Optimisation of a Simultaneous Saccharification and Fermentation Process for use with Steam Pretreated Sweet Sorghum Bagasse

by

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Thesis presented in partial fulfillment
of the requirements for the Degree

of

MASTER OF SCIENCE IN ENGINEERING
(CHEMICAL ENGINEERING)

in the Faculty of Engineering
at Stellenbosch University

Supervised by

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December 2013

DECLARATION

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Abstract

Global warming and greenhouse gas (GHG) emissions are serious issues of our time. One of our greatest sources of pollution originates from the combustion of fossil fuels where the largest contributor, on a global scale, is the transport sector. Renewable sources of liquid fuels have been investigated with bioethanol being the most widely used worldwide. Production of first generation bioethanol from food crops raised concerns over food supplies being channelled for biofuel production leading to possible increases in food prices. Second generation or lignocellulosic bioethanol from the residual plant material does not compete with food crops while dedicated energy crops could be cultivated on land that is not suited for food production by agriculture. Of the various crops investigated, sweet sorghum appears to be a viable candidate for lignocellulosic ethanol production since this crop can be cultivated in a range of soil conditions whilst low water requirements make it ideal for arid regions. The sweet juice extracted from the sweet sorghum stem can be utilised for first generation biofuel production in conjunction with the bagasse in a second generation plant. In order to ensure economic feasibility for industrial-scale lignocellulosic ethanol production, it is critical that the process as a whole be optimised to minimize expenditure while maximising yields.

In the current study a fed-batch simultaneous saccharification and fermentation process was developed for use with steam-pretreated sweet sorghum bagasse at high solid loadings. Two separate studies were conducted in parallel with the first focusing on selecting the preferred sweet sorghum varieties and optimising the steam pretreatment conditions while the second focussed on optimising the enzyme cocktail required for hydrolysis, which occurs simultaneously with fermentation. The developed SSF process was investigated by utilising the preferred pretreatment conditions and enzyme cocktails and further optimised in terms of solid loadings and feeding to achieved 40 g/L of ethanol.

Preliminary fermentation runs were performed to identify the mains issues with the SSF process. For these runs sweet sorghum bagasse of variety MSJH13, pretreated dry (7.3% moisture) at 200 °C for 5 minutes, with an enzyme dosage of 0.167 ml Cellic CTec2/g dry WIS, was utilised. Investigation of the three process options of whole slurry, pressed WIS and washed WIS resulted in low ethanol concentrations and accumulation of glucose. The performance of three recombinant strains of *Saccharomyces cerevisiae* (D5A^{+}, D5A^{+H} and TMB3400) and one wild-type industrial strain (MH1000) were investigated under SSF conditions. Strain MH1000 delivered the highest ethanol concentration of 34.5 g/L from a cumulative solids loading of 20% using pressed WIS compared to 32.2 g/L for

strain D5A^{+H} under the same conditions. At a solid loading of 20%, yeast growth and fermentation inhibition coupled with glucose accumulation was observed, due to inhibitor concentrations reaching critical levels. Strain MH1000 showed increased tolerance to the inhibitor concentrations and was only inhibited after 94h compared to 76h for strain D5A^{+H}.

To optimise the SSF process the preferred sweet sorghum varieties SS27 and AP6 were pretreated with water-impregnation at 205 °C for 5 minutes while variety SS27 was also pretreated after impregnation with 3% SO_2 at 185 °C for 8 minutes. Two enzyme cocktails of 0.15 ml Cellic CTec2/g dry WIS and containing either 0.32 ml Cellic HTec2/g dry WIS or 0.017 ml Cellic HTec2/g dry WIS (referred to as Cocktail 1 and Cocktail 2) were also investigated. At cumulative solid loadings of 20% in fed-batch SSF using the water-soaked material, yeast inhibition and glucose accumulation was observed, irrespective of the enzyme cocktail utilised. The 18-fold increase in the Cellic HTec2 concentration from Cocktail 1 did not significantly increase the ethanol productivity or ethanol concentration obtained, compared to Cocktail 2 which resulted in 43.6 g/L of ethanol. By reducing the cumulative solids loading to 16% and 13%, glucose accumulation was reduced and avoided for the respective loadings while the maximum ethanol concentration only decreased to 41.4 g/L and 38.9 g/L respectively. An ethanol yield of 82% of the theoretical maximum, based on the glucose added to the fermentation broth, was calculated for fermentation with the cumulative solid loading of 13% using the water-soaked material form variety SS27. In literature a similar yield of 75% has been reported for a solids loading of 16%, but washing of the pretreated material prior to SSF was required. Using pretreated material from variety SS27, impregnated with 3% SO_2 as catalyst during steam pretreatment, at a solids loading of 13% and Cocktail 2 resulted in a maximum ethanol concentration of 36.8 g/L and a productivity of 0.298 g/L.h with a yield of 79% of the theoretical maximum (based on glucose). The differences, compared to the water-soaked only pretreatment, were not statistically significant. Similarly sweet sorghum varieties AP6, pretreated after water soaking, under SSF conditions also exhibited no significant differences compared to variety SS27 with regards to the maximum ethanol concentration (35.8 g/L) and productivity (0.289 g/L.h) obtained and a yield of 75% of the theoretical maximum (based on glucose) was calculated.

Samevatting

In die moderne era word groot klem gelê op die effekte van aardverwarming weens kweekhuisgasse. Op 'n wêreldwye skaal is die grootste bron van kweekhuisgasse afkomstig vanaf die verbranding van fossielbrandstowwe soos gebruik deur die vervoerindustrie. Van die alternatiewe brandstowwe wat ondersoek word, blyk bio-etanol die belowendste te wees. Eerstegenerasie bio-etanol afkomstig vanaf voedselbronne soos mielies het gelei tot etiese kwessies vanweë die wêreld voedseltekort. Tweedegenerasie of lignosellulose etanol maak gebruik van residuele plant materiale of energiegewasse was verbou kan word op grond wat ongeskik is vir boerdery bedrywighede en hou dus geen bedreig vir voedselverskaffing in nie. In Suid-Afrika blyk soetsorghum 'n ideale energiegewas te wees vir die produksie van tweedegenerasie bio-etanol aangesien hierdie gewas verbou kan word 'n wye reeks grond kondisies. Die sap afkomstig vanaf soetsorghum kan gebruik word vir etanol produksie in 'n eerstegenerasie proses terwyl die oorblywende plantmateriaal gebruik kan word in die tweedegenerasie proses. Om die ekonomiese lewensvatbaarheid van die proses te verseker, moet proses optimering ondersoek word.

Dit was genoodsaak om die gelyktydige hidroliese en fermentasie (GHF of *SSF* in Engels) van stoom behandelde soetsorghum bagasse in 'n semi-enkellading proses te ondersoek. Studies wat in parallel uitgevoer was, het gefokus op die seleksie van soetsorghum kultivars en optimering van die kondisies vir stoom behandeling en die bepaling van die ensiempreparaat vir hidroliese. Die optimeerde stoom behandelings kondisies en ensiempreparaat was ondersoek tydens *SSF* tesame met verder optimering van die SSF proses om ten einde 'n etanol konsentrasie van 40 g/L te verkry.

Vir ontwikkeling van die SSF proses was monsters van soetsorghum kultivar MSJH13 met 'n vog-inhoud van 7.3%, behandel teen 200 °C vir 5 minute en 'n ensiem lading van 0.167 ml Cellic CTec2/gram droë materiaal was gebruik. Ondersoek van die drie moontlike proses opsies van *slurry, pressed WIS en washed WIS* was onbevredigend vanweë die lae etanol konsentrasies en akkumulasie van glukose. Drie rekombinante gis rasse van *Saccharomyces cerevisiae* ($D5A^{+}$, $D5A^{+H}$ en TMB3400) en een industriële ras (MH1000) was ondersoek. Ras MH1000 het die hoogste etanol konsentrasie gelewer (34.5 g/L) vanaf 'n materiaallading van 20% en ook die stertste weerstand teen inhibitore gebied. 'n Akkumulasie van glukose weens inhibisie van die gis was waargeneem vir 'n materiaallading van 20%, vanweë die konsentrasie van inhibitore in die proses.

Vir optimering van die SSF proses was monsters van soetsorghum kultivars SS27 en AP6 behandel na water-benatting teen 205 °C vir 5 minute en 'n tweede monster van SS27 was ook behandel met 3% SO_2 teen 185 °C vir 8 minute. Twee ensiemdosisse was ondersoek en beide het 0.15 ml Cellic

CTec2/gram droë materiaal bevat met onderskeidelik 0.32 ml Cellic HTec2/gram droë materiaal of 0.017 ml Cellic HTec2/gram droë materiaal (*Cocktail 1* en *Cocktail 2*).

Met ‘n materiaallading van 20% was inhibisie van die gis en akkumulasie van glukose ondervind, ongeag die ensiemdosis wat gebruik was. Die hoë ensiemdosis van *Cocktail 1* het nie ‘n beduidende verhoging in die etanol konsentrasie veroorsaak nie, in vergelyking met *Cocktail 2* wat 43.6 g/L etanol gelewer het. Deur die materiaallading te verlaag tot 13% kon inhibisie van die gis voorkom word en is ‘n etanol konsentrasie van 38.9 g/L verkry teen ‘n etanol opbrengs van 82% van die teoretiese maksimum (gebaseer op die glukose in die fermentasie). ‘n Vergelyking met SO_2 behandelde materiaal (teenoor water-benatte materiaal van kultivar SS27) teen ‘n materiaallading van 13% het geen statistiese beduidende verskille opgelewer met betrekking tot die maksimum etanol konsentrasie (36.8 g/L) of produktiwiteit (0.298 g/L.h) nie terwyl ‘n opbrengs van 79% bereken was. Vergelyking met soetsorghum AP6, ook water-benat, in *SSF* met ‘n materiaallading van 13% het ook geen beduidende verskille opgelewer in terme van maksimum etanol konsentrasie (35.8 g/L) of produktiwiteit nie (0.289 g/L.h).

Acknowledgements

The following people are thanked for their contribution to this project:

Prof. J.F. Görgens for his knowledge, insight and guidance over the past 3 years in helping me to complete this project.

Justin Smith and Drs. Eugéne van Rensburg and **María del Prado García-Aparicio** and for their inputs, knowledge and comments relating to my work.

The Lignocellulosic Ethanol Research Group for their interests and recommendations relating to the work.

Mrs. M. Rossouw and **Ms. L. Hamerse** for analyses of all the samples.

The **National Research Foundation** (NRF) for financial support

I dedicate this work to my parents,

Wilhelm and Alda Dreyer

Without your love, support and encouragement this project would never have seen completion.

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1. INTRODUCTION

In the 21st century, global warming and the effects thereof is one the most serious topics affecting our existence. One of the main contributors to global warming is the use of fossil fuels with the global transport sector being one of the largest contributors [1,2]. Continuously soaring oil prices and political instability in many of the OPEC countries further underline the fact that an alternative source of liquid fuel has to be found [1]. Numerous alternative sources were investigated, including bioethanol, biodiesel and hydrogen. Of the aforementioned fuels, bioethanol is by far the most widely used biofuel worldwide, accounting for nearly 2% of the worldwide fuel supplies [2,3].

The use of biofuels, particularly bioethanol, was widespread in both Europe and the United States until the early twentieth century. For instance vegetable oils, either pure or blended, were used in diesel engines, while ethanol was investigated in the Otto engine [4]. Since the end of World War 2, it became cheaper to produce petroleum-based fuels and potential of bioethanol was ignored [2]. Currently most vehicles that run on gasoline can utilise a maximum blend of 10% ethanol (E10), while in countries such as the USA, Canada and Brazil, so-called flexi-fuel vehicles can utilise a blend with up to 85% ethanol (E85) [2,5]. In the United States the incentives for buying flexi-fuel vehicles are low since there are only a few service stations that stock E85 gasoline [5]. One area where the use of ethanol would be advantageous is as a fuel additive/oxygenate as replacement for carcinogenic MTBE (methyl tertiary butyl ether) presently used to improve the combustion of the hydrocarbons in gasoline [5]. Ethanol would not only be a more environmentally-friendly alternative, but can be used in current vehicles without modifications, while simultaneously reducing our dependence on imported fossil fuels. Combustion of bioethanol also generates lower levels of nitrogen oxides and volatile organic compounds but increased emissions of aldehydes, which can be controlled through equipping vehicles with catalytic converters [6].

During the oil crisis of the 1970's, the use of alternative fuel sources were encouraged and bioethanol production reached a peak of 15 billion litres in 1985 [7]. Afterwards, the level of biofuel production was relatively stable. Currently new targets for renewable fuels are being set, such as the EU Renewable Energy Directive that requires biofuels to account for 10% of transport fuel by 2020 [8] and the U.S. Energy Independence and Security Act of 2007 which calls for a biofuel standard of 36 billion gallons by 2022 [5]. In 2006, a biofuel study conducted in South Africa concluded that a biofuel target of 3.4% (only liquid fuels) in 2013 seemed to be a reasonable target [9]. In the following year (2007) the National Biofuel Industrial Strategy was released with the aim of achieving a 2% target of the national fuel supply [10]. Further regulations relating to the blending of

biofuels with petrol and diesel were passed in 2012, but these regulations did not enforce mandatory blending [11].

First generation biofuels are produced from food sources, such as corn and wheat [8]. This led to debates regarding the ethical aspects of using food for fuel and fears of increasing food prices, which would be felt most strongly in developing countries [5,8,12]. As an alternative, second generation biofuels, produced from residues of plant material (lignocellulosics), pose no risk to food security [13]. Growing dedicated energy crops for second generation biofuel production on land of marginal quality which is not current being utilised, would minimise the impact on land usage for food crop production. Whilst it was found in 2008 that industrial-scale production of second generation bioethanol was not feasible, future improvements and developments of the production process could potentially enhance the economic feasibility [4].

The recalcitrant nature of the lignocellulosic (second generation) substrates makes it difficult to unlock the fermentable sugars required for ethanol fermentation. The first step in the production of 2nd generation ethanol is thus pretreatment where the fibrous plant material is treated, mostly though thermochemical means, to render the material amenable to enzymatic digestion. The resulting sugars are readily fermented to ethanol, mostly using *Saccharomyces cerevisiae* (brewer's yeast), followed by a distillation step to remove ethanol from the fermentation broth [2]. During pretreatment a portion of the sugars in the fibres are degraded to form by-products such as organic acids, furans and phenolic compounds, which are inhibitory to the enzymes and yeast [14–17].

A great deal of literature is available on integrating enzymatic hydrolysis and fermentation steps into a single unit operation known as simultaneous saccharification and fermentation (SSF), as seen in Section 2.5 of this report. This process configuration provides several benefits over separate hydrolysis and fermentation (SHF), including a decrease in feedback inhibition on the enzymes through fermentation of the liberated sugars during hydrolysis. Feedback inhibition of the hydrolysing enzymes is characterised by a slowing in the rate of hydrolysis in response to increased sugar concentrations in the medium. The ethanol produced and low residual sugar concentrations also contribute to combating microbial infections within the process vessel [18]. Furthermore, the yeast have also been shown to assimilate the inhibitory products from pretreatment when present at sub-toxic levels [14,19], thereby relieving the inhibition on the enzymes [15,20]. However, the main disadvantage of SSF over SHF is a compromise with regards to the operating temperature since enzymatic hydrolysis has an optimum temperature around 50 °C compared to 30 – 37 °C for the fermenting organisms [16].

To ensure the feasibility of the lignocellulosic ethanol process it is important that the process as a whole, i.e. pretreatment, hydrolysis and fermentation, be optimised as an integrated whole to ensure that, first and foremost, maximum yields are obtained. Higher productivities, without sacrificing yield, would increase the throughput of the process which would allow for capital savings in terms of the capacity of the process equipment required. Simultaneous saccharification and fermentation processes incorporate the hydrolysis and fermentation steps in a single processing vessel, thereby reducing capital costs by as much as 20% [17]. Regarding the production costs of lignocellulosic ethanol, the pretreatment step alone can account for as much as 33% of the processing cost (energy, labour etc.) of ethanol production [21]. The use of optimised pretreatment methods and feedstock material or varieties most susceptible to degradation is thus imperative. A higher enzyme dosage could lead to increased productivity and possibly higher sugar yield but would result in an increase in process costs [22,23]. An enzyme cocktail (or cocktails) containing the optimum combination of activities to ensure maximum sugar liberation should thus be found.

This study formed one part of a three-part study on the investigation of lignocellulosic ethanol production from sweet sorghum bagasse. The additional in-depth studies were conducted by other researchers in parallel to the present study, and focussed on optimising the steam pretreatment and enzymatic hydrolysis steps. From these studies the most suitable sweet sorghum cultivars were selected on the basis of agronomic factors, chemical composition and pretreatment response of a small-scale dilute-acid process [24]. Steam pretreatment of the selected cultivars was optimised with regards to the digestibility and chemical composition (sugar content) of the pretreated fibres, while minimizing the amount of inhibitors formed [24]. Therefore, the pretreatment conditions selected in the present study were based on optimisations performed in parallel studies by other researchers. Enzymatic hydrolysis optimisation was based on the effect of different volumes and combinations of enzymes on the hydrolysis of sweet sorghum bagasse samples. Using a central composite design (CCD) the minimum volume of enzymes required for 80% hydrolysis of the polysaccharides in the pretreated material was identified [25]. Regarding the current study, with the focus on fermentation, the overall aim was to obtain 40 g/L of ethanol in the final fermentation broth of a process based on SSF of steam pretreated sweet sorghum bagasse. To ensure feasibility the ethanol yield obtained from the total glucose in the fermentation broth should also be maximised while ensuring the process operates with high ethanol productivity. To achieve these goals, the study was divided into two parts, with the first focussing on developing an SSF process for use with steam-pretreated sweet sorghum bagasse to ferment the high solid loadings required in order to obtain 40 g/L of ethanol. Factors such as the yeast strain utilised and the solid material feed rate during fed-batch fermentations were investigated. The combination of best performing

parameters was used as the basis for the second part of the study which required optimisation of the process to achieve the required concentration of ethanol. A comparison between two pretreatment methods of using either water or SO_2 as catalyst was performed. Utilising two preferred varieties of sweet sorghum bagasse, the performance of two optimised enzyme cocktails were investigated under SSF conditions. Different solid loadings of 20, 16% and 13% were investigated to determine the minimum loading required to obtain 40 g/L with minimal residual glucose.

1.1 LAYOUT OF THESIS

Chapter 1 provides an introduction to lignocellulosic ethanol production and covers the requirements for this process.

Chapter 2 provides a review of the literature covering the aspects relating to the lignocellulosic ethanol production process with the focus on utilisation of simultaneous saccharification and fermentation (SSF).

Chapter 3 states the research question(s) that will serve as the basis for the research strategy.

Chapter 4 contains the first part of the work regarding the screening of process parameters. The work involved the development of a protocol for a SSF process for use with high solid loadings. During this part of the study one sweet sorghum variety and one enzyme dosage was selected.

Studies by McIntosh [24] and Pengilly [25] were conducted in parallel with this project. McIntosh [24] selected the most suitable sweet sorghum cultivars and optimised the pretreatment conditions for pilot-plant steam pretreatment. Pengilly [25] optimised enzymatic hydrolysis by investigating the effects of various dosages and combinations of two types of cellulase mixtures produced by Novozymes, namely Cellic CTec2 and Cellic HTec2, on the sugars released during hydrolysis of one steam pretreated sweet sorghum bagasse sample. The outputs from the work by McIntosh [24] and Pengilly [25] were incorporated into optimisation of the SSF process in the present study.

Chapter 5 contains the second part of the work and designated as 'Process optimisation'. The work involved optimisation of the SSF process by investigating two optimised enzyme cocktails and the performance of selected sweet sorghum cultivars and pretreatment conditions to obtain 40 g/L of ethanol.

Chapter 6 provides concluding thoughts and summarises the answers to the research question posed earlier in Chapter 3.

Chapter 7 contains recommendations for future work to expand upon the current project, followed by the references sited in **Chapter 8**.

2. Literature review

2.1 First Generation Biofuels

The so-called "first generation" biofuels are produced mainly from starch- or sugar-crops, such as corn and sugarcane [3,16]. In the developed countries (especially the United States, Brazil and some European countries), production of biofuels has greatly increased during the last 10 years [3,5,8,12]. However, given their widespread availability, high energy densities and efficient production processes, fossil fuels can still be produced relatively cheaply, making it difficult for biofuels to obtain a substantial foothold in the global liquid fuel market. A further concern preventing the widespread implementation of first generation biofuels is the possible competition arising between fuel and food. Table 2.1 provides a summary of the five main ethanol producing nations which accounted for 80% of the worldwide bioethanol production in 2005 together with the feedstocks utilised. It is worth noting that all of the ethanol was produced using first generation processes.

Table 2.1: Summary of the five largest bioethanol producers in 2005 and the feedstock utilised.

Country	Litres produced (millions)	Feedstock
Brazil	16 489	Sugarcane
USA	16 217	Corn
China	1 998	Corn, wheat
European Union	950	Sugar beet, wheat, sorghum
India	299	Sugarcane

Data obtained from Escobar et al. [4].

The main advantages of first generation biofuels, accounting for their increased interest, include a decrease in the volume of imported foreign oil, especially with the high and constantly fluctuating oil price and environmental concerns regarding possible oil spills [3]. Since first generation biofuels are produced from renewable resources, there is larger security regarding the availability of supply [2]. In the developing countries or countries with a strong agricultural sector, where there is a greater availability of land, there is a widespread availability of the required biomass [2]. Whilst ensuring greater supply security and widespread availability, crops cultivated in rural areas would also provide farmers with an increase in disposable income, leading to the strengthening of regional economies. Most importantly, preferred types of biofuels contribute to the mitigation of greenhouse gas (GHG) emissions and sustainable development [2].

To ensure the availability of land for the cultivation of crops for first generation biofuel, increased rates of deforestation have become evident. This has led some to question the GHG emission benefits of these fuels. Indeed, it has been revealed that some fuels produced from cereals actually produce more GHGs than gasoline [3]. Corn and wheat as feedstock can only reduce greenhouse gas emission by a maximum of 20% and 50% respectively compared to gasoline, whereas lignocellulosic- and sugarcane ethanol can result in GHG reductions of up to 90% [2].

Besides increased deforestation and minimal reductions in GHG emissions, first generation biofuels also cause concerns because of competition for available resources which contribute to a higher overall food price [3]. Increased production of first generation biofuels could have been one of the factors contributing to the increase in food prices observed from 2008-2009 [5]. The impact of the increased food prices would be felt most strongly in developing countries where the low-income communities have to spend a greater fraction of their disposable income on food [5]. On the other-hand Ajanovic [8] concluded that first generation biofuel production did not have a significant impact on feedstock prices and that there was a far stronger correlation between the oil price and feedstock prices, due to the mechanisation in planting and harvesting and the increased fertiliser requirements. In 2011 a study was done on several first generation biofuels to determine the effect of biofuel production on food prices. It was concluded that biofuel had a modest effect (3 – 30% increase) on food prices [12]. Whilst first generation biofuels may or may not have contributed to current increases in food prices, the growing global population will in the future require larger volumes of both food and fuel which will negatively affect the viability of first generation biofuel production. Additional issues such as land and fresh water utilisation have also contributed to preventing a universal acceptance of these fuels [26].

In 2007, worldwide biofuel production accounted for only 1.8% of the total fuel requirements for transport, in energy terms [8]. Furthermore, in this year it was estimated that about 1% of the world's arable land was already used for first-generation biofuel production [8]. It would thus be impossible for first-generation biofuels alone to produce the volumes of fuel required worldwide. To increase the volume of ethanol produced, it is thus essential that production of 2nd generation biofuels from lignocellulosic material, including ethanol, be incorporated into existing production regimens, especially since much of this feedstock can be produced from waste products from current agricultural activities and from crops that can be cultivated on land of marginal quality (i.e. not suited for food crops or livestock production).

2.2 Second Generation Biofuels

Many of the limitations of first generation biofuels can be addressed by utilising alternative feedstocks such as agricultural and forest residues, municipal solid waste, waste paper and energy crops [3,7,13,16,27]. Lignocellulosic or second generation biofuel derived from these feedstocks may avoid competition with the production of food crops and may therefore be more easily accepted with regards to the third world and developing countries. However, competition might still exist for available land to grow food crops and feedstock for second generation biofuel. If energy crops can be grown on land that is not suited for food production through agriculture (marginal quality, inadequate rainfall etc.) the impact on food crops can be minimised. Utilisation of these options will also provide the farmers with an alternative market which could be beneficial to low-income and rural areas.

Fossil fuels still serve as cheap and concentrated forms of carbon and as such it would be difficult for biofuels to compete on an economic level. It was furthermore estimated that second generation biofuels, specifically, could not be produced economically because of certain technical barriers that first have to be overcome [13]. The authors were vague as to what these barriers were, but would most likely include high processing costs and low yields obtained from the lignocellulosic material. The incentive for biofuels should thus be the drive to reduce the environmental impact of fossil fuels and fossil fuel imports through local, sustainable development. It is however still important to minimize the cost of biofuel production to minimize the economic impact on society. As a first step it is thus imperative that the raw materials be obtained as cheaply as possible. Lignocellulosic material, since it is essentially a waste-product, serves as a cheap and highly abundant feedstock obtainable from renewable sources [2].

The chemical composition of the lignocellulosic material is an important factor influencing the production of bioethanol. However, due to variation in varieties and geographic location, large differences even in the same species are often observed [2]. A typical lignocellulosic feedstock consists out of 48 % (w/w) carbon, 6 % (w/w) hydrogen, 45 % (w/w) oxygen with the remainder made up of inorganic matter [28]. These elements constitute the three main constituents of lignocellulose, namely cellulose, hemicellulose and lignin [29]. Together these three constituents account for roughly 90% of the plant dry matter with the remainder comprised of extractives and ash [30]. Cellulose and hemicellulose are the most abundant throughout the material and constitute nearly two-thirds of the cell-wall dry-matter [2,30,31]. The compositions of the three main types of lignocellulosic materials are shown in Table 2.2.

