POTABLE ETHANOL PRODUCTION FROM RAW CORN USING SIMULTANEOUS SACCHARIFICATION AND FERMENTATION

by

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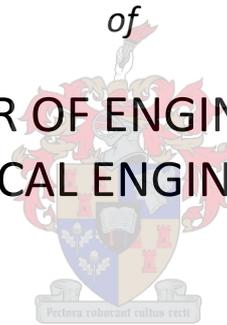
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ABSTRACT

Corn starch is one of the most widely used substrates for the production of potable ethanol, such as Scotch grain whisky or South African single grain whisky. High energy demands in these processes led to extensive research on the development of more cost-effective production methods with lower energy demands and higher corn-to-ethanol efficiency. Therefore, finding and optimising less energy intensive methods are of utmost importance. In this study 30 South African corn cultivars were used as substrate to perform a comprehensive process comparison in 1 L shake flask cultures between cooked starch hydrolysis (CSH) and raw starch hydrolysis (RSH) ethanol production processes, where STARGEN^{TM} 002 was used as a raw starch hydrolysing enzyme (RSHE). Information based on optimisation experiments were used in an Aspen Plus® process simulation to predict the energy requirements and cost per litre ethanol for both the CSH and RSH processes. Furthermore, the RSH process was investigated to establish weather bacterial contamination had a significant impact on process performance.

 Similar final ethanol concentrations and ethanol yields as fraction (%) of theoretical maximum were observed in both methods, with final ethanol concentrations of 9.82% and 9.63% (v/v) for the CSH and RSH processes, respectively. Ethanol productivity for the RSH process was beyond any doubt higher than that of the CSH process, with the highest RSH process productivity of 1.3 g/L.h, which was 20% higher than the highest productivity of the CSH process. The absence of starch gelatinization during the pre-treatment section of the RSH process led to the opportunity for very higher gravity fermentations.

Small-scale optimisation of the RSH process showed a maximum solids loading of 40% during pre-treatment, due to the inability to obtain homogenously mixed slurries. Surface response models with final ethanol concentration, ethanol productivity and ethanol yield as fraction (%) of theoretical maximum as dependent variables, were successfully used to find an optimum solids loading (37.5%) and an enzyme dosage (1.4 g/kg corn) for the RSH process. Scale-up of the preferred RSH process to pilot-scale achieved a final ethanol concentration of 13.12% (v/v) at a productivity of 1.23 g/L.h, with a solids loading not higher than 37.5% and at an enzyme dosage of 1.4 g/kg, indicating that the process may be applicable under industrial conditions.

Aspen Plus® simulations, based on the industrial ethanol production process at the James Sedgwick distillery, together with optimum process parameters for the RSH process, were used to predict and compare the energy requirements for the CSH and RSH processes. The Aspen Plus® simulation

predicted an energy requirement of 1.97 kg steam per litre ethanol produced for the RSH process, while the value of 2.8 kg steam per litre ethanol was predicted for the CSH process. The RSH process was more energy efficient, due to the lower pre-treatment temperatures, when compared to the CSH process. A cost model developed for each process, based on the performance fixtures of the Aspen Plus® simulations, showed that the RSH process had higher enzyme costs, when compared to the CSH process, which was due to high STARGEN[TM] 002 dosage requirements and high STARGEN[TM] 002 price. However, the lower energy requirements and lower water consumption by the RSH process outweighed the drawbacks of STARGEN[TM] 002 dosage and price. The cost models predicted a total cost of R 7.70 per litre ethanol produced for the RSH process, while the CSH process had a predicted value of R 8.97 per litre ethanol.

All the experimental and simulation work show that the STARGEN[TM] 002 is ready to be tested and as a raw RSHE at an industrial ethanol production process, such as the James Sedgwick distillery. It is recommended that the industrial-scale testing should be at solids loading not higher than 37.5% and at an STARGEN[TM] 002 dosage of 1.4 g/kg.

OPSOMMING

Mieliestysel is een van die algemeenste substrate wat gebruik word tydens die produksie van drinkbare etanol soos Skotse graan whisky of Suid-Afrikaanse enkelgraan whisky. Die hoë energie vereistes wat benodig word tydens hierdie prosesse het gelei tot 'n aanvraag vir navorsing om sodoende hierdie proses meer koste-effektief te maak. Dit kan bereik word deur meer energie effektiewe metodes te implementeer, wat ook 'n hoër mielie-na-etanol opbrengs het. Dit is daarom uiters belangrik om energie effektiewe metodes te optimaliseer. In hierdie verslag was 30 Suid-Afrikaanse mieliekultivars as substrate gebruik om 'n omvattende vergelyking tussen verskillende prosesse te tref. 'n Een liter skudfles was gebruik tydens die vergelyking tussen gaar stysel hidrolise (GSH) en rou stysel hidrolise (RSH) met STARGEN™ 002 as die rou-stysel-hidroliserings-ensiem (RSHE). Inligting aangaande die optimalisering van die eksperimente was gebruik in 'n Aspen Plus® proses simulasie om die energie behoeftes en koste per liter etanol, vir die GSH en RSH prosesse, te voorspel.

Soortgelyke finale etanol konsentrasies en etanol opbrengs as funksie (%) van die teoretiese maksimum was in beide GSH en RSH metodes waargeneem. Die etanol konsentrasie vir die GSH en RSH prosesse was onderskeidelik 9.82% en 9.63% (v/v). Die etanol produktiwiteit vir die RSH proses was, sonder twyfel, aansienlik hoër in vergelyking met die GSH proses. Die hoogste produktiwiteit vir die RSH proses was 1.3 g/L.h wat 20% hoër was as die hoogste GSH waarde. Die afwesigheid van stysel gelatinisasie tydens die behandelings aspek van die RSH proses het die geleentheid geskep vir baie hoë gravitasie fermentasie.

Die optimalisering van die RSH proses op klein skaal het aangedui dat 'n maksimum vaste stof hoeveelheid van 40% gedurende die behandeling gebruik moet word, aangesien 'n homogene mengsel nie verkry kan word met 'n hoër persentasie vaste stof nie. Reaksie oppervlak modelle met 'n finale etanol konsentrasie, etanol produktiwiteit en etanol opbrengs as funksie (%) van die teoretiese maksimum was as afhanklike veranderlikes gebruik. Die modelle het die 'n optimale vaste stof hoeveelheid bepaal (37.5%), asook die ensiem hoeveelheid van 1.4 g/kg mielies, vir die RSH proses. Tydens die uitvoering van die RSH proses op 'n 150 L skaal was 'n finale etanol konsentrasie van 13.12% (v/v) teen 'n produktiwiteit van 1.23 h/L.h bereik. Die vaste stof hoeveelheid was egter nie hoër as 37.5% nie en die ensiem hoeveelheid was 1.4 g/kg wat aandui dat die proses wel tydens industriële omstandighede 'n noemenswaardige opsie kan wees.

Aspen Plus® simulasies was gebaseer op die industriële etanol produksie by die James Sedgwick distilleerdery, asook optimale proses parameters was gebruik om die energie vereistes van die GSH en RSH prosesse te voorspel en te vergelyk. Die Aspen Plus® simulasie het 'n energie vereiste van 1.97 kg stoom per liter etanol voorspel tydens die RSH proses, waar die waarde van die GSH proses 2.8 kg stoom per liter etanol was. Die RSH proses was dus meer energie effektief, aangesien laer behandelings temperature gebruik was in vergelyking met die GSH proses. 'n Koste model wat saamgestel was vir elke proses, na aanleiding van die Aspen Plus® simulasie, het aangedui dat die RSH proses 'n hoër ensiem koste het in vergelyking met die GSH proses. Dit was as gevolg van die hoë STARGEN^{TM} 002 hoeveelhede en koste. Die laer energie vereiste en laer water verbruik van die RSH proses dui egter aan dat die voordele van die RSH proses steeds die nadele van STARGEN^{TM} 002 oortref. Die koste model voorspel 'n totale koste van R 7.70 per liter etanol wat geproduseer word, terwyl die GSH proses 'n voorspelde waarde van R 8.97 per liter etanol het.

Hierdie simulasie en eksperimentele resultate dui aan dat STARGEN^{TM} 002 gereed is om getoets te word as 'n RSHE tydens 'n industriële etanol produserings proses, soos by die James Sedgwick distilleerdery. Dit word aanbeveel dat die vaste stof hoeveelheid op industriële skaal nie 37.5% oorskry nie en dat 'n STARGEN^{TM} 002 hoeveelheid van 1.4 g/kg gebruik moet word.

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NOMENCLATURE

ANOVA   -   Analysis of variance

KPI   -   Key Performance Indicator

HT   -   High-Temperature

LT   -   Low-Temperature

SSF   -   Simultaneous Saccharification and Fermentation

CSH   -   Cook Starch Hydrolysis

CSHE   -   Cooked Starch Hydrolysing Enzyme

RSH   -   Raw Starch Hydrolysis

RSHE   -   Raw Starch Hydrolysing Enzyme

TS   -   Total Starch

RS   -   Resistant Starch

PFD   -   Process Flow Diagram

VHG   -   Very High Gravity

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1. **INTRODUCTION**

1.1. **Background**

Starches are the most abundant form of storage polysaccharides in plants and are commonly used as sources of fermentable sugars in the food and beverage industries, as well as for the production of bioethanol (Robertson et al., 2006). The most commonly used agricultural sources of starch include corn, wheat and sorghum, which have starch contents ranging between 60 to 75% on a dry basis (Nigam & Singh, 1995). For potable ethanol specifically, such as Scotch grain whisky or South African single grain whisky, corn is one of the preferred cereals used as a substrate, due to its high starch content (Jacques et al., 2003). The production methods of these whiskies - and for potable ethanol in general - are governed by the required flavour profiles and legal process constraints. These constraints prohibit or regulate the use of urea as yeast nutrition and antibiotics for bacterial contamination regulation in the production methods (Jacques et al., 2003; Olmstead, 2012). Therefore, these characteristics need to be considered during the selection and optimisation of such an ethanol production process.

