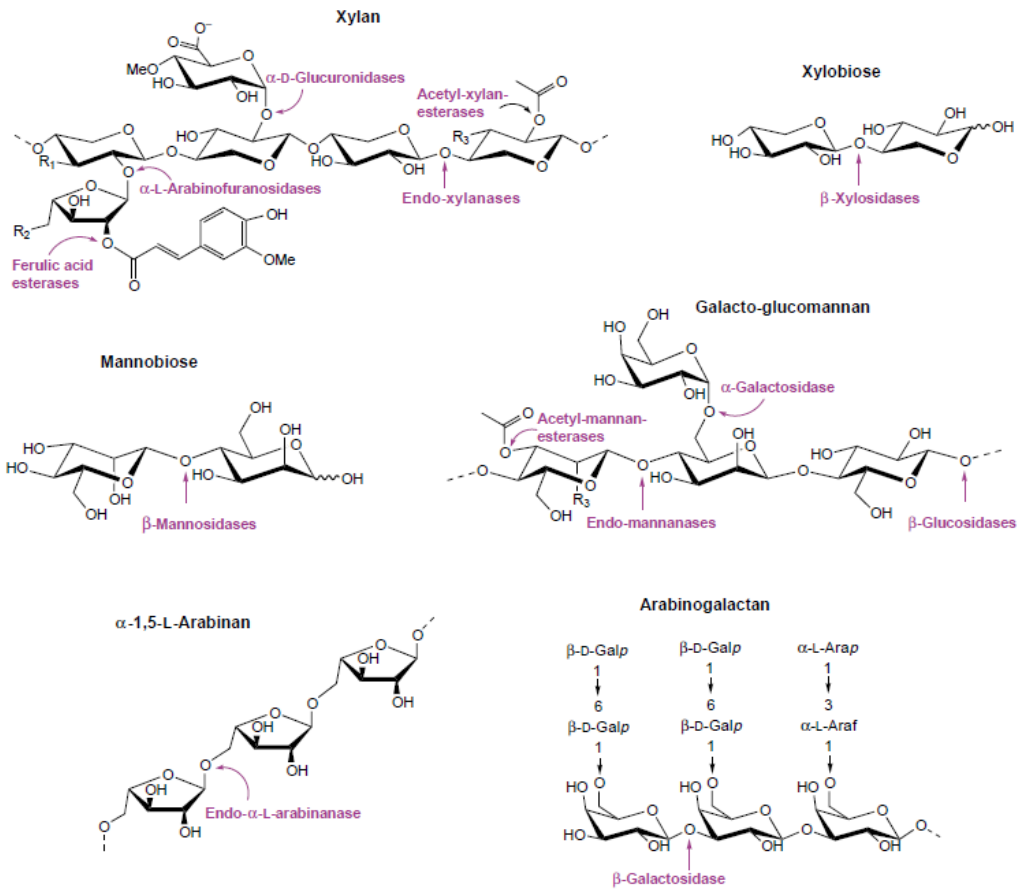


Figure 2. 6 Hemicellulose degrading enzyme (Shallom and Shoham, 2003)

Hemicellulose degrading enzyme requires enzymes in the following list.

1. 1-4,-β-D- xylan xylanohydrolase or endo-1-4,-β-xylanase (EC.3.2.1.8)

This enzyme has an ability to break down the glycosidic bonds in xylans into β-D-xylopyranosyl oligomers.

2. α-D-glucuronidase (EC 3.2.1.139)

This hydrolase breaks the α-1,2 glycosidic bondin glucuronic acid sidechains in xylan. The product from this reaction is D-glucuronic acid. D-glucuronic acid and glucose have been reported to repress the glucosonidase activity.

3. α-L-arabinofuranosidase (EC 3.2.1.55)

This enzyme hydrolyse α-(1-2) and α-(1-3) link between arabinofuranosyl residues and the xylan backbone. It is specific to (1-2)-, (1-3)- and (1-5)-α-arabinofuranosyl linkages of arabinan and arabinoxylan.

4. Ferulic acid esterase or feruloyl esterase (EC 3.1.1.73)

This hydrolase breaks the ester bond that links arabinoxylan and ferulic acid. (Moreira, 2008) suggested that ferulic acid esterase plays an important role in releasing hemicellulose from lignin.

5. 1-4,-β-D-xylan xylohydrolase or β-D-xylosidase (EC.3.2.1.37)

This enzyme is specifically binds to the non-reducing ends of short xylooligomers (Barker *et al*., 2010). The xylo-oligasaccharide and xylobiose molecules are then hydrolysed and converted into β-D –xylopyranosyl residues.

6. β-1,4-Mananase or Endo mannanase (EC 3.2.1.78)

This enzyme randomly cleaves β-1,4-D-mannosidic linkages in mannans, galactomannans and glucomannans polymers. The product from this reaction is manno-ligomer or mannobiose

7. Acetyl-mannan-esterase (EC 3.1.1.6)

This enzyme removes the acetyl substitution group on galacto-glucomannan structure by breaking the ester bond.

8. β-galactosidase (EC 3.2.1.22)

This enzyme removes the galactosyl substitution group on galactoglucomannan.

9. β -1,4-mannosidase (EC 3.2.1.25)

This enzyme hydrolyses β-1,4-D mannosyl groups from the reducing end of mannobiose.

10. α-arabinanase (EC 3.2.1.99)

This enzyme has the ability to remove L-arabinose residues from hemicellulose polymers such as arabinoxylans.

11. Acetyl xylan esterase (EC 3.1.1.6)

This enzyme is specific for the acetyl group in xylan polymers. It has de-acetylation activity so this enzyme can degrade esterified acetyl group in xylan to acetic acid.

Ligninase

Although lignin is a complex polymer it can be hydrolysed by some fungal and bacterial strains that have the ability to produce ligninase or ligninolytic enzymes. This enzyme group is comprised of following activities:

1. Laccases or phenol oxidase (EC 1.10.3.2)

This enzyme is an oxidative enzyme that oxidizes aromatic amines and phenolic compounds. The oxidized phenolic compounds are then converted into p-quinone (Thurston, 1994). It is a nonspecific phenol oxidase enzyme (Madhavi and Lele, 2009).

2. Lignin peroxidase (EC. 1.11.1.14)

This enzyme is an oxidative enzyme that oxidizes aromatic amines and phenolic compounds (Fernández-Fueyo and Martínez). It is activated by veratryl alcohol (Piontek *et al*., 2001).

3. Manganese peroxidase (EC. 1.11.1.13)

This enzyme is an oxidative enzyme that oxidizes aromatic amines and phenolic compounds. They need manganese ions (Mn2+) as their coenzyme.

In enzymatic hydrolysis, there are many factors that affect the efficacy of enzymatic hydrolysis. One of the major factors that affect enzymatic hydrolysis is substrate concentration. High concentrations of substrate can cause various problems which lead to unequally distributed mass transfer. Besides this it may cause product inhibition which can suppress cellulolytic enzyme activity.

Hydrolysis yield is highly affected by characteristics and components of the substrate. The compact biomass structure without pretreatment does not provide satisfactory hydrolysis yield. The selection of pretreatment method should be compatible with the selection of hydrolysis. Enzyme loading is another factor influencing hydrolysis. Higher enzyme loading clearly results in higher sugar liberation but it requires larger amounts of enzyme leading to increased processing cost. The hydrolysis conditions such as temperature, mixing rate and pH are important issue impacting enzyme efficiency. The optimal condition for lignocellulolytic enzymes depends on its source of production. For example, the optimal temperature of fungal cellulase is in the range of 45-55˚C, however thermophilic microorganism, such as, *Hypocrea jecorina* can produce thermotolerant cellulase that has an optimal temperature at 70˚C. Clearly, higher temperature hydrolysis with efficient enzymes leads to an improvement in hydrolysis yield. The thermostable cellulase can increase the sugar yield around 3 times compared to the wild type enzyme which operates at 60 °C (Trudeau *et al*., 2014). The optimal pH for fungal cellulase is in the range of 4 to 5.5. The cellulases may lose their activity if a pH above that range is applied.

Enzyme production

Since enzymatic hydrolysis is very important for bioethanol production. Enzyme cost becomes one of the major barriers to an economically viable bioethanol production process. In order to reduce bioethanol production costs, on-site enzyme production is an attractive option. The on-site enzyme production must be a cost effective process. Low cost sources of carbohydrate such as agricultural wastes are normally used as a substrate for enzyme

production. Beside the issue of substrate cost, another issue that catch an attention is the ability of microorganism to produce active lignocellulolytic enzymes. Lignocellulolytic enzyme production has been studied in various types of microorganism especially in fungal strains (see Table 2.9) because fungi are the main cellulose degradation microorganisms in natural ecosystem. *Trichoderma reesei* and *Aspergillus niger* are predominant strain that have been used for cellulolytic enzyme production because they can produce extracellular cellulase enzyme with a high level of activity even in wild type strains.

Trichoderma sp. is a complement cellulase production strain. It can produce high levels of endo-glucanase and exoglucanase but it generates low level of β-glucosidase enzyme. *Aspergillus* strain can produce various types of enzymes depending on the substrate in the culture condition. (Kim et al., 1997) have reported that 84 FPU/g and 9100 U/g of xylanase activity were produced from rice hull by *A. niger* KKS. The *A. niger* NS-2 produced 310 U/g of CMCase,17 U/g of Filter paper activity and 33 U/g of β-glucosidase using kitchen waste as a substrate (Bansal *et al*., 2012). The *A. niger* NS-2 has also been studied for enzyme production using wheat brand as a substrate. The results show that carboxymethylcellulase (333 U/g), filter paper activity (15 U/g), β-glucosidase (30 U/g), xylanase (1679 U/g), mannanase (160 U/g), α-amylase (19698 U/g), glucoamylase (474 U/g) and pectinase (110 U/g) were detected after 96 hours of fermentation (Bansal *et al*., 2011).

Aspergillus niger can produce a high level of β-glucosidase activity. The most commercial used enzymes for hydrolysing feedstock are usually produced from *T.reesei* supplimented with β-glucosidase from *A.niger*. Gutierrez-Correa *et al*.

(1999) has reported that 6.5-10 U/g of filter paper activity was obtain from sugarcane bagasse using *T. reesei* LM-UC4 and the filter paper activity has improved to 14.7-15.5 U/g when *A. niger* was applied.

Table 2. 9 Lignocelluloslytic enzymes produced by different fungal strains and their substrates (Dashtban *et al*., 2009, Rodriguez Couto and Sanromán, 2005)

Group	Fungal strain	Enzymes	Substrate
Ascomycetes	*Trichoderma reesei*	Cellulases (CMCase, CBH, BGL) Hemicellulase (xylanase)	Wheat straw
	Trichoderma harzianum	Cellulases (CMCase, CBH), β-1,3-glucanases	Wheat bran,wheat straw
	Trametes versicolor	MnP, laccase	Bagasse
	Aspergillus niger	Cellulases, Xylanases, glycosidasse	Sugar cane bagasse,grape
	Pestalotiopsis sp.	Cellulases (CMCase, CBH), Laccase	Forest litter
	Botryosphaeria sp.	Laccase	Ballico seed
Basidiomycetes	*Penicillium chrysosporium*	Cellulases (CMCase, CBH, BGL), Ligninase (LiP, MnP, Hemicellulase (xylanases)	Red oak, grape seeds, barley bran, woodchips
	Fomitopsis palustris	Cellulases (CMCase, CBH, BGL)	Microcrystalline cellulose
	Pleurotus ostreatus	Laccase, MnP, LiP	Bagasse,
	P.sajor-caju	Laccase, LiP	Banana waste
	Lentinus edodes strain CS-495	Laccase	Corn

Note: CMCase is Carboxymethyl cellulase, CBH is exoglucanase, BGL is β-glucosidase, MnP is manganese peroxidase and LiP is lignin peroxidase.

Enzymes production can be carried out in both solid state fermentation and submerged fermentation. Both of these have their own benefits and drawbacks. Most enzyme production procedures are carried out in submerged fermentation because culture conditions in submerged fermentation are a lot easier to control

compared to solid state fermentation. However, solid state fermentation dose have some advantages over submerged fermentation, which are (i) its condition is close to the nature habitat (ii) it has a lower energy consumption and (iii) the operating cost is less expensive.

2.2.3 Fermentation

Bioethanol fermentation is a process that converts hexose or pentose sugar into alcohol and carbon dioxide by alcoholic fermentation microorganism. The microorganism that is most commonly used in ethanol fermentation is yeast, particularly *Saccharomyces cerevisiae*. Wild type strains of *S. cerevisiae* metabolise glucose (C6 sugar) via the Embden–Meyerhof pathway or glycolysis into pyruvate. In anaerobic condition, this yeast strain produces pyruvate decarboxylase and alcohol dehydrogenase enzymes which converts pyruvate to alcohol and carbon dioxide (Chang *et al*., 1983). The yeast will completely oxidise pyruvate into carbon dioxide and water when oxygen is presented. It is known that *S. cerevisiae* can generate a high yield of ethanol from hexose sugar, but that it cannot metabolise pentose sugars which are normally also found in lignocellulose hydrolysates. *Candida shehatae* (Ge *et al*., 2011), *Pachysolen tannophilus* (Slininger *et al*., 1987), *Kluyveromycess marxianus* (Margaritis and Bajpai, 1982), *Pichia stipites* (Agbogbo and Coward-Kelly, 2008a) and *Zymomonas mobilis* (Delgenes *et al*., 1996) have been reported to ferment pentose to ethanol. Some xylose utilising yeast strains such as *K. marxianus SUB-80-S* can produce ethanol from xylose under aerobic condition, but the ethanol yield is still low at 55% of theoretical yield (Margaritis and Bajpai, 1982). Strain improvement by genetic engineering has improved the ethanol yield. *Kluyveromyces marxianus* IMB4 has been

engineered allowing alcohol to be produced under high temperature conditions. It can grow at temperatures up to 52°C and can tolerate ethanol at concentrations greater than 7.5% (w/v).this strain can be used in simultaneous saccharification and fermentation processes due to the temperature of fermentation being close to cellulase optimum temperature (Suryawati *et al*., 2009).

Even though yeast is a robust microorganism it can still be susceptible to inhibitor compounds. The inhibitor compounds can be classified in 3 groups 1) furans, 2) weak acids and 3) phenolic compounds

Furans (Furfural and hydroxymethyl furfural)

Presence of Furans in fermentation media can prolong the lag phase during cell growth since it has been found to deactivate cell respiration (Chandel, 2011). High level of furan can inhibit ethanol production. Hydroxymethyl furfural is less toxic than furfural. Furfural and hydroxymethyl furfural are generated from pentose and hexose sugar degradation respectively under severe condition. Pretreatment processes at high temperature and with long reaction times leads to furan formation (McKillip *et al*., 2000).

Weak acids

Acid compounds, such as, acetic acid, formic acid and levulinic acid can reduce pH in fermentation media. This may decrease cell activity since weak acids can disrupt the cell membrane and dissociate in the cytosol. Moreover it may interrupt the enzymes involved in sugar metabolism. Acetic acid in hydrolysate is generated from de-acetylation of hemicellulose while formic acid and levulinic acid are generated from furan degradation. Larsson *et al*.

(1999) have reported that the ethanol yield and productivity were both decreased when concentrations of acetic acid, formic acid, and levulinic acid were higher than 100 mM. Concentration of weak acid less than 100 mM can enhance the ethanol production (Larsson *et al*., 1999). It has been reported that 0.5 g/L of formic acid does not affect the ethanol production but formic acid at 1 g/L can prolong the ethanol production rate (Fu *et al*., 2014).

Phenolic compounds

Phenolic compounds, such as, vanillin and syringaldehyde are generated from depolymerisation of lignin. Phenolic compounds are inhibitory compounds that have negative effects on cell growth by disrupting the cell membrane (Jönsson et al., 2013b). These inhibitors can also decrease the ethanol yield.

In order to avoid inhibitory compound formation, pretreatment process must be carried out under as mild a condition as possible. However some inhibitory compounds like acetic acid are difficult to prevent, since this compound originates from the biomass (Harmsen et al., 2010, Jönsson et al., 2013b). There are several methods to remove inhibitory compounds such as evaporation, membrane technology and ion exchange resins.

The simplest detoxification can be done by overliming treatment. Overliming treatment has raise pH to 10 by addition of alkali reagent (calcium hydroxide). This pH adjustment results in precipitation of toxic compound. After overliming the pH of hydrolysate needs to readjust to desired value again by acid reagent. Even overliming treatment is one of the most efficacy methods but it can be attributed to sugar degradation during treatment (Jönsson et al., 2013b).

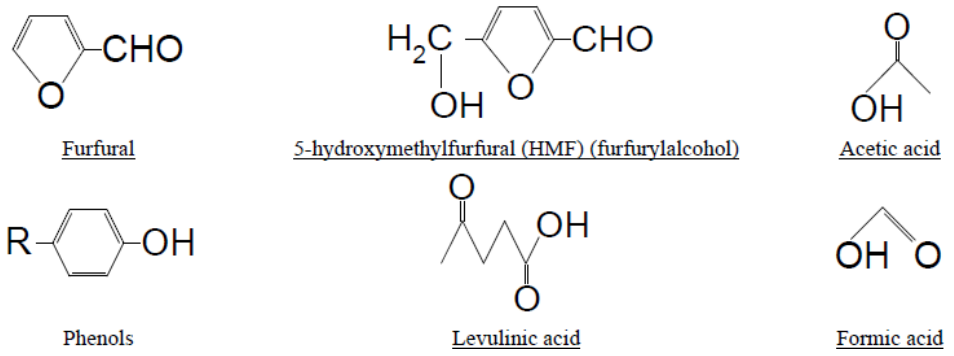


Figure 2. 7 The structure of some typical inhibitor compounds generated in various pretreatment of lignocellulosic raw materials

Table 2. 10 List of inhibitory concentration to yeast strain

Compound	Inhibitor concentration	Minimal inhibitory concentration
Furfural	1-8 mM	15 mM
HMF	0.8 mM	10 mM
Vanillin	60 µM	10 mM
Syringaldehyde	18 µM	>20 mM
Acetic acid	25-100 mM	50 mM
Formic acid	20-100 mM	20 mM
Levulinic acid	ND	75 mM
Coumaric acid	60 µM	>20 mM
Feroic/ferulic acid	50 µM	>20 mM

2.2.4 Challenges in bioethanol production

Production cost of lignocellulosic bioethanol is a major concern in this field. It is related to feedstock supply costs, pretreatment costs, operating technologies and operating time (Sims *et al*., 2010). The main obstacle to bioethanol production is the energy consumption in the pretreatment technologies and the fact that some of the pretreatment technologies produce inhibitor compounds. Furthermore, the cost of production of the enzymes required to hydrolyse lignocellulose biomass into fermentable sugar is also very expensive. These enzyme and pretreatment cost were accounted as a major cost of lignocellulosic bioethanol production (Vincent, 2010b, Klein-Marcuschamer et

al., 2012). The challenge would be to minimize or replace the most costly part of the lignocellulosic bioethanol process i.e. pretreatment step. Besides the pretreatment technology issue, the cost of commercial enzymes used in hydrolysis step must be reduced. This can be done by generating on-site enzymes.

2.3 Biorefining processes to convert lignocellulose to biochemical

The increasing worldwide concern of sustainable production not only affects the energy industry, but also impacts the chemical industry. Currently, the majority of fine chemicals, polymers, fibres and plastics are produced from fossil resources, such as crude oil, coal and natural gas. The global shortage of energy supply and the "finite" nature of the fossil resources drive the research of developing a biorefining process to use renewable materials for the production of biochemicals. Nowadays, several platform chemicals have already been widely produced via bioprocesses, such as lactic acid, succinic acid, 1,3-propanediol and itaconic acid (Koutinas *et al*., 2014)

2.3.1 Succinic acid production

Succinic acid and its derivatives have wide applications in surfactant, detergent, electroplating, food and pharmaceutical industries (McKinlay *et al*., 2007). Currently, it is produced predominantly from petrochemical precursors (maleic anhydride). The production of succinic acid from renewable resources would be expected to lower the production costs and reduce dependence on petroleum. More promisingly, 1 mole of glucose could convert to 2 moles of succinic acid, consuming 2 moles of carbon dioxide, which could contribute to a reduction of greenhouse gas emissions.

Succinic acid could be produced using several bacterial strains, such as *Actinobacillus succinogenes, Anaerobiospirillum succiniciproducens, Mannheimia succiniciprodu- cens and Escherichia coli* (Du *et al*., 2008). In Prof. Colin Webb's lab in the University of Manchester, a wheat-based biorefinery strategy was developed and applied to fermentative succinic acid production (Du *et al*., 2008, Lin *et al*., 2011), Up to 90 g/L succinate was produced using a wild bacterial strain (Lin *et al*., 2011). However, these results were obtained based on the glucose derived from starch materials, which restrict the commercial potential of the research. Besides that, the use of prokaryotic hosts, such as *E. coli* and *Actinobacillus succinogenes* generate succinic salts rather than succinic acid. Most specialty and commodity based applications of succinic acid require the free acid form rather than the salt form. This necessitates additional chemical processing, surplus equipment and perhaps most importantly extra energy to convert the salt into the desired succinic acid (Nevoigt, 2008, Chen and Nielsen, 2013).

A promising alternative approach is to produce succinic acid using a yeast strain (Raab *et al*., 2010, Otero *et al*., 2013). Yeast has been widely used as a chassis in synthetic biology. Yeast strains, *Saccharomyces cerevisiae* for example, are well proven industrial microorganisms. They are biologically safe, and have been widely explored for bioethanol fermentations using non-food materials (Nevoigt, 2008). Yeast has been used for bioethanol production for thousands of years. As discussed in previous sections, intensive investigations have been carried out recently to utilise *S. cerevisiae* to convert lignocellulosic raw materials into bioethanol. This suggests that a lignocellulosic based succinic acid production process could be relatively

easily built up in the yeast system. The availability of a comprehensive genome sequence for multiple species and stains and the well-established genetic engineering tools presents the potential of yeast as an excellent succinic acid producer. More promisingly, yeast e.g. *Saccharomyces cerevisiae*, produce succinic acid in acid form instead of salt form because they can be grown at pH 4.0 and the pK_{a1} of succinic acid is 4.2. The major metabolic pathways involved in the fermentative succinic acid synthesis in *Saccharomyces cerevisiae* are shown in Figure 2.8.

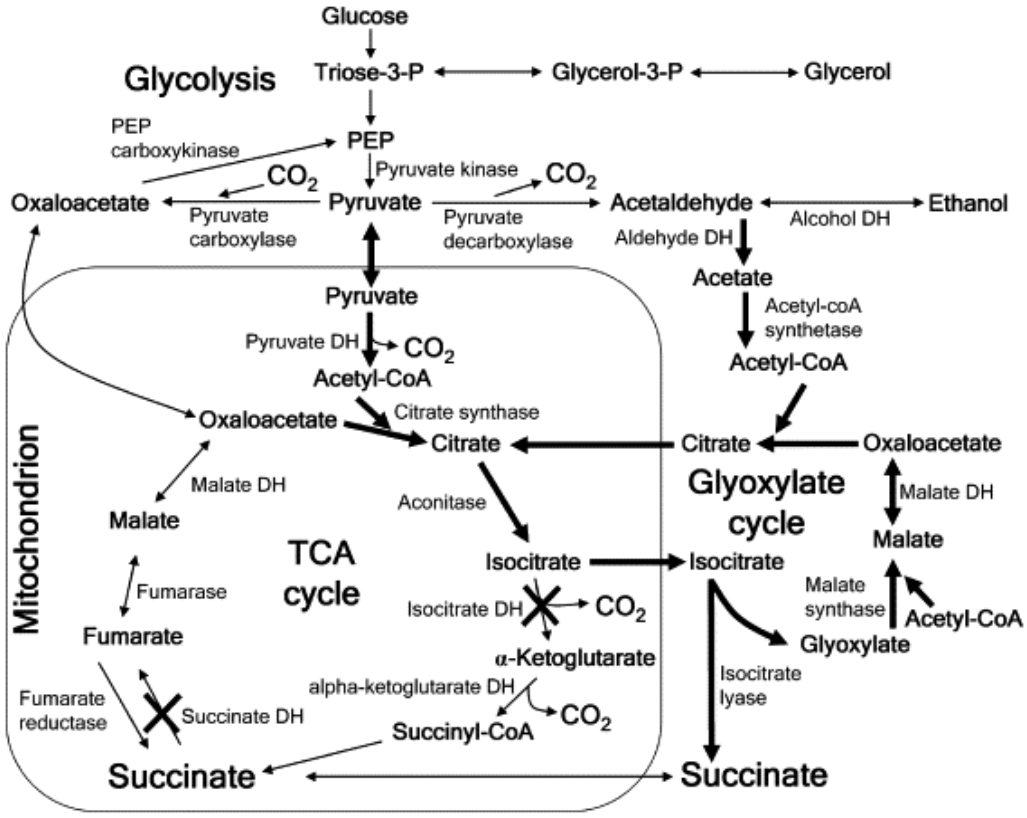


Figure 2. 8 Major metabolic pathways of succinic acid synthesis in *S. cerevisiae.* Not all enzymatic steps or intermediates are shown (PEP, phosphoenolpyruvate; P, phosphate; DH, dehydrogenase) (Raab *et al*., 2010).

Using *S. cerevisiae* for organic acid production, e.g. succinic acid, has a distinct advantage over bacterial processes due to its high tolerance of low pH (Nevoigt, 2008). This could potentially reduce the requirement for downstream processing by producing succinic acid rather than the salt (Lin *et al.*, 2011).

However, the high ethanol fermentation activity in natural *S. cerevisiae* drains off metabolic flux, preventing other metabolites from being synthesised to a significant concentration.

Research on yeast based succinic acid production has only recently commenced with the first paper published in 2010 (Raab *et al*., 2010). Although there are only two publications available using recombinant *Saccharomyces cerevisiae* for the production of succinic acid, encouraging levels of succinate production were achieved. Raab *et al*. (2010) constructed a yeast strain with four gene deletions (*Δsdh1Δsdh2Δidh1Δidp1*, encoding succinate dehydrogenase (SDH) subunits 1, 2 and isocitrate dehydrogenase isoenzymes), leading to 3.6 g/L succinic acid production ($Y_{x/s}$ = 0.11 mol succinic acid/mol glucose). This was 4.8 times higher than the parent strain. Otero *et al*. (2013) blocked SDH3 (SDH subunit 3) and 3-phosphoglycerate dehydrogenase, resulting in a *Saccharomyces cerevisiae* strain that produced 30 folds higher succinic acid (0.9 g/L) than its parent strain (Otero *et al*., 2013). However, compared with the best result obtained in bacterial fermentations (McKinlay *et al*., 2007), these succinate concentrations are very low. This is because only limited genetic modification were done in the *Saccharomyces cerevisiae* strains at this stage. No attempt was made to stop other end-metabolites e.g. ethanol, glycerol, acetate and no attempt was made to overexpress enzymes related to PEP/pyruvate carboxylation. Therefore, in Raab's fermentations (Raab *et al*., 2010), ethanol was still the main product (10-20 g/L, ethanol concentration was not reported in the other study (Otero *et al*., 2013)). Furthermore, limited fermentation optimisation was done to improve succinic acid production using recombinant yeast strains. These facts

indicated that succinic acid production from yeast strains could potentially play a major role in future succinic acid industry.

2.3.2 Itaconic acid production

Itaconic acid is also an important platform chemical, which could be used to replace crude oil derived acrylic acid (El-Imam and Du, 2014). Itaconic acid and poly-itaconic acid have been used or potentially could be used in the production of ion exchange resins, fabric binders, glass fiber, adhesives, plastics, drug coating and paints (Okabe *et al*., 2009, Willke and Vorlop, 2001). *Aspergillus terreus* is the main itaconic acid producer, although several other strains, such as *Aspergillus itaconicus* and *Ustilago zeae* (Haskins *et al*., 1955) could also synthesis and accumulate itaconic acid in the fermentation broth. Major metabolic pathways leading to the formation of itaconic acid in *A. terreus* are shown in Figure 2.9.

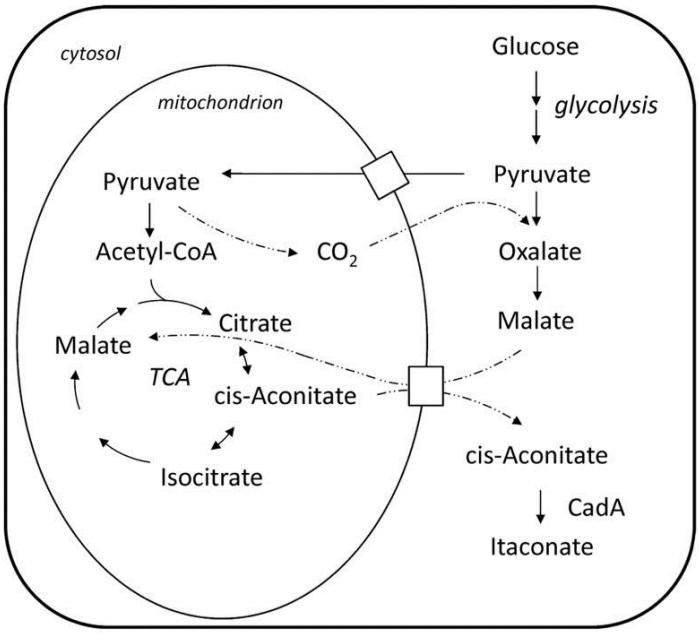


Figure 2. 9 Biosynthesis pathway of itaconic acid in *A. terreus* cell (Steiger *et al*., 2013)

Early research in itaconic acid fermentation focused on the improvement of itaconic acid titre. Therefore, glucose based semi-synthesis medium have been widely used. Under best fermentation condition using *Aspergillus terreus,* up to 91 g/L itaconic acid could be produced (Kuenz *et al*., 2012). Similarly to the development of bioethanol production process, with the growing concern of competing with food application, increasing number of research groups turned their focuses to produce itaconic acid using waste biomass or lignocellulosic raw materials, such as Jatropha cake, olive waste and palm oil mill effluent. Fermentative itaconic acid production was recently reviewed by (El-Imam and Du, 2014).

Table 2. 11 Itaconic acid production using waste biomass and lignocellulosic raw materials. (adapted from (El-Imam and Du, 2014))

Microorganism	**Substrate**	**Concentration (g/l)**
A. terreus	Jatropha cake	48.7
A. terreus CECT 20365	Olive & beet waste	44
A. terreus 282743	POME	5.76
A. terreus	Glycerol	30.2
A. terreus MJL05	Glycerol	27.6
A. terreus	Jatropha cake	24.5

2.4 Wheat straw as lignocellulosic ethanol feedstock

Wheat (Triticum aestivum L.) is originally grown in Middle East 10,000 years ago. Nowadays this crop has grown in many countries around the world. The trend of global wheat production keeps increasing every year. In 2012, we can produce around 670.9 metric tons per year around the world (FAO, 2014). Approximately 70% of global production of wheat is for human food and

around 17% of global production goes through feed production. With the huge production of this crop, it will leads to a large quantity of wheat straw left after harvesting. In term of wheat straw utilisation, some of wheat straw are used in feed production industry, pulping and packaging industry, furniture manufacturing. However there are still some left over every year. The wheat straw is usually removed from the field by open air burning. This improper waste management can cause serious environmental pollution. Petrik *et al*. (2013b) have suggested that wheat straw can serve as resource for value added product. The main advantages of utilising wheat straw as substrate for ethanol production are the huge amount of wheat straw that is available for the production. It does have less sugar decomposition during storage period unlike other sugar material, so it does not need to be proceeded immediately after harvesting. Ideally using abundant raw material like wheat straw to produce ethanol should reduce production cost but due to the recalcitrant character of biomass, the pretreatment is essentially required.

There are numerous studies that investigated the pretreatment technology for bioethanol production from wheat straw (Saha *et al*., 2005, Petersen *et al*., 2009, Ballesteros *et al*., 2006). Littlewood *et al*. (2013) reported that steam explosion, dilute acid, hot water and wet oxidation are efficacious pretreatment method on wheat straw. These pretreatment technologies are energy intensive process and they require high stiffness and corrosive resistant reactor which lead to high investment cost. Furthermore the cost of enzyme for hydrolysis is major variable cost for ethanol production from wheat straw. In order to produce bioethanol that has potential to compete with gasoline without subsidies, it is necessary to reduce energy consumption of pretreatment and

decrease in the cost of enzyme for hydrolysis. Therefore biological pretreatment is an alternative route to reduce energy consumption and production cost. Since it offers low capital cost, low energy consumption and mild environmental conditions. The biological pretreatment with a high potential of cellulase production microorganism strain is another advantageous in production of bioethanol when used for break down wheat straw structure and also for cellulase production. However, the main disadvantage of biological pretreatment is long retention time of the process compared to other technologies. Therefore it is necessary to carry on studying microorganisms for their ability to treat plant material quickly and efficiently.

In this study, a solid state fermentation based biorefining strategy was developed and a wheat straw derived fermentation feedstock was generated. The wheat straw hydrolysate contained high sugar contains, indicating it could be used for itaconic acid fermentation. And the utilisation of wheat straw hydrolysate will provide another example of utilising wheat straw, an abundant agriculture waste biomass stream into valuable chemicals.

Chapter 3 Materials and Methods

3.1 Wheat straw

Cordiale wheat straw (*Triticum aestivum L.*) was obtained from the University Farm (University of Nottingham, Sutton Bonington, UK). The air-dried wheat straw was Knife-milled and passed through a 2-mm screen sieve (Fritsch, Idar-Oberstein, Germany). The wheat straw was collected and stored in a cold room until use.

3.2 Wheat straw analysis

3.2.1 Sugar analysis

Sugar standard solution preparation

Standard solution containing arabinose, galactose, glucose and xylose were prepared as follows. 25 mg of sugars (arabinose, galactose, glucose and xylose) were dissolved in 1000 ml deionized water as "sugar stock solution". Sugar stock solution was diluted with deionized water to final concentrations as presented in Table 3.1

Table 3. 1 Sugar standard preparation

Concentration (mg/L)	Sugars stock solution (µl)	Deionized water (µl)	Total volume (µl)
25	1000	0	1000
12.5	500	500	1000
6.25	250	750	1000
3.125	125	875	1000
1.5625	62.5	937.5	1000
0.78125	31.25	968.75	1000
0	0	1000	1000

Sample preparation

Total sugar analysis was performed by total acid hydrolysis method (Saeman *et al*., 1945). 30 mg of biomass was weighted out and put into a 50 ml Teflon cap tube (Pyrex, UK). 1 ml of 12 M sulphuric acid was added and incubated at 37°C for 1 hour. 11 ml of distilled water was added to dilute acid solution to 1M and the hydrolysis continued at 100°C for 2 hours. The total acid hydrolysate was then cooled to room temperature.

Dionex Ion Chromatography (Dionex)

The amounts of sugars were quantified by HPLC. Prior to HPLC analysis, all samples and standards were filtered using 0.2 μm pore size Whatman GD/X syringe filters (GF/C 25 mm filter diameter; Whatman International Ltd., UK). Monosaccharides (arabinose, galactose, glucose and xylose) were analysed using Dionex ICS-3000 Reagent-FreeTM Ion Chromatography equipped with Dionex ICS-3000 system, electrochemical detection using ED 40 and computer controller. The CarboPacTM PA 20 column (3 x 150 mm/; Dionex, USA) was used and the mobile phase was 10 mM NaOH with a flow rate of 0.5 mL/min. The injection volume was 10 μl and the column temperature was 30°C. The sample analysis was completed in 14 minutes. The chromatogram of standards and retention time of each sugar are shown in Figure 3.1. The peak area of sugar in standard solution was plotted against sugar concentration to draw a standard curve. Sugar concentration of sample was calculated by interpolation on the standard curve.

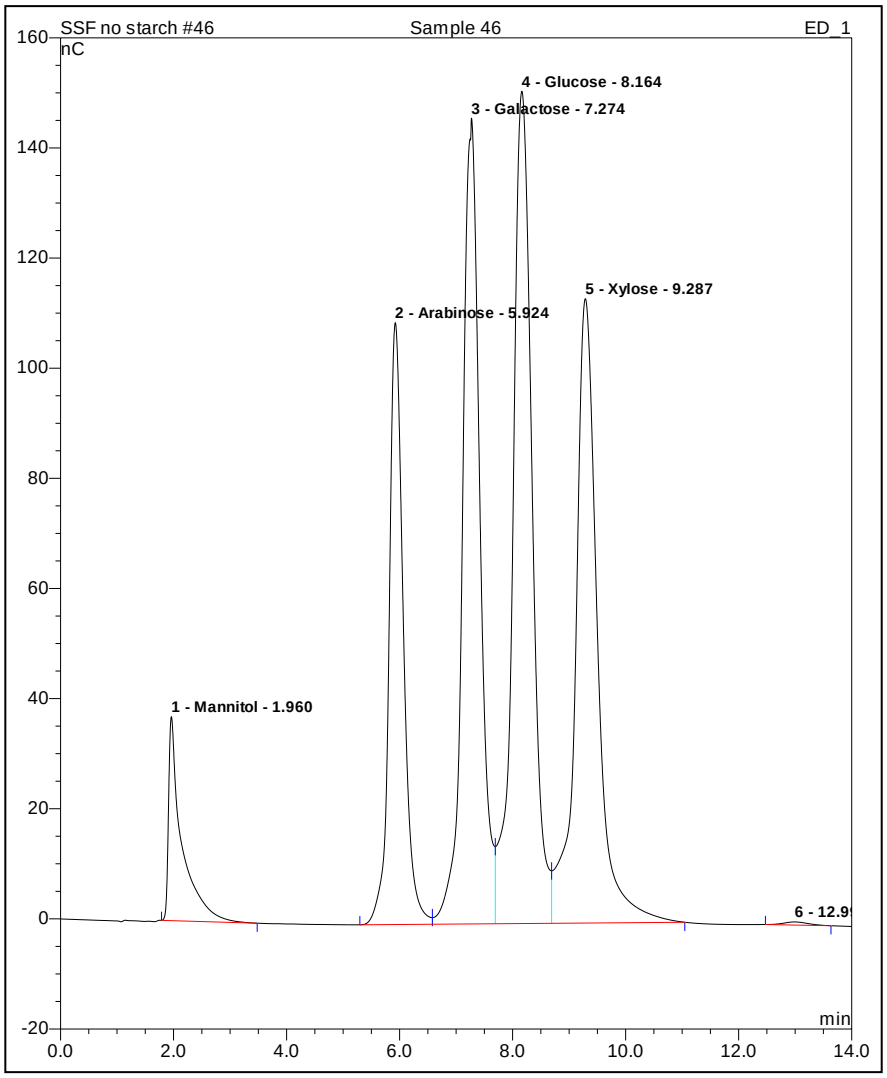


Figure 3. 1 The chromatogram of standard sugars as detected by Dionex. Sugars are (i) mannitol, arabinose, galactose, glucose and xylose with retention times of 1.960, 5.924, 8.164 and 9.287 minutes, respectively.

3.2.2 Lignin analysis

Standard preparation

Lignin standard solution was prepared by weighted out 10 mg of lignin (Sigma Aldrich, UK) and dissolving in 5 ml of 80% v/v dioxane (Simax, USA). Lignin standard solution 0.2, 0.3, 0.4, 0.5 and 0.6 ml was pipetted into 50 ml Teflon tubes (Pyrex, UK). Then 0.5 ml of 25%v/v acetyl bromide in glacial acetic acid was added to each tube and thoroughly mixed. The reaction tubes were incubated at 50°C for 30 minutes then cooled down to room temperature.

After cooling, 2.5 ml of glacial acetic acid, 1.5 ml of 0.3 M NaOH (2.4 g NaOH dissolved in 100 ml of distilled water and made up to 200 ml) and 0.5 ml of 0.5 M hydroxylamine HCl (6.95 g hydroxylamine HCl dissolved in 100 ml of distilled water and made up to 200 ml) were added. The final standard solution was made up to 10 ml with glacial acetic acid.

Sample preparation

Lignin analysis was performed using the acetyl bromide method (Fukushima and Hatfield, 2001). 100 mg of biomass was weighted out and put into a 50 ml Teflon cap tube (Pyrex, UK). 4 ml of 25% v/v acetyl bromide in glacial acetic acid was added and incubated in a water bath at 50°C for 2 hours with occasional mixing. The reaction tube was cooled down to room temperature. Then 12 ml of glacial acetic acid was added into the reaction tube. The reaction solution was thoroughly mixed and centrifuged at 5000 rpm (4,696 g) for 5 minutes.

Lignin analysis

0.5 ml of sample solution was added into Teflon tube (Pyrex, UK). 2.5 ml of glacial acetic acid, 1.5 ml of 0.3 M NaOH and 0.5 ml of 0.5 M hydroxylamine HCl were added and thoroughly mixed. The final solution was made up to 10 ml by adding glacial acetic acid. The optical density of all samples and standard solution were measured (OD) at 280 nm. The OD reading of standard solution was plotted against lignin concentration and concentration of lignin in sample calculated from this lignin standard curve.

3.2.3 X-ray diffraction

Crystalline structure of biomass was measured using X-ray diffraction (D5000, Siemens / Bruker Gmbh) with a copper Kα X-ray irradiation source (λ = 1.541 Angstroms) (Ibbett et al., 2013a, Delmas et al., 2012). Samples were prepared by milling to a fine particle size and sieving through a 200 mesh (74 μm). Fine sample was put into a circulator holder. The 2θ scans were performed from 5 to 50° with 0.05° increments. Baseline was adjusted by using ball milled wheat straw. Sample crystallinity was calculated based on the method proposed in Ibbett *et al* (2013). The reflexed beam from the sample was measured as the function of incident angle. This reflexion profile was curved fitted with Gaussian shapes with linear baseline interpolation and calculated using an Excel programme.

3.2.4 Scanning electron microscopy (SEM)

Biomass structure was imaged by scanning electron microscopy (JEOL UK, UK). The biomass sample was dried at 40ºC for 24 hours in order to fix the morphological shape. After that the sample was pinned onto carbon discs (part no. G3347N) and sputter coated with gold by Leica EM SCD005 and put into JEOL 6060LV SEM instrument (JEOL UK., Ltd, UK).

3.3 Particle size distribution

Particle size distribution analysis was modified from (Miao et al., 2011). 100 g of milled wheat straw was sieved with AS 200 control Retsch vibratory sieve shaker (F-Kurt Retsch Gmbh & Co.KG, Germany) equipped with 6 levels of metal sieves (75-425 μm) and a dust pan. Sieving was run at 1 mm amplitude for 30 hours. Wheat straw from each sieve was collected and weighted. The

amount of wheat straw from each sieve was compared to initial weight of wheat straw and calculated as a percentage.

3.4 Solid state fermentation

3.4.1 Microorganisms

Aspergillus niger N402 (Bos *et al*., 1988) and *Trichoderma reesei* were obtained from the School of Life sciences, the University of Nottingham . Procedures for storing and cultivating *A. niger* were as described by (Delmas et al., 2012).

3.4.2 Fungal pre-culture

The *A. niger* N402 was supplied as a culture slant. This was incubated at 28°C for around 5 days to form conidia. 2 ml of sterile tween 80 were added to wash off the *A. niger* spores. 10 µl of *A. niger* spore suspension was spotted on the middle of a Potato Dextrose agar plate (90 mm-size). *A. niger* culture was incubated at 28°C for 5-7 days. This fungal pre-culture was ready to use for the preparation of the spore suspension.

For strain preservation, 500µl of fungal spore solution and 50% glycerol were added into a cryogenic vial and kept at -80°C.

The *T. reesei* came with silica gel preservation. 4-5 glaze of silica gel were inoculated into PDA slant culture. The PDA culture slant was turned over 2-3 times and incubated at 28°C for 5 days. 2 ml of sterile tween 80 was added to wash *T. reesei* spores. After that 10 µl of *T .reesei* spore suspension was spotted on the middle of a Potato Dextrose agar plate (90 mm-size).

The *T. reesei* culture was incubated at 28°C for 5-7 days. Then the pre-culture was ready to use for the preparation of the spore suspension.

3.4.3 Spore suspension preparation

After 7 days of pre-culture, 10 ml of sterile water was added into the culture plate to prepare a fungal spore suspension. Fungal spores were detached by swirling and scratching with a sterile spatula. Spore suspension was harvested and collected in a sterile tube. An aliquot of fungal spore suspension was then sampled for spore counting using a haemocytometer.

3.4.4 Media preparation for solid state fermentation

16 g of wheat straw and 2.4 g of starch (2% w/v starch at relative volume) were put into 500 ml Duran bottle. Moisture content was adjusted by adding 120 ml distilled water. This fungal media was autoclaved at 121 °C for 15 minutes to sterilise it and subsequently cooled to ambient temperature before inoculation.

3.4.5 Solid state fermentation and sampling

Solid state fungal fermentation was carried out in 90 mm petri-dish. Solid state fermentation was started by adding 10^6 spores/g of substrate onto sterile solid state fermentation media and mixing. After that 2 g of biomass were separated into individual petri-dishes and incubated for 5 days at 28°C. The samples were taken at day 1, 3 and 5.

3.5 Enzyme extraction

The fermented wheat straw mash from each Petri dish was transferred into a blender (Waring commercial blender BN974, USA). Then, 30 ml of 50 mM sodium citrate buffer pH 4.8 per Petri dish was added. The mash was then blended at “high” power for 10 seconds. The mixture was transferred to a

beaker and the contents were stirred by using a magnetic stirrer at 4°C, 300 rpm for 30 minutes. Then the mixture was centrifuged at 5000 rpm (4,696 g) for 10 min. The clear supernatant (fungal extract) was used as the crude enzyme. Note that in the moisture content experiments, no blender was used. The fermented wheat straw mash was directly transferred to a beaker containing 30 ml of 50 mM sodium citrate buffer pH 4.8 per Petri dish. It was then stirred using a magnetic stirrer 4°C, 300 rpm for 30 min. After centrifuging at 5000 rpm for 10 min, the supernatant was collected as the "fungal extract" or "crude enzyme". The fungal extract from cultured media was kept at 4°C until use.

3.6 Enzyme assays

3.6.1 Filter paper activity

Cellulase activity was determined as filter paper units (FPU) according to NREL Laboratory Analytical Procedure (Adney and Baker, 1996). 0.5 ml suitable diluted enzyme solution was mixed with 1 ml sodium citrate buffer (50 mM, pH 4.8) in a test tube containing a Whatman No. 1 filter paper strip (1.0 x 6.0 cm, around 50 mg). The reaction mixtures were incubated at 50°C for 60 min. The released reducing sugar (as glucose) was measured by using Dinitrosalicylic (DNS) method (Miller, 1959). The reaction was terminated by addition of 3 ml of dinitrosalicylic acid solution (DNS) and boiling for 5 minutes. The boiled reaction mixture was colorimetric measured at 540 nm after cooling. The filter paper activity (U/ml) was calculated using the following equation (Adney and Baker, 1996)

$$Filter\ paper\ activity, (\frac{U}{ml}) = \frac{0.37}{concentration\ of\ enzyme\ that\ release\ 2.0\ mg\ glucose} \quad (1)$$

Then it was then converted to U per gram of dry weight wheat straw using the following equation.

$$Filter\ paper\ activity, \left(\frac{U}{g}\right) = \frac{\text{Filter paper activity,}\left(\frac{\text{U}}{\text{ml}}\right)\times\text{Total volume of the fungal extract,(ml)}}{Dry\ weight\ of\ the\ wheat\ straw\ used\ in\ SSF,(g)} \quad (2)$$

3.6.2 Carboxymethyl cellulase activity

Carboxymethyl cellulase activity was determined by the method of the International Union of Pure and Applied Chemistry (Ghose, 1987). 0.5 ml suitabled diluted enzyme solution was mixed with 0.5 ml of 2% (w/v) Carboxymethyl cellulose (Sigma-aldrich, UK) dissolved in 50 mM sodium citrate buffer, pH 4.8 in a test tube. The reaction mixtures were incubated at 50°C for 30 minutes. The released reducing sugar (as glucose) was measured by DNS colorimetric observation at 540 nm (Miller, 1959). One unit of enzyme was defined as the amount of enzyme required to release 1µmol of glucose from carboxymethyl cellulose per minute.

3.6.3 Avicelase activity

Avicelase activity was determined by a modified method of (Ghose, 1987). 0.5 ml of 1% (w/v) Avicel (Sigma-Aldrich, UK) dissolved in 50 mM sodium citrate buffer pH 4.8 was incubated with 0.5 ml of crude enzyme solution. This mixture was incubated at 50 °C for 30 min. The released reducing sugar (as glucose) was measured by DNS colorimetric observation at 540 nm (Miller, 1959). One unit of enzyme was defined as the amount of enzyme required to release 1µmol of glucose from Avicel per minute.

3.6.4 β-glucosidase activity

β-glucosidase activity was determined by the method of Herr (1979). The reaction required 1 ml of 2mM p-nitrophenyl-β-glucopyranoside in 0.05M sodium acetate buffer pH 5.0 incubated with 100 µl of crude enzyme at 50°C for 5 minutes. The reaction was stopped by adding 2 ml of 1M sodium carbonate (Na_2CO_3). The released p-nitrophenol was measured by colorimetric observation at 405 nm. One unit of enzyme was defined as the amount of enzyme required to release 1µmol of p-nitrophenol from p-nitrophenyl-β-glucopyranoside per minute.