Table 2.2: Structural composition of the three main types of lignocellulosic biomass used for second generation biofuel production.

Material	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Softwoods	41 – 50	11 – 33	19 – 30
Hardwoods	39 – 53	19 – 36	17 – 24
Herbaceous material	24 - 50	12 - 38	6 – 29

Data obtained from Klinke et al. [30]

Cellulose $(C_6H_{10}O_5)_x$ is a linear polymer of glucose, a six-carbon sugar, linked by β-1,4 bonds. [2,16]. The cellulose chains are further grouped together to form micro fibrils [30]. These structures are responsible for imparting the strength in woods and are quite rigid and difficult to break down with higher degrees of crystallinity severely impacting on the degree of digestibility[2] [2,27,32]. Cellulose is broken down to glucose through an acid- or enzyme-catalysed process by the addition of water molecules in a process known as hydrolysis or saccharification [33].

Hemicellulose $(C_5H_8O_4)_m$ differs from cellulose in that it is a highly branched polymer consisting of both five- and six-carbon sugars [2,27]. The branched nature of the polymer makes it more susceptible to hydrolysis [16,34]. Hemicellulose is bound to cellulose through hydrogen bonds and to lignin through covalent bonds [29]. Importantly, the hydroxyl groups of the sugars can be substituted with acetyl groups, leading to the formation of acetic acid upon hemicellulose hydrolysis [31]. In hardwoods and herbaceous material, such as sorghum bagasse, the main constituent of hemicellulose is xylose as oppose to mannose and galactose in softwoods [35]. Interestingly, it has been found that herbaceous material contains lower amounts of acetyl groups compared to hardwoods [16].

Lignin $(C_9H_{10}O_3(OCH_3)_{0.9\text{-}1.7})_n$ is linked to the cellulose and hemicellulose in the fibres, forming a barrier to prevent enzymatic degradation, effectively preventing hydrolysis and overall ethanol production from the material [16,27]. Absorption of the enzymes onto the lignin also further limits the degree of hydrolysis. Lignin also prevents the cellulose from swelling, limiting the surface-area available for hydrolysis [24]. Lignin is an aromatic polymer that is comprised of three main compounds, namely p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol to form a complex matrix [2,16].

2.2.1 Sweet Sorghum

Sweet sorghum [*Sorghum bicolor* L. Moench] is but one of many 'new crops' (compared to the 'traditional crops' such as sugarcane and corn) that are currently being investigated for the production of first and second generation bioethanol [36]. Sorghum can broadly be classified into three types, namely sweet sorghum, grain sorghum and forage sorghum [37]. Compared to grain sorghum, sweet sorghum produces less grain but the stem can be crushed, similar to sugarcane, to yield sugar-rich syrup. After crushing, the bagasse remains which can be used for second generation bioethanol production in combination with first generation ethanol produced from the juice. Approximately 0.46 ton of sorghum bagasse is produced per ton of sorghum grain harvested [38]. The grains, syrup and resultant lignocellulosic material of the bagasse allows the whole sweet sorghum plant to be used for bioethanol production without leaving waste [27]. Overall, sorghum is the fifth most widely grown cereal crop in the world behind wheat, corn, rice and barley [39,40]. The structural composition of the lignocellulosic residues of these five crops can be seen in Table 2.3.

Table 2.3: Structural composition of the lignocellulosic residues of the five most widely grown cereal crops.

Material	Cellulose	Hemicellulose	Lignin	Reference
[A]**Wheat**	30 - 38	20 - 29	17 - 23	[34,41–43]
[B]**Corn**	34 - 41	26 - 34	12 - 26	[1,44,45]
[A]**Rice**	37 - 39	24 - 27	12 - 14	[34,46]
[A]**Barley**	35 - 41	19 - 26	15 - 23	[47–49]
[C]**Sorghum**	32 - 43	18 - 24	19 - 25	[37,50–52]

[A] as straw
[B] as corn stover
[C] as bagasse

Sweet sorghum utilises C_4 carbon-fixing together with a high photosynthetic efficiency to assimilate carbon dioxide, leading to a high accumulation of biomass and sugars [36,53,54]. This leads to sweet sorghum having one of the highest dry matter accumulation rates, calculated on a day-to-day basis [36]. Furthermore, sweet sorghum has one-third of the water requirements of sugarcane and should thus be well suited for cultivation in arid countries such as South Africa [53,55]. Importantly, whereas sugarcane has a seven month growing period (allowing for only one harvest per year), sweet sorghum requires only four months in tropical regions [53]. The shorter growing period would allow for up to three harvests per year, resulting in an ample supply of raw material year round and ensure greater land usage. Sweet sorghum can also be cultivated in a wide variety of soil conditions and with lower fertilizer and overall crop management requirements it is also suited for cultivation in rural area [53,54].

2.3 Pretreatment

Due to the complex structure of the lignocellulosic material and the association of lignin and hemicellulose with the cellulose, a suitable pretreatment step must be employed to reduce the recalcitrant nature of the material and render the fibres more amenable to enzymatic hydrolysis [54,56]. Pretreatment also reduces the crystallinity of the cellulose, further improving the enzymatic hydrolysis [57]. However, it is important to optimise both the chosen technology and conditions used, not just from a process point-of-view, but also from an economic perspective. If the chosen pretreatment is not efficient enough, the yield from the successive enzymatic hydrolysis and fermentation steps will be poor, but if the severity is increased, degradation of the structural components releases toxic compounds that will inhibit the enzymes and microorganisms in subsequent process steps (discussed in Section 2.3.2.) [2,14].

Excluding the pretreatment step would constitute costs savings and prevent the formation of degradation products, but would ultimately results in an enzymatic hydrolysis yield of less than 20%, whereas pretreatment could improve the yields to greater than 90% and increase the rate of hydrolysis, thereby vastly improving the viability of the process [33]. Taherzadeh and Karimi [35] summarised that the ideal pretreatment option should generate fibres that are accessible for enzymatic degradation whilst minimising degradation of the cellulose and hemicellulose structures to prevent formation of inhibitory products. From an economic perspective the energy and process chemical requirements should also be minimised. To limit capital costs, the pretreatment conditions should also not necessitate the use of exotic alloys for construction of the pretreatment vessel.

2.3.1 Steam pretreatment

Steam pretreatment is the most widely used and cost-effective pretreatment option [43,58]. Steam pretreatment involves heating the raw material with saturated steam to temperatures ranging from 160 to 260 °C under pressures ranging between 6.9 and 48 bar. These conditions are maintained for a certain time period, ranging from seconds to several minutes after which the material is rapidly depressurised to atmospheric conditions [2,59]. The high temperatures cause hydrolysis of the hemicellulose and the sudden drop in pressure causes the water inside the material to instantly vaporise, resulting in physical disruption of the crystalline structure and rendering the cellulose polymers accessible to the hydrolytic enzymes. Palonen et al. [60] observed that the lignin is redistributed and a portion of it removed from the material during steam pretreatment [2,16]. Various studies also made use of an acid-catalysed steam pretreatment step to reduce the temperatures and pretreatment times required [24,52,61]. In an attempt to quantify the severity of

various steam pretreatment conditions, relating to different temperatures and residence times, Overend et al. [62] have introduced a severity factor (Eq. 1) :

$$\log_{10} R_0 = \log_{10} \left[t \times \exp\left(\frac{T-100}{14.75}\right)\right] \qquad (1)$$

Where R_0 is the severity factor, t the time in minutes and T the temperature in °C. Alfani et al. [18] have reported that the optimal severity, for a maximum sugar yield, is between 3.0 and 4.5. However, what was not mentioned in other papers [2,16] was that all the conditions that were considered by Alfani et al. [18] lay between severity factors of 3.0 and 4.5 and that only wheat-straw was considered as the feedstock. Due to difference in the composition of the lignocellulosic feedstocks (Tables 2.2 & 2.3) the optimum pretreatment conditions (severity) would also differ depending on the feedstock utilised.

Some of the advantages of steam pretreatment, compared to other pretreatment methods discussed below, include the potential for lower capital investments, a larger potential for increasing energy efficiencies while the use of less hazardous process chemicals and milder process conditions, also contribute to a lower environmental impact [2]. Steam pretreatment has also been found to work well with (and is a cost effective option for) hardwoods and agricultural residue, but is less effective for softwoods [27].

2.3.2 Other pretreatment methods

Physical techniques such as **milling** [2,35] can be used to improve the susceptibility of the lignocellulosic material to enzymatic hydrolysis by reducing the material size and crystallinity. While the energy requirements for comminution are dependent on the degree of milling (final particle size required) and the characteristics of the feedstock it was concluded that these techniques are too time-consuming and energy intensive for pretreatment on commercial scale [16]. However, a degree of milling is required to reduce the size of bulky material (straw, branches etc.) to be loaded into the steam pretreatment unit. It could also be beneficial to utilise milling to partially reduce the particle size to allow for better steam pretreatment [63].

Ammonia Fibre Explosion (AFEX) is quite similar to steam pretreatment, with the only difference being the use of liquid ammonia as opposed to water/steam. The raw material, mixed with liquid ammonia, is subjected to high temperatures and pressures followed by rapid decompression [2,16]. Typical dosages of ammonia range between 1-2 kg ammonia/kg dry biomass with temperatures of around 363 K (90 °C) for residence times of 30 minutes [64,65]. It is also stated that the AFEX

method does not directly liberate any sugars, but rather allows the cellulose and hemicellulose to be hydrolysed by enzymatic means. Due to the milder processing conditions this process has the benefit of not generating inhibitors [42,64].

It has been reported that AFEX is well suited for treating herbaceous and agricultural residue, but is less effective for hard- and softwoods because of their high lignin content [66]. In a paper by Salvi et al. [67], it was stated that ammonia showed success in the delignification process, since it was a selective reagent for lignin [68]. It was also stated [68] that ammonia is a less expensive chemical, compared to sulphuric acid, and is non-corrosive but this conflicts with the data given on the Material Safety and Data Sheets (MSDS) for both ammonia [69] and aqueous ammonia (ammonium hydroxide) [70].

The main problem with the AFEX process is the toxicity of the ammonia meaning it can't be released to the environment and thus has to be recovered [35]. Ammonia is also more expensive to use than water (both from a chemical and materials of construction viewpoint). One would have to recover the ammonia by some means in order to ensure the process remains viable – possibly by evaporation [2]. Ammonia is corrosive to certain metal surfaces and would thus require an exotic alloy to be used in the construction of the treatment vessel, increasing the capital costs of the process.

In the pulp industries, the process of cooking the lignocellulosic material in **Liquid Hot Water** (LHW) has been used for decades. In this process, the biomass is 'cooked' in the hot liquid water at a high pressure for a fixed duration which allows for high recoveries of pentose sugars while generating low concentrations of inhibitors [2]. For temperatures between 473 – 503 K (200 – 230 °C) and a duration of 15 minutes around 40-60% of the biomass was dissolved with 4-22% of the cellulose, 35-60% of the lignin and nearly all of the hemicellulose removed [71].

The main problem that one would encounter with the LHW process would be the large quantities of water required. This, combined with the energy required to heat the water, could make the process economically unfeasible in countries with high water- and electricity tariffs. However, this process (together with AFEX and steam pretreatment) has been reported as cost-effective [42].

For **ozonolysis**, ozone gas is utilised, usually at room temperature, to break down the lignin and hemicellulose while increasing the degradability of the cellulose. The main drawback of this process is the large quantities of ozone required which impact on the economic viability of the process [41].

Alkaline pretreatment involves utilising alkaline solutions to remove the lignin from the raw material and increase the digestibility of the cellulose with less degradation of the sugars [2,59].

Alkali pretreatment has the benefit of being carried out at ambient conditions, thereby reducing costs involved with high temperature and pressure applications. However, the drawback of such conditions is that the reaction time required is in the order of hours or days, rather than minutes or seconds [42].

Chemicals such as sodium-, potassium- and calcium hydroxide have been deemed acceptable for the process with NaOH being the most widely investigated [65]. Calcium hydroxide (or lime) has a lower cost compared to the rest of the chemicals and is not as hazardous. It also has the added benefit of being able to recover it from the hydrolysate – the carbonates precipitate after reacting with CO_2 [72]. Due to the processing chemical costs involved and the long pretreatment times, it was found that the above processes were not competitive on large scale [16].

Through the use of chemicals such as sulphuric, nitric or hydrochloric acid, **acid pretreatment** removes the hemicellulose portion of the raw material, thereby exposing the cellulose for hydrolysis [2]. The dilute acid process (high temperature, low acid concentration and shorter residence time) has been reported to work fairly well as a pretreatment prior to enzymatic hydrolysis on many agricultural feedstocks, including corn stover and wheat straw, however the costs involved are reportedly higher than those of physico-chemical pretreatments, such as steam explosion [2]. The dilute acid process also offers significantly higher xylose yields compared to steam pretreatment [73]. Disadvantages of this pretreatment included the need for expensive materials of construction due to the corrosive nature of the acids and the need to neutralise the pretreated material before fermentation [42,59]. The formation of a wide variety of degradation products that inhibit enzymatic hydrolysis and fermentation have also been reported [73]. How these concentrations compare to those generated from steam pretreatment would most likely depend on the substrate as well as the processing conditions utilised.

The concentrated acid process (low temperature, high acid concentration and longer residence time) cannot be considered a pretreatment method, but rather a complete hydrolysis step. Besides the need for neutralisation prior to fermentation, the high energy consumption and corrosion of the equipment are also major drawbacks of this process. The high acid consumption and the need for an acid-recovery step also impact negatively on the process economics [59].

Biological pretreatment requires the use of organisms such as white-, brown- and soft-rot fungi to degrade the lignin and hemicellulose in the raw material [16]. The main benefit of such a biological process is the low energy requirements and the mild environmental conditions. However, the

drawbacks of the process are the fact that most fungi mainly attack the cellulose fraction of the material and the rate of biological hydrolysis is very low [2].

2.3.3 Degradation products

Various degradation products can be formed during pretreatment of lignocellulose prior to hydrolysis-fermentation, which might inhibit subsequent enzymatic hydrolysis and fermentation steps. The degradation of each of the sugars from the three main components of the raw material (cellulose, hemicellulose and lignin) yields different inhibitory products (furans, weak acids and phenolic compounds) with each exhibiting different inhibitory effects, as discussed below. The yeast *S. cerevisiae* has been found to be highly robust to inhibition by the inhibitors and capable of assimilating several of these compounds when present at sub-toxic levels [74–76].

The main degradation product from glucose (mainly from the cellulose fraction) is the furan derivative 5-Hydroxymethyl furfural (HMF) while furfural is generated from the degradation of pentose in the hemicellulose fraction [74,77]. Both products are formed in higher concentrations under acidic pretreatment conditions, such as when an acid catalysts is used [30]. The presence of both furans during fermentation has been shown to decrease ethanol productivity and yield and also lead to a longer lag phase [16,74]. A synergistic effect between these two inhibitors has also been noted [74]. Whilst furfural was found to be more inhibitory to the yeast, its rate of assimilation by the yeast was also higher compared to HMF [74]. Almeida et al. [74] compiled a list of several *S. cerevisiae* strains that exhibit higher tolerance to both HMF and furfural and it was concluded that the industrial yeast strains appeared to be the most tolerant.

Acetic acid is released during the hydrolysis of the acetyl-groups in the hemicellulose while formic acid is produced from further degradation of HMF and furfural [19,74,77]. Larsson et al. [19] have noted that there are no synergistic effects between these acids while both reduce biomass production and ethanol yield through uncoupling of the yeast metabolism [74]. At pH values higher than the pKa of the acids (4.75 for acetic acid and 3.75 for formic acid [77]), the acid-base equilibrium shifts towards predominance in the conjugate base or deprotonated form, which do not readily cross the cellular membrane. On the other hand, a decrease in medium pH would result in a higher level of the protonated form, which permeates more readily over the cell membrane [78]. Once in the neutral environment of the cytosol, this acid again dissociates, resulting in the acidification of the cytosol. Subsequently, ATP is expended in pumping protons out of the cells, resulting in the uncoupling of the metabolism since the ATP molecules would not be available for the cellular energy metabolism. Furthermore, should the intracellular pH decrease below critical levels,

cellular function would cease, resulting in cell death [74]. Under micro-aerobic conditions, ethanol fermentation leads to ATP production which suggests that low concentrations of these acids can lead to an increase in ethanol production by *S. cerevisiae* [79].

Phenolic compounds are generated due to the degradation of lignin [30,74,77]. Due to the complex structure of lignin, various different phenolic compound can be formed, as summarised by Jönsson et al. [76] and Klinke et al. [30]. While these phenolics are considered to be highly inhibitory to the yeast, there is evidence to suggest that the low-molecular weight compounds are the most inhibitory by impacting on the growth rate and ethanol productivity of the yeast [14,19,74,76,80].

The data in Table 2.4 shows the concentrations of inhibitors in the hydrolysate fractions of several herbaceous materials following steam pretreatment. Most notably, while pretreatment at 180 °C and 10 minutes (with 2% SO_2) resulted in similar concentrations of inhibitors from sweet sorghum bagasse and sugarcane bagasse, an increase in temperature to 190 °C resulted large variation in inhibitor concentrations between sweet sorghum bagasse and sugarcane bagasse.

In an attempt to reduce the impact of the inhibitors, various methods of detoxifying the hydrolysate prior to enzymatic hydrolysis or fermentation have been investigated. Since all of these processes require additional processing-time or expensive chemicals, they will all impact negatively on the economics of the process and should be avoided if possible. Palmqvist et al. [80] provided a summary of various detoxification methods, including:

- Biological detoxification where fungi (or enzymes obtained from the fungi) such as *Trametes versicolor* or *Trichoderma reese*i are employed to detoxify the slurry [80]. Jönsson et al. [76] have found that treatment of hydrolysate with laccase and peroxidase increased the glucose uptake and ethanol productivity of the yeast.
- Physical methods, such as the use of ion exchange resins or activated carbon, evaporation of the volatile inhibitors or liquid-liquid extraction with compounds such as diethyl ether [30,80]. However, these processes require extra chemicals or possibly large amounts of energy.
- Overliming of the hydrolysate with $Ca(OH)_2$ or NaOH and then readjusting the pH with H_2SO_4 have also been investigated [80].

Table 2.4: Inhibitors concentrations in the hydrolysate fraction following steam pretreatment of different lignocellulosic substrates.

	Pretreatment conditions			By-product concentrations in hydrolysate fraction (g/L)				
Material	**Temperature**	**Time (min)**	**Catalyst**	**Acetic acid**	**Formic acid**	**HMF**	**Furfural**	**Reference**
Sweet sorghum bagasse	180 °C 190 °C	10	2% SO_2	1.66 4.95	3.30 3.56	0.12 0.36	0.23 1.00	Sipos et al. [52]
Sweet sorghum bagasse	190 °C	10	2% SO_2	0.7	0.8	0.1	0.4	Gyalai-Korpos et al. [61]
Sugarcane bagasse	180 °C 190 °C	10	2% SO_2 Water 2% SO_2 Water	~1.9 ~0.1 ~2.6 ~0.2	N.R. N.R. N.R. N.R.	N.R. N.R. N.R. N.R.	~0.35 0.0 ~0.55 ~0.1	Carrasco et al. [81]
Corn stover	200 °C	5	2% SO_2	1.6	N.R.	0.06	1.1	Ohgren et al. [1]

~ - estimated from graphs
N.R. – not reported

2.4 Enzymatic Hydrolysis

In order to produce the monomeric sugars required by the micro-organisms for fermentation, a hydrolysis or saccharification step is required. This can be achieved through chemical (concentrated acid process, section 2.3.2) or enzymatic means [27,82]. The use of an enzymatic hydrolysis step, as used in this study, offers certain advantages over an acid hydrolysis including lower costs due to milder processing conditions (pH 4.8 and 45 to 50 °C [64]), preventing corrosion of the equipment and produces a less toxic hydrolysate [17,27].

Specific enzyme cocktails are required since there are at least three main groups of cellulases involved in the complete hydrolysis process. Endoglucanases (endo-1,4-b-glucanases) attack the amorphous region of the cellulose, cleaving the β-1,4 bonds to create new chain ends. Exoglucanases (cellobiohydrolases) cleave the cellulose chains from the free ends, incrementally releasing free units of cellobiose or glucose. β-glucosidases hydrolyse the cellobiose units (from hydrolysis or pretreatment) to release two glucose units, preventing cellobiose-accumulation and inhibition of the enzymes [17,27]. Factors such as the type of substrate, temperature, pH and cellulase activities all impact on the overall effectiveness of the hydrolysis steps [64]. Supplementation of the enzyme cocktail with xylanase and β-xylosidases will allow for hydrolysis of the xylan fraction of the hemicellulose and xylooligomers in the pretreatment hydrolysate [83]. The enzyme activities of several commercially available enzyme preparations are summarised in Table 2.5.

Table 2.5: Comparison of the enzyme activities and protein concentrations of commercial enzyme preparations.

Enzyme preparation	FPU/ml	B-glucosidase (IU/ml)	Xylanase activity (IU/ml)	Protein content (mg/ml)	Reference
Cellic CTec2	120.5	2731	--	161	[84]
Cellic CTec2	177	N.R.	--	N.R.	[85]
Cellic CTec2	100	3950	--	N.R.	[86,87]
Cellic HTec2	--	--	1300	N.R.	[86,87]
Novozyme 188	--	231	--	220	[84]
Spezyme CP	65 - 74	48 - 53		115 - 137	[88]
Celluclast 1.5L	62.0	15.0	--	127	[84]

N.R. – not reported

To ensure the economic viability of the ethanol production process the costs involved, specifically the cost of the enzymatic hydrolysis steps should be kept to a minimum, while maximizing throughput. Ethanol production costs can be decreased by up to 20% if the substrate loading is increased from 5% to 8% and even higher saving are possible if the loadings are further increased [89]. However, high substrate loadings exhibit an inhibitory effect, leading to lower hydrolysis rates but inhibition can be partially overcome by utilising a higher enzyme dosage [90]. The economic viability of the process will however be adversely effected by the high enzyme dosage [64]. Typical enzyme loadings are between 7 and 33 FPU (filter paper units)/ g cellulose, depending on the nature of the substrate used but for laboratory experiments loadings of 10 FPU/g cellulose are mostly used, since it proves a reasonable rate of hydrolysis at a reasonable cost [64,91].

2.5 Fermentation of the pretreated material

While pretreatment can result in material with a higher sugar concentration and amenability to degradation, the main structural constituents are still in polymeric form and can thus not be readily fermented to ethanol. The hydrolysis and fermentation steps are critical to the success of the ethanol production process. There are several processes configurations available, as will be discussed, for hydrolysing the polymers and fermenting to resulting monomeric sugars to ethanol. The selection of the appropriate configuration is in part dependent on the microorganism selected for fermentation.

2.5.1 Yeast

The ideal microorganism for fermentation of the pretreated lignocellulosic material should be able to produce ethanol at a high yield, be able to tolerate the high concentrations of ethanol required for an economically feasible distillation step and show resistance to the inhibitors generated during pretreatment [17]. Selecting a suitable microorganism or strain is thus crucial to the success of the fermentation process and lignocellulosic ethanol production process as a whole.

In order to maximize the ethanol concentration obtained from the lignocellulosic feedstock, the fermenting organism should be able to ferment both hexose and pentose sugars as xylose are abundant in the hemicellulose fraction of agricultural residues. Yeast strains such as *Scheffersomyces* (*Pichia) stipitis* and *Candida shehatae* poses natural xylose-fermenting capabilities, but the inhibitor tolerances of these species have been found to be poor [92–94]. Bacteria such as *Zymomonas mobilis* and *Escherichia coli* have also been investigated, but have also been deemed unsuitable. Besides having low inhibitor tolerances, *Z. mobolis* lacks pentose fermenting capabilities and *E. coli* exhibits poor ethanol production [17]. *Saccharomyces cerevisiae* (baker's yeast) fulfils several of the requirements required for lignocellulosic ethanol production, but lacks pentose-fermenting capabilities. Through genetic engineering, *S. cerevisae* strains have been transformed with the xylose isomerase (XI) gene from fungi such as *Piromyces* sp. [95,96] or xylose reductase (RD), xylitol dehydrogenase (XDH) and xylulokinase (XK) from yeast such as *Scheffersomyces (Pichia) stipitis* [96–98]. The selection of a suitable yeast strain for use with SSF would thus have to be carefully considered since a recombinant strain could allow for xylose-fermentation while the industrial strains (presumably without xylose-fermenting capabilities) as discussed in Section 2.3.2 appear to be more resistant to inhibitors.

2.5.2 Fermentation process configurations

Separate hydrolyses and fermentation (SHF)

Traditionally the hydrolysis step would be performed first followed by the fermentation of the released glucose perhaps even in a separate vessel with each step taking up to 72 hours to complete. Although very time-consuming it does offer certain advantages such as that the hydrolysis and fermentation steps can each be performed at their optimum conditions of pH and temperature (around 45 to 50 °C for hydrolysis and 30 °C for fermentation [64]) to achieve a higher ethanol yield of closer to the theoretical maximum [18,27,82,99]. The main disadvantages of SHF include a slowdown in the rate of hydrolysis as the enzymes are inhibited by high concentrations of glucose, known as feedback inhibition [27], which requires higher enzyme dosages to overcome inhibition, compared to SSF. The high glucose concentration also increases the chances for bacterial contamination [99]. Furthermore, the inhibitors generated during pretreatment can also adversely affect the performance of the enzymes if a washing or detoxification step is not included prior to hydrolysis. A flow sheet for the SHF process can be seen in Figure 2.1.