Saccharomyces cerevisiae is the most popular microorganism used globally for potable ethanol production, due to its outstanding capacity to produce ethanol with high productivity and ethanol yield as fraction (%) of theoretical maximum. Furthermore, *S. cerevisiae* can tolerate low pH, high sugar and ethanol concentrations, all of which are conditions for low contamination risk and high process productivity, while also being fairly resistant to inhibitors present in biomass hydrolysates (Nevoigt, 2008).

Conventional ethanol production from starch is done through a dry-grind process utilising a high-temperature (HT) pre-treatment step in the presence of a thermal stable endo-activity α-amylase, followed by simultaneous saccharification and fermentation (SSF) in the presence of an exo-activity glucoamylase and yeast. During HT pre-treatment the corn starch is gelatinized (cooked), which fully hydrate the starch granules and allow the α-amylase to partially hydrolyse the long chain polysaccharides into short chain oligosaccharides. Subsequently, during SSF the glucoamylase converts oligosaccharides to glucose and maltose, while the yeast simultaneously produces ethanol from these fermentable sugars (Robertson et al., 2006; Kwiatkowski et al., 2006). This conventional ethanol production process, defined the cooked starch hydrolysis (CSH) process, is one of the most widely used methods for the production of potable and fuel ethanol form corn starch. The CSH process is also currently implemented by the James Sedgwick distillery in Wellington, South Africa,

which produces high quality single grain whisky. However, the CSH process remains a costly process due to high energy inputs, which is intensified by process temperatures above corn starch gelatinization during HT pre-treatment. Additionally, the HT pre-treatment restricts the CSH process to low gravity fermentations (≤ 30% solids during liquefaction), due to viscosity limitations of the pre-treated slurry, which is a direct cause of corn starch gelatinization (Li et al., 2012; Cinelli et al., 2015).

High energy demands in the CSH process have encouraged the development of enzymes with the ability of hydrolysing starch granules at sub-gelatinization temperatures to fermentable sugars, which is also known as raw (uncooked) starch hydrolysing enzymes (RSHEs) (Uthumporn et al., 2010). The utilization of RSHEs eliminates the requirement of starch gelatinization during pre-treatment, thus only sub-gelatinization pre-treatment or low-temperature (LT) pre-treatment is necessary. The dry-grind process that includes the use of RSHEs with LT pre-treatment is known as the raw starch hydrolysis (RSH) process. In 2005 a RSHE, namely STARGEN™, was developed by Genencor International Inc. (today DuPont), which is an enzyme cocktail of an endo-activity α-amylase and an exo-activity glucoamylase that hydrolyse raw starch granules through synergistically breaking down starch polysaccharides to glucose (Robertson et al., 2006; Cinelli et al., 2015). Enzyme cocktails STARGEN™ 001 and 002 have successfully been used in the RSH process on lab scale and have proven to achieve competitive ethanol concentrations and ethanol yields when using corn starch and Indian broken rice as substrates (Gohel & Duan, 2011; Sharma et al., 2007). POET (a bioethanol producing company) in the USA is currently producing fuel ethanol from corn on industrial scale using a RSHE (BPX™) developed Novozymes (POET, 2015; Schill, 2013). It has been reported by POET that such a RSH process utilizing BPX™ as a RSHE can lead to a 15% reduction in energy consumption, compared to the conventional CSH processes (POET, 2015). The elimination of starch gelatinization during RSH process gives the potential for very high gravity fermentation (> 30% solids during liquefaction), due to avoidance of high-viscosity slurries (Puligundla et al., 2011). However, the absence of starch gelatinization may cause a vulnerability to high levels of bacterial contamination (Wang et al., 2007). None the less, the production of ethanol from raw starch is an industrially mature technology for fuel ethanol production – a process that is very similar to whisky production.

Limited information is available in literature where the upper limit has been defined for solids loading in very high gravity fermentations when using STARGEN™ as a RSHE in the RSH process. A comparison between the CSH and RSH processes with the same enzyme dosage showed that the RSH process delivered 10% lower ethanol concentrations when using corn with 20% amylose starch as substrate, compared to the CSH process (Sharma et al., 2007). Therefore, optimising the

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STARGEN^{TM} dosage and solids loading for the RSH process are crucial requirements to ensure an economically viable process that can compete with conventional processes like the CSH.

1.2.   Research Aims

The general focus of this study is to optimise the production of potable ethanol from raw corn using simultaneous saccharification and fermentation, together with raw starch hydrolysing enzyme, STARGEN^{TM} 002.

The aims of this study can be summarised by the following:

- To compare the CSH and RSH ethanol production methods, based on the ethanol production performance, using 30 different South African yellow dent corn cultivars as substrates. The ethanol production performance criteria will be the final ethanol concentration (expressed as % v/v), ethanol productivity and ethanol yield as fraction (%) of theoretical maximum, which are measured for each of the cultivars, allowing a wide comparison of the relative performances of CSH and RSH processes.

- To determine whether ethanol production with the RSH process will not be affected significantly by bacterial contamination

- To optimise the RSH process with regards to maximising the solids loading and minimising STARGEN^{TM} 002 dosage, while still maintaining acceptable fermentation performance.

- To investigate whether the performance on small-scale (1 L Erlenmeyer flask) of the RSH process can be replicated on pilot-scale (150 L bioreactor).

- To develop process simulations of the CSH and RSH processes, and use these to determine the energy requirements of the CSH and RSH processes. An associated cost model will be used to calculate the cost per unit ethanol produced for the both processes.

1.3.   Research Approach

The study consists of five chapters. The first chapter is an introduction to the thesis with a background, research aims and approach subsections. The second chapter is a detailed literature review of the cooked and uncooked corn to ethanol production processes. The third chapter defines the materials, methods, economic model and calculations. The results of the experimental work and the economic model are stated in the fourth chapter, while the discussion of these results is in fifth chapter. In the final chapter, the work is summarised with conclusions drawn and recommendations made.

The mind map shows the sequential research approach that is followed in order to complete all the required experimental and simulation work.

Small-scale (1 L) experiments using both the CSH and RSH processes

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Small-scale (1 L) optimisation of the RSH process

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RSH process scale-up with 5 L and 150 L experiments

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Process simulation
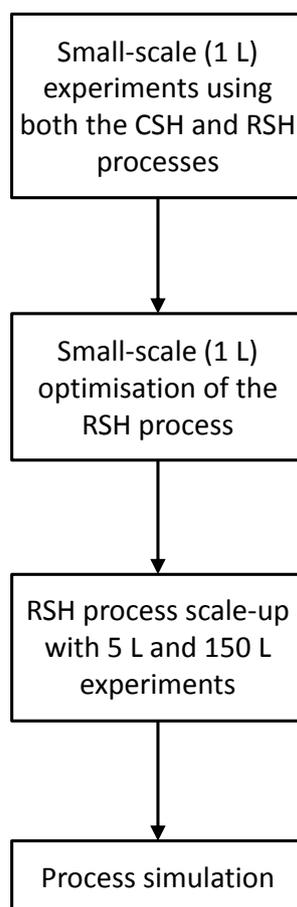
Figure 1-1: Mind map that shows the research approach for completion of experimental and simulation

2. LITERATURE REVIEW

2.1. Starch

Starch granules consist out of two polymers namely, amylose and amylopectin that are densely packed in a semi-crystalline structure with inter- and intra-molecular bonds. This specific structure causes the starch granules to insoluble in cold water and resistant to degradation by chemicals and enzymes (Uthumporn et al., 2010). The ratio between amylose/amylopectin within the starch granule is also an important property that influences the quality of starch as a fermentation substrate. This ratio directly correlates to starch gelatinization temperatures and resistant starch content (Robertson et al., 2006; Sharma et al., 2010).

The amylose molecule has a linear structure, where the glucose molecules are linked in a straight chain by α-1,4 glycosidic linkages. This linear structure of the amylose polymer can be up to 1000 glucose units, and have an estimated molecular weight of up to 1×10^6 g/mol (Zou et al., 2012). The molecular structure of the amylose polymer with α-1,4 linkages between glucose molecules are shown in Figure 2-1. The second molecule, amylopectin, has a highly branched structure with short linear α-1,4 linked chains, which are connected with α-1,6 linkages that occur approximately once every 25 glucose units. The branched structure of the amylopectin molecule can be up to 10 000 glucose units that have an estimated molecular weight of 1×10^8 g/mol (Curá et al., 1995; Zou et al., 2012). The molecular structure of the amylopectin polymer, together with the location of both α-1,4 and α-1,6 linkages are shown in Figure 2-2a. A simplified overview of this polymer, with debranching chains joint by α-1,6, is also visible in Figure 2-2b. The content of amylose and amylopectin in corn starch is dependent on the corn cultivar properties, but typical values are from 10% to 25% amylose and from 75% to 90% amylopectin.
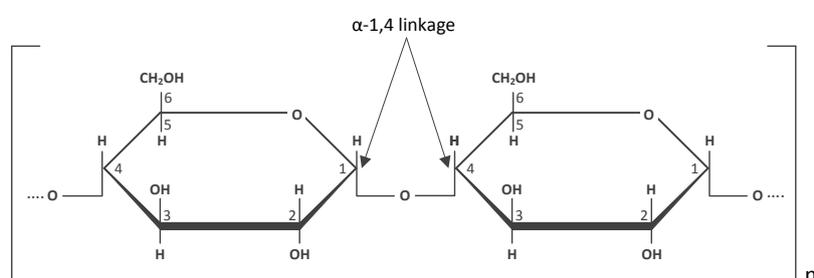