3.6.5 Xylanase activity

Xylanase activity was determined by the method of (Bailey et al., 1992).The reaction was carried out with 0.5 ml of 1% (w/v) Birchwood xylan dissolved in 50 mM sodium citrate buffer pH 4.8 incubating and 0.5 ml of crude enzyme at 50°C for 5 minutes. The released reducing sugar (as xylose) in all reaction tube was measured by dinitrosalicylic acid method (DNS) (Miller, 1959). One unit of enzyme was defined as the amount of enzyme required to release 1µmol of xylose from birchwood xylan per minute.

3.7 Protein determination

3.7.1 Reagent preparation

- Complex-forming reagent: 2% (w/v) Na_2CO_3, 1% (w/v) $CuSO_4.5H_2O$ and 2% (w/v) sodium potassium tartrate, all in distilled water and mixed at a ratio of 100:1:1 respectively.

- 2N NaOH prepared by weighing out 80 g of NaOH and dissolving into 800 ml of distilled water. After NaOH has completely dissolved, topped up the final volume to 1000 ml.
- Folin-Ciocalteu solution was prepared by diluted Folin-Ciocalteu phenol reagent with distilled water at a ratio of 1:1. This solution is light sensitive and needs to be freshly prepared.

3.7.2 Standard protein preparation

Standard bovine serum albumin fraction V was prepared by weight out 0.2 g and dissolving in 100 ml distilled water to give a standard stock solution of 2 mg/ml. The protein stock solution was diluted with distilled water to final concentrations as shown in Table 3.2.

3.7.3 Protein measurement

Protein content was measured by a modified Lowry method (Lowry *et al*., 1951). 0.3 ml of crude enzyme or standard protein was mixed with 0.3 ml of 2N NaOH and incubated at 100°C for 10 minutes. After that the reaction mixture was cooled down to room temperature. 3 ml of Complex-forming reagent was added. The reaction mixture was incubated for 10 minutes at room temperature. Then 0.3 ml of folin-ciocalteu solution was added and incubated 30 min. The optical density of mixture was measured at 750 nm.

Table 3. 2 Protein standard preparation

Concentration (mg/ml)	Protein stock solution (µl)	Deionized water (µl)	Total volume (µl)
2	1000	0	1000
1	500	500	1000
0.5	250	750	1000
0.2	100	900	1000
0.1	50	950	1000
0.05	25	975	1000
0.02	10	990	1000
0.01	5	995	1000
0	0	1000	1000

3.8 Glucosamine determination

Glucosamine is a monomer of chitin and chitosan with founded fungal cell wall component. Therefore it could be used as an indirect method to measure fungal growth during fermentation. Glucosamine content was determined by a colorimetric method (Elson and Morgan, 1933).

3.8.1 Reagent preparation

- 4% (v/v) acetyl acetone reagent

4 ml of acetyl acetone was added into 100 ml of 1.25 N Na_2CO_3.

- Ehrlich reagent

1.6 g of N-N dimethyl-p-aminobenzaldehyde was added to 60 ml solution containing 50:50 (v/v) of absolute ethanol: concentrated HCl.

3.8.2 Sample preparation

0.5 g dry weight of sample was hydrolyzed in 2 ml of concentrated sulphuric acid (98%) at room temperature for 24 hours. The mixture was diluted to 1 N sulphuric acid solution (18.3 time dilution by volume) then autoclaved at 121˚C for 15 minutes. Then it was neutralized with NaOH to pH 7 and further diluted with water to final volume of 100 ml.

3.8.3 Glucosamine measurement

The glucosamine concentration was analysed based on the method reported by Sakurai *et al*. (1977). The glucosamine was determined as follows: 1 ml of the above sample solution was transferred into a test tube. 1 ml acetyl acetone reagent (4% (v/v) acetyl acetone in 1.25 N Na_2CO_3) was added then incubated at 100˚C for 20 minutes. After cooling to room temperature, 6 ml of absolute ethanol was added and then mixed gently. 1 ml of Ehrlich reagent (1.6 g of N-N dimethyl-p-aminobenzaldehyde added into 60 ml solution containing 50:50 (v/v) of absolute ethanol: concentrated HCl) was added. The mix was incubated at 65˚C for 10 minutes and the absorbance value was determined at 530 nm).

3.9 Enzymatic hydrolysis

Pretreated wheat straw samples were hydrolysed using commercial enzyme Cellic® CTec2 (Novozyme, Denmark) or fungal enzyme (crude enzyme) at a ratio of 30 FPU per gram of dry substrate. The hydrolysis was performed in a water bath shaker at 50 °C, 150 rpm for 72 hours, where the solid to liquid ratio was varied. The slurry was centrifuged at 5,000 rpm for 10 minutes. Solid residue was discarded and hydrolysate was collected and kept in 4°C.

3.9.1 Response Surface Methodology

Response surface Methodology was investigated in order to determine the optimal conditions for acid hydrolysis and enzymatic hydrolysis. STATGRAPHICS Centurion XVI software (StatPoint Technologies, Inc., Virginia, D.C., USA, version 16.1.11) was used in design experiment.

3.9.2 Hydrolysate analysis

Sugar analysis

Sugar concentrations of hydrolysate were determined by Dionex Ion chromatography as described in 3.2.1.

Saccharification yield was calculated as:

$$Saccharification\ yield\ (\%) = \left(\frac{Sugar\ released\ (g) x\ 0.9}{Initial\ carbohydrate\ content\ (g)}\right) \text{x } 100 \qquad (3)$$

Where 0.9 is the hydration factor of cellulose (glucan polymer) to glucose.

Inhibitory compound analysis

Inhibitory compounds in wheat straw hydrolysate were determined by Waters HPLC. The inhibitory compounds investigated were hydroxymethyl furfural, furfural and vanillin.

Standard preparation

0.2 g of hydroxymethyl furfural, 1.72 ml of furfural and 0.2 g of vanillin were dissolved in 50 ml of distilled water and made up to the final volume of 100 ml with water. Stock inhibitory solutions were store at -20°C until required.

Sample preparation

Hydrolysate was centrifuged at 5000 rpm for 10 minutes. The supernatant was filtered through a 0.2µm syringe filter (Whatman, UK) and put into a glass vial (Chromacol, UK).

Inhibitory compound analysis

Inhibitory compounds in the fermentation sample were measured by Waters 2996 HPLC. The HPLC system has equipped with waters 2996 photodiode array detector, pump and computer controller. The Techsphere ODS-2 column (Thermo Fisher Scientific Inc., UK) was used and mobile phase was 20% (v/v) ethanol with a flow rate of 1.0 ml/min. The injection volume was 10µl. The chromatogram was processed by Empower PDA pro software (Waters, UK). For the standard curve, the peak areas of the standards were plotted against concentration. The concentration of inhibitory compounds in the sample were calculated by interpolation from the standard curve.

3.10 Succinic acid fermentation

3.10.1 Media

YPD broth and YPD agar preparation

YPD broth was prepared by dissolving 10 g of yeast extract, 20 g of peptone and 20 g of glucose in 1000 ml of distilled water. YPD agar was prepared by adding 20 g of agar into YPD broth. The media was sterilised at 121°C for 15 minutes.

Succinic acid production media (synthetic) for Batch fermentation

60 g of glucose and 5 g of yeast extract were dissolved in 500 ml of distilled water. The solution was stirred until the compounds were completely dissolved. 20 g of calcium carbonate ($CaCO_3$) was added. And the final volume was adjusted to 888 ml by topped up with distilled water. This solution was sterilised using an autoclave at 121°C for 15 minutes. After sterilisation, the solution was cooled down to room temperature. 100 ml of YNB solution (6.7 g

of Yeast nitrogen base (YNB) dissolved in 100 ml of distilled water), 10 ml of $ZnCl_2/Na_2HPO_4$ solution (2 g of Zinc chloride ($ZnCl_2$) and 3.1 g of Sodium Phosphate dibasic (Na_2HPO_4) were dissolved with 100 ml of distilled water and adjusted to pH 2.5 with phosphoric acid); 1 ml of Biotin solution (0.002 g of Biotin dissolved in 10 ml of distilled water) and 1 ml of Vitamin B12 solution (0.001 g of Cobalamin (B12) dissolved in 10 ml of distilled water) were added. These solutions were prepared separately since they were temperature-sensitive compounds. A syringe with filter pore size of 0.45µm was used to sterile the solution. All solutions were kept at -20°C until use. Succinic acid production media composition is shown in Table 3.3.

Table 3. 3 Succinate fermentation medium composition

Final media components	**Per 1000 ml**	**Volume (ml)**
Glucose	60 g	888
Calcium carbonate ($CaCO_3$)	20 g	
Yeast extract	5 g	
Zinc Chloride ($ZnCl_2$)	0.2	10
$NaHPO_4$	0.31	
Yeast nitrogen base (YNB)	6.7	100
Biotin	200 µg	1
Vitamin B12	10 µg	1

3.10.2 Microorganisms

Saccharomyces cerevisiae D2 and *S. cerevisiae* D2, a single mutant strain, were obtained from Ed Louis's Laboratory (Leicester University). *S.cerevisiae* D2S2S2 was manipulated by Dr.Abhishek Somani, the University of Nottingham. *Saccharomyces cerevisiae* BY4741 and its mutant strain were also obtained from Ed Louis's Laboratory (Leicester University).

The microorganisms were cultured in a slant of PDA at 30°C. After that the yeast slant was stored at 4°C for 3-6 months and used as working stock of yeast culture in further experiments.

Pre-inoculum preparation

Pre-inoculum yeast was prepared by the following procedure. 100 µl of cryogenic yeast solution was added into a 250 ml Erlenmeyer flask containing 50 ml of YPD medium. The culture yeast was grown for 48 hours at 30°C, 150 rpm in a shaking incubator.

3.10.3 Cell propagation

The cell propagation media was prepared by adding 100 µl of pre-inoculum yeast into a 250 ml Erlenmeyer flask containing 50 ml of YPD medium. The culture yeast was grown for 48 hours at 30°C, 150 rpm in a shaking incubator. Cell density measurement of propagated yeast was performed by using a WPA Biowave S2100 Diode array spectrophotometer (WPA, UK).

Cell viability was determined using the methylene blue staining method and a haemocytometer (Painting and Kirsop, 1990). Methylene blue solution was prepared by dissolving 0.01 g of methylene blue in 10 ml distilled water. Then 2 g of sodium citrate dehydrate was added. The solution was thoroughly mixed until all compounds were completely dissolved. Distilled water was added to adjust the final volume to 100 ml. The propagated yeast, at an appropriate dilution, was mixed with methylene blue solution in a ratio of 1:1. The cell mixture was incubated at room temperature for 5 minutes. After that cell counting was performed on a haemocytometer. Methylene blue stains dead cells while living cells have an enzyme that decolourised methylene blue. The

percentage of cell viability was calculated by the following equation. Note that propagated yeast need to have at least 95% viability.

$$\text{Percentage viability} = \frac{Live\ cell\ count}{Total\ cell\ count} \times 100 \quad (4)$$

3.10.4 Succinic acid fermentation process (Batch)

Succinic acid fermentation was carried out in 500 ml Erlenmeyer flasks. The flasks contained 50 ml of succinate fermentation media and fermentation was initiated by the addition of an appropriated inoculum volume to get a final optical density of 1. Fermentations were carried out at 30°C, 150 rpm for 72 hours. Samples were kept at -20°C until the time of analysis.

3.10.5 Fed-batch fermentation for succinic acid production

Fermentation was performed in 500 ml Erlenmeyer flasks containing 50 ml of media. All components in the media were as described in succinic acid production media except for the glucose concentration. In fed batch fermentation, the initial media was prepared with 10 g/L of glucose and the fermentation conditions were the same as for batch fermentation. 2 ml of fermentation sample was sampled at regular time intervals. After sample withdrawal, 2 ml of 25% (w/v) glucose was added to achieve a final glucose concentration of 10 g/L and to maintain the same working volume. The fermentation sample was kept in -20°C until the time of analysis.

3.10.6 Fed-batch Succinate fermentation in bioreactor

All components in the media were prepared as described in 3.10.1. Fermentation was performed in 2L bioreactors (Electrolab Biotech Ltd., UK) with 1000 ml working volume. The bioreactor was equipped with a 2L Electrolab bottle, fermentation jacket, stirrer control, gas flow system control

and FerMac 360 pump controller. Electrolab Fermentation Manager Software (version Life-Plus Rev. 1.2 was applied to monitor the condition during fermentation.

Fermentation was initiated by adding an appropriated volume of inoculum as described in 3.10.4. Fermentation conditions were set as 30°C, 1 volume per volume per minute, VVM (min/ml) aeration rate with agitation at 200 revolutions per minute. The pH was controlled at 5 by 1 M sulphuric acid and 1M sodium hydroxide. 12.5 ml of fermentation sample was collected at regular time interval through the sampling port. After the sampling, 12.5 ml of 80% (w/v) glucose solution was added to maintain the level of glucose at 10 g/L. The fermentation sample was centrifuged at 4000 rpm for 5 minutes. The supernatant was then filtered through a 0.45 µm Syringe filter (Sartorius Stedim, UK) and kept in -20°C until the time of analysis.

3.10.7 Fermented sample analysis

Standard preparation for succinic fermentation

Standard solution was prepared as shown in Table 3.4 with 50 ml of distilled water. The solution was mixed until all compounds were completely dissolved then adjusted to a final volume of 100 ml. The standard solution for fermentation sample analysis was kept at -20°C until the time of analysis.

Table 3. 4 The composition of the standard solution for succinic acid fermentation sample analysis

Compounds	**Weight (g)**
Glucose	7.49 g
Succinate	0.5
Glycerol	0.51
Formic acid	0.51
Acetic acid	0.5
Ethanol	5.00

Sample preparation

Fermentation sample was centrifuged and filtered through Whatman GD/X syringe filters (GF/C 25 mm filter diameter 0.2 μm pore size; Whatman International Ltd., Banbury, UK). 1 ml of filtered fermentation sample was transferred to 2ml amber vial (Chromacol Ltd., Herts, UK). All samples and standards were analysed by HPLC.

Jasco HPLC

The fermentation sample was analysed by HPLC. The HPLC system was equipped with Jasco AS-2055 intelligent auto sampler (Jasco, Japan), Jasco PU-1580 intelligent pump, Jasco RI 2031 intelligent refractive index detector and computer controller. The Hi-Plex H column (7.7 × 300mm; Agilent Technologies, Inc., USA) was used and mobile phase was 0.005 N H_2SO_4 with a flow rate of 0.4 ml/min. The injection volume was 10μl and the column temperature was 35°C. The sample analysis was completed in 35 minutes. The chromatogram was processed by Data Azur software (version 4.6.0.0, DATALYS, Azur, France). The chromatogram of standards and retention time of each compound are shown in Figures 3.2 and 3.3.

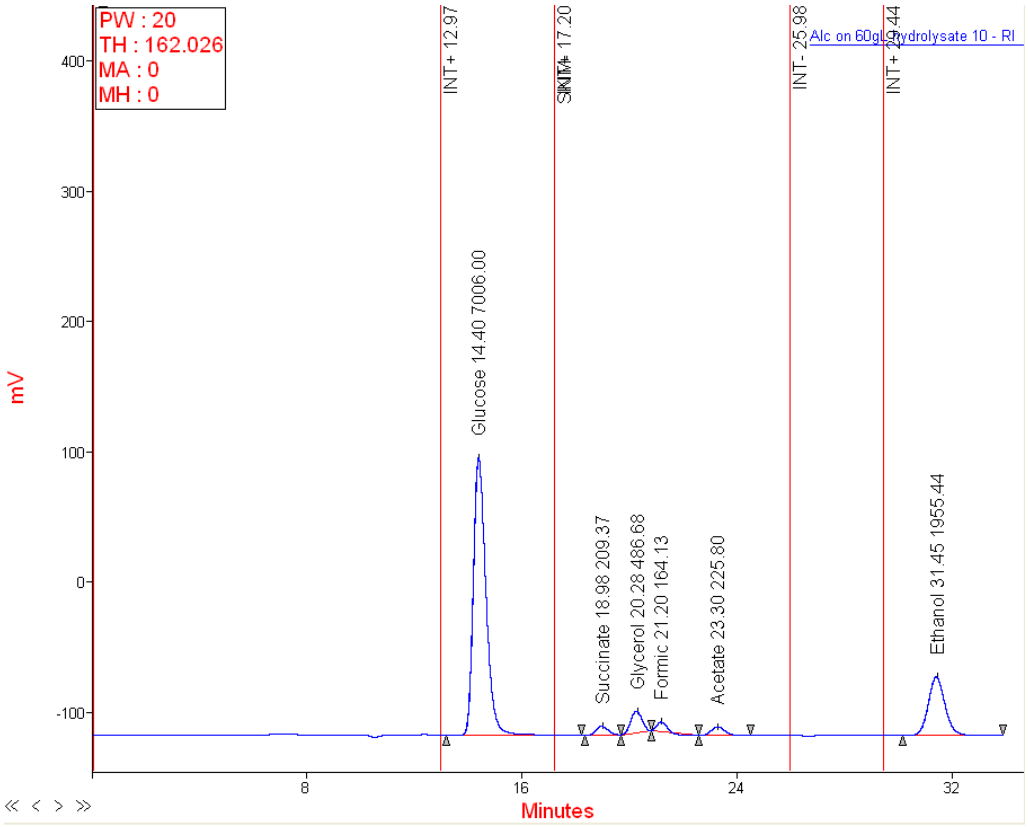


Figure 3. 2 Chromatogram of standard solution detected by Jasco HPLC. Compounds are glucose, succinate, glycerol, formic acid, acetate and ethanol which were detected at retention times of 14.40, 18.98, 20.28, 21.20, 23.30 and 31.45 minutes, respectively.

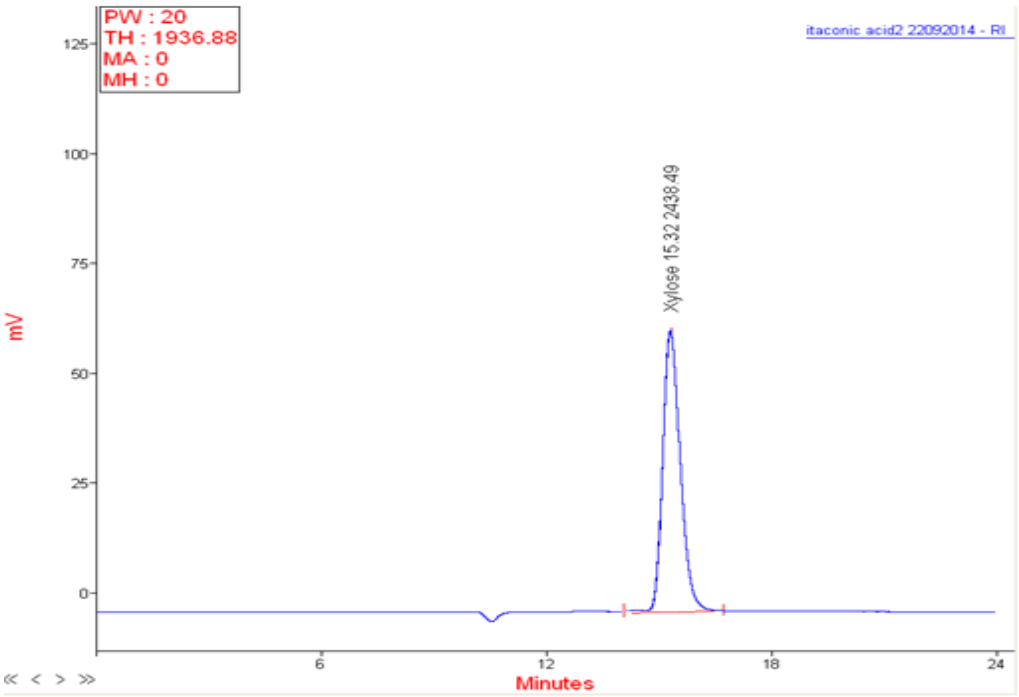


Figure 3. 3 Chromatogram of xylose which was detected by Jasco HPLC at a retention time of 15.32 minutes.

3.11 Alcohol fermentation

The purpose of this experiment was to examine ethanol production from different yeast strains using hydrolysate. The control for this experiment was a synthetic media.

3.11.1 Media

YPD broth was used as a synthetic media in alcohol fermentation. This YPD was prepared as described in 3.10.1, but glucose concentration in media was increased to 60 g/L.

Glucose concentration of the hydrolysate obtained from enzymatic hydrolysis was measured by Dionex. Then the hydrolysate was filter sterilised by using 0.45μm stericup vacuum filter (Millipore, UK). The hydrolysate was stored at 4°C until use.

3.11.2 Microorganisms

Saccharomyces cerevisiae NCYC 2592, *S. cerevisiae* D2, *Scheffersomyces stipitis* CBS 6054 and *Scheffersomyces anomalous* CBS 5759 were obtained from Lallemand Inc. *S.cerivisiae* D2S1S2 was manipulated by Dr. Abhishak Somani, the University of Nottingham. The microorganisms were grown on YPD slants at 30°C for 48 hours. After that the yeast slant was stored at 4°C for 3-6 months and used as a working stock of yeast culture in further experiments.

3.11.3 Pre- inoculum preparation

A single loop of yeast was transferred to a 250 ml Erlenmeyer flask containing 50ml of YPD broth. The culture yeast was incubated at 30°C for 48 hours in a shaking incubator at 150 rpm.

Note that stock culture can be prepared from this step. The stock culture preparation was performed by transferring 0.5 ml of pre-inoculum yeast into a cryogenic preservative vial. Then 50% glycerol was added at an equal volume. This stock culture was stored at -80°C until required.

3.11.4 Cell propagation

100 µl of pre-inoculum yeast was added to a 250 ml Erlenmeyer flask containing 50 ml of YPD medium. The culture yeast was grown for 48 hours at 30°C, 150 rpm in a shaking incubator. After that the culture yeast was transferred to a 50 ml centrifuge bottle and centrifuged (J2-21 Beckman/; Beckman, UK) at 5,000 rpm for 10 minutes. The supernatant was then discarded. The precipitated cells were collected and washed with 50 ml sterilised distilled water. The solution was thoroughly mixed and centrifuged again. This washing step was repeated 3 times. After precipitated cells were free from media, 25 ml of sterilised distilled water was added. This cell solution was used as inoculum, it therefore required cell counting and checking the viability of the cells as described in 3.10.3.

3.11.5 Alcohol fermentation process

Alcohol fermentation was carried out at miniature scale due to limitation of hydrolysate. The fermentation vessel was furnished with a 25 ml glass bottle, magnetic stirrer and the bottle was sealed with a rubber septum and metal crimp. A hypodermic needle was attached to a Bunsen valve to allowed CO_2 release.

Fermentation was started by inoculating 1×10^7 cells/ml into 25 ml of media (YPD broth or hydrolysate). Fermentation conditions were set at 30°C, 300

rpm. The fermentation vessel was weighted at regular intervals and the weight loss recorded. Fermentation samples were collected when fermentation has finished and centrifuged (Eppendrof, UK) at 4000 rpm for 5 minutes. The supernatant was filtered through a 0.45 µm Syring filter (Sartorius Stedim, UK) and kept at -20°C until the time of analysis. Fermentation sample was analysed using the same procedure as described in 3.10.7.

3.12 Phenotypic microarray analysis

Phenotypic microarray analysis was applied to investigate the metabolic profile of different yeast strain on wheat straw hydrolysate. 5 yeast strains were tested on the Phenotype MicroArray (OmniLog® PM) system. The Biolog system has equipped with Biolog Omnilog automatic incubator and reader. Each system contained OmniLog instrument software to determine metabolism profile of the cells.

3.12.1 Cell culture preparation

Saccharomyces cerevisiae NCYC 2592, *S. cerevisiae* D2, *S.cerivisiae* D2S1S2, *Scheffersomyces stipitis* CBS 6054 and *Scheffersomyces anomalous* CBS 5759 were cultured in 5 ml of YPD for 48 hours. Precipitated cells (refer to section 3.11.4.) were added to 20×100 mm test tubes containing 10 ml of sterilised distilled water until they reached 62% transmittance (~5×10^6 cells/ml) on the Biolog turbidimeter (Biolog).

3.12.2 Redox reaction analysis

The redox reaction was performed in a 96 well plate. 0.5 ml of cell solution was mixed with 2.5 ml of IFY buffer™ (Biolog). 90 µl of mixture was added to each well containing 30µl of media (6.0% glucose solution, 0.67% Yeast

nitrogen base, 0.2µl of tetrazolium dye D (Biolog) and the final volume adjusted to 30µl with sterilised distilled water). After that, the plate was put into the Biolog Omnilog incubator and incubated for 96 hours at 30°C. During the reaction, if the cells metabolise the sugar and NADH is produced. The amount of purple redox dye formed corresponds to the amount of NADH generated. The OmiLog instrument captures images of the developing colour on each well every 15 minutes. The colour density was converted to a signal value. The signal value was then converted in to Microsoft Excel using biology software.

3.13 Itaconic fermentation

3.13.1 Media

A semi-synthetic media for itaconic acid production was prepared by the protocol proposed by (Kuenz *et al*., 2012). The components of the fermentation media are shown in Table 3.5. All compounds were dissolved in 800 ml of distilled water. The semi-synthetic media was adjusted to pH 3.1 by 1 M sulphuric acid and the final volume was topped up to 1000 ml. Then the media was sterilised at 121°C for 15 minutes.

Table 3. 5 The components in the semi-synthetic medium for itaconic fermentation.

Compound	**Weight (g)**
Glucose	60
Magnesium sulphate ($MgSO_4.7H_2O$)	1.0
Copper sulphate ($CuSO_4.5H_2O$)	0.015
Potassium dihydrogen phosphate (KH_2PO_4)	0.1
Ammonium nitrate (NH_4NO_3)	3.0
Calcium chloride ($CaCl_2$)	5.0
Iron (III) chloride hexahydrate ($FeCl_3.6H_2O$)	0.00167
Zinc sulphate heptahydrate ($ZnSO_4.7H_2O$)	0.008
Total volume	1000 ml

3.13.2 Microorganism

Aspergillus terreus DSM 826 was obtained from DSMZ. The fungal was cultured in 90 mm-size PDA plate at 35°C for 5 days.

3.13.3 Pre-inoculum preparation

An isolated colony was sampled onto a PDA plate and incubated at 35°C for 72 hours. After 72 hours of incubation, the fungal culture had sporulated and the culture plate was used for preparation of a spore suspension (refer to section 3.4.3)

3.13.4 Itaconic production

The itaconic acid production was carried out in 500 ml Erlenmeyer flasks containing 50 ml of fermentation media. Fermentations were initiated by inoculating spores at a concentration of 1×10^6 spore/ml. Fermentation conditions were set at 33°C and 200 rpm. Fermentation samples were collected every 24 hours for 144 hours. The collected samples were centrifuged. The supernatant was filtered through a 0.2 μm syringe filter (Whatman International Ltd, UK). Filtrate was kept at -20°C until the time of analysis.

3.13.5 Fermented sample analysis

Standard preparation for itaconic acid fermentation

Standard solution was prepared by dissolving all the compound in Table 3.6 in 50 ml of distilled water. The solution was mixed until all compounds were completely dissolved then adjusted to a final volume of 100 ml with water. Standard solution was kept in -20°C until the time of analysis.

Table 3. 6 The composition in the standard solution for itaconic fermentation sample analysis

Compounds	Weight (g)
Oxalic acid	0.63
Glucose	8.58
Xylose	3.98
Succinic acid	0.99
Itaconic acid	6.19

Sample preparation

Fermentation samples were centrifuged and filtered through Whatman GD/X syringe filters (GF/C 25 mm filter diameter 0.2 μm pore size; Whatman International Ltd., Banbury, UK). 1 ml of filtered fermentation sample was transferred to a 2ml amber vial (Chromacol Ltd., Herts, UK). All samples and standard were analysed by HPLC

Jasco HPLC

The fermentation sample was analysed by HPLC. The HPLC system has equipped with Jasco AS-2055 intelligent auto sampler (Jasco, Japan), Jasco PU-1580 intelligent pump, Jasco RI 2031 intelligent refractive index detector and computer controller. The Hi-Plex H column (7.7 × 300mm; Agilent Technologies, Inc., USA) was used and mobile phase was 0.005 N H_2SO_4 with a flow rate of 0.4 ml/min. The injection volume was 10μl and the column temperature was 35°C. The sample analysis was completed in 24 minutes. The chromatogram was processed by Data Azur software (version 4.6.0.0, DATALYS, Azur, France). The chromatogram of itaconic acid and retention is shown in Figure 3.4.

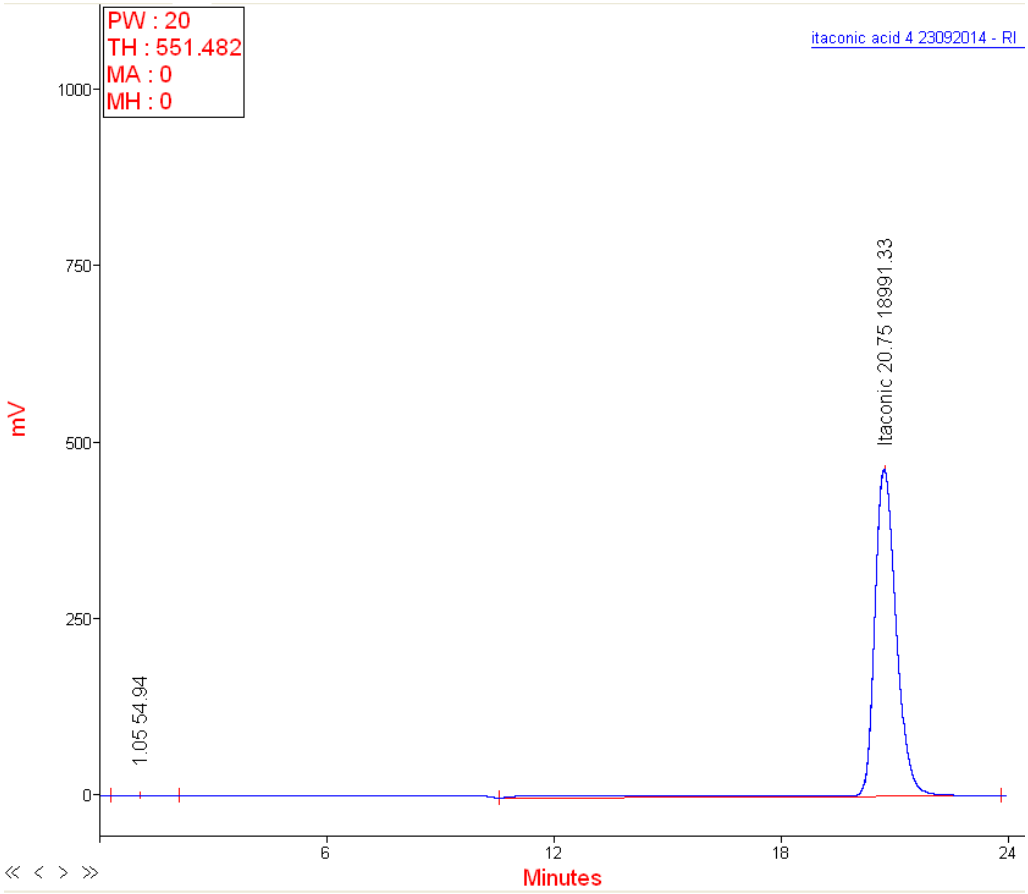


Figure 3. 4 Chromatogram of itaconic acid as detected by Jasco HPLC at retention time of 20.75 minutes.

3.14 Statistical analysis

Note that all experiment in this thesis have been carried out at least in triplicates; therefore the data values reported are the mean values with standard deviations. The statistical analysis was carried out using Excel (Microsoft, USA) and STAT GRAPHICS (StatPoint Technologies, USA).

Chapter 4 Biological Pre-treatment of Wheat Straw

Biological pretreatment is usually operated under milder conditions than physical and chemical pretreatment processes, therefore it would in theory generate fewer inhibitor compounds. However, due to the long retention time of the process, this makes it unfavourable compared with the other methods. Therefore the development of a new biological pretreatment process that reduces the retention time could make industrial implementation more practical.

In this chapter, a study of biological pretreatment has been carried out on wheat straw. Characterisation of the biological pretreatment (fermented) wheat straw was investigated, which was then compared with the raw wheat straw. The aims were to study the feasibility of using fungal strains to carry out biological pretreatment on wheat straw using solid state fungal fermentation; and to explain influences of solid state fermentation on wheat straw structure. All experiments shown here were performed at least in triplicate.

In order to utilise wheat straw for ethanol production; it is necessary to understand the properties of the biomass, which are namely chemical composition and physical properties. These will be explained in detail with chemical composition analysis, x-ray diffraction analysis and scanning electron microscope (SEM) images in the following sections.

4.1 Cell wall compositions of wheat straw

The chemical composition of raw wheat straw influences greatly the conversion of biomass into fermentable sugars. In this study, wheat straw was knife milled (Fritsch, Idar-Oberstein, Germany) and screened to a particle size of 2 mm. The chemical composition of milled wheat straw was analysed and presented in Table 4.1. Carbohydrates and lignin, were analysed by the methods described in (Saeman, 1954) and (Lacerda et al., 2006), respectively.

Table 4. 1 Carbohydrates and lignin contents (wt%dry basis) in wheat straw

Compositions	Content (%)
Lignin	20.250 ± 0.055
Carbohydrates	
Arabinose (g/g)	0.024 ± 0.000
Galactose (g/g)	0.008 ± 0.000
Glucose (g/g)	0.352 ± 0.004
Xylose (g/g)	0.203 ± 0.003
Cellulose	31.676 ± 0.386
Hemicellulose	17.862 ± 0.283
Other (crude protein, ash and silica)	30.200 ±0.636

Note: the moisture content of dry wheat straw was 9.98±0.02% and same sample was used for chemical composition analysis.

There are numerous studies analysing wheat straw composition (Chang *et al*., 2012, Huang *et al*., 2009, Satyanarayana and Johri, 2005, Yang *et al*., 2008, Hansen *et al*., 2011). The results show that the chemical compositions of wheat straw can vary depending on their genetic variations, geological variations in planting and storage site, harvesting method, cultivation period and even the analytical method used. Collins *et al*. (2014) have investigated the variation in the chemical compounds of 6 wheat straw varieties; *Avalon, Cadenza, Charger, Paragon, Robigus* and *Savannah* under the same cultivation condition. The result also shows that each variety has its unique morphology and chemical composition. Not only that, since the straw consists of 3 parts, which are (i) the leaves (ii) the stem and (iii) the nodes, even within the same

sample the composition analysis could show different chemical composition results depending on which of the wheat component are being sampled.

Zhang *et al*. (2012) have also reported a similar story in that different varieties of wheat straw grown under the same conditions also have different physical properties. Moreover even the same variety cultivated in different places, can show different physical properties. Their results indicate that variations in climatic conditions, nutrients in cultivation area, variety of wheat also have an influence on physical properties of wheat straw in addition to the other factors discussed earlier.

Table 4.2 compares the results obtained in this study with the data reported in the literature. Generally, cellulose content is in the range of 30% to 50%, hemicellulose content is in the range of 16% to 49% and lignin content of wheat straw varies from 8% to 25%. As shown in Table 4.2, the results do agree well with the literature.

Table 4.2 Chemical compositions of the wheat straw used in this study compared with values reported in literature.

Substrate	Originated	Cultivation period	Cellulose	Hemicellulose	Lignin	Reference
Wheat straw	Italy	n.a	36.0	49.0	15.0	Curreli *et al.* (1997)
Riband Wheat straw	Llangefni, UK	n.a	39.0	38.9	17.0	Sun *et al.* (2004)
Milled wheat straw	USA	n.a	48.5 ± 0.3	27.7 ± 0.1	8.2 ± 0.9	Saha *et al.* (2005)
Wheat straw	Hubei, China	n.a	41.2 ± 0.5	25.8 ± 0.5	21.3 ± 0.4	Zhu *et al.* (2006b)
Wheat straw	Sweden	n.a	39.8	n.a	22.6	Kristensen *et al.* (2008)
Wheat straw	Beijing, China	n.a	38.7	33.3	23.3	Chen *et al.*, (2008)
Wheat straw	Netherlands	September 2006	32.6	16.7	25.5	Kootstra *et al.* (2009)
Wheat straw	Denmark	n.a	35.1	n.a	18.9	Kaparaju and Felby, (2010)
Apache wheat straw	Pomacle, France	n.a	37.1 ± 0.2	23.0 ± 0.5	19.1 ± 0.1	Rémond *et al.* (2010)
Wheat straw	Denmark	n.a	42.1	20.6	21.6	Hansen *et al.* (2011)
Wheat straw	Spain	n.a	31.1±1.0	18.9±0.7	15.1 ± 0.1	Huijgen *et al.* (2012)
Wheat straw	Estonian	August 2011	46.5	31.0	7.9	Tutt *et al.* (2012)
Wheat straw	Denmark	Summer 2008	36.2	19.6	18.6	Petrik *et al.* (2013a)
Cordial wheat straw	Sutton bonington, UK		31.7 ± 0.4	17.9 ± 0.3	20.2±0.1	This study

4.2 Physical properties of wheat straw

4.2.1 Morphology and crystallinity

Not only chemical compositions of wheat straw has an effect on the release of sugar from biomass, but also the physical properties; for example, moisture content, particle size and cellulose crystallinity of the biomass. These properties also affect the efficiency of enzymatic hydrolysis; especially in the crystalline regions, where the cellulose may be tightly bound by hemicellulose and lignin, which results in low accessibility of cellulolytic enzymes to the cellulose polymer. A dense crystalline cellulose structure can lead to a low saccharification rate (Arantes and Saddler, 2010).

In this section, a scanning electron microscope (SEM) was used as an imaging tool to study the surface morphology of the wheat straw samples including the untreated wheat straw (milled wheat straw not being treated by any physiochemical or biological treatment); the SEM image of the untreated wheat straw is shown in Figure 4.1. Note that in order to make a clear comparison between the morphology of the untreated wheat straw and the biological treated wheat straw, the SEM images of the treated wheat straw were also taken and explained in detail later in section 4.3.3.

Figure 4.1 shows that the morphology of the untreated wheat straw was tightly packed making a strong and sturdy structure. This form of structure leads to a low hydrolysis yield because it has a very limited surface area for enzyme to access.

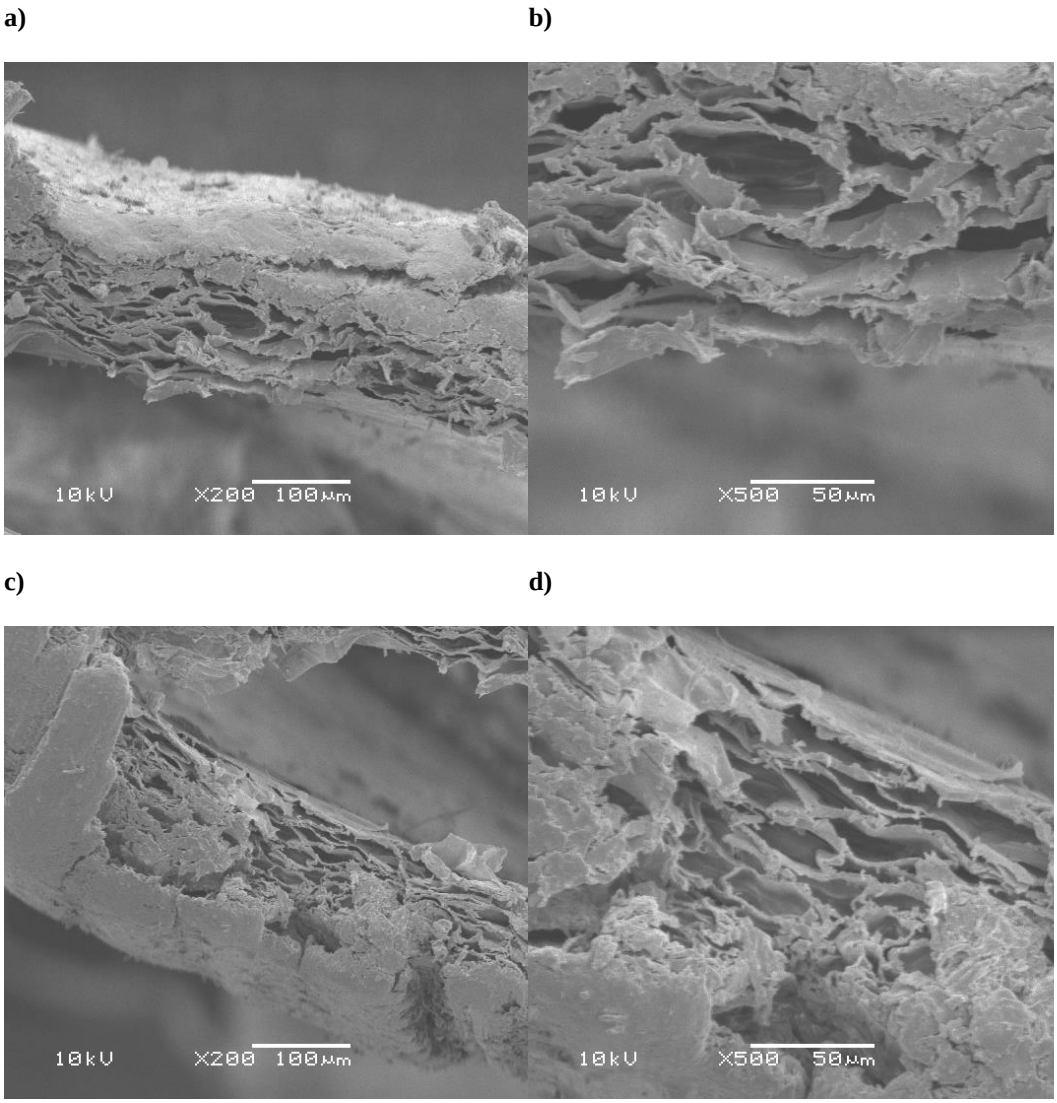


Figure 4. 1 Scanning electron microscopic images of untreated wheat straw taken with 10kV electron potential and 200 times 500 times magnifications.

The crystalline cellulose of the untreated wheat straw was then analysed by X-ray diffraction (XRD) and the result is shown in Figure 4.2.

Figure 4.2 shows that the XRD profile (shown in red) of untreated wheat straw. The experimental data were then fitted with Gaussian deconvolution interpolation as explained in Ibbett *et al*. (2013a).

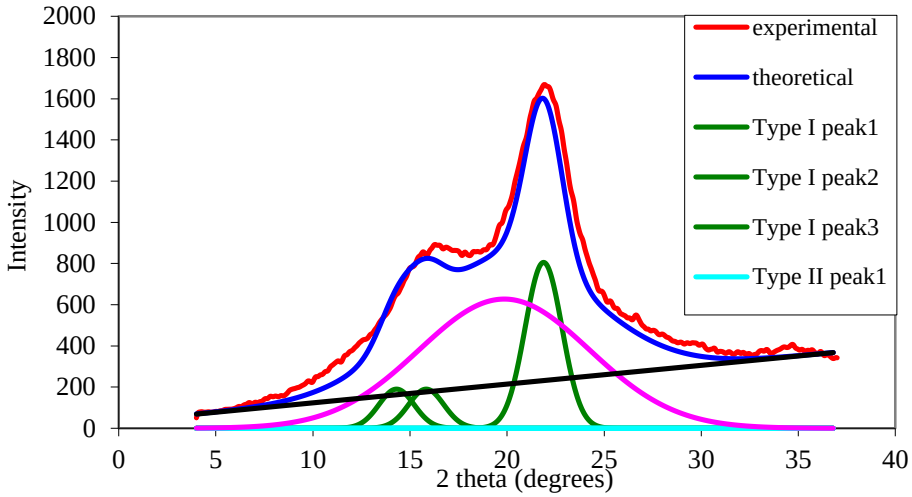


Figure 4.2 X-ray diffraction result of the untreated wheat straw. The red line shows the experimental result; the blue line shows the curve fitted data computed where the curve can be treated as a combination of 4 sub peaks shown in green and pink using peak deconvolution method.

The result shows 4 sub-peaks within the XRD pattern at 20 degrees, 14.14 degrees, 16.21 degrees and 22.35 degrees; these peaks are similar to the values reported by a number of other studies (Park *et al*., 2010, He *et al*., 2008, Garvey *et al*., 2005). These peaks correspond to the crystal planes 101 (14.14 degrees in figure 4.2), 10ī (16.21 degrees in Figure 4.2), 002 (22.35 degrees in figure 4.2) and 040 (20 degrees in Figure 4.2). Researchers believe that the XRD curves should in fact show more than 4 crystallinity planes because of the complexity of the cellulose structure expecting to see a large number of narrower XRD peaks rather than 4 boarder ones, they however believe that since there is also some amorphous crystal structure of the cellulose this makes it very difficult to distinguish between each of the crystal planes and each of the narrow peaks are superimposed on each other forming four to five distinguishable board peaks.

To simplify this, the amount of crystalline cellulose was then calculated as a ratio between biomass fraction and the crystallinity results from XRD as described by Ibbett *et al*. (2013b). The untreated wheat straw used in this study had a crystallinity index of 26.50% of dry biomass.

4.2.2 Impact of moisture content and particle size

Moisture content and particle size may affect the cellulosic hydrolysis rate as well. In the hydrolysis process, some cellulase proteins are adsorbed to the surface of the wheat straw, making them unavailable for the hydrolysis (Yu *et al*., 2009). As some of the adsorption is irreversible, these enzymes lose their activity. It is important to determine these properties of the wheat straw used in this study; (i) the initial moisture content and (ii) particle size. The initial moisture content was determined and the particle size distribution of untreated (milled) wheat straw was analysed as described earlier in chapter 3.3. The result shows that the moisture content in untreated wheat straw was 9.987±0.023% and the particle size distribution is shown in Figure 4.3.

According to the particle size distribution analysis, 70.68% by weight of the milled wheat straw had a particle size within the range of 2 mm - 425 μm. The wheat straw particle size distribution was within the range of 250-425 μm for 14.51% by weight, 106-250 μm for 7.94% by weight and 75-106 μm for 3.77% by weight, respectively. The amount of particles smaller than 75 μm was only 2.43%. The wheat straw particle sizes were statistically tested with t-test to show that they were statistically different ($P<0.05$). The majority of the wheat straw was bigger than 0.425 mm which was similar to the size of 0.64 mm for ground wheat straw reported by Mani *et al*. (2004).

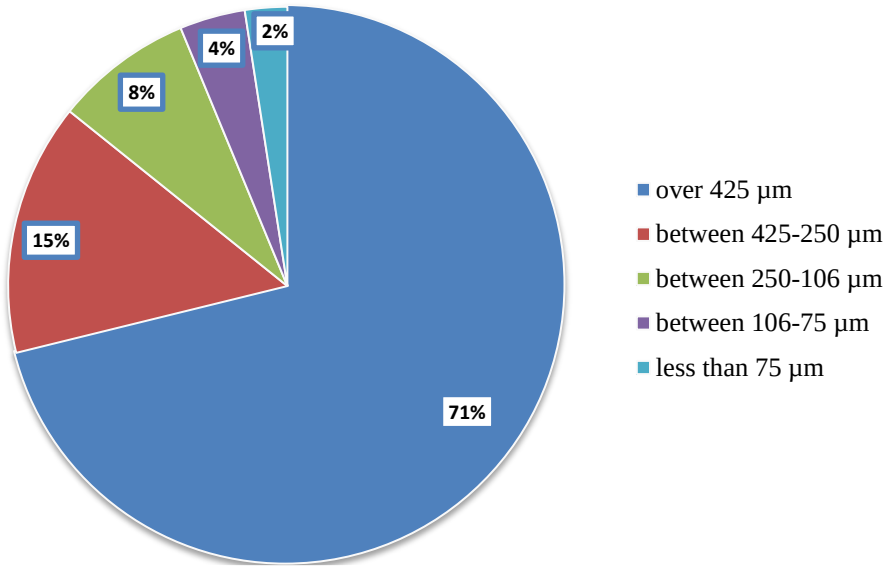


Figure 4. 3 Particle size distribution of milled untreated wheat straw

4.3 Characterization of biologically pretreated wheat straw

Solid state fermentation was carried out in this study as a means of "biological pretreatment". In this initial screening experiment, two fungal strains (*Aspergillus nig*er and *Trichoderma reesei*) were investigated to see whether they were effective strains for biological pretreatment processes. These fungi were selected because of their known high cellulase production (Mrudula and Murugammal, 2011). The chemical composition and structural changes occurring after the pretreatment and the enzyme production arising from the fungal fermentation were also determined. The efficacy of pretreatment was evaluated by sugar release in enzymatic hydrolysis.

4.3.1 Fungal growth

The experiment commenced by culturing fungal strain in solid state fermentation (refer to 3.4). Figures 4.4 and 4.5 show microscopic images of the fungi, it can be clearly seen that the hypha grew on both inside and outside of the wheat straw as indicated by red circles in the two figures; especially in

figure 4.5 *T. reesei* formed conidia after 24 hours of fermentation. This implied that these two fungi grew well under a moisture content of 89.5%, 28°C and static condition.