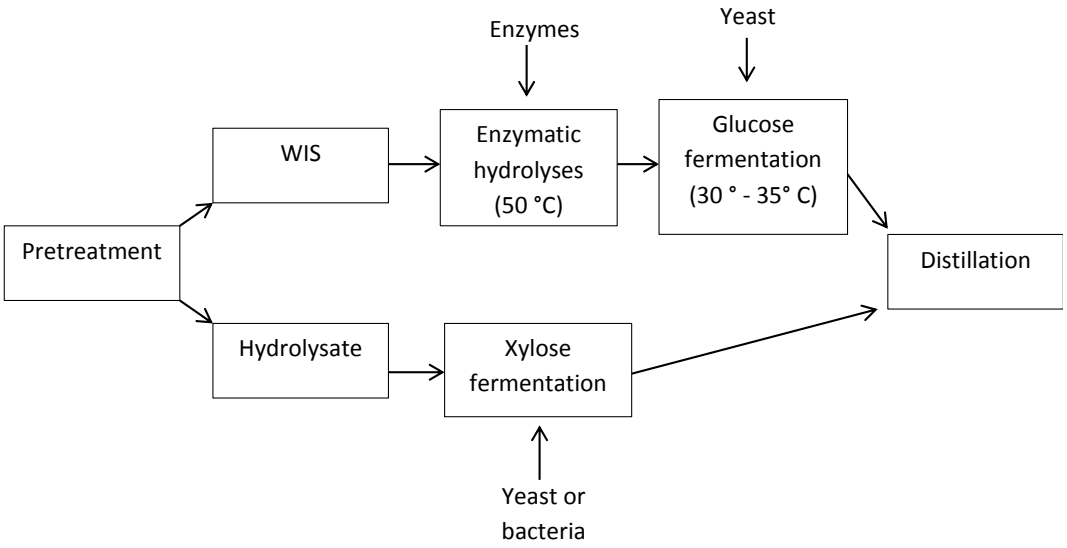

Figure 2.1: Schematic representation of the separate hydrolysis and fermentation (SHF) process for the production of lignocellulosic ethanol with separate fermentation of the hydrolysate steam. Adapted from Den Haan et al. [99].

Simultaneous saccharification and fermentation (SSF)

One way in which end-product inhibition during hydrolysis can be overcome is to utilise the sugar products from enzymatic hydrolysis as soon as they are formed, thereby ensuring that the residual sugar concentration in the reaction mixture remains low [100]. Rather than removing the formed product (glucose) from the vessel and adding it to another vessel for fermentation, the enzymes and pretreated material are added directly into the fermentation vessel, together with the fermenting organism (Figure 2.2). Besides removal of end-product inhibition, SSF also allows for a lower enzyme dosage to be used, compared to SHF and allow for shorter processing times while the ethanol produced also helps combat microbial contamination in the processing vessel. The yeast has also been shown to assimilate the inhibitors generated during pretreatment which would decrease the inhibitory effect on the enzymes. Equally important, by using only one process vessel capital cost expenditure is greatly reduced [17,18,64,99]. The disadvantages of SSF include a compromise with regards to the operating temperature which would lead to slower rate of hydrolysis which would lead to lower ethanol productivity. The ethanol produced during fermentation also impacts on, and reduces the activity of, the enzymes [101].

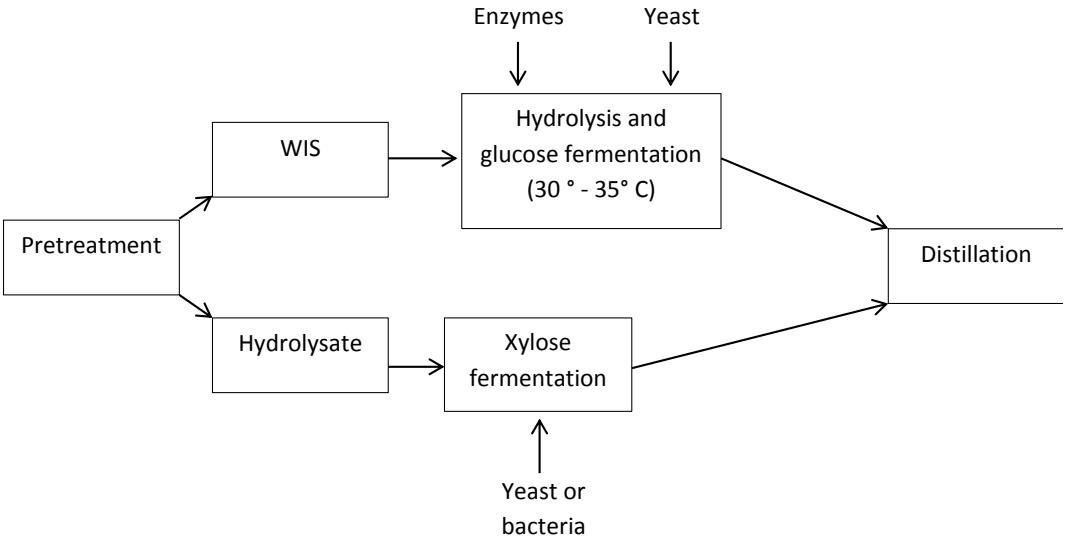

Figure 2.2: Schematic representation of the simultaneous saccharification and fermentation (SSF) process for the production of lignocellulosic ethanol with separate fermentation of the hydrolysate stream. Adapted from Den Haan et al. [99].

Table 2.6: Comparison between SHF and SSF cultures of lignocellulosic material with regards to maximum ethanol concentration obtained.

Material	Configuration	Detoxification	Temperature	Solid loading	Max ethanol (g/L)	Ethanol yield	Reference
Wheat straw	SHF (batch) SSF (batch)	NaOH and distilled water	45 & 37 °C 37 °C	10% (w/v)	32 25	81% 68%	Alfani et al. [18]
Wheat straw	SHF (batch) SSF (batch)	None	50 & 37 °C 37 °C	10% (w/v)	22.6[A] 24	-- 0.43 g/g	Tomás-Pejó et al. [98]
Sorghum straw	SHF SHF SSF (batch) SSF (fed-batch)	$Ca(OH)_2$ None	50 & 32 °C 50 & 32 °C 32 °C 32 °C	10% (w/w)	23.0 23.0 25.2 26.3	N.R. N.R. N.R. N.R.	Mehmood et al. [51]

[A] - Theoretical, based on glucose content
N.R. – not reported

The data in Table 2.6 showed that Alfani *et al.* found SHF to be the preferred process option while Tomas-Pejo *et al.* found SSF to deliver higher ethanol concentrations. The discrepancy between the results can be attributed to the detoxified step employed by Alfani et al. [18]. When inhibitors are also present in the fermentation (as in the case of Tomás-Pejó et al. [98]), SSF would the preferred option because of the ability of the yeast to assimilate the inhibitors thereby relieving the inhibition on the enzymes to allow for better hydrolysis. Mehmood et al. [51] found the performance of SSF (without detoxification) to be superior to that of the SHF experiments, both with and without detoxification.

Simultaneous saccharification and co-fermentation (SSCF)

During SSF only the glucose fraction of the cellulose and hemicellulose are fermented. When a recombinant organism with both glucose and xylose fermenting capabilities is utilised for fermentation, the process configuration is referred to as SSCF (Figure 2.3). The SSCF process offers the same advantages as SSF, compared to SHF, but with the additional benefit of a possibly higher ethanol concentration by fermentation of both the glucose and xylose fractions [27]. With current yeast strains the co-fermentation of both glucose and xylose is limited due to the reported 200-fold

higher affinity of the yeast for glucose [102]. It is thus crucial to maintain a low (but non-zero) concentration of glucose during SSCF to facilitate the uptake of xylose [103]. A fed-batch protocol is thus required to allow for low levels of residual glucose that would enhance xylose uptake [104,105].

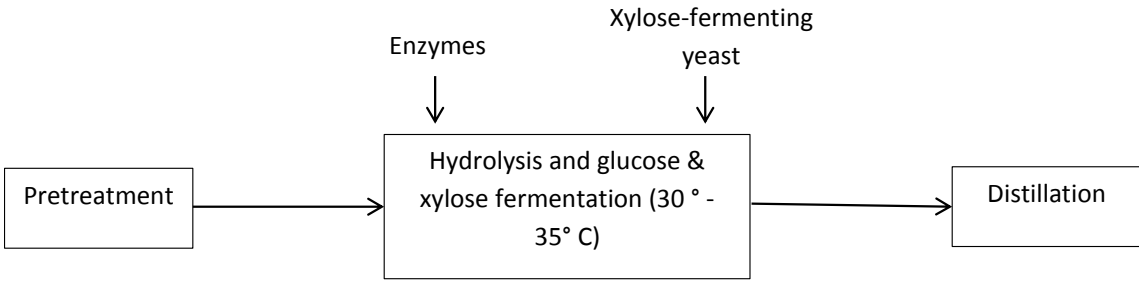

Figure 2.3: Schematic representation of the simultaneous saccharification and co-fermentation (SSCF) process for the production of lignocellulosic ethanol. Adapted from Den Haan et al. [99].

Consolidated bioprocessing (CBP)

The processes of SHF, SSF and SSCF all require the purchase or on-site production of enzymes which constitutes additional expenditure. Development in lignocellulosic ethanol production suggests a process where enzyme production, hydrolysis of the pretreated material and fermentation of the hexose and pentose sugars are all performed by a single microorganism or consortium of organisms [100]. This integrated ethanol production process is referred to as CBP and would constitute significant cost saving compared to the currently employed processes [106] (Figure 2.4). Costs saving of up to 75% from using CBP compared to SSCF have been estimated [106].

There is currently no natural-occurring microorganism that display the characteristics required for CPB, thus genetically engineered strain would have to be produced. The yeast *S. cerevisiae* exhibits favourable characteristics as a host organism for CBP, including easy and stable manipulation of its genome [100]. It does however seem unlikely to engineer an organism capable of expressing all the required enzymes for complete hydrolysis [100]. An organism capable of expressing only the major cellulase would already reduce the volume of enzyme required.

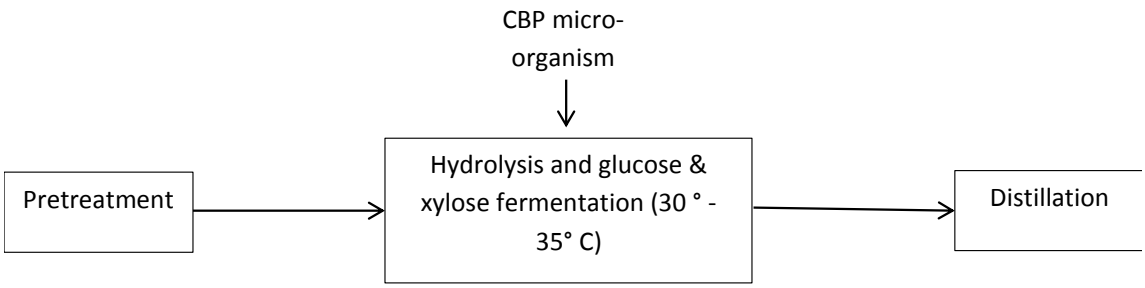

Figure 2.4: Schematic representation of consolidated bioprocessing (CBP) for the production of lignocellulosic ethanol. Adapted from Den Haan et al. [99].

2.5.3 Parameters of the SSF process

From the comparisons in Table 2.6 it was decided to utilise a SSF configuration in the present study. To develop the protocol for an SSF process to obtain a sufficiently high ethanol concentration, various aspects of the process should be investigated. These include the option to include a pre-saccharification step prior to fermentation, the operating temperature, the solids loading, the process option with regards to the condition of the WIS, and the enzyme cocktail and dosage. In the following sections several of these aspects are discussed to determine which parameters were selected based on literature and which were experimentally investigated.

2.5.3.1 Pre-saccharification

In a pre-saccharification step the enzymes and solids are added to the fermenter a few hours prior to yeast inoculation to allow the enzymes to partially hydrolyse the material. By utilising a pre-saccharification step, the viscosity can be reduced prior to fermentation, allowing for higher solid loadings to be used (>10%), while still ensuring adequate mixing. Pre-saccharification would also ensure a higher initial concentration of glucose to stimulate ethanol production in the yeast through the induced Crabtree-effect, whereby the yeast ferments the available glucose to ethanol, even in the presence of sufficient oxygen.

An important consideration for pre-saccharification is the optimum duration, since enzymes experience end-product inhibition from the released glucose (and possibly xylose if it contains xylanase activity). It would therefore be required to add the yeast cells before end-product inhibition reduces the rate of hydrolysis, during pre-saccharification, to lower than the rate of hydrolysis during SSF. Furthermore, longer pre-saccharification would also lead to a greater release

of inhibitors from the fibres, requiring the yeast to be inoculated into a more toxic environment which could potentially result in poorer performance [87].

2.5.3.2 Operating temperature

In order to optimise the process of SSF, a compromise between the optimum temperatures for enzymatic hydrolysis (45 to 50 °C) and fermentation (30 to 35 °C) have to be found [64]. In an attempt to increase the fermentation temperature as close to the optimum temperature for hydrolysis, the use of a wide range of temperatures were reported, reaching as high as 38 °C [18,64,103]. Whereas 40 °C was found to be too high for the strains of *S. cerevisiae* used, increasing the temperature above the recognised optimum of 30 °C for *S. cerevisiae* resulted in a substantially longer lag phase, detracting from process performance [18]. At operating temperature of 35 °C has previously been determined to be the optimum operating temperature for SSF of cellulose with *S. cerevisiae* [107]. It was also postulated that the inhibition effects of the inhibitors might be more pronounced at the higher temperatures [103].

2.5.3.3 Material process options

The inhibitors from pretreatment will impact on the performance of the yeast and enzymes, as discussed in Section 2.3.2., but several methods of detoxification have also been reported. The use of the whole pretreatment slurry would thus be the most cost-effective option, but could result in poor performance. To overcome possible limitation from inhibitors, various authors [105,108] resorted to washing the pretreated material with water to remove the inhibitors. While such a step should generate more fermentable fibres, it would also lead to a loss of potentially fermentable sugars from the hydrolysate. When using washed solids at a loading of 5% during enzymatic hydrolysis, the energy demand can be twice as high as when the whole slurry was used due to the loss of sugars [6]. The large volumes of wash water and the need for water treatment would also further increase processing costs. Another drawback of a washing step could be possible formation of lactic acid due to the difficulty in avoiding bacterial contamination during SSF experiments, which would lead to lower ethanol yields [105,108]. An intermediate option could be the utilisation of WIS from which a portion of the hydrolysate had been removed, since such a configuration had been shown to result in superior performance to that of the whole slurry and washed WIS [105]. To minimize water usage, pressing of the pretreated material in a dead-end press would be preferred but with such a configuration the material would still contain low levels of inhibitors. The

hydrolysate obtain from the pressed WIS could also be fermented in a separate step at potentially more favourable processing condition of temperature and pH (see Figures 2.1 & 2.2). The selection of the appropriate process option would thus have to be investigated to determine if a detoxification or washing step would be required.

2.5.3.4 PRE-CONDITIONING OF THE YEAST

From the assimilation profiles from Delgenes et al. [14], it was seen that *S. cerevisiae* can be conditioned to tolerate the toxicity of the hydrolysate, since it was able to assimilate the inhibitors to varying degrees. By incorporating a pre-conditioning step, whereby the yeast inoculum for SSF is cultivated in the presence of inhibitors, the inhibitory effect of these compounds during fermentation could potentially be reduced and allow for increased rates of assimilation. Investigations into the effect of pre-conditioning found that at solids loadings of 3% and 5%, using the whole slurry, there was little difference in the ethanol yield between the control yeast (no pre-conditioning) and yeast that had been pre-conditioned for 17 hours during fed-batch cultivation with 50% hydrolysate [109]. When the solid loading was increased to 8% solids, however, the control yeast was not able to match the performance of the pre-conditioned yeast. Linde et al. [110] found that by cultivating baker's yeast on the hydrolysate from pretreatment for 14 hours, the lag phase encountered by the yeast during SSF at 7.5% solids loading was removed. When the yeast was not pre-conditioned a maximum ethanol yield of 82% of the theoretical maximum could be obtained with 5% WIS and yeast loading of 5 g/L. With pre-conditioning, a yield of 76% was obtained from a WIS loading of 7.5% (a 50% increase) and a yeast loading of 2 g/L (a 60% decrease), both of which will benefit the economics of the process. These results illustrated the importance of pre-conditioning the yeast culture, especially at the high loadings required (> 10% solids) to achieve 40 g/L of ethanol. The concentration of hydrolysate utilised during pre-conditioning would have to be based on the steam pretreatment conditions with high severity pretreatment requiring lower concentrations of hydrolysate to prevent complete inhibition of the yeast.

2.5.3.5 ENZYME FEED STRATEGY

The selection of the optimum enzyme dosage was conducted in a separate study by Pengilly [25] and would thus not form part of this study. Different strategies regarding the feeding of the enzymes, such as feeding all enzymes at the start of the fermentation or adding the enzymes throughout the cultivation were investigated, but no trend between feed strategy and ethanol yield was observed

[105]. A decrease in enzyme activity, due to factors including enzyme deactivation or irreversible binding to the substrate [17], will occur during the fermentation with the result that, if all the enzymes are added upfront, fewer enzymes would be available to hydrolyse the latter feeds of WIS, possibly resulting in a decrease in yield. Feeding of the enzymes and substrate together at each feed interval, would ensure a portion of the enzymes in the fermentation would always be highly active. However, this would also mean that the volume of enzyme within the fermentation at each time point would be lower, compared to feeding all the enzymes at the start of the fermentation. The final enzyme additions would also not be optimally utilised since the fraction of unhydrolysed material remaining in the reactor would be low. If enzyme recycling cannot be implemented economically, enzyme utilisation should be maximised which would require addition of the enzymes early during the fermentation; the procedure that was also selected for use during SSF in the current study.

2.5.3.6 SOLID LOADING AND FEED RATE

In order to obtain an ethanol concentration of 4%, a high solid loading is required depending on the composition and digestibility of the pretreated material. Due to an increase in viscosity and resulting mixing problems associated with solid loadings higher than 10%, ethanol yields tend to decrease with increasing solid loadings [17]. Ethanol production costs as a function of the solid loading used during SSF clearly showed the importance of working at higher solid loadings, but also seemed to suggest that for loadings higher than 10% the production costs start to level off, possibly due to the reduction in yield [111]. A solid loading of 14 to 15% (w/v) has been reported to be required to ensure economic feasibility [112]. Wang et al. [50] used response surface methodology to optimise the solid loading for sweet sorghum bagasse in SSF. The optimisation was done with regards to maximizing the ethanol yield from batch SSF of washed WIS, which would entail using a low solids loading. The optimum solid loading was found to be 7% (w/v) solids, which resulted in a maximum ethanol concentration of 25.5 g/L.

A solution to overcome the limitation of a low yields coupled to high solids loadings is the use of a fed-batch SSF process where the solid material is fed at intervals to allow a fraction of the material in the reactor to be hydrolysed before additional material is fed. The inhibitor concentrations will also increase incrementally and would allow sufficient time for the yeast to assimilate a portion of the inhibitors to maintain sub-critical concentrations. With such a process the solid loading inside the reactor can be kept low to increase the rate of hydrolysis while simultaneously allowing for a higher final solid loading (>10% solids) to be reached [17]. Different solid loadings and feed strategies

would have to be investigated to find the combination most suitable for sweet sorghum bagasse. Various solid loadings and feed rates have been utilised in the literature and the results are summarised in Table 2.7. The results showed that while 40 g/L of ethanol have been obtained with forage sorghum at a loading of 16% (w/w), washed WIS was utilised while using the whole slurry from wheat straw at a lower solid (11%, w/w) loading delivered almost similar results. There is thus a possibility of decreasing the solids loading by using whole slurry or pressed WIS from sweet sorghum bagasse, but would entail using a fed-batch approach to limit the effect of the inhibitors. Various feed strategies have been investigated for wheat straw and spruce in fed-batch SSF (Table 2.7), but have not been applied to sweet sorghum bagasse. Therefore different feed strategies would thus have to be investigated.

Table 2.7: Summary of the results from batch and fed-batch SSF cultures of various steam-pretreated lignocellulosic materials

Material	Pretreatment conditions	Process option	Yeast and temperature	Solids loading	Feed rate	Maximum ethanol (g/L)	Ethanol yield [A]	Reference
Forage sorghum	220 °C, 7 min, 9% moisture	Washed WIS	*S. cerevisiae*, 35 °C	9% (w/w) 13% (w/w) 16% (w/w)	Batch	25 33 43	80% 77% 75%	Manzanares et al. [37]
Sweet sorghum bagasse	210 °C, 2 min	WIS (filtered)	*K. marxianus* CECT 10875, 42 °C	10 % (w/v)	Batch	16	61%	Ballesteros et al. [113]
Wheat straw	190 °C, 8 min					18	63%	
Poplar	210 °C, 4 min					19	71%	
Wheat straw	180 °C, 10 min, water (80% moisture)	Washed WIS	*K. marxianus* CECT 10875, 42 °C	10% (w/v)	Batch	N.R.	~65%	Ballesteros et al. [43]
	180 °C, 10 min, 0.9% H_2SO_4					N.R.	~69%	
	190 °C, 10 min, water (80% moisture)					N.R.	~71%	
	190 °C, 10 min, 0.9% H_2SO_4					N.R.	~80%	

Wheat straw	211 °C ,2.5 min, H_2SO_4	Whole slurry	*S. cerevisiae* TMB3400, 34 °C	7%	Batch	33	75%	Olofsson et al. [103]
				9%	Batch	33	59%	
				4% initial and 7% final (w/w)	4 additions in 12h	35	78%	
				4% initial and 7% final (w/w)	8 additions in 24h	33	78%	
				6% initial and 9% final (w/w)	4 additions in 12h	38	71%	
				6% initial and 9% final (w/w)	8 additions in 24h	37	69%	
Wheat straw	190 °C, 10 min, 0.2% H_2SO_4	Whole slurry	*S. cerevisiae* TMB3400, 34 °C	8% initial and 11% final (w/w)	Additions after 6, 12, 18 and 24h	33 – 37	61 – 68%	Olofsson et al. [114]
Spruce	210 °C, 5 min, 2% SO_2	Whole slurry	*S. cerevisiae*, 37 °C	10% (w/w)	Batch	~18	77%	Hoyer et al. [105]
				6% initial and 10% final (w/w)	Additions after 4, 5.5, 7, 8.5h	~17	69%	

		Pressed WIS		14% (w/w)	Batch	~26	~72%	
				9% initial and 14% final	Additions after 4, 5.5, 7, 8.5h	~25	~65%	
		Washed WIS		14% (w/w)	Batch	~15[B]	~52%[B]	
				9% initial and 14% final (w/w)	Additions after 4, 5.5, 7, 8.5h	~18[B]	~61%[B]	
Spruce	215 °C, 5 min, 3% SO_2	Whole slurry	*S. cerevisiae*, 37 °C	10% (w/w)	Batch	43[C]	82%[C]	Rudolf et al. [115]
				6% initial and 10% final (w/w)	1% every 1.5h	44[C]	84%[C]	
					1% every 3h	40[C]	78%[C]	
					1% every 5h	44[C]	84%[C]	
Sugarcane bagasse	190 °C, 5 min, 2% SO_2	Whole slurry	*S. cerevisiae* TMB3400, 32 °C	5% (w/w)	Batch	21	0.35 g/g	Rudolf et al. [116]
				7.5% (w/w)		27	0.30 g/g	

A – % of theoretical maximum or gram ethanol/gram sugar
B – after compensating for lactic acid formation
C – after compensating for ethanol loss due to nitrogen sparging
~ - estimated from graph
N.R. – not reported

3. OBJECTIVES

3.1 AIMS

From the literature in Section 2, two main aims were identified for this project. A list of intermediate questions that would assist in reaching the desired outcomes was also compiled:

1. **Develop a generic SSF process for use with steam-pretreated sweet sorghum bagasse at solid loadings higher than 10%**.

Additional questions that had to be answered during this work included:

1. What process option, with regards to the pretreated material, should be selected for the final process – whole slurry, pressed material or washed material?
2. What strain of *Saccharomyces cerevisiae* would deliver the best SSF performance and is there a benefit from using a laboratory strain with xylose-fermenting capabilities?
3. How should the feed strategy for use with a fed-batch SSF be applied to ensure optimum performance?

2. **Optimise the generic process, through utilisation of an integrated approach to achieve 40 g/L of ethanol in the reaction broth.**

Additional questions that had to be answered during this work included:

- How do the preferred enzyme cocktails that differ in composition affect the performance of the SSF process and can the selection of more than one cocktail be justified for future work?
- When utilising the preferred varieties pretreated under optimum conditions, could the solid loadings for SSF be reduced to increase the ethanol yield from the pretreated material while still achieving the required 40 g/L of ethanol?
- If multiple optimum pretreatment conditions have been identified, how do these performances translate to SSF in terms of ethanol production?
- If more than one optimum sorghum variety was identified, do the performance of these varieties translate to SSF such that a recommendation for a variety(ies) can be made for use on industrial scale?

3.2 Scope

This project was limited to working only with steam-pretreated sweet sorghum bagasse.

Several yeast strains were investigated and the best performing strain selected. The development of a yeast strain specifically for use in SSF was beyond the scope of this project.

A standard enzyme dosage was selected to conduct work relating to the development of the SSF process. The enzymatic cocktail was optimised simultaneously in a different study within the research group and the findings applied to the latter part of the present study.

A standard pretreatment condition and sweet sorghum variety was selected for use during the development phase of the study. To save on limited material resources, a non-preferred (lower performing) variety was used. A separate study within the research group focussed on selecting the preferred sorghum varieties (based on sugar content, digestibility of the pretreated material and yields per hectare) and optimising the pretreatment conditions to obtain high sugar content with low concentration of inhibitors.