Figure 2-1: Molecular structure of an amylose polymer in starch with α-1,4 glucosidic linkages between glucose molecules. Figure redrawn from Jacques et al., 2003
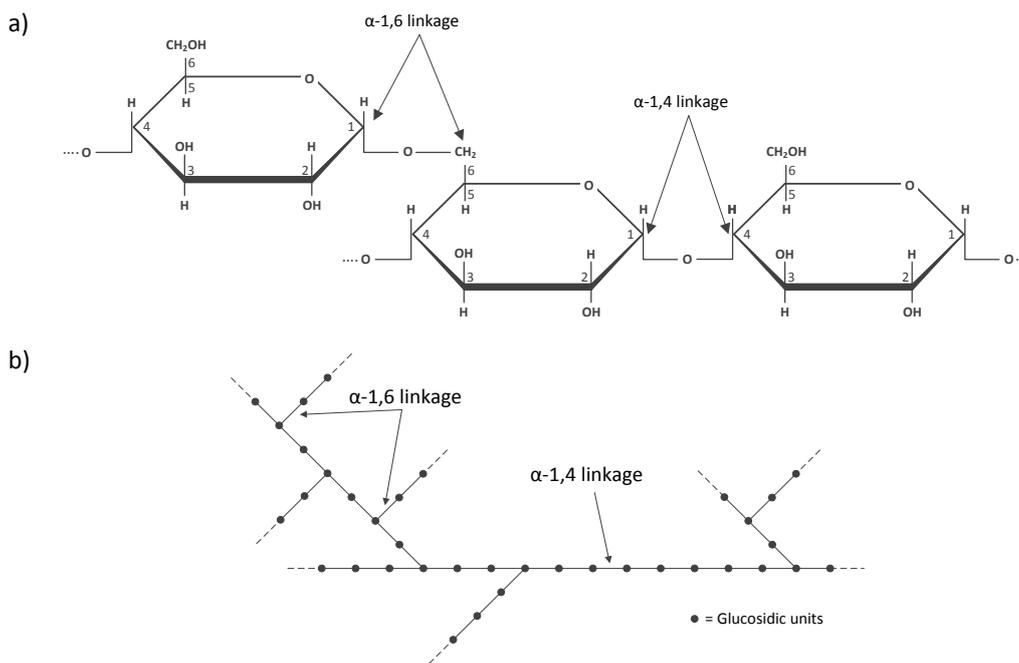
Figure 2-2: a) Molecular structure of an amylopectin polymer in starch, b) simplified overview of the amylopectin polymer with branched linear chains connected with α-1,6 linkages. Figure redrawn from Jacques et al., 2003

2.2.   Ethanol production from cooked corn

The dry-grind process, together with the application of cooked starch hydrolysing enzymes (CSHEs) is a widely used method for the production of potable and bioethanol. Unfortunately, a major concern when using the cooked starch hydrolysing (CSH) ethanol production process is the intensive water and energy consumption per litre ethanol produced. (Robertson et al., 2006). Important factors that have an influence on the ethanol production performance are the substrate type, operational temperature & pH, solids percentage in slurry, incubation time, enzyme type, yeast type and added nutrients (Cinelli et al., 2015). The process flow diagram for the dry-grind process that utilises cooked starch hydrolysis, with the required process steps, is shown in Figure 2-3.

Corn is grinded and water is added to form a slurry/mash with 30% solids. The following process unit is the pre-treatment (partial hydrolysis) section, which includes gelatinization and pre-treatment at 90°C and a pH of 6 using thermostable α-amylase enzyme. The CSH process is restricted to low gravity fermentations (<30% solids), due to viscosity limitations, which is a direct cause of corn starch gelatinization (Li et al., 2012; Cinelli et al., 2015). Industrial scale pre-treatment can be done in

a jet cooker with direct injection of superheated stream (105-120 ^{o}C) for 2 to 7 minutes (Wrenn, 2008). Alternatively, the pre-treatment step can be carried out at 90°C over a time period of 60 minutes. The subsequent process step is the simultaneous saccharification and fermentation (SSF) of the fermentable sugars at 30 °C, pH of 4.2 and time duration from 72 to 96 hours, with addition of glucoamylase enzyme and yeast. The exit stream of the SSF is split into ethanol and co-product DDGS through distillation (Wang et al., 2005). The distillers dried grain with solubles (DDGS) mainly consists out of protein, fat and carbohydrates which is sold to the animal feed industry. Figure 2-3 is a typical process flow diagram for the above mentioned process.
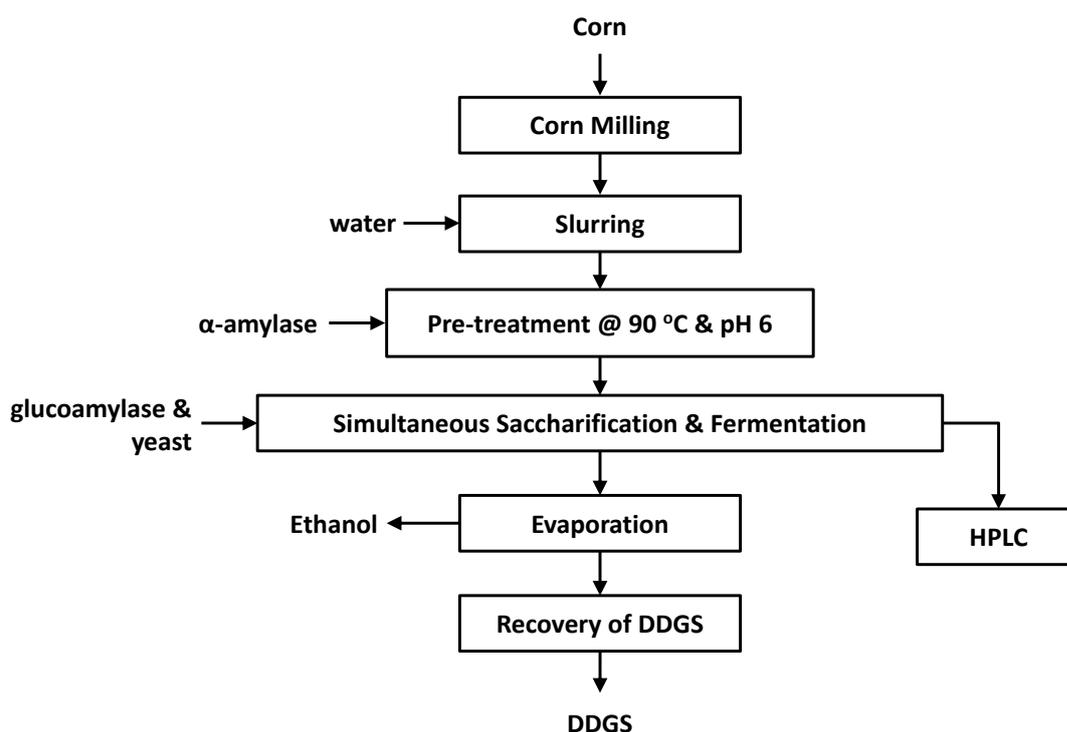
Figure 2-3: Process flow diagram for the dry-grind ethanol production method from corn, utilising cooked starch hydrolysis. Figure redrawn from Wang et al., 2005

2.2.1. High-temperature pre-treatment

For CSHEs to have access to the amylose and amylopectin polymers, the native starch granules need to be fully hydrated (Zou et al., 2012). This hydration is achieved through cooking the corn slurry (containing milled grains and water) in the high-temperature (HT) pre-treatment step above the gelatinization temperature of corn, which may vary from 62 to 72 ^{o}C. During cooking, the starch granules absorb water and start to swell, which leads to the polymer losing its crystalline structure as it fills with gel. As each of the gel-filled pocket starts to enlarge, it takes up more space, which in turn causes an increase in viscosity (Jacques et al., 2003). Depending on the granule size, granule

structure and amylose/amylopectin ratio, the gelatinized starch can have a viscosity up to 20 times larger than that of the original slurry. A higher viscosity results in smaller percentage solids in the initial slurry which have a negative impact on the yield of the process, final ethanol concentration and ethanol production costs (Robertson et al., 2006).

Once CSHEs have access to the amylose and amylopectin polymers, it can convert the long chain polysaccharides into dextrins and α-limited dextrins. This conversion is done using an endo-activity α-amylase that is produced mainly by *Bacillus* species added during pre-treatment as an exogenous component. The formed dextrins are short linear chain polymers of glucose molecules (mostly oligosaccharides) produced through the random hydrolysis of α-1,4 linkages between adjacent glucose units by the α-amylase. The random hydrolysis of α-1,4 linkages take place in the amylose and the amylopectin polymers. The α-limit dextrins are the remaining branched chains of the amylopectin with α-1,6 linkages that cannot be hydrolysed through the α-amylase enzyme (Jacques et al., 2003). Additionally, during the hydrolysis of the polysaccharides the viscosity of the gelatinized starch is reduced in order to have a manageable slurry viscosity for following processing units (Saha et al., 2011).

2.2.2.   Saccharification

Saccharification is the step in which glucose units, which are bound in the oligosaccharides (dextrins and α-limit dextrins chains), are released as monomeric units through the use of an exo-activity glucoamylase (Nigam & Singh, 1995). Glucoamylase, which mainly produced from fungal sources such as *Aspergillus niger*, is less thermo-tolerant than α-amylase and the preferred operation temperature is well below that of pre-treatment. This exo-activity enzyme, in contrast to the endo-activity α-amylase, is capable of hydrolysing the α-1,4 and α-1,6 linkages. These two linkages that are present between two glucose units can be seen in Figure 2-2. However, not all glucoamylase are capable to hydrolyse α-1,6 linkages; some require accessory enzymes. (Robertson et al., 2006). The above mentioned dextrin chain lengths are depended on the activity of the α-amylase during the pre-treatment step. Furthermore, it is difficult to estimate the chain lengths due to the fact that HPLC analysis cannot distinguish between a 4 unit and a 14 unit chain. The dextrin chain length is very important due to the fact the amount of work that needs to be done by die glucoamylase, to release units, increases with the increase of chain length (Jacques et al., 2003). If the chains are too long, then the exo-activity enzyme will not be able not hydrolyse all dextrins which will lead to smaller ethanol yields.