(a) **(b)**

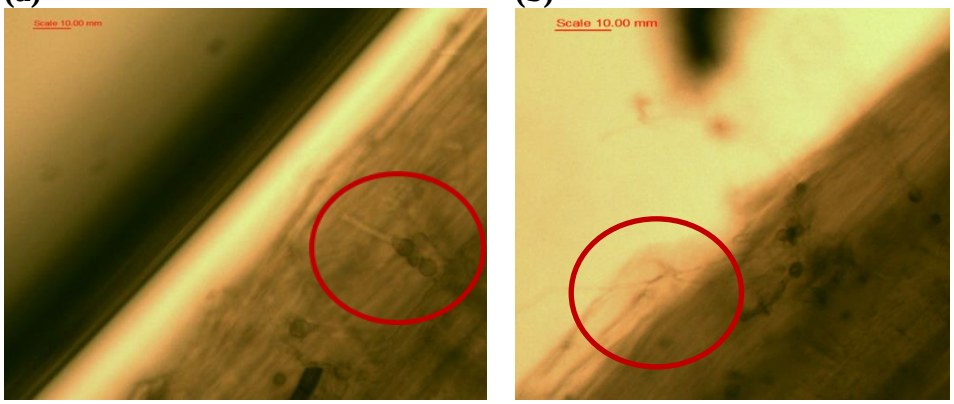


Figure 4.4 Microscopic images of *A. niger* growth on solid state fermentation

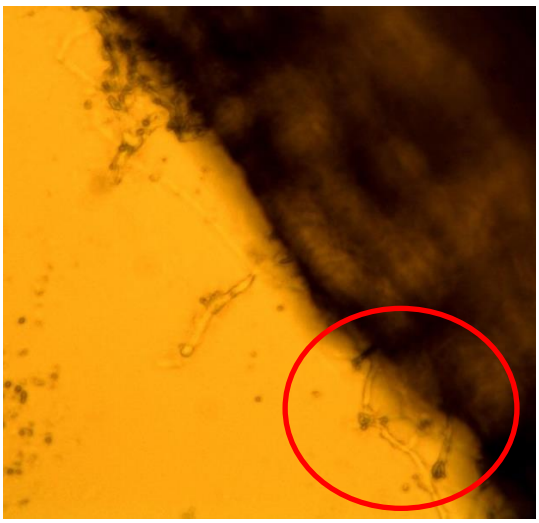
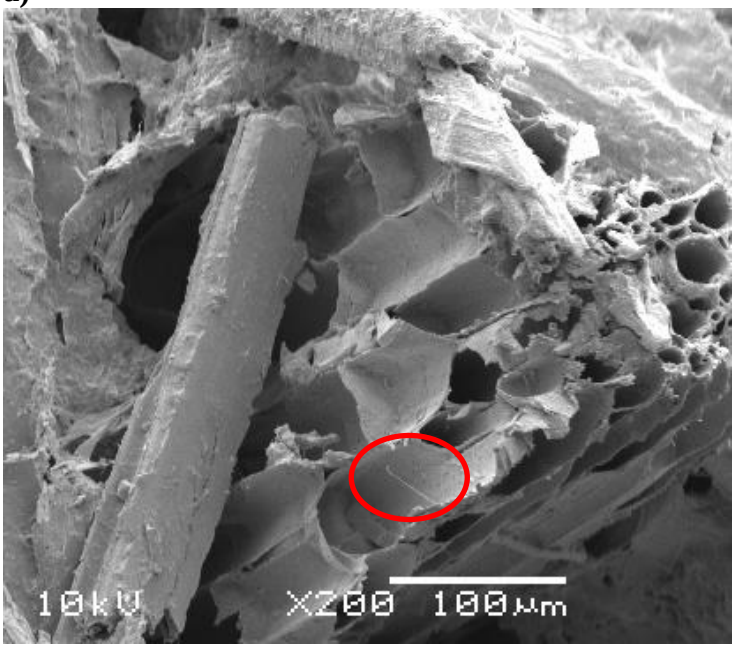


Figure 4. 5 Microscopic images of *T. reesei* growth on solid state fermentation

Figure 4.6 shows that mycelium growth and sporulation of *A. niger* can be observed on wheat straw. The growth of *A. niger* mycelium on wheat straw particle was non-uniform. It developed not only on the surface, but also accessed inside the wheat straw tissues by day 1 as seen by scanning electron microscopy. The mycelium penetrated throughout the wheat straw particle by day 3. The spore formation started after 24 hours of fermentation and the

number of spores increased over the culture period. The figure also shows that the wheat straw structure was disrupted and partially broken down during *A. niger* fungal fermentation.

a)



b)

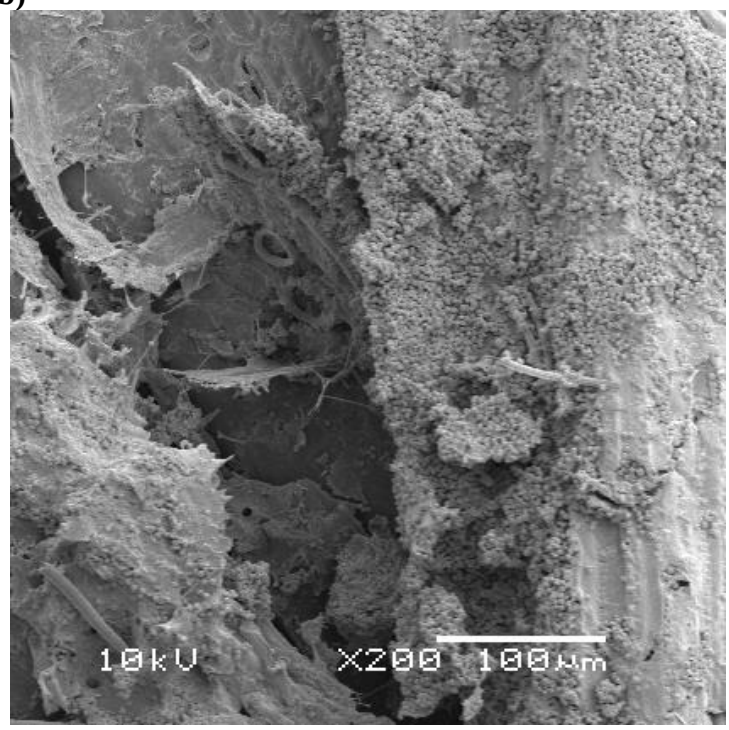


Figure 4. 6 Scanning electron microscopic images of wheat straw pretreated with *A. niger* for 1 day (a) and b) 3 days. Fungal fermentation condition was carried out at 28˚C under static condition.

Similarly for the *T. reesei* treated wheat straw, *T. reesei* hyphae colonized the wheat straw structure within 24 hours of fermentation as shown in Figure 4.7. The mycelia also increased with fermentation time.

a) **b)**

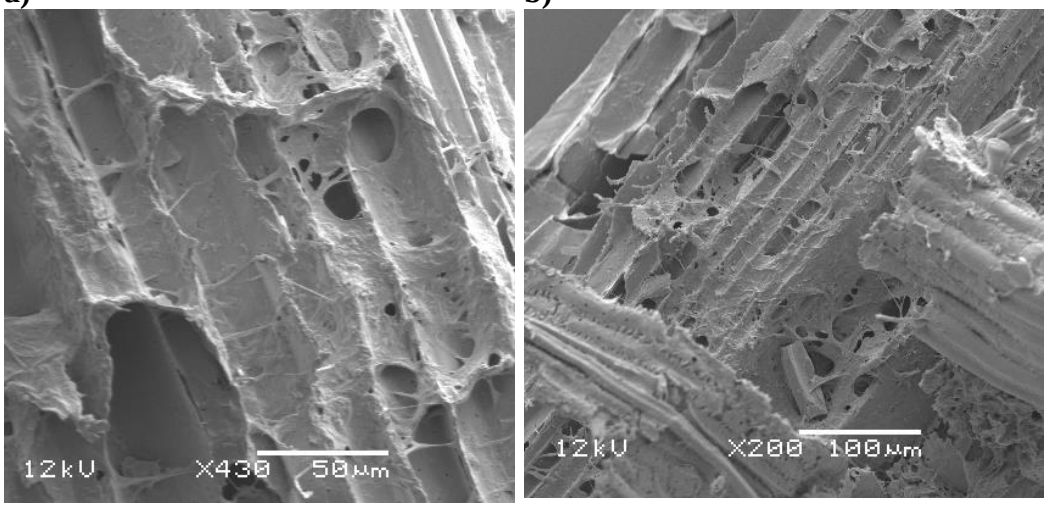


Figure 4. 7 Scanning electron microscopic images of wheat straw pretreated with *T. reesei*. a) *T. reesei* for 1 day and b) 3 days

4.3.2 Effect of solid state fermentation on wheat straw composition

In this section, the change in the chemical composition and physical properties of these fungal treated wheat straws were examined and the rate of enzymatic hydrolysis using fermented wheat straw was also measured.

The total weight loss of wheat straw during solid-state fermentation cannot be explicitly determined because the increase of fungal biomass may interfere with the accuracy of weight loss measurement. The lignin degradation was not monitored due to the fact that these two fungi are not known to be lignin degrading microorganisms (Levasseur *et al*., 2010, Hamed, 2013).

Table 4. 3 Cellulose and hemicellulose loss of fungal treated wheat straw

Sample	Cellulose loss (%)	Hemicellulose loss (%)
A. niger treated wheat straw	4.498±0.134	8.913±0.037
T. reesei treated wheat straw	2.067±0.114	6.071±0.529

Note that the loss was calculated by comparing the cellulose or hemicellulose component in the fungal treated wheat straw with autoclaved untreated wheat straw.

The chemical composition changes of wheat straw during the fungal fermentation are shown in Table 4.3. From the table, it can be seen that both cellulose and hemicellulose were degraded during the fermentation with both types of fungi. During the fungal growth, *A. niger* and *T. reesei* produce lignocellulosic enzymes, which hydrolyse carbohydrate polymers in wheat straw converting them into soluble sugar monomers. The released sugar monomers are then consumed by the fungi. Previous research on lignocellulosic biomass has demonstrated carbohydrate losses during pretreatment process, especially when long fermentation periods are carried out (Lee *et al*., 2007, Ray *et al*., 2010, Monrroy *et al*., 2011, Saritha and Arora, 2012).

4.3.3 Effect of solid state fermentation on physical properties of wheat straw

The XRD analysis was performed to study the crystallinity of wheat straw before and after solid-state fermentation. Figure 4.8 shows the XRD profiles of the untreated wheat straw in (a), autoclaved wheat straw (b), *T. reesei* treated wheat straw (c), and *A. niger* treated wheat straw (d). The crystallinity of the raw wheat straw, autoclaved wheat straw, *T. reesei* treated wheat straw and *A. niger* treated wheat straw were 28.5%, 24.64%, 21.07% and 19.63%, respectively as shown in Table 4.4.

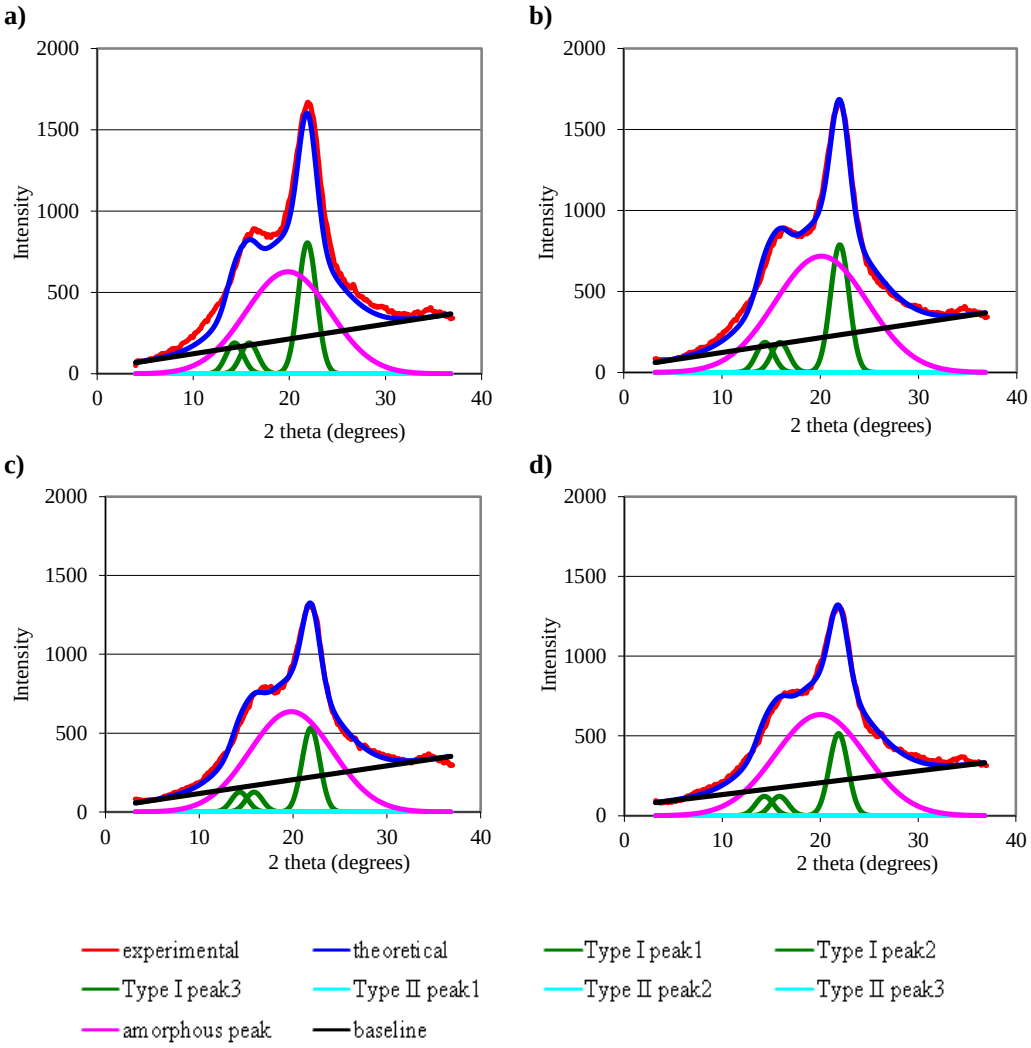


Figure 4. 8 XRD profile of untreated wheat straw and pretreated wheat straw. a) XRD profile of untreated wheat straw, b) autoclaved wheat straw, c) *T. reesei* treated wheat straw and d) *A. niger* treated wheat straw.

This indicates that sterilisation using the autoclave has partially broken down the crystalline structure in wheat straw. Furthermore, both *T. reesei* and *A. niger* altered the crystalline structure of wheat straw during solid state fermentation. This might be because the fungal hyphae penetrated the crystalline structure. This leads to reduction of crystallinity in wheat straw structure and might improve the subsequent enzymatic hydrolysis rate.

Table 4. 4 Crystallinity of wheat straw before and after solid state fermentation

Sample	Crystallinity (%)
Untreated wheat straw*	28.502±0.271
Autoclaved wheat straw	24.644±0.472
T. reesei treated wheat straw	21.069±0.550
A. niger treated wheat straw	19.630±0.071

Note: this data are mean values ± standard deviation of three replicates. * The crystallinity of untreated wheat straw analysed by method proposed in Ibbett *et al* (2013) was 26.50%.

4.3.4 Cellulase enzyme production on wheat straw by A. niger

Cellulase production

The cellulase production by *A. niger* during solid state fungal fermentation of wheat straw was investigated. The solid state fermentation was carried out for 7 days. Cellulase activity, protein content and glucosamine concentration were measured. Glucosamine content is generally used as an indicator for cell growth in fungal fermentation experiments. As shown in Figure 4.9, glucosamine content increased consistently during the 7 days of culture. Cellulase enzyme production reached the highest activity of 5.342±0.626 FPU/g on the third day of fermentation and then decreased afterward. The protein production had a similar trend to cellulase activity (see Figure 4.9). Couri *et al.* (2000) have also reported a similar reduction of cellulase and protein concentration after 3 days. They believe that this was due to the formation of protease enzymes, which hydrolysed cellulase and other proteins and led to the reduction of protein level and cellulase activity.

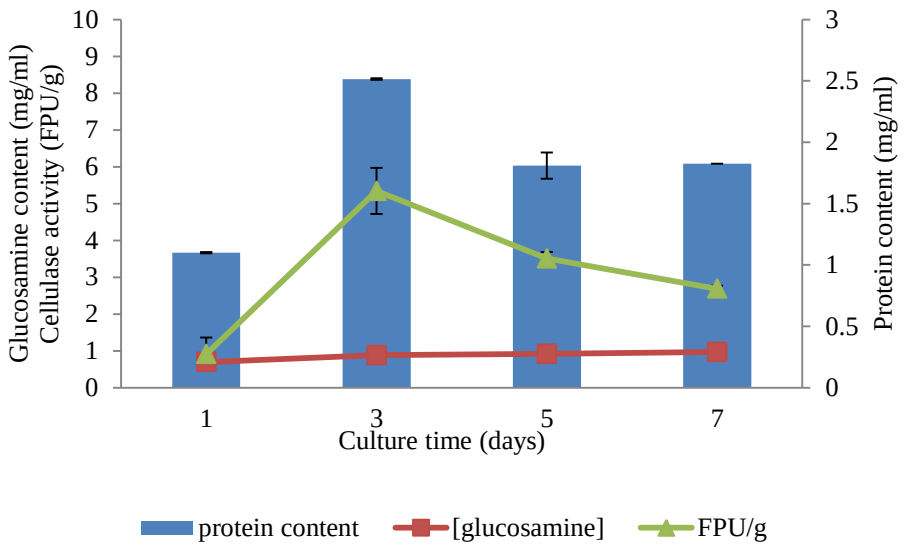


Figure 4. 9 The time courses for fungal growth (glucosamine), protein content in the fungal filtrate and cellulase production during solid state fermentation of *A. niger* on wheat straw. Solid state fermentation was supplemented by 0.24 g of starch and it was carried out at initial moisture content of 89.5%, 28˚C for 7 days. Data are mean values of three replicates and error bars indicate standard deviations (SDs)

Extract method

Comparing with other similar research on cellulase production using agriculture waste substrates (Bansal *et al*., 2012, Kang *et al*., 2004), the cellulase activity detected in this experiment was relatively low. It was suspected that the enzymes were produced but they were somehow trapped inside the biomass matrix. Therefore several methods were investigated in order to improve enzyme extraction efficacy and as shown later it was found that cellulase production can be improved by applying the optimal conditions.

Effect of different extraction solutions

Four different extraction solutions, including (i) distilled water, (ii) 0.05 M sodium citrate buffer at pH 4.8, (iii) 0.05 M sodium citrate buffer at pH 4.8 with 1% tween 80 and (iv) 0.1 M sodium sulphate were applied as extraction solutions for enzyme extraction. The solid state fermentation of wheat straw

was carried out using *A. niger* under moisture content of 89.5%, 28˚C and static condition for 5 days.

The result shows that the crude enzyme shows a statistically significant difference in cellulase activity at the 95.0% confidence level in different extracts (see Table 4.5, 4.6 and Figure 4.10). The 0.05 M sodium citrate buffer (pH 4.8) shows a better result in term of cellulase recovery. The cellulase activity increased by 68.82% compared to enzyme extraction using only water. The addition of tween 80 into the sodium citrate buffer extracted 80.58% protein from the solid state fungal fermentation (Table 4.5); however, the cellulase activity in the fungal extraction was reduced.

Table 4.5 The effect of extraction solutions on cellulase activity.

Extraction solution	**Cellulase activity (FPU/ml)**	**Cellulase activity (FPU/g)**	**Protein (mg/ml)**
Water	0.180±0.025	3.002± 0.413	1.082±0.016
Sodium Citrate Buffer	0.304±0.070	5.068± 1.175	1.215±0.195
Sodium Citrate Buffer with tween 80	0.165±0.044	2.749± 0.730	2.194±0.265
Sodium sulphate	0.226±0.027	3.761± 0.450	1.096±0.006

Note: Cellulase production by *A. niger* grown in solid state fermentation at 28˚C for 3 days. Data are mean values ± standard deviation of four replicates.

Table 4. 6 ANOVA table for the effect of extraction solution on cellulase activity experiment

Source	***Sum of Squares***	***Df***	***Mean Square***	***F-Ratio***	***P-Value***
Between groups	0.0390723	3	0.0130241	7.26	0.0059
Within groups	0.0197281	11	0.00179346		
Total (Corr.)	0.0588004	14			

Note: Degree of freedom (Df)

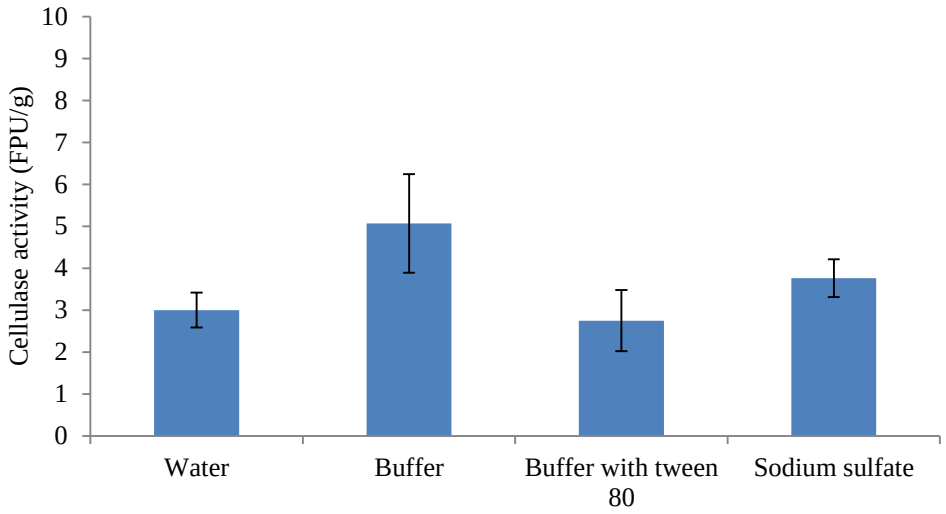


Figure 4.10 The effect of extraction buffer on cellulase activity.
Note:Cellulase production by *A. niger* grown in solid state fermentation at 28˚C for 3 days. Data are mean values ± standard deviation of four replicates and error bars indicate standard deviations (SDs)

Effect of blending before enzyme extraction

An additional blending step before the enzyme extraction was investigated in order to improve the extraction efficacy. This has been done to address the hypothesis that the cellulase enzyme was trapped in the wheat straw matrix. In this experiment, 10 second blending using a commercial fruit blender (Waring commercial blender BN974, USA) was employed to open the wheat straw.

The result shows that after applying a blending step for just 10 seconds, the cellulase activity reached 9.51 U/g at day 3 of fermentation (Table 4.7). This was 71% higher than that obtained using the enzyme extraction method without a blending step (5.57 U/g). This clearly indicates that some of the cellulase enzyme was trapped in the wheat straw matrix. The effect of the extraction method on enzyme recovery was also investigated by Dhillon *et al*. (2012), they used three different shaking conditions, (i) wrist action shaker, (ii) incubation shaker and (iii) vortex shaker. The three methods showed

different enzyme extraction levels. This confirms that the amount of enzyme recovered could be affected by the extraction method.

Table 4.7 The effect of blending on cellulase activity (FPU/g) from *A.niger*.

Time (day)	Without blending step	With blending step
1	1.105±0.001	4.330±0.381
3	5.573±0.073	9.515±1.644
5	4.520±0.126	5.552±0.539

Note: Cellulase production by *A. niger* in solid state fermentation of wheat straw with 0.24 g of starch as supplement. Solid state fermentation was carried out at 28˚C for 5 days. Sodium citrate buffer pH 4.8 was used as extraction buffer in enzyme extraction. Data are mean values ± standard deviation of four replicates.

4.3.5 Cellulase production on wheat straw by T. reesei

Unlike *A. niger*, the glucosamine content from *T. reesei* did not increase over the culture time. The cellulase activity from *T. reesei* reached 5.573±0.624 FPU/g (0.334±0.037 FPU/ml) on the third day of fermentation. It then decreased slightly in day 5 (see Figure 4.11). However, the change was not statistically different at the 95.0% confidence level (Table 4.8). This result is similar to the results reported by Esterbauer *et al*. (1991), where they show that cellulase production from 1% cellulose and 2% grain husks by a *T. reesei* mutant reached the highest cellulase activity after 72 hours of fermentation. The protein content of *T. reesei* increased gradually along with the cultivation (Figure 4.11).

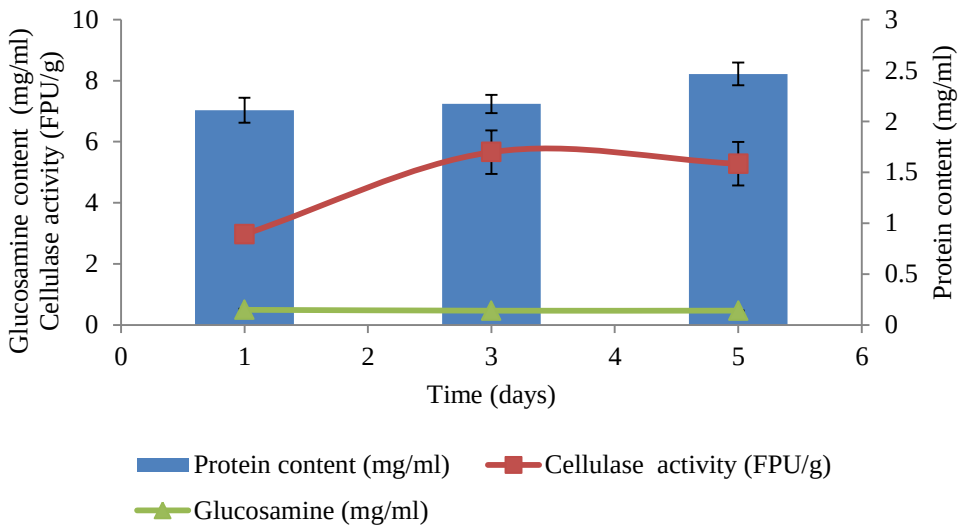


Figure 4. 11 The time courses for fungal growth (glucosamine), protein content in the fungal filtrate and cellulase production during solid state fermentation of *T. reesei* on wheat straw. Solid state fermentation of wheat straw was supplemented by 0.24 g of starch and it was carried out at initial moisture content of 89.5%, 28˚C for 5 days. The blending process was applied before the enzyme extraction process. Data are mean values of three replicates and error bars indicate standard deviations (SDs)

Table 4.8 ANOVA table of cellulase production form wheat straw by *T.reesei.*

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	13.9488	2	6.97441	19.82	0.0023
Within groups	2.11098	6	0.35183		
Total (Corr.)	16.0598	8			

Note: Degree of freedom (Df)

4.3.6 Cellulase activity of co-culture of A. niger and T. reesei

The cellulase activity obtained from solid state fermentation of *T. reesei* in this study was relative low compared with other reports (Persson *et al*., 1991, Sukumaran *et al*., 2009). Chahal (1985) investigated cellulase production by *T. reesei* QMY-1 on wheat straw and achieved a cellulase activity of 6.7 IU/ml after 14 days of fermentation. Mekala *et al*. (2008) also reported a higher cellulase activity (25.6 FPU/g) than this study. (Sohail et al., 2009) reported that there are many factors affecting the cellulase activity, for example, the microorganism used, medium composition, temperature, pH, moisture content

and incubation time. Moreover the level of the individual enzymes - cellulase (endo-glucanase, exo-glucanase and β-glucosidase) in fungal filtrate also influences the total cellulase activity. *A. niger* has been studied for cellulase production using many types of feedstock. This strain produces higher amounts of β-glucosidases and endo-glucanases. On the other hand, it does not produce a sufficient amount of exo-glucanase, which plays an important role in cellulose hydrolysis (Hanif *et al*., 2004a). While *T. reesei* produces high amounts of endo-glucanase and exo-glucanase but a lower level of β-glucosidase. With an insufficient amount of β-glucosidase, the product of exo-glucanase will accumulate and inhibit endo-glucanase and exo-glucanase, such effects would slow down the cellulose degradation (Ahamed and Vermette, 2008).

In order to provide balanced endo-glucanase, exo-glucanase and β-glucosidase concentrations, co-culturing of *A. niger* and *T. reesei* on wheat straw for cellulase production was investigated. Co-culture fermentation was carried out by adding 0.5×10^6 spores of *A. niger* per gram of substrate into a wheat straw fermentation media and then adding the same amount of *T. reesei* spores. The co-culture fermentation was then incubated at 28°C for 4 days. The cellulase activity was analysed and the results are shown in Figure 4.12. The cellulase activity reached 11.138±0.511 FPU/g after 3 days of fermentation. This cellulase activity value was significantly ($p<0.005$) higher than single culture with *T. reesei* (5.658±0.715) around 2-fold. This indicated that co-culture improved cellulase activity compared with the fermentation using *T.reesei* only. This improvement of enzyme activity resulting from a co-culture method agreed with several research papers (Duenas *et al*., 1995, Massadeh *et al*.,

2001, Ahamed and Vermette, 2008, Gutierrez-Correa and Tengerdy, 1998, Gutierrez-Correa *et al*., 1999). Their studies reported that co-culture, or mixed culture, led to higher cellulase activity than pure culture. The increase of cellulase activity was generally considered to be related to the increased amount of β-glucosidase produced by *A. niger* strain, which balanced the ratio of individual enzyme in the cellulase consortium (Brijwani *et al*., 2010).

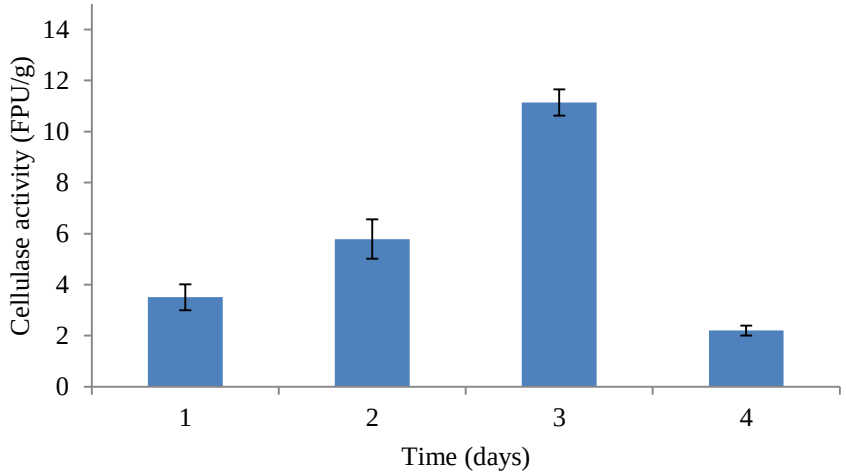


Figure 4.12 Cellulase activity of fungal extract from co-culture on wheat straw via solid state fermentation. Solid state fermentation was supplemented by 0.24 g of starch and it was carried out at initial moisture content of 89.5%, 28˚C for 4 days. The blending process was employed before the enzyme extraction process. Sodium citrate buffer pH 4.8 was applied as extraction buffer during enzyme extraction. Data are mean values of three replicates and error bars indicate standard deviations (SDs)

It can be seen that although the cellulase activity of the co-culture fermentation was higher than the cellulase activity detected in the fermentation of pure *A. niger*, this was not statistically significant (see table 4.9). The enzyme activity from the co-culture was higher than the average enzyme activity of the mono-culture of *T. reesei* around 1.97 fold and *A. niger* around 1.17 fold.

Table 4. 9 Cellulase activity in fungal extract from *A. niger, T. reesei* and Co-culture.

Time (days)	*A.niger*	Time (days)	*T.reesei*	Time (days)	Co-culture
1	4.330±0.381	1	2.970±0.134	1	3.508 ±0.506
3	9.515±1.644	3	5.658±0.715	2	5.784 ±0.769
5	5.552±0.539	5	5.278±0.715	3	11.138±0.511
7	n.a	7	n.a	4	2.201±0.193

Note: Cellulase activity of fungal extract from mono-culture and co-cultures of *A. niger* and *T. reesei* on wheat straw via solid state fermentation. Solid state fermentations were supplemented by 0.24 g of starch and carried out at initial moisture content of 89.5% at 28˚C. The blending process was employed before the enzyme extraction process. Sodium citrate buffer pH 4.8 was applied as extraction buffer during enzyme extraction. Data are mean values ± standard deviation of at least three replicates.

4.4 Biological pretreatment efficiency

Besides cellulase production, the efficacy of biological pretreatment could be evaluated by the amount of glucose released in a subsequent enzymatic hydrolysis step. In order to test whether the solid state fungal fermentation improves the following enzymatic hydrolysis, the untreated wheat straw, the autoclaved wheat straw and the *A. niger* treated wheat straw were hydrolysed using a commercial cellulase, Cellic® CTec 2 (Novozyme, Denmark) . The enzyme loading rate was 30 FPU/g substrate with 5% substrate loading at 50°C. After 6 hours of hydrolysis 2.101±0.272, 2.545±0.129 and 2.700±0.125 g/L of glucose were liberated from the untreated wheat straw, the autoclaved wheat straw and the *A. niger* treated wheat straw, respectively (see Figure 4.13). By comparing the raw wheat straw with the autoclaved wheat straw as a mean of sterilising the substrate, it can be observed that autoclaving has enhanced the sugar released from wheat straw. Moreover, with the *A. niger* treatment, the sugar concentration in the wheat straw hydrolysate increased even more. This result agreed with the pretreatment studies on wheat and rice straws (Patel *et al*., 2008). In Patel *et al*'s report, the sugar yield obtained from the fungal treated wheat straw was better than the untreated wheat straw.

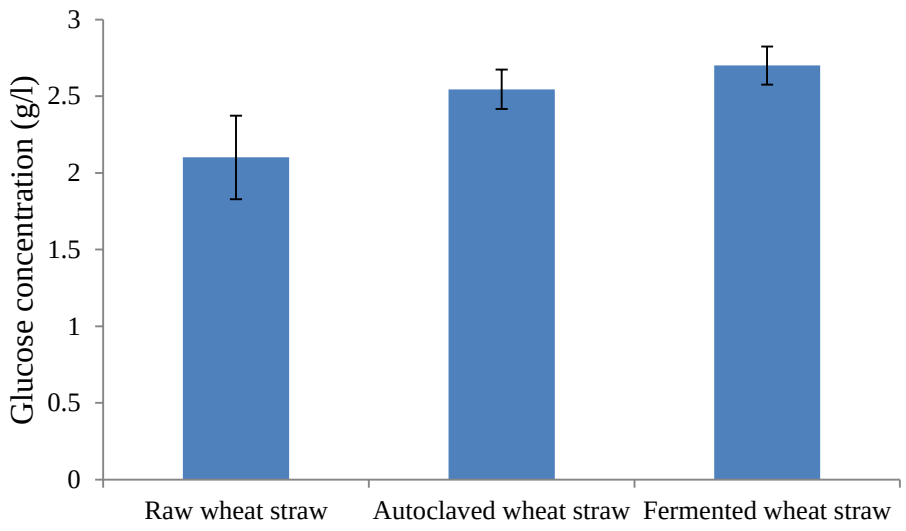


Figure 4. 13 Glucose concentration after 6 h of enzymatic hydrolysis of untreated wheat straw, autoclaved wheat straw and *A. niger* treated wheat straw. Hydrolysis was carried out at 50°C, 150 rpm for 6 hours. The enzyme loading was 30 FPU/g and solid loading was 5%. Data are mean values of three replicates and error bars indicate standard deviations (SDs)

4.5 Conclusion

Biological pretreatment of wheat straw by culturing two cellulase producing fungi, *A. niger* and *T. reesei* was carried out. The objective is to reduce the recalcitrance of wheat straw and to produce cellulase at the same time. The results showed that after biological pretreatment (SSF), both the chemical composition and the physical properties of wheat straw changed. The crystallinity of wheat straw after biological pretreatment by *A. niger* and *T. reesei*, dropped to 21.0% and 19.6%, respectively from 28.5% in the raw wheat straw.

In comparison with *T. reesei*, SSF of *A. niger* on wheat straw produced higher cellulase. The culture time required for the cellulase production by *A. niger* was only 3 days. After 5 days, the cellulase production dropped. Co-culture of *A. niger* and *T. reesei* resulted in further increased cellulase production but that the difference of cellulase activity from co-culture and cellulase activity from mono-culture of *A. niger* was not statistically significant. Furthermore co-

culture may not suitable for large–scale industrial process. Due to the fact that system of co-culture fermentation is operated by two or more microorganisms so the co-culture fermentation requires more considerations than mono culture fermentation. For example, it requires a well understanding on compatibility of each co-cultivation. The difference between the microorganisms can also have different ecological relationship, such as competitors or co-operators. Secondly, it is difficult to control population size of each strain. Thirdly, it has a high possibility of mutation on microorganism during co-culturing. With these complexities of the co-culture system, it is not normally picked up by the industrial sector.

A. niger was selected as the microorganism for the following investigation on the biological pretreatment of wheat straw.

Chapter 5 Evaluation of Culture Conditions for Cellulase Production by *A. niger*

In the previous chapter, the biological pretreatment of wheat straw by *A. niger* has been shown to improve the wheat straw structure and the saccharification efficacy. Moreover during the biological pretreatment step *A. niger* produced significant amounts of cellulase enzyme. The cellulolytic enzymes play an important role in the conversion of lignocellulosic biomass to bioethanol in the production process. So far however, the culture condition in *A. niger* fermentations has not been optimised and it would be interesting to see how much optimisation could increase the amount of cellulase enzyme. Therefore, this chapter focuses on optimising the culture condition for *A. niger* in solid state fermentation.

5.1 Cellulase production using raw wheat straw

Fungal cellulase production has been studied on various types of agricultural waste, such as corn stover, rice straw, banana waste and potato waste (Reddy *et al*., 2003, Deswal *et al*., 2011, dos Santos *et al*., 2012). The optimal condition for cellulase production varies in these different studies due to the complexity of interaction between substrate and fungal strain. Kawamori *et al*. (1986) proposed that cellulase productivity could be improved by strain screening and/or optimizing fermentation conditions. Dos Santos *et al*. (2012) experimentally demonstrated that *Aspergillus* strain is efficient in cellulase production and this results presented in the previous chapter also showed that *A. niger* can provide a high cellulase yield.

The factors that normally affect solid state fermentation are initial moisture content, carbon source, the ratio of carbon to nitrogen provided, trace elements, pH and aeration rate. In this research, the following parameters were investigated: (i) the effect of initial moisture content, (ii) effect of wheat straw modification techniques, (iii) the level of nitrogen source and (iv) the addition of mineral elements.

5.1.1 Effect of initial moisture content of wheat straw on cellulase production in solid state fermentation

The moisture content plays one of the most important roles in solid state fermentation along with cultivation time, inoculum size and concentration of substrate (Park *et al*., 2002).

In this experiment, the solid state fermentation substrate was prepared as described in chapter 3.4.4 but the initial moisture content was varied by adding water to wheat straw with ratio from 5:1 to 9:1 (refer Table 5.1). This solid state fermentation was carried out as described in chapter 3.4.5. The culture time for this experiment was 5 days. Samples were taken at day 1, 3 and 5 of the fermentation for further analysis.

Table 5. 1 Water to wheat straw ratio 5:1 to 9:1 (v/w) were equalling to 85.1 to 91.1 %

Liquid to solid ratio (w/w)	Initial moisture content (%)
5:1	85.1
6:1	87.2
7:1	88.8
7.5:1	89.5
8:1	90.1
9:1	91.1

Note: the initial moisture content of solid state fermentation substrate calculation was based on dry basis

The results show that for every moisture content the cellulase activities in day 3 and day 5 were significantly increased ($p<0.05$) from the first day of

incubation (see Figure 5.1). However, the cellulase activities at day 3 were not significantly different to those obtained at day 5 for all conditions. Therefore from this point onward, the experiments were only carried out with the biomass fermented for three days.

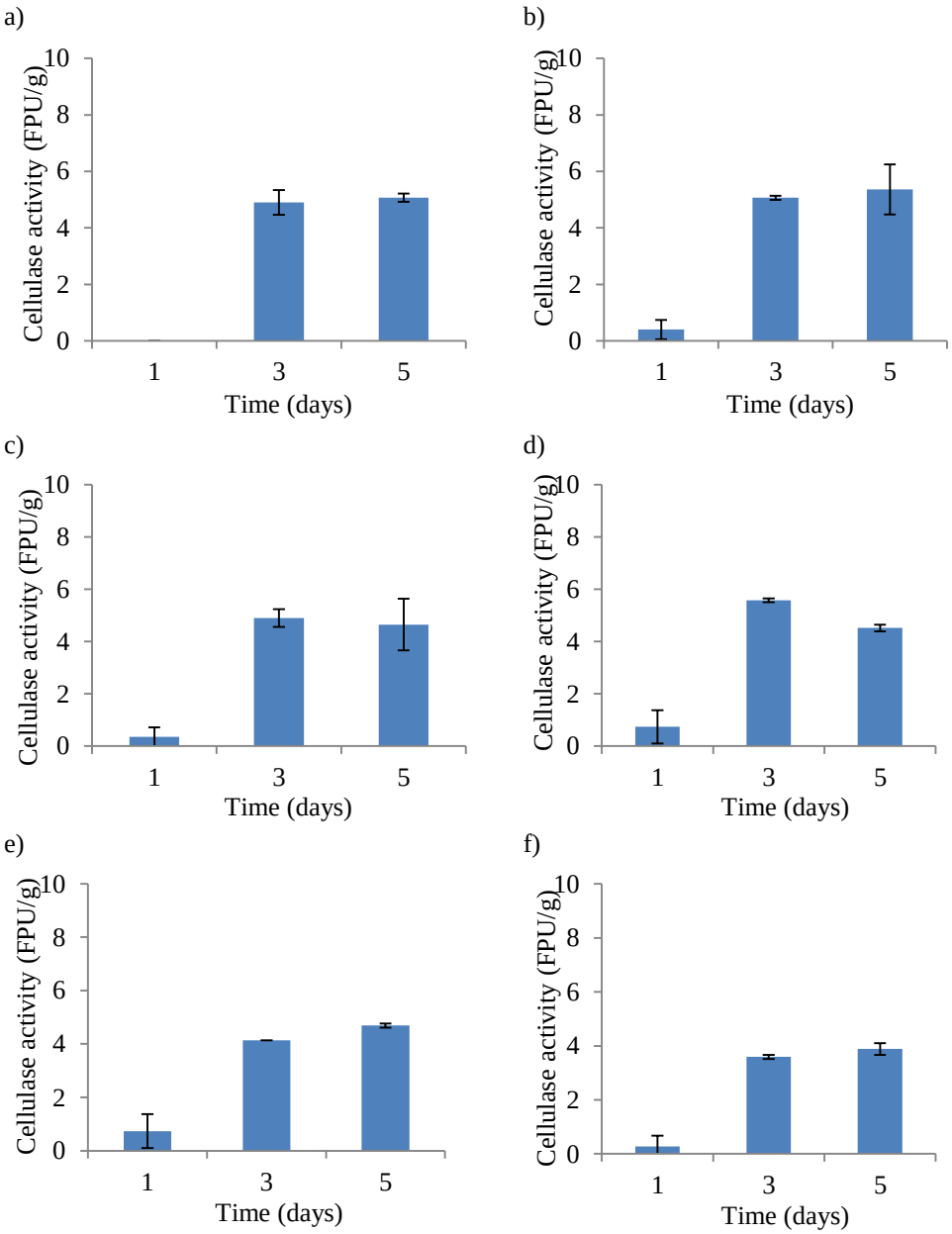


Figure 5. 1 The effect of initial moisture content of solid state fermentation on cellulase activity. Solid state fermentation was carried out at 28˚C for 5 days. Liquid to solid ratio in solid state fermentation was 5:1 (a); 6:1 (b); 7:1 (c); 7.5:1 d); 8:1 (e) and 9: 1 (f). The blending process was employed before the enzyme extraction process. Sodium citrate buffer pH 4.8 was used as extraction buffer in enzyme extraction. Data are mean values of three replicates and error bars indicate standard deviations (SDs).

The cellulase activities obtained from solid state fermentation at 5:1 to 7:1 showed no significant differences at the 95.0% confidence level. The highest cellulase activity was obtained from solid state fermentation at the liquid to solid ratio of 7.5:1 or 89.5 % moisture content. The cellulase activity decreased from the moisture content of 7.5:1 to 8:1 and further decreased at 9:1, (Figure 5.2.)

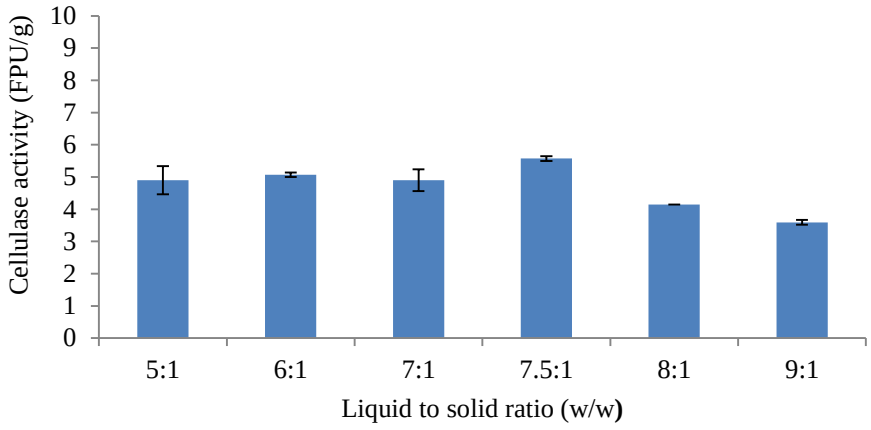


Figure 5. 2 The effect of initial moisture content of solid state fermentation on cellulase activity after 3 days incubation. Data are mean values of three replicates and error bars indicate standard deviations (SDs).

Figure 5. 3 Spore formation in solid state fermentations with difference initial moisture contents. *A. niger* was cultured in solid state fermentation for 1 to 5 days with liquid to solid ratio of A1-A5) 5:1, B1-B5) 6:1, C1-C5) 7:1, D1-D5) 7.5:1, E1-E5) 8:1 and F1-F5) 9:1.

The spore formation was also influenced by the moisture content in solid state fermentation as shown in Figure 5.3. The higher initial moisture content in solid state fermentation appeared to form more spores.

The influence of the initial moisture content on enzyme production has been studied by various researchers (Bansal *et al*., 2012, Narra *et al*., 2012, Ito *et al*., 2014). In solid state fermentation, water has an important role on nutrient mass transfer (Pandey, 2003). Normally the solid state fermentation is operated under low moisture conditions as fungi are capable of growing at low water activity. Low moisture content in solid state fermentation is believed to be good for the prevention of bacterial contamination. However, if the moisture content is too low, the fungal growth and enzyme production would be actually limited. Similarly, a high moisture content in solid state fermentations could also show negative effects on enzyme production. Deswal *et al*. (2011) worked out the optimal conditions for cellulase production by *Fomitopsis sp. RCK2010* (brown rot fungi). The results showed that cellulase activity increased along with the moisture content ratio when it was lower than 3.5:1 (liquid: solid), however when the moisture content was increased further the cellulase activity seemed to decrease. They believe that this was because the water blocked some spaces in the material structure (Satyanarayana and Johri, 2005). This could lead to oxygen transfer limitation which reduces microorganism growth and enzyme production.

The optimal moisture content for enzyme production was also dependent on the behaviour of strain and substrate. Delabona *et al*. (2013) investigated the effect of moisture content on cellulase production from *A. niger* and *A. fumigatus* growing on wheat bran, soybean bran and orange bagasse. The

results revealed that moisture content in solid state fermentation strongly affected enzyme production. For *A. niger*, the highest value of cellulase activity (2.9 FPU/g) was obtained at 50% moisture content with wheat bran, while the highest cellulase activity from *A. fumigatus* (5.0 FPU/g) was obtained under the same conditions. Moreover different substrates showed different optimal moisture content conditions. The highest cellulase activity of *A. niger* from wheat bran, soybean bran and orange bagasse were achieved at initial moisture content of 50%, 60% and 70%, respectively.

In this project, for solid state fermentation using the wheat straw sample with the liquid to solid ratio of 1:7.5 (w/v) was used in further experiments to maximise cellulase production.

5.2 The effect of modified wheat straw on cellulase production

Many studies reported that substrate has a significant influence on enzyme production (Mandels and Reese, 1960, Jecu, 2000, Kalogeris *et al*., 2003, Gupte and Madamwar, 1997). Gupte and Madamwar (1997) reported several biomass modification methods that could increase the accessibility of cellulose to the microorganism thus enhancing enzyme production.

In this study, chemical agents and thermal treatment of wheat straw were used as modification approaches. The aim of modification is to alter wheat straw structure in order to improve enzyme production during solid state fermentation. The conditions for each of the modification processes employed here are listed in Table 5.2.

Table 5. 2 Operation conditions for various modification processes used in this study.

Modification technique	Chemical	Temperature (°C)	Retention time (minute)
Hydrothermal modification	Water	121	15
Acid soaking	1% H_2SO_4	50	30
Diluted acid	1% H_2SO_4	121	30
Hot alkali	1% NaOH	121	30
Alkali soaking	1% NaOH	25	24 hour

Note: Hydrothermal modification also called autoclave modification

The effect of each modification technique was evaluated through chemical composition analysis in comparison with the non-treated biomass sample. The cellulose, hemicellulose and lignin compositions of raw straw and the various types of modified wheat straws are shown in Table 5.3. Each of the modification methods result in different final compositions, for example, the diluted acid modification targets the hemicellulose, therefore the acid modified wheat straw shows low hemicellulose content, while the alkali modification targets decomposition of lignin, therefore the alkali modified wheat straw contains lower amount of lignin.