3.3 Methodology

This project had two main objectives: Firstly, to develop a protocol for an SSF process for use with sweet sorghum bagasse at solids loadings higher than 10%. Secondly, to optimise this process with regards to pretreatment, the nature of the enzyme cocktail and final solid loadings in SSF culture to obtain a final ethanol concentration of 40 g/L in the fermentation broth. A flow sheet outlining the methodology by which the research work was done is shown in Figure 3.1 with the three distinct phases of the project highlighted in green.

The process screening involved the investigation of several process parameters to determine the preferred combination that will allow for a high cumulative solids loading (20% solids) to be utilised during SSF. To save on limited material resources, a non-preferred (lower performing) sorghum variety, MSJ13, was utilised. The end result of this work was a protocol for a generic SSF process. The results for the screening are presented in Chapter 4.

Process optimisation was the culmination of three separate parts of the study. Using an integrated approach, pretreatment and enzymatic hydrolysis was each optimised in separate studies and the final results integrated into the SSF process developed. The results are compiled in Chapter 5.

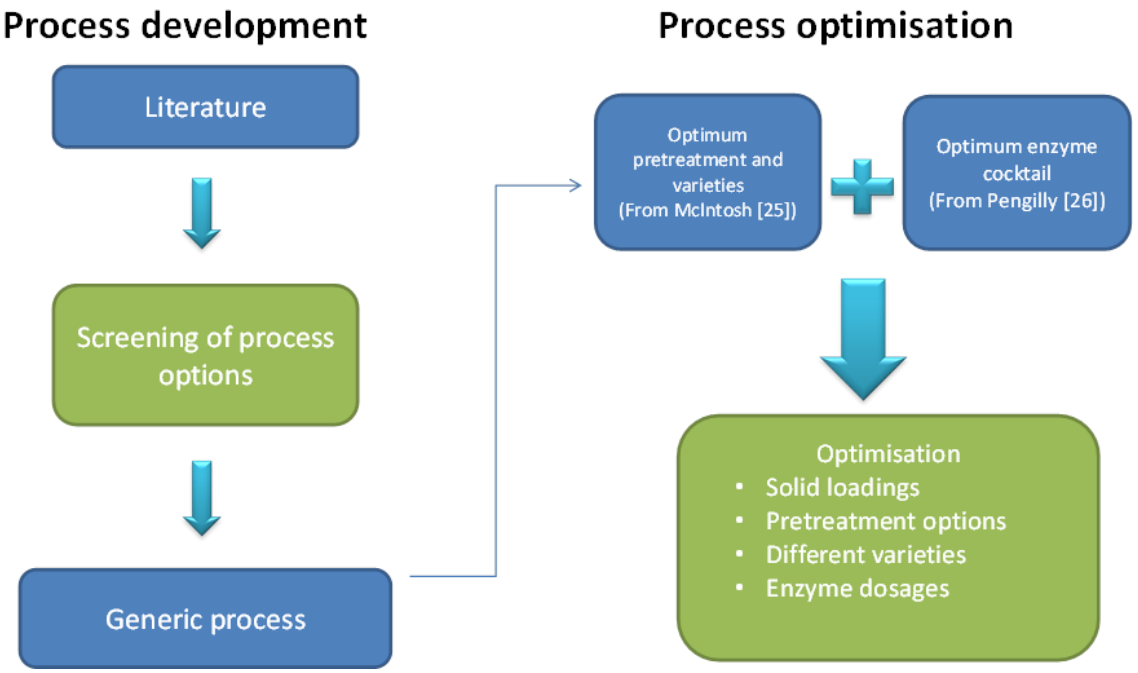

Figure 3.1: Flowsheet outlining the sweet sorghum SSF project, showing how the results from the studies conducted in parallel by McIntosh and Pengilly feature in the overall process.

4. Screening of process options to identify preferred combinations for subsequent optimisation

Abstract

During ethanol production from lignocellulose, the fermentation step is critical in obtaining the desired ethanol concentration before distillation. Whilst pretreatment and the selection of the enzyme cocktail focus on generating sugars for fermentation, the parameters of the fermentation process have to be investigated to ensure that a minimum ethanol concentration of 40 g/l is achieved, while simultaneously ensuring maximum ethanol yield from the available sugars. To develop a protocol for simultaneous saccharification and fermentation (SSF), pressed WIS from one variety of sweet sorghum bagasse, MSJH13, pretreated dry at 200 °C for 5 minutes was selected together with an enzyme cocktail consisting of 0.167 ml Cellic CTec2/g dry WIS. Investigations showed the performance of a hardened *Saccharomyces cerevisiae* strain D5A^{+H} to be superior to the parental D5A^{+} strain, and comparable to that of strain TMB3400, in terms of the maximum ethanol concentration produced, but at reduced ethanol productivity. Strain D5A^{+H} produced a maximum ethanol concentration of 32.2 g/L from a solids loading of 20% using pressed WIS, but was outperformed by strain MH1000 which produced 34.5 g/L of ethanol. At this high solid loading (20%), yeast inhibition was observed for both strain D5A^{+H} and MH1000 as evident from an accumulation of residual glucose in the culture broth. An increase in the solid feed rate confirmed that the inhibition was related to the concentrations of inhibitors in the fermentation broth. Strain MH1000 showed increased tolerance to the inhibitor concentrations since it was only inhibited after 94h, compared to 76h for strain D5A^{+H}. Although an ethanol concentration of 40 g/L was not obtained, optimum process parameters for the yeast strain, solid feed rate and solid loadings were identified to be utilised in the subsequent process optimisation work.

Keywords

Simultaneous saccharification and fermentation, Fed-batch cultures, Sweet sorghum bagasse, *Saccharomyces cerevisiae*, Yeast inhibition.

4.1 Introduction

The work in this chapter focussed on identifying the process parameters of an SSF process that would allow for an ethanol concentration of 4% (40 g/L) to be obtained in the final fermentation broth to reduce the associated costs during the subsequent distillation step [1]. In order to achieve this ethanol titre, a cumulative solids loading of 20% was required but with solids loadings higher than 10% viscosity and mixing becomes problematic which leads to a reduction in the overall ethanol yields obtained [17]. One solution was the utilisation of a fed-batch process where the solids were fed in increments to limit the material inside the reactor. Furthermore, factors such as the process options of using whole pretreatment slurry, pressed WIS or washed WIS was investigated, along with the selection of the best performing yeast strain and the feed rate of solids material to identify the best performing configuration.

4.2 Materials and Methods

Raw material

Selected sweet sorghum cultivars collected from the Ukhulinga experimental research farm in Pietermaritzburg (Kwa-Zulu Natal, South Africa) were processed by the Sugar Milling Research Institute (SMRI, Durban, South Africa). The processing of the fresh sweet sorghum was achieved through the use of a Jeffco cutter grinder (Jeffress Engineering, Dry Creek, Australia) for initial preparation followed by juice removal and subsequent dewatering in a Walkers 3 roller mill (Bundaberg Walkers, Queensland, Australia). Afterwards the material (bagasse) was washed three times with warm water before being dried at 40 ^{o}C and transported to the University of Stellenbosch (Stellenbosch, South Africa). Further processing of the bagasse was achieved through use of an impact mill (Condux LV15M ,Netzch-Condux GmbH, Hanau, Germany) and an ultra-centrifugal mill (Retch ZM 200, Monitoring and Control Laboratories, Parkhurst, RSA). The milled material was sieved in a pilot plant sieve shaker and the fraction between 680µm and 6.5mm was collected. Using cone-and-quarter subsampling the materials was divided into 600 gram aliquots into plastic bags and stored in a shipping container with adequate ventilation until pretreatment.

Pretreatment

Throughout this study steam pretreatment was used for fibre pretreatment. The six hundred gram aliquots were pretreated in a steam pretreatment unit as described by McIntosh [24] consisting of a

40 bar electric boiler, a 19 L high pressure reactor and a cyclone for collection of the pretreated material. Samples from sweet sorghum variety MSJH13 were selected for development of the SSF process. This variety was pretreated dry, i.e. without any additional soaking beforehand and moisture content of 7.3%, at a temperature of 200 °C for 5 minutes. The variety and conditions were selected based on recommendations from McIntosh [24].

The slurry recovered after pretreatment consisted of the water insoluble fibres/solids (WIS) and the liquid hydrolysate containing most of the inhibitors and solubilised sugars. Three SSF process options were investigated, namely the use of whole (pretreated) slurry, pressed WIS and washed WIS as feedstock. The whole slurry consisted of the WIS and hydrolysate obtained after pretreatment. For pressed WIS, the slurry was pressed in a dead-end hydraulic shop press press (50 ton hydraulic shop press, Eurotool, South Africa) at a pressure of 15 tons (7.4 MPa) to a moisture content of between 50% and 55% (w/w). Following pressing, the resulting material was washed with RO-water in a 1:1 mass-ratio and pressed again to remove the excess water and most of the remaining inhibitors to produce the washed WIS. The WIS or slurry was stored in plastic bags at -20 °C and the hydrolysate in glass bottles at 4 °C. A schematic of the three process options can be seen in Figure 4.1.

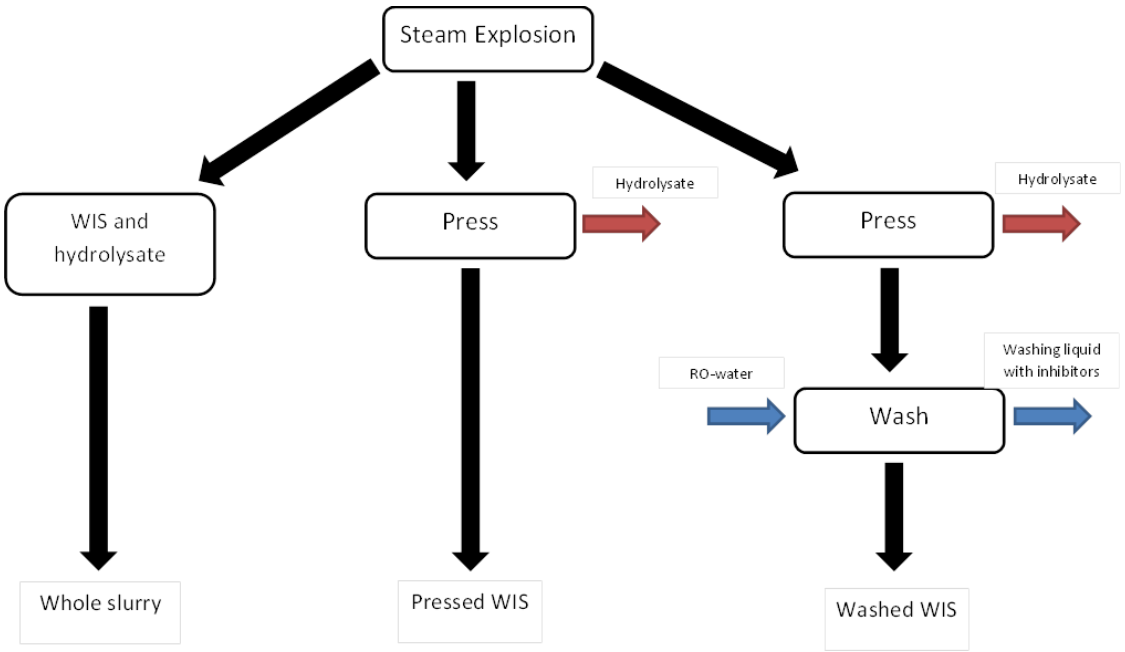

Figure 4.1: Schematic representation of the three process options to be investigated in SSF.

Microorganisms

To achieve a maximum ethanol yield during SSF, the microorganism responsible for the fermentation should have the capability to ferment both glucose and xylose. The microorganism selected was a genetically-modified strain of *Saccharomyces cerevisiae* designated as strain D5A^{+}. This strain was transformed with a bacterial xylose isomerase (XI) gene, thus imparting the ability to assimilate xylose via the pentose phosphate pathway.

In a separate study by other researchers within the research group this strain was subjected to random mutagenesis and continuous cultivation on hydrolysate with xylose as carbon source to improve resistance against high inhibitor concentrations (manuscript prepared for submission). This mutated strain resulting from this long-term selection/adaptation process was designated strain D5A^{+H}.

A third strain labelled TMB3400, encoding with xylose reductase (XR) and xylitol dehydrogenase (XDH) for xylose conversion and introduction into the central carbon metabolism, was used as benchmark [97]. This strain is known for its strong ethanol-producing capability and robustness in hydrolysate liquor [104,116].

A wild-type industrial strain, MH1000, deposited in the yeast culture collection of the Dept. of Microbiology, Stellenbosch University was also investigated. This strain however lacks the ability to ferment xylose.

An enzyme cocktail of Cellic CTec2, (Novozymes A/S, Bagsvaerd, Denmark) was used to hydrolyse the cellulose and hemicellulose. An enzyme loading of 0.167 ml /g dry WIS was selected upon recommendation from Pengilly [25] for preliminary process development work. This dosage was volumetrically equivalent to a Spezyme dosage of 10 FPU/g dry WIS.

Fermentations

The growth-medium was a modified form of the mineral medium described by Verduyn *et al.* [117] and contained per litre: 20 g yeast extract, 7.5 g $(NH_4)_2SO_4$, 3.4 g KH_2PO_4, 0.8 $MgSO_4.7H_2O$, 0.05 g $CaCL_2.2H_2O$, 0.5 g citric acid and 1 ml of a trace element stock solution containing per litre: 4.5 g $ZnSO_4.7H_2O$, 0.3 g $CoCl_2.6H_2O$, 1.5 g $MnSO_4.H_2O$, 0.3 g $CuSO_4.5H_2O$, 3 g $FeSO_4.7H_2O$, 0.4 g $NaMoO_4.2H_2O$, 1 g H_3BO_3, 0.1 g KI, 0.1 g $Al_2(SO_4)_3$. The medium was autoclaved for 15 minutes at 121 °C. The carbon-source (glucose or xylose) was autoclaved separately at a concentration of

125 g/l before aseptic addition to the growth medium to reach a final concentration of 20 g/L. All reagents were ordered from Sigma-Aldrich, Manheim, Germany.

Yeast strains were inoculated from freezer stocks or fresh agar-plates aseptically into 50 ml medium in 250 ml Erlenmeyer-flasks and incubated at 30 °C and 150 rpm for 24 hours. The culture was used to inoculate a second set of 1000 ml flasks containing 500 ml medium supplemented with 20% (v/v) sterilised hydrolysate for an initial optical density (OD_{600}, measured with a spectrophotometer [Cecil Instruments, Cambridge, England]) of 0.2 and incubated at 30 °C until and OD_{600} of between 5.0 and 5.5 was reached.

The cultures were harvested by centrifugation at 8000 rpm for 5 minutes and washed twice with sterile phosphate buffered saline (PBS) containing per litre: 8.01 g NaCl, 0.2 g KCl, 1.78 g $Na_2HPO_4.2H_2O$, 0.27 g KH_2PO_4, pH 7.4. After harvesting the cells were resuspended in 50 ml conical centrifuge tubes and the suspension added to the reactor to reach the required biomass concentration of 1.34 g/L

Fermentations were performed in computer-controlled 5 L glass-jacketed reactors (Sartorius Stedim, BBI, Göttingen, Germany) equipped with an exhaust condenser perfused with water at 6 °C, a marine impeller, temperature probe and a combination pH electrode (Hamilton AG, Bonaduz, Switzerland). The temperatures and pH levels were maintained at 35°C and pH 5.0 with automatic titration of 3 M KOH solution. SSF experiments were conducted with a final mass of the culture broth of 2000 g (ca. 1 600 ml). The culture broth was comprised of four main constituents, namely the yeast solution, enzymes, the pretreated material and mineral medium (without carbon-source) to provide buffering capacity and additional nutritional resources to the yeast cells. Initial SSF experiments were conducted with continuous nitrogen sparging through the reaction broth at a flow rate of 100 ml/min.

The reactors with medium (prepared as a concentrated to account for dilution of adding WIS, enzymes and yeast suspension) were autoclaved at 121 °C for 15 minutes to minimize sources of contamination in subsequent fermentations. A pre-saccharification step at 35 °C was included whereby the non-sterile enzymes together with the initial feed of pretreated material were added to the reactor at 2 hours prior to addition of the yeast. The lower temperature of 35 °C was selected due to concerns regarding the release of inhibitors from the unwashed WIS [87](see also Table 5.5 in section 5.3.2). Single fermentation experiments were performed in the present section of preliminary fermentation process development.

Hydrolysate fermentation in shake flask cultures

The schematic in Figure 2.2 shows the hydrolysate stream to be fermented separately by xylose-fermenting yeast or bacteria. This process was investigated in shake flask fed-batch cultures using yeast strain D5A^{+H}. Prior to fed-batch fermentation the hydrolysate was concentrated, through boiling, to obtain a concentration of xylose sufficient to achieve 4% (40 g/L) ethanol. A acid hydrolysis as described in the NREL protocol [118] was employed and the pH adjusted to pH 5.0 with 3 M KOH. Due to the volume of hydrolysate available, the fermentations were performed in 500 ml shake flask cultures with a starting volume of 250 ml at conditions of 30 °C and 120 rpm. Concentrated hydrolysate was fed in 39 ml increments once every 24 hours and samples were taken twice a day, between 6 and 8 hours apart.

Analyses

To generate metabolite profiles, samples were withdrawn at various stages of the fed-batch SSF culture, each time just before the addition of pretreated feedstock. The samples were centrifuged at 14 000 rpm for 5 minutes and the supernatant collected and treated with 35% perchloric acid (PCA) to precipitate the proteins. The PCA was neutralised with 7M KOH and the samples left on ice overnight before filtering through 0.22 µm filters prior to analyses by HPLC (Water Breeze, Waters Corporation, Milford, MA, USA).

Sugars, ethanol, glycerol, acetic acid and formic acid present in the liquid resulting after fermentation were analysed on an Aminex HPX-87H Column equipped with a Cation-H Micro-Guard Cartridge (Bio-Rad, Johannesburg, South Africa) with column temperature set to 65 °C with a mobile phase of 5 mM sulphuric acid and a flow rate of 0.6 ml/min. Sugar, glycerol, ethanol, acetic acid and formic acid peaks were detected with an RI detector (Shodex, RI-101) operated at 45 °C. The by-products HMF and furfural were analysed on a Luna C18(2) reversed phase column equipped with a Luna C18(2) precolumn (Phenomenex, Separations, Johannesburg, South Africa) with column temperature set to 25 °C and a flow rate of 0.7 ml/min. The mobile phases used for elution were 5 mM trifluoroacetic acid in water (A) and 5mM trifluoroacetic acid in acetonitrile (B). Separation was carried out by gradient elution from 5% mobile phase B, increasing to 11% B over 14 minutes and then increasing to 40% B over 3 minutes. The mobile phase composition was then kept constant at 40% for 2 minutes, followed by a decrease to 5% B over 5 minutes and ending with a final step of constant composition at 5% B for 4 minutes in order to equilibrate. HMF and furfural peaks were detected with a Dionex Ultimate 3000 diode array detector at 215 nm and 285 nm.

Calculations

The maximum volumetric ethanol productivity (g/L.h) was calculated from a linear regression of the ethanol concentration curve plotted as a function of time where a constant increase in the ethanol concentration was evident with a minimum of five data points used. This represents the combined hydrolysis and fermentation rates with low residual glucose concentrations. The overall ethanol productivity was calculated as the maximum ethanol concentration obtained divided by the time taken to attain that concentration. The ethanol yields were calculated based on the ethanol concentrations and liquid volumes at the end of the cultivations and expressed as a percentage of the theoretical maximum based on the available glucose in the fermentation, using a theoretical maximum conversion of 0.51 gram ethanol produced per gram glucose consumed.

4.3 Results

4.3.1 Pretreatment

The chemical composition of the WIS and hydrolysate following steam pretreatment at 200 °C for 5 minutes is shown in Table 4.1 and was determined according to the NREL protocol [119]. The low xylose content of the WIS pointed to substantial hydrolysis of the hemicellulose during pretreatment, as seen in the high concentration of solubilised xylose in the hydrolysate. However, more than 90% of the solubilised glucose and xylose in the hydrolysate were present in oligomeric form. The concentration of acetic acid in the hydrolysate, due to the liberation of the acetyl groups from the hemicellulose during pretreatment, is much higher than those reported Table 2.4 for different substrates. The high acetic acid concentration also attests to the high concentration of xylose in the hydrolysate, liberates from the hemicellulose.

Table 4.1: Composition of the WIS and hydrolysate fractions from steam pretreated sweet sorghum variety MSJH13. The monomeric sugar concentration in the hydrolysate is shown in parenthesis. Standard deviations were calculated from duplicate samples.

Variety	**MSJH13**	
Pretreatment conditions	200 °C, 5 minutes	
Fraction	**WIS (g/100g)**	**Hydrolysate (g/L)**
Glucose	49.4 ± 0.09	7.05 ± 0.06 (0.32)
Xylose	4.13 ± 0.05	43.1 ± 0.25 (4.00)
Arabinose	0.12 ± 0.12	1.66 ± 0.13
Cellobiose	ND[a]	0.74 ± 0.01
Acetic acid		8.12 ± 0.24
Formic acid		2.00 ± 0.07
HMF		0.16 ± 0.01
Furfural		0.60 ± 0.04

[a]N.D. = none detected

Based on the glucose content of the WIS in Table 4.1, a final solids loading of close to 20% would be required to achieve the required ethanol threshold concentration of 40 g/L. However, this would be a very high solids loading in a batch culture and due to the high viscosity of such a mixture effective mixing of the substrate, enzymes and yeast would be problematic [17]. It was therefore required to

investigate and develop a protocol for fed-batch fermentations to minimize viscosity effects, yet allow a high overall solids loading.

The data in Table 4.2 shows the contribution of the WIS and liquid fractions to the concentration of glucose and xylose in the final fermentation liquid. It is important to note that the solid loading during SSF is based on the dry matter content of the fibres, but will also be influenced by the soluble sugars still contained in the hydrolysate fraction of the pressed WIS and whole slurry.

Table 4.2: Comparison of the glucose and xylose concentrations in the final fermentation liquid, as obtained from the WIS and liquid (hydrolysate) fractions respectively.

	Cumulative solid loading			
	20%		10%	
	From WIS	From liquid	From WIS	From liquid
Washed WIS	123.5 g/L glucose 10.3 g/L xylose	0.0 g/L glucose 0.0 g/L xylose	61.8 g/L glucose 5.16 g/L xylose	0.0 g/L glucose 0.0 g/L xylose
Pressed WIS	123.5 g/L glucose 10.3 g/L xylose	0.11 g/L glucose 1.41 g/L xylose	61.8 g/L glucose 5.16 g/L xylose	0.06 g/L glucose 0.71 g/L xylose
Whole slurry	123.5 g/L glucose 10.3 g/L xylose	0.24 g/L glucose 2.94 g/L xylose	61.8 g/L glucose 5.16 g/L xylose	0.12 g/L glucose 1.47 g/L xylose

4.3.2 Comparison of three recombinant yeast strains in batch culture

For selection of the yeast strain most suitable for SSF the ethanol production of three recombinant strains of *S. cerevisiae* were compared in batch SSF. The whole slurry from steam pretreatment was utilised at a solids loading of 10% (w/w). This process configuration would ensure high concentrations of inhibitors during SSF to evaluate the inhibitor tolerance of the three strains. The performance of strain D5A^{+H} was gauged against that of the parental strain of D5A^{+}, with strain TMB3400 included as a reference.

As seen from the trends in Figures 4.2A, C & E, batch cultures with whole slurry at 10% solid loadings of all three yeast strains showed increases in the ethanol concentration up to a maximum value before decreasing until the end of the cultivation. Overall the ethanol concentrations obtained from all three yeast strains were very low. The maximum ethanol concentration achieved with strain D5A^{+} was the lowest between the strains evaluated at 6.1 g/L. Strain TMB3400 produced a maximum ethanol concentration of 6.6 g/L, which is similar to the 6.8 g/L obtained with strain

D5A^{+H}. These concentrations equated to yields of 18.2% for D5A^{+}, 20.2% for D5A^{+H} and 19.6% for TMB3400 of the theoretical maxima. In terms of ethanol produced, strain D5A^{+H} outperformed the others, but it required nearly 50 hours to reach this maximum concentration, whereas the other two strains did so in half the time (Figures 4.2 A, C & E). This led to variations in the overall volumetric ethanol productivities, calculated as 0.233 g/L.h for strain D5A^{+}, 0.134 g/L.h for strain D5A^{+H} and 0.251 g/L.h for strain TMB3400. Strain D5A^{+H} produced roughly half the concentration of glycerol recorded for the other two strains while all three yeast strains were capable of assimilating the inhibitors HMF and furfural to negligible concentrations (Figures 4.2 B, D & F). Whilst none of the three yeast strains were capable of delivering an ethanol yield greater than 20%, it was decided to continue with strain D5A^{+H} in further investigations. This decision was made based on the fact that D5A^{+H} deliver the greatest ethanol concentration.