2.3.   Ethanol production from raw corn

When considering high energy usages (which are associated with the use of the CSH process) and viscosity limitations, starch hydrolysis at low temperatures (sub-gelatinization) is desirable. The dry-grind process with raw starch hydrolysing enzymes (RSHEs), which is defined as the RSH process, is similar to the CSH process, with the exception of the enzyme type and pre-treatment conditions. For the RSH process, corn is grinded and water is added to form a corn slurry with 30% solids. The pre-treatment section includes only partial pre-treatment with no starch gelatinization, at 48°C, pH of 4.2 and an incubation time period of 60 minutes (Uthumporn et al., 2010). The subsequent unit is the SSF of the fermentable sugars at 30°C, pH of 4.2 and time duration from 72 to 96 hours. The SSF process is performed with a raw starch hydrolysing enzyme (RSHE) such as STARGEN[TM] 001 or 002, which contains both α-amylase and glucoamylase activities for complete hydrolysis of pre-treated starch to fermentable sugars. The exit stream of the SSF is split into ethanol and by-product DDGS through distillation (Wang et al., 2005). The dry-grind ethanol production method, utilising raw starch hydrolysis together with a RSHE are shown in Figure 2-4.
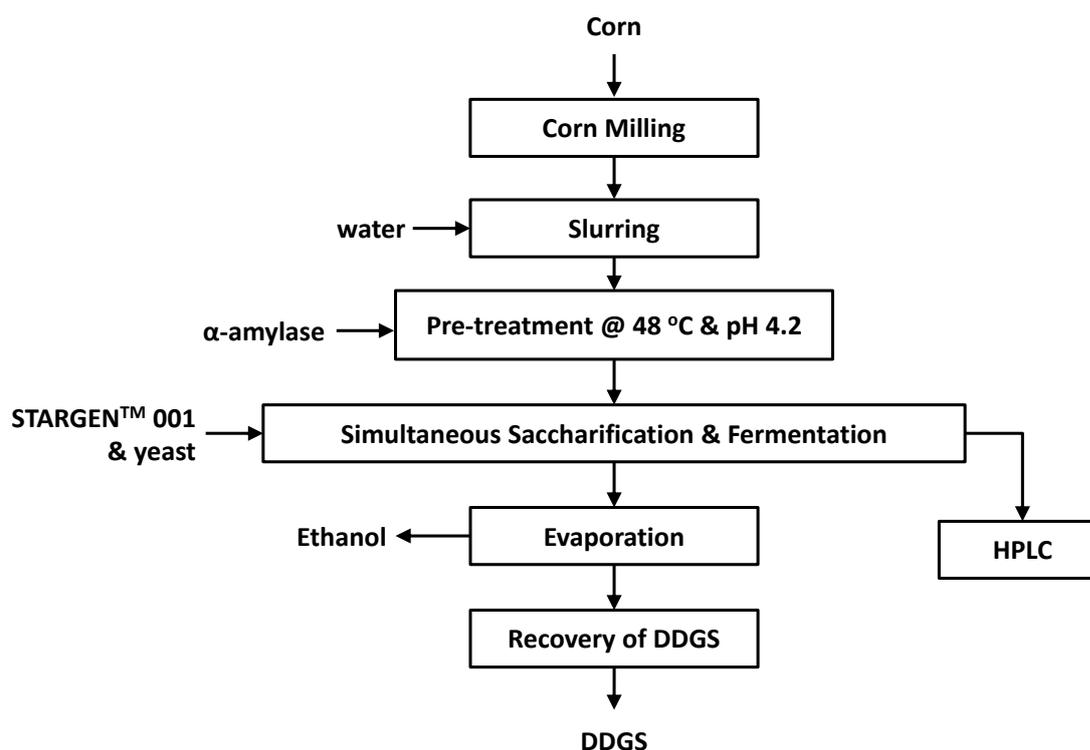

Figure 2-4: Process flow diagram for the RSH process. Figure redrawn from Wang et al, 2005

2.3.1. Raw Starch Hydrolysing Enzymes (RSHEs)

Raw starch hydrolysing enzymes (RSHEs) are able to hydrolyse raw starch granules without the need for a gelatinization step. This ability, together with other advantages, holds a promise of a more cost-effective ethanol production method due to improved efficiency. More than 80 RSHEs have been identified since 1972, with the rate of research on these enzymes growing considerable over the last 10 years. The identification of the superior RSHE cocktails depends on kinetic capabilities, intrinsic activity, stability, inhibition, thermal stability and the pH stability of the specific enzyme components (Robertson et al., 2006). Furthermore, enzymatic synergies by endo- and exo-activity enzymes have been reported as a very important source for raw starch hydrolyses. In conventional CSHEs processes, the α-amylase and glucoamylase enzymes are added separately to the pre-treatment slurry in order to convert the starch to glucose. In the case where the endo-activity enzyme (α-amylases) acts alone, the number substrate sites, together with their concentration, decreases each time a α-1,4 linkage is hydrolysed. In the case where the exo-activity enzyme (glucoamylase) acts alone, there is no increase in the number of substrate sites until the amylose or amylopectin is hydrolysed to the last unit. On the other hand, when the endo- and exo-activity enzymes work in synergy, as is the case for RSHEs where these enzymes are combined in a single cocktail, the number of substrate sites will increase with time (Wang et al., 1996; Robertson et al., 2006). This increase in substrate sites will lead to an enhanced rate of conversion, which possibly means a higher ethanol yield (Robertson et al., 2006). Since 2005 an RSHE named STARGEN[TM] 001 has been produced. This consists out of endo and exo-activity enzymes, namely α-amylase form Aspergillus kawachii and a gluco-amylase from Aspergillus niger. Further research from the same company produced the second generation RSHE (STARGEN[TM] 002), which has the same endo-activity enzymes but a different exo-activity enzyme, namely gluco-amylase *Trichoderma reesei*. During raw starch hydrolyses, the exo-activity enzymes drill deep pin-like holes into the surface of the granules, which allow access for the endo-activity enzyme to hydrolyse the starch granule from within. This pin-like hole will be at the location of a previous existing cavity (which is the centre of enzymatic attack) on the surface, which is enlarged by the endo-activity enzyme as the degree of hydrolyses increases.

Figure 2-5 is an example of such pin-like holes which forms on the surface of the starch granule. Figure 2-5a and Figure 2-5b are SEM micrographs of corn granules before and after hydrolysis respectively. Hydrolysis was done at 35 °C for 24 hours using STARGEN[TM] 001. Figure 2-5b shows that even though the hydrolysis was carried out at sub-gelatinization temperatures (35 °C), the exo-activity enzyme was still able to attack and create access holes at existing cavities. Furthermore,

after 24 hours the granules have been hydrolysed through the large number of holes that gave access for the endo-activity enzymes (Uthumporn et al., 2010).

a)
b)
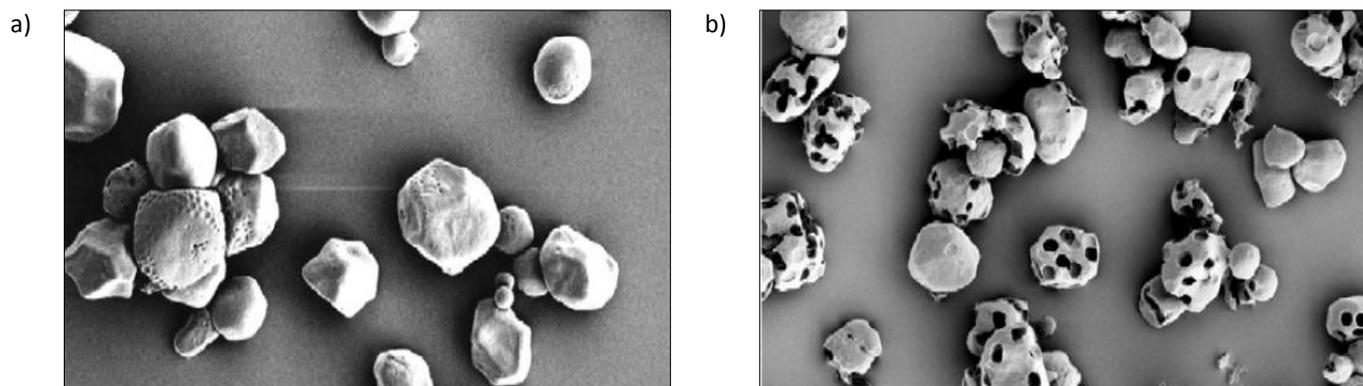
Figure 2-5: SEM micrographs for a) control and b) hydrolysed starches with RSHEs at 35°C for 24 hours. Figure reprinted with permission from Elsevier

Adams et al. (2011) has confirmed the enzymatic synergy capabilities of STARGEN[TM] 001 through the hydrolysis of different mutants of corn at different dosages at 32°C. In Figure 2-6a, the hydrolysed starch granule can be seen with no STARGEN[TM] 001 addition to the treatment. In Figure 2-6b the same mutant corn was hydrolysed with the addition of 600 µg STARGEN[TM] 001 enzyme per 25 mg corn.

a)
b)
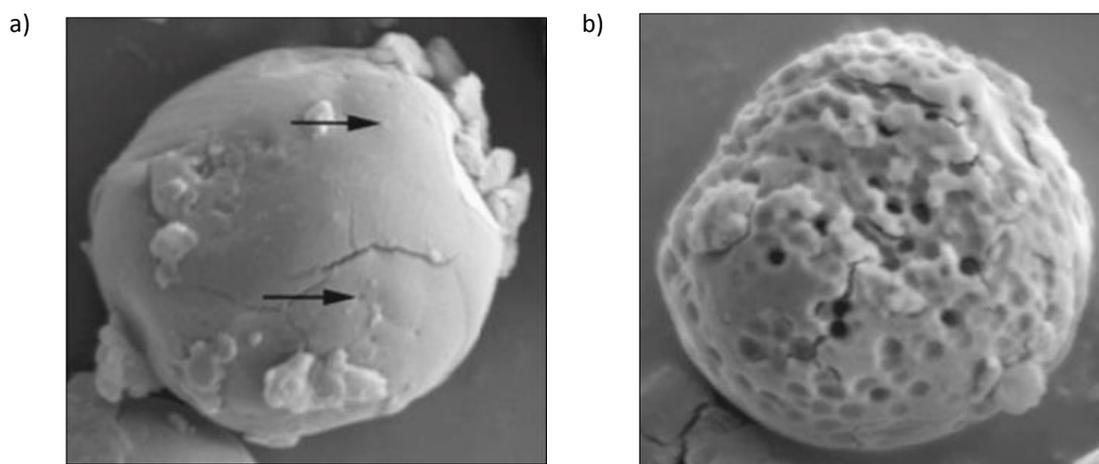
Figure 2-6: Starch mutants that were incubated a) without and b) with STARGEN[TM] 001 enzyme at 32°C for 4 hours. Figure reprinted with permission from Springer

2.4.   Current Research on RSHEs

2.4.1.   Amylose/Amylopectin Ratio

The amylose/amylopectin ratio in starch has a major impact on the performance of that specific corn cultivar during CSH and RSH processes. The amylose/amylopectin mass ratios in corn starch results in classification into three groups namely: waxy, regular and high-amylose corn, which have approximate ratios of 1/99, 25/75 and 50/50 respectively.