Table 5. 3 Characteristic of various modified wheat straw

Compositions Content (wt. %)	Non treated	Hydrothermal	Acid soaking	Dilute acid	Hot alkali	Alkali soaking
Lignin	20.25±0.06	15.30±0.43	19.07±0.64	14.26±0.50	10.27±0.31	10.44±0.27
Cellulose	31.68±0.39	34.05±1.77	30.40±2.36	53.31±2.06	46.64±1.22	51.54±0.85
Hemicellulose	17.86±0.28	14.81±1.12	15.59±1.60	5.53±0.21	21.2±0.01	24.37±0.50
Other	30.21±0.64	35.84±0.02	34.94±4.60	26.90±2.36	21.89±0.92	13.65±1.60
Crystallinity	26.50	24.64	22.76	25.10	32.28	28.69

Note: Hydrothermal modification also called autoclave modification

In hydrothermal or autoclaved modification, weak organic acid released from the biomass matrix at high temperature functioned as a chemical catalyst to disrupt the biomass structure. The composition analysis shows that crystalline cellulose and hemicellulose content were reduced (Table 5.3). As shown in Table 5.3, the crystallinity was reduced from 26.5% to 24.6% in comparison with the non-treated wheat straw, which is equivalent to a 1.9% reduction. The hemicellulose content in the hydrothermal modified wheat straw was decreased

from 17.86% to 14.81%. The acid modification reduced the amount of the hemicellulose as well. The result showed a 69% hemicellulose removal after the dilute acid modification. The alkali modification mainly targeted lignin degradation. Taherzadeh and Karimi (2008) explained how the alkali would degrade the ester and glycosidic side chains of the biomass. This caused lignin degradation and cellulose swelling. The lignin content in wheat straw was reduced by around 50% after it was treated by 1% NaOH.

5.2.1 Cellulase production from non-treated wheat straw

In this experiment, non-treated wheat straw was used to produce cellulase. Note that the non-treated wheat refers to the milled wheat straw dried at 60°C for 24 hours. This substrate was used in solid state fungal fermentation under the condition described in section 3.4.5. After the fungal fermentation, a blending process was then applied before the enzyme extraction process described in section 4.3.4.

As indicated in Table 5.4, cellulase activity of 4.04 FPU/g was detected just after 1 day of fermentation. The highest cellulase activity was obtained on the 3rd day of fermentation and decreased afterward (Table 5.4). However, the cellulase activity in this experiment was low in comparison with the results using the autoclaved wheat straw. This might be due to contamination from other microorganisms, since the medium was not sterilised in this preliminary experimental study.

Table 5. 4 Cellulase production from *A. niger* growing on non-treated wheat straw

Time (day)	Cellulase activity (FPU/g)
1	4.037±1.178
3	5.662±0.573
5	3.660±0.861

Note: Cellulase production by *A. niger* in solid state fermentation of non-treated wheat straw with 0.24 g of starch as supplement. Solid state fermentation was carried out at 28˚C for 5 days. The blending process was employed before the enzyme extraction process. Sodium citrate buffer pH 4.8 was used as extraction buffer in enzyme extraction. Data are mean values ± standard deviation of four replicates

5.2.2 Cellulase production from Hydrothermal or Autoclaved wheat straw

In this experiment, Autoclave modified wheat straw was prepared as described in 3.4.4. The cellulase production profile was then examined. After 24 hours of incubation (1 day), significant cellulase activity was detected. The cellulase activity reached a maximum of 9.245±1.294 FPU/g, which was equal to 0.568±0.068 U/ml after 3 days of fermentation. Then cellulase activity was reduced to 5.668±0.405 FPU/g on day 5 (Table 5.5).

Table 5. 5 Cellulase production from autoclaved wheat straw.

Time (day)	Cellulase activity (FPU/g)
1	4.258±0.404
3	9.245±1.294
5	5.668±0.405

Note: Cellulase production by *A. niger* in solid state fermentation of autoclave wheat straw with 0.24 g of starch as supplement. Solid state fermentation was carried out at 28˚C for 5 days. The blending process was employed before the enzyme extraction process. Sodium citrate buffer pH 4.8 was used as extraction buffer in enzyme extraction. Data are mean values ± standard deviation of nine replicates

5.2.3 Cellulase production from Acid modified wheat straw

There were two methods applied in the acid modification processes. (1) The acid treatment at relatively high temperature and short reaction time; this method was named ***dilute acid modification***. (2) The acid treatment at relatively low temperature; this was named ***acid soaking modification***.

The dilute acid modified wheat straw was prepared by adding 1% (v/v) H_2SO_4 solution into dried wheat straw at a solid to liquid ratio of 1:10 (w/v), the solid

suspension was heated to 121°C for 30 minutes. The treated wheat straw was adjusted to pH 7 by the addition of 1 M NaOH s and the solid residue rinsed with distilled water to remove acid residues. The collected biomass was then dried in an oven at 40 °C until the weight was constant. The solid state fermentation of *A. niger* on dilute acid modified wheat straw was then carried out as described in 3.4.4.

The cellulase production profile in solid state fermentation of dilute acid modified wheat straw was then investigated. The highest cellulase activity was obtained on day 3, which was 10.435±0.742 FPU/g and after that the cellulase activity gradually decreased. However when considering the cellulase activity at day 1, the cellulase activity from diluted acid modified wheat straw was 78.42% higher than the cellulase activity obtained from autoclaved wheat straw (Table 5.6).

Table 5. 6 Cellulase production from *A. niger* on wheat straw treated with dilute acid at high temperature and short retention time.

Time (day)	Cellulase activity (FPU/g)
1	7.597±1.271
3	10.435±0.742
5	4.772±0.596

Note: Cellulase production by *A. niger* in solid state fermentation of diluted acid modified wheat straw with 0.24 g of starch as supplement. Solid state fermentation was carried out at 28˚C for 5 days. The blending process was employed before the enzyme extraction process. Sodium citrate buffer pH 4.8 was used as extraction buffer in enzyme extraction. Data are mean values ± standard deviation of three replicates

The acid soaking modification was prepared by the following procedure. The wheat straw was mixed with 1% H_2SO_4 solution at a solid to liquid ratio of 1:10 (w/v); incubated in a water bath at 50°C for 30 minutes. The treated wheat straw was adjusted to pH 7 by the addition of 1 M NaOH and then rinsed with distilled water to remove acid residue. The collected biomass was dried in an oven at 40 °C until the weight was constant.

As shown in Table 5.7, the cellulase activity of the acid soaking modified wheat straw on day 1 showed similar values to the cellulase activity obtained from the dilute acid modified wheat straw at the same retention time. This might be because during acid modification some small residues from acid hydrolysis are inducing cellulase production. The cellulase activity then decreased rapidly after 72 hours; it might be because the secreted cellulase generated glucose in the fermentation medium. Lynd *et al.* (2002) have explained that the cellulase is produced when there is sufficient level of cellulose, however the cellulase production itself could be suppressed by high levels of glucose. Hence the cellulase production was consequently reduced.

Table 5. 7 Cellulase production from *A. niger* on acid soaking modified wheat straw reported in cellulase activity (FPU/g)

Time (day)	Cellulase activity (FPU/g)
1	7.512±1.195
3	5.110±1.076
5	4.730±0.932

Note: Cellulase production by *A. niger* in solid state fermentation of acid soaking modified wheat straw with 0.24 g of starch as supplement. Solid state fermentation was carried out at 28˚C for 5 days. The blending process was employed before the enzyme extraction process. Sodium citrate buffer pH 4.8 was used as extraction buffer in enzyme extraction. Data are mean values ± standard deviation of three replicates

In ANOVA table (Table 5.8) shows that the cellulase activity obtained from each group are statistically significant difference.

Table 5. 8 ANOVA Table of cellulase activity obtained from non-treated wheat straw, autoclaved modified wheat straw, diluted acid modified wheat straw and acid soaking modified wheat straw

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	16.7017	2	8.35083	8.11	0.0017
Within groups	27.7979	27	1.02955		
Total (Corr.)	44.4996	29			

Note: The F-test in the ANOVA table tested whether there were any significant differences amongst the means. Since the P-value of the F-test was less than 0.05, there was a statistically significant difference between the means of the samples at the 95.0% confidence level.

The effects of these modifications on cellulase production are summarised in Figure 5.4. The cellulase activity from the non-treated wheat straw was

significantly lower when compared to the modified wheat straw. The higher cellulase activity in the fungal filtrate from the dilute acid modified wheat straw might be due to the higher proportion of cellulose in the substrate compared to non-treated wheat straw (Table 5.3). This explanation is similar to that proposed by Rezaeian *et al*. (2005). By comparing the cellulase activity in fungal filtrate using different substrates, it can be seen that the cellulase activity from the autoclaved modified wheat straw was significantly less than the dilute acid modified wheat straw and the acid soaking modified wheat straw on the first day of fermentation. This might be due to the acid modification, which has altered the wheat straw structure. In this case, the crystalline cellulose from the dilute acid modified wheat straw was reduced to 25.10% (Table 5.3). Furthermore a large amount of hemicellulose was removed by the acid modification (Table 5.3), which means that the cellulose was then likely to be exposed to the *A. niger*. Asimilar explanation has been proposed by Ibbett *et al*. (2011). The exposed cellulose was then degraded to oligomeric sugars by constitutive enzyme and the oligomer would then induce cellulase production. The mechanism has also been studied and reported by Hanif *et al*. (2004b). The induction of cellulase by wheat straw was a rapid process in liquid culture, taking around 6 hours (Delmas *et al*., 2012). Although the fermentation conditions in this study were different from the liquid culture, the induction of cellulase in solid state fermentation could be fast as well (El-Metwally, 2014). This agrees well with the experimental data in this study, *A. niger* growing in the acid modified wheat straw secreted a higher amount of cellulase than the fermentations using the autoclave modified wheat straw and the non-treated wheat straw within the first 24 hours of incubation.

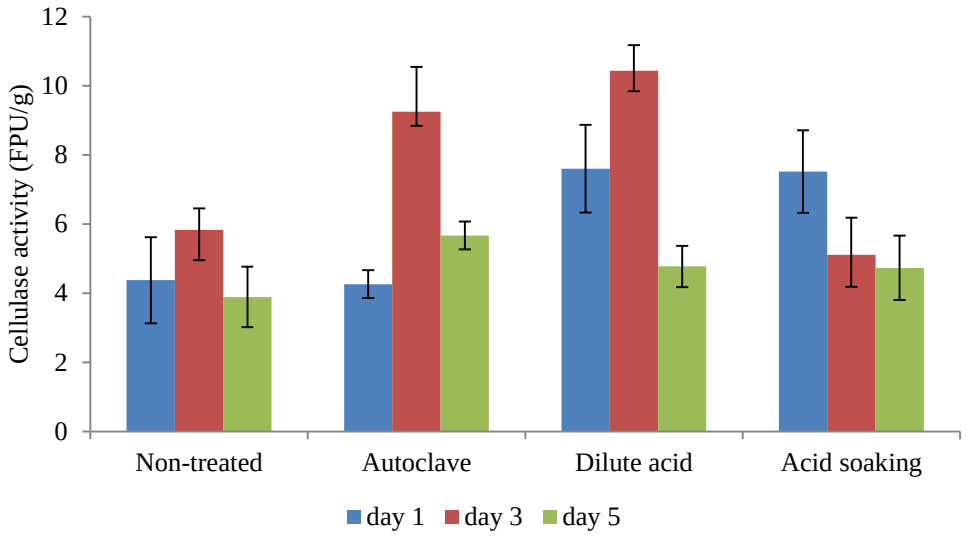


Figure 5. 4 The cellulase activity obtained from solid state fermentation of non-treated wheat straw, autoclaved modified wheat straw, dilute acid modified wheat straw and acid soaking modified wheat straw. Solid state fermentation was supplemented by 0.24 g of starch and it was carried out at 28˚C for 5 days. The blending process was employed before the enzyme extraction process. Sodium citrate buffer pH 4.8 was used as extraction buffer in enzyme extraction. Data are mean values of at least three replicates and error bars indicate standard deviations (SDs).

However, for all the conditions, lower cellulase activity was observed at day 5. In the dilute acid experiment, the enzyme activity dropped to 4.77 U/g at day 5 which was only 54% of the peak cellulase activity at day 3. Singh *et al.* (2011) believe that the decrease in cellulase activity might be because of the reduction in cellulose content and nutrient depletion. It could also be caused by protease activity and unbalancing of individual enzyme. It has been reported that the high level of glucose liberated from hydrolysed substrate could show a negative effect on cellulase productivity. The utilisation of the released glucose would result in the increase of cell growth and enzyme production until 96 hours of culturing. After that the cellulase production decreased (Hanif *et al.*, 2004a). Similar to this study, it was observed that the cellulase activity from *A. niger* reduced on day 5 of fermentation.

The highest cellulase activities obtained from autoclaved modified wheat straw and the dilute acid modified wheat straw were 9.25 and 10.44 FPU/g respectively. The enhanced cellulase activity in the dilute acid modification experiment might be because the acid solution at high temperature increased the amount of accessible cellulose in wheat straw. However the autoclaved modified wheat straw shows a greater cellulase activity than that of acid soaking modified wheat straw. This indicates that temperature in modification process plays an important role in altering wheat straw structure. The cellulose proportion in the substrate would also affect cellulase activity in solid state fermentation and, in this case, the wheat straw treated with high temperature shows better result than the acid chemical treatment itself.

Modification of the lignocellulosic materials by acid for fungal fermentations was also explored by a number of researchers as a way to improve cellulase production, such as, Bansal *et al*. (2012) where they modified wheat straw with 1% (v/v) sulphuric acid leading to 19.2 FPU/g from solid state fermentations using *A. niger* NS-2. This was 8 times higher than that using the autoclaved wheat straw.

In this experiment, the cellulase activity obtained from the dilute acid modified wheat straw was 12% higher than the autoclaved modified wheat straw, however there was no statistically significant difference. Note that the autoclaved modification operation is rather simple and cost effective, I therefore chose the autoclave modified wheat straw as the substrate for further experiments.

Effect of nitrogen source on cellulase production

In general, fermentation medium should contain carbon source, nitrogen source and trace elements that are necessary for the microorganism to grow. For cellulolytic enzyme production from lignocellulose material, cellulose or hemicellulose are normally used as a carbon source and an inducer. Since lignocellulosic materials like wheat straw have only 3.9% protein content, the wheat straw based fermentation substrate will, of course, require an additional supply of nitrogen. Many enzymes including cellulase produced from *A. niger* could be stimulated by nitrogen source. A number of different nitrogen sources have been studied for their impact on cellulase production such as ammonium sulphate, sodium nitrate, peptone and yeast extract (Hanif *et al*., 2004a, Kachlishvili *et al*., 2006, Gao *et al*., 2008, Membrillo *et al*., 2008). The level of increase in cellulase synthesis varied with the type of nitrogen source (Hanif *et al*., 2004a). Deswal *et al*. (2011) has reported that the cellulase activity from *Fomitopsis* sp. RCK2010 was stimulated by adding organic nitrogen sources; peptone, yeast extract, casein and urea. On the other hand, inorganic nitrogen sources such as ammonium chloride, sodium nitrate and potassium nitrate did not show any positive effect on cellulase accumulation.

The majority of reports show that the addition of nitrogen has a positive impact on cell growth, protein production and cellulase productivity. Moreover, it has been reported that the supplementation of nitrogen into lignocellulosic raw material did not show any repressive effect on cellulase production (Hanif *et al*., 2004a). In other words, the amount to be added is only limited by cost issues.

The experiments in this section were designed to test the hypothesis that nitrogen source supplement could improve cellulase production from *A. niger* on the autoclave modified wheat straw. The cellulose and hemicellulose in autoclave modified wheat straw were used as the carbon source, yeast extract was selected to be used as the nitrogen source.

As shown in Figure 5.5, cellulase production was significantly affected by the addition of the nitrogen source. The cellulase activities increased to 15.10 FPU/g and 18.64 FPU/g after 3 and 5 days culture, respectively in solid state fermentation with 0.5% (w/w) yeast extract. When 5% (w/w) yeast extract was added, a cellulase activity of 38.80 FPU/g was obtained after 5 days incubation. This was in good agreement with various studies that have shown that the addition of nutrients can improve cellulase production (Kachlishvili *et al*., 2006, Gao *et al*., 2008, Han and Chen, 2010). However, Ncube *et al*. (2012) cultured *A. niger* FGSCA733 on a Jatropha curcas-based substrate; it was found that the addition of a nitrogen source did not enhance cellulase production. *A. niger* was found to grow on a medium containing yeast extract and minerals (0.5%, in a liquid culture), but the cellulase activity was only 0.15 ± 0.01 U/ml after 5 days culture.

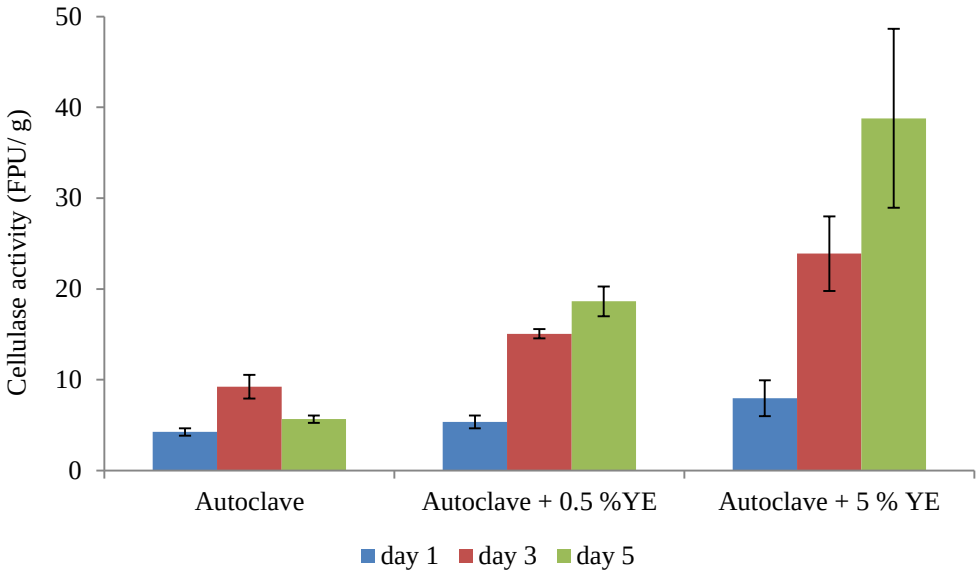


Figure 5. 5 Effect of yeast extract on cellulase production by *A. niger* on autoclaved modified wheat straw. Solid state fermentation was carried out on autoclaved wheat straw 0.24 g of starch as supplement. It was run at 28˚C for 5 days. The blending process was employed before the enzyme extraction process. Sodium citrate buffer pH 4.8 was used as extraction buffer in enzyme extraction. Data are mean values of at least three replicates and error bars indicate standard deviations (SDs).

In this experiment, it can be seen from figure 5.5 that the enzyme activity from *A. niger* as grown on autoclaved modified wheat straw can be stimulated by yeast extract and that the level of yeast extract concentration can greatly influence cellulase production.

Effect of additional micronutrients on cellulase production

Besides nitrogen, phosphorus and micronutrients also have an important role in fungal growth and thus enzyme production. As a phosphorus source, Potassium di-hydrogen phosphate (KH_2PO_4) is usually added to the fermentation medium. This is because phosphorus stimulates phospholipid bilayer formation in cell membranes supporting cell growth (Yoon *et al*., 2013). Trace elements, for example, Copper, Manganese, Magnesium and Zinc can also stimulate cell growth and enzyme synthesis. On the other hand, in some cases, some trace elements, such as some heavy metal ions can inhibit cell reproduction.

Therefore an experiment was carried out to test the hypothesis that inclusion of a mineral solution could improve cellulase productivity. The mineral solution was composed of the following chemicals (Table 5.9). The solid state fermentation was carried out as described in 3.4.4. Note that mineral solution was used instead of distilled water to adjust initial moisture content in the media preparation procedure for solid state fermentation.

Table 5. 9 The composition of a mineral solution used in SSF of wheat straw.

Chemical	Amount (g)
$(NH_4)_2SO_4$	1
KH_2PO_4	0.5
K_2HPO_4	0.5
$MgSO_4$	0.2
Distilled water	1000 ml

Similarly to the addition of nitrogen source discussed above, the addition of the mineral solution also boosted cellulase activities up to 16.70 and 24.03 FPU/g after 3 and 5 days culture, respectively. This result demonstrated that the cellulase production from *A. niger* has been highly influenced by the mineral solution which is in agreement with the finding of many other researchers. Several reports have shown that micro-nutrients, such as, zinc, nickel, manganese and copper, are capable of enhancing cellulase production (Yoon *et al*., 2013, Deswal *et al*., 2011, Levin *et al*., 2008, Shi *et al*., 2009). This is probably due to the fact that certain enzymes need these metals as their cofactors. However, there are other reports that show that some trace elements suppress cellulase production if their concentrations are too high. The reduction of enzyme activity might be because the trace element reacts with the active groups of the enzyme or may bind with the enzyme-substrate complex (Deng and Tabatabai, 1995). In our experiment, the minerals only look to

clearly increase cellulase production by day 5 relative to the series with yeast extract but no mineral supplementation.

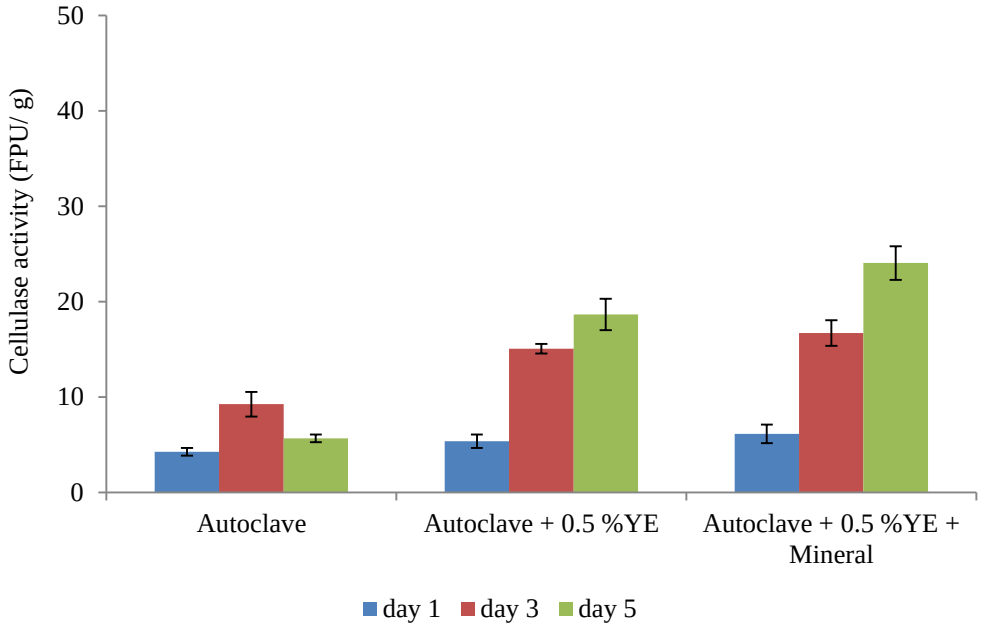


Figure 5. 6 Effect of micronutrient on cellulase production by *A. niger* on autoclaved modified wheat straw. Solid state fermentation was supplemented by 0.24 g of starch. It was carried out at 28˚C for 5 days. The blending process was employed before the enzyme extraction process. Sodium citrate buffer pH 4.8 was used as extraction buffer in enzyme extraction. Data are mean values of at least three replicates and error bars indicate standard deviations (SDs)

5.2.4 Cellulase production from alkali modified wheat straw

Alkali treatment is an effective technique for the removal of lignin and also increases the surface area of lignocellulosic biomass. Mild alkali solution, such as, 1M sodium hydroxide is commonly used in alkali pretreatment. The alkali breaks the ester bond in lignin polymer (Narra *et al*., 2012) and increases the swelling capacity of the material, which increases the accessibility of the substrate for digestion by microorganisms (Awafo *et al*., 2000).

Here a set of experiments were carried out to test the hypothesis that alkali modification of wheat straw can improve cellulase production in *A. niger*. Alkali modification had divided into 2 groups.

(i) Dilute alkali treated with high temperature and short time; this method is named ***hot alkali modification***.

(ii) Dilute alkali treated at low temperature and long retention time; this method is named ***alkali soaking modification.***

The hot alkali modified wheat straw was prepared by adding 1% (w/w) NaOH solution into dried wheat straw at a solid to liquid ratio of 1:10 (w/v), and autoclaving at 121°C for 30 minutes. The autoclaved wheat straw was then adjusted to pH 7 with 1 M H_2SO_4 and the biomass was rinsed with distilled water to remove alkali residues. The biomass was collected and dried in an oven at 40 °C until the weight was constant. Then hot alkali modified wheat straw was used as a substrate in solid state fermentation of *A .niger* (refer to 3.4.4) and 0.5% yeast extract with mineral solution was added to the fermentation medium to stimulate the cellulase productivity.

As shown in Table 5.11, the cellulase production profile in solid state fermentation using alkali modified wheat straw was significantly different to that observed using the acid modified wheat straw. The cellulase activity from hot alkali modified wheat straw shows the highest activity after 24 hours of fermentation (17.29 FPU/g). Then the activity gradually decreased. A 25.36% reduction of cellulase activity was observed after 3 days of fermentation. The rapid secretion of cellulase on day 1 might be due to the fact that hot alkali modification removed a large amount of lignin from the wheat straw making cellulose more immediately accessible. The amount of lignin content was decreased from 20.25% to 10.27%. However, after 72 hours of fermentation, the activity dramatically dropped. This might be because the glucose released during fermentation influenced the secretion of the inducible enzymes (Hanif *et*

al., 2004a). Glucose is known to act as a cellulase repressor. The active cellulase on the first day of fermentation may generate a high level of glucose, which inhibited cellulase synthesis at the later stages of the fermentation.

Table 5. 10 Cellulase production from *A. niger* grown on hot alkali modified wheat straw.

Time (day)	Cellulase Activity (FPU/g)
1	17.292±1.891
3	12.906±1.363
5	10.236±1.212
7	7.211±1.092

Note: Cellulase production by *A. niger* in solid state fermentation of hot alkali modified wheat straw with 0.24 g of starch as supplement. Solid state fermentation was carried out at 28˚C for 5 days. The blending process was employed before the enzyme extraction process. Sodium citrate buffer pH 4.8 was used as extraction buffer in enzyme extraction. Data are mean values ± standard deviation of three replicates

The alkali soaked modified wheat straw was prepared by mixing wheat straw with 1% NaOH to at a solid to liquid ratio of 1:10 (w/v), and incubating in a water bath at 25°C for 24 hours. The treated wheat straw was adjusted to pH 7 with 1 M H_2SO_4 and then rinsed off with distilled water to remove acid residue. Collected biomass was dried in an oven at 40 °C until the weight was constant.

The cellulase production profile in solid state fermentation using alkali soaked modified wheat straw was similar to the cellulase production profile obtained from hot alkali modified wheat straw. The cellulase activity was 21.84 FPU/g on the first day of fermentation and it then decreased to 21.84 FPU/g to 15.68 FPU/g and 9.80 FPU/g after 3 days and 5 days of fermentation respectively (Table 5.10). The alkali soaked modified wheat straw showed a higher cellulase production capacity than that of hot alkali modified wheat straw. This might be because cellulose content in the alkali soaked modified wheat straw was higher than in the hot alkali modified wheat straw (Table 5.3). The high temperature during hot alkali modification can also degrade some of the

cellulose. Since the cellulose acted as carbon source in fermentation and the level of carbon source also affected the enzyme production, it is expected that the cellulase production from alkali soaked modified wheat straw is better than the hot alkali modified wheat straw. It can be clearly seen from the experimental results that alkali soaked modified wheat straw was, in fact, better than the hot alkali modified wheat straw as expected.

Table 5. 11 Cellulase production from *A. niger* on alkali soaked modified wheat straw

Time (day)	Average
1	21.845±1.865
3	15.689±1.625
5	9.795±0.886
7	7.122±0.930

Note: Cellulase production by A. niger in solid state fermentation of alkali soaked modified wheat straw with 0.24 g of starch as supplement. Solid state fermentation was carried out at 28˚C for 5 days. The blending process was employed before the enzyme extraction process. Sodium citrate buffer pH 4.8 was used as extraction buffer in enzyme extraction. Data are mean values ± standard deviation of three replicates

In comparison with the acid or autoclaved modified wheat straw methods mentioned in the previous section, the cellulase productivity from both alkali modified wheat straw techniques has significantly been improved (Table 5.11). The improvement of cellulase activity may be because of their higher cellulose content and the larger accessible area of alkali modified wheat straw for *A. niger*. Therefore it led to a better result on cellulase production compared to other modification methods (Table 5.12). In contrast, Yoon *et al.* (2013) has reported that alkali-treated biomass decreased cellulase production. They explained that the reduction of cellulase generation was due to the fact that the alkali-pretreated biomass had higher protein adsorption ability compared to non-treated biomass. Therefore the biomass matrix could adsorb the cellulase produced and resulted in a lower cellulase activity.

Table 5. 12 Comparison of cellulase production by *A. niger* as grown on different modified wheat straws

Time (days)	Non-treated	Autoclaved modification	Dilute acid modification	Acid soaked modification	Hot alkali modification	Alkali soaked modification
1	4.037±1.178	4.258±0.404	7.597±1.271	7.512±1.195	17.2921±1.891	21.845±1.865
3	5.662±0.573	9.245±1.294	10.435±0.742	5.110±1.076	12.9055±1.363	15.689±1.625
5	3.660±0.861	5.668±0.405	4.772±0.596	4.730±0.932	10.2356±1.212	9.795±0.886
7	n.a	n.a	n.a	n.a	7.211±1.092	7.122±0.930

Note: Cellulase production by *A. niger* in solid state fermentation of various modified wheat straw with 0.24 g of starch as supplement. Solid state fermentation was carried out at 28˚C for 5 days. The blending process was employed before the enzyme extraction process. Sodium citrate buffer pH 4.8 was used as extraction buffer in enzyme extraction. Data are mean values ± standard deviation of at least three replicates

Effect of starch on cellulase production from alkali modified wheat straw

This experiment was set to test the hypothesis that the addition of starch could improve cellulase production. Hot alkali modified wheat straw was used as substrate and solid state fermentation was performed as described in 3.4.4; note that the level of starch was varied in this experiment.

As shown in Figure 5.7 and Table 5.13, the highest cellulase production from a starch-free fermentation medium was only 4.61 FPU/g which was very small compared to the cellulase activity obtained from fermentation medium with 0.2% (w/w) starch. When the starch level was increased to 2% (w/w), the cellulase activity after 24 hours of fermentation increased from 17.29 to 21.05 FPU/g, which was 17% higher.

These results indicate that starch plays an important role in cellulase production. This result agrees with many other reports (Chen and Wayman, 1992, Taj-Aldeen, 1993, Chen and Wayman, 1991, Sun *et al*., 2010). Inoue *et al*. (2013) have suggested that starch induced the expression system of cellulolytic enzyme which led to improvement in enzyme activity. Khokhar *et al*. (2013) believe that the starch in fermentation medium improves fungal growth which might support enzyme production.

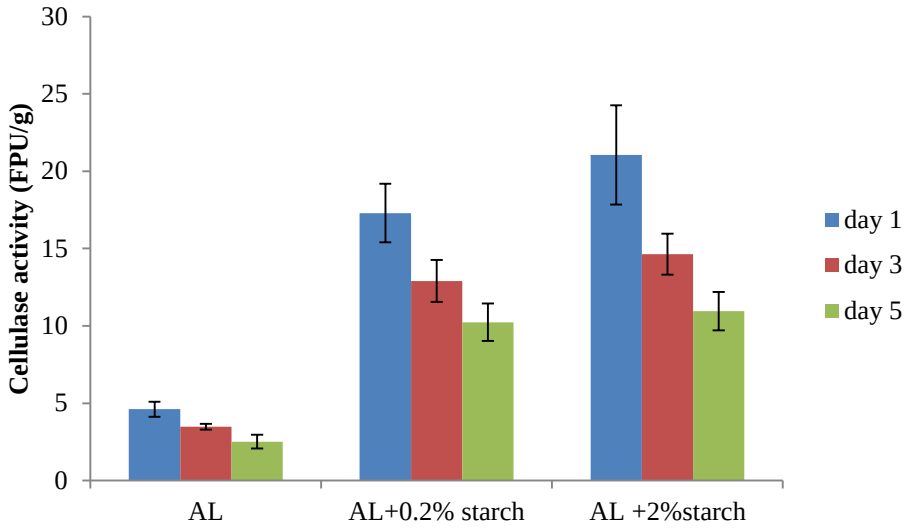


Figure 5. 7 Effect of starch on cellulase production from *A. niger* on hot alkali modified wheat straw. Solid state fermentation was carried out at 28˚C for 5 days. The blending process was employed before the enzyme extraction process. Sodium citrate buffer pH 4.8 was used as extraction buffer in enzyme extraction. Data are mean values of three replicates and error bars indicate standard deviations (SDs). AL is represent hot alkali modified wheat straw and ALS is represent alkali soaked modified wheat straw.

Table 5. 13 Effect of starch on cellulase production from *A. niger* on alkali modified wheat straw

Time (day)	AL	AL+0.2% starch	AL +2%starch
1	4.610±0.488	17.292±1.891	21.052±3.206
3	3.487±0.186	12.906±1.363	14.643±1.324
5	2.517±0.441	10.236±1.212	10.950±1.239
7	1.579±0.872	7.211±1.092	
Time (days)	**ALS**	**ALS+0.2% starch**	
1	6.599±0.639	21.845±1.865	
3	4.221±0.332	15.689±1.625	
5	1.379±0.245	9.795±0.886	
7	0.431±0.073	7.122±0.930	

Note: Cellulase production by *A. niger* in solid state fermentation of alkali modified wheat straw. Solid state fermentation was carried out at 28˚C for 5 and 7 days. The blending process was employed before the enzyme extraction process. Sodium citrate buffer pH 4.8 was used as extraction buffer in enzyme extraction. Data are mean values ± standard deviation of at least three replicates. AL is represent hot alkali modified wheat straw and ALS is represent alkali soaked modified wheat straw.

Effect of additional nutrient on cellulase production from alkali modified wheat straw by A. niger

Although the alkali modified wheat straw contained a high proportion of cellulose as carbon source, it may not provide sufficient nutrients needed for *A. niger* for optimal enzyme production. From our previous experiment using various acid modified wheat straw, the addition of nutrients significantly

improve cellulase production. Therefore, it is expected that supplying some nutrient would further promote cellulase production in fermentations using various alkali modified wheat straw as well. Hence, the effect of addition of yeast extract and minerals on cellulase production from both hot alkali and alkali soaking modified wheat straw was also investigated.

The addition of nutrients shows a positive effect on both hot alkali and alkali soaking modified wheat straw. For the hot alkali modified wheat straw, the highest cellulase activity increased from 17.29 to 20.70 FPU/g (Table 5.14). Similarly the alkali soaking experiment shows an increase of the cellulase activity, which increased by 10% from 21.84 to 24.17 FPU/g. This might be because the additional nutrient supported fungal cell growth and stimulated cellulase synthesis. This result was correlated with the previous experiments, in which the autoclaved modified wheat straw was used.

Based on the experiments mentioned above, the alkali soaking modified wheat straw is a preferable substrate in solid state fermentation of *A .niger* and 0.5% yeast extract with mineral solution added to the fermentation medium in order to stimulate cellulase productivity. The highest cellulase activity was 24.17 FPU/g obtained within 24 hours.

In order to further understand the cellulase catalysis mechanism, the enzyme activities of endo-glucanase, exo-glucanase and beta-glucosidase were then determined (refer to section 3.6.2-3.6.4).

The results show that the total cellulase and the exo-glucanase (or avicelase) shared the same trend. They reached the highest activity after 24 hours, and then the activity dropped gradually.

Table 5. 14 Effect of additional nutrient on cellulase activity from alkali modified wheat straw by *A. niger*

Time (day)	AL+0.2% starch	AL+0.2% starch + 05.%YE +M
1	17.2921±1.891	20.697±6.666
3	12.9055±1.363	12.466±1.695
5	10.2356±1.212	7.988±1.098
7	7.211±1.092	4.601±1.116
Time (day)	**ALS+0.2% starch**	**ALS +0.2% starch + 05.%YE +M**
1	21.845±1.865	24.170±1.917
3	15.689±1.625	10.817±1.512
5	9.795±0.886	7.730±0.852
7	7.122±0.930	3.065±0.541

Note: Cellulase production by *A. niger* in solid state fermentation of alkali modified wheat straw. Solid state fermentation was carried out at 28˚C for 7 days. The blending process was employed before the enzyme extraction process. Sodium citrate buffer pH 4.8 was used as extraction buffer in enzyme extraction. Data are mean values ± standard deviation of at least three replicates. AL represents hot alkali modified wheat straw, ALS represents alkali soaked modified wheat straw, YE represents yeast extract and M represents mineral solution.

On the other hand, the activities of the endo-glucanase (carboxy methycellulase), the beta-glucosidase and the xylanase increased over time. The activity of these enzymes could generate high level of glucose in fermentation medium, which might cause cellulase repression.

Table 5. 15 The production of individual enzyme on alkali soaking modified wheat straw with additional nutrient

Time (day)	Total cellulase	CMCase	Avicelase	β-glucosidase	Xylanase
1	24.170±1.917	45.163±3.825	39.515±3.717	63.714±9.066	100.551±4.722
3	10.817±1.512	95.607±11.014	6.060±3.716	426.884±16.562	278.053±4.490
5	7.730±0.852	86.100±4.530	5.695±2.012	399.504±13.096	259.030±6.251
7	3.065±0.541	86.438±7.919	7.872±1.704	273.798±2.734	206.204±1.995

Note: Solid state fermentation of alkali soaked modified wheat straw was carried out at 28˚C for 7 days. The blending process was employed before the enzyme extraction process. Sodium citrate buffer pH 4.8 was used as extraction buffer in enzyme extraction. Data are mean values ± standard deviation of at least three replicates.

5.3 Conclusion

The fermentation conditions for solid state fermentation of *A. niger* on wheat straw were evaluated for cellulase production. The best moisture content for cellulase production on autoclaved modified wheat straw was at a liquid to solid ratio of 7.5:1. The acid modified wheat straw increased cellulase production, but that difference was not statistically significant compared to cellulase production by autoclaved wheat straw. Addition of yeast extract and a

mineral solution improved cellulase production from around 10 FPU/g to over 18 FPU/g in solid state fermentation of autoclaved wheat straw in 5 days. Furthermore, alkali modified wheat straw led to a high cellulase production of over 21 FPU/g with a significant shortened culture time of 1 day only. Further addition of yeast extract and the mineral solution (KH_2PO_4, $(NH_4)_2SO_4$, K_2HPO_4 and $MgSO_4$) enhanced cellulase production to 24.2 FPU/g in solid state fermentation of alkali soaking modified wheat straw.

It is hypothesized that alkali modification technique increased wheat straw surface area by removing lignin content from the biomass. The exposed cellulose structure induced *A. niger* activity, making cellulase produced faster.

Chapter 6 Wheat Straw Hydrolysis

In chapter 5, the optimal condition for cellulase production in solid state fermentation by *A. niger* was investigated. The initial moisture content showed an important role in cellulase production. The modification of wheat straw was shown to significantly enhance cellulase production. The addition of a nitrogen source and trace elements also improved cellulase production. Based on these results, the following conditions were selected for a large quantity cellulase production: solid liquid ratio of 7.5:1, alkali soaking wheat straw, 0.5% yeast extract and with mineral solution. The fungal mash after solid state fermentation namely fermented wheat straw, was then used as a substrate for hydrolysis and compared with raw wheat straw.

Hydrolysis is an essential step for bioethanol and biochemical production. As summarised in the literature review, both acid hydrolysis and enzymatic hydrolysis were applied at industrial scale for the conversion of lignocellulose to glucose rich hydrolysate. In this chapter, direct acid hydrolysis of raw and fermented wheat straw was investigated. The results were then compared with enzymatic hydrolysis using the fresh fungal filtrate produced via the process developed in Chapter 5. A statistical programme based on response surface methodology was applied to optimise the conditions for the wheat straw hydrolysis.

6.1 Direct acid hydrolysis

Using acid to hydrolyse lignocellulosic biomass can be tracked back to 1883, when it was found that acid had then ability to depolymerise cellulose into oligosaccharide (Yoon *et al*., 2014, Shahbazi and Zhang, 2010). In the latest

lignocellulosic bioethanol research, acid hydrolysis has been mainly used as a "pretreatment" method to remove hemicellulose and to "purify" cellulose. Then the cellulose will be further hydrolysed to glucose by mainly enzymes, or occasionally an acid. Nevertheless, direct acid hydrolysis of lignocellulosic biomass does generate a hydrolysate containing sugars. In this chapter, direct acid hydrolysis of raw wheat straw and fermented wheat straw (biological pre-treated wheat straw) was explored with a hope that a suitable sugar solution could be generated.

6.1.1 Acid hydrolysis of non-treated wheat straw

A set of trial experiments using acid to hydrolyse non-treated wheat straw was carried out. This experiment was performed by using a microwave reactor, (Monowave Anton Paar Ltd, UK) and 1% (w/w) H_2SO_4 as the catalytic reagent. The effect of wheat straw particle size, retention time and temperature was investigated. In each experiment, 0.5 g wheat straw was added to a reactor tube and sulphuric acid solution was added to achieve a solid to liquid ratio of 1:10. A multi-level factorial design experiment was applied in this study. The ranges of factor and experimental design are shown in Tables 6.1 and 6.2. Non-treated wheat straw was fractionated by passing through sieves with the sizes of 75, 250 and 425 mm, respectively. Biomass powders that have a particle size less than 75 mm, between 75 to 250 mm and between 250 to 425 mm were designated as wheat straw with a particle size of 75, 250 and 425 mm, respectively. The retention time and temperature of hydrolysis were chosen from the design table below, and both were automatically controlled by the Monowave machine reactor. After treatment the levels of glucose, xylose and selected inhibitors (HMF and Furfural) were determined.

Table 6. 1 Factor and level of variable used in the multi-level factorial design for acid hydrolysis.

Factors	Symbol	Code level		
		-1	0	+1
		Actual level		
particle size (mm)	A	75	250	425
Time (min)	B	5	10	15
Temperature (˚C)	C	160	180	200

Table 6. 2 Multi-level factorial experimental design.

Block	Particle size (mm)	Time (min)	Temperature (˚C)	Block	Particle size (mm)	Time (min)	Temperature (˚C)
1	425	10	200	2	425	15	160
1	250	5	180	2	250	5	180
1	425	15	160	2	425	5	200
1	250	10	200	2	75	5	160
1	75	15	160	2	75	15	200
1	75	5	160	2	250	10	180
1	250	5	200	2	425	15	180
1	75	15	180	2	250	15	180
1	250	5	160	2	250	10	160
1	250	10	180	2	75	15	180
1	425	10	180	2	425	5	160
1	250	15	160	2	75	5	200
1	250	15	200	2	250	10	200
1	425	15	200	2	425	10	200
1	75	5	180	2	75	10	200
1	425	15	180	2	75	10	160
1	425	5	180	2	250	5	200
1	250	10	160	2	250	5	160
1	250	15	180	2	75	15	160
1	75	10	180	2	250	15	200
1	75	15	200	2	425	10	160
1	425	10	160	2	425	10	180
1	425	5	160	2	425	15	200
1	425	5	200	2	425	5	180
1	75	5	200	2	75	10	180
1	75	10	200	2	75	5	180
1	75	10	160	2	250	15	160

Unfortunately this experimental design could not be fully accomplished because of the upper pressure limit of the Monowave. The reactions at high temperature build up pressure. In this case, when the temperature over 180˚C was set in the Monowave, the machine automatically terminated the run. So this experiment was run at only 36 conditions with temperatures at 160˚C and 180˚C. The order of the experiments was fully randomized.

The effect on glucose and inhibitor production is shown in Table 6.3. The responses of the multi-level factorial were fitted with an orthogonal design (show in Equation 6.1 below for glucose as an example).

Equation 6.1:

$$Glucose\ concentration\ (Y, \frac{g}{L}) = -29.48 - 0.03A + 1.35B + 0.22C + 0.01A^2 + 0.01AB + 0.01AC - 0.02B^2 - 0.01BC$$

Where Y is glucose concentration and A is particle size of substrate, B is retention time and C is temperature. The R-Squared was 97.23%, indicating that the model fitted the experimental results well.

Furthermore the statistical significance of the equation was evaluated using the P-value of analysis of variance (ANOVA). It then tested the statistical significance of each effect by comparing the mean square against an estimation of the experimental errors. In this case, particle size, retention time and temperature had P-values of less than 0.05, indicating that they were significantly different at the 95.0% confidence level. (The ANOVA table for multi-level factorial design on glucose releasing can be found in the appendix Table 6.14)

Table 6. 3 Experimental design and results of multi-level factorial design on glucose, xylose, HMF and furfural production

Row	Actual level			Glucose concentration (g/L)	Xylose concentration (g/L)	HMF concentration (g/L)	Furfural concentration (g/L)
	Particle size (mm)	Time (min)	Temperature (˚C)				
1	425	15	160	8.416	14.238	0.208	1.906
2	75	10	160	8.819	15.751	0.521	2.190
3	425	15	180	12.305	0.477	0.367	2.091
4	425	5	160	6.529	19.765	0.050	1.373
5	250	15	160	8.479	14.394	0.201	1.680
6	75	15	160	9.480	14.044	0.272	1.969
7	75	5	180	12.469	8.081	0.280	1.938
8	250	5	180	11.757	8.163	0.199	1.808
9	250	10	160	8.071	18.108	0.460	2.064
10	425	5	180	10.882	7.831	0.306	2.031
11	425	10	160	7.744	17.219	0.385	2.025
12	250	5	160	6.988	19.364	0.203	1.525
13	425	10	180	13.428	4.094	0.523	2.037
14	75	15	180	12.441	0.887	0.144	1.852
15	250	10	180	12.803	4.337	0.330	1.915
16	250	15	180	12.024	0.911	0.146	1.656
17	75	10	180	12.395	4.190	0.401	2.122
18	75	5	160	7.866	18.338	0.450	2.187
19	75	15	160	9.487	14.144	0.172	1.969
20	250	10	180	12.703	4.070	0.530	1.915
21	425	15	180	12.105	0.777	0.467	2.091
22	250	15	180	12.024	0.911	0.146	1.656
23	425	5	160	6.629	19.765	0.050	1.073
24	75	15	180	12.441	0.880	0.144	1.852
25	75	5	180	12.469	8.481	0.210	1.838
26	75	5	160	7.866	18.338	0.450	2.187
27	425	15	160	8.416	14.238	0.168	1.906
28	250	5	180	10.663	8.178	0.673	2.100
29	75	10	160	8.829	15.751	0.521	2.190
30	425	5	180	10.782	7.831	0.516	2.231
31	250	10	160	8.071	18.178	0.460	2.064
32	425	10	180	13.528	4.194	0.123	1.537
33	425	10	160	7.764	17.329	0.585	2.225
34	75	10	180	12.385	4.291	0.441	2.122
35	250	15	160	8.479	14.394	0.231	1.680
36	250	5	160	6.984	14.238	0.103	1.525

The effect of the investigated factors on glucose production are shown in Figures 6.1 and 6.2. The glucose production increased with both temperature and retention time. In contrast wheat straw particle size did not affect glucose production.

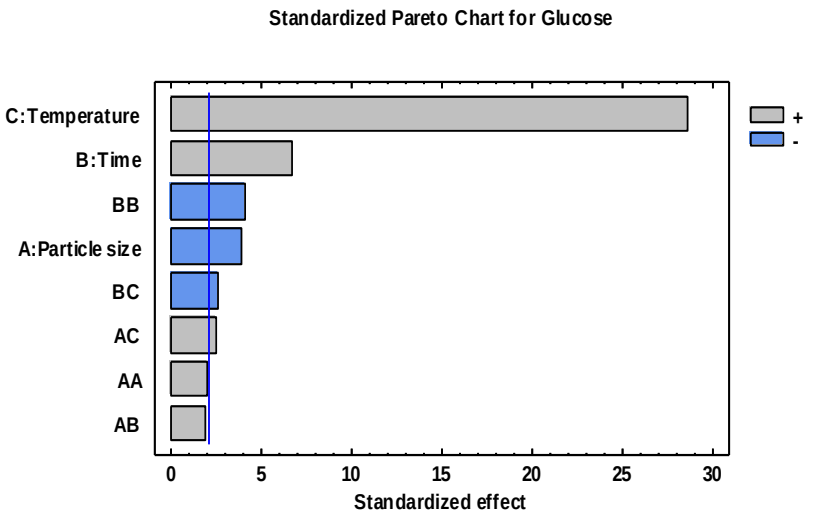


Figure 6. 1 Pareto chart for glucose production in multi-level factorial design.