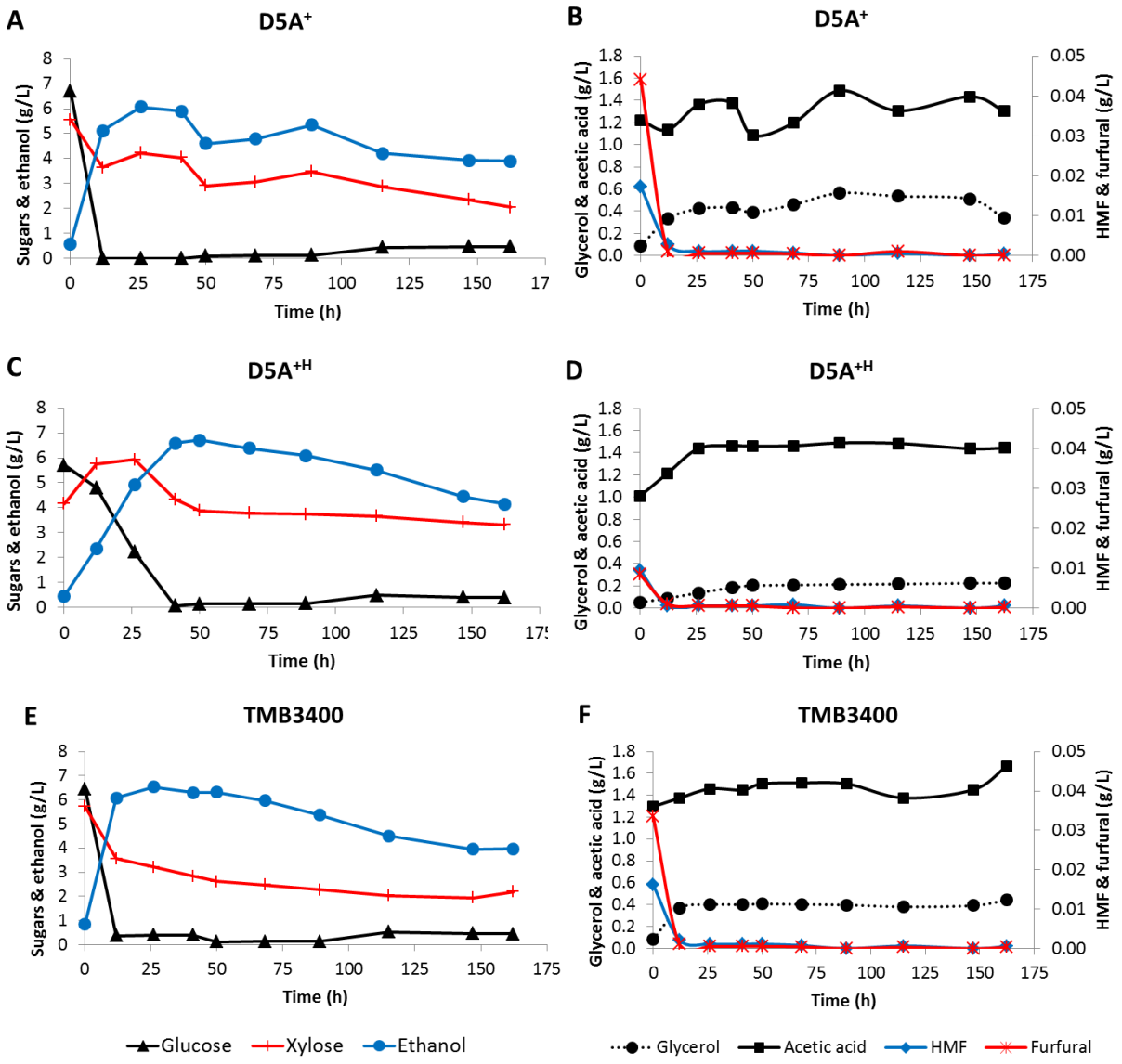

Figure 4.2: Ethanol and by-product graphs for batch SSF cultivations with the whole slurry at solid loadings of 10% using three *S. cerevisiae* strains. Temperature of 35 °C and pH 5.0 was utilised with an enzyme dosage of 0.167 ml Cellic CTec2/g dry WIS.

4.3.3 Evaluation of three process options in fed-batch culture

Due to time and feedstock constraints, at least one of the process options of whole slurry, pressed WIS or washed WIS had to be eliminated, with the focus remaining on the optimisation of the remaining two processing options. All three process options were investigated under fed-batch SSF conditions with final solid loadings of 20% using only the D5A^{+H} yeast strain. A solid feed rate of 5% solids per day, fed in 2%, 1% and 2% increments (roughly 4 hours apart, with the feed intervals indicated on the graphs) was used to achieve a final, cumulative solids loading of 20%. Due to concerns regarding the concentrations of inhibitors, the fermentation with the whole slurry was started with a lower initial solids loading of only 1% solids, compared to 3% for the pressed and washed WIS. The same feed rate was utilised for all three fermentations.

Fermentation of the washed WIS at a final solids loading of 20 % in SSF resulted in the highest ethanol concentration of 7.8 g/l, and based on the glucose content of the WIS (Table 4.1) this concentration can be equated to a yield of only 12% of the theoretical maximum. Maximum ethanol concentrations from the pressed WIS and whole slurry fermentations were lower at 3.6 g/L and 2.7 g/L respectively; equating to yields of 5.4% and 4% of the theoretical maxima (Figures 4.3C & E). The low yields achieved were in part due to the high accumulation of glucose within the fermentations, reaching concentrations up to 29, 64 and 49 g/L for the washed WIS, pressed WIS and whole slurry fermentations respectively (Figures 4.3 A, C & E). With both the pressed WIS and whole slurry (Figures 4.3C & E), discontinuities in the ethanol production were observed and occurred after 26 h and 19h respectively. Following this, the ethanol concentrations in both cultures decreased. With the washed WIS ethanol production continued until the end of the cultivation, but at a low rate.

An acetic acid concentration of 8.12 g/L was recorded with the washed WIS which was nearly 13% higher than when the whole slurry (7.21 g/L) and 48% higher than when pressed WIS (5.47 g/L) was used (Figures 4.3B, D &F). Concentrations of HMF and furfural for the pressed WIS and whole slurry fermentations showed concentrations of less than 0.07 g/L and 0.16 g/L, respectively, while the washing step reduced these concentrations to negligible levels.

When utilising either pressed WIS or washed WIS, the separated hydrolysate (Figure 4.1 and Table 4.1) can be fermented under more optimum conditions compared to SSF so as to maximise the ethanol yield on, predominantly, the xylose.

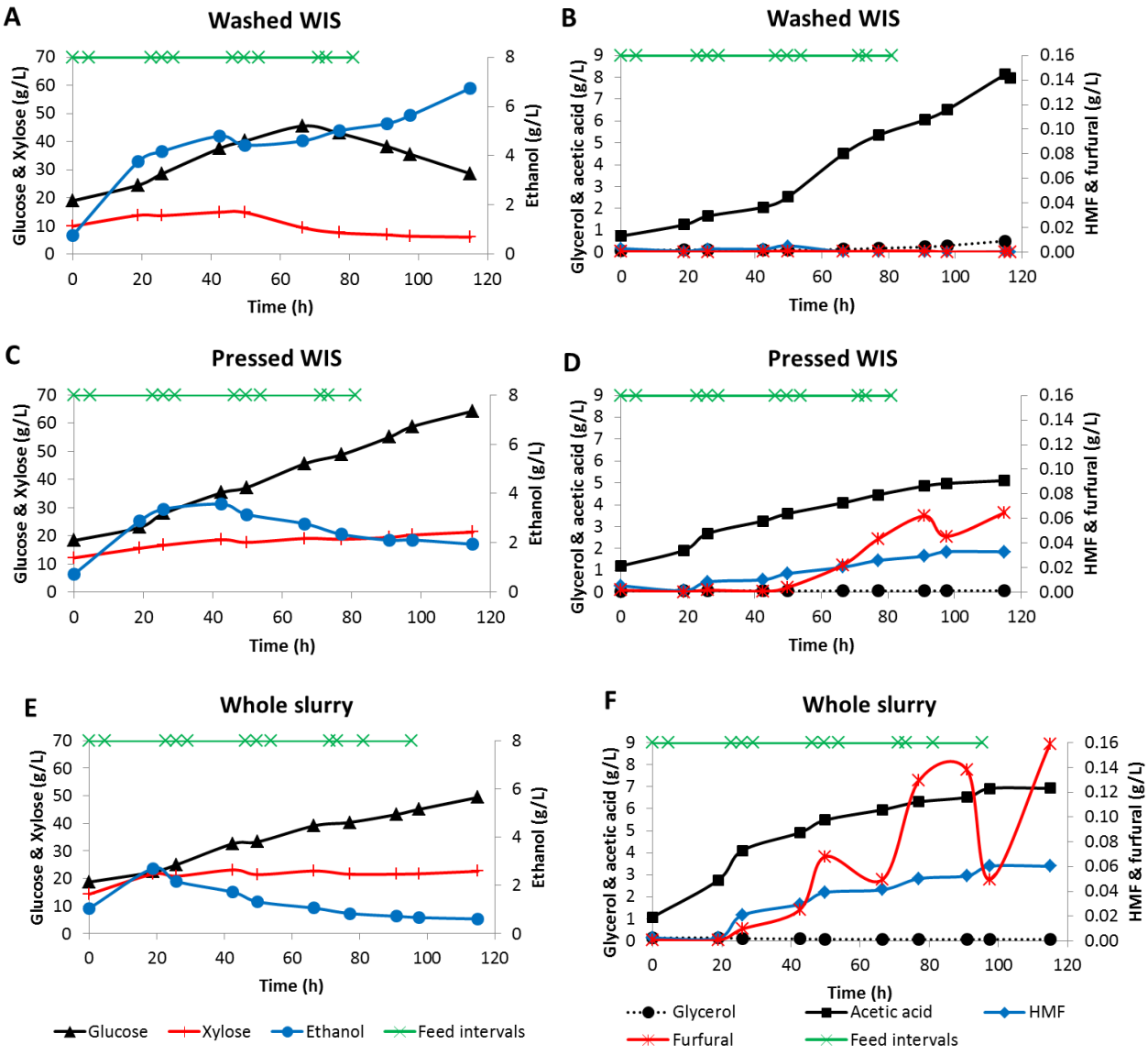

Figure 4.3: Ethanol and by-product graphs for fed-batch SSF cultivations with washed WIS (A &B), pressed WIS (C & D) and whole slurry (E & F) at final solid loadings of 20% using *S. cerevisiae* strain D5A^{+H}. Temperature of 35 °C and pH 5.0 was utilised with an enzyme dosage of 0.167 ml Cellic CTec2/g dry WIS.

4.3.4 Fermentation of the hydrolysate

Since the whole slurry from pretreatment retains all of the sugars present in the hydrolysate, a valid comparison between the different process options could only be made if the hydrolysates obtained from the pressed WIS and washed WIS were also fermented. Only *S. cerevisiae* strain D5A^{+H} was employed for these fermentations and the fermentations were performed under more ideal temperature of 30 °C in shake flask cultures. No nitrogen sparging was utilised.

In Figure 4.4 it was seen that xylose consumption was most noticeable during the first 20 hours of the fermentation, although the ethanol concentration showed a consistent increased until the end of the cultivation period and reached a maximum value of 5.4 g/L. The yield of ethanol on the total xylose in the fermentation broth was very low at only 23% of the theoretical maximum while the yield on only the xylose consumed by the yeast was slightly higher at 46% of the theoretical maximum. Glycerol production was low during the cultivation, while the concentration of acetic acid increased for the duration of the cultivation.

From the data in Figures 4.2 & 4.3 it appeared that xylose was not fermented in these cultures. If the xylose had been fermented and assuming a yield of 46%, the ethanol concentration for the whole slurry fermentation with D5A^{+H} (Figure 4.2) would have increased by only 0.78 g/L while increases of 1.42 g/L, 5.0 g/L and 5.3 g/L would have been obtained in the ethanol concentrations from the washed WIS, pressed WIS and whole slurry fermentations (Figure 4.3).

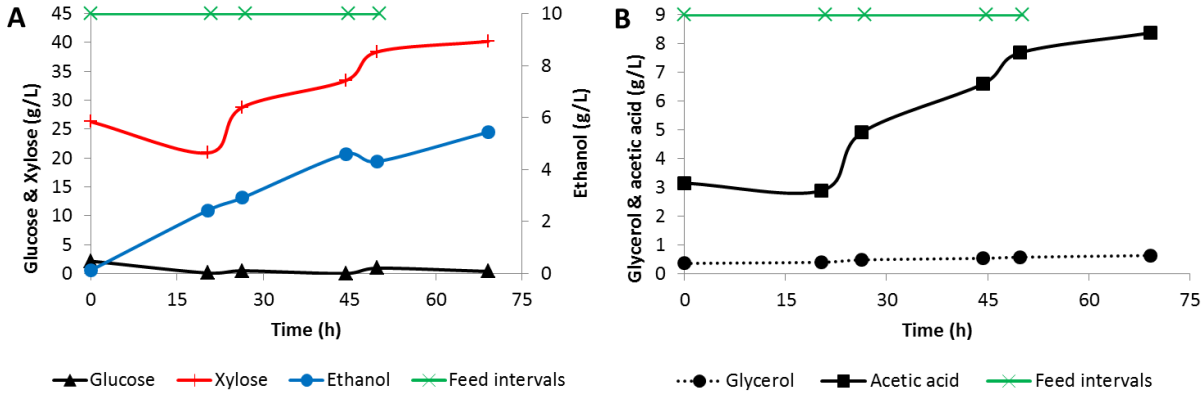

Figure 4.4: Ethanol (A) and by-product (B) graphs for the hydrolysate fermentation in fed-batch shake flask cultures using *S. cerevisiae* strain D5A^{+H} at 30 °C with the feed intervals indicated on the graphs. The hydrolysate was concentrated through boiling, followed by an acid-hydrolysis and the pH adjusted to 5.0 prior to fermentation.

4.3.5 Selection of the preferred yeast strain in fed-batch culture

Previous results in Section 4.3.2 showed the performance of strain D5A^{+H} to be comparable or even superior to that of TMB3400 with regards to the ethanol concentration obtained. This strain did however exhibit a lower overall ethanol productivity due to a substantial lag phase which would impact negatively on the process productivity. A second *S. cerevisiae* strain was thus introduced, namely strain MH1000 which does not poses xylose-fermenting capabilities. The performance of strains D5A^{+H} and MH1000 were investigated in a series of fed-batch fermentations with final solid

loadings of 10% and 20% using pressed WIS, as presented below. Nitrogen sparging was not utilised during these cultivations.

4.3.5.1 CULTIVATIONS WITH 10% SOLID LOADINGS

The fermentations (Figure 4.5) were started with initial solid loadings of 3% and with subsequent feed additions of 2% and 2% (for 4% per day), 6 to 8 hours apart. The feed intervals are indicated on the graphs in Figure 4.5. Fermentation experiments with both strains with final solid loadings of 10% resulted in ethanol concentrations in excess of 20 g/l, where 23.8 g/L and 21 g/L were produced by strains D5A^{+H} and MH1000, respectively (Figure 4.5A & C). Strain D5A^{+H} did however exhibit a substantial lag phase lasting for approx. 53 hours (Figure 4.5A). However, after 101 hours the cultivations with both yeast strains showed similar ethanol concentration with 22.6 g/L and 20.2 g/L for strain D5A^{+H} and MH1000 respectively, indicating that the extended lag phase for strain D5A^{+H} did not impact on the overall productivity of the process. During active fermentation, both yeast strains were able to assimilate HMF and furfural to negligible concentrations (Figure 4.5B & D). In these experiments it seems that neither of the organic acids was assimilated.

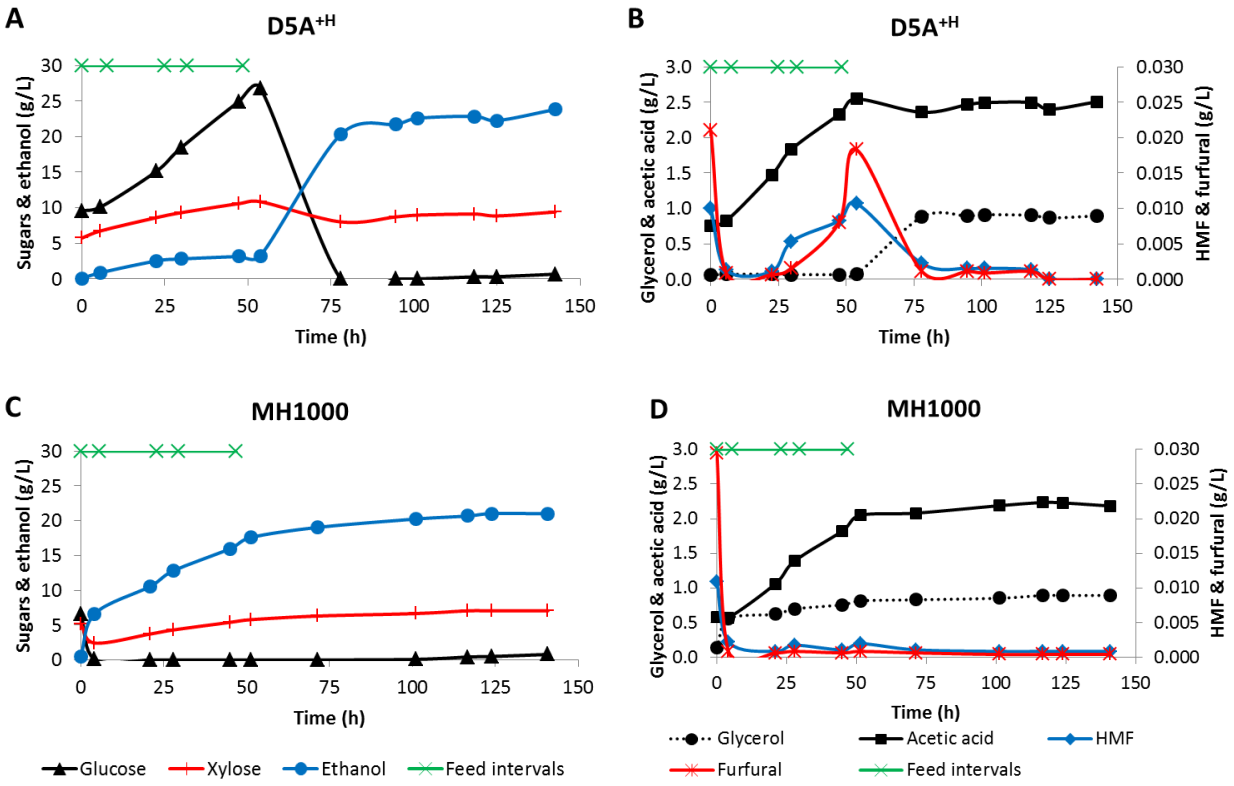

Figure 4.5: Ethanol and by-product graphs for fed-batch SSF cultures with 10% pressed solids comparing *S. cerevisiae* strains D5A^{+H} (A & B) and MH1000 (C & D). Fermentation temperature of 35 °C and pH 5.0 were utilised with an enzyme dosage of 0.167 ml Cellic CTec2/g dry WIS. The feed intervals are indicated on the graphs.

4.3.5.2 CULTIVATIONS WITH 20% SOLID LOADINGS

The capabilities and inhibitor tolerance of strains D5A^{+H} and MH1000 were also investigated in fed-batch fermentations with a final (cumulative) solids loading of 20%. The solids were fed in 1% and 2% increments between 6 and 8 hours apart for a total of 3% solids per day, as indicated in Figure 4.6.

With pre-culturing and pre-conditioning performed with glucose as carbon source, strain D5A^{+H} still exhibited a noticeable lag phase of 28 hours (Figure 4.6A) as evident from the lag in the production of ethanol. Once fermentation had started the process ethanol productivities for both strains were identical with 0.255 g/L.h for strain D5A^{+H} and 0.253 g/L.h for strain MH1000. Strain MH1000 produced a maximum ethanol concentration of 34.5 g/L compared to 32.2 g/L from strain D5A^{+H} (Figure 4.6A & C). In cultures of both strains, the residual glucose concentration increased, which pointed to inhibition of the yeast. For D5A^{+H} this inhibition occurred sooner, after 76 hours compared to 94 hours for MH1000 (Figure 4.6 A & C). At the point of yeast-inhibition the concentrations of acetic acid were 3.5 g/L for MH1000 and 3.0 g/L for D5A^{+H} (Figure 4.6 B & D).

Since strain MH1000 did not exhibit any lag phase, also at the lower solids loading in Figure 4.5C, it was concluded that the lag observed with strain D5A^{+H} was not the result of the conditions during SSF, but rather a strain-specific phenomenon. Similar results were also observed in Section 4.3.2, but since the parental D5A^{+} strain did not exhibit such a long lag, one has to conclude that the extended lag phase was a by-product of the mutagenesis and hardening processes. These observations made the convincing argument that strain MH1000 should be used for future fermentations since it delivered more stable and consistent results.

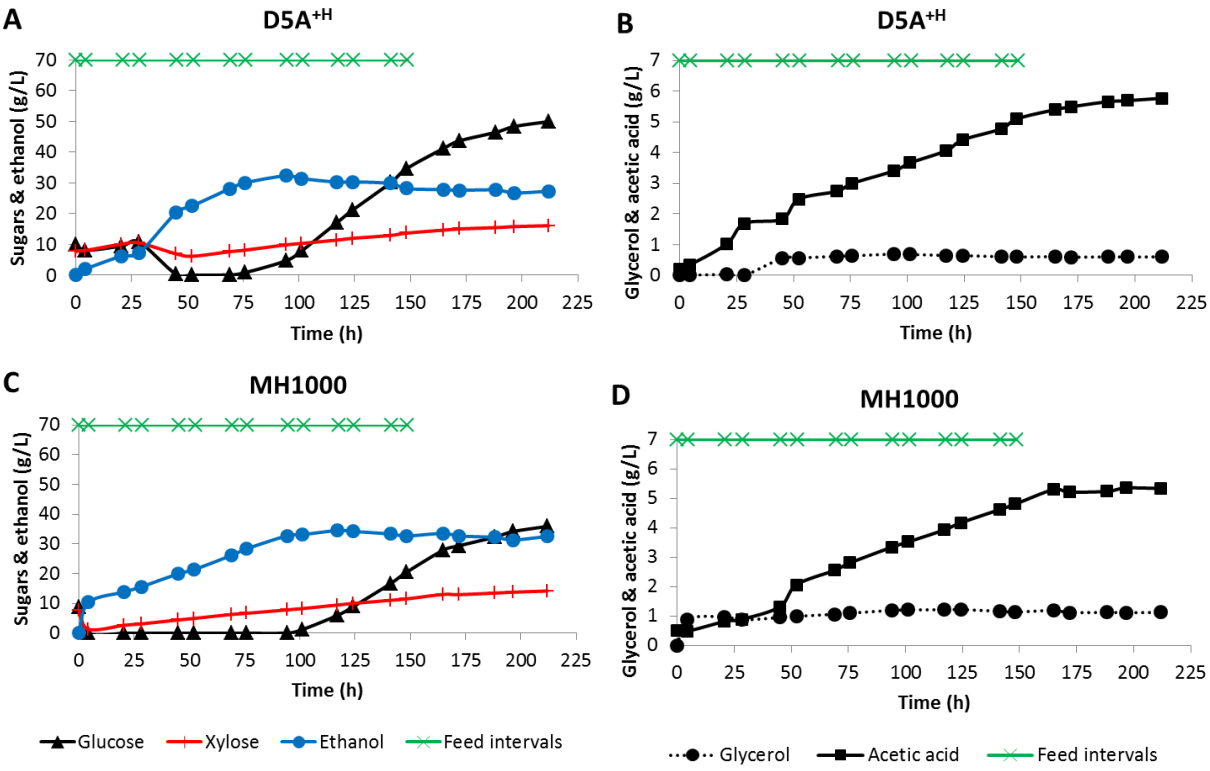

Figure 4.6: Ethanol and by-product graphs for fed-batch SSF cultures with 20% pressed solids comparing *S. cerevisiae* strains D5A^{+H} (A & B) and MH1000 (C & D) with solid feed rates of 3% per day. Fermentation temperature of 35 °C and pH 5.0 were utilised with an enzyme dosage of 0.167 ml Cellic CTec2/g dry WIS. The feed intervals are indicated on the graphs.

4.3.7 Effect of continuous nitrogen sparging on SSF performance

Continuous sparging of nitrogen was investigated as a means to ensure anaerobic conditions during SSF to promote ethanolic fermentation and increase the ethanol concentration obtained from the pretreated material. Continuous sparging with nitrogen through the culture broth was investigated using yeast strain MH1000 and nitrogen flow rates of 100 ml/min and 50 ml/min (Figure 4.7). Previous results (Figures 4.2 & 4.3) have shown that sparging strips ethanol from the broth, but ethanol production during those fermentations was very low and hence the benefits from nitrogen sparging were perhaps not evident.

Fermentation runs indicated that the process ethanol productivities were reduced due to the stripping of ethanol from the fermentation broth by the nitrogen sparging: With 50 ml/min the productivity was reduced by 13% (0.214 g/L.h) and with 100 ml/min by 29% (0.175 g/L.h) compared to the control with no sparging (0.246 g/L.h, Figure 4.7). After 50 hours, sparging at 50 ml/min

resulted in an ethanol concentration in the broth that was 10% lower (16.3 g/L) and 100 ml/min in a concentration that was 19% lower (14.8 g/L) than the control fermentation (18.2 g/L). By the end of the fermentations these differences had increased to 24% (50 ml/min, 16.7 g/L) and 39% (100 ml/min, 13.4 g/L) respectively compared to the control fermentation at 21.8 g/L of ethanol (Figure 4.7).

Whereas the control fermentation without sparging showed a gradual increase in ethanol after production levelled off at 70 hour, both fermentations with sparging showed a decrease in ethanol after the period of maximum productivity ended. After that point the loss due to stripping was greater than the ethanol production rates of the yeast. If one assumes that the difference in productivities was only due to the stripping effect of the sparging, the rates of ethanol loss due to stripping could be calculated as 0.032 g/L.h at 50 ml/min and 0.071 g/L.h at 100 ml/min. The loss of ethanol due to the stripping effect brought on by the continuous sparging of nitrogen demonstrates that nitrogen sparging should be avoided.