The gelatinization temperature of a corn cultivar is the first characteristic that is affected by the amylose/amylopectin ratio. A corn cultivar with a higher amylose/amylopectin ratio will have a higher gelatinization temperature, since amylose molecules have closer proximity than that of amylopectin molecules. This occurrence is due to the linear and branched structures of amylose and amylopectin respectively. The closer proximity will lead to stronger inter-molecular hydrogen bonding and thus more energy will be required to break these bonds (Robertson et al., 2006; Cinelli et al., 2015).

In RSHE research, done by Adams et al. (2011), a hypothesis has been formed that amylopectin is the preferred granule component for hydrolysis by the STARGEN^{TM} 002 enzyme. STARGEN^{TM} 002 is a second generation RSHE, which was developed by Genencor International Inc. (today DuPont) specifically for the hydrolysis of higher amylopectin starches. Saccharification and fermentation experiments were done on different corn cultivars, which also differed in amylose/amylopectin ratios. Residual starch assays and ethanol yield calculations were carried out and the results were as follows (Adams et al., 2011):

- Corn cultivars with a higher amylopectin composition had lower residual starch after fermentation (residual starch is the starch polymers that have not been hydrolysed).
- The waxy cultivar (high amylopectin) resulted in the highest ethanol yield.

The first result is an indication that the RSHE hydrolysed the high amylopectin starch to a higher degree than the other corn cultivars, which had lower amylopectin compositions. The higher ethanol yield can be due to the fact that there were more fermentable sugars available because of the lower residual starch level or an indication that there was a higher sugar conversion. Both these results support the hypothesis that amylopectin is the preferred granule component for the RSHE STARGEN^{TM} 002 (Adams et al., 2011).

2.4.2. Enzyme Dosages

Since the development of STARGEN™ 001 & 002 there have been numerous comparisons between the performance abilities of CSHEs and RSHEs. One determining factor, which is very important in this comparison, is the dosage required for each enzyme type, to maintain the desired hydrolysis-fermentation performance (Kimura & Robyt, 1995). The supplier provides an upper and lower limit for the dosages, which must be used in each specific process step (pre-treatment and SSF). In Table 2-1 the upper and lower limits can be found for a group of CSHEs (Termamyl SC and Saczyme) and RSHEs (GC 626 and STARGEN™ 002). The upper limit for the recommended STARGEN™ 002 dosage is 140 µL per 100g substrate, which is double the recommend dosage of the CSHE (Saczyme) used during SSF. This shows the large difference between recommended dosages between CSHEs and RSHEs, which will have an impact on the operational costs of the production method.

Table 2-1: Average supplier recommended dosages for CSHEs and RSHEs

Enzyme Type	CSHE		RSHE	
Enzyme type	Endo-activity (α-amylase)	Exo-activity (glucoamylase)	Endo-activity (α-amylase)	Endo- & Exo-activity (α-amylase & glucoamylase)
Process step	Pre-treatment	SSF	Pre-treatment	SSF
Enzyme name	Termamyl SC	Saczyme	GC 626	STARGEN™ 002
Dosage lower limit (µL/100g)	12	39	-	70
Dosage upper limit (µL/100g)	36	65	140	140

Wang et al (2007) compared dry-grind ethanol production using a RSHE (STARGEN™ 001) at high dosages with the performance of the same process using two other CSHEs at regular (lower) dosages. RSHEs dosages were added in excess, due to the fact that the study wanted to compare enzyme performances and not to optimise dosages. In each experimental run the ethanol, glucose, organic acid and glycerol profiles were taken over the fermentation period to determine the performance accurately. It was concluded by Wang et al (2007) that the residual glucose concentration in the fermenter vessel of the SSF process was lower for the RSHEs than for the other

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two CSHEs. Furthermore, the final ethanol concentrations were similar for all of the three enzymes, under conditions of excessive enzyme dosages

Sharma et al. (2010) also compared STARGEN[TM] 001 with one RSHE (Spezyme Xtra) and two CSHE on two variations of corn starch which have 0% and 30% amylose respectively. The operation conditions for the pre-saccharification step can be found in Table 2-2. Only the pre-treatment conditions are reported, since the SSF conditions is the same for all four enzymes. The ethanol concentration results can be found in Table 2-3. The corn cultivar with the lower percentage amylose yielded higher ethanol concentrations, compared to the 30% amylose cultivar. This result is expected, since it was concluded in the section 2.4.1 that amylopectin is the preferred granule for enzymatic hydrolysis. Furthermore, the STARGEN[TM] 001 delivered the second highest ethanol concentration with the 0% amylose cultivar, but was outperformed by both CSHEs when the 30% amylose cultivar was used as substrate. This suggests that STARGEN[TM] 001 is able to hydrolyse more starch when the cultivar contains less amylose, but even at high dosages the RSHEs couldn't hydrolyse 30% amylose cultivars sufficiently.

Table 2-2: Pre-treatment operation conditions for the a study done by Sharma et al. (2010)

Enzyme name	Solids %	Enzyme dosage (µL)	Pre-treatment time (h)	Pre-treatment pH	Pre-treatment temperature (°C)
STARGEN[TM] 001	15	140	2	4.2	48
Spezyme Xtra	15	140	2	5.5-6.0	60
Ultra-Thin 100L	15	140	2	4.5	90
Liquozyme SC	15	140	2	5.5-6.0	90

Table 2-3: Ethanol concentrations results for the comparison of four different enzymes and two different cultivars for the study by Sharma et al. (2010)

Enzyme type	EtOH (% v/v) 0% amylose	EtOH (% v/v) 30% amylose
STARGEN[TM] 001	9.62	6.12
Spezyme Xtra	9.49	5.97
Ultra-Thin 100L	10.16	8.72
Liquozyme SC	9.44	8.29

2.4.3. Protease and Urea addition to fermentation

A study by Wang et al (2007) indicated that application of the RSHEs may result in energy-conservation. This has led to further research being done on RSHEs and specifically to lower the required enzyme dosages without the reduction of ethanol yield. Urea is able to disrupt the intermolecular bonding rather than intramolecular hydrogen bonding in amylose and reduce the strength by decreasing the intermolecular network formation between water and amylose. A study by Li et al (2012) indicates that urea breaks hydrogen bonds in starch molecules effectively at a sub-gelatinization temperature. Wang et al (2009) investigated the effects of protease and urea on the RSH process. Firstly, only the effects of protease on ethanol concentration were investigated. Subsequently, the dosages of two types of protease and RSHEs were varied and the corresponding ethanol concentrations determined. In the Table 2-4 & Table 2-5 results can be found for the variation of an endo-protease and an exo-protease respectively on ethanol concentration. The effect of protease on ethanol productivity is also essential to the optimization of RSHEs use during starch fermentation; unfortunately literature was deficient in reporting this specific process variable.

Table 2-4: Final ethanol concentrations (expressed as % v/v) with varying endo-protease and RSHE dosages

		Endo-protease (mL)			
		0	0.1	0.2	0.4
RSHE (mL)	0.1	15.10	16.30	16.30	16.70
	0.2	15.70	16.90	17.40	17.80
	0.4	16.20	17.60	17.80	18.00

Table 2-5: Final ethanol concentrations (expressed as % v/v) with varying exo-protease and RSHE dosages

		Exo-protease (mL)			
		0	0.1	0.2	0.4
RSHE (mL)	0.1	15.20	15.20	15.60	15.70
	0.2	16.00	16.30	16.60	16.60
	0.4	16.40	16.70	17.00	17.20

From Table 2-4 & Table 2-5 it is clear that the addition of protease has a positive effect on the final ethanol concentration. The ethanol concentration increased with the increase of protease dosage for every one of the three different RSHE dosages. With the RSHE dosage of 0.1 mL and the addition of only 0.1 mL of endo-protease, a superior ethanol concentration can be achieved than in the case of 0.4 mL CSHE with no protease addition. Furthermore, the addition of endo-protease resulted in higher ethanol concentrations compared to the exo-protease. Secondly the effects of dosage variation in protease and urea on ethanol concentrations were investigated. In Table 2-6 the ethanol concentration results can be found for these dosage variations.

Table 2-6: Final ethanol concentration (expressed as % v/v) with varying urea, protease and CSHE dosages

Urea (g/100g)	RSHE (mL/100g)	Protease (mL/100g)			
		0	0.05	0.1	0.2
	0.1	13.3	13.7	14.3	14.5
0	0.2	14.8	15.4	16.2	16.2
	0.4	15.4	15.8	16.5	16.7
	0.1	12.9	13.3	13.6	13.8
0.125	0.2	15.2	15.3	15.8	15.9
	0.4	15.7	15.8	16.0	16.4

From Table 2-6 it can be seen that the addition of urea, with no protease addition, have a positive effect on ethanol concentration with higher RSHE dosages. In contrast, the addition of urea & protease did not resulted in superior ethanol concentrations. This is clear from the ethanol concentrations with only protease addition being higher than in the case of protease & urea addition.