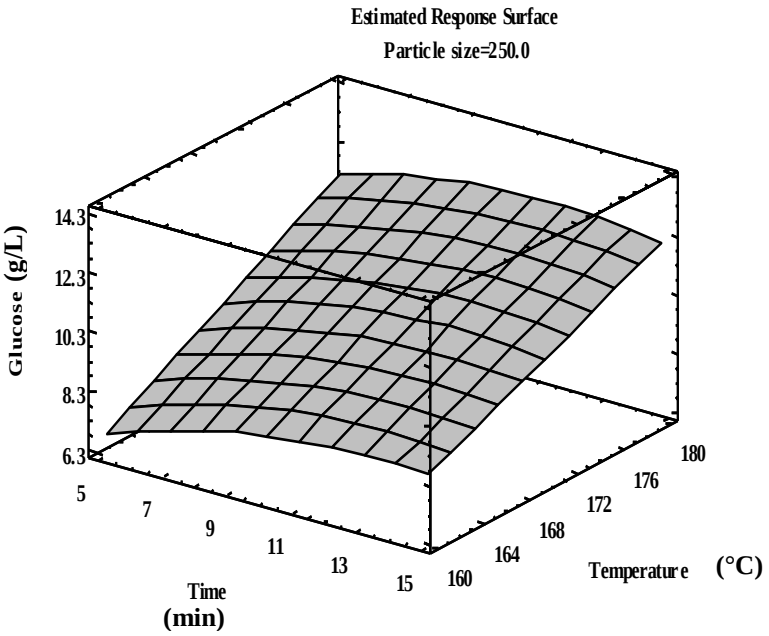


Figure 6. 2 The forecast model of combination factor (time and temperature) of glucose liberation

Xylose liberation is shown in Table 6.4. The experimental data fitted well with the predicted model (R^2 = 99.74%) shown in Equation 6.2 below. And the results showed that each parameter had a significant effect at the 95.0% confidence level.

Equation 6.2:

$$Xylose\ concentration(Y, \frac{g}{L}) = 93.08 + 0.04A + 1.49B - 0.46C - 0.01A^2 + 0.01AB - 0.01AC - 0.01B^2 - 0.01BC$$

Statistical analysis of the experimental data showed that all three independent variables (particle size, retention time and temperature) significantly influenced xylose production in the acid hydrolysis. These effects are shown in Figures 6.3 and 6.4. The result shows that highest xylose production of 19.76 g/L was achieved at 160˚C for 5 min. This Pareto chart indicated that higher temperatures and longer retention times both had negative affects on xylose production. This may be because hemicellulose in wheat straw is not rigidly structured therefore it does not require high reaction rates to release xylose from the structure. This result was also supported by Quintero-Ramirez (2008) who suggested that xylose production required less harsh conditions than cellulose in acid hydrolysis due to hemicellulose is an amorphous polymer which could be easily degraded by acid (Quintero-Ramirez, 2008). Thus the xylose was probably released rapidly, the subsequent reduction may be related to the fact that the xylose could then be degraded further into a range of inhibitory products.

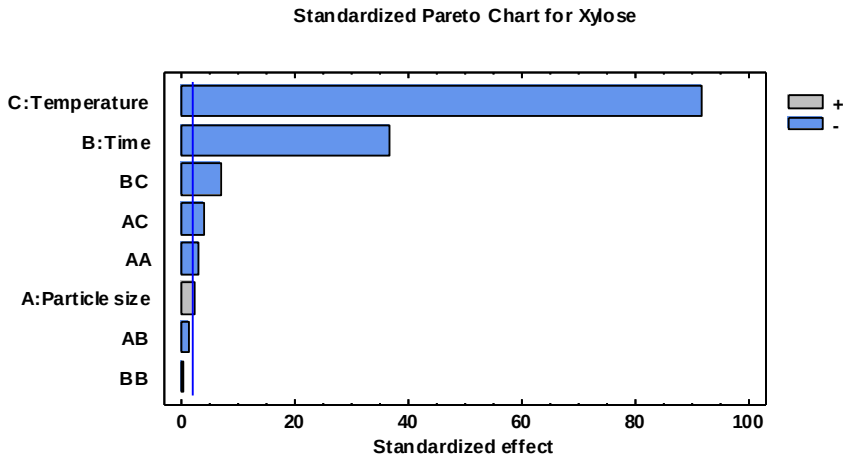


Figure 6. 3 Pareto chart for xylose production in multi-level factorial design.

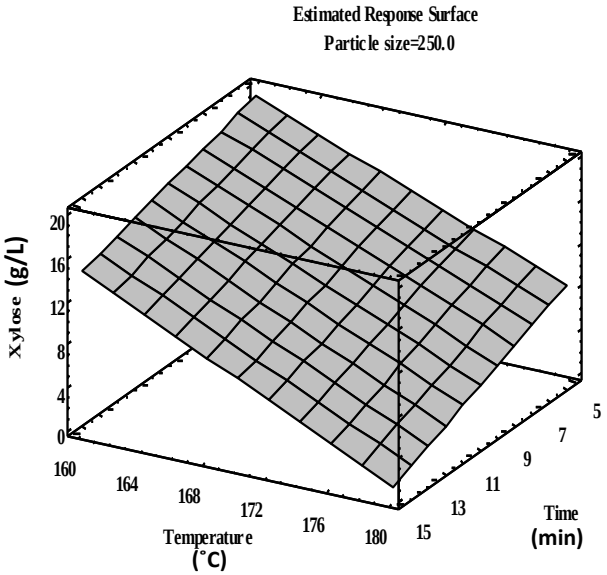


Figure 6. 4 The forecast model of combination factor (time and temperature) of xylose production

HMF formation during the acid hydrolysis process is shown in Table 6.4. The estimation of each parameter's effect on HMF generation is shown in Figures 6.5 and 6.6. Retention time had a negative impact on HMF formation. This may be because HMF was further degraded at high temperature and long

retention time. Contrastingly, HMF formation was positively influenced by temperature. This result is in agreement with the finding of many other researchers (Montané *et al*., 2002, Roberto *et al*., 2003). Dussán *et al*. (2014) has also reported that higher temperatures in the acid hydrolysis of sugarcane bagasse increased HMF formation and Karimi *et al.* (2006) claimed that in acid hydrolysis pressure also had a positive influence on HMF generation.

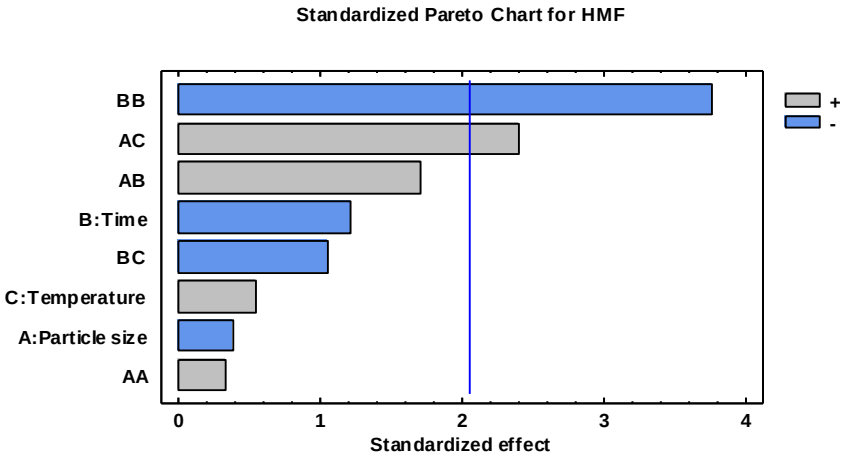


Figure 6. 5 Pareto chart for HMF formation during acid hydrolysis

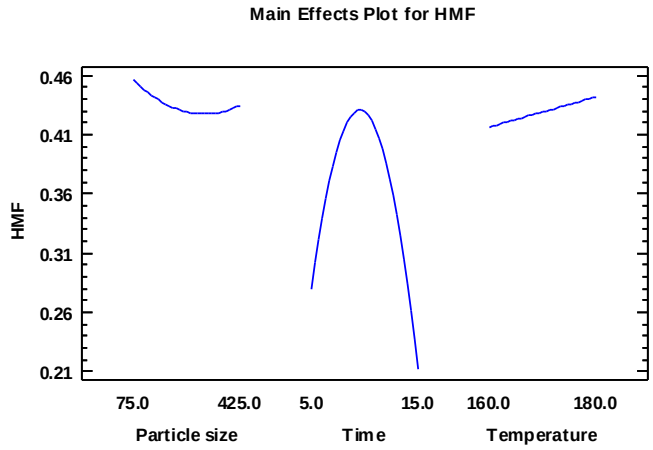


Figure 6. 6 Main effects plot for HMF formation during acid hydrolysis

Furfural formation during acid hydrolysis is shown in Table 6.4. The effect of each parameter on furfural generation is presented in Figures 6.7 and 6.8. The furfural formation showed a similarl trend to that for HMF. The increase in temperature led to higher furfural formation and the length of retention time encouraged furfural generation. The long retention time in catalytic reaction facilitate the degradation of this compound to weak acid residues (Jönsson et al., 2013a).

There was no significant difference in inhibitor formation in experiments using wheat straw with different particle sizes in the range investigated in this study. A similar result was reported by Yemiş and Mazza (2012) who found that temperature and retention time had positive effects on furfural yield. However, these inhibitors generated were not stable. HMF and furfural could turn into formic acid under acid conditions (Ertas *et al*., 2014, Larsson *et al*., 1999). In this study, they were degraded when the reaction run at long retention time and high temperature. This result was also supported by O'Neill *et al.* (2009) who reported furfural production was sensitive to high temperature.

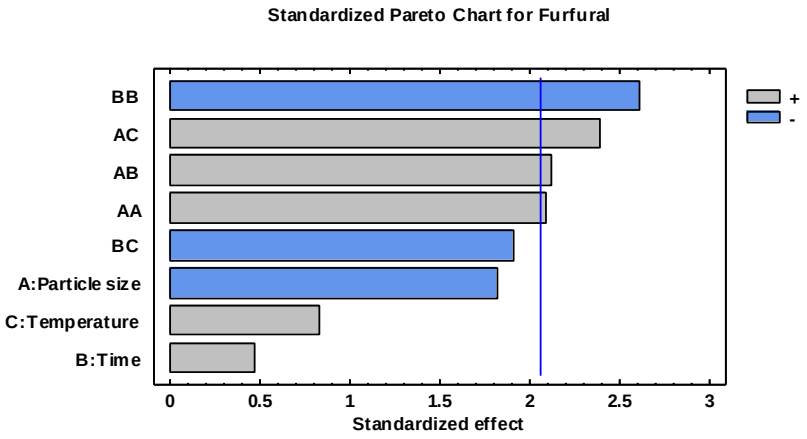


Figure 6. 7 Pareto chart for furfural formation during acid hydrolysis

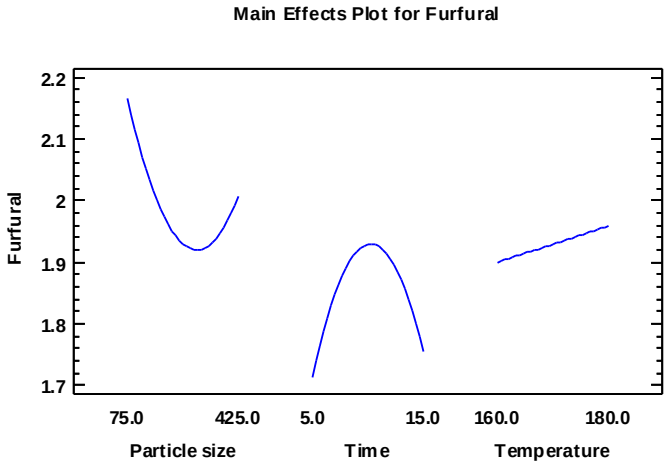


Figure 6. 8 Main effect plot for furfural formation during acid hydrolysis

In this experiment, the highest glucose concentration of 13.43 g/L obtained was using 1% (w/w) H_2SO_4 at 180˚C for 10 min. This equates to cellulose hydrolysis yield of 42.39%. Under the same conditions, 4.09 g/L xylose was generated. High amounts of HMF (0.52 g/L) and furfural (2.04 g/L) were found compared with other acid hydrolysis conditions in this experiment. This indicated that high temperature in acid hydrolysis, as expected, boosted the catalytic reactions for both sugar hydrolysis and inhibitor formation.

Since acid hydrolysis at high temperature led to high amount of HMF and furfural formation and glucose releasing from the biomass (around 13.48 g/L from treated at 180°C for 10 min) was less than amount required in fermentation, further investigation was necessary to develop an efficient process to hydrolyse the wheat straw.

6.1.2 Acid hydrolysis of fermented wheat straw

It is possible that the fermentation of the wheat straw described in previous chapters may have impacted the recalcitrance of the residual polysaccharides to acid hydrolysis. The aim of this experiment was to thus compare the performance of raw and fermented wheat straw through acid hydrolysis. As for the raw straw, Respond Surface Methodology (RSM) was again used. The ranges and levels of factors are shown in Table 6.4.

Table 6. 4 Factors and code level and actual level of acid hydrolysis experimental design

Independent variables	Symbols	Coded level				
		-1.68	-1	0	+1	+1.68
		Actual level				
Temperature (˚C)	X_1	52.8	80	120	160	187.2
Acid concentration (% w/w)	X_2	0.0034	0.51	1.255	2	2.5066
Time (min)	X_3	6.36	10	15	20	23.24

This experimental design had 24 runs including 9 replications of the centre point. The order of the experiments was fully randomized. In each experiment, 0.5 g of dried fermented wheat straw was added to a reactor tube and sulphuric acid solution was added to achieve a solid/liquid of 1:10. This hydrolysis volume was kept constant. The glucose or other sugar concentrations in hydrolysate were determined by Dionex Ion Chromatography.

The response of central composite design experiment was analysed to optimise acid hydrolysis condition and the behaviour of acid hydrolysis on fermented wheat straw was explained with a second order polynomial equation.

Equation 6.3:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{22}x_2^2 + b_{23}x_2x_3 + b_{33}x_3{}^2$$

where Y is predicted response (glucose concentration) and b_0 is constant while b_1, b_2, b_3 are linear effects, b_{11}, b_{22}, b_{13} are interaction terms.

The experimental design and results of acid hydrolysis on glucose production are shown in Table 6.5.

Table 6. 5 Experimental design and results of acid hydrolysis of fermented wheat straw using RSM

Run	Factor			Glucose concentration (g/L)	
	Actual level			Experiment	Predicted
	X_1	X_2	X_3		
1	120	1.255	15	0.698	0.657431
2	160	0.51	10	1.733	1.10877
3	120	1.255	15	0.71	0.657431
4	120	0.0034	15	0.111	-0.830225
5	187.2	1.255	15	4.9035	6.21755
6	80	2	10	0.097	-1.32214
7	160	0.51	20	1.017	1.50407
8	120	1.255	15	0.686	0.657431
9	52.8	1.255	15	0.142	0.146082
10	160	2	10	4.82	4.17921
11	80	0.51	10	0.119	1.37467
12	120	1.255	15	0.671	0.657431
13	120	1.255	6.60	0.271	0.671036
14	120	1.255	15	0.668	0.657431
15	120	1.255	23.40	1.056	1.9741
16	80	0.51	20	0.0765	-0.214779
17	120	1.255	15	0.684	0.657431
18	120	1.255	15	0.668	0.657431
19	120	1.255	15	0.692	0.657431
20	160	2	20	9.506	7.31826
21	120	1.255	15	0.666	0.657431
22	80	2	20	0.14	-0.167836
23	120	2.5066	15	0.631	1.79118
24	120	2.5066	15	0.692	1.79118

Then response surface optimisation analysis was applied to the experimental data to investigate the relationships between these 3 factors (temperature, acid concentration and retention time) on glucose production. The prediction model of optimisation point for glucose production was created which is shown in Equation 6.4 below.

Equation 6.4:

$$\text{Glucose concentration, Y } \left(\frac{\text{g}}{\text{L}}\right) = 53.92 - 0.55x_1 - 25.57x_2 - 2.08 + 0.01x_1^2 + 0.15x_1x_2 + 0.01x_1{x_3}^2 + 1.14x_2^2 + 0.59x_2x_3 + 0.02x_3^2$$

The experimental data fitted the model with a R^2 of 89.05%. The experimental results and predicted results from the model for glucose concentration are compared

A model that describes relationship of these 3 factors (temperature, acid concentration and time) on glucose production is shown in Table 6.6. Temperature and acid concentration both shown significant influence on glucose generation from acid hydrolysis at the 95.0% confidence level, since their P-value were less than 0.05 (Table 6.6).

Table 6. 6 ANOVA for response surface design in acid hydrolysis of fermented wheat straw.

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
X_1:Temperature	422.367	1	422.367	49.23	0.0000
X_2:Acid concentration	132.215	1	132.215	15.41	0.0017
X_3:Time	20.9183	1	20.9183	2.44	0.1424
X_1^2	101.097	1	101.097	11.78	0.0045
X_1X_2	161.191	1	161.191	18.79	0.0008
X_1X_3	21.2878	1	21.2878	2.48	0.1392
X_2^2	6.35303	1	6.35303	0.74	0.4051
X_2X_3	38.3163	1	38.3163	4.47	0.0545
X_3^2	4.10414	1	4.10414	0.48	0.5013
Total error	111.542	13	8.58017		
Total (corr.)	1018.71	22			

Note

R-squared = 89.0506 percent — R-squared (adjusted for d.f.) = 81.4703 percent

Standard Error of Est. = 2.92919 — Mean absolute error = 1.43196

Durbin-Watson statistic = 1.61726 (P=0.1516 — Lag 1 residual autocorrelation = 0.130505

In order to explore the effect of acid hydrolysis condition on glucose production, the response surface mesh plot was created by plotting the response value against three independent variables. The predicted values were presented by points in various colours. The high values of predicted data were spotted in red colour shown in Figure 6.9. It is shown that all 3 factors have intensely affected glucose production.

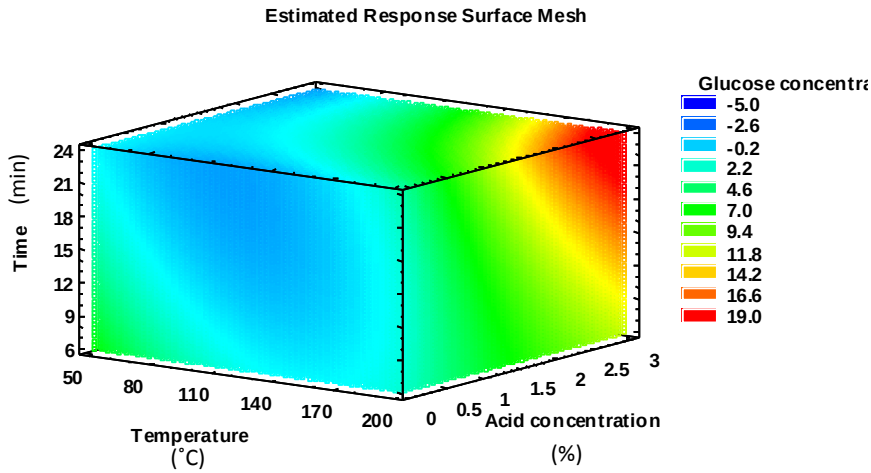


Figure 6. 9 Response surface mesh plot on glucose concentration from fermented wheat straw by acid hydrolysis.

The optimal condition for glucose production from fermented wheat straw in acid hydrolysis was predicted by RSM. It was proposed that 2.51% (w/w) H_2SO_4 at 187˚C for 23 min was the optimal condition for acid hydrolysis of fermented wheat straw. It was predicted that under this condition 15.65 g/L of glucose could be produced. The actual acid hydrolysis experiment at this condition was not performed due to the fact that this condition is too harsh for the Monowave.

This predicted result was different from Guerra-Rodriguez *et al* (2012) who proposed that the optimal condition of acid hydrolysis of wheat straw was at 130°C for 180 min with 5% (w/w) H_2SO_4. Only 7.3 g/L of glucose was presented in hydrolysate when 5% solid loading rate was employed. With this condition, the saccharification yield was 18% and 4.0 g/L of furfural was found (Guerra-Rodríguez *et al*., 2012). The difference may be because the character of wheat straw was different to fermented wheat straw used in this study. This suggestion was supported by El-Zawawy *et al*. (2011) who reported that the glucose production from the acid hydrolysis depended on the character of biomass.

Based on the previous experimental results obtained from acid hydrolysis of non-treated wheat straw, 0.52 g/L of HMF and 2.04 g/L of furfural were obtained in a process using 1% H_2SO_4 at 180˚C for 10 min. Generally, high temperature and high acid concentration in acid hydrolysis would increase HMF and furfural formation (Taherzadeh and Karimi, 2007a). Hence acid hydrolysis by 2.51% (w/w) H_2SO_4 at 187˚C for 23 min on fermented wheat straw may generate furfural higher than 2.04 g/L. Hydrolysate contains furfural at this level are not favourable for alcohol fermentation. Wikandari *et al.*,

(2010) reported that only 0.5 g/L of furfural and HMF reduced both ethanol yield and ethanol production. It was also showed that 1.0 g/L of furfural presented in medium were decreased ethanol yield around 26% and ethanol productivity was decreased by 73%.

Beside inhibitor formation in acid hydrolysis, the concentration of fermentable sugar obtained from acid hydrolysis was still less than that required for fermentation. The direct acid hydrolysate of fermented wheat straw was not suitable for glucose generation.

6.2 Enzymatic hydrolysis

Enzymatic hydrolysis is another method to convert cellulose and hemicellulose into fermentable sugars. Compared with direct acid hydrolysis, enzymatic hydrolysis process has several advantages, such as it is carried out at relatively low temperature (around 55^{o}C) and it does not generate inhibitor compounds. Therefore enzymatic hydrolysis has been widely applied for biomass hydrolysis (Table 6.7).

Table 6. 7 Typical operation conditions for enzymatic hydrolysis of various biomass.

Substrate	Hydrolysis condition	Reference
Algal biomass	5% SL, 19.5 FPU/g at 50 °C for 72 h. (150 rpm)	(Chen et al., 2012)
Corn cob	20 FPU/g at 50˚C , pH 4.8 for 48 h	(Chen et al., 2007)
Corn stover	25% SL , 20 FPU/g	(Chen et al., 2011)
Oil palm empty fruit bunch	5% SL, 20 FPU/g at 50 °C for 72 h	(Zakaria et al., 2015)
Spruce	10%SL at 45 °C (170 rpm)	(Soudham et al., 2014)
Sugarcane bagasse	10 FPU/g at 50 ˚C pH 4.8 for 96 h (180 rpm)	(Batalha et al., 2015)
Wheat straw	2% SL, at 55˚C pH 4.5 for 90 h (160 rpm)	(Zhang et al., 2015)

Note: SL is solid to liquid ratio in enzymatic hydrolysis.

6.2.1 Enzymatic hydrolysis of fermented wheat straw by using commercial enzyme

Commercial cellulolytic enzyme was used in enzymatic hydrolysis of wheat straw in order to find out the optimum enzymatic hydrolysis condition.

A trial experiment was set up to investigate the effect of substrate characteristics on glucose production during enzymatic hydrolysis using a commercial cellulase, Ctec 2(Novozyme, Denmark). Fermented wheat straw, autoclaved wheat straw and non-treated wheat straw were used in this experiment. Biomass (2g) was weighted and added to a 100 ml Erlenmeyer flask containing 30 FPU/g in 40 ml of buffer. The enzymatic hydrolysis was performed at 50˚C for 49 h. The hydrolysis samples were taken at certain time intervals until 49 h. The results were shown in Figure 6.10.

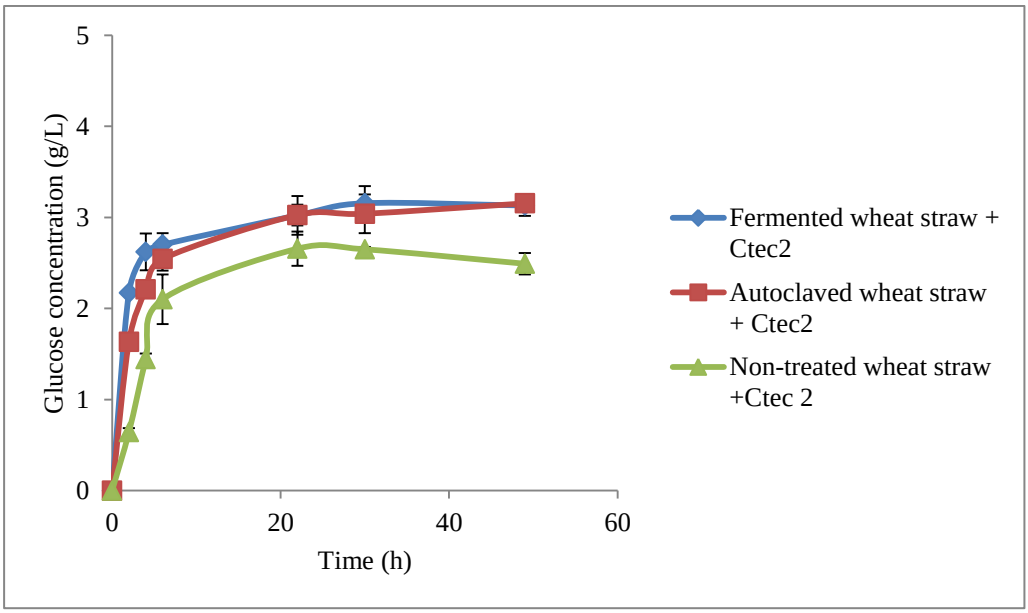


Figure 6. 10 Hydrolysis profile on various modified wheat straw at 5% solid loading rate, enzymatic loading rate 30 FPU/g, 50˚C for 49 h. Data are mean values of three replicates and error bars indicate standard deviations (SDs)

In the first 6 h of enzymatic hydrolysis, glucose was rapidly released no matter which substrate was used. After that, the glucose concentrations in the

hydrolysis experiments using both fermented wheat straw and autoclaved wheat straw increased along with reaction time; while glucose concentration in the hydrolysis using non-treated wheat straw decreased after 22 h. The reduction of glucose might be due to contamination with microorganisms as a wide range of fungi were used in the lab while this experiment was running. The final glucose concentrations obtained in experiments using both fermented wheat straw and autoclaved wheat straw were higher than that obtained from non-treated wheat straw. This result might lead to the conclusion that the structure of biomass affected glucose release during enzymatic hydrolysis (Kumar *et al*., 2009). As cellulose in raw biomass is covered with hemicellulose and lignin, it is not directly available for hydrolysis by enzymes (Zheng et al., 2009b). In this experiment, the lignin content in pretreated wheat straw was significantly lower than that in non-treated wheat straw (Table 5.3). The lignin removal in biomass would increase accessible surface area for enzymes to hydrolyse cellulose, which enhanced the hydrolysis rate. Yan *et al* studied the effect of lignin content in sweet sorghum on enzymatic hydrolysis rate. They reported that lignin content had shown negative correlation to hydrolysis rate (Yan *et al*., 2014). Zhang *et al*. (2007) also found that rubber wood pretreated by white rot fungi, which had lower lignin and hemicellulose content in biomass showed improved enzymatic hydrolysis.

Contrastingly, Siti Aisyah *et al.* (2014) reported an enzymatic hydrolysis of lignin-free biomass which was generated from hydrothermal pre-treatment with the addition of alkali. This biomass had a lower enzymatic hydrolysis rate than control. It was explained that the biomass was covered with phenolic compound which had been generated during pretreatment step.

Although hydrolysis using fermented wheat straw produced higher amount of glucose than the raw wheat straw in this experiment, the glucose level is too low for a fermentation process. In order to improve glucose concentration, the optimisation of enzymatic hydrolysis on fermented wheat straw was investigated. Besides the characteristics of biomass, solid loading rate, enzyme loading rate and hydrolysis condition were considered to be key factors for enzymatic hydrolysis (Taherzadeh and Karimi, 2007b). In this case, hydrolysis condition are mixing rate only due to optimum temperature and pH for enzymatic hydrolysis were already provided by the reference document from Novozyme.

6.2.2 Optimisation for enzymatic hydrolysis on fermented wheat straw

Experimental design was carried out as above to study the effect of 3 factors: substrate concentration, enzyme loading rate and mixing rate on glucose production by enzymatic hydrolysis. The range and level of the factors are shown in Table 6.8.

A total 23 sets of experiments including 9 central points were designed and run in a single block as shown in Table 6.9. The order of the experiments was fully randomized.

Table 6. 8 Factors, code level and actual level of experimental design for enzymatic hydrolysis of fermented wheat straw.

Independent variables	Symbols	Coded level				
		-1.68	-1	0	+1	+1.68
		Actual level				
Substrate concentration (%)	X_1	1.6	5	10	15	18.4
Enzyme loading rate (U/g)	X_2	4.8	15	30	45	50.1
Mixing rate (rpm)	X_3	99.6	120	150	180	200.4

The prediction model of optimisation point for glucose production is shown in an Equation 6.5 below.

Equation 6.5:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{22}X_2^2 + b_{23}X_2X_3 + b_{33}X_3^2$$

where Y is the predicted response (glucose concentration), and b_0 is a constant. While b_1, b_2, b_3 are linear effects, b_{11}, b_{22}, b_{13} are interaction terms.

The relationships between these 3 factors are shown in Equation 6.6.

Equation 6.6:

$$Glucose\ concentration, Y\ \left(\frac{g}{l}\right) = -87.44 + 0.30x_1 + 0.33x_2 + 1.10x_3 + 0.01x_1^2 + 0.05x_1x_2 + 0.01x_1x_3 - 0.02x_2^2 + 0.01x_3 - 0.01x_3^2$$

The predicted responses of glucose concentration using Equation 6.6 are given in Table 6.10, and compared with actual experimental data. One additional centre point (0,0,0) was added in order to ensure that the model has accounted for measurement noise error and the assumption of the region around the centre point must be flat is valid. The easiest way to test this hypothesis is of course to add the centre point to model.

Table 6. 9 Experimental design and results of RSM on glucose production by enzymatic hydrolysis

Run	Factor			Glucose concentration (g/L)	
	Actual level			Experiment	Predicted
	X_1	X_2	X_3		
1	5	15	120	6.41	5.71
2	5	15	180	6.52	4.73
3	5	45	120	11.16	8.51
4	5	45	180	26.16	20.17
5	15	15	120	18.77	23.92
6	15	15	180	23.64	25.45
7	15	45	120	41.10	42.05
8	15	45	180	56.36	56.22
9	1.6	30	150	3.66	9.87
10	18.4	30	150	60.52	55.50
11	10	4.8	150	6.50	3.44
12	10	50.1	150	27.42	31.67
13	10	30	99.6	17.72	15.69
14	10	30	200.4	23.56	26.78
15	10	30	150	35.17	31.78
16	10	30	150	37.84	31.78
17	10	30	150	31.91	31.78
18	10	30	150	33.30	31.78
19	10	30	150	30.24	31.78
20	10	30	150	30.67	31.78
21	10	30	150	28.65	31.78
22	10	30	150	28.67	31.78
23	10	30	150	28.47	31.78
24	10	30	150	29.76	31.78

Table 6. 10 Analysis of variance for glucose concentration in the optimisation of enzymatic hydrolysis using fermented wheat straw

Source	***Sum of Squares***	***Df***	***Mean Square***	***F-Ratio***	***P-Value***
X_1:Substrate concentration	2512.76	1	2512.76	125.28	**0.0000**
X_2:Enzyme loading rate	962.041	1	962.041	47.97	**0.0000**
X_3:Mixing rate	148.685	1	148.685	7.41	**0.0174**
X_1^2	1.63595	1	1.63595	0.08	0.7797
X_1X_2	117.504	1	117.504	5.86	**0.0309**
X_1X_3	3.15005	1	3.15005	0.16	0.6983
X_2^2	401.757	1	401.757	20.03	**0.0006**
X_2X_3	79.8848	1	79.8848	3.98	0.0674
X_3^2	220.748	1	220.748	11.01	**0.0056**
Total error	260.742	13	20.0571		
Total (corr.)	4705.49	22			

Note

R-square	94.4588 percent	R-square (adjust for d.f.)	90.6225 percent
Standard Error of Est.	4.47851	Mean absolute error	2.82549
Durbin-Watson statistic	2.17027 (P=0.6889)	Lag 1 residual autocorrelation	-0.115121

The impact of each factor on the glucose concentration is shown in Table 6.10. It also shows the statistical significance of each effect by comparing the mean square against an estimated experimental error. In this case, all individual 3 factors had P-values less than 0.05, indicating that they were significantly different at the 95.0% confidence level.

The R-Squared statistic indicated that the model fitted 94.46% of the variability in glucose concentration while the coefficient of variation (CV %) and the standard deviation (SD) were 4.48 and 2.83, respectively.

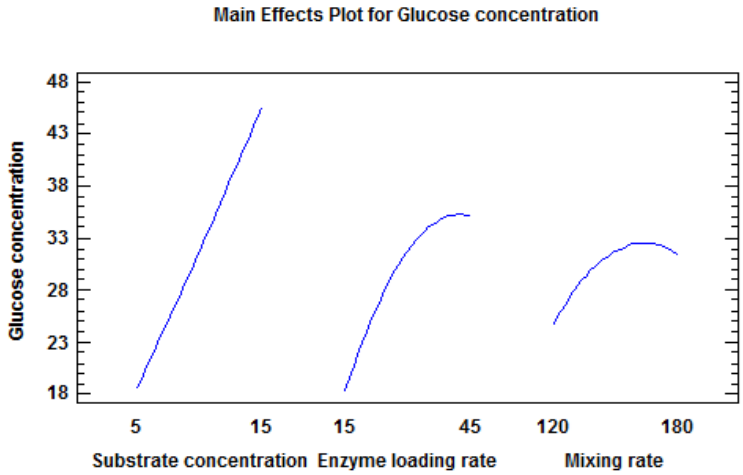


Figure 6. 11 The relationship between individual factors with glucose released during hydrolysis.

The trend of each factor's effect on glucose liberation is shown in Figure 6.12. The substrate concentration showed a linear relationship with glucose liberation, which means the substrate concentration significantly affected the glucose concentration that was obtained during the enzymatic hydrolysis. While the relationship between enzyme loading rate and mixing rate on glucose liberation fitted with polynomial trend lines.

The improvement of glucose production by increasing solid loading rate was in agreement with Chen *et al* (2007) who reported that rising solid loading rate of

corn cob from 5 to 15% led to an increased glucose concentration from around 25 g/L to almost 60 g/L after 48 hours of hydrolysis. Contrastingly, the high solid loading rate showed negative effect on sugar yield of hydrolysis of corn cob. The sugar yield was reduced from 70% to 50% when solid loading rate was increased from 5 to 15% (Chen *et al*., 2007). A similar behaviour was found when chipped spruce was used as substrate. It was also reported that when solid loading rate was increased from 5 to 10%, glucose concentration was nearly doubled from 23 to 45 g/L (Tengborg *et al*., 2001).

Regarding the hydrolysis rate, some papers concluded that lower solid loading rate could benefit glucose conversion rate (Galbe and Zacchi, 2002). Zhong *et al*. (2009) found that when glucan loading was increased from 1 to 3%, it led to a 3.1% decrease in conversion rate. The reduction of hydrolysis rate might have been because the limitation of mass transfer during hydrolysis (Hodge *et al*., 2008) or cellulase adsorption onto biomass (Ma *et al*., 2008). In another report, Kristensen *et al*. (2009) showed that increasing solid loading rate from 5 to 30% led to a linearly decreasing hydrolysis rate. They suggested that the reduction of hydrolysis rate may not come from the insufficient mixing, but it may be because that enzyme was inhibited by high sugar concentrations in the hydrolysis system.

It needs to be pointed out that low hydrolysis rate does not necessarily mean low glucose concentration in the hydrolysate. On the contrary, high solid loading rate normally results in high sugar concentration but low hydrolysis yield.

Generally, increasing enzyme loading rate can increase both hydrolysis yield and final glucose concentration. Galbe and Zacchi (2002) claimed that concentration of enzyme in the hydrolysis system had a huge affected on the hydrolysis rate, but leading to higher production costs.

The overall interaction between 3 factors on glucose production was plotted in a 3-dimensional (3D) plot. The high value of predicted data was presented in red colour as shown in Figure 6.12. This response surface mesh figure showed that enzyme loading rate strongly affected glucose production and it also indicated that high substrate concentration and hydrolysis with high cellulase activity in thoroughly mixed conditions would generate a higher concentration of glucose. The model predicted that the optimal enzymatic hydrolysis condition for the maximum glucose production was: substrate concentration (solid loading rate), 18.38 %; enzyme loading rate, 55.23 FPU/g; and mixing rate at 189 rpm. With this condition, the glucose concentration was predicted to achieve 72.4 g/L at 50°C in 72 hours. Then a set of experiment was carried out at this condition to validate the predicted model. The result showed that a glucose concentration of 59.8±2.1 g/L was obtained from the predicated optimal condition.

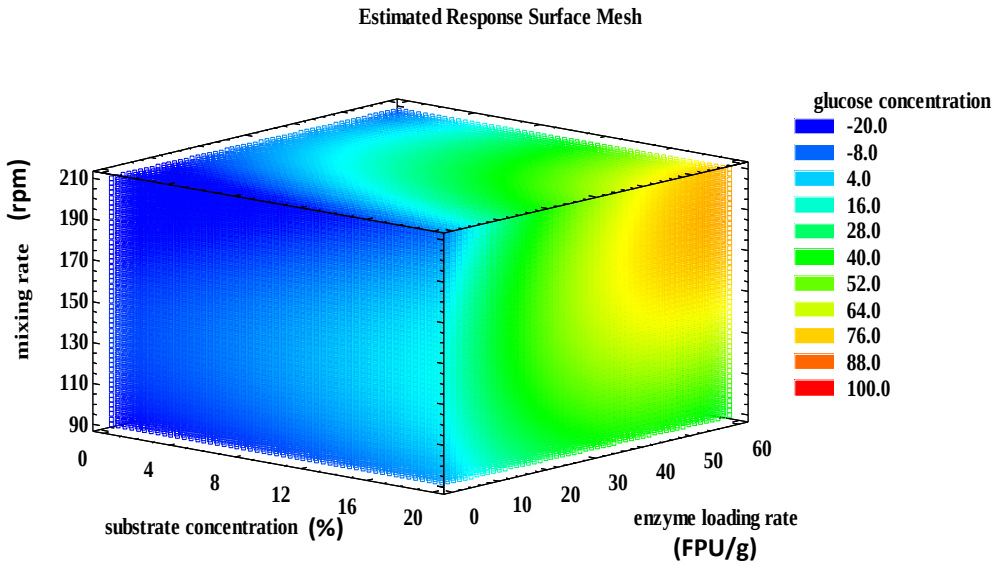


Figure 6. 12 Response surface mesh plot on glucose concentration from fermented wheat straw by enzymatic hydrolysis

Furthermore the saccharification yield from the experimental design of glucose production from fermented wheat straw through enzymatic hydrolysis was studied. The experimental data were fitted into the polynomial equation show below

Equation 6.7:

$$Saccharification\ yield(\%) = -1.30 + 0.05x_1 + 0.01x_2 + 0.02x_3 - 0.01x_1^2 - 0.01x_1x_2 - 0.01x_3 - 0.01x_2^2 + 0.01x_3 - 0.01x_3^2$$

While x_1, x_2 and x_3 are substrate concentration, enzyme loading rate and mixing rate, respectively. The response value and predicted value are shown in Table 6.11. The statistical information showed that the model was fitted with experimental data at an R-Squared of 85.86%. It confirmed that the enzyme loading rate had significantly affected saccharification yield. The prediction

model showed an optimal point for saccharification yield (70.73%) at substrate concentration of 1.65 % with 55.23 FPU/g, 200 rpm. Even though the enzymatic hydrolysis at this condition may reach 70.73 % conversion yield, but with the low substrate concentration it would definitely result in a small amount of fermentable sugar released to the hydrolysate.

Table 6. 11 Experimental design and results of RSM on Saccharification yield by enzymatic hydrolysis

Run	Factor						Saccharification yield (%)	
	Coded level			Actual level			Experiment	Predicted
	X1	X2	X3	X1	X2	X3		
1	-1	-1	-1	5	15	120	0.180	0.148
2	-1	-1	1	5	15	180	0.183	0.188
3	-1	1	-1	5	45	120	0.313	0.310
4	-1	1	1	5	45	180	0.733	0.606
5	1	-1	-1	15	15	120	0.175	0.268
6	1	-1	1	15	15	180	0.221	0.191
7	1	1	-1	15	45	120	0.384	0.345
8	1	1	1	15	45	180	0.526	0.524
9	-1.68	0	0	1.6	30	150	0.320	0.398
10	1.68	0	0	18.4	30	150	0.461	0.431
11	0	-1.68	0	10	4.8	150	0.091	0.053
12	0	1.68	0	10	50.1	150	0.384	0.469
13	0	0	-1.68	10	30	99.6	0.248	0.220
14	0	0	1.68	10	30	200.4	0.330	0.405
15	0	0	0	10	30	150	0.492	0.444
16	0	0	0	10	30	150	0.530	0.444
17	0	0	0	10	30	150	0.447	0.444
18	0	0	0	10	30	150	0.466	0.444
19	0	0	0	10	30	150	0.424	0.444
20	0	0	0	10	30	150	0.430	0.444
21	0	0	0	10	30	150	0.401	0.444
22	0	0	0	10	30	150	0.402	0.444
23	0	0	0	10	30	150	0.400	0.444
24	0	0	0	10	30	150	0.417	0.444

Although this optimal condition for saccharification yield might not be applicable in practical work, it confirmed that low solid concentration has increased saccharification yield. Chen *et al.* (2007) have investigated effect of substrate concentration on sugar yield during batch enzymatic hydrolysis of

corncob. Their result indicated that when corncob concentration rose, sugar concentration would normally show contrasting trend to hydrolysis yield.

Then the predicted optimal conditions for glucose production were rounded up to substrate concentration at 18% with enzyme loading rate of 55 FPU/g at 50˚C, 190 rpm, which was used to prepare hydrolysate for further fermentations. This condition was also applied to non-treated wheat straw in order to re-test the biological pretreatment efficiency on the biomass. The result showed that non-treated wheat straw adsorbed most of the liquid in the enzyme solution causing a low hydrolysis efficiency. After 72 hours of hydrolysis, only10.6±1.1 g/L glucose was obtained from non-treated wheat straw. This indicated that fermented wheat straw had significantly improved hydrolysis efficiency. It also worth pointing out that the solid loading rate can be scaled up to a solid loading rate of 18%; while in other studies only 5-15% solid loading rate were applied (Wang *et al*., 2011, Pryor *et al*., 2011, Han *et al*., 2012, Jin *et al*., 2013, Ertas *et al*., 2014, Zheng *et al*., 2014).

Although enzymatic hydrolysis is better than acid hydrolysis in terms of environmental friendliness, it has its own drawback. Enzymatic hydrolysis requires longer retention time than acid hydrolysis. In terms of catalyst reagent in the hydrolysis process, the price of commercial enzymes is much higher than for sulphuric acid. In order to minimise the cost of enzymatic hydrolysis, economical cellulase production should be achieved. Normally it is considered that cellulase produced from agricultural waste via solid state fermentation could reduce the cellulase production cost. Kovacs *et al* (2009) suggested that fungal cellulase from a type of given biomass would have specificity on particular substrate (Pensupa *et al*., 2013). Therefore we carried out a set of

experiments in order to test the possibility of using fungal extracts that we obtained in fungal fermentation as a catalyst in enzymatic hydrolysis. In this experiment, fungal extract obtained from solid state fermentation in the previous chapter was applied as a catalyst reagent and fermented wheat straw was used as the substrate. Hydrolysis was performed at 12% solid loading rate with 20 FPU/g, 50°C, 150 rpm for 120 h. The results showed that the hydrolysis profile was different from the normal hydrolysis curve obtained using the commercial enzymes (Figure 6.13). The glucose concentration was gradually increased in the first 12 h then it dropped after 12 h and after 120 h. This might be due to contamination in the process. The contamination may have occurred during the cellulase preparation process, where some spores may have been left in the fungal filtrate. In order to avoid contamination, sodium azide was used in the media to prevent microbial contamination (Batalha *et al*., 2015, Olsson and Hahn-Hägerdal, 1996, Srilekha Yadav *et al*., 2011). And 2.5 mg of tetracycline was also added into the hydrolysis process to prevent fungal contamination.

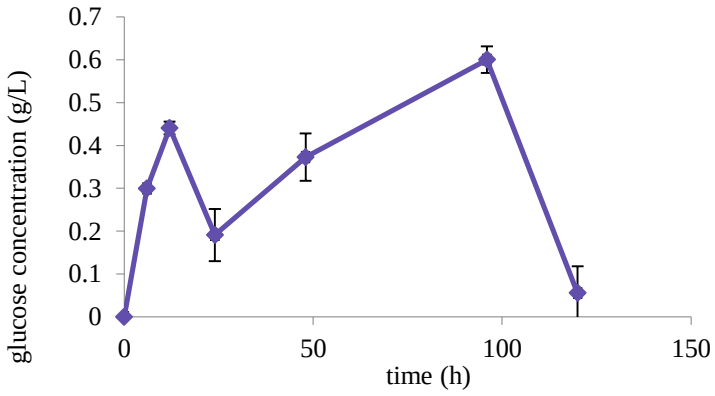


Figure 6. 13 Profile of enzymatic hydrolysis on fermented wheat straw. Hydrolysis was carried out at 50°C, 150 rpm for 120 hours. The enzyme loading was 20 FPU/g and solid loading was 12%. Data are mean values of three replicates and error bars indicate standard deviations (SDs)

A comparative study using fungal extract and commercial enzyme in enzymatic hydrolysis was carried out. Hydrolysis of different biomass was performed at 5% solid loading rate, enzyme loading rate of 30 FPU/g, 50°C 150 rpm for 72 h and 2.5 mg of antibiotic was added into hydrolysis solution since hydrolysis started.

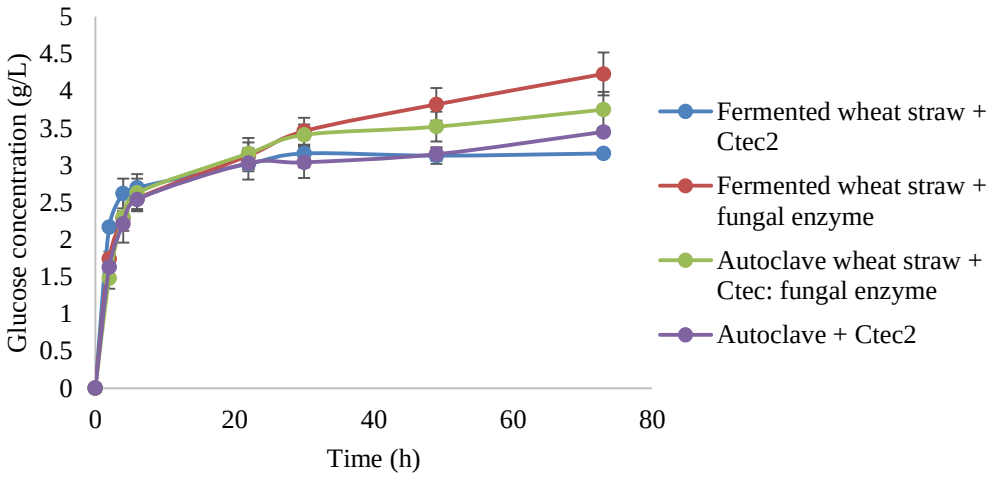


Figure 6. 14 Hydrolysis profile of fermented wheat straw and autoclaved wheat straw by fungal extract and commercial cellulase enzyme. Hydrolysis was carried out at 50°C, 150 rpm for 72 hours. The enzyme loading was 30 FPU/g and solid loading was 5%. Data are mean values of three replicates and error bars indicate standard deviations (SDs)

Hydrolysis with the presence of antibiotic in the process, the glucose concentration from all conditions was similar to a typical hydrolysis profile obtained using commercial cellulases. Glucose was rapidly released in the first 6 h and then the level of glucose gradually increased until it reached a steady state. The final glucose concentration obtained from the hydrolysis of autoclaved wheat straw by using commercial enzymes (CTec2) was equal to 3.45 g/L. and when fungal extract was added to support CTec2 to hydrolyse autoclaved wheat straw, it can further increased glucose concentration to 3.75 g/L. this has indicated that cellulase in fungal extract can be used as an enzyme support in enzymatic hydrolysis. In case of hydrolysis of fermented wheat straw by CTec2, the final glucose concentration was equal to 3.16 g/L and

when fungal extract applied as substitutive enzyme the final glucose concentration reached 4.23 g/L. This has indicated that cellulase in fungal extract was tailored for hydrolyse fermented wheat straw it does release more sugar compared with CTec2.

Furthermore the effect of solid loading rate on fermentable sugar production by using fungal extract in enzymatic hydrolysis was investigated. Solid loading rate in enzymatic hydrolysis was varied from 10 to 20% and hydrolysis condition was performed at 50°C, 150 rpm for 72 h with the fungal extract containing cellulase of 0.5 FPU/ml (total activity is equal to 20 FPU/g). The result is summarised in Table 6.12. It demonstrated that increasing substrate concentration enhanced sugar concentration in hydrolysate. However, the final glucose concentration in hydrolysate was still very low in this experiment. This may be because that the cellulase in this particular batch of fungal extract had a low enzymatic activity. The highest cellulase activity obtained from solid state fermentation from wheat straw in chapter 5 was only 1.462 FPU/ml (24.374 FPU/g) which might not be enough to generate a glucose-rich hydrolysate.

Table 6. 12 The effect of solid loading rate on fermentable sugar production by using fungal extract to substitute enzyme solution in enzymatic hydrolysis

Solid liquid ratio (%)	Hydrolysate volume (ml)	Glucose (g/L)	Xylose (g/L)
10	20.750±0.354	0.799±0.0113	2.360±0.0740
15	15.250±1.061	1.085±0.046	3.150±0.096
20	9.625±0.177	1.366±0.006	3.793±0.018

Note: Hydrolysis was carried out at 50°C, 150 rpm for 72 hours. The enzyme loading was 30 FPU/g. Data are mean values of three replicates and error bars indicate standard deviations (SDs)

Another problem which occurred when high solid loading rate was applied during hydrolysis was the lack of free water. The biomass normally adsorbed most of free water in the hydrolysis system, which led to insufficient mixing

and reduced hydrolysis rate. Therefore fed-batch model was proposed in the enzymatic hydrolysis. Tai *et al*. (2015) studied fed-batch strategy on enzymatic hydrolysis of corn stover and the result showed that glucose concentration obtained from fed-batch process was 108.76% higher than in batch hydrolysis under the same hydrolysis conditions. Hodge *et al*. (2009) has reported that 25% solid loading rate of corn stover could be achieved in a fed-batch hydrolysis system. And they claimed that with fed-batch strategy, it could increase hydrolysis yield up to 80%.