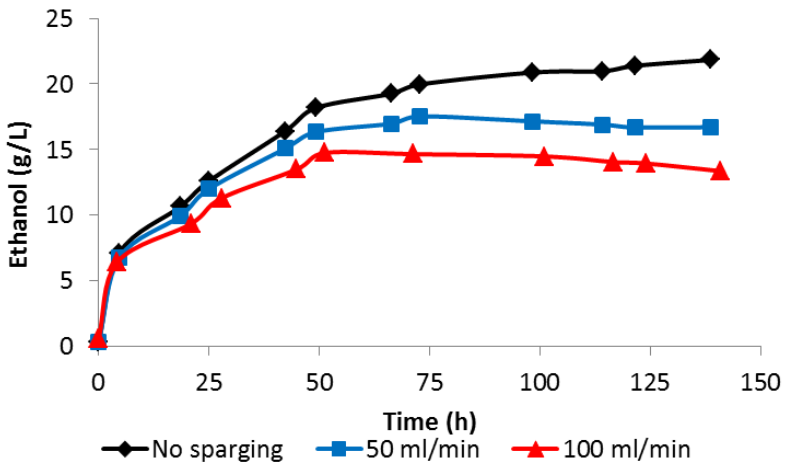

Figure 4.7: Ethanol trends comparing the effect of continuous nitrogen sparging during fed-batch SSF with 10% solids using *S. cerevisiae* strain MH1000. Fermentation temperature of 35 °C and pH 5.0 were utilised with nitrogen flow rates of 50 ml/min and 100 ml/min compared to the control fermentation with no sparging.

4.3.6 Comparison of solid feed-rates during fed-batch SSF

With a solid feed rate of 3% solids per day, as utilised for the cultivations shown in Figure 4.6, a processing time of 150 hours was required to feed all the material. With a higher feed rate, the processing time could be reduced and the productivity of the process possibly increased. A comparison between two possible feeds rates to be used for fed-batch fermentations with the required 20% solids loading was thus investigated. Feed rates of 3% per day (in 1% and 2% increments as shown in Figures 4.6C & D)) and 5% per day (2% and 3% increments, Figures 4.8A & B) were compared using yeast strain MH1000. No nitrogen sparging was utilised.

The differences in feed rate had a substantial effect on the performance of the yeast, as evident from the accumulation in ethanol and residual glucose. Firstly, the rate of ethanol production did not correlate with the feeding rate. In Figure 4.8A, the feeding rate per day increased by 66% over that in Figure 4.6C (5% compared to 3% per day), but the process ethanol productivity only increased by 52% (0.38 g/L.h with 5% per day compared to 0.25 g/L.h with 3% per day). Secondly, with the increased feed rate (Figure 4.8A) the onset of glucose accumulation and yeast inhibition was seen to occur sooner, after 53 hours compared to 101 hours with the slower feed rate (Figure 4.6C).

The data suggested that a slower feeding strategy is preferential since the maximum ethanol concentration was 16% higher at 34.5 g/L with the slower feed rate (Figure 4.6C) compared to 29.7 g/L where a higher feed rate was used (Figure 4.8A). However, a longer residence time is required with the slower feeds rates, which could have an impact on the process economics.

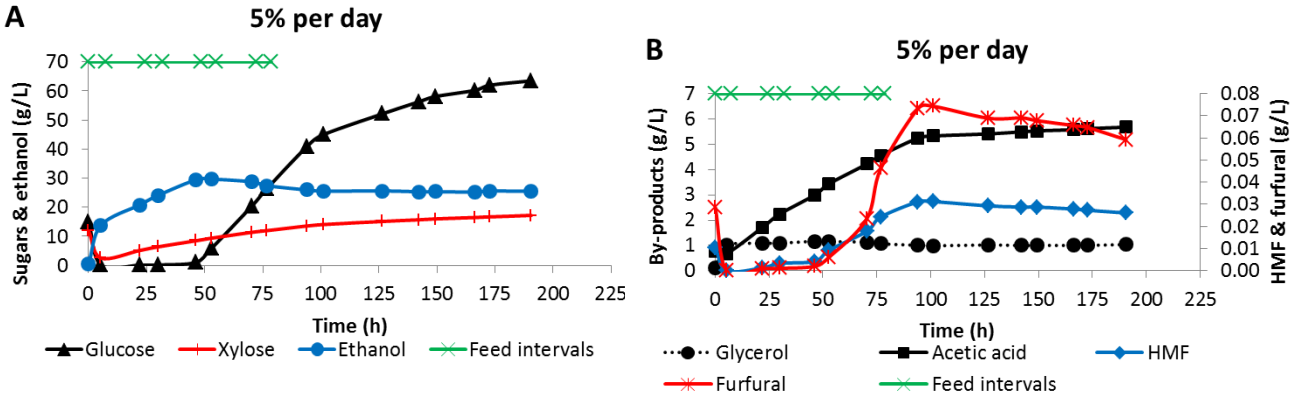

Figure 4.8: Ethanol (A) and by-product (B) graphs for fed-batch SSF culture with 20% pressed solids using *S. cerevisiae* strain MH1000 with a solid feed rate of 5% per day. Fermentation temperature of 35 °C and pH 5.0 were utilised with an enzyme dosage of 0.167 ml Cellic CTec2/g dry WIS. The feed intervals are indicated on the graphs.

4.4 Discussion

Pre-treatment and by-products

The main aim for this work was to develop an SSF process for use with solid loadings up to 20% (w/w) to obtain a final ethanol concentration of 40 g/L. The conditions used for steam pretreatment would thus have a substantial impact on the performance of the process. Manzanares et al. [37] investigated steam explosion pretreatment without an additional catalyst (dry material), but utilised forage sorghum at different pretreatment conditions. At conditions of 190 °C and 10 minutes (which also equates to a severity factor of 3.65 as calculated in Section 2.3.1), the glucose content of the WIS was similar to that shown in Table 4.1, but the xylose content was higher at 15 g/100 g dry weight. The discrepancy in xylose content can be attributed to a greater hydrolysis of the hemicellulose fraction for variety MSJH13, as seen from the high xylose concentration in the hydrolysate in Table 4.1. The authors made no mention of the concentrations of inhibitors.

McIntosh [24] also investigated steam explosion pretreatment of dry sweet sorghum bagasse but utilised different cultivars and pretreatment conditions, with pretreatment conditions of 205 °C and 5 minutes found to be optimum. Attesting to the higher severity, the optimised conditions (severity factor of 3.79 compared to 3.65 for the pretreatment data shown in Table 4.1) resulted in higher sugar concentrations in the WIS but also resulted in higher concentrations of inhibitors with increases of up to 50% recorded compared to the data in Table 4.1. Overall it was concluded that steam pretreatment of dry material was not well suited for ethanol production, as steam pretreatment with both water-soaking and SO_2-impregnation of lignocellulose resulted in higher sugar concentrations and lower inhibitors. However, by utilising sub-optimum pretreated material for screening of the process parameters ('worst-case scenario') one can be sure the chosen parameters should also be well suited when utilising material from optimised pretreatment conditions (Chapter 5).

In fermentations in this study (Figures 4.3, 4.6 & 4.8) it was seen that ethanol production ceased and glucose started to accumulate within the cultures. Since this was observed with both strains D5A^{+H} and MH1000 (Figure 4.6A & C), it was concluded that the inhibition was not a strain specific phenomenon. When different feed rates were utilised (3% per day in Figure 4.6C and 5% per day in Figure 4.8A) inhibition and glucose accumulation occurred at different time points, but in both cases after 12% to 13% solids had been fed. It would thus seem that inhibition is not a function of the duration of the fermentation, but rather of the concentration of inhibitors which was dependent on the cumulative amount of solids fed, and not directly on the feed rate. Furthermore, when washed

WIS was utilised (Figure 4.3A) inhibition was avoided since ethanol production was seen to continue until the end of the cultivation period.

It has been previously reported that inhibitors trapped within the WIS are released upon hydrolysis and that a variety of enzymatic activities, including xylanases, are responsible for this release [120]. The initial increase in residual glucose with continued ethanol production (seen in Figures 4.6A & C & 4.8A) represented an initial decrease in the yeast metabolic activity as the inhibitor concentrations increased. Complete inhibition of the yeast followed after which no ethanol production occurred as a result of disruption of the metabolism. In previous reports where glucose accumulation was also encountered the authors also resorted to washing of the pretreated material to reduce the concentrations of inhibitors. Cantarella *et al.* [121] found fermentation to be completely inhibited and result in an accumulation of glucose up to a concentration of 44 g/L when the whole pretreatment slurry for steam pretreated poplar (214 °C, 6 min) was used at a solid loading of 10% (w/v) in batch SSF. Following a thorough washing step, the fermentability of the material was greatly increased and an ethanol concentration of 22 g/L was obtained in 48 hours. Hoyer *et al.* [105] was able to prevent glucose accumulation during batch SSF of 14% (w/w) solids by removing 20% of the hydrolysate fraction. The authors provided no explanation for the glucose accumulation and no mention of the inhibitor concentrations was made.

Of the inhibitors analysed the furans HMF and furfural were assimilated during active ethanol fermentation and glucose uptake (Figures 4.3B, D &F & 4.5B & D & 4.8B). Only once the concentration of residual glucose was seen to increase did the concentrations of these furans also increase. Previous reports [14,15,19,75] have indicated that both HMF and furfural can be assimilated by *S. cerevisiae* from concentrations as high as 4.0 to 5.0 g/L HMF [14,122] and 1.6 to 2.0 g/L furfural [14,122]. These concentrations were significantly higher than those encountered in Figures 4.3, 4.5 and 4.8, although these studies [14,122] were carried out with single inhibitory compounds which would disregard potential synergistic effects. Palmqvist *et al*. [123] found that the non-volatile fraction of the hydrolysate from steam pretreated willow inhibited the enzymatic hydrolysis and fermentation steps to a greater extent than the volatile fraction. Although acetic acid exhibited the greatest inhibitory effect in the volatile fraction, in the non-volatile fraction (containing both acetic acid and lignin-derived compounds) it was found that the synergistic effects between the acid and the other compounds resulted in the inhibition. Ding *et al*. [124] studied the effects of furfural, phenol and acetic acid on *S. cerevisiae* at 30 °C in YPD medium. Whilst neither furfural (1.3 g/L) nor phenol (0.5 g/L) had any significant effect on the growth rate of the yeast, acetic acid (5.3 g/L) had a profound effect by inducing a lag phase and significantly lowering the growth rate.

However, it was the combination of furfural, phenol and acetic acid at the above concentrations that resulted in the lowest culture growth rate. The data thus suggest that the yeast inhibition observed in the present study was due to the synergistic effect of the inhibitors present in the pressed WIS. Data in the current study showed yeast inhibition and glucose accumulation to coincide with an acetic acid concentration of between 3.0 and 3.4 g/L (Figures 4.6B & D & 4.8B) and it was postulated that the concentration of acetic acid within the SSF culture could be used to predict the possible onset of yeast inhibition but it was seen from previous report on whole slurry SSF of corn stover [1] and unwashed fibres from sugarcane bagasse [81] that this observation did not hold true, although these studies were conducted at a lower temperature of 30 °C which would reduce the toxicity of the inhibitors [103].

Comparison of batch and fed-batch fermentations

The low ethanol concentrations and yields (<20% of theoretical maximum yield) obtained from the batch fermentations (Figure 4.2A, C & E) for all three yeasts strains investigated pointed to ineffective utilisation of the available sugars in the pretreated material. However, it is known [125] that the performance of batch SSF is usually lower compared to fed-batch. Nilsson *et al.* [126] proved that even when using detoxified slurry, where no inhibitors are present, the results from fed-batch fermentations were still superior. In order to obtain the required ethanol concentration of 40 g/L, the solids loadings would have to be increased, which would result in high initial concentrations of inhibitors that could completely inhibit ethanol fermentation. The increased solid loadings would also prevent effective mixing of the substrate and enzymes.

Several of the shortcomings of the batch process could be addressed through the use of a fed-batch protocol. For the fed-batch fermentation using the whole slurry (Figure 4.3F) the initial concentrations of HMF and furfural were reduced while the concentration of acetic acid remained similar although the final solid loadings were doubled. However, the ethanol concentrations and yields obtained were lower than those of the batch fermentation. With all the fed-batch fermentations significant levels of residual glucose were present from the start of the fermentations (Figure 4.3A, C & E)). While this showed the enzymes to be suited to the protocol utilised, further investigation into selecting a suitable yeast strain was still required in order to obtain an effective fermentation process.

Investigation of the process options

Whereas washing the solids before fermentation was shown to remove the sugar degradation products [87], possibly enhancing the ethanol production, results from the present study showed that this process step resulted in high concentrations of acetic acid during fermentation; much greater than when either the whole slurry or pressed solids were used (Figure 4.3B, D & F). A possible reason for this observation could reside in the fact that solubilised sugars are contained in the hydrolysate of the pressed WIS and whole slurry and could contribute to a higher dry matter content of these materials, measured after oven drying (Table 4.2). The washed WIS would thus have a lower dry matter content, which would require a larger amount of the washed WIS to be added to the fermentation for the same solids loading, compared to the pressed WIS or whole slurry. The larger amount of material would thus lead to a higher concentration of acetic acid.

The use of washed WIS does however have the advantages of removing the inhibitors such as HMF, furfural (Figure 4.3B) and the phenolic compounds which effectively prevented the onset of yeast inhibition and a discontinuity in the ethanol production. However, a reduction in the inhibitor concentrations could increase the risk of bacterial contamination which would affect the process performance and lower the ethanol concentration obtained [105,108]. Besides the additional processing costs involved in implementing a washing step, the loss of fermentable sugars in the wash water and hydrolysate (Figure 4.1) would also reduce the ethanol yield of the raw material. Although mostly xylose, low concentrations of glucose are also lost.

The use of the whole slurry would potentially be the most economical option since not additional processing steps are required [86]. This option would also include the fermentable sugars present in the hydrolysate fraction, ensuring maximum usage of the raw material. However, this process option would also lead to the greatest concentration of inhibitors in the SSF cultures and would require sufficiently robust yeast to ensure continual ethanol production. A xylose-fermenting yeast strain would also be required to effectively utilise the xylose contained in the hydrolysate fraction. Since strain MH1000 was selected for further investigation in the present study, it would negate the benefit from using the whole pretreatment slurry since this strain does not have xylose-fermenting capabilities.

The intermediate solution of utilising the pressed WIS does seem to be a plausible process option since the concentrations of inhibitors are lowered, but not eliminated, which could limit bacterial growth providing the yeast is sufficiently robust or has been pre-conditioned on the hydrolysate fraction prior to SSF. A fraction of the separate hydrolyse stream can be used for pre-conditioning of the yeast while the remainder can be fermented, following an acid hydrolysis, under more ideal

processing conditions of temperature and pH. Hoyer et al. [105] also found that using WIS from which 20% of the hydrolysate had been removed (similar to the pressed WIS utilised in this study) delivered results superior to that of the whole slurry or washed WIS. With the whole slurry significant glucose accumulation was observed (up to 45 g/L), while the washed WIS resulted in significant lactic acid production of up to 18 g/L [105]. Although the results in Figure 4.3 showed the washed WIS to deliver the highest ethanol concentration, further investigation into the use of pressed WIS (Figures 4.5, 4.6 & 4.8) showed it (pressed WIS) to be a viable option to investigate use WIS from the preferred varieties and pretreated under optimised conditions.

Continuous sparging and ethanol evaporation (stripping)

Generally, fermentations were characterised by a decrease in ethanol production subsequent to the inhibition of yeast performance due to toxicity. The data in Figure 4.3C & E for the pressed WIS and whole slurry, clearly shows that ethanol concentration increased to a maximum but once the ethanol production stopped, the concentration decreased until the end of the experiments. A plausible explanation would be the stripping of ethanol due to the continuous nitrogen sparging. The data from investigation into continuous sparing, shown in Figure 4.7, clearly demonstrated that the stripping of ethanol, as a result of sparing, was noticeable and that nitrogen sparging should rather be avoided. Continuous sparging was initially investigated as a means to ensure anaerobic conditions to enhance fermentation. However, as evident by the ethanol production in Figures 4.5 and 4.6 A & C, the conditions during SSF were sufficiently anaerobic to induce ethanol production, even though oxygen-impermeable tubing was not utilised and oxygen was introduced into the fermentations during sampling and feeding.

A second explanation for the decrease in ethanol concentration could be as a result of bacterial contamination of the culture broth since the cultivations were not performed under aseptic conditions and bacterial contamination was observed on microscopy slides. However, the conditions during cultivation (with regards to oxygen) would not have been favourable for the growth of *Acetobacter* or *Clostridium* bacteria [127].

Yeast strains and inhibitor tolerances

To optimise the ethanol yield on the pretreated material, it is preferable to use yeast strains with the ability to utilise both glucose and xylose as carbon sources to maximum the ethanol yield from the

raw material. Three xylose-utilising yeast strains were investigated during this study and included strains D5A^{+}, D5A^{+H} and TMB3400. With low solid loadings of 10% (Figure 4.2) all three strain produced nearly identical concentrations of ethanol, showing similar levels of tolerance to the inhibitor concentrations. However, higher solid loadings of 20% would be required in order to obtain 40 g/L of ethanol and would thus exhibited higher inhibitor stresses on the yeasts. A further concern was the low ethanol productivity of strain D5A^{+H} which was 60% of that of strains D5A^{+} and TMB3400.

Further investigation of strain D5A^{+H} (Figures 4.3, 4.5A & 4.6A) showed xylose accumulation within the cultures of up to 16 g/L. Fermentation of the hydrolysate (Figure 4.4A) showed that while strain D5A^{+H} was capable of producing ethanol from xylose in the presence of inhibitors, the yields were very low (46%) and it is thus doubtful whether fermenting of the xylose fraction would contribute significantly to the ethanol concentration obtained during SSF. In comparison, Kuyper *et al*. [128] recorded a yield for ethanol on xylose of 82% for a recombinant *S. cerevisiae* strain in a synthetic medium at a temperature of 30 °C whilst Madhavan *et al*. [129] reported an ethanol yield on xylose of 84% in a synthetic medium at a temperature of 35 °C, although both studies were conducted without the presence of inhibitors. Furthermore, the high concentrations of residual glucose, most notable during the cultivations shown in Figure 4.3A, C & E, would supress the uptake of xylose due to the greater glucose affinity of the yeasts [102].

In an attempt to increase the xylose fermenting capabilities of strain D5A^{+H} during SSF, the pre-cultures and pre-conditioning was performed with xylose as carbon source (Figure 4.5A). During the fermentation xylose uptake was not improved noticeably, but a substantial lag phase of 53 hours was induced. Since strain D5A^{+H} had to switch from xylose-metabolism (pre-conditioning) to glucose-metabolism during SSF the possibility of a lag phase does therefore exist but since glucose consumption is inherent to all organisms, one would not expect the lag phase to continue for very long. Impaired growth on glucose by recombinant *S. cerevivisae* strains after an extended selection process on xylose, have also been reported elsewhere [130]. After the lag ended, all the accumulated glucose was consumed within 25 hours.

In comparison, strain MH1000 did not exhibited any lag phase and was able to ferment all the available glucose. At a solid loading of 10% solids (Figure 4.5A & C) strain D5A^{+H} outperformed strain MH1000 in terms of maximum ethanol produced, but at the higher solids loading of 20% (Figure 4.6A & C) strain MH1000 produced the highest ethanol concentration. With pre-culturing and pre-condition performed with glucose as carbon source, strain D5A^{+H} still exhibited a lag phase of 35 hours, as evident by the initial accumulation of glucose although the overall ethanol productivities

were similar for the two strains. The higher ethanol concentration produced by strain MH1000 in the fermentation with 20% solids was in part due to the onset of yeast inhibition and glucose accumulation occurring 25 hours earlier with strain D5A^{+H} (Figure 4.6A & C).

Fed-batch process development

In order to obtain a fed-batch protocol that would deliver the required 40 g/L of ethanol at a high volumetric productivity, two solid feed rates were investigated. The increase in ethanol productivity (Figure 4.8A compared to Figure 4.6C for stain MH1000) did not corresponded with the increase in feed rate. Since the higher feed rate would cause a higher concentration of solids in the reactor (relative to the lower feed rate) it was postulated that this would in turn lead to a lower yield and consequently the lower ethanol productivity. The lower maximum ethanol concentration obtained from the higher feed rate of 5% solids per day (Figure 4.8A) was in part due to the onset of yeast inhibition occurring 50 hours sooner than with the slower feed rate of 3% per day (Figure 4.6C) due to the increased rate of inhibitor accumulation. The increase in acetic acid did correspond with the feed rates where the increase in concentration from the slower feed rate was 61% (0.031 g/L.h from Figure 4.6D) of that of the higher feed rate (0.051 g/L.h from Figure 4.8B) – the same relative difference as that of the feeding rates of 3% per day compared to 5% per day. Due to the faster onset of yeast inhibition, the higher feed rate resulted in a substantial increase in residual glucose, which would translate to a notable loss of fermentable carbon from the pretreated material.

The use of strain MH1000 with a slower fed-batch feed rate of 3% solids per day, in 1% and 2% increments, would thus serve as the basis for the SSF process at a temperature of 35 °C and pH 5.0. This process resulted in an ethanol concentration of 34.5 g/L but with a residual glucose concentration of 35.9 g/L from a final solids loading of 20% pressed WIS. The main problem preventing a higher ethanol concentration from being obtained was the onset of yeast inhibition, which was observed to coincide with an acetic acid concentration of roughly 3 g/L. It was understood that the preferred sweet sorghum varieties pretreated at the optimised pretreatment conditions, as were available for the work completed in Chapter 5, should result in WIS with higher glucose content and lower concentrations of inhibitors. It is thus feasible that for the optimised process a solids loading of 20% could deliver 40 g/L of ethanol or that a lower solids loading could be utilised, while still resulting in 40 g/L of ethanol.

4.5 Conclusions

One of the proposed outcomes of the fermentations was to eliminate the process option(s) that would not deliver suitable ethanol concentrations. From the data in Figure 4.3F it can be seen that the whole slurry resulted in the lowest ethanol concentration and the highest concentrations of HMF and furfural, while the acetic acid concentrations was also higher then when the pressed WIS was used. Washed WIS (Figure 4.3A & B) produced the highest ethanol concentration but also resulted in substantially higher levels of acetic acid (13% higher than whole slurry and 48% higher than pressed WIS). Furthermore, from an economic perspective it is preferable to avoid the use of washed WIS and eliminate the additional processing costs associated with this option. Pressed WIS thus presents an ideal opportunity for further optimisation since it contains lower concentrations of inhibitors, without incurring additional processing expenses.

Yeast inhibition was encountered at solid loadings of 20% pressed WIS. The evidence suggests that the inhibition is related to the concentration of inhibitors during the cultivations. Inhibition was not observed when washed WIS was utilised. Using pressed WIS or the whole slurry the inhibition coincided with an acetic acid concentration of between 3.0 and 3.5 g/L (Figures 4.6B & D & 4.8B). While the exact cause of the inhibition could not be identified, it was concluded that the concentration of acetic acid could be used to predict the onset of yeast inhibition in fermentation using either pressed WIS or whole slurry.

The conditions during SSF and especially at higher solid loadings appear to be very harsh and would require careful control of feed strategies to ensure feasibility. Slower feed rates slow the increase in inhibitors which in turn promotes the longevity of the yeast, leading to a higher maximum ethanol concentration, but at a reduced productivity.

Summary of the project thus far

The performance of four strains of *Saccharomyces cerevisiae* were investigated under SSF conditions and it was found that the industrial strain MH1000 delivered the highest ethanol concentration and displayed the greatest inherent resistance to inhibitor concentrations. It was also seen that a slower solid feed rate of 3% solids per day (compared to 5% solids per day) resulted in a higher ethanol concentration from a cumulative solid loading of 20%, but a significant level of glucose accumulation was also observed due to inhibition of the yeast by inhibitor concentrations reaching critical levels. Whilst the use of washed WIS did reduce the concentrations of the inhibitors HMF and furfural, it also resulted in the highest concentration of acetic acid.

The basic fed-batch SSF process thus consisted of yeast strain MH1000 with a final solids loading of 20%, using pressed WIS, and fed at a slower feed rate of 3% solids per day, in 1% and 2% increments, at a temperature of 35 °C and pH 5.0. This process resulted in an ethanol concentration of 34.5 g/L but with a residual glucose concentration of 35.9 g/L from a final solids loading of 20% pressed WIS.

Further optimisation of the SSF process was thus required in order to obtain 40 g/L of ethanol without the loss of fermentable sugars due to glucose accumulation. It was understood that the preferred sweet sorghum varieties pretreated at the optimised pretreatment conditions should result in WIS with higher glucose content and lower concentrations of inhibitors. The optimised enzyme cocktails should also result in an improved hydrolysis. It was thus feasible that for the optimised process a solids loading of less than 20% could deliver the required 40 g/L of ethanol, which was the target set for subsequent process optimisation, as reported in Chapter 5.

5. Optimisation of the SSF process

Abstract

Sweet sorghum bagasse is a potential substrate for lignocellulosic ethanol production, possibly in combination with ethanol production from the sweet juice extracted from the stems. The aim of this study was to obtain 40 g/L of ethanol in the fermentation broth using pressed WIS from steam pretreatment, without washing of the WIS prior to SSF. Previous optimisation work provided the conditions for steam pretreatment of the bagasse and two enzyme cocktails of Cellic CTec2 and HTec2 to be utilised. In fed-batch SSF with a cumulative solids loading of 20%, an 18-fold increase in the concentration of Cellic HTec2 enzymes (Cocktail 1) did not significantly improve the maximum ethanol concentration or productivity compared to Cocktail 2. Further investigation into different cumulative solid loadings using Cocktail 2 showed yeast inhibition and glucose accumulation to occur with cumulative solid loadings of 20% and 16%, due to inhibitor concentrations reaching critical levels. With a cumulative solid loading of 13%, yeast inhibition was avoided and a final ethanol concentration of 39 g/L was obtained at an ethanol yield of 82% of the theoretical maximum. Fed-batch SSF with Cocktail 2 at cumulative solid loadings of 13% showed no significant difference in the ethanol concentrations and productivity obtained from material impregnated with water or 3% SO_2 as catalyst during steam pretreatment. Similarly, an investigation of two selected sweet sorghum varieties, SS27 and AP6, under SSF conditions also exhibited no significant differences.