Further research by Wang et al. (2010) in the following year has supported the two above suggestions namely:

- protease addition to pre-saccharification can decrease RSHE dosage requirements without a decrease in ethanol yields

- protease addition alone will result in superior ethanol yields when compared with the addition of protease and urea

2.4.4. Contamination

The conventional CSH process is not affected significantly by bacterial contamination, due to the high temperature pre-treatment step that causes starch gelatinization. The temperature of the pre-treatment step is at least 90 °C, which is higher than the thermal tolerance limit of Lactobacilli, therefore bacterial growth is impossible (Narendranath et al., 2001). In the case of the RSH process the pre-treatment of raw starch granules is carried out temperatures typically lower than 50 °C, which cause the absence of high temperature gelatinization that acts as sterilisation. This makes the RSH process vulnerable for bacterial contamination that may lead to a reduced ethanol yield (Narendranath et al., 2001). The reduction in yield is due to, not only the excess production of lactic and acetic acid that inhibit yeast growth but also a decrease the amount of substrate available for ethanol production (Narendranath et al., 1997; Broda & Grajek, 2009).

Three other common solutions to reduce bacterial growth are the usage of antibiotics, grain disinfection with an ammonia solution and low pH process conditions (Broda & Grajek, 2009; Robertson et al., 2006). Antibiotics is an effective method of bacterial contamination reduction, although it is not always the desired action to be taken, since residues present in process products create problems in markets where antibiotics have been banned. A study by Broda & Grajek (2009) found that disinfecting corn grain with an ammonia solution can effectively reduce bacterial contamination. Lastly, low pH conditions during SSF can be favourable solution, since RSHEs such as STARGEN[TM] 001 & 002 have a very low pH tolerance. It must be noted that the pH at which optimal yeast growth occurs will still play a crucial role in the operating pH value (Jacques et al., 2003); (Robertson et al., 2006).

2.4.5. Viscosity

The amount of solids present in the slurry is dependent on the allowable viscosity of the slurry. With this in mind, technology which is capable of very high gravity (VHG) fermentation has been developed (Puligundla et al., 2011). The increase in percentage solids will lead to very high gravity fermentation during the SSF, which is desirable due to the resulting process benefits (Puligundla et al., 2011; Kollaras et al., 2011). These benefits are as follow:

- Decrease in process water requirements

- Reduction in distillation costs due to higher final ethanol concentrations

- Reduction in effluent treatment cost due to lower qualities of process water

- Increased productivity in fermentation vessels

The first three benefits are directly related to the lower volume water that is added per process batch. Since the desirable solids loading in VHG slurries can increase up to 35%-40%, compared with the 20-25% in normal gravity operation, the water volume reduction is enough to cut cost significantly (Kollaras et al., 2011). The increase in productivity is due to a larger amount of corn starch that can be added per volume of processing batch, which results in a higher production capacity of fermenters. During CSH process, the viscosity increases considerably due to the gelatinization of the starch granules. Therefore, most production plants already operate at their maximum allowable viscosity which has the result of a process cannot be changed to accommodate VHG fermentation. A high slurry viscosity can lead to handling difficulties, resistance to solid-liquid separation, incomplete starch hydrolysis and finally low process efficiency (Puligundla et al., 2011).

The usage of RSHEs in the dry-grind process enables the process to eliminate the gelatinization step. Furthermore, the slurry viscosity of the RSHEs process will be lower, which will consequently result in VHG fermentation possibilities for the RSH process. These capabilities further increase the potential for RSHEs to be a cost-effective alternative over the currently used CSHEs.

2.4.6. Resistant Starch

The desired conversion of starch to fermentable sugars through enzymatic hydrolysis is 100%. Unfortunately, there will always be a percentage residual starch that stays unconverted which is then unavailable for fermentation. The percentage of starch that will be unconverted by enzymatic hydrolysis is dependent on the specific corn cultivar that is used, as well as the process conditions such as temperature, pH, enzyme type and pre-treatment duration (Sharma et al., 2010; Xie et al., 2006; Haralampu, 2000). Furthermore, this unconverted residual starch that will be recovered in the DDGS can be divided into two fractions, namely: solubilisable starch (SS) and resistant starch (RS). The RS is unavailable for enzymatic hydrolysis, while the SS can be hydrolysed enzymatically to produce fermentable sugars that can be converted into ethanol. Further studies have shown that the amylose/amylopectin ratio has an influence on the fraction of RS, where cultivars with high amylose content will have a high RS content (Berry, 1986; Evans & Thompson, 2004).

Sharma et al. (2010) completed a study where the performance of resistant starch hydrolysis was analysed using two RSHEs and two CSHEs with two different corn cultivars as substrate. The two

corn cultivars had 0% and 30% amylose, respectively. The weight of the RS was determined on three different intervals, namely: before pre-treatment (Initial), after pre-treatment (Pre-treatment) and after fermentation (SSF). With these values it is possible to determine the effect that each process step has on the percentage RS. In Figure 2-7 and Figure 2-8 the RS values can be found for each interval with 30% and 0% amylose cultivars as substrate, respectively.
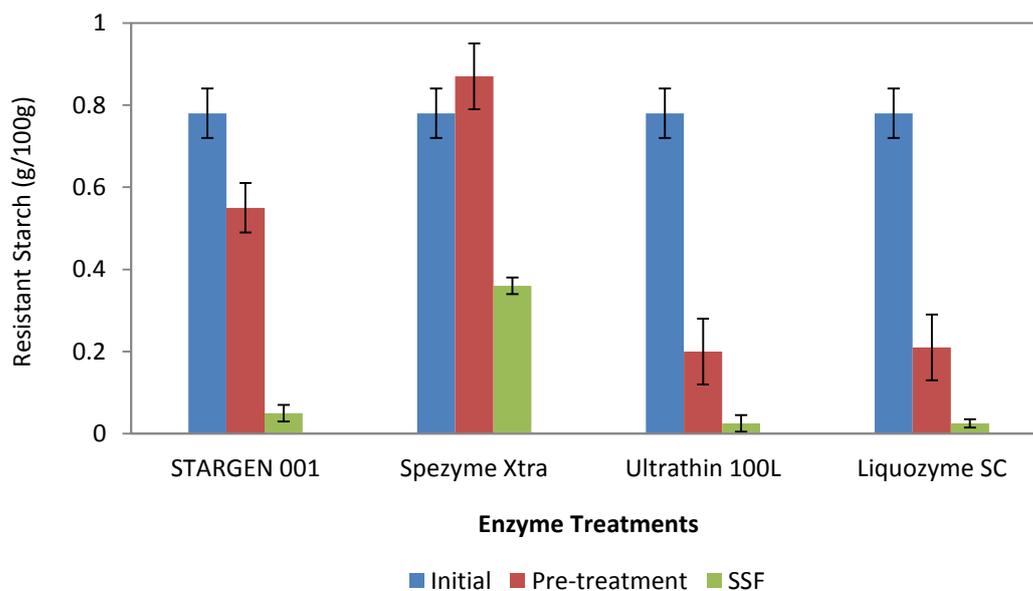

Figure 2-7: Resistant starch content for the four different enzymes with 0% amylose. Figure redrawn from (Sharma, et al., 2010)
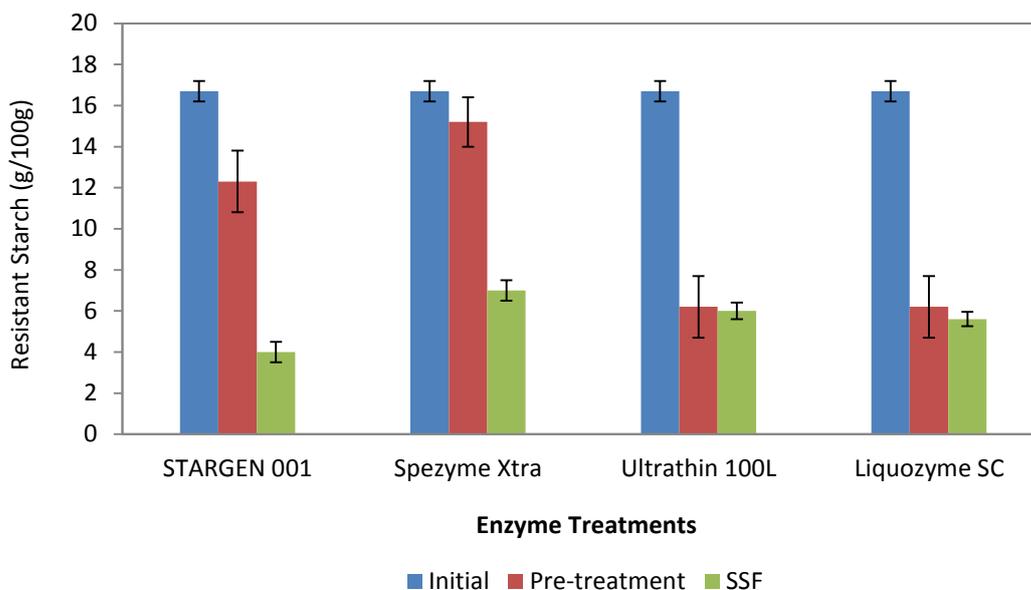
Figure 2-8: Resistant starch content for the four different enzymes with 0% amylose. Figure redrawn from (Sharma et al., 2010)

When comparing the results of the two cultivars, the 0% amylose cultivar had an average initial SR 0.8%, while the 30% amylose cultivar a much larger RS value of 17%. This relates to results by Berry et al. (1986), which states that waxy corn has a low RS content. In seven of the eight treatments the RS content decreased after each process step (Pre-treatment & SSF). Furthermore, a higher pre-treatment temperature resulted in a larger RS portion being hydrolysed.