Then fed-batch hydrolysis on the fermented wheat straw by using CTec2 was studied, and it was compared with a batch fermentation operated at the same condition. The batch hydrolysis was started with a solid loading rate of 12%, an enzyme loading rate of 30 FPU/g at 50˚C, 150 rpm for 6 h. For fed-batch hydrolysis, it was performed at the same conditions except that 2% of solid biomass was added every hour until the experiment finished at 6h. The results showed that fed-batch hydrolysis resulted in increase the mean value of glucose concentration (3 replicates) but that the difference was not statistically significant compared to batch hydrolysis (Figure 6.15). These results agreed with (Cui et al., 2014) who reported that in fed-batch hydrolysis process the cellulose conversion rate was relatively stable compared with the batch hydrolysis process, so it could lead to a higher glucose concentration.

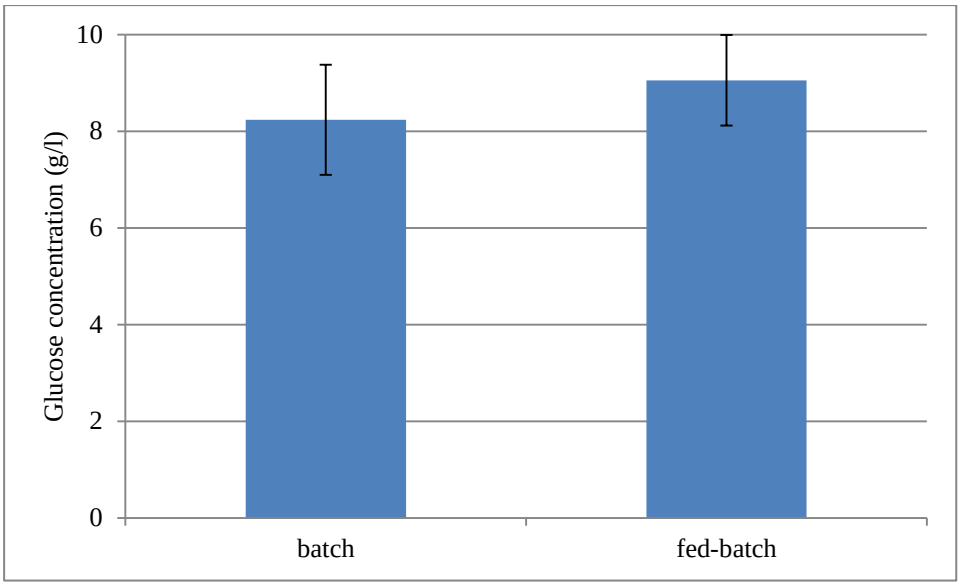


Figure 6. 15 Final glucose concentration obtained from fermented wheat straw hydrolysis for 6 h in a batch hydrolysis and a fed-batch hydrolysis. Hydrolysis was carried out at 50°C, 150 rpm. The enzyme loading was 30 FPU/g and total solid loading was 12%. Data are mean values of three replicates and error bars indicate standard deviations (SDs).

6.3 Conclusion

The aim of the study in this chapter was to develop a cost-effective process for the production of a glucose rich hydrolysate. Acid hydrolysis might not be suitable for fermented wheat straw even though high acid concentration led to high glucose release (Tichagwa, 2012) under harsh condition like acid the monosaccharides could turn into organic acids via furans (Yoon *et al*., 2014) and higher temperatures in acid hydrolysis could also encourage the degradation of the sugar (Yoon *et al*., 2014). Enzymatic hydrolysis is another effective way to produce glucose-rich hydrolysate. According to the cellulolytic enzyme cocktail from the company are pricey therefore fungal extract was applied in order to produce cost effective hydrolysate. Then the conclusion of this chapter was listed below

In this chapter, the process of hydrolysing fermented wheat straw into a glucose rich hydrolysate was investigated.

Direct acid hydrolysis of raw wheat straw was carried out using an orthogonal design to examine the impact of particle size, temperature and retention time. The results showed that the temperature had the most significant impact while the particle size had no impact on glucose generation from direct acid hydrolysis of wheat straw. In terms of direct acid hydrolysis of fermented wheat straw, reaction temperature, acid concentration and retention time were studied. The model predicted that a glucose concentration of 15.7 g/L could be produced in a hydrolysis at 187˚C for 23 min using 2.51% (w/w) H_2SO_4. These data indicated that direct acid hydrolysis alone will not produce a hydrolysate with a commercially interesting amount of glucose (e.g. over 50 g/L glucose).

The impact of solid loading rate, enzyme loading rate and agitation on the enzymatic hydrolysis of fermented wheat straw was explored. Within the range investigated, all of the above three parameters have positive impact on the glucose production. A glucose concentration of up to 72.4 g/L could be reached at 18.3% solid loading rate, 55.23 FPU/g (Ctec2) and a mixing rate of 189 rpm in the enzymatic hydrolysis. A confirmation hydrolysis experiment operated at the above mentioned condition resulted in a hydrolysate with 59.8 g/L glucose. When raw wheat straw was used in a hydrolysis at a round up condition (18% solid loading rate, 55 FPU/g and a mixing rate of 190 rpm), only 10.6 g/L glucose was produced.

The replacement of Ctec2 with fresh fungal filtrate was attempted. Due to the low cellulase concentration in the fungal filtrate, the enzyme loading rate of 55 FPU/g could not be achieved. However, when Ctec2 was diluted to the same FPU activity as the fungal filtrate, hydrolysis using fungal filtrate led to higher glucose production.

Chapter 7 Succinic Acid Fermentation using Genetically Modified Yeast Strains

In chapters 4-6, a solid state fungal fermentation strategy has been developed to convert wheat straw into a sugar rich hydrolysate. In the following chapters, the sugar rich hydrolysate was used as a generic fermentation feedstock for the production of biofuels and biochemicals. In this chapter (chapter 7), an approach of using recombinant yeast strains for the fermentative succinic acid production was described. Then succinic acid fermentations using semi-synthetic medium were carried out to optimise the fermentation conditions. Finally, actual wheat straw hydrolysate was tested for succinic acid fermentation using the selected recombinant yeast strains in section 8.1.

7.1 Introduction

Fermentative succinic acid production has been widely investigated. However, most of the studies utilised bacterial strains, such as *Actinobacillus succinogenes, Anaerobiospirillum succiniciproducens, and Escherichia coli* for succinic acid production (McKinlay *et al*., 2007, Du *et al*., 2008). The aim of this work was thus to construct recombinant *Saccharomyces cerevisiae* strains to enable them to convert glucose to succinic acid. As pointed out in the literature review (chapter 2.8.1), succinic acid fermentation using yeast strains has received limited attention. The potential benefits of using recombinant yeasts for the succinic acid production include: (i) It is a biologically safe process; (ii) It is potentially a high productive process. Yeast could produce ethanol at a productivity of over 5 g/L/h at industrial scale. By comparison, the current highest succinic acid productivity in a batch fermentation was only 2

g/L/h at lab scale (Cheng *et al*., 2012); (iii) Potentially, this process could use lignocellulosic hydrolysate, including both xylose and glucose; (iv) Succinic acid is produced in the acid form instead of the salt form.

7.2 Yeast strain development

Genetically modified yeast that had previously been selected for enhanced succinate production under either aerobic or anaerobic fermentation were available for this project. Four yeast clones containing a single deletion in the succinate dehydrogenase encoding gene SDH1 and four other clones containing a single deletion in the succinate dehydrogenase encoding gene SDH2 from the parent strain S. cerevisiae D2 were provided by Dr. Abhishek Somani (The University of Nottingham).

7.3 Succinic acid fermentation using S. cerevisiae D2 and its derivatives

7.3.1 Succinic acid fermentation using single deletion strains

These strains were fermented synthetic medium in mini FVs under anaerobic conditions and in shake flasks under aerobic conditions (refer to section 3.10.1-3.10.4). The fermentations were carried out for 24 hours. As shown in Figure 7.1A, there was no difference in terms of cell growth between the control (parent strain) and all 8 recombinant yeasts both in aerobic fermentations and in anaerobic fermentations.

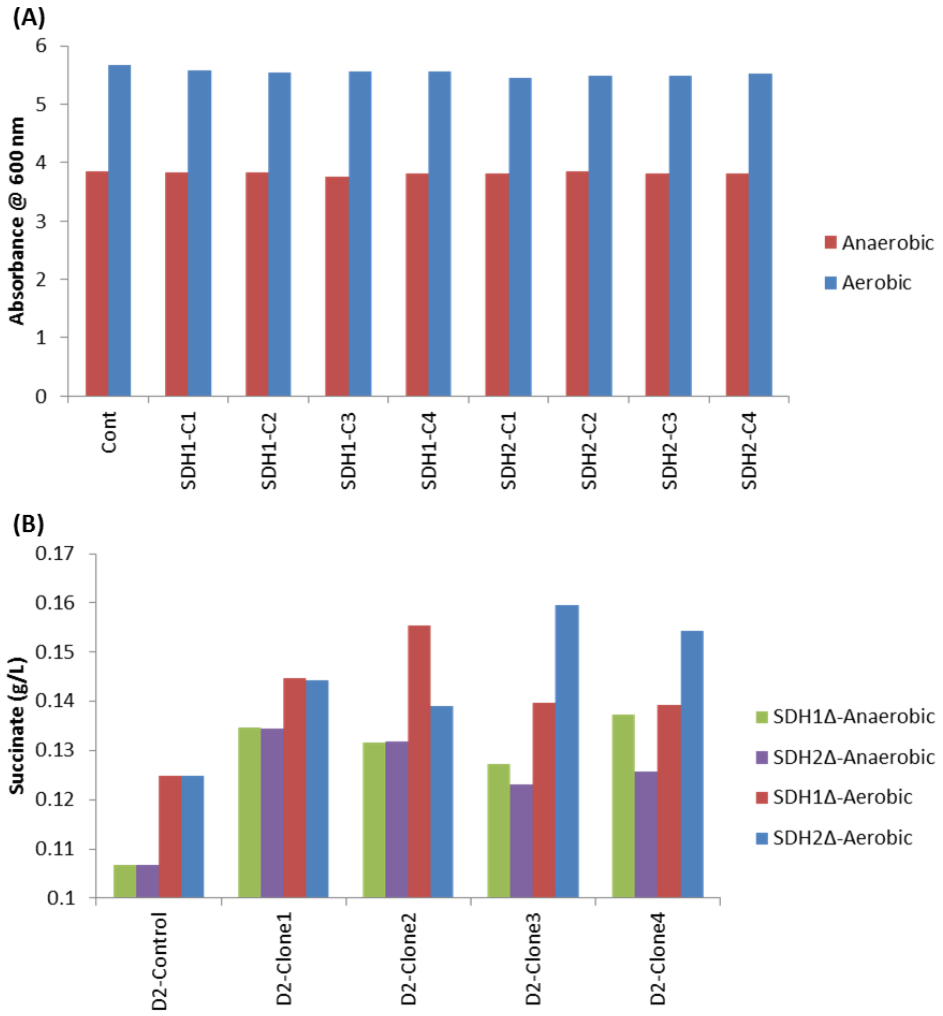


Figure 7. 1 Cell growth (A, measured as OD_{600}) and succinic acid production (B) in fermentation using wild and recombinant yeast strains. Succinic acid fermentation was carried out 30°C, 150 rpm for 24 hours.

Figure 7.1B indicated that the single deletion of either SDH1 or SDH2 gene improved succinic acid production in anaerobic fermentations, from around 0.106 g/L to around 0.115 - 0.138 g/L. For fermentations under aerobic condition, the succinic acid production also increased from 0.125 to the range of 0.140 - 0.160 g/L. The highest succinic acid production was observed using strain *S. cerevisiae* D2S2-clone3 under aerobic fermentation condition. Compared with its control, the succinic acid production was enhanced by 28%.

7.3.2 The impact of fermentation time and initial glucose concentration

The impact of fermentation time and the substrate (glucose) concentration on succinic acid production using the control strain and two selected single mutation strains were investigated. As shown in Table 7.1, both extending fermentation time simultaneously and increasing initial glucose concentration improved succinic acid accumulation in the fermentation broth. But both the fermentation yield (Table 7.2) and fermentation productivity (Table 7.3) did not change, indicating that the strain production capacity did not change along with fermentation time and glucose concentration in the range investigated. However, compared with the wild type strain, the single deletion of either SDH1 or SDH2 led to increases in both succinic acid production yield and productivity.

Table 7. 1 Succinic acid production in fermentations terminated at different time using *S. cerevisiae* D2 and its derivative strains

Time (h)	Initial glucose (g/L)	D2 wild type (g/L)	D2S1 clone2, (g/L)	D2S2 clone3, (g/L)
24	20	0.125 ±0.001	0.158 ±0.006	0.160 ±0.005
48	40	0.236 ±0.001	0.313 ±0.011	0.299 ±0.001
72	60	0.375 ±0.009	0.484 ±0.004	0.493 ±0.001

Note: succinic acid fermentation was carried out at 30°C and 150 rpm. Data are mean values ± standard deviation of three replicates

Table 7. 2 Succinic acid yield in fermentations terminated at different time using *S. cerevisiae* D2 and its derivative strains

Time (h)	Initial glucose (g/L)	D2 wild type	D2S1 clone2	D2S2 clone3
24	20	0.64%	0.79%	0.80%
48	40	0.57%	0.80%	0.74%
72	60	0.63%	0.81%	0.82%

Table 7. 3 Succinic acid productivity in fermentations terminated at different time using *S. cerevisiae* D2 and its derivative strains

Time (h)	Initial glucose (g/L)	D2 wild type (g/L/h)	D2S1 clone2 (g/L/h)	D2S2 clone3 (g/L/h)
24	20	0.0052	0.0066	0.0067
48	40	0.0051	0.0067	0.0062
72	60	0.0052	0.0067	0.0068

7.3.3 Succinic acid fermentation using double deletion strains

In the next step, both *S. cerevisiae* D2S1 (clone 2) and *S. cerevisiae* D2S2 (clone 3) were used as the parent strains for the construction of double deletion strains by further deleting SDH2 and SDH1, respectively. Strain *S. cerevisiae* D2S1S2 was successfully constructed from the parent strain *S. cerevisiae* D2S1, while there was no success in obtaining *S. cerevisiae* D2S2S1. Succinic acid fermentation using the double deletion strain *S. cerevisiae* D2S1S2 was carried out and compared with succinic acid production from *S. cerevisiae* wild type and its single deletion strains (Figure 7.2). The initial glucose concentration was 60 g/L while the fermentation time was 72 hours.

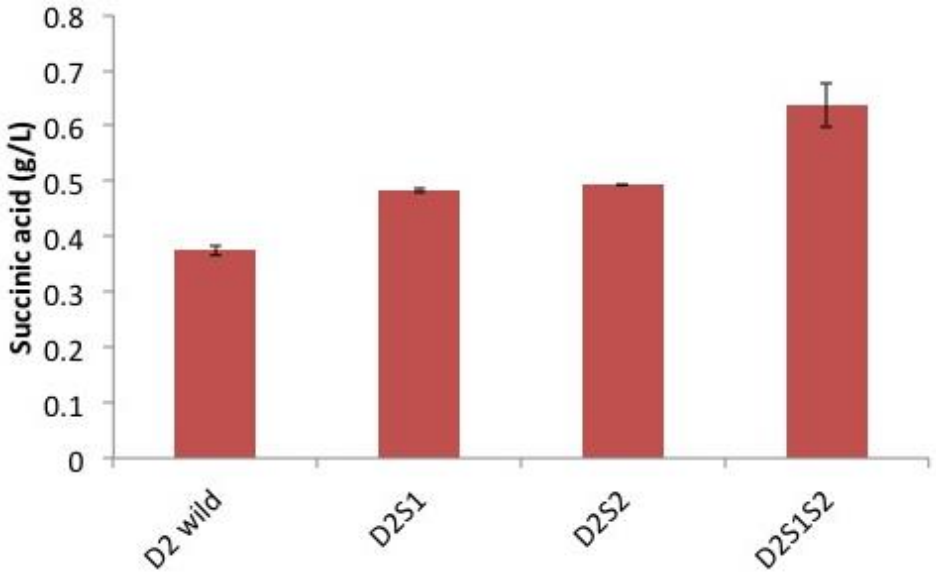


Figure 7. 2 Succinic acid production using double mutation strains *S. cerevisiae* D2S1S2 and compared with D2 wild type and its single mutation strains. Succinic acid fermentation was carried out at 30°C and 150 rpm for 72 hours. Data are mean values of three replicates and error bars indicate standard deviations (SDs).

As illustrated in Figure 7.2, the deletion of both SDH1 and SDH2 in *S. cerevisiae* D2 resulted in further increased succinic acid production. 0.64 g/L succinic acid was obtained after 72 hours fermentation. This equals to an increase of succinic acid accumulation of 69.7%.

7.3.4 The impact of oxygen transfer on succinic acid production

In the previous screening experiments, fermentation was carried out using 100 ml shake flasks with a liquid load of 50 ml in each flask. These results indicated that succinic acid was accumulated under aerobic conditions. Therefore, it was natural to consider whether an increase in oxygen transfer rate would improve succinic acid production using these yeast strains. In order to investigate the impact of oxygen transfer rate on succinic acid production, fermentations with *S. cerevisiae* D2S1S2 strain were carried out in 500 mL shake flasks and 1000 mL shake flasks. The fermentation volume was kept at 50 mL, the initial glucose concentration was 20 g/L and the fermentation was carried out for 72 hours. By comparing typical fermentation profiles in Figures 7.3-7.6, the increase of fermentation flask volume from 500 ml to 1000 ml led to a significant increase in succinic acid production from 1.35 g/L to 2.04 g/L. As a contrast, ethanol production in the fermentation in the 500 mL shake flask was around 9.16 g/L, which dramatically reduced to 2.02 g/L in the fermentation using the 1000 mL shake flask. Lowering fermentation volume and using a larger fermentation vessel increased the surface area, which enhanced oxygen transfer. The availability of oxygen in the fermentation strongly depressed the synthesis of ethanol in yeast fermentation. As a result, the formation of glycerol and succinic acid was enhanced.

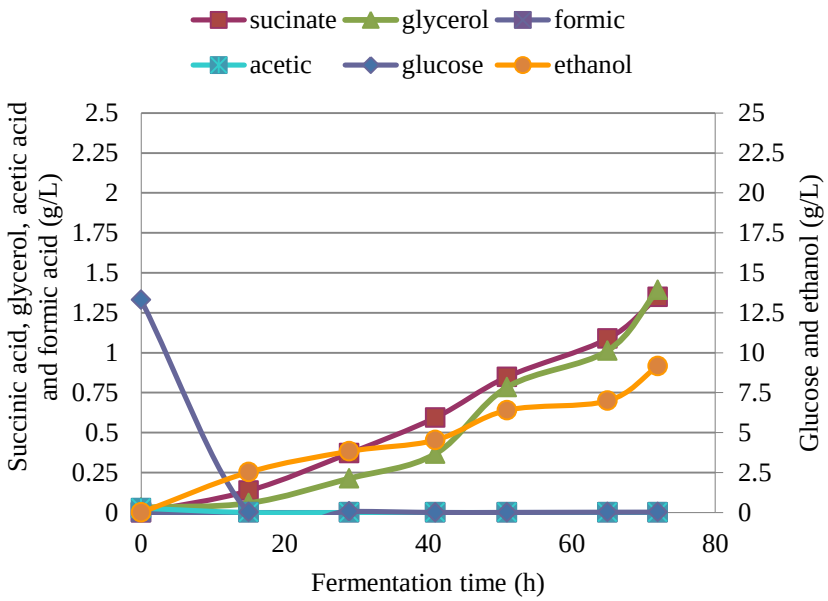


Figure 7. 3 Succinic acid, glycerol, acetic acid, formic acid and ethanol production, together with glucose consumption in fermentations using *S. cerevisiae* D2S1S2 strain. Fermentation volume was 50 mL in a 500 mL shaking flask. Succinic acid fermentation was carried out at 30°C and 150 rpm for 72 hours. Data are mean values of three replicates

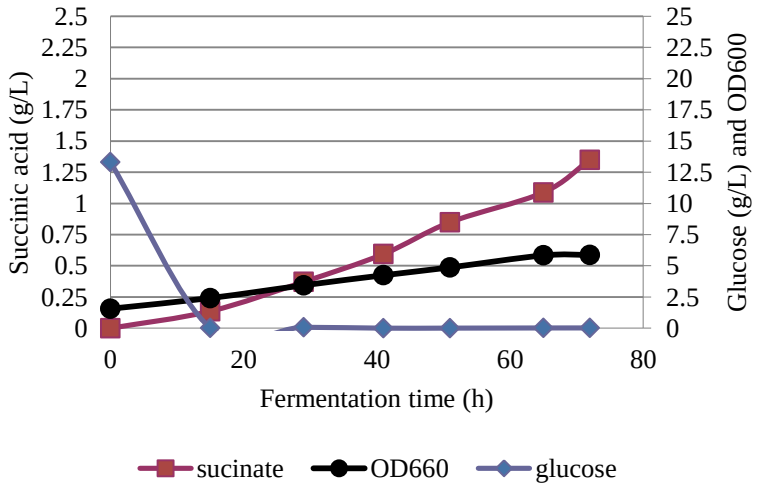


Figure 7. 4 Succinic acid production, glucose consumption and cell growth curve in fermentations using *S. cerevisiae* D2S1S2 strain, with 50 mL fermentation volume in a 500 mL shaking flask

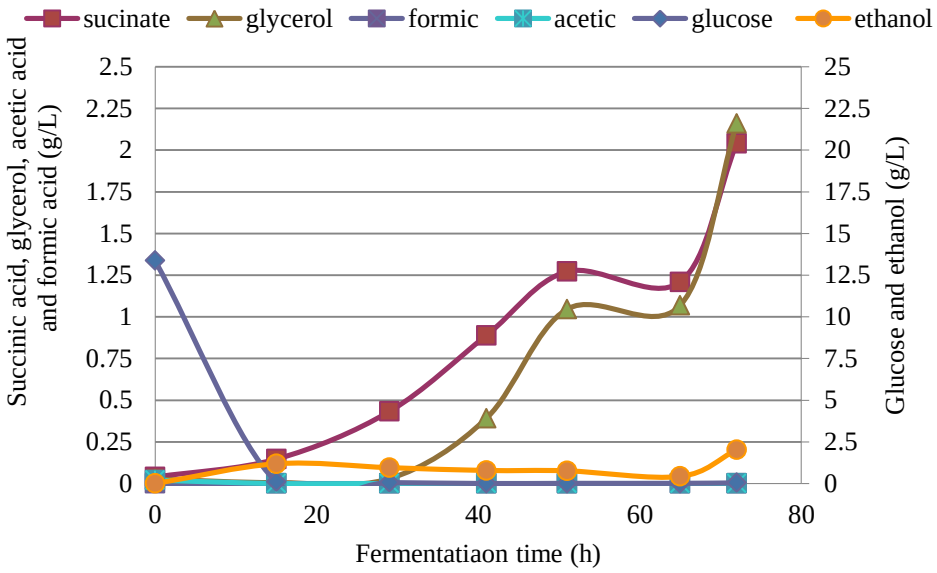


Figure 7. 5 Succinic acid, glycerol, acetic acid, formic acid and ethanol production, together with glucose consumption in fermentations using *S. cerevisiae* D2S1S2 strain. Fermentation volume was 50 mL in a 1000 mL shaking flask. Succinic acid fermentation was carried out at 30°C and 150 rpm for 72 hours. Data are mean values of three replicates

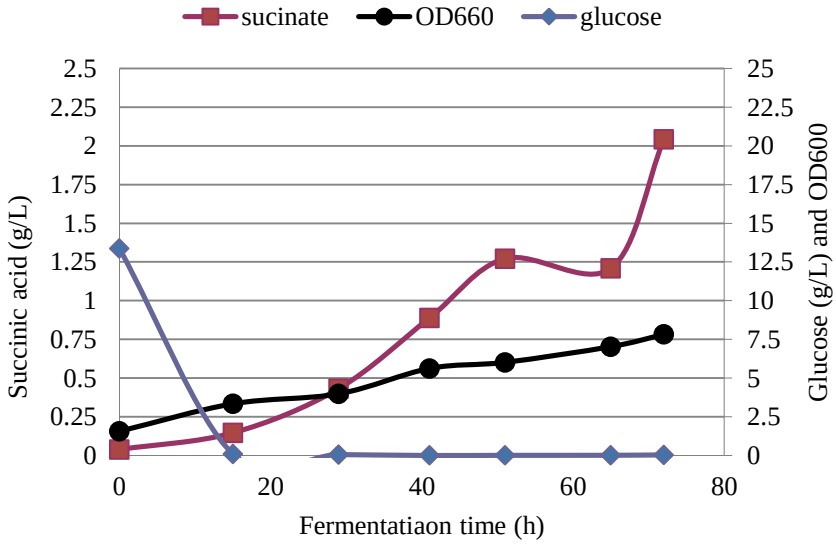


Figure 7. 6 *S. cerevisiae* D2S1S2 Succinic acid production, glucose consumption and cell growth curve in fermentations using *S. cerevisiae* D2S1S2 strain, with 50 mL fermentation volume in a 1000 mL shaking flask

These results also show that with high availability of oxygen, the yeast cell growth was encouraged as well. The OD_{600} of the recombinant yeast in the fermentation using the 1000 mL flask reached 7.8 (Figure 7.6), which was 33% higher than that in the fermentation using the 500 mL flask (OD_{600} 5.87, Figure

7.4). The availability of more yeast cells may be one of the reasons that succinic acid production was enhanced. The specific succinic productions for 500 mL and 1000 mL flasks were 0.23 g/OD and 0.26 g/OD, respectively. This suggested that in fermentations at a high oxygen transfer efficiency (using 1000 mL flask), the unit yeast cell produced more succinic acid than those at low oxygen transfer efficiency (using 500 mL flask).

Figure 7.7 plotted succinic acid production data at different fermentation volumes. Although the first data point did not match exactly the conditions using to generate the other data, it clearly showed the increase of oxygen transfer (shake flask volume) increased succinic acid accumulation.

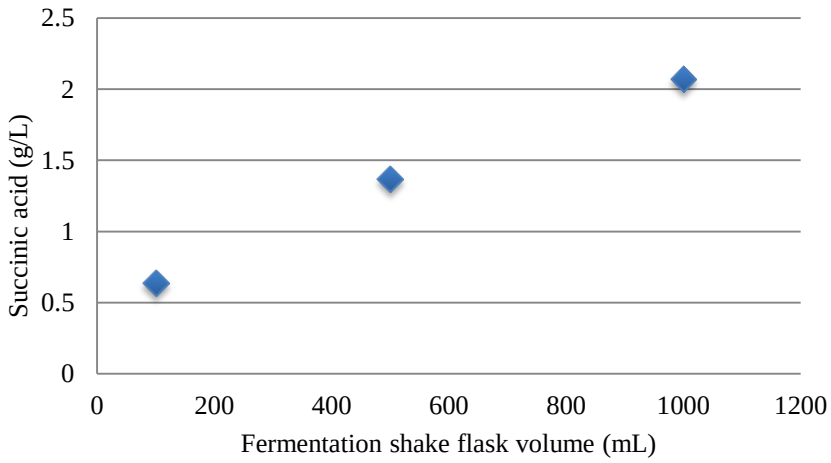


Figure 7. 7 Comparing succinic acid production in fermentations carried out using different sizes of shake flask. The fermentation using 100 ml had an initial glucose concentration of 60 g/L, while the other two experiments started with 20 g/L glucose. The data represented the average of three replicates.

7.3.5 Fed batch fermentation using S. cerevisiae D2S1S2 strain

In order to further increase succinic acid production, fed batch fermentation was carried out using strain *S. cerevisiae* D2S1S2. The result is shown in Figure 7.8. The initial glucose concentration was lowered to 10 g/L.

Approximately 10 g/L glucose was added into the flasks each time when a sample was taken after 24 hours (no glucose feed in the first 24 hours). At the end of fermentation, around 2.1g/L succinic acid was produced. Although this succinic acid concentration was slightly higher than that obtained in the batch fermentation, the enhancement was not significant. This may be due to the active ethanol production, which directed most of the sugars into ethanol. The glycerol production was also high in comparison with batch fermentation.

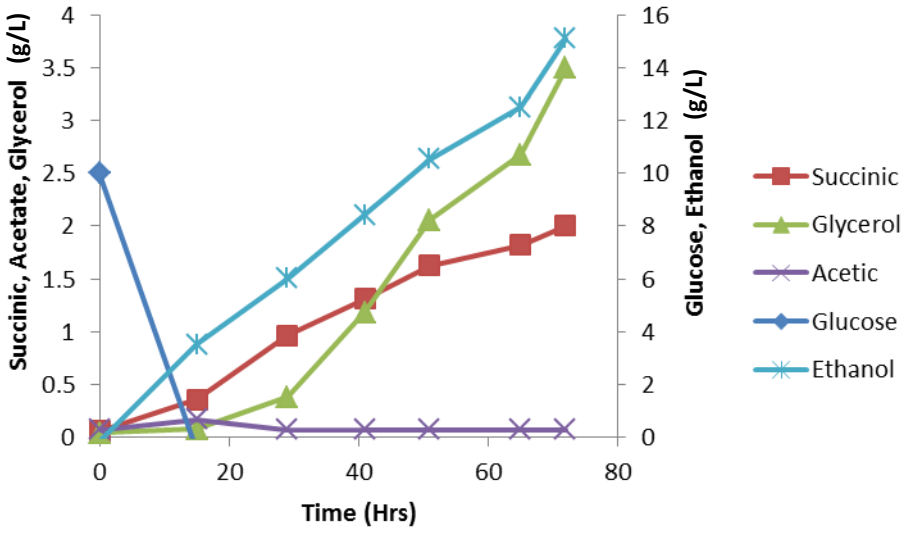


Figure 7. 8 Fed batch fermentation using *S. cerevisiae* D2S1S2 for succinic acid production, initial glucose concentration was 10 g/L and the fermentation volume was 1000 ml. The total addition of glucose was 60 g/L. Succinic acid fermentation was carried out at 30°C and 150 rpm for 72 hours. Data are mean values of three replicates.

7.3.6 Succinic acid fermentation using S. cerevisiae D2S1S2 at bench top fermenter scale

In order to further increase the oxygen transfer and to control the fermentation pH, succinic acid fermentation using *S. cerevisiae* D2S1S2 at a 2 L fermentation scale was carried out. The initial glucose concentration was 10 g/L. The working volume was around 1 L, and the agitation speed was 200 rpm. Compressed air was spurged into the fermentation with a flow rate of around 1 vvm (volume/volume/minute). Approximately 10 g/L glucose was added into the fermenter each time when a sample was taken. The fermentation

pH was automatically controlled to 5.0 by adding 2 M NaOH. As shown in Figure 7.9, around 3.15 g/L succinic acid was produced within 72 hours, which was significantly higher than that (around 2.0 g/L, Figure 7.7, Figure 7.8) obtained in fermentations using shake flasks. The strict control of pH, the steady purging of air and the agitation may all contribute to the increased succinic acid production.

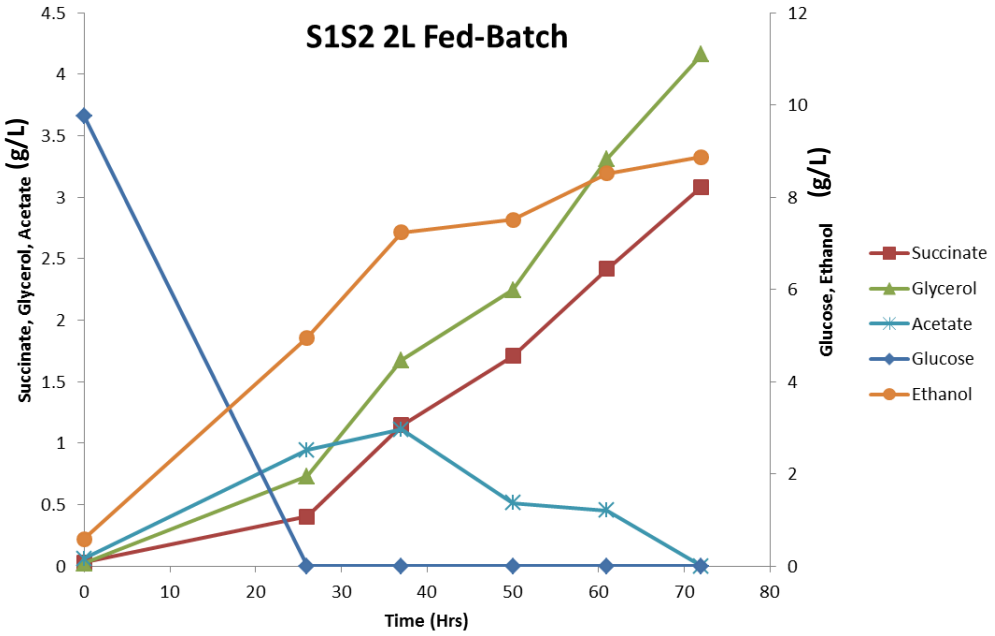


Figure 7. 9 *S. cerevisiae* D2S1S2 fermentation in a 2 L bench top fermenter. Succinic acid fermentation was carried out at 30°C, 200 rpm with aeration rate of 1 vvm for 72 hours. Data are mean values of three replicates.

It can be seen from Figure 7.9 that by the end of 72 hours, succinic acid production had not stopped. This fermentation was terminated because the succinic acid analysis was carried out a few days later than the fermentation experiment due to the unavailability of the HPLC. Therefore, a second experiment was planned and the fermentation time was extended to 14 days. The fermentation results are shown in Figures 7.10 and 7.11.

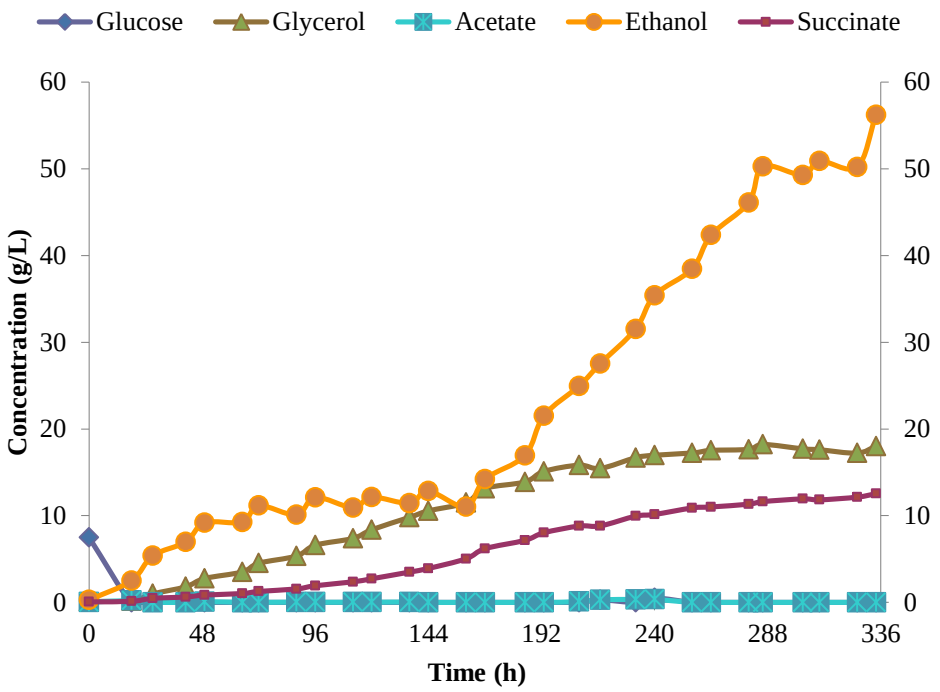


Figure 7. 10 *S. cerevisiae* D2S1S2 fed batch fermentation in a 2 L bench top fermenter for 14 days. Succinic acid fermentation was carried out at 30°C, 200 rpm with aeration rate of 1 vvm for 72 hours. 10 g/L of glucose was added after every sampling time point. Data are mean values of three replicates.

As shown in Figure 7.10, the final succinic acid concentration reached 12.5 g/L, which was 3.04 times higher than that obtained in fermentations for 3 days, and was 99 times higher than the initial succinic acid concentration observed in wild type *S. cerevisiae* D2 in screening experiments (0.125 g/L, in Table 7.4).

Figure 7.11 illustrated the fermentation profiles for the first 72 hours. Within this period, succinic acid accumulation was steadily increased, while the ethanol concentration was kept lower than 14.5 g/L. The glucose concentration was not detected except for the first sample. The feeding was made after the sampling, and the additional glucose was consumed completely within the 8-16 hour gaps between sampling. Glycerol was produced continuously alongside succinic acid. No acetate was detected. After 168 hours, ethanol production increased, while succinic acid accumulation rate dropped. It was interesting to

find out that the cell growth (Figure 7.12) slowed down after 168 hours. There may be a link between the yeast cell growth and succinic acid production.

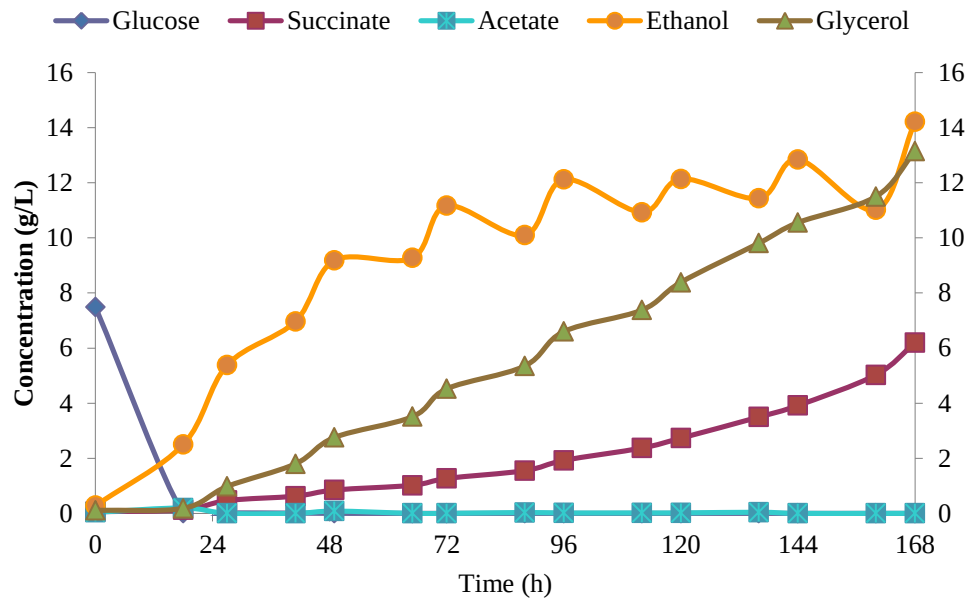


Figure 7. 11 The fermentation profile of the first 7 days of *S. cerevisiae* D2S1S2 in a 2 L bench top fermenter. Succinic acid fermentation was carried out at 30°C, 200 rpm with aeration rate of 1 vvm for 168 hours. Data are mean values of three replicates.

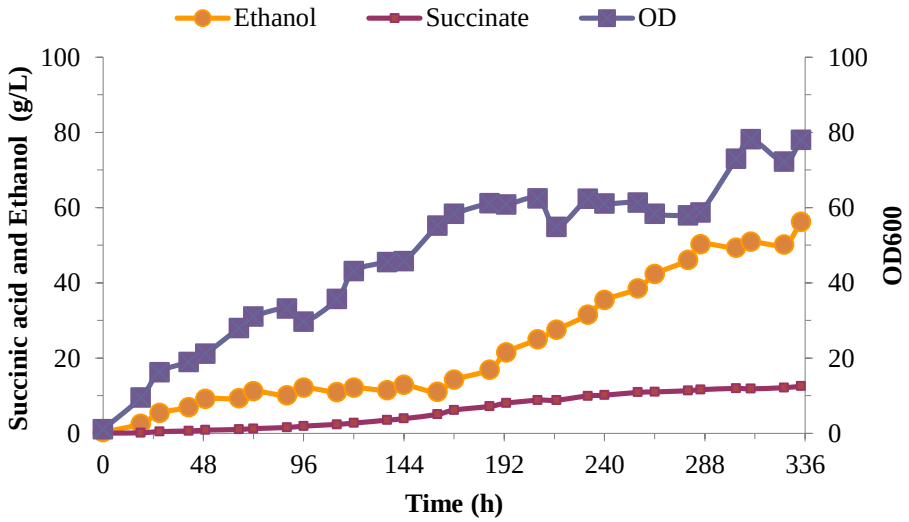


Figure 7. 12 *S. cerevisiae* D2S1S2 fermentation in a 2 L bench top fermenter. The cell growth curve compared with ethanol production and succinic acid accumulation.

Following the previous fermentation, another fermentation was carried out in order to confirm that the *S. cerevisiae* D2S1S2 strain could produce up to 12 g/L succinic acid. In this experiment, the air aeration rate was increased to 2 VVM. The fermentation results are shown in Figure 7.13. In this fermentation, the ethanol production was limited to no more than 10 g/L. The succinic acid accumulation was even faster than the previous experiment and 11.8 g/L succinic acid was produced within 7 days of fermentation. Unfortunately, although succinic acid continued to be produced, the experiment was terminated after 7 days. Figure 7.14 showed the cell growth profile compared with ethanol and succinic acid production. Similar to Figure 7.12, succinic acid production had a positive correlation with yeast cell growth.

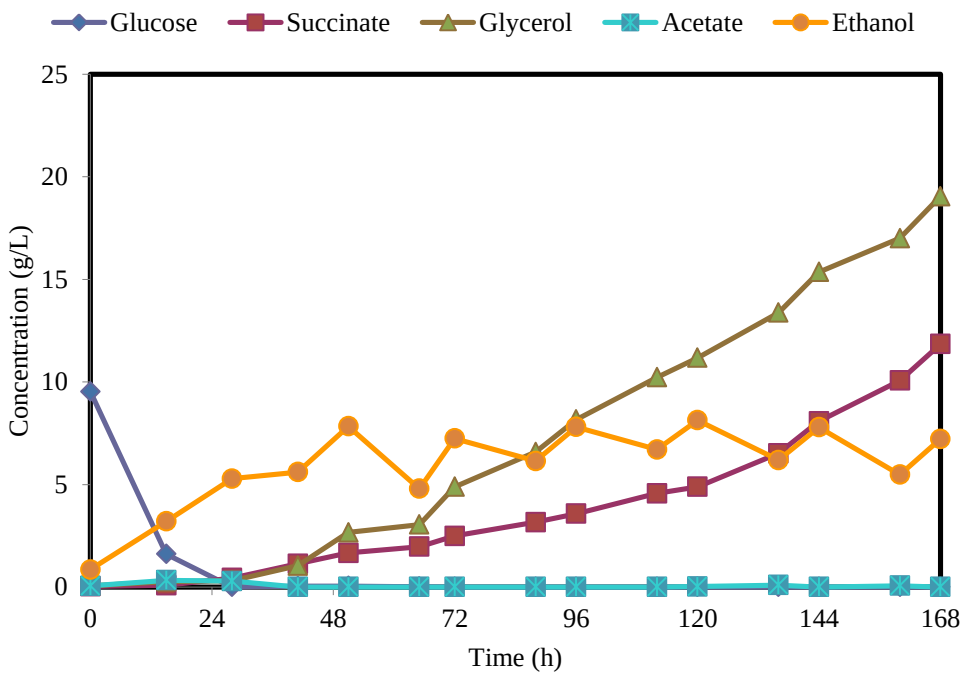


Figure 7. 13 *S. cerevisiae* D2S1S2 fermentation in a 2 L bench top fermenter. Succinic acid fermentation was carried out at 30°C, 200 rpm with aeration rate of 2 vvm for 168 hours. Data are mean values of three replicates.

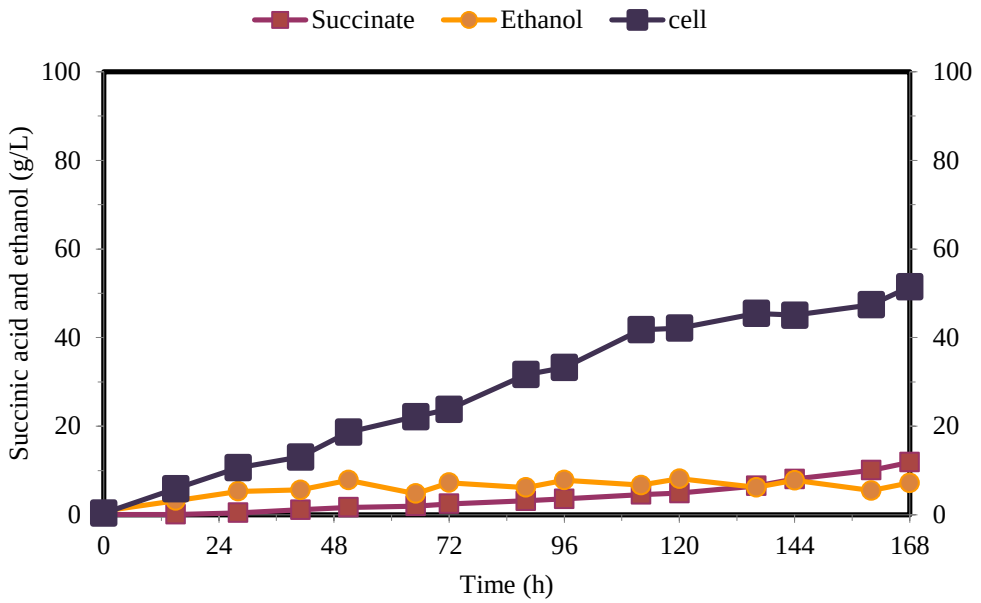


Figure 7. 14 *S. cerevisiae* D2S1S2 fermentation in a 2 L bench top fermenter with an air aeration rate of 2 vvm. The cell growth curve compared with ethanol production and succinic acid

7.4 Succinic acid fermentation using *S. cerevisiae* BY4741 and its derivatives

In order to investigate the impact of gene deletion on the succinic acid production in recombinant yeast strains, a laboratory yeast strain *S. cerevisiae* BY4741 and its derivatives were also investigated. The recombinant *S. cerevisiae* BY4741 strains with single deletion of various known genes were originally obtained from EUROSCARF collection, and provide by Prof. Ed Louis.

7.4.1 Succinic acid production using various single mutant S. cerevisiae BY4741 strains.

Initial screening of succinic acid production using parent and various recombinant *S. cerevisiae* BY4741 strains were carried out in 100 mL shake flask, with an initial glucose concentration of 60 g/L. The fermentation was carried out for 72 hours in aerobic conditions with a fermentation volume of 50

ml. The succinic acid, ethanol, glycerol, acetic acid production and the residential glucose are shown in Tables 7.4, 7.5 and Figures 7.15- 7.17.

Alcohol dehydrogenases (ADH) are a set of dehydrogenase enzymes, which are encoded by seven different genes in yeast strains. As indicated in Table 7.5, the removal of only 1 ADH gene did not prevent ethanol synthesis. On the contrary, the ethanol production was increased compared with the parent strain. In terms of succinic acid production, the deletion of a copy of ADH gene had little impact on succinic acid accumulation with the exception of ADH3 gene. By deleting ADH3, succinic acid production increased from 0.31 g/L to 0.56 g/L. This may be due to the direction of carbon flux from the acetic acid synthesis to the succinic acid synthesis, as the acetic acid production was significantly reduced (Table 7.8).

Table 7. 4 Residue glucose, succinic acid and glycerol concentrations in screening fermentations of various *S. cerevisiae* BY4741 strains.

Strain	Glucose (g/L)	Succinic acid (g/L)	Glycerol (g/L)
S. cerevisiae BY4741	0.00 ± 0.00	0.31 ±0.01	1.93 ±0.03
S. cerevisiae BY4741 ADH1	0.00 ± 0.00	0.29 ±0.00	1.45 ±0.03
S. cerevisiae BY4741 ADH2	0.00 ± 0.00	0.39 ±0.01	2.24 ±0.07
S. cerevisiae BY4741 ADH3	0.00 ± 0.00	0.56 ±0.02	2.44 ±0.08
S. cerevisiae BY4741 ADH4	0.00 ± 0.00	0.32 ±0.00	2.22 ±0.10
S. cerevisiae BY4741 ADH5	0.00 ± 0.00	0.38 ±0.00	1.70 ±0.03
S. cerevisiae BY4741 ADH6	0.00 ± 0.00	0.39 ±0.01	2.29 ±0.04
S. cerevisiae BY4741 ADH7	0.00 ± 0.00	0.42 ±0.01	2.11 ±0.04
S. cerevisiae BY4741 PDC-1	9.80 ± 0.27	0.14 ±0.01	4.70 ±0.08
S. cerevisiae BY4741 PDC-5	0.70 ± 0.41	0.36 ±0.01	2.23 ±0.10
S. cerevisiae BY4741 PDC-6	0.00 ± 0.00	0.36 ±0.00	2.13 ±0.01
S. cerevisiae BY4741 SDH1	0.00 ± 0.00	0.62 ±0.03	1.86 ±0.06
S. cerevisiae BY4741 SDH2	0.00 ± 0.00	0.59 ±0.02	1.80 ±0.04

Table 7. 5 Acetic acid and ethanol concentrations in screening fermentations of various *S. cerevisiae* BY4741 strains.