Keywords:

Sweet sorghum bagasse, Simultaneous saccharification and fermentation, Fed-batch, Ethanol, Yeast inhibition

5.1 Introduction

The purpose of the present study was to optimise an SSF process to obtain 40 g/L of ethanol from steam pretreated sweet sorghum bagasse without the inclusion of a washing step prior to SSF. For this purpose, two different enzyme cocktails were evaluated during SSF with a solids loading of 20% (w/w). Both cocktails contained 0.15 ml Cellic CTec2/g dry WIS and were supplemented with 0.32 ml Cellic HTec2/g dry WIS (Cocktail 1) or 0.017 ml Cellic HTec2/g dry WIS (Cocktail 2), respectively. To reduce the effect of inhibitors during SSF to increase the ethanol yield, lower cumulative solid loadings of 16% (w/w) and 13% (w/w) were also investigated. Two pretreatment options of using either water or SO_2 as catalyst and two selected sweet sorghum varieties, SS27 and AP6, were also compared to determine the process configuration that would result in the highest ethanol yield.

5.2 Materials and Methods

Raw material

The sweet sorghum bagasse was obtained from the same source and processed using the same procedures and machinery as discussed under 'Raw material' in Chapter 4. Two varieties of sweet sorghum were utilised during this part of the study, namely SS27 and AP6. These varieties were identified by McIntosh [24] to deliver the highest combined sugar yield from enzymatic hydrolysis subsequent to steam pretreatment.

Steam-pretreatment and enzyme cocktails

Six hundred grams of sweet sorghum bagasse samples (moisture content of 7.3%) was impregnated with either water or a combination of water and SO_2 as catalysts (Table 5.2). For water impregnation, the material was soaked overnight in 12 L of reverse-osmosis (RO) water followed by spin drying for 3 minutes to a moisture content of 70% (w/w). For SO_2-impregnation, water-soaked samples after spin-drying were loaded into plastic bags to which 3% (w/w) high purity (99.9%) SO_2-gas was added, determined gravimetrically, and sealed. After a 45 minute exposure time, the bags were vented in a fume hood, allowing excess SO_2 to dissipate. Pretreatment was performed in a steam pretreatment unit as described by McIntosh [24]. The pretreatment conditions in terms of time and temperature are summarised in Table 5.2.

A portion of the hydrolysate (roughly 33%, determined from dry weights before and after pressing) was removed from the slurry after pretreatment using a dead-end press (50 ton hydraulic shop press, Eurotool, South Africa) at a pressure of 15 tons (7.4 MPa) to final moisture content of between 45% and 55% (w/w). The latter "pressed" solids were used as feedstock for SSF runs. The hydrolysate was stored in glass bottles at 4 °C and used for conditioning the yeast inoculums used in fermentation experiments. The composition of the two preferred varieties of sweet sorghum prior to steam pretreatment has previously been determined by McIntosh [24] is and reported in Table 5.1.

The pressed solids from multiple pretreatment runs, at each of the conditions shown in Table 5.1, were combined and divided in aliquots in plastic bags for sterilisation by irradiation. The mass of each aliquot was pre-determined based on the mass of material required for one complete fed-batch run. Aliquots were sterilised by irradiation using a cobalt-60 radiation source (HEPRO, Cape Town, South Africa) at a dosage of 5 kGy (kilogray) and subsequently stored at 4 °C. To prevent heat damage to the material, this radiation dosage was selected as the minimum effective dosage to completely sterilise the material. Subjecting the material to dosages of up to 60 kGy confirmed that radiation exerted no significant effect on the digestibility of the material (data not shown).

Table 5.1: Chemical composition of the two preferred sweet sorghum, varieties prior to steam pretreatment, as determined by McIntosh [24].

Component (per 100g oven dry material)	Variety SS27	Variety AP6
Glucose	41.1 ± 0.10	40.7 ± 0.49
Xylose	21.5 ± 0.09	20.6 ± 1.10
Arabinose	1.34 ± 0.19	1.59 ± 0.06
Lignin	18.4 ± 0.43	17.8 ± 0.04
Ash	1.91 ± 0.03	1.67 ± 0.09
Extractives	7.13 ± 0.18	7.4 ± 0.12

Two enzyme cocktails were used where Cocktails 1 and 2 both contained 0.15 ml Cellic CTec2 /g dry WIS (Novozymes, Bagsvaerd, Denmark), but were supplemented with 0.32 ml and 0.017 ml Cellic HTec2/g dry WIS (Novozymes), respectively. Cocktail 1 was optimised by Pengilly [25], while Cocktail 2 was supplemented with a lower dosage of Cellic HTec2 to be volumetrically equivalent to a Spezyme dosage of 10 FPU/g dry WIS. The enzyme preparations of Cellic CTec2 and Cellic HTec2 were specifically designed for processes with high substrate loadings [87].

Yeast strain and culture media

Only *Saccharomyces cerevisiae* strain MH1000 was selected for this work based on performance during the process development work (Chapter 4). This strain was preserved as 1 ml freezer stocks at -80 °C using 30% glycerol as cryoprotectant. All other medium and cultivation procedures were similar to those utilised during the work in Chapter 4.

Table 5.2: Summary of sweet sorghum varieties, pretreatment conditions, enzyme cocktails and solid loadings used during fed-batch SSF cultures of *S. cerevisiae* at 35 °C and pH 5.0. Feeding of the WIS was performed twice daily in 1% and 2% increments.

Variety	**SS27**	**SS27**	**AP6**
Pretreatment catalyst	Water-soaked	SO_2-impregnated	Water-soaked
Pretreatment Conditions [24] (Temp, time)	205 °C, 5 min	185 °C, 8 min	205 °C, 5 min
Enzymes and solids loading in SSF			
[a] **Cocktail 1**	20% solids		
[b] **Cocktail 2**	20% solids		
Cocktail 2	16% solids		
Cocktail 2	13% solids	13% solids	13% solids

[a] Cocktail 1: 0.15 ml Cellic® CTec2 /g dry WIS; 0.32 ml Cellic® HTec2/g dry WIS

[b] Cocktail 2: 0.15 ml Cellic® CTec2 /g dry WIS; 0.017 ml Cellic® HTec2/g dry WIS

Fermentations

All fermentations were performed according to the procedure discussed under 'Fermentations' in Chapter 4. A solids feed rate of 3% solids per day in 1% and 2% increments, as identified to be the preferred feed rate in Chapter 4, was used. Only additional changes were the exclusion of the continuous nitrogen sparging and all fermentations were performed in duplicate. A summary of the fermentations performed is shown in Table 5.2.

All fermentations were preceded by a 2 hour pre-saccharification step, performed at 35 °C. A comparison between the sugar and acetic acid concentrations following pre-saccharification at 35 °C and 50 °C with Cocktails 1 and 2 are shown in Table 5.5. These investigations were performed in

triplicate 250 ml Erleynmeyer shake flasks with identical loadings and enzyme cocktails as those used for the fermentations shown in Figure 5.1, but scale-down by a factor of 10 for final weights of only 200 g. Only sweet sorghum variety SS27, pretreated after water-soaking was utilised. The shake flask cultures were incubated in a shaker water-bath at 90 rpm and temperatures of either 35 °C or 50 °C.

Analyses

Analyses were performed according to the same procedures as discussed in Chapter 4.

Calculations

The maximum volumetric ethanol productivity (g/L.h) was calculated from a linear regression of the ethanol concentration curve plotted as a function of time where a constant increase in the ethanol concentration was evident with a minimum of five data points used. This represents the combined hydrolysis and fermentation rates with low residual glucose concentrations. The overall ethanol productivities were calculated from the maximum ethanol concentration and the time taken to achieve this concentration. The ethanol yields were calculated based on the ethanol concentrations and liquid volumes at the end of the cultivations and expressed as a percentage of the theoretical maximum based on the glucose added to the fermentation broth, using a stoichiometric conversion factor of 0.51 gram ethanol produced per gram glucose consumed. Data was analysed on Microsoft Excel 2010 by means of a one-way analysis of variance (ANOVA) and a probability value (*P*) of less than 0.05 was considered as statistically significant.

5.3 Results

5.3.1 Chemical composition after pretreatment using water and SO_2 impregnation

The chemical composition of the water insoluble solids (WIS) and the hydrolysate fractions following steam pretreatment of the samples from the two sorghum bagasse varieties at the preferred conditions selected from previous reports [24], are shown in Table 5.3. The water-soaked material was pretreated at 205 °C for 5 min and the material impregnated with SO_2 at 185 °C for 8 min; conditions that were identified by McIntosh [24] to give the greatest combined sugar yields. Combined sugar is defined as the total of glucose and xylose release from both pretreatment itself and subsequent enzymatic hydrolysis. An acid hydrolysis was performed on the hydrolysate fraction from pretreatment, to provide the total monomeric concentrations of glucose and xylose, and these concentrations are shown in parenthesis.

There was no significant difference between the glucose and xylose content of the WIS from pretreatment with water or SO_2-impregnation of bagasse from variety SS27. The WIS from varieties SS27 and AP6, pretreated after water-soaking, also showed no statistically significant difference in terms of glucose or xylose content (Table 5.3). Conversely, impregnation with SO_2 resulted in a significantly higher concentration of monomeric glucose and xylose in the hydrolysate from pretreatment. For pretreatment with water only using sweet sorghum variety SS27 or AP6, 20% of the glucose and xylose in the hydrolysate was in monomeric form, compared to nearly 80% when using SO_2 as catalyst. Although glucose concentrations in the hydrolysates were low, the hydrolysate after pretreatment with SO_2-impregnation contained a higher concentration of glucose (oligomeric and monomeric combined) compared to water-only pretreatment, while the combined monomeric and oligomeric xylose concentrations in the hydrolysates were similar. Concentrations of the inhibitors acetic acid, HMF and furfural in the hydrolysate were all significantly higher with SO_2-impregnation as catalyst in pretreatment, compared to water-only pretreatment. The hydrolysate fraction from variety AP6 did contain a lower concentration of acetic acid compared to variety SS27.

The glucose and xylose content of the WIS (Table 5.3) from varieties SS27 and AP6 following steam pretreatment after water-soaking were lower than those reported by McIntosh [24]. The glucose content was on average 16 g/100 g lower and the xylose 4.5 g/100g lower. The chemical composition of the WIS after steam pretreatment with SO_2 as catalyst was not provided by McIntosh [24], but the glucose content of the WIS was in-line with that reported by Sipos et al. [52] using 2%

SO_2 and pretreatment conditions of 190 °C for 5 or 10 minutes, but the xylose content was on average 12 g/100 g lower.

Table 5.3: Composition of the WIS and hydrolysate fractions of sweet sorghum bagasse samples after steam pretreated using either water or SO_2 as catalysts at the optimal conditions shown in Table 5.2. Standard deviations were calculated from duplicate samples and the concentrations of monomeric glucose and xylose in the hydrolysate shown in parenthesis.

	SS27		**SS27**		**AP6**	
Catalyst	Water		3% SO_2		Water	
Fraction	WIS (g/100g)	Hydrolysate (g/L)	WIS (g/100g)	Hydrolysate (g/L)	WIS (g/100g)	Hydrolysate (g/L)
Glucose	53.5 ± 1.11	5.93 ± 0.01 (0.58)	52.2 ± 0.08	7.1 ± 0.14 (4.85)	53.1 ± 0.28	3.12 ± 0.59 (0.17)
Xylose	2.96 ± 0.01	29.9 ± 0.04 (7.05)	2.56 ± 0.07	31.4 ± 0.52 (25.5)	2.74 ± 0.07	28.4 ± 0.52 (5.34)
Arabinose	0.25 ± 0.00	1.5 ± 0.01	0.34 ± 0.00	2.52 ± 0.11	0.24 ± 0.00	1.12 ± 0.01
Cellobiose	[a] N.D.	0.27 ± 0.01	N.D.	0.18 ± 0.00	[a] N.D.	0.00 ± 0.01
Acetic acid		5.48 ± 0.12		6.54 ± 0.35		4.83 ± 0.01
Formic acid		0.53 ± 0.03		0.24 ± 0.01		0.56 ± 0.00
HMF		0.13 ± 0.01		0.38 ± 0.00		0.08 ± 0.00
Furfural		0.58 ± 0.03		1.45 ± 0.00		0.47 ± 0.01

[a] N.D. = none detected

The data in Table 5.4 shows the contribution of the WIS and hydrolysate fractions for the different varieties and pretreatment conditions to the total glucose and xylose concentrations in the fermentation broth. Due to differences in the varieties and the pretreatment methods, the moisture content of the material after pressing varied between 52 and 58 %. In general the contribution of the hydrolysate fraction to the sugar concentrations in the fermentation broth was less than 2 g/L, except in the case of the SO_2-catalyst steam pretreatment, due to the higher concentration of monomeric sugars.

Table 5.4: Comparison of the glucose and xylose concentrations in the final fermentation liquid, as obtained from the WIS and liquid (hydrolysate) fractions respectively.

	Cumulative solid loading					
	20%		16%		13%	
	From WIS	From liquid	From WIS	From liquid	From WIS	From liquid
SS27 – water soaked	133.8 g/L glucose	0.15 g/L glucose	107 g/L glucose	0.12 g/L glucose	86.9 g/L glucose	0.09 g/L glucose
	7.4 g/L xylose	1.77 g/L xylose	5.92 g/L xylose	1.42 g/L xylose	4.81 g/L xylose	1.15 g/L xylose
SS27 – SO_2 impregnated					84.8 g/L glucose	1.08 g/L glucose
					4.16 g/L xylose	5.69 g/L xylose
AP6 – water soaked					86.3 g/L glucose	0.03 g/L glucose
					4.45 g/L xylose	1.03 g/L xylose

5.3.2 Influence of enzyme cocktail on fermentation performance

Two enzyme cocktails (Cocktails 1 and 2, Table 5.2) containing different concentrations of Cellic HTec2 together with CTec2 were investigated under SSF conditions for selecting the cocktail that delivered the best trade-off between ethanol yield and enzyme volume to be used in subsequent fermentations. Comparative fed-batch profiles for the two enzyme cocktails with final solid loadings of 20% with solid feed rates of 3% per day (in 1% and 2% increments) are shown in Figure 5.1. Due to the similarity of the material composition of the two pretreatment options (Table 5.3), these experiments were conducted using only water-soaked material.

The higher Cellic HTec2 content of Cocktail 1 compared to Cocktail 2 (Table 5.2) resulted in a 2-fold higher initial concentration of glucose and resulted in an ethanol concentration after 6 hours that was 14 g/L higher than when Cocktail 2 was used (Figure 5.1A). However, the rate of ethanol production when using either cocktail was markedly similar, as well as the final ethanol concentration and ethanol yield. Respective process ethanol productivities for Cocktails 1 and 2,

calculated from the slope of the ethanol production curve, were 0.355 and 0.361 g/L.h, with the difference not statistically significant. The overall ethanol productivities for cultivations with both cocktails were calculated as 0.660 g/L.h and 0.472 g/L.h after 69 and 92 hours for Cocktail 1 and 2 respectively. Whilst the peak ethanol concentration in the presence of Cocktail 1 (45.8 g/L) was 5% greater than when Cocktail 2 (43.6 g/L) was used, final ethanol concentrations at the end of the 190 h cultivation period differed by less than 2% (Figure 5.1A). The final ethanol concentrations in the broth corresponded to 44% and 50% of the maximum theoretical yield for Cocktails 1 and 2 respectively.

During cultivations with both Cocktails 1 and 2 the concentrations of residual glucose in the fermentation broth was close to zero. However, after 69 and 92 hours with Cocktails 1 and 2 respectively, the concentration of residual glucose in the cultivations was seen to increase (Figure 5.1A). This accumulation of glucose coincided with a decrease in the ethanol concentrations, indicating inhibition of the yeast metabolism. From the onset of glucose accumulation, ethanol production continued for a further 16 hours before production ceased, resulting in a distinct decrease in the ethanol concentration and the low ethanol yields observed (Figure 5.1A). Whilst the onset of glucose accumulation coincided with an acetic acid concentration of 2.6 g/L, irrespective of the enzyme cocktail used, ethanol production only ceased once acetic acid reached a concentration of between 3.0 and 3.2 g/L (Figure 5.1B). With both Cocktails 1 and 2, HMF and furfural were assimilated by the yeast and only after yeast inhibition did the concentrations of these furans increase (Figure 5.1C).

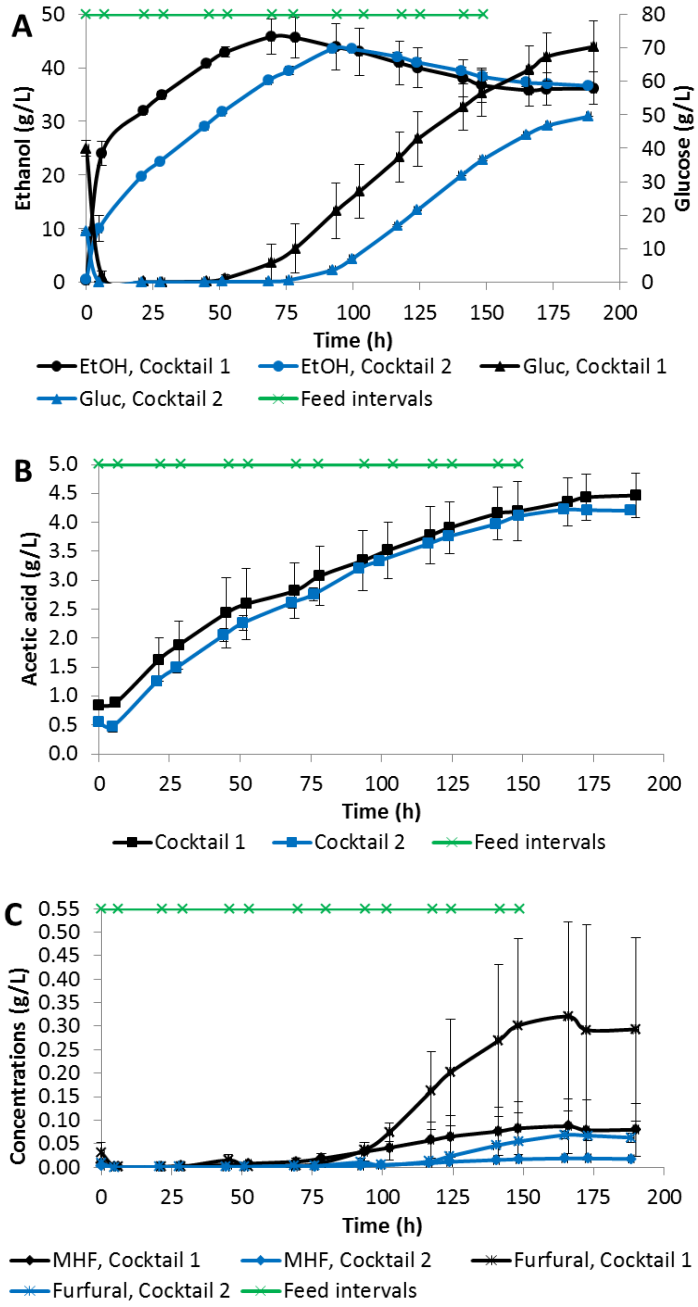

Figure 5.1: Comparison of the two optimised enzyme cocktails in fed-batch SSF cultures at 35 °C and pH 5.0 using the water-soaked, variety SS27 at 20% final solid loadings. Graphs indicate ethanol and glucose (A), acetic acid (B) and by-product (C) concentrations. Feed intervals of 1% and 2% per day are indicated on the graphs and the standard deviations calculated from duplicate fermentations.

5.3.3 Influence of temperature on pre-saccharification with the optimised enzyme cocktails

Enzymatic hydrolysis was investigated with both Cocktails 1 and 2 at 1% solid loadings of variety SS27 (water-soaked) at temperatures of 35 °C and 50 °C to determine the effect of the enzyme cocktail and temperature on sugars and acetic acid released during pre-saccharification (Table 5.5).

For Cocktail 1, with the higher dose of HTec2, the initial concentrations of both glucose and acetic acid were significantly higher compared to when Cocktail 2 was utilised (Table 5.5). With Cocktail 1 a significant increase in the glucose concentration was observed after the 2 hour pre-saccharification period at both 35 °C ($p = 0.042$) and 50 °C ($p = 0.012$). With Cocktail 2 only pre-saccharification at 50 °C resulted in a significant increase in the glucose concentration ($p = 0.026$). After pre-saccharification at 35 °C the glucose content in the broth increased by 9% for Cocktail 1, while an increase of only 5% was observed with Cocktail 2. However, pre-saccharification at 50 °C resulted in increases of 20% and 29% from Cocktails 1 and 2 respectively. Both the xylose and acetic acid concentrations decreased slightly during pre-saccharification at both 35 °C and 50 °C.

Table 5.5: Comparison between the glucose, xylose and acetic acid concentrations following 2 hour pre-saccharification in shake flask cultures at 35 °C and 50 °C with enzymes cocktails 1 and 2 using sweet sorghum variety SS27, pretreated after water-soaking (Table 5.2). Experiments were performed with initial solids loadings of 1%, identical to the fermentations shown in Figure 5.1. Standard deviations were calculated from triplicate samples.

	35 °C				**50 °C**			
	Cocktail 1		**Cocktail 2**		**Cocktail 1**		**Cocktail 2**	
	T0	T1	T0	T1	T0	T1	T0	T1
Glucose	37.5 ± 1.07	40.7 ± 1.07	12.9 ± 1.30	13.6 ± 0.32	37.0 ± 1.57	44.4 ± 1.79	13.5 ± 0.38	17.4 ± 1.56
Xylose	8.28 ± 0.14	7.12 ± 0.04	7.95 ± 0.66	6.74 ± 0.19	8.14 ± 0.22	7.35 ± 0.27	8.18 ± 0.18	7.03 ± 0.11
Acetic acid	0.62 ± 0.01	0.46 ± 0.02	0.30 ± 0.01	0.28 ± 0.00	0.59 ± 0.04	0.49 ± 0.01	0.30 ± 0.01	0.30 ± 0.00

5.3.4 Optimisation of solids loading during fed-batch SSF

In fed-batch cultivations with final cumulative solid loadings of 20% (Figure 5.1A) yeast inhibition was observed. Feed additions after the onset of yeast inhibition did not increase the ethanol concentration and only increased the concentration of residual glucose. By decreasing the cumulative solids loading less carbon would be lost as unfermented sugars in the broth. Fed-batch fermentations with lower final solid loadings of 16% and 13% solids will also result in lower concentrations of inhibitors during the cultivations that could postpone the onset of yeast inhibition. Due to the similarities in the ethanol concentrations and ethanol production rates, only Cocktail 2,

with low levels of HTec2 supplementation, was selected for SSF cultivations at the lower solid loadings.

Solid loadings of 20% and 16% resulted in inhibition of the fermentative performance of the yeast as evident from glucose accumulation, in each case at an acetic acid concentration of approximately 3.0 g/L (Figures 15A & B) while a maximum acetic acid concentration of only 2.6 g/L was analysed for the fermentation with 13% solid loading. However, the lower solids loading resulted in the onset of yeast inhibition occurring 18 hours later with a solids loading of 16%, compared to 20% solids, although without any effect on the rate of ethanol production. Glucose accumulation up to 49.5 g/L was observed for SSF with 20% solids loading, whilst these residual glucose concentrations were reduced to 16.1 g/L and 1.4 g/L with loadings of 16% and 13% solids, respectively. No significant differences were observed between the final ethanol concentrations or process ethanol productivities for the different solid loadings (Figure 5.2A). Compared to the 20% solids loading, which resulted in a peak ethanol concentration of 43.6 g/L, the lower loadings of 16% and 13% solids resulted in respective peak ethanol concentrations that were only 5% (41.4 g/L) and 11% (38.9 g/L) lower (Figure 5.2A). A 35% decrease in the solids loading (13% compared to 20%) therefore had a marginal effect on the ethanol concentration but resulted in a significant increase in the ethanol yield on total glucose, apparently due to decreased inhibitor concentrations. Whereas with 20% solids the final ethanol concentration corresponded to yield of 50% of the theoretical maximum, this yield increased to 71% and 82% of the theoretical maximum using 16% and 13% solids, illustrating more effective substrate utilisation. The ethanol productivities were calculated as 0.361 g/L.h with 20% solids compared to 0.329 g/L.h with 16% solids and 0.309 g/L.h with 13% solids. The overall productivities were calculated as 0.472 g/L.h, 0.350 g/L.h and 0.304 g/L.h for loadings of 20%, 16% and 13% solids after 92, 117 and 126 hours respectively.

Fluctuations in the concentrations of furfural following feed additions were observed for solid loadings of 16% and 13%. Following yeast inhibition and glucose accumulation, accumulation of HMF and furfural were observed in the fermentations with solid loadings of 16% and 20%, but not with 13% (Figure 5.2C).