After SSF the residual starch can be found in the DDGS with a specific SS/RS ratio. From this ratio certain conclusions can be made on the enzyme dosages and enzyme activity. In Figure 2-9 & Figure 2-10 the SS and RS fractions in the residual starch can be found for 30% and 0% amylose cultivars, respectively. From Figure 2-9 & Figure 2-10 it can be seen that the amount of SS for both RSHEs are much higher when compared to the SS for both CSHEs, which is almost zero. The fact that the SS content in the residual starch is so high is an indication of low enzyme activity or that a higher enzyme dosage is required. Finally, the fact that the SS for the 0% amylose cultivar is much lower, is a confirmation that amylopectin is the preferred granule for hydrolysis by a CSHE.
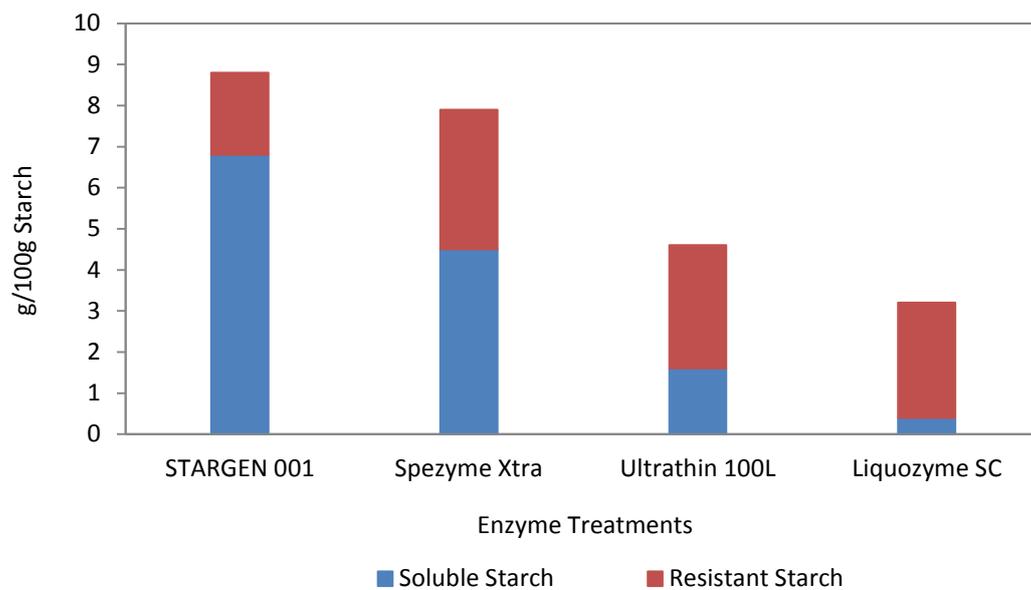
Figure 2-9: Solubilisable and Resistant starch in the residual starch for 30% amylose content. Figure redrawn from (Sharma et al., 2010)
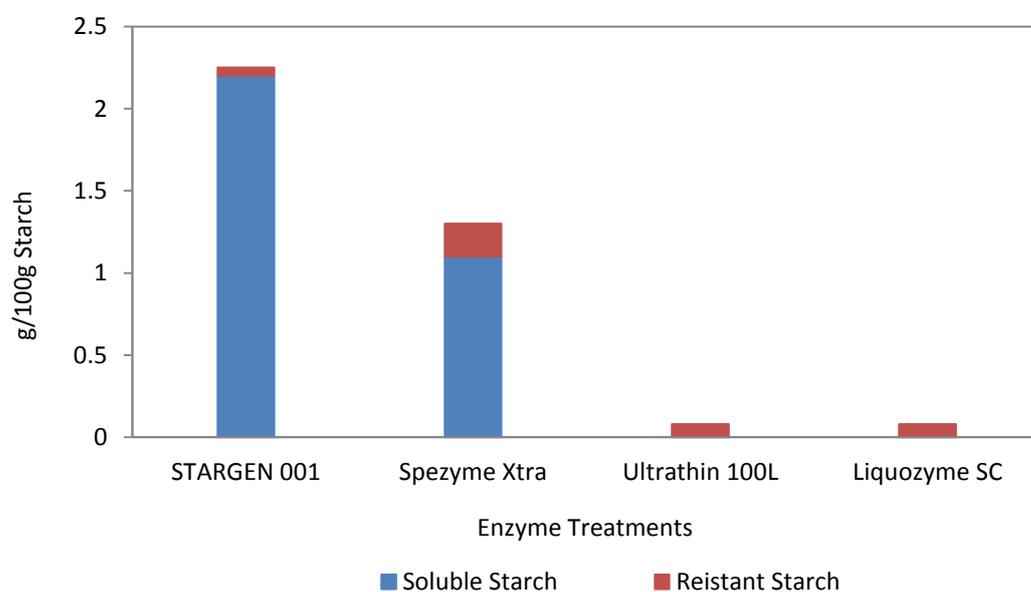

Figure 2-10: Solubilizable and Resistant starch in the residual starch for 0% amylose content. Figure redrawn from (Sharma et al., 2010)

2.5.   General Conclusion

The raw starch hydrolysing ethanol production, using RSHEs (specifically STARGEN^TM 002) has shown to be able to deliver competitive ethanol concentrations, yields and productivities, compared to cooked starch processes (Robertson et al., 2006). The RSH process is inherently vulnerable to microbial contamination ethanol production performance; although numerous sources have reported that the RSH process did not show significant contamination during fermentations (Robertson et al., 2006; Wang et al., 2007; Sharma et al., 2010). The mechanism behind the RSH process is not fully understood and need to be investigated, in order to remove any doubt that the RSH process is able to competitively. The comparison of the two processes using 30 different cultivars will be the opportunity to investigate and answer the questions the bacterial contamination.

There is a lack of information available on the upper limit for solids loading during high gravity fermentation for the RSH process. No optimization for STARGEN^TM 002 dosage on corn as a substrate has been reported. Once superior corn cultivars have been selected in the first part of the experimental work, small-scale (1 L) and bioreactor-scale (5 L) optimization can be carried out to determine optimum solids loading and STARGEN^TM 002 dosages. Viscosity measurements of both the LT and HT pre-treatment step would provide improved understanding of where the upper limit for solids loading may be for the RSH process. Energy recruitments or operational costs for a typical RSH process using STARGEN^TM 001 or 002 have not been estimated by literature. This estimation is crucial to provide proof beyond any doubt that the advantageous of the RSH process, such as higher gravity fermentations and lower energy requirements, will outweigh the drawbacks, which is reported in literature (high RSHE dosage requirements and unknown enzyme costs). The Aspen Plus® simulation that will be based on an industrial ethanol production will serve as the foundation to answer these questions.

3. **MATERIALS AND METHODS**

3.1. **Materials**

Thirty South African yellow dent corn cultivars were supplied by Griekwaland-Wes Kooperatief Ltd (Douglas, Northern Cape, South Africa). As control, a proprietary blend consisting of several different cultivars currently used for industrial production of potable ethanol, which was supplied by the James Sedgwick distillery (Wellington, Western Cape, South Africa). All corn samples were frozen prior to use in order to limit spoilage. Coning and quartering was used to ensure a representative sample of the supplied material, which was milled with a universal laboratory disk mill (Bühler type DLFU, Johannesburg, South Africa) to a particle size smaller than 800 µm. A particle size distribution analysis, using a sieve shaker (Retch type AS 200, Johannesburg, South Africa), showed that 70% of the milled material was smaller than 425 µm.

Saccharomyces cerevisiae strain DY10 was supplied by Anchor Yeast[TM] (Randburg, Gauteng, South Africa) in the form of dry active yeast and stored at 4 °C. Storage at 4°C was to minimum the reduction in yeast viability. The inoculum for fermentation was prepared by dissolving dried yeast pellets (1 g/L fermentation slurry) in water at 38 °C for 15 min, where the water volume was 1% of the final volume of the fermentation slurry. This concentration of dry yeast corresponded to 18×10^6 CFUs/mL fermentation slurry through agar plating in YPD media.

Cooked starch hydrolysing enzymes Termamyl® SC and Saczyme® were supplied by Novozymes (Johannesburg, Gauteng, South Africa). The Termamyl® SC is an endo-activity α-amylase from *Bacillus licheniformis* with a declared activity of 120 KNU-S/g and a specific gravity of 1.15 g/mL. The Saczyme® is an exo-activity glucoamylase from *Aspergillus niger* with a declared activity of 750 GAU.g^{-1} and a specific gravity of 1.13 g/mL. The raw starch hydrolysing enzymes GC 626 and STARGEN[TM] 002 were supplied by Genencor International (Palo Alto, CA, USA). The GC 626, which was added to reduce mash viscosity and to activate starch granules, is an *Aspergillus kawachii* acid α-amylase with a specific gravity of 1.15 g/mL (Genencor 2009). The STARGEN[TM] 002 enzyme contains *Aspergillus kawachii* acid α-amylase expressed in *Trichoderma reesei* and a glucoamylase from *Trichoderma reesei* that synergistically hydrolyse the granular starch. STARGEN[TM] 002 had an activity of 570 GAU/g and specific gravity of 1.14 g/mL (Genencor 2009).