Strain	Acetic acid (g/L)	Ethanol (g/L)
S. cerevisiae BY4741	1.53 ± 0.02	19.69 ± 0.29
S. cerevisiae BY4741 ADH1	1.34 ± 0.01	20.17 ± 0.47
S. cerevisiae BY4741 ADH2	1.22 ± 0.03	21.66 ± 1.34
S. cerevisiae BY4741 ADH3	0.47 ± 0.02	20.03 ± 1.26
S. cerevisiae BY4741 ADH4	1.36 ± 0.05	21.48 ± 0.89
S. cerevisiae BY4741 ADH5	0.91 ± 0.03	21.44 ± 0.98
S. cerevisiae BY4741 ADH6	1.26 ± 0.03	21.75 ± 0.57
S. cerevisiae BY4741 ADH7	1.18 ± 0.03	20.49 ± 0.62
S. cerevisiae BY4741 PDC-1	0.81 ± 0.01	16.60 ± 0.52
S. cerevisiae BY4741 PDC-5	1.22 ± 0.03	20.74 ± 0.22
S. cerevisiae BY4741 PDC-6	1.02 ± 0.02	19.68 ± 0.54
S. cerevisiae BY4741 SDH1	1.66 ± 0.09	19.89 ± 0.62
S. cerevisiae BY4741 SDH2	1.70 ± 0.11	20.37 ± 1.89

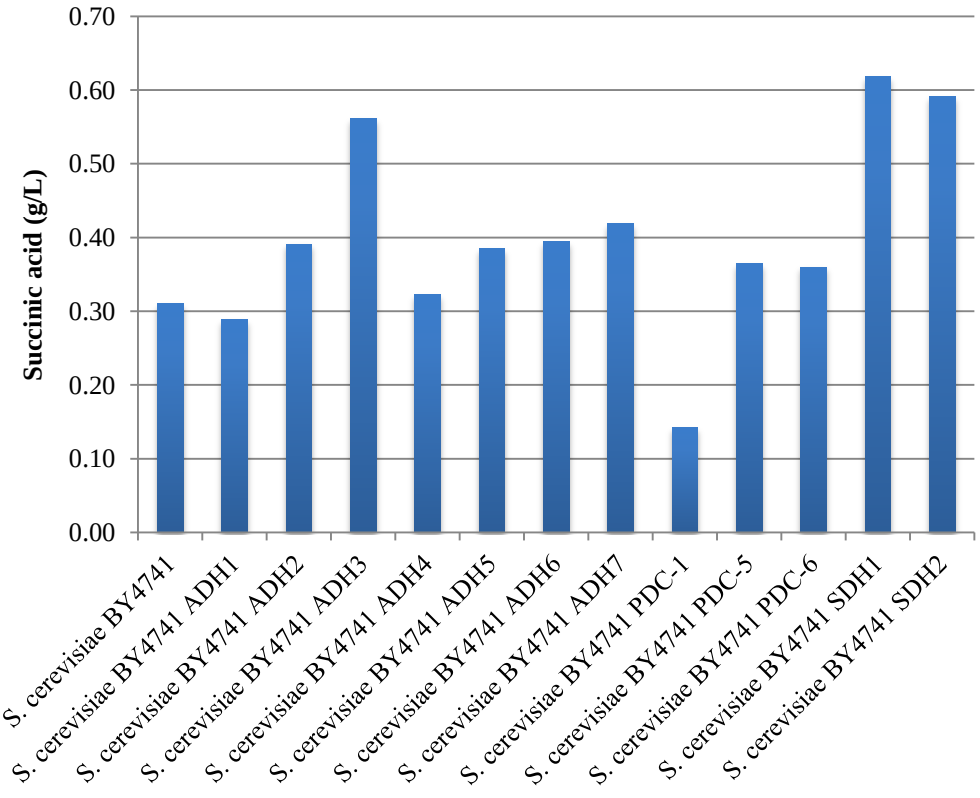


Figure 7. 15 Succinic acid production using various *S. cerevisiae BY4741* strains. Initial glucose concentration was 60 g/L in 100 ml shake flask. Succinic acid fermentation was carried out at 30°C, 150 rpm for 72 hours. Data are mean values of three replicates.

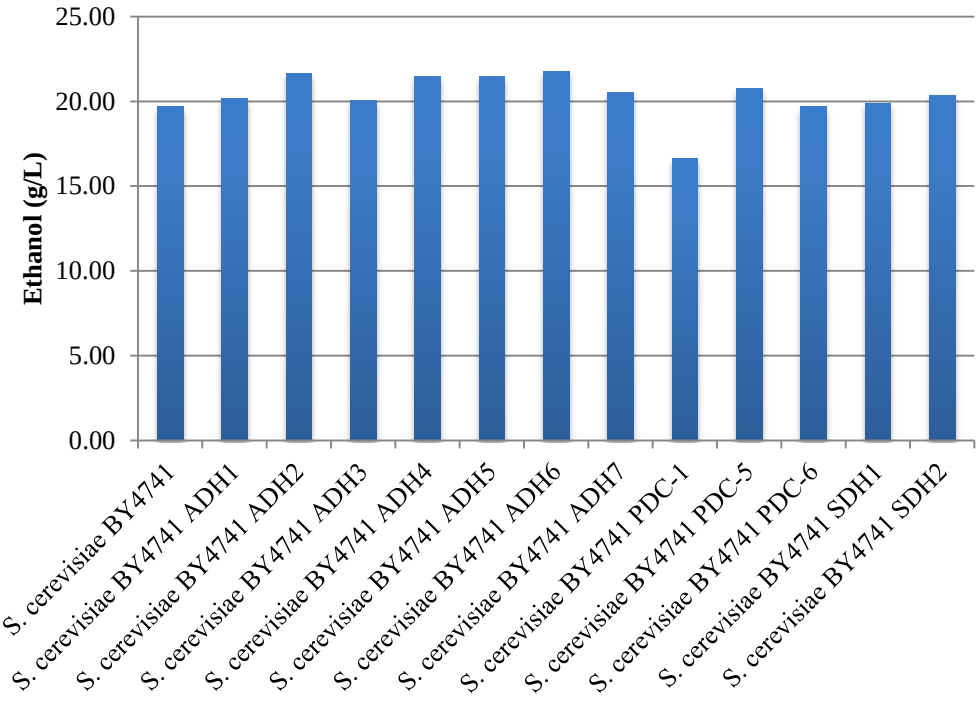


Figure 7. 16 Ethanol production using various *S. cerevisiae* BY4741 strains.

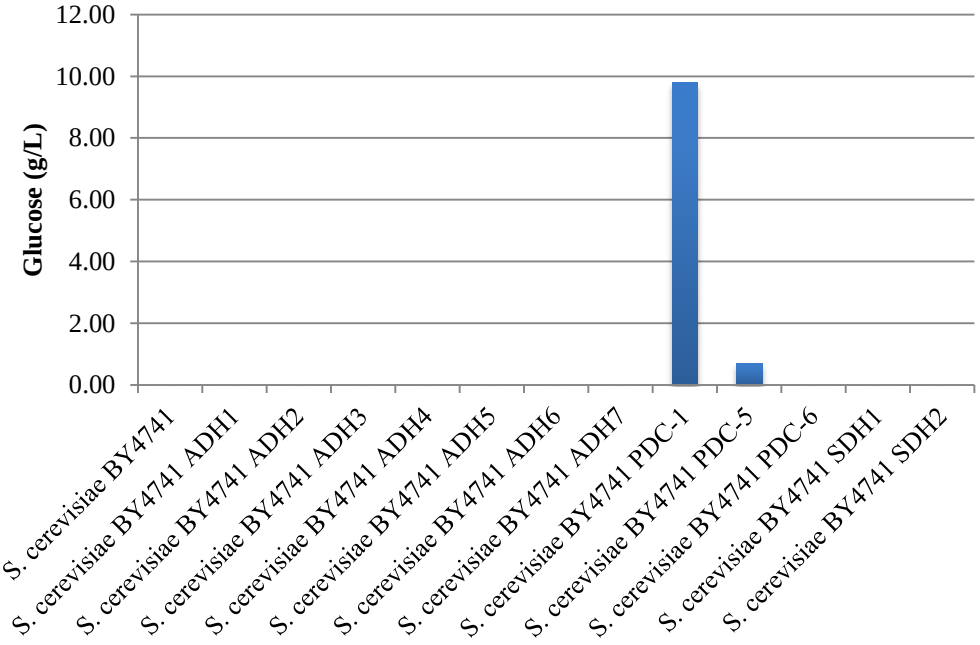


Figure 7. 17 Residual glucose concentration in fermentations using various *S. cerevisiae* BY4741 strains.

Deletion of SDH1 gene or SDH2 gene also showed a significant increase in succinic acid production (Table 7.7, Figure 7.15). In fermentation using those two strains, around 0.62 g/L and 0.59 g/L succinic acid productions were

observed. These results confirmed that similar to succinic acid production using recombinant *E. coli* strain (Cheng et al., 2012), the deletion of SDH genes could improve succinic acid production in yeast as well.

The removal of PDC5 or PDC6 genes had little impact on succinic acid production. But the deletion of PDC1 gene led to a reduction of cell activity, which can be seen by the high resident glucose concentration (Figure 7.17) and low ethanol formation.

7.4.2 Succinic acid production using various double and triple mutant S. cerevisiae BY4741 strains.

Based on the initial screening of fermentations using various single mutation strains, strains with the deletion of ADH3, SDH1 or SDH2 exhibited enhanced succinic acid production. Therefore, double *S. cerevisiae* BY4741 mutant strains were developed by deleting ADH3 and SDH1, ADH3 and SDH2, SDH1 and SDH2. A triple *S. cerevisiae* BY4741 mutant strain based on strain *S. cerevisiae* BYA3-S2 was also obtained, which had ADH3, SDH1 and SDH2 genes all removed. Figure 7.18 showed the succinic acid formation using various clones of the double deletion strains.

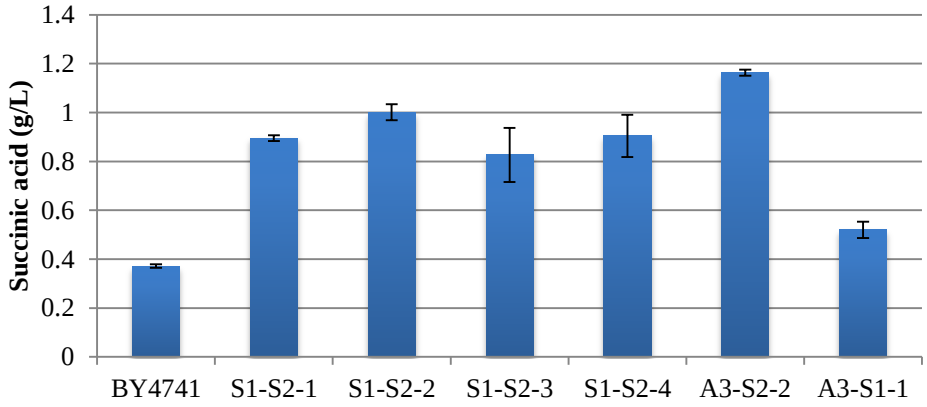


Figure 7. 18 Succinic acid production using various double mutations of *S. cerevisiae* BY4741 strains. Initial glucose concentration was 60 g/L in 100 ml shake flask. Succinic acid fermentation was carried out at 30°C, 150 rpm for 72 hours. Data are mean values of three replicates.

At this shake flask scale experiment scale, strain *S. cerevisiae* BY4741 A3-S2-2 showed the highest succinic acid production among various double deletion *S. cerevisiae* BY4741 strains. The succinic acid concentration reached 1.16 g/L, which was 2.14 fold higher than that of the parent *S. cerevisiae* BY4741 strain. Therefore, it was selected for further deletion of SDH1 gene. Another double deletion strain, *S. cerevisiae* BY4741 A3-S2-4 was also used as a starting strain for the construction of triple deletion strains in order to increase the chance of obtaining a high succinic acid producer. The succinic acid fermentation screening results is shown in Figure 7.19. The strain (*S. cerevisiae* BY4741 A3-S2-S1-3) gave the highest succinic acid production and reached 1.68 g/L, which was 44% higher than its parent strain. This strain was then selected for fermentation at 2 L scale.

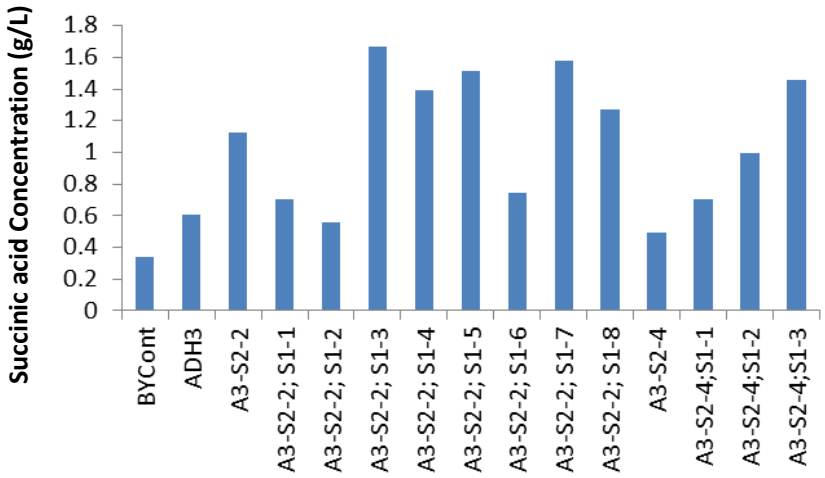


Figure 7. 19 Succinic acid production using various triple mutations of *S. cerevisiae* BY4741 strains. Initial glucose concentration was 60 g/L in 100 ml shake flask. Succinic acid fermentation was carried out at 30°C, 150 rpm for 72 hours. Data are mean values of three replicates.

7.4.3 Succinic acid production using various S. cerevisiae BY4741 strains at bench top fermenter scale.

Bench top fermentations using 2 L fermenters with various *S. cerevisiae* BY4741 strains were carried out. Figure 7.20 illustrated the fermentation profiles using the wild type *S. cerevisiae* BY4741 strain, while the

fermentation profiles using a double deletion strain, *S. cerevisiae* BY4741 A3-S2 is shown in Figure 7.21. The initial glucose concentration was 60 g/L and no further additions were made. The aeration rate was 1 VVM, and the pH was automatically adjusted control to 5.0 by adding 2 M NaOH.

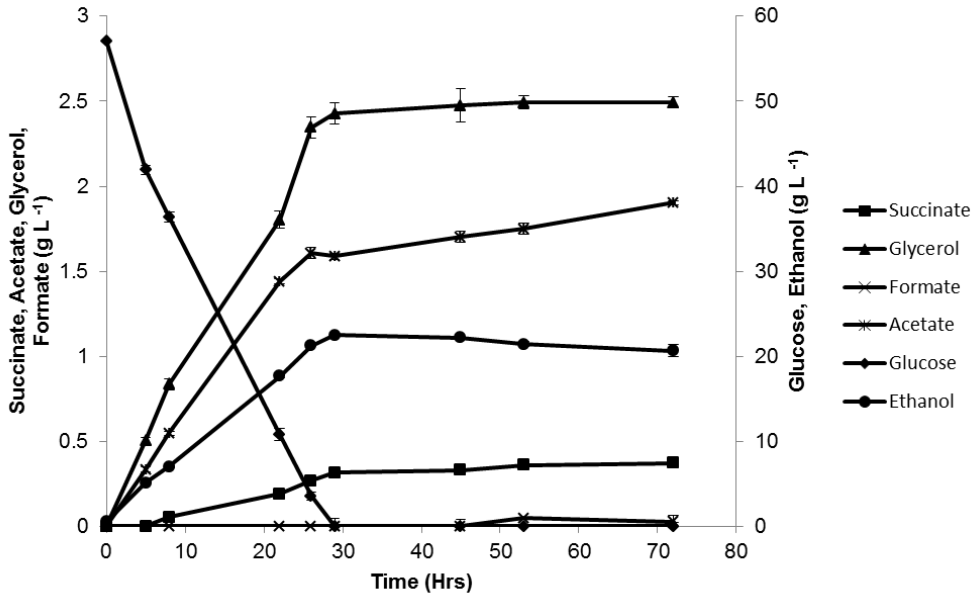


Figure 7. 20 Batch fermentation using wild type *S. cerevisiae* BY4741 strain. Initial glucose concentration was 60 g/L in 2 L fermenter. Succinic acid fermentation was carried out at 30°C, 200 rpm and aeration rate was 1 vvm for 72 hours. Data are mean values of three replicates.

The performance of *S. cerevisiae* BY4741 in the 2 L fermenter was slightly better than or similar to that in the shake flask screening experiment in term of succinic acid production. The fermentation profile did indicate that succinic acid was quickly produced in the first 30 hours while glucose was not completely consumed , then it further accumulated at the expense of ethanol. By the end of the fermentation, around 0.37 g/L succinic acid, 20 g/L ethanol, 2.5 g/L glycerol, 1.8 g/L acetic acid were produced from 60 g/L glucose.

Similarly, the double mutation strain *S. cerevisiae* BY4741 A3-S2 produced slightly higher succinic acid than in the shake flask experiments. By the end of

fermentation, around 1.31 g/L succinic acid, 16.8 g/L ethanol, 2.2 g/L glycerol was produced from 60 g/L glucose.

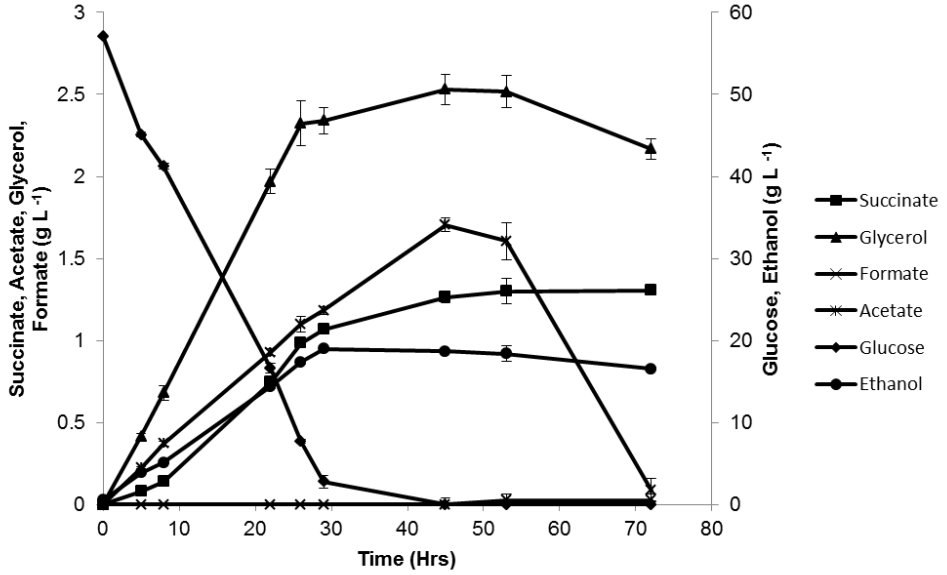


Figure 7. 21 Batch fermentation using *S. cerevisiae* BY4741 A3-S2. Initial glucose concentration was 60 g/L in 2 L fermenter. Succinic acid fermentation was carried out at 30°C, 200 rpm and aeration rate was 1 vvm for 72 hours. Data are mean values of three replicates.

Two clones of the triple mutation strains of *S. cerevisiae* BY4741 A3S2S1 (3) and *S. cerevisiae* BY4741 A3S2S1 (7) were also investigated in batch fermentations using the 2-L fermenter. The triple mutation strain *S. cerevisiae* BY4741 A3S2S1 (3) only led to a succinic acid production of 1.01 g/L (Figure 7.22), which was less than the double mutation strain (Figure 7.21) and to what was obtained in the screening experiment (1.68 g/L, Figure 7.19). The other triple mutation strain *S. cerevisiae* BY4741 A3S2S1 (7) resulted in even lower succinic acid production of only 0.55 g/L (Figure 7.23). The reasons were unclear.

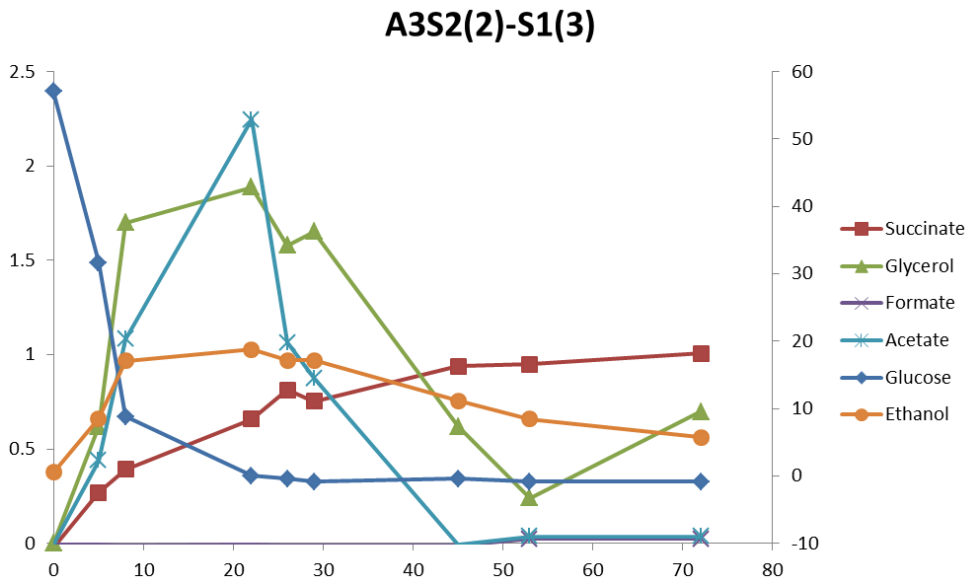


Figure 7. 22 Batch fermentation using triple mutation strain *S. cerevisiae* BY4741 A3S2S1 (3). Initial glucose concentration was 60 g/L in 2 L fermenter. Succinic acid fermentation was carried out at 30°C, 200 rpm and aeration rate was 1 vvm for 72 hours. Data are mean values of three replicates.

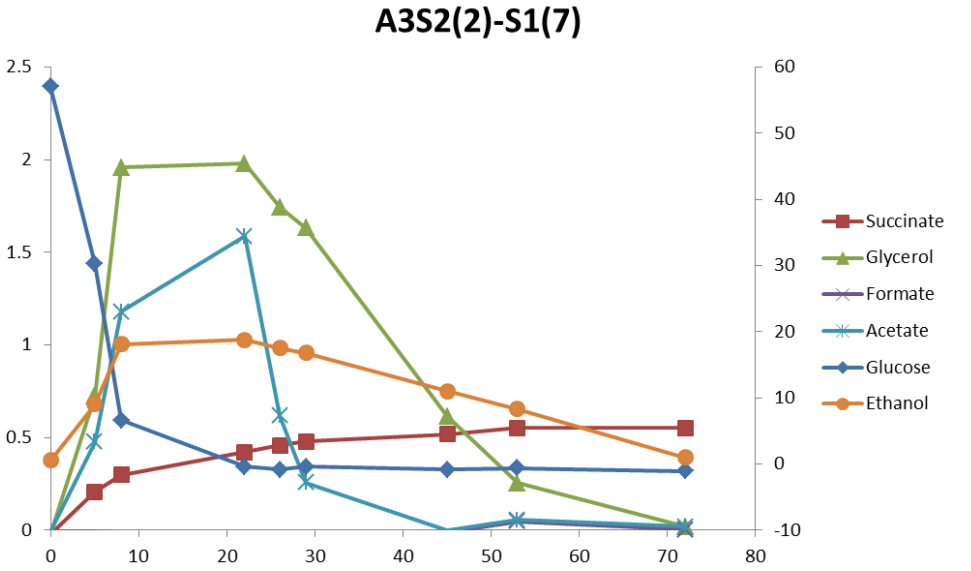


Figure 7. 23 Batch fermentation using triple mutation strain *S. cerevisiae* BY4741 A3S2S1(7). Initial glucose concentration was 60 g/L in 2 L fermenter. Succinic acid fermentation was carried out at 30°C, 200 rpm and aeration rate was 1 vvm for 72 hours. Data are mean values of three replicates.

7.5 Conclusion

In this chapter, the utilisation of recombinant yeast strains for succinic acid production was investigated. The succinic acid production using various *S. cerevisiae* D2 strains and *S. cerevisiae* BY4741 strains is summarised in Tables 7. 9 and 7.10, respectively.

Table 7. 6 Succinic acid production in fermentations using various wild type and recombinant *S. cerevisiae* D2 strains.

Strain	Succinic acid (g/L)	Initial glucose (g/L)	Total glucose (g/L)	Time (h)	Yield (g/g)	Batch or Fed-batch	Fermenter size (mL)
S. cerevisiae D2	0.13	20	20	24	0.006	Batch	100
S. cerevisiae D2	0.24	40	40	48	0.006	Batch	100
S. cerevisiae D2	0.38	60	60	72	0.006	Batch	100
S. cerevisiae D2S1	0.48	60	60	72	0.008	Batch	100
S. cerevisiae D2S2	0.49	60	60	72	0.008	Batch	100
S. cerevisiae D2S1S2	0.64	60	60	72	0.011	Batch	100
S. cerevisiae D2S1S2	1.37	20	20	72	0.068	Batch	500
S. cerevisiae D2S1S2	2.07	20	20	72	0.104	Batch	1000
S. cerevisiae D2S1S2	3.08	10	60	72	0.051	Fed-batch	2000
S. cerevisiae D2S1S2	12.5	10	270	336	0.046	Fed-batch	2000
S. cerevisiae D2S1S2	11.8	10	130	168	0.091	Fed-batch	2000

For *S. cerevisiae* D2, the single deletion of either SDH1 or SDH2 increased succinic acid production by around 27%, while double deletion of both SDH1 and SDH2 enhanced succinic acid production by 68%. The succinic acid yield was increased up to 1.6 fold compared to wild type strain. Similar result was reported by (Kregiel, 2012). The single deletion gene of SDH1 of *S. cerevisiae* led to a1.6-fold increase of succinic acid production. The increment of succinic acid yield of the mutant strain is because disruption of gene encoding succinate dehydrogenase subunit. This mutation leads to reduce activity of succinate dehydrogenase enzyme which involves oxidation of succinate to fumarate activity (Rashid *et al*., 2013). Furthermore the deletion of SDH2 in yeast cell showed greatly accumulation of succinate (Kregiel, 2012). This study also found that evaluation of fermentation condition, e.g. increase oxygen, extended fermentation time, using fed-batch fermentation strategy led to a succinic acid production of around 12 g/L, which was nearly 100 times higher than succinic acid production achieved using the wild *S. cerevisiae* D2 strain at initial

fermentation conditions. Since oxygen molecule act as a co-factor of α-ketoglutarate dependent enzyme which hydroxylate succinyl CoA to succinate. Therefore the high oxygen transfer was improved succinic acid production

Table 7. 7 Succinic acid production in fermentations using various wild type and recombinant *S. cerevisiae* BY4741 strains.

Strain	Succinic acid (g/L)	Initial glucose (g/L)	Time (h)	Yield (g/g)	Batch or Fed-batch	Fermentation size (mL)
S. cerevisiae BY4741	0.31	60	72	0.005	Batch	100
S. cerevisiae BY4741 ADH3	0.56	60	72	0.009	Batch	100
S. cerevisiae BY4741 SDH1	0.62	60	72	0.010	Batch	100
S. cerevisiae BY4741 SDH2	0.59	60	72	0.010	Batch	100
S. cerevisiae BY4741 A3S2	1.16	60	72	0.016	Batch	100
S. cerevisiae BY4741 A3S2S1	1.68	60	72	0.028	Batch	100
S. cerevisiae BY4741	0.37	60	72	0.006	Batch	2000
S. cerevisiae BY4741 A3S2	1.31	60	72	0.022	Batch	2000
S. cerevisiae BY4741 A3S2S1	1.01	60	72	0.017	Batch	2000

Since succinic acid is an intermediate of tricarboxylic acid (TCA) cycle. Succinic acid production was involved with many chemical pathways such as, oxidative TCA cycle, reductive TCA cycle and glyoxylate pathway (Cheng *et al*., 2012, Cheng *et al*., 2013). The effect of gene disruption (Δ*sdh* and Δ*adh*) on succinic acid accumulation on haploid strain was studied. The *S. cerevisiae* BY4741 is haploid strains, the impact of gene deletion was more significant. The best clones of single, double and triple deletions of ADH3, SDH1, SDH2 genes increased succinic acid production by approximately 90%, 2.7 times and 4.4 times respectively. However, the change of fermentation condition did not result in any further improvement in succinic acid production.

Chapter 8 Biofuel and Biochemical Fermentations using Wheat Straw Hydrolysate

In chapter 7, production of succinic acid from semi-synthetic media by using recombinant *Saccharomyces cerevisiae* D2 strains and *Saccharomyces cerevisiae* BY4741 strains was investigated. In this chapter, the utilisation of wheat straw hydrolysate as produced in Chapter 6 for succinic acid production was explored. In addition fermentation of the wheat straw hydrolysate for the production of ***Bioethanol*** and another biochemical Itaconic Acid were also described, using yeast strains and *Aspergillus terreus, respectively*.

8.1 Succinic acid production from wheat straw hydrolysate

In the past decades, a large numbers of research papers on succinic acid production from semi-synthetic media using various bacteria have been published (Lee *et al*., 2000, Song and Lee, 2006, de Barrosa *et al*., 2013, Meynial-Salles *et al*., 2008). Recently, several studies investigated using lignocellulosic hydrolysate for succinic acid production (Lee *et al*., 2003, Jiang *et al*., 2014, Chen *et al*., 2010, Zheng *et al*., 2009a). Utilising lignocellulose derived sugars would not compete with food use of land-based crops. Therefore, it would potentially benefit the environment.

Succinic acid fermentation using wheat straw hydrolysate was carried out as described previously in chapter 3.10.5. The fermentation was performed in 500 ml or 1000 ml Erlenmeyer flasks containing 50 ml of wheat straw hydrolysate with an initial pH of 4.8. The wheat straw hydrolysate consisted of 5.55 g/L glucose. The *S. cerevisiae* D2 wild type strain and the double deletion strain *S. cerevisiae* D2S1S2 were used. The experiment was carried out in triplicate. As

shown in Figure 8.1, the glucose in the hydrolysate was completely consumed after 72 hours of fermentation. 0.199±0.001 g/L of succinic acid was produced from *S. cerevisiae* D2. The yield was 0.035 g of succinic acid/g of glucose. The succinic acid yield was improved to 0.043 g of succinic acid/g of glucose when 1000 ml shake flasks were used, in which oxygen transfer rate was increased. This result was in agreement with the conclusion drawn from fermentations using semi-synthetic media (section 7.3.4). When the double mutation strain *S. cerevisiae* D2S1S2 was tested for succinic acid production, 0.576 g/L succinic acid was accumulated. Compared with the wild type *S. cerevisiae* D2 strain, the succinic acid production was significantly different ($p < 0.05$).

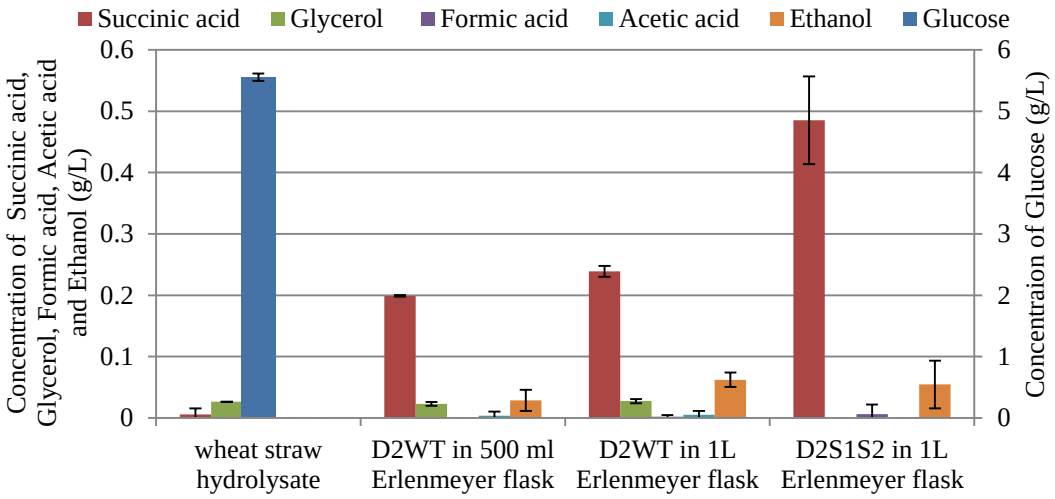


Figure 8. 1 Succinic acid production using wheat straw hydrolysate as the carbon resource by using *S. cerevisiae* D2 and *S. cerevisiae* D2S1S2 for 72 hours. Note: the first series column showed wheat straw hydrolysate compositions pior to fermentation.It contained glucose 5.5 g/L, succinic acid 0.01 g/L and glycerol 0.03 g/L. Formic acid and acetic acid were not found in the wheat straw hydrolysate. D2WT 500 represented *S. cerevisiae* D2 wild type cultured in 500 ml of Erlenmeyer flasks containing 50 ml of wheat hydrolysate., D2WT 1L represented *S. cerevisiae* D2 wild type cultured in 1000 ml of Erlenmeyer flasks containing 50 ml of wheat hydrolysate and D2S1S2 1L represented recombinant yeast *S.cerevisiae* D2S1S2 cultured in 1000 ml of Erlenmeyer flasks containing 50 ml of wheat hydrolysate.

This result agreed with Kubo *et al* (2000). They investigated the effect of succinate dehydrogenase gene disruption on succinic acid production using sake yeast strains. Their results indicated that the succinate dehydrogenase

gene deletion improved succinic acid production up to 1.9 fold compared with the wild type strain (Kubo *et al*., 2000). Compared with fermentation using semi-synthetic media in 1000 mL flasks (Figure 7.6, Table 7.9), where around 2.07 g/L succinic acid was produced from 20 g/L glucose, the succinic acid yield (0.104 g/g glucose) was exactly the same. This indicated that the wheat straw hydrolysate based medium could replace the commercial glucose based medium, without any potential inhibitory effect. The only limitation was the glucose concentration in the wheat straw hydrolysate.

8.2 Ethanol fermentation from lignocellulosic hydrolysate

As described in the literature review, bioethanol production from lignocellulosic raw materials has several advantages over the 1st generation bioethanol production. Chapters 4 to 6 described an alternative process to produce cellulase and to generate wheat straw hydrolysate. In this chapter, the fermentation of wheat straw hydrolysate to bioethanol using yeasts was explored. Lignocellulosic hydrolysates generally contain glucose and xylose depending on the raw material. Thus pentose utilising yeast like *Scheffersomyces sp.* or *Pichia sp.* were used in this study. These have a xylose metabolic pathway in the cell so can ferment xylose once glucose is depleted.

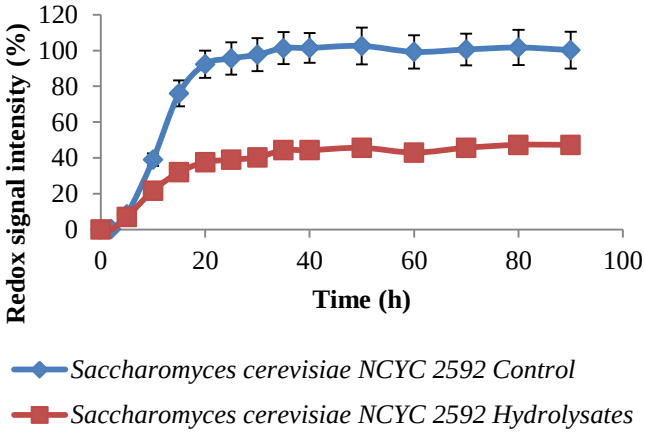
In chapter 6 optimal conditions for the enzymatic hydrolysis of biologically pretreated wheat straw to produce a sugar rich hydrolysate were determined. These conditions, were hydrolysis at 50˚C, 200 rpm for 72 hours, with a solid loading rate of 18% and an enzyme loading of 55 FPU/g of substrate. The hydrolysate obtained was centrifuged to separate the solid biomass from the liquid fraction. The liquid fraction was then filtered through a 0.45 μm filter paper for sterilisation.

This ethanol fermentation was performed using 5 different yeast strains. Since wheat straw hydrolysate contained glucose and xylose then *Saccharomyces cerevisiae* NCYC 2592, *Saccharomyces cerevisiae* D2 and *Saccharomyces cerevisiae* D2S1S2 were investigated as hexose utilisation yeasts and *Scheffersomyces stipitis* CBS 6054 and *Scheffersomyces anomalous* CBS 5759 were selected as pentose utilisation yeasts. Phenotypic microarray was applied to measure metabolic activities of these yeast strains. The ethanol fermentation from wheat straw hydrolysate was carried out as described in section 3.11.5 and weight loss was monitored throughout the fermentation process.

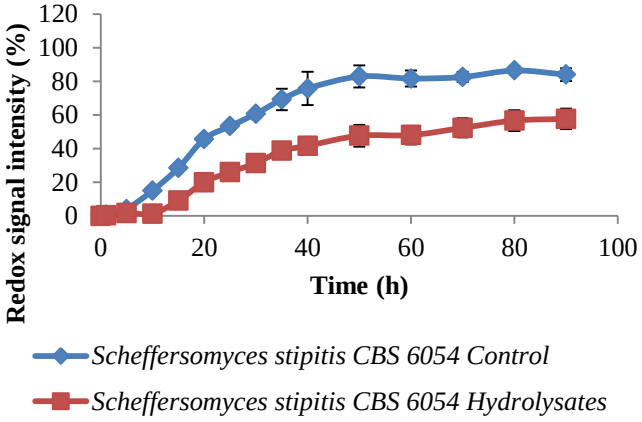
8.2.1 Phenotypic microarray

Phenotypic microarray analysis was used to measure metabolic activity of different yeast strain's growth on wheat straw hydrolysate. The conversion of a redox sensitive dye from an oxidized state to a reduced state was detected and recorded as the metabolic output. As shown in Figure 8.2, all yeast strains except *S. cerevisiae* D2S1S2 gave relatively high metabolic activities in the control media. The metabolic activities of all yeast strains on wheat straw hydrolysate were relatively low compared to that obtained in the control media. This was probably due to the fact that control media comprised of 6% glucose and 0.67% Yeast nitrogen base (YNB) while wheat straw hydrolysate contained no YNB. However phenotypic microarray results confirmed that all 5 yeast strains can metabolise wheat straw hydrolysate.

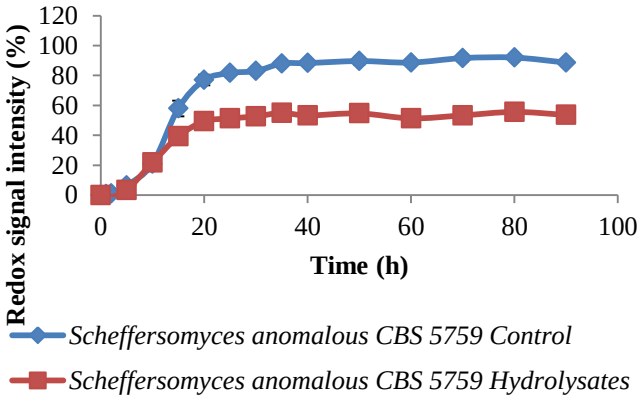
a)


Redox signal intensity (%)
120
100
80
60
40
20
0
0
20
40
60
80
100
Time (h)
Saccharomyces cerevisiae NCYC 2592 Control
Saccharomyces cerevisiae NCYC 2592 Hydrolysates

b)


Redox signal intensity (%)
120
100
80
60
40
20
0
0
20
40
60
80
100
Time (h)
Scheffersomyces stipitis CBS 6054 Control
Scheffersomyces stipitis CBS 6054 Hydrolysates

c)


Redox signal intensity (%)
120
100
80
60
40
20
0
0
20
40
60
80
100
Time (h)
Scheffersomyces anomalous CBS 5759 Control
Scheffersomyces anomalous CBS 5759 Hydrolysates

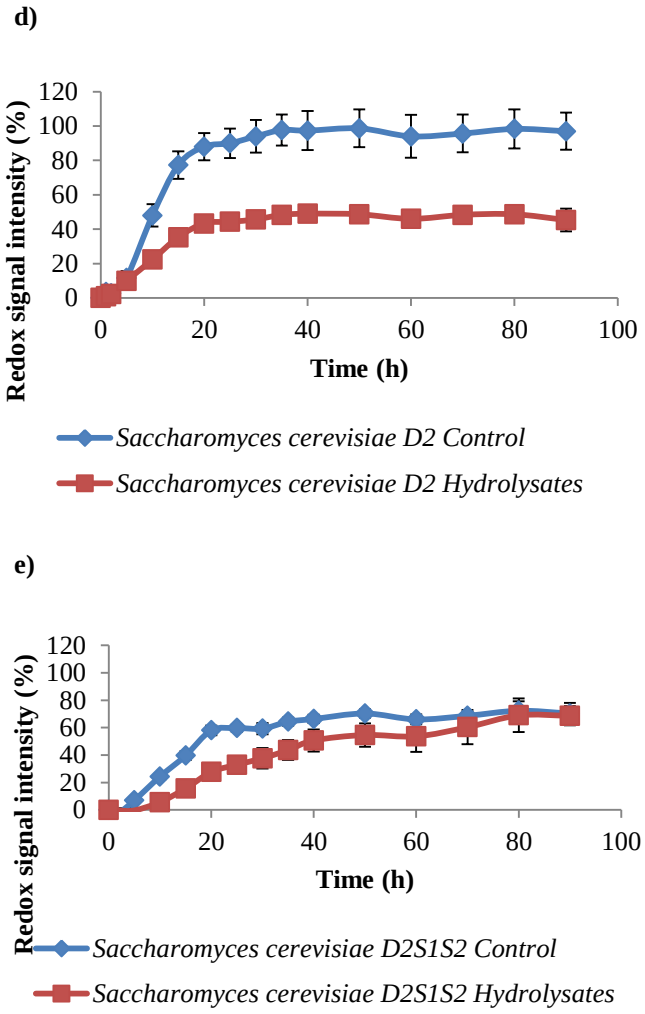


Figure 8. 2 Phenotypic microarray analysis (redox signal intensity) of 5 yeast strains on wheat straw hydrolysate for ethanol production. Yeast were grown in control medium containing 6 % glucose and 0.67% YNB at 30°C and metabolic activity was measured for 90 hrs. Results are plotted as% of RSI (redox signal intensity). The blue lines show redox signal intensity of yeast strain on control medium and the red lines show redox signal intensity of yeast strain in wheat straw hydrolysate. a) *Saccharomyces cerevisiae* NCYC 2592, b) *Scheffersomyces stipites* CBS 6054, c) *Scheffersomyces anomalous* CBS 5759, d) *Saccharomyces cerevisiae* D2 and e) *Saccharomyces cerevisiae* D2S1S2

8.2.2 Yeast fermentations in shake flask

Five selected yeast strains were cultured on YPD medium and wheat straw hydrolysate based medium. Wheat straw hydrolysate consisted of 59.8±2.1 g/L glucose, 10.51±0.10 g/L xylose, 0.02 ± 0.01 g/L glycerol, 0.182±0.034 g/L acetic acid, and there was no HMF and furfural detected in the wheat straw hydrolysate. During ethanol fermentation under anaerobic condition, CO_2 was produced as a by-product. (Wimalasena et al., 2014) has proposed that sugar

utilisation was linearly related to the weight loss of the fermentation system, therefore the weight loss could be used as an indicator of fermentation progress. The most notable weight loss occurred in the first 48 hours and increased slowly overtime. The increase of weight loss proved that these yeast cells metabolized sugars in hydrolysate and converted it to ethanol and CO_2. As shown in Figure 8.3, the weight losses from *S. cerevisiae* NCYC 2592, *S. cerevisiae* D2, *S. cerevisiae* D2S1S2 and *Sc. anomalous* CBS 5759 was almost complete after 72 hours of fermentation. The weight loss from *Sc. stipitis* CBS 6054 developed much more slowly as compared to others (Figure 8.3). This result is in agreement with (Agbogbo and Coward-Kelly, 2008b) who also found that *Sc. stipitis* had a slower sugar utilisation rate compared to *S. cerevisiae*.

Ethanol concentration and ethanol yield

Ethanol production on wheat straw hydrolysate from 5 yeast strains is presented in Table 8.2. Surprisingly, the ethanol concentration obtained from the wheat straw hydrolysate was better than that obtained from YPD. This was probably due to the presence of other unidentified sugars in the wheat straw hydrolysate. Acetic acid in the hydrolysate may also contribute to the increase in ethanol production. Acetic acid has been defined as an inhibitor of ethanol fermentation in many studies (Nigam, 2001, Graves *et al*., 2007, Casey *et al*., 2010). However, ethanol production could be improved when a small amount of acetic acid was present in the hydrolysate. It has been reported that acetic acid could enhance transportation capacity across the plasma membrane. The sugar uptake rate would thus increase which could encourage ethanol formation (Taherzadeh *et al*., 1997). The highest ethanol production was

28.6±0.0 g/L obtained from fermentation using wheat straw hydrolysate by *S. cerevsiae* D2S1S2, which equated to 93.36% of the glucose to ethanol theoretical yield.

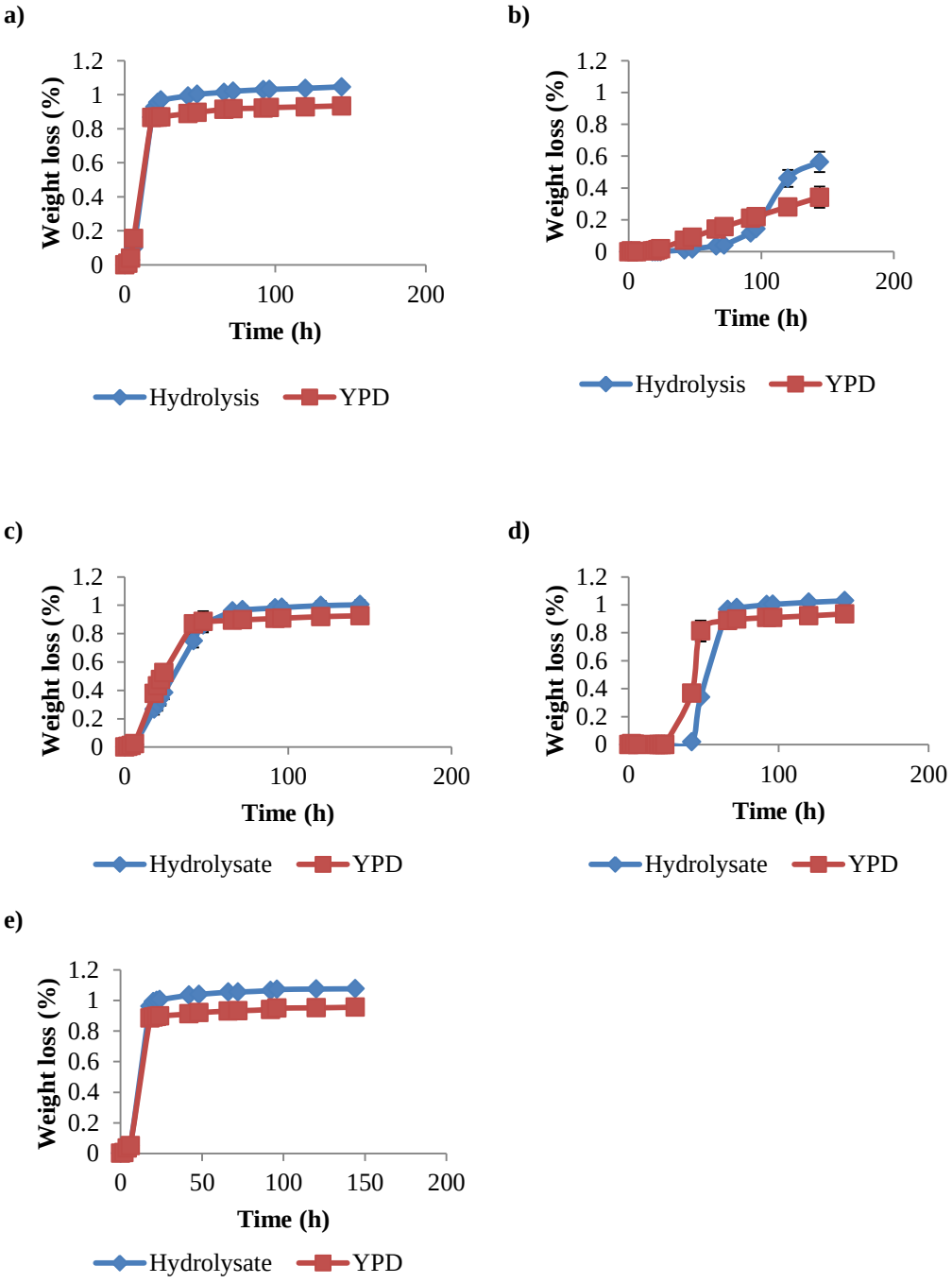


Figure 8. 3 The fermentation performance of 5 yeast strains on wheat straw hydrolysate in term of weight loss due to CO_2 production. The weight loss from ethanol fermentation on wheat straw hydrolysate was presented in blue line and glucose synthetic media was presented in red line by a) *Saccharomyces cerevisiae* NCYC 2592, b) *Scheffersomyces stipitis* CBS 6054, c) *Scheffersomyces anomalous* CBS 5759, d) *Saccharomyces cerevisiae* D2 and e) *Saccharomyces cerevisiae* D2S1S2

As shown in table 8.2, the glucose to ethanol conversion efficiency of *S. cerevisiae* D2S1S2 using YPD was lower than other yeast strains but there was no statistically significant difference amongst the 5 yeast strains. However, the result showed significant difference in ethanol yield ($p<0.5$) level between fermentations using semi-synthetic media or wheat straw hydrolysate based media.