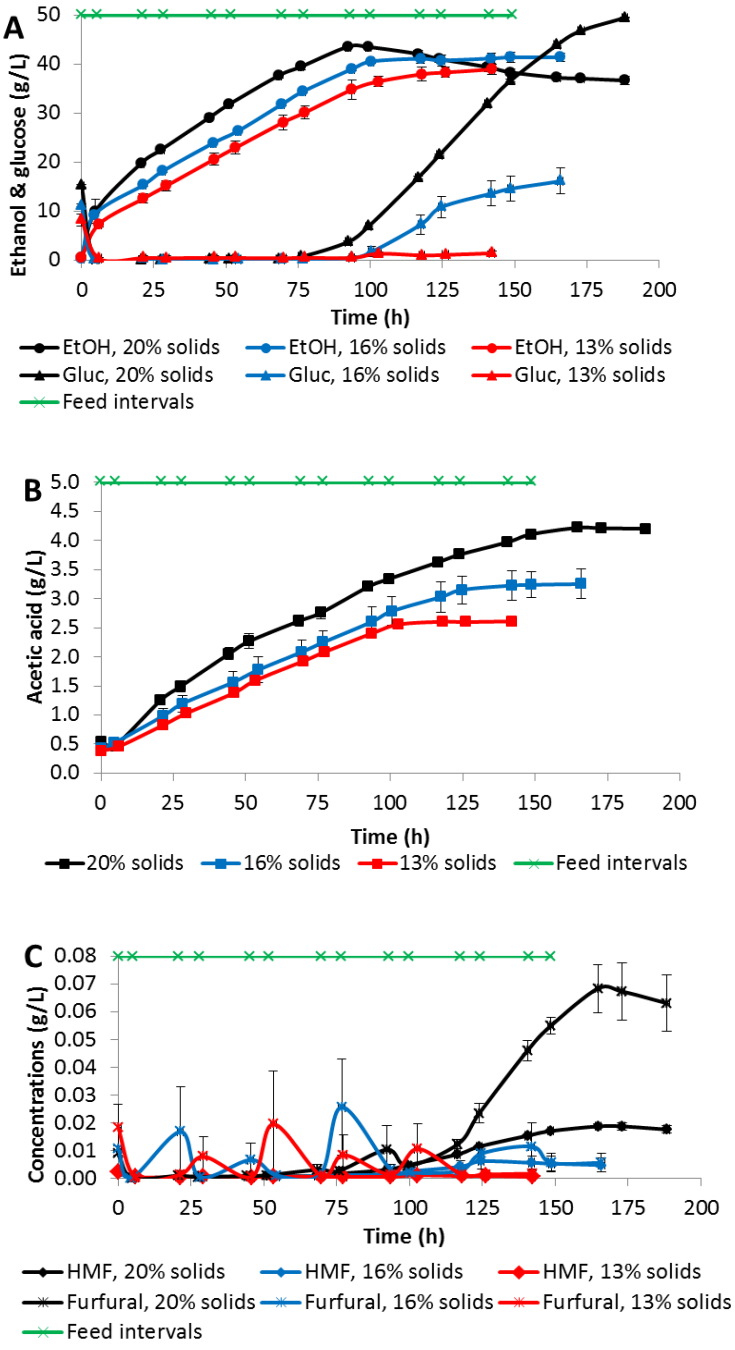

Figure 5.2: Comparisons of the trends for the different final solid loadings of 20%, 16% and 13% investigated in fed-batch SSF cultures at 35 °C and pH 5.0. Water-soaked variety SS27 and enzyme cocktail 2 were utilised. Graphs indicate ethanol and glucose (A), acetic acid (B) and by-product (C) concentrations. Feed intervals of 1% and 2% are indicated on the graphs and the standard deviations calculated from duplicate fermentations.

5.3.5 Effect of the catalyst used during steam explosion pretreatment on SSF performance

The use of a lower solids loading of 13% solids after water-impregnated steam pretreatment resulted in an ethanol concentration of 39 g/L, which was very close to the required ethanol concentration of 40 g/l, with little accumulation of residual glucose (Figure 5.2A), resulting in a high ethanol yield. Due to the similarities in the chemical composition of the WIS (Table 5.3), the effect of the two pretreatment catalysts (water and SO_2) was investigated in fed-batch SSF with 13% solid loadings using enzyme cocktail 2.

There was no statistically significant difference between the water-soaked and SO_2-impregnated material in terms of ethanol productivities or maximum ethanol concentrations obtained. From the trends in Figure 5.3A, an ethanol productivity of 0.309 g/L.h was calculated for the water-soaked material and 0.298 g/L.h for SO_2-impregnated material. Final ethanol concentration of 38.9 g/L from the water-soaked material and 36.8 g/L from the material impregnated with SO_2 equated to yields of 82% with the water-soaked and a yield of 79% with SO_2-impregnation, respectively (Figure 5.3A). The overall ethanol productivities were calculated as 0.304 g/L.h and 0.289 g/L.h for the water-soaked and SO_2-impregnated material after 126 hours. While impregnation with SO_2, compared to water only, significantly increased the concentration of monomeric xylose in the hydrolysate fractions (Tables 5.3 & 5.4) there was no significant difference between the xylose concentrations from the two pretreatment catalysts during SSF with 5.5 g/L for the water-soaked material and 7.2 g/L for the SO_2-impregnated material (data not shown). Fermentation of the pretreated material from both catalysts resulted in low concentrations of residual glucose with 1.4 g/L for the water-soaked pretreatment and 2.8 g/L for the SO_2-impregnated pretreatment (Figure 5.3A). Concentrations of acetic acid were below 2.6 g/L and 2.9 g/L for the water-soaked and SO_2-impregnated material respectively (Figure 5.3B), while concentrations HMF and furfural were below 0.006 g/L and 0.02 g/L during the cultivations (data not shown).

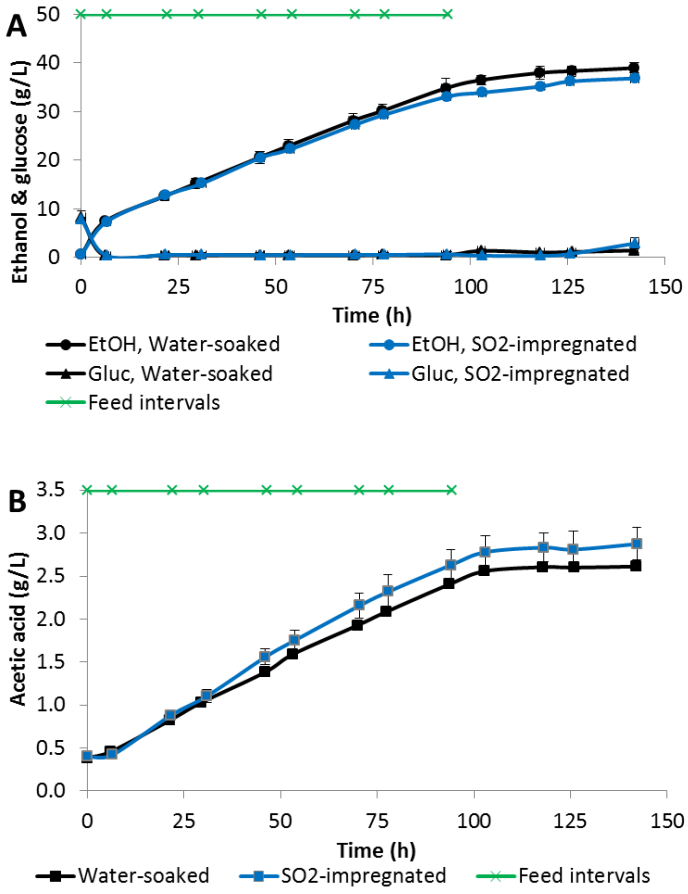

Figure 5.3: Comparison of water-soaked and SO_2-impregnated steam pretreatment material from variety SS27 in SSF fed-batch cultures at 35 °C and pH 5.0 with final solid loadings of 13% using enzyme cocktail 2. Graphs indicate ethanol and glucose (A) and acetic acid (B) concentrations. Feed intervals of 1% and 2% are indicated on the graphs and the standard deviations calculated from duplicate fermentations.

5.3.6 Effect of the sweet sorghum variety utilised in SSF fed-batch culture

The chemical compositions of the WIS from the two selected sweet sorghum varieties following steam pretreatment after water-soaking (Table 5.3) show marked similarities and were thus investigated in fed-batch SSF with cumulative solid loadings of 13% to gauge the similarities in performance. Only enzyme cocktail 2 was utilised during these fermentations. On industrial scale it would be ideal to have access to multiple varieties of sweet sorghum that would deliver acceptable results. This would conserve genetic diversity while guarding against the impact of a variety specific disease to reduce the overall risk involved with investing in the project.

In general, small differences between the performances of the two sweet sorghum varieties in terms of ethanol productivity or ethanol concentration were observed (Figure 5.4A). Variety SS27 did result in slightly higher process productivity of 0.309 g/L.h compared to 0.289 g/L.h for variety AP6 but the difference was not statistically significant. The overall productivities were also similar at 0.304 g/L.h and 0.297 g/L.h after 126 and 118 hours for varieties SS27 and AP6, respectively. Fermentation of variety SS27 resulted in a higher maximum ethanol concentration and ethanol yield of 38.9 g/L at 82% of the theoretical maximum compared to 35.8 g/L and 75% of the theoretical maximum from variety AP6, with the differences not statistically significant (Figure 5.4A). Final concentrations of residual glucose of 1.4 g/L and 0.96 g/L were analysed for the fermentations with varieties SS27 and AP6 respectively (Figure 5.4A) while the concentrations of acetic acid showed no significant differences were below 2.6 g/L (Figure 5.4B).

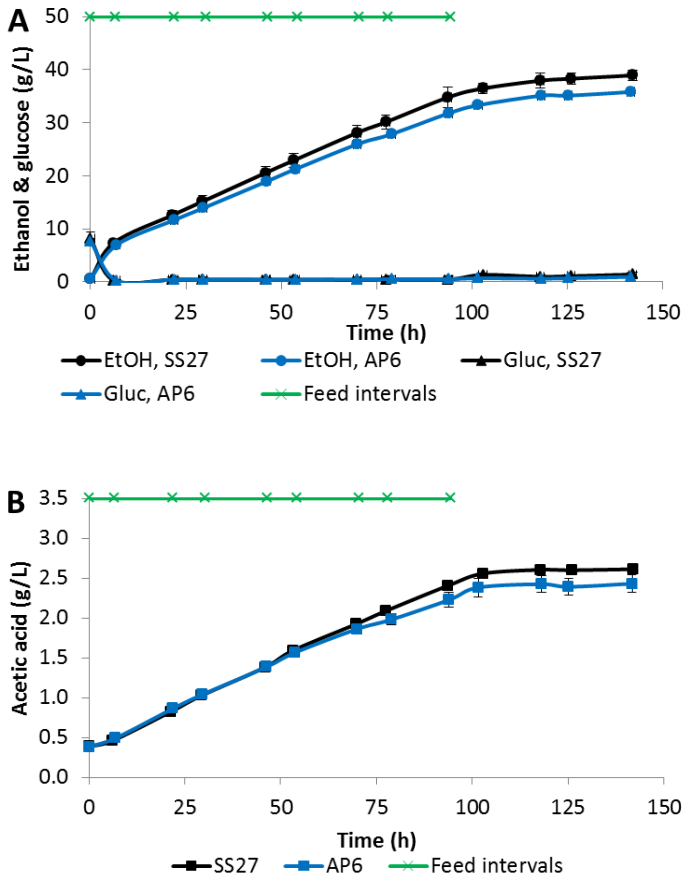

Figure 5.4: Comparison of samples from varieties SS27 and AP6, pretreated after water-soaking, in SSF fed-batch cultures at 35 °C and pH 5.0 with final solid loadings of 13% using enzyme cocktail 2. Graphs indicate ethanol and glucose (A) and acetic acid (B) concentrations. Feed intervals of 1% and 2% are indicated on the graphs and the standard deviations calculated from duplicate fermentations.

5.4 Discussion

Optimisation was performed on an SSF process using steam pretreated sweet sorghum bagasse to obtain a final ethanol concentration of 40 g/L without the inclusion of a washing step prior to SSF. When considering the process economics, one would prefer to minimize the running costs of the process which would entail using the lowest possible enzyme dosage, providing it still delivers acceptable results during SSF. Data from the present study suggest that supplementation of Cellic CTec2 with the high concentration of Cellic HTec2 in Cocktail 1, as opposed to the 18-fold lower dosage in Cocktail 2 did not significantly affect the rate of glucan hydrolysis during SSF, while also resulting in the onset of yeast inhibition and glucose accumulation occurring sooner (Figure 5.1A). Pengilly [25] also found the effect of HTec2 on glucose hydrolysis of steam pretreated sweet sorghum bagasse to be less significant (p-value = 0.0473) than that of CTec2 (p-value = 0.0035). Jung et al. [131] found that supplementation of 0.09 ml CTec2 /g biomass with 0.01 ml Htec2/g biomass did not increase the glucose yield compared to using only 0.1 ml CTec2/g biomass from a solids loading of 2.5% hydrothermally treated sunflower stalks at 50 °C. With higher enzyme loadings it was found that supplementation of 0.5 ml CTec2/g biomass by 0.5 ml HTec2/g biomass increased the glucose yield by 44% compared to only 0.5 ml CTec2/g biomass after 24 hours but after 48 hours the yields were identical. From the results of the pre-saccharification experiments (Table 5.5), the higher initial concentration of glucose in the SSF cultivation with Cocktail 1 compared to Cocktail 2 (Figure 5.1A) could be attributed to the significant concentration of glucose in the enzyme preparation of HTec2. No literature could be found in which the sugar concentrations in the enzyme preparations of Cellic CTec2 and HTec2 were reported, but Alvira et al. [87] did report that they corrected for the glucose content of these preparations in their enzymatic hydrolysis results. The increased dosage of HTec2 did result in greater degree of xylan-hydrolysis since the xylose concentration by the end of the fermentation in the presence of Cocktail 1 was 22% higher compared to Cocktail 2, while the initial xylose concentrations with both cocktails were similar (data not shown).

Fed-batch fermentation with cumulative solid loadings of 20% (Figure 5.1A) resulted in the onset of yeast inhibition and glucose accumulation, irrespective of the enzyme cocktail utilised. However, it was observed that for Cocktail 1 with the higher dosage of HTec2, yeast inhibition occurred 23 hours sooner compared to when Cocktail 2 was utilised. Data from the pre-saccharification experiment (Table 5.5) also indicated that Cocktail 1 resulted in an increased rate of glucan hydrolysis compared to Cocktail 2 at a temperature of 35 °C which would result in greater quantities of inhibitors to be released from the hydrolysed fibres [87]. Furthermore, the data in Table 5.5 also suggests that the higher dosage of HTec2 will lead to higher concentrations of acetic acid in the fermentation broth

which is also evident from the acetic acid trends in Figure 5.1B. In Figures 4.6B & D and 4.8B, yeast inhibition and glucose accumulation was seen to coincide with acetic acid concentrations of between 3.0 and 3.4 g/L, which were slightly higher than the inhibitory concentration of between 2.8 and 3.2 g/L observed in the data from Figure 5.1B.

By decreasing the solids loading to 13% the concentrations of inhibitors was reduced and an ethanol concentration of 39 g/L was obtained with minimal residual glucose of 1.4 g/L, indicating a higher degree of material utilisation (Figure 5.2A). While this ethanol concentration was obtained from fermentation of only the glucose fraction, the final ethanol concentration could be increased to as high as 41.3 g/L if the yeast strain exhibited the ability to ferment xylose with a conversion of 84% as reported at a temperature of 35 °C [129]. The ethanol yield on the pretreated material is limited by the onset of yeast inhibition at higher solid loadings and consequently it is not viable to investigate higher loadings with the current process configuration and yeast strain employed. However, the ethanol yield of 82% from the cumulative solids loading of 13% agrees well with data that has been reported for SSF with high solid loadings (see also data summarise in Table 2.7). Microwave pretreatment has successfully been utilised with sweet sorghum bagasse and resulted in an ethanol concentration of 48 g/L from a solids loading of 18% (w/v) and a yield of 71% of the theoretical maximum [132]. Steam pretreatment (220 °C, 7 min) has also been utilised for forage sorghum to obtain an ethanol concentration of 43 g/L from a solids loading of 16% (w/w) and a yield of 75%, but a washing step was included prior to SSF [37]. Due to similarities in the ethanol productivities and final ethanol concentrations obtained during SSF from the two catalysts employed during steam pretreatment (Figure 5.3A) and the two preferred varieties of sweet sorghum (Figure 5.4A), neither of these changes would allow for utilisation of a solids loading higher than 13% without the occurrence of yeast inhibition.

An alternative solution regarding yeast inhibition would be the utilisation of a more robust yeast strain which had been hardened against high concentrations of inhibitors. Such a strategy has been successfully employed to obtain strain with higher inhibitor tolerance and, in some cases, increased xylose utilisation [94,133–135]. If such a hardened strain was able to ferment the residual glucose in the current fermentations with the higher solid loadings, maximum final ethanol concentrations of 61.9 g/L and 49.6 g/L could be obtained from the fermentations with 20% and 16% solids respectively.

From the final ethanol concentrations for the water-soaked and SO_2-impregnated steam pretreatments for variety SS27, the possible ethanol yields were calculated as 184 L ethanol/ton bagasse and 181 L ethanol/ton bagasse for the water-soaked and SO_2-impregnated material

respectively, which were obtain only from the glucose in the fermentations. These values agree well with the maximum theoretical ethanol yields of 235 L/ton bagasse and 238 L/ton bagasse calculated from the data of McIntosh [24], based only on the glucose yields from enzymatic hydrolysis of the pretreated material from variety SS27. From the data of Pengilly [25], a theoretical ethanol yield of 213 L/ton bagasse was calculated based on the glucose yield from enzymatic hydrolysis but a different variety, MSJH16 pretreated dry at 200 °C for 5 minutes, was utilised in this study.

The use of SO_2-impregnation prior to pretreatment resulted in a significantly higher concentration of monomeric sugars (Table 5.3), compared to water-soaking, which would be advantages for whole slurry SSF with a sufficiently robust yeast strain with xylose fermenting capabilities, since no additional enzymes are required. However, the process costs of the SO_2 together with the associated materials of construction and safety concerns would also have to be considered.

5.5 Conclusions

When unwashed WIS (pressed WIS or whole slurry) is utilised during SSF, the cumulative solid loadings should be controlled to prevent the onset of yeast inhibition and glucose accumulation due to increases in the concentrations of inhibitors. In this study an acetic acid concentration of between 2.8 g/L and 3.4 g/L was used as indicator of possible onset of yeast inhibition. The data suggests that solid loadings higher than 13% will result in yeast inhibition and a loss of fermentable carbon.

A lower dosage of Cellic HTec2 promotes the longevity of the yeast due to a slower release of inhibitors during enzymatic hydrolysis which in turn will result in a higher ethanol concentration and yield from the raw material.

Utilisation of a sufficiently robust yeast strain with xylose-fermenting capabilities together with whole slurry fermentation would constitute simultaneous saccharification and co-fermentation (SSCF) which would increase the ethanol yield from the raw material.

6. RESEARCH QUESTIONS ANSWERED

From the results and conclusions of chapters 4 and 5, the research questions posed in chapter 3 could be answered and are summarised below:

Main aim:

Develop a generic SSF process for use with steam-pretreated sweet sorghum bagasse at solid loadings higher than 10%.

Already stated in Section 4.5, fed-batch SSF with a solid feed rate of 3% solids per day, in 1% and 2% increments, allowed for a final cumulative solids loading of 20% to be achieved. *Saccharomyces cerevisiae* strain MH1000 delivered the best results in this process configuration with a maximum ethanol concentration of 34.5 g/L from a non-preferred sweet sorghum variety that was pretreated dry at 200 °C for 5 minutes. Substantial accumulation of glucose up to 35.9 g/L was seen with this process configuration prior to optimisation.

Intermediate questions:

What process option, with regards to the pretreated material, should be selected for the final process – whole slurry, pressed material or washed material?

Washed WIS at a final solid loading of 20% resulted in a maximum ethanol concentration of 7.8 g/L and an acetic acid concentration of 8.1 g/L. Pressed WIS and whole slurry resulted in lower ethanol concentration of 3.6 g/L and 2.7 g/L respectively, but also substantially lower concentrations of acetic acid at 5.5 g/L and 7.2 g/L respectively. The discrepancy in the acetic acid concentrations was most likely due to the contribution of the soluble sugars (Table 4.2), in the case of the pressed WIS and whole slurry, to higher dry matter content compared to the washed WIS. Resultantly, more washed WIS was loaded into the reactor to obtain the same cumulative solid loading. Pressed WIS was selected for further optimisation since it would be more economical than washed WIS and should results superior to that of whole slurry due to the lower inhibitor concentration.

What strain of *Saccharomyces cerevisiae* would deliver the best SSF performance and is there a benefit from using a laboratory strain with xylose-fermenting capabilities?

The performance of the hardened *Saccharomyces cerevisiae* strain D5A^{+H} was comparable to that of TMB3400 and superior to the parental D5A^{+} strain during batch fermentation of the whole slurry at a

solids loading of 10%. Strain D5A^{+H} did however exhibit a noticeable lag phase and resulted in a lower maximum ethanol concentration when compared to strain MH1000 in fed-batch SSF with a cumulative solid loading of 20% pressed WIS. Xylose-fermentation by strain D5A^{+H} was limited with an ethanol yield of 46% of the theoretical maximum during fermentation of hydrolysate and no observable xylose consumption during SSF. Strain MH100 was thus selected as the preferred strain.

How should the feed strategy for use with fed-batch SSF be applied to ensure optimum performance?

At a final cumulative solids loading of 20% in fed-batch SSF, a higher feed rate of 5% solids per day (in 2% and 3% increments) resulted in a higher ethanol productivity compared to a feed rate of 3% solids per day (in 1% and 2% increment), but a lower maximum ethanol concentration. The increased productivity did not correlate to the same increase in feed rate due to a lower rate of hydrolysis as a result of the higher solids concentration in the reactor. Yeast inhibition with glucose accumulation was observed with both feed rates after 13% solids had been fed. The lower feed rate of 3% solids per day would thus promote the longevity of the yeast and result in an increased ethanol concentration.

Main aim:

Optimise the generic process, through utilisation of an integrated approach to achieve 40 g/L of ethanol in the reaction broth.

Pressed WIS from sweet sorghum variety SS27 steam pretreated at 205 °C for 5 minutes after water-soaking resulted in a maximum ethanol concentration of 38.9 g/L from fed-batch SSF with a cumulative solids loading of 13%. The fed-batch protocol of 3% solids per day with yeast strain MH1000 and an enzyme cocktail consisting of 0.15 ml Cellic CTec2/g dry WIS and 0.017 ml Cellic HTec2/g dry WIS was utilised. Sweet sorghum variety AP6 also water-soaked and pretreated at the same conditions and variety SS27 pretreated after impregnation with 3% SO_2 at 185 °C for 8 minute showed no significant difference in the ethanol productivity and maximum ethanol concentrations obtained.

Intermediate questions:

How do the preferred enzyme cocktails that differ in composition affect the performance of the SSF process and can the selection of more than one cocktail be justified for future work?

Two enzyme cocktails were identified, both containing 0.15 ml Cellic CTec2/g dry WIS and either 0.32 ml Cellic HTec2/g dry WIS or 0.017 ml Cellic HTec2/g dry WIS for Cocktail1 and Cocktail 2 respectively. At cumulative solid loadings of 20% pressed WIS from water-soaked variety SS27, Cocktail 1 resulted in a higher maximum ethanol concentration of 45.8 g/L compared to 43.6 g/L from Cocktail 2 with no significant difference in the ethanol productivities. Cocktail 1 also resulted in the onset of yeast inhibition occurring sooner and resulted in a higher residual glucose concentration of 70 g/L compared to 50 g/L with Cocktail 2. Due to the greater volume of enzyme in Cocktail 1 with no significant improvement in performance, it was decided to continue with Cocktail 2.

When utilising the preferred varieties pretreated under optimum conditions, could the solid loadings for SSF be reduced to increase the ethanol yield from the pretreated material while still achieving the required 40 g/L of ethanol?

Maximum ethanol concentrations of 43.6 g/L and 41.1 g/L were obtained from solid loadings of 20% and 16% using pressed WIS from water-soaked variety SS27. A reduced loading of 13% resulted in an ethanol concentration of 38.9 g/L. With loadings of 20% and 16% yeast inhibition followed by significant glucose accumulation was observed. A solid loading of 13% was thus sufficient to achieve the required ethanol concentration at a yield of 82% of the theoretical maximum.

If multiple optimum pretreatment conditions have been identified, how do these performances translate to SSF in terms of ethanol production?

Pretreatment conditions of 205 °C for 5 minutes were identified to be optimum for water-soaked sweet sorghum bagasse and resulted in a maximum ethanol concentration of 38.9 g/L from a solids loading of 13%. Pretreatment with SO_2 as catalyst was performed at 185 °C for 8 minutes and resulted in 36.8 g/L of ethanol from 13% solids. The ethanol yields per ton raw material was similar for the two pretreatment conditions were similar.

If more than one optimum sorghum variety was identified, do the performance of these varieties translate to SSF such that a recommendation for a variety(ies) can be made for use on industrial scale?

Fed-batch fermentation of the pressed WIS from varieties SS27 and AP6, both water-soaked and pretreated at 205 °C for 5minutes, showed no significant difference with respect to maximum ethanol concentration and ethanol productivity.

7. RECOMMENDATIONS

1. The data in this project suggested that the yeast inhibition was caused by inhibitor concentrations reaching critical levels. In order to utilise solid loadings higher than 13% or to utilise the whole pretreatment slurry containing higher concentrations of inhibitors, a sufficiently roust yeast strain should be utilised. In order to maximize ethanol yields from available sugars, the yeast strains should further be engineered to impart the ability to ferment xylose. Co-fermentation of xylose would be preferred since this would not require additional processing time during the fermentation and thus improve the volumetric ethanol productivity.

2. With a solids loading of 13%, only one feed rate of 3% solids per day was investigated. Since yeast inhibition was not observed at this solid loading, higher feed rates should also be investigated to potentially increase the volumetric ethanol productivity of the process. However, as seen from the data in this study, an increase in feed rate might not correlate with an equivalent increase in productivity and could potentially lead to a lower overall yield. An economic assessment would thus be required to select the preferred feed rate.

3. Steam pretreatment was the only method of pretreatment investigated using this study. It would be advantages to investigate the performance of additional pretreatment options such as ammonia fibre explosion (AFEX), dilute acid and perhaps also fractionation methods under SSF conditions to further identify the preferred pretreatment option.

4. In this study the performance of only 2 preferred sweet sorghum cultivars were investigated during SSF. It is recommended that the performances of additional cultivars, both preferred and non-preferred be investigated under SSF conditions to generate a database of cultivars that can be utilised on industrial scale. A wider range of cultivars would allow farmers to select the appropriate cultivars most suited to the climate and environmental conditions of their area.

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