Table 8. 1 Ethanol concentration and relative ethanol yield obtained from yeast fermentations using wheat straw hydrolysate or YPD media. Fermentations were carried out at 30˚C for 6 days.

Strain	Medium	Ethanol (g/L)	Yield (%)
S.cerevisiae NCYC 2592	Hydrolysate	28.056±0.200	91.430±0.652
	YPD	24.645±0.645	80.316±2.101
Sc. stipitis CBS 6054	Hydrolysate	26.589±0.001	86.648±0.001
	YPD	23.967±0.001	78.104±0.001
Sc. anamalous CBS 5759	Hydrolysate	27.640±1.822	90.074±5.937
	YPD	24.723±0.688	80.569±2.241
S.cerevisiae D2	Hydrolysate	26.615±0.297	86.734±0.969
	YPD	24.110±0.605	78.570±1.972
S.cerevisiae D2S1S2	Hydrolysate	28.648±0.001	93.361±0.001
	YPD	23.765±0.816	77.446±2.658

8.3 Itaconic acid production

In this chapter, the utilisation of wheat straw hydrolysate for the production of itaconic acid by *Aspergillus terreus* was investigated. The commercial glucose based semi-defined media was used to set up a benchmark. Then, wheat straw hydrolysate was used to replace the glucose based media for itaconic acid production.

8.3.1 Itaconic acid production from semi-synthetic media

Firstly, the effect of pH on itaconic acid production using semi-synthetic medium was investigated. The fermentation experiment was carried out at 3

different pH levels (pH 2.0, 3.0 and 4.0). The fermentation procedure was as described in section 3.13. The fermentation samples were collected every 2 days over 6 days' incubation. Then the itaconic acid concentrations were determined by HPLC as described in section 3.10.8.

The results are shown in Figure 8.4. It was found that 6.264±0.185 g/L of itaconic acid was produced from the glucose synthetic medium at pH 2.0 within 6 days of fermentation. Itaconic acid concentration was increased to 20.298±0.137 g/L at pH 3.0 and it was slightly decreased when the pH of fermentation medium rose to 4.0. Results indicated that the optimal pH for itaconic acid production by *A. terreus* was pH 3. At this condition, the itaconic acid yield was 0.312 g of itaconic acid/ g of glucose. This result did not agree with many reports (Rychtera and Wase, 1981, Goshadrou *et al*., 2011, Tichagwa, 2012, Riscaldati *et al*., 2000). Their results showed that the optimum pH for itaconic acid production was in the range of 2.0-2.4. However the result from this experiment is supported by Meena *et al*. (2010), who also found that the optimal pH for itaconic acid production by *A. terreus* was pH 3.5. They also suggested that pH control during fermentation was necessary. Since the pH of fermentation media decreases naturally to a pH of 1.85 during itaconic acid fermentation (Riscaldati *et al*., 2000), lack of pH control may reduce itaconic acid production (Ekman, 2010). Low pH levels in fermentation medium has been shown to negatively impact the production of secondary metabolites and it could suppress fungal growth (Riscaldati *et al*., 2000, Tevž et al., 2010). (Hevekerl et al., 2014) reported that itaconic acid fermentation with pH control to pH 3 produced 129 g/L of itaconic acid after 122 hours fermentation (Carvalheiro et al., 2008). However, the higher pH over 3.1 was

considered to have negatively impacted itaconic acid production. It was reported in one paper that when the pH was over 3.1, the mycelia of *A. terreus* formed a pellet (Rychtera and Wase, 1981).

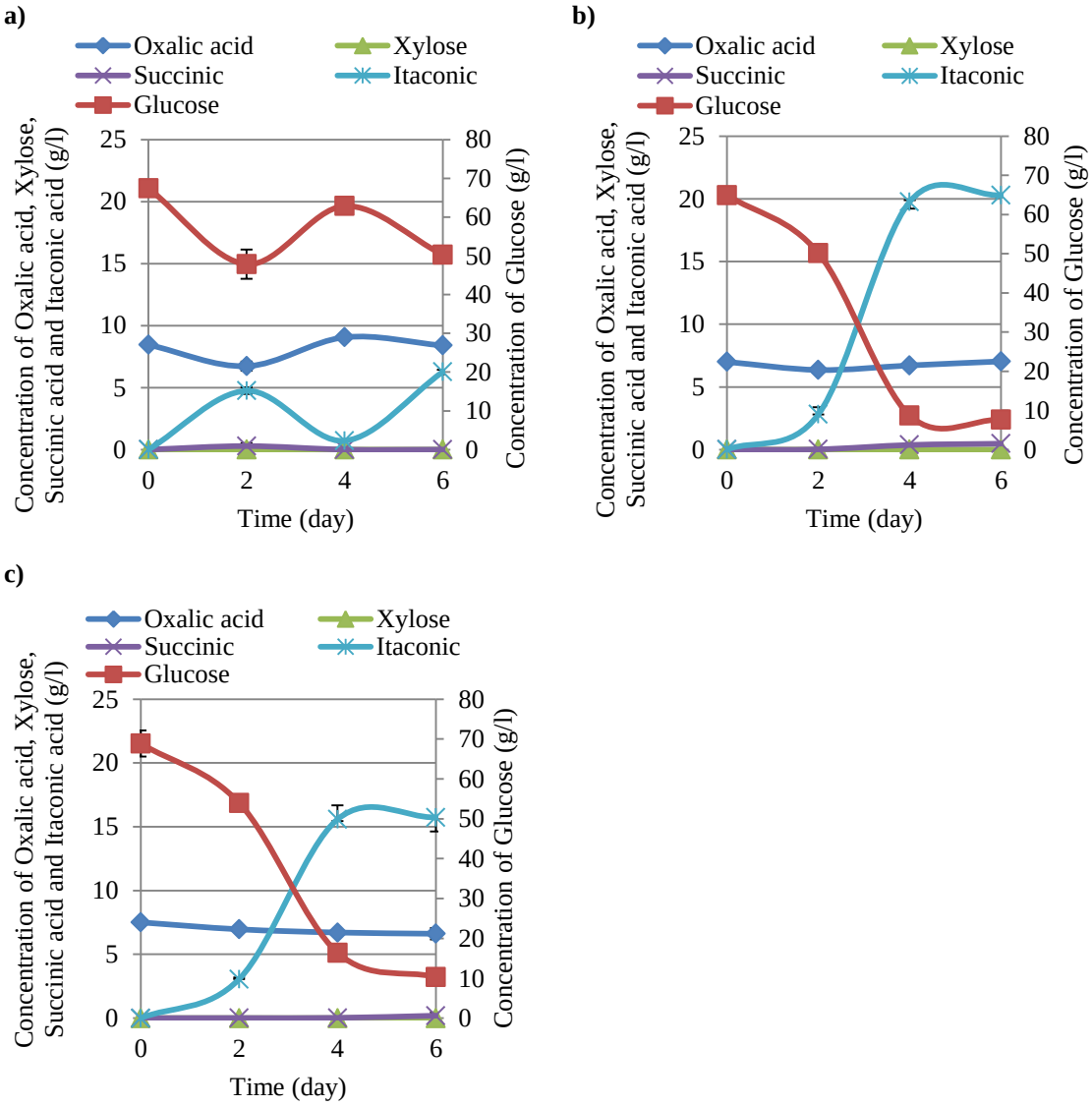


Figure 8. 4 Concentration profiles of Oxalic acid, Glucose, Xylose, Succinic acid and Itaconic acid during fermentation of synthetic medium. a) pH 2, b) pH 3 and c) pH 4.

In the next step, the effect of oxygen transfer rate on itaconic acid production was investigated. As the oxygen transfer rate is related to surface area of the medium, the use of a larger fermentation bottle would lead to a high oxygen transfer rate (Zhang *et al*., 2010b, Lee *et al*., 2014). Itaconic acid fermentations were carried out in 3 different size Erlenmeyer flasks (250 ml, 500 ml and

1000 ml) with the same volume of fermentation medium. Besides the change of flask size, all the other experimental conditions were the same as described in chapter 3.13. Results are illustrated in Figure 8.5. The highest itaconic acid production (26.746±0.386 g/L) was obtained from fermentations using *A. terreus* in 1000 ml Erlenmeyer flasks. This result confirmed that oxygen transfer rate had a positive influence on itaconic acid production. This conclusion is in agreement with several other reports (Guo *et al*., 2008). Moreover, Gyamerah (1995) claimed that *A. terreus* NRRL 1960 stopped producing itaconic acid when the aeration stopped for just 5 minutes (Jacobsen and Wyman, 2000).

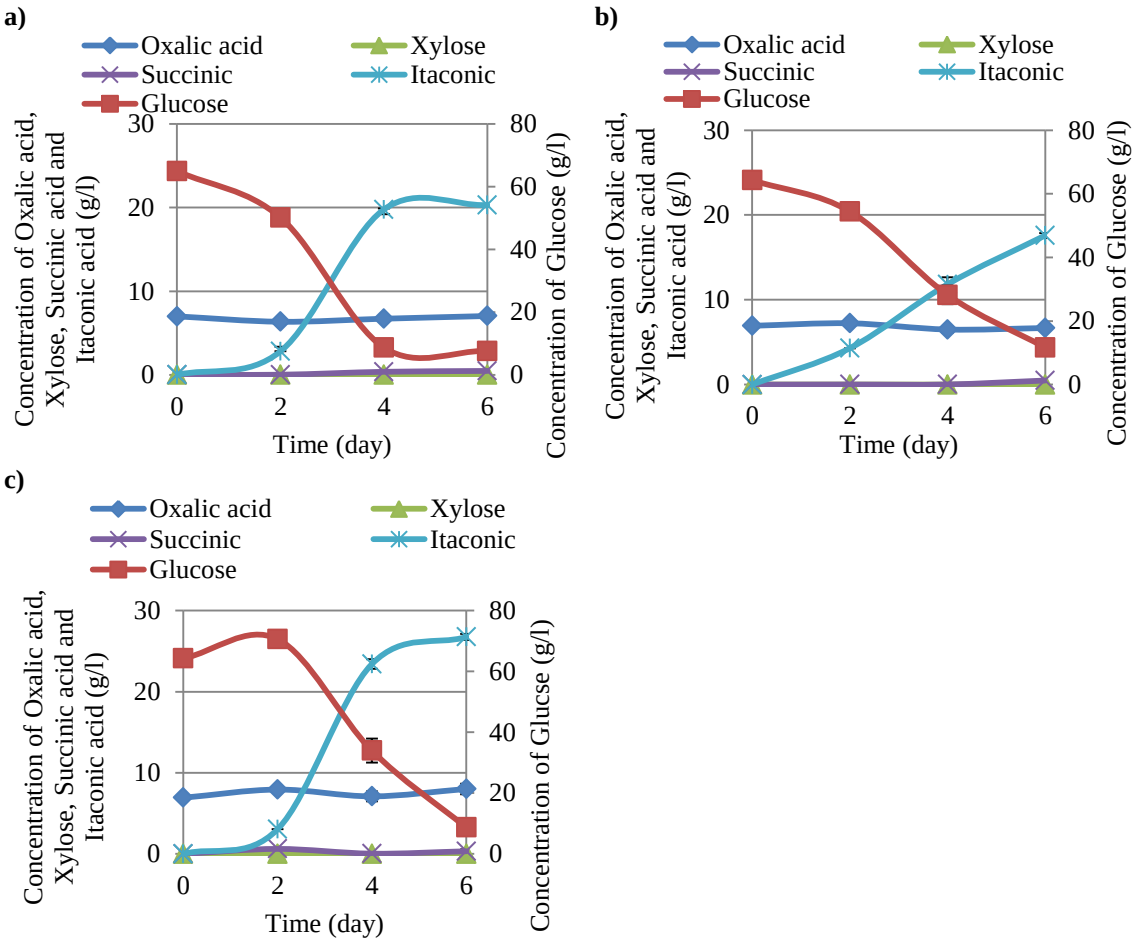


Figure 8. 5 Concentration profiles of Oxalic acid, Glucose, Xylose, Succinic acid and Itaconic acid during fermentation of synthetic medium at pH 3 with different flask size. a) 250 ml. Erlenmeyer flask, b) 500 ml. Erlenmeyer flask and c) 1000 ml. Erlenmeyer flask.

8.3.2 Itaconic acid fermentation using wheat straw hydrolysate

From the above scouting experiments, the highest itaconic acid production was obtained in fermentations using 50 ml of fermentation medium in 1000 ml Erlenmeyer flasks at pH 3.0 at 30˚C. This condition was then further applied to the experiments using wheat straw hydrolysate for itaconic acid production. The itaconic acid fermentation using wheat straw hydrolysate was carried out as described in section 3.13 except wheat straw hydrolysate replaced the semi-synthetic medium. Wheat straw hydrolysate was obtained from enzymatic hydrolysis of fermented wheat straw at 18% solid loading rate, and enzyme loading rate of 55 FPU/g of substrate, 50˚C for 96 hours. It comprised of 70.234±1.628 g/L of glucose, 8.798±0.107 g/L of xylose, 2.022±0.062 g/L of oxalic acid, 0.140±0.177 g/L of succinic acid. The pH of the wheat straw hydrolysate was adjusted to 3.0. Due to the pH adjustment, the sugar content in hydrolysate was diluted to 67.498±0.277 g/L glucose and 8.361±0.048 g/L xylose.

Table 8. 2 The profile of fermentations using wheat straw hydrolysate by *A. terreus*. The fermentation was carried out in 1000 ml of Erlenmeyer flasks containing 50 ml of wheat straw hydrolysate at pH 3.0.

Time (days)	Oxalic acid	Glucose	Xylose	Succinic acid	Itaconic acid
0	7.251±0.163	67.498±0.277	8.361±0.04	0.408±0.048	0.000±0.000
2	8.711±0.230	68.218±1.688	7.600±0.000	0.146±0.168	0.973±0.117
4	7.383±0.339	33.426±0.921	7.001±0.160	0.000±0.000	0.000±0.000
6	8.168±0.194	21.005±0.292	6.517±0.027	0.000±0.000	0.000±0.000

As shown in table 8.3, only trace amount of itaconic acid were produced. This was very low when compared to the itaconic acid production in fermentations using semi-synthetic media. This may be because wheat straw hydrolysate did not contain certain nutrients which are essential for itaconic acid production.

(Kautola et al., 1985) investigated the effect of trace element on itaconic acid production by *A. terreus* TKK 200-5-1. Their results indicated that magnesium sulphate was essential for mycelium growth and itaconic acid production. At least 0.95 g/L of magnesium sulphate was normally added into synthetic medium or hydrolysate for itaconic acid production purposes (Kuenz *et al*., 2012, Dwiarti *et al*., 2007, Yahiro *et al*., 1997, Juy *et al*., 2010). Beside Magnesium, phosphorous is also important for fungal growth (Zhang *et al*., 2010a). Li *et al.* (2012) found that phosphorous and copper had a positive impact on itaconic acid production. Similarly for the production of other organic acids, itaconic acid production is sensitive to nitrogen content in the media (Juy *et al*., 2010). It was suspected that certain nutrients should be added into wheat straw hydrolysate in order to enable it to be used for itaconic acid production. However, due to time limit, these experiments were not carried out.

8.4 Conclusions

In this chapter, the conversion of wheat straw hydrolysate to various biofuels and biochemicals has been investigated. The recombinant yeast strain *S. cerevisiae D2S1S2* was able to produce succinic acid using a wheat straw hydrolysate based medium. The maximum succinic acid yield was 0.10 g succinic acid/ g glucose.

The feasibility of utilising five yeast strains, *S. cerevisiae* NCYC 2592, *S. stipites* CBS 6054, *S. anomalous* CBS 5759, *S. cerevisiae* D2 and *S. cerevisiae* D2S1S2 for the bioethanol synthesis were confirmed. Surprisingly, compared with fermentations using the YPD medium, fermentations using the wheat straw hydrolysate based medium led to higher bioethanol concentrations and higher bioethanol yield with all five strains. Moreover, the results indicated

that strain *S. cerevisiae* D2S1S2 was able to produce succinic acid under aerobic condition and produce ethanol under anaerobic condition.

Itaconic acid production from glucose synthetic media using *A. terreus* was sensitive to oxygen transfer rate and pH. The optimal pH for itaconic acid production was 3.0. No itaconic acid was detected in fermentations using a medium containing only wheat straw hydrolysate, indicating certainly nutrient, e.g. nitrogen source need to be supplied to enable wheat straw hydrolysate to be used for itaconic acid production.

Chapter 9 Conclusion and Future Work

9.1 Conclusion

In this study, a solid state fungal fermentation based strategy has been development for biological pretreatment of wheat straw for the production of a glucose-rich hydrolysate. Then wheat straw hydrolysate has been used for ethanol and value added biochemical synthesis.

In the first step, the feasibility of pretreating wheat straw using a solid state fungal fermentation based biological method was investigated. Two filamentous fungi, *T. reesei* and *A. niger* were cultured on the wheat straw for 2 purposes: (i) to reduce the recalcitrance of wheat straw and (ii) to produce cellulase for the following enzymatic hydrolysis. Initial screening experiments showed that both these two strains were able to reduce the crystallinity of wheat straw and to produce cellulase.

Compared with *T. reesei*, *A. niger* accumulated higher concentration of cellulase in solid state fermentation. And the culture period required to achieve high cellulase production by *A. niger* was only 5 days, two days shorter than that for *T. reesei*. Therefore *A. niger* was selected as the main microorganism for the biological pretreatment of wheat straw in the following study.

The optimisation of SSF condition for the cellulase production by *A. niger* using wheat straw was carried out. The optimum moisture content of SSF was found to be at a liquid to solid ratio of 7.5:1 (w/w). Addition of nutrient like yeast extract and a mineral solution significantly increased cellulase production. Modification of wheat straw before SSF by either, autoclave, acid or alkali encouraged cellulase accumulation. More interestingly, in a SSF using

alkali soaking modified wheat straw, around 21.8 FPU/g cellulase activity was detected after just 1 day fermentation.

Enzymatic hydrolysis of biologically pretreated wheat straw was optimised using a response surface methodology. The model predicted that 72.4 g/L glucose could be produced at a substrate loading rate of 18.3%, an enzyme loading rate of 55.23 FPU/g substrate and a mixing rate of 189 rpm. Experiments carried out at the above mentioned condition resulted in a hydrolysate consist of 59.8 g/L glucose. Enzymatic hydrolysis of a non-treated raw wheat straw at a round up condition (18% solid loading rate, 55 FPU/g and 190 rpm) only led to a hydrolysate containing 10.6 g/L glucose.

In the next step, the wheat straw hydrolysate was explored for the production of bioethanol and biochemicals, including succinic acid and itaconic acid. Prior to utilising wheat straw hydrolysate, a glucose based semi-synthesis medium was used for the investigation of succinic acid synthesis using a range of recombinant yeast strains. Compared with the succinic acid production by native *S. cerevisiae* D2 strain, the single deletion of either SDH1 gene or SDH2 gene increased the succinic acid production by 27%. Encouragingly, the double deletion of both SDH1 and SDH2 enhanced succinic acid production by 68%. Further evaluation of fermentation conditions, including increase oxygen transfer, extend fermentation time, using fed-batch fermentation strategy led to a succinic acid production to around 12 g/L, which was nearly a 100-folds increase over the succinic acid production obtained in fermentations using the wild *S. cerevisiae* D2 strain at the initial fermentation conditions. For *S. cerevisiae* BY4741 strains, the best clones of single, double and triple deletions of ADH3, SDH1, SDH2 genes increased succinic acid production by

approximately 90%, 2.7-times and 4.4-times respectively. However, the change of fermentation condition did not result in further improvement in succinic acid production. Replacement of the semi-synthetic medium by a wheat straw hydrolysate containing around 5.6 g/L glucose led to a succinic acid production of 0.57 g/L succinic acid. The succinic acid yield was 0.10 g succinic acid/ g glucose, which was exactly the same as that observed in fermentations using the semi-synthetic medium.

Replacement of semi-synthetic medium with wheat straw hydrolysate did not affect the growth of bioethanol yeast strains. Moreover, increased in the bioethanol accumulation was observed in all fermentations using the wheat straw hydrolysate. The best ethanol yield obtained was 93.36% of the theoretical yield, which was observed in fermentations using wheat straw hydrolysate by *S. cerevisiae* D2S1S2.

For itaconic acid fermentation by *A. terreus*, itaconic acid prodution using the wheat straw hydrolysate was relatively low in comparison with that using the semi-synthetic medium. This may be due to the lack of nutrients in wheat straw hydrolysate.

9.2 Future work

One of the challenges of biological pretreatment is to find an effective microorganism that can break down lignocellulosic structures in a short culture period with less carbohydrate consumption. Although *A. niger* reduced the recalcitrance of wheat straw and provided fresh cellulase, it consumed cellulose which reduced the overall glucose yield. Strategies to reduce cellulose consumption could be investigated. For example, genetic engineering

could be a possible approach to improve the cellulase production during biological pretreatment. The exploration of underlying theory of biological pretreatment is also an interesting topic, which may provide guidance on further optimisation of the process.

The challenge in enzymatic hydrolysis process is to find a cheap enzyme with high efficacy of biomass conversion to fermentable sugar. Ideally, this enzyme should applicable to all biomass but due to the individual character of each biomass, the enzymes of particular biomass are produced. Although this study demonstrated that the cellulase in the fresh fungal filtrate generated higher glucose concentration than commercial cellulase Ctec2 at the same level of cellulase activity. However the cellulase concentration of fungal filtrate was still low. In order to increase a titre of cellulase in the enzyme solution, cellulase purification and concentration should be investigated. This would enable us to use our own fresh cellulase enzyme to hydrolyse the fermented wheat straw at the optimised hydrolysis condition (18% solid loading rate and 55 FPU/g substrate). This study also demonstrated that the activity of cellulases were increased when additional nutrient presented. Therefore cheap nitrogen source such as soybean mill, corn steep liquor or wheat bran may apply in solid state fermentation to enhance cellulases activity. An alternative way to achieve high efficacy cellulolytic enzyme is build up an enzyme cocktail formula that suitable for the biomass and the enzymes will still need to be produced on in expensive material.

In succinic acid production using genetically modified yeast strains (*S. cerevisiae* D2) on semi synthetic media study, the succinic acid yield of mutant strains were increased up to 67% compared to wild type strain. Moreover this

study found that oxygen transfer rate showed highly influenced on succinic acid. These succinic acid yield improvements of were achieved without optimising the fermentation. Therefore further optimisation of fermentation condition is required for improve succinic acid yield.

Conversion of wheat straw hydrolysate to succinic acid was an interesting topic. However, due to the time limit, the utilisation of a hydrolysate containing a higher glucose concentration was not tested. It would be sound if a fermentation using a wheat straw hydrolysate based medium could generate around 12 g/L succinic acid.

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Appendix

Appendix A

Table A. 1 Particle size distribution of milled wheat straw

Particle size	**Amount (%)**
mixed 2 mm	100.000±0.000
over 425 µm	70.680 ±2.659
between 425-250 µm	14.510 ±0.806
between 250-106 µm	7.940 ±2.517
between 106-75 µm	3.770 ±0.806
less than 75 µm	2.430 ± 0.467

Table A. 2 Characterisation of *A. niger* on cellulase production

Time (day)	**Cellulase activity (FPU/ml)**	**Cellulase activity (FPU/g)**	**Protein content (mg/ml)**	**Glucosamine (mg/ml)**
1	0.055±0.027	0.915±0.447	1.101±0.006	0.697±0.006
3	0.320±0.038	5.342±0.626	2.515±0.007	0.883±0.002
5	0.210±0.011	3.508±0.179	1.809±0.108	0.917±0.003
7	0.161±0.005	2.686±0.089	1.825±0.001	0.969±0.016

Table A. 3 Characterisation of *T. reesei* on cellulase production

Time (day)	**Cellulase activity (FPU/ml)**	**Cellulase activity (FPU/g)**	**Protein content (mg/ml)**	**Glucosamine (mg/ml)**
1	0.178±0.022	2.833±0.365	2.109±0.123	0.497±0.037
3	0.334±0.037	5.573±0.624	2.171±0.089	0.469±0.009
5	0.322±0.043	5.362±0.730	2.466±0.112	0.470±0.016

Table A. 4 Cellulase activity of fungal extract from co-culture on wheat straw via solid state fermentation

Time (day)	Cellulase activity (FPU/ml)	Cellulase activity (FPU/g)
1	0.210±0.030	3.508 ±0.506
2	0.347±0.046	5.784 ±0.769
3	0.668±0.031	11.138±0.511
4	0.132±0.012	2.201±0.193

Table A. 5 Glucose concentration from hydrolyse untreated wheat straw, autoclaved wheat straw and *A. niger* treated wheat straw were hydrolysed by Cellic® CTec 2 (30 FPU/g) with 5% solid loading at 50°C

Sample	Glucose concentration (g/l)
Raw wheat straw	2.101±0.272
Autoclaved wheat straw	2.545±0.129
Fermented wheat straw	2.700±0.125

Appendix B

Table B. 1 The effect of initial moisture content of solid state fermentation on cellulase activity (FPU/g)

Liquid to solid ratio (w/w)	Time (day)	Cellulase activity (FPU/g)
5:1	1	0.000±0.001
	3	4.899±0.438
	5	5.068±0.146
6:1	1	0.340±0.346
	3	5.068±0.073
	5	5.363±0.888
7:1	1	0.357±0.363
	3	4.899±0.334
	5	4.646±0.988
7.5:1	1	0.737±0.638
	3	5.573±0.073
	5	4.520±0.126
8:1	1	0.737±0.638
	3	4.140±0.001
	5	4.688±0.073
9:1	1	0.273±0.395
	3	3.592±0.073
	5	3.887±0.219

Table B. 2 ANOVA Table of the effect of initial moisture content of solid state fermentation on cellulase activity (FPU/g)

Source	***Sum of Squares***	***Df***	***Mean Square***	***F-Ratio***	***P-Value***
Between groups	231.411	17	13.6124	70.14	0.0000
Within groups	6.79309	35	0.194088		
Total (Corr.)	238.204	52			

Note: The ANOVA table decomposes the variance of the data into two components: a between-group component and a within-group component. The F-ratio, which in this case equals 70.1352, is a ratio of the between-group estimate to the within-group estimate. Since the P-value of the F-test is less than 0.05, there is a statistically significant difference between the means of the 18 variables at the 95.0% confidence level.

Table B. 3 The effect of initial moisture content of solid state fermentation on cellulase activity,(FPU/g) after 3 days of incubation.

Liquid to solid ratio (w/w)	***Cellulase activity (FPU/g)***
5:1	4.899±0.438
6:1	5.068±0.073
7:1	4.899±0.334
7.5:1	5.573±0.073
8:1	4.140±0.001
9:1	3.592±0.073

Table B. 4 ANOVA Table the effect of initial moisture content of solid state fermentation on cellulase activity,(FPU/g) after 3 days of incubation

Source	***Sum of Squares***	***Df***	***Mean Square***	***F-Ratio***	***P-Value***
Between groups	7.22663	5	1.44533	24.85	0.0000
Within groups	0.639691	11	0.0581537		
Total (Corr.)	7.86632	16			

The ANOVA table decomposes the variance of the data into two components: a between-group component and a within-group component. The F-ratio, which in this case equals 24.8535, is a ratio of the between-group estimate to the within-group estimate. Since the P-value of the F-test is less than 0.05, there is a statistically significant difference between the means of the 6 variables at the 95.0% confidence level.

Table B. 5 Cellulase production from *A. niger* on non-treated wheat straw reported in cellulase activity (FPU/g)

Time (day)	Trial 1	Trial 2	Trial 3	Average
1	4.988±1.137	3.550±0.809	3.255±0.276	4.037±1.178
3	5.826±0.775	5.826±0.499	4.395±1.002	5.397±0.979
5	3.698±0.657	3.792±1.214	3.128±0.657	3.660±0.861

Table B. 6 ANOVA Table of Cellulase production from *A. niger* on non-treated wheat straw

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	16.7017	2	8.35083	8.11	0.0017
Within groups	27.7979	27	1.02955		
Total (Corr.)	44.4996	29			

The StatAdvisor

The ANOVA table decomposes the variance of the data into two components: a between-group component and a within-group component. The F-ratio, which in this case equals 8.11113, is a ratio of the between-group estimate to the within-group estimate. Since the P-value of the F-test is less than 0.05, there is a statistically significant difference between the means of the 3 variables at the 95.0% confidence level

Table B. 7 Cellulase production from *A. niger* on autoclaved wheat straw reported in cellulase activity (FPU/g)

Time (day)	Trial 1	Trial 2	Average
1	4.330±0.381	4.210±0.434	4.258±0.404
3	9.515±1.644	9.090±1.167	9.245±1.294
5	5.552±0.539	5.784±0.196	5.668±0.405

Table B. 8 Cellulase production from *A. niger* on diluted acid modified wheat straw reported in cellulase activity (FPU/g)

Time (day)	Trial 1	Trial 2	Trial 3	Average
1	8.651±0.584	8.566±0.001	6.322±0.418	7.597±1.271
3	10.012±0.126	11.348±0.126	9.747±0.365	10.435±0.742
5	4.435±0.073	5.278±0.334	4.604±0.842	4.772±0.596

Table B. 9 Summarised acid and hydrothermal modification reported in cellulase activity (FPU/g)

Time (day)	Non-treated	Autoclave	Diluted acid	Acid soaking
1	4.372±1.248	4.258±0.404	7.597±1.271	7.512±1.195
3	5.826±0.622	9.245±1.294	10.435±0.742	5.110±1.076
5	3.887±0.876	5.668±0.405	4.772±0.596	4.730±0.932

Table B. 10 Effect of yeast extract on cellulase production (FPU/g) by *A. niger* on autoclaved modified wheat straw

Time (day)	Autoclave	Autoclave + 0.5 %YE	Autoclave + 5 % YE
1	4.258±0.404	5.373±0.703156	7.962±1.97414
3	9.245±1.294	15.058±0.511084	23.896±4.101583
5	5.668±0.405	18.641±1.644311	38.790±9.853582

Note: YE is yeast extract

Table B. 11 Effect of mineral solution on cellulase production (FPU/g) by *A. niger* on autoclaved modified wheat straw

Time (day)	Autoclave	Autoclave + 0.5 %YE	Autoclave + 0.5 %YE + Mineral
1	4.258±0.404	5.373±0.703156	6.142±0.964478
3	9.245±1.294	15.058±0.511084	16.702±1.328338
5	5.668±0.405	18.641±1.644311	24.037±1.76366

Note: YE is yeast extract, M is mineral solution

Table B. 12 Cellulase production from hot alkali modified wheat straw reported in cellulase activity (FPU/g)

Time (day)	Trial 1	Trial 2	Average
1	17.028±1.907	17.777±1.932	17.292±1.891
3	13.482±0.723	12.328±1.643	12.906±1.363
5	10.736±1.193	9.307±0.508	10.236±1.212
7	n.a	7.211±1.092	7.211±1.092

Table B. 13 Cellulase production from alkali soaking modified wheat straw reported in cellulase activity (FPU/g)

Time (day)	Trial 1	Trial 2	Trial 3	Average
1	22.340±1.448	21.855±2.534	20.833±1.329	21.845±1.865
3	16.196±0.896	15.532±2.060	15.725±1.909	15.689±1.625
5	9.473±0.773	10.410±0.928	9.755±0.998	9.795±0.886
7	6.827±0.976	7.565±0.648	n.a	7.122±0.930

Table B. 14 Cellulase production from hot alkali modified wheat straw by *A. niger* without adding starch and nutrient (FPU/g)

Time (day)	Trial 1	Trial 2	Average
1	4.583±0.521	4.646±0.551	4.610±0.488
3	3.634±0.126	3.339±0.073	3.487±0.186
5	2.370±0.438	2.665±0.479	2.517±0.441
7	1.189±0.479	1.780±0.949	1.579±0.872

Table B. 15 Cellulase production from alkali soaking modified wheat straw by *A. niger* without adding starch and nutrient (FPU/g)

Time (day)	Trial 1	Trial 2	Average
1	6.669±0.876	6.564±0.584	6.599±0.639
3	4.140±0.001	4.282±0.457	4.221±0.332
5	1.358±0.126	1.400±0.365	1.379±0.245
7	0.431±0.073		0.431±0.073

Table B. 16 Cellulase production from hot alkali modified wheat straw by A. niger with adding 2% starch (FPU/g)

Time (day)	Trial 1	Trial 2	Trial 3	Average
1	21.086±3.506	20.169±3.196	21.613±3.438	21.052±3.206
3	14.468±1.011	13.962±1.223	15.501±1.402	14.643±1.324
5	11.538±0.490	10.033±1.548	11.506±0.656	10.950±1.239

Table B. 17 Cellulase production from hot alkali modified wheat straw by A. niger with adding 0.5% yeast extract and mineral solution (FPU/g)

Time (day)	Trial 1	Trial 2	Trial 3	Average
1	35.713±2.466	17.283±0.820	18.582±1.675	20.697±6.666
3	15.037±0.727	11.348±0.935	12.470±1.606	12.466±1.695
5	9.704±0.126	8.456±0.644	7.567±0.987	7.988±1.098
7	4.182±0.448	4.962±0.831	4.485±1.370	4.601±1.116

Table B. 18 Cellulase production from alkali modified wheat straw by A. niger with adding 0.2% starch, 0.5% yeast extract and mineral solution (FPU/g)

Time (day)	Trial 1	Trial 2	Trial 3	Average
1	26.144±2.070		23.182±0.811	24.170±1.917
3	10.969±0.829	12.183±1.337	9.777±1.042	10.817±1.512
5	8.018±1.248	7.808±0.876	7.365±0.494	7.730±0.852
7	2.876±0.759	3.255±0.219		3.065±0.541

Appendix C

Table C. 1 Experimental design and results of multi-level factorial design on glucose releasing

Row	Actual level			Glucose concentration (g/L)	
	Particle size (mm)	Time (min)	Temperature (˚C)	Experiment	Predicted
1	425	15	160	8.416	8.43548
2	75	10	160	8.819	9.17896
3	425	15	180	12.305	12.5599
4	425	5	160	6.529	6.38119
5	250	15	160	8.479	8.49604
6	75	15	160	9.480	9.17185
7	75	5	180	12.469	12.1019
8	250	5	180	11.757	11.4609
9	250	10	160	8.071	8.30058
10	425	5	180	10.882	11.4351
11	425	10	160	7.744	8.03746
12	250	5	160	6.988	6.84687
13	425	10	180	13.428	12.6267
14	75	15	180	12.441	12.4165
15	250	10	180	12.803	12.4499
16	250	15	180	12.024	12.1806
17	75	10	180	12.395	12.8883
18	75	5	160	7.866	7.92781
19	75	15	160	9.487	9.10124
20	250	10	180	12.703	12.3793
21	425	15	180	12.105	12.4893
22	250	15	180	12.024	12.110
23	425	5	160	6.629	6.31058
24	75	15	180	12.441	12.3459
25	75	5	180	12.469	12.0313
26	75	5	160	7.866	7.8572
27	425	15	160	8.416	8.36487
28	250	5	180	10.663	11.3903
29	75	10	160	8.829	9.10835
30	425	5	180	10.782	11.3645
31	250	10	160	8.071	8.22997
32	425	10	180	13.528	12.556
33	425	10	160	7.764	7.96685
34	75	10	180	12.385	12.8177
35	250	15	160	8.479	8.42543
36	250	5	160	6.984	6.77626

Table C. 2 ANOVA table for multi-level factorial design on glucose releasing

Source	***Sum of Squares***	***Df***	***Mean Square***	***F-Ratio***	***P-Value***
A:Particle size	2.95332	1	2.95332	15.54	0.0005
B:Time	8.41706	1	8.41706	44.28	0.0000
C:Temperature	154.949	1	154.949	815.12	0.0000
AA	0.757065	1	0.757065	3.98	0.0565
AB	0.656505	1	0.656505	3.45	0.0745
AC	1.16116	1	1.16116	6.11	0.0203
BB	3.16639	1	3.16639	16.66	0.0004
BC	1.29596	1	1.29596	6.82	0.0148
blocks	0.0448734	1	0.0448734	0.24	0.6311
Total error	4.94243	26	0.190093		
Total (corr.)	178.343	35			

Table C. 3 Experimental design and results of multi-level factorial design on xylose production

Row	Actual level			Xylose concentration (g/L)	
	Particle size (mm)	Time (min)	Temperature (˚C)	Experiment	Predicted
1	425	15	160	14.238	14.5616
2	75	10	160	15.751	16.1745
3	425	15	180	0.477	0.273312
4	425	5	160	19.765	19.7476
5	250	15	160	14.394	14.5909
6	75	15	160	14.044	13.8026
7	75	5	180	8.081	7.82798
8	250	5	180	8.163	8.23908
9	250	10	160	18.108	17.0989
10	425	5	180	7.831	7.83269
11	425	10	160	17.219	17.2059
12	250	5	160	19.364	19.5045
13	425	10	180	4.094	4.10425
14	75	15	180	0.887	0.813354
15	250	10	180	4.337	4.64683
16	250	15	180	0.911	0.952083
17	75	10	180	4.190	4.37192
18	75	5	160	18.338	18.4439
19	75	15	160	14.144	13.8539
20	250	10	180	4.070	4.69806
21	425	15	180	0.777	0.324535
22	250	15	180	0.911	1.00331
23	425	5	160	19.765	19.7989
24	75	15	180	0.880	0.864576
25	75	5	180	8.481	7.8792
26	75	5	160	18.338	18.4951
27	425	15	160	14.238	14.6128
28	250	5	180	8.178	8.29031
29	75	10	160	15.751	16.2258
30	425	5	180	7.831	7.88391
31	250	10	160	18.178	17.1502
32	425	10	180	4.194	4.15547
33	425	10	160	17.329	17.2571
34	75	10	180	4.291	4.42314
35	250	15	160	14.394	14.6421
36	250	5	160	19.364	19.5558

Table C. 4 ANOVA table for multi-level factorial design on xylose production

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
A:Particle size	0.87478	1	0.87478	5.27	0.0301
B:Time	223.284	1	223.284	1344.27	0.0000
C:Temperature	1395.5	1	1395.5	8401.50	0.0000
AA	1.33661	1	1.33661	8.05	0.0087
AB	0.296753	1	0.296753	1.79	0.1929
AC	2.5311	1	2.5311	15.24	0.0006
BB	0.0210125	1	0.0210125	0.13	0.7250
BC	8.44907	1	8.44907	50.87	0.0000
blocks	0.0236134	1	0.0236134	0.14	0.7092
Total error	4.31862	26	0.166101		
Total (corr.)	1636.63	35			

R-squared = 99.7361 percent R-squared (adjusted for d.f.) = 99.6448 percent
Standard Error of Est. = 0.407555 Mean absolute error = 0.239411
Durbin-Watson statistic = 2.153 (P=0.6408) Lag 1 residual autocorrelation = -0.0928808

Table C. 5 ANOVA table for multi-level factorial design on HMF formation

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
A:Particle size	0.0027735	1	0.0027735	0.15	0.7062
B:Time	0.0282907	1	0.0282907	1.48	0.2344
C:Temperature	0.005776	1	0.005776	0.30	0.5870
AA	0.0021125	1	0.0021125	0.11	0.7421
AB	0.0559323	1	0.0559323	2.93	0.0989
AC	0.10962	1	0.10962	5.74	0.0241
BB	0.269378	1	0.269378	14.11	0.0009
BC	0.0213607	1	0.0213607	1.12	0.2999
blocks	0.00822044	1	0.00822044	0.43	0.5175
Total error	0.496412	26	0.0190928		
Total (corr.)	0.999876	35			

R-squared = 50.3527 percent R-squared (adjusted for d.f.) = 33.167 percent
Standard Error of Est. = 0.138177 Mean absolute error = 0.0794352
Durbin-Watson statistic = 2.55485 (P=0.9510)Lag 1 residual autocorrelation = -0.299978

Table C. 6 Enzymatic hydrolysis on difference biomass by using commercial enzyme (Cellic ® CTec2) at solid loading rate of with enzyme loading 30 FPU/g ,50˚C for 73 h

Time (h)	Fermented wheat straw + Ctec2	Autoclaved wheat straw + Ctec2	Milled wheat straw +Ctec 2
0	0.000±0.000	0.000±0.000	0.000±0.000
2	2.173±0.013	1.634±0.020	0.642±0.044
4	2.622±0.202	2.209±0.090	1.442±0.062
6	2.700±0.125	2.545±0.129	2.101±0.272
22	3.022±0.212	3.026±0.114	2.656±0.189
30	3.156±0.189	3.040±0.214	2.650±0.028
49	3.131±0.114	3.154±0.094	2.492±0.117
73	3.160±0.007	3.449±0.037	1.496±0.279

Table C. 7 Analysis of Variance for saccharification yield of enzymatic hydrolysis on fermented wheat straw

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
X_1:substrate concentration	0.00131966	1	0.00131966	0.26	0.6172
X_2:enzyme loading rate	0.20917	1	0.20917	41.57	0.0000
X_3:mixing rate	0.0410513	1	0.0410513	8.16	0.0135
X_1^2	0.00181931	1	0.00181931	0.36	0.5580
X_1X_2	0.00358586	1	0.00358586	0.71	0.4138
X_1X_3	0.00690375	1	0.00690375	1.37	0.2625
X_2^2	0.0666703	1	0.0666703	13.25	0.0030
X_2X_3	0.0330243	1	0.0330243	6.56	0.0237
X_3^2	0.0344547	1	0.0344547	6.85	0.0213
Total error	0.0654166	13	0.00503205		
Total (corr.)	0.462506	22			

R-squared = 85.8561 percent
R-squared (adjusted for d.f.) = 76.0641 percent
Standard Error of Est. = 0.0709369
Mean absolute error = 0.0422328
Durbin-Watson statistic = 1.20962 (P=0.0278)
Lag 1 residual autocorrelation = 0.369784

Appendix D

Table D. 1 Concentrations of glucose, succinic acid, glycerol, formic acid, acetic acid and ethanol (g/L) in succinic acid fermentations using *S. cerevisiae* D2 and *S. cerevisiae* D2S1S2 for 72 hours.

	Glucose	**Succinate**	**Glycerol**	**Formic**	**Acetate**	**Ethanol**
Initial hydrolysate	5.552±0.060	0.006±0.010	0.026±0.000	0.000±0.000	0.000±0.000	0.000±0.000
D2WT 500	0.000±0.000	0.199±0.001	0.023±0.003	0.000±0.000	0.004±0.006	0.029±0.017
D2WT 1L	0.000±0.000	0.239±0.009	0.027±0.003	0.002±0.003	0.005±0.006	0.062±0.012
D2S1S2 1L	0.000±0.000	0. 576±0.020	0.000±0.000	0.006±0.015	0.000±0.000	0.055±0.005

Table D. 2 Concentration of Glucose, Xylose, Succinic acid, Glycerol, Acetic acid, Formic acid and Ethanol during ethanol fermentation.

Strains	Fermentation Medium	Glucose	Succinate	Glycerol	Acetate	Ethanol
n/a	Hydrolysate	59.810±2.062	0.005±0.001	0.024±0.003	0.182±0.034	0.008±0.001
S.cerevisia NCYC 2592	Hydrolysate	0.000±0.000	0.676±0.031	3.010±0.000	0.676±0.147	28.055±0.200
	YPD	0.002±0.003	0.386±0.049	2.636±0.009	0.810±0.051	24.645±0.645
Sc. stipitis CBS 6054	Hydrolysate	0.000±0.000	0.506±0.066	4.361±0.001	1.044±0.728	26.589±0.001
	YPD	7.453±0.000	0.149±0.039	0.172±0.099	0.286±0.105	23.967±0.001
Sc. anamalous CBS 5759	Hydrolysate	0.000±0.000	0.804±0.024	3.114±0.417	0.846±0.134	27.640±1.822
	YPD	0.000±0.000	0.518±0.068	3.345±0.189	0.707±0.079	24.723±0.688
S.cerevisiae D2	Hydrolysate	0.000±0.000	0.858±0.013	3.650±0.039	0.480±0.033	26.615±0.297
	YPD	0.000±0.000	0.543±0.045	2.849±0.054	0.410±0.274	24.110±0.605
S.cerevisiae D2S1S2	Hydrolysate	0.000±0.000	0.614±0.001	3.249±0.001	1.388±0.001	28.648±0.001
	YPD	0.000±0.000	0.000±0.001	1.462±0.557	1.087±0.022	23.765±0.816

Note : Xylose concentration in wheat straw hydrolysate was 10.509±0.018 g/L

Table D. 3 Ethanol concentration and Ethanol yield from different media and different yeast strain.

Strains	Fermentation Medium	Ethanol	Ethanol yield (%)
n/a	Hydrolysate	0.008±0.001	n/a
S. cerevisiae NCYC 2592	Hydrolysate	28.055±0.200	91.430±0.652
	YPD	24.645±0.645	80.316±2.101
Sc. stipitis CBS 6054	Hydrolysate	26.589±0.001	86.648±0.001
	YPD	23.967±0.001	78.104±0.002
Sc. anamalous CBS 5759	Hydrolysate	27.640±1.822	90.074±5.937
	YPD	24.723±0.688	80.569±2.241
S. cerevisiae D2	Hydrolysate	26.615±0.297	86.734±0.969
	YPD	24.110±0.605	78.570±1.972
S. cerevisiae D2S1S2	Hydrolysate	28.648±0.001	93.361±0.002
	YPD	23.765±0.816	77.446±2.658

Table D. 4 Concentration profiles of Oxalic acid, Glucose, Xylose, Succinic acid and Itaconic acid during fermentation of synthetic medium at pH2-pH4.The itaconic fermentation was carried out in 250ml Erlenmeyer flask with 50 ml of synthetic medium.

		Oxalic acid		
pH	Day 0	Day 2	Day 4	Day 6
2	8.452±0.000	6.729±0.374	9.056±0.086	8.395±0.153
3	6.997±0.054	6.352±0.000	6.715±0.001	7.037±0.033
4	7.504±0.018	6.959±0.000	6.709±0.054	6.613±0.434
		Glucose		
pH	Day 0	Day 2	Day 4	Day 6
2	67.427±0.000	47.847±3.804	62.844±0.707	50.283±0.758
3	64.949±0.642	50.140±0.000	8.715±0.020	7.629±0.027
4	68.862±3.290	53.996±0.000	16.416±0.265	10.276±0.395
		Xylose		
pH	Day 0	Day 2	Day 4	Day 6
2	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
3	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
4	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
		Succinic		
pH	Day 0	Day 2	Day 4	Day 6
2	0.000±0.000	0.285±0.230	0.000±0.000	0.000±0.000
3	0.000±0.000	0.020±0.000	0.3690.013±	0.470±0.005
4	0.000±0.000	0.008±0.000	0.021±0.001	0.190±0.264
		Itaconic		
pH	Day 0	Day 2	Day 4	Day 6
2	0.000±0.000	4.743±0.264	0.713±0.018	6.264±0.185
3	0.000±0.000	2.813±0.000	19.773±0.557	20.298±0.137
4	0.000±0.000	3.051±0.000	15.580±0.127	15.731±1.107

Table D. 5 Concentration profiles of Oxalic acid, Glucose, Xylose, Succinic acid and Itaconic acid during fermentation of synthetic medium at pH 3 with different flask size

Flask size	**Oxalic acid**			
(ml)	Day 0	Day 2	Day 4	Day 6
250	6.997±0.054	6.352±0.000	6.715±0.001	7.037±0.033
500	6.931±0.065	7.226±0.171	6.493±0.398	6.670±0.059
1000	6.931±0.065	7.938±0.026	7.102±0.594	8.013±0.659
Flask size	**Glucose**			
(ml)	Day 0	Day 2	Day 4	Day 6
250	64.949±0.642	50.140±0.000	8.715±0.020	7.629±0.027
500	64.268±0.585	54.464±0.330	28.202±2.949	11.667±0.041
1000	64.268±0.585	70.637±0.246	33.968±3.980	8.679±0.028
Flask size	**Xylose**			
(ml)	Day 0	Day 2	Day 4	Day 6
250	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
500	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
1000	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
Flask size	**Succinic**			
(ml)	Day 0	Day 2	Day 4	Day 6
250	0.000±0.000	0.020±0.000	0.369±0.013	0.470±0.005
500	0.000±0.000	0.007±0.001	0.012±0.001	0.478±0.016
1000	0.000±0.000	0.620±0.008	0.037±0.007	0.332±0.251
Flask size	**Itaconic**			
(ml)	Day 0	Day 2	Day 4	Day 6
250	0.000±0.000	2.813±0.000	19.773±0.557	20.298±0.137
500	0.000±0.000	4.288±0.003	11.820±0.847	17.584±0.298
1000	0.000±0.000	3.044±0.001	23.413±0.584	26.746±0.386