3.2.   Simultaneous Saccharification and Fermentation

Simultaneous saccharification and fermentation (SSF) experiments were performed on small (1 L), medium (5 L) and pilot-scale (150 L), while using any one of two possible pre-treatment methods. The first ethanol production method was the cooked starch hydrolysis (CSH) process, which utilized high-temperature (HT) pre-treatment to cook the corn, followed by SSF. The second method was the raw starch hydrolysis (RSH) process, which kept the corn starch raw (uncooked) with low-temperature (LT) pre-treatment, followed by SSF. The CSH and RSH processes also differed on the following parameters: pre-treatment enzyme type, pre-treatment enzyme dosage, pre-treatment temperature, pre-treatment pH, SSF enzyme type and SSF enzyme dosage. Furthermore, the corn solids loading during pre-treatment for the RSH process varied between 30, 35 and 40%, compared to only 30% during pre-treatment for the CSH process. Corn solids of 30, 35 and 40% during pre-treatment corresponded to corn solids loading of 21, 26 and 31% during SSF. The parameters for CSH process were identical to the conditions of the industrial scale James Sedgwick production process, while the initial RSH process conditions were the same as the Genencor's pre-determined optimal conditions (Genencor 2009). The details for each method's parameters are shown in Figure 3-1.
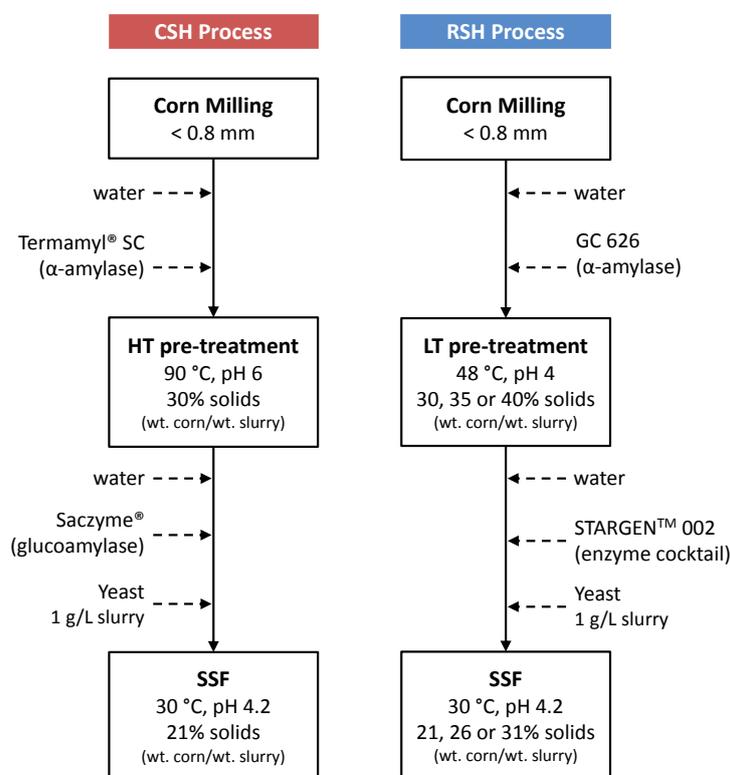

Figure 3-1: SSF process diagram for both the CSH and RSH processes, together with the process parameters for each method

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The 9% drop in solids loading from pre-treatment to SSF (30% to 21%) was due to the CSH process design at the James Sedgwick distillery. This industrial-scale production process utilises a series of vessels for pre-treatment and SSF steps, which improves the plant throughput compared to design where every pre-treatment and SSF step occurs in the same vessel. This multi-vessel design, together with the use of a shell and tube heat exchanger for heating and cooling purposes, requires a relative large amount of wash water to rinse any residual material. This rinse water, containing a substantial amount of residual material is, therefore, added to the fermentation, thus lowering the solids loading. This 9% is a very large drop and will add additional energy requirements during distillation. However, since the litre ethanol yield per kg substrate won't be affected, it was decided to implement the 9% drop for the RSH process as well for the sake of method comparability to industrial practise.

3.2.1. Small-Scale experiments (1 L)

The following generic recipe was used to carry out small-scale experiments for both the CSH and RSH processes. For pre-treatment, slurry was produced by adding milled corn and distilled water into a 1 L Erlenmeyer flask. The pH of the corn slurry was adjusted with 72% H_2SO_4 and the pre-treatment enzyme was added (0.29 and 0.16 g/kg corn for CSH and RSH process, respectively). The flask was then placed into a water bath and kept at a constant pre-treatment temperature for 60 min, while the slurry was constantly mixed with an overhead straight blade impeller at a rate of 150 rpm. At the end of the 60 min period, the flask was placed into an ice bath to cool down to 35 ^{o}C. Subsequently, the pH of the slurry was adjusted to 4.2 with 72% H_2SO_4, the SSF enzymes were added (0.33 and 1.6 g/kg corn for CSH and RSH processes, respectively) and the slurry was inoculated with an activated dry active yeast solution (see section 2.2). After inoculation, distilled water was added to ensure a final fermentation volume of 500 mL and the flask was sealed with a rubber stopper. The rubber stopper was drilled to allow insertion of a glass tube for CO_2 venting. For the fermentation step, the flask was incubated in a rotary shaker at 30 ^{o}C and 120 rpm for 120 hours, with regular sampling.

3.2.2. Medium-Scale Fermentations (5 L)

Medium-scale RSH process experiments were carried out in jacketed BIOSTAT® Bplus-5 L CC twin bioreactors (Sartorius BBI Systems GmbH, Switzerland) with a final working volume of 3 L. Pre-treatment slurry was produced through mixing water and milled corn in the 5 L vessel to achieve the required solids loading ranging from 30% to 40%. Subsequent pH adjustment to 4 and the addition of pre-treatment enzyme (GC 626) at a dosage of 0.16 g/kg corn were carried out. Vessel was heated

to 48°C (via water heating jacket) and kept at a constant temperature for 60 min, while continuously stirred homogenously at a rate of 1200 rpm with Rushton blade impeller. After the pre-treatment period, the slurry was cooled down to a temperature of 35°C, with subsequent pH adjustment to 4.2 and the addition of SSF enzyme (STARGEN^{TM} 002) at a dosage ranging from 1.0 to 1.4 g/kg corn. For fermentation, the slurry was inoculated with a dry active yeast solution and distilled water was added to ensure final fermentation volume of 3 L, with a final fermentation solids loading ranging from 21% to 31%. During fermentation, the vessel was kept at a constant temperature of 30°C for a period of 120 hours while being continuously stirred, with regular sampling.

3.2.3. Pilot-Scale Fermentations (150 L)

Pilot-scale RSH process experiments were carried out in a jacketed 150 L bioreactor (New Brunswick Scientific, Enfield, CT, USA) with a final working volume of 90 L. Pre-treatment slurry preparations were carried out to ensure a pre-treatment solids loading of 37.5% at a pH of 4 and GC 626 dosage of 0.16 g/kg corn. The pre-treatment slurry was heated to a temperature of 48°C (via steam heating jacket) and kept at a constant temperature for a pre-treatment period of 60 min, while the slurry was continuously stirred with two Rushton impellers at a rate of 300 rpm. After the pre-treatment period, the slurry was cooled down to a temperature of 35°C, with subsequent pH adjustment to 4.2 and the addition of STARGEN^{TM} 002 at a dosage of 1.4 g/kg corn. Subsequently, the slurry was inoculated with a dry active yeast solution and distilled water was added to ensure final fermentation volume of 90 L, with a final fermentation solids loading of 28.5%. During fermentation, the vessel was kept at a constant temperature of 30°C for a period of 120 hours while being continuously stirred, with regular sampling.

The recipes for small, medium and pilot-scale experiments were not carried out under sterile conditions, as the experiments focussed on replicating industry standards. Therefore, contamination monitoring was required through monitoring of lactic acid levels in the slurry during the full fermentation period. Samples were taken at regular intervals to determine the: ethanol, glucose, maltose, maltotriose, fructose, glycerol, acetic acid and lactic acid concentrations. A 2 mL sample was centrifuged at 14 000 rpm for 10 min, subsequently a diluted supernatant was filtered through a 0.45 µm membrane before it was analysed on a HPLC system.

3.3.   Analytical Methods

3.3.1.   Viscosity

Viscosity measurements of the corn slurry during a typical 60 min pre-treatment (either HT pre-treatment (gelatinization) for the CSH process or LT pre-treatment for the RSH process) were performed in a rheometer (Anton Paar type Physica MCR 501, Germany) with a cup-shaped reactor vessel with a working volume of 45 mL. The mixture in the reactor was stirred with an overhead cross blade impeller at a rate of 300 rpm to keep it homogeneous during the entire run. In a typical run the slurry temperature was increased from 30 $^{\circ}$C to the desired pre-treatment temperature (48 $^{\circ}$C/90 $^{\circ}$C) at a heating rate of 4 $^{\circ}$C per min. Subsequently, the temperature was kept constant for 60 min with a viscosity measurement taking place every 15 seconds. The corn solids % during these viscosity tests were 30% for the CSH process, while 30, 35 and 40% for the RSH process.

3.3.2.   Starch Assay

Starch content present in each corn cultivar was determined using a total starch assay kit (Megazyme International Ireland Ltd, Wicklow, Ireland). The determination of the soluble starch portion was based on enzymatic hydrolysis using thermostable α-amylase and glucoamylase, while the total starch (soluble and resistant) were converted to glucose with an alkaline hydrolysis process using 2M KOH. The resistant starch portion was the difference between the total and soluble starch. The kit quantified the glucose formed based on a quinoneimine dye assay, performed at a 510 nm wavelength as described previously by Adams (Adams et al., 2011).

3.3.3.   Moisture

Corn samples were weighed before and after drying at 104°C for duration of five hours. The moisture content was determined as follow:

$$Moisture\ \% \left(\frac{w}{w}\right) = \left(\frac{weight\ of\ wet\ sample\ (g) - weight\ of\ dry\ sample\ (g)}{weight\ of\ dry\ sample\ (g)}\right) \times 100$$

3.3.4.   High Performance Liquid Chromatography (HPLC)

The specifications for the HPLC system that was used to analyse all fermentation samples was as follow: a HPLC system, model HP 100, equipped with a refractive index detector (model HP 1047 A)

and an organic acid column (Biorad Aminex HPX-87H Ion Exclusion column). A solution of 5 mM H_2SO_4 was used as the mobile phase with flow rate of 0.6 mL/min. The column had a temperature of $50^{\circ}C$.

3.4. Calculations

3.4.1. KPI Calculation for Process Comparison

The success of the of two ethanol production methods were based on the following three KPIs (key performance indicators): (1) the final ethanol concentration, expressed as % v/v, after which no significant increase was detected during the 120 hour fermentation period, (2) ethanol productivity and (3) the ethanol yield as a fraction (%) of theoretical maximum yield.

$$Ethanol\ Productivity\ =\ \frac{Max\ EtOH\ Conc.}{SSF\ Time}$$

Where Max EtOH Conc. is the maximum achievable ethanol concentration during fermentation - in grams per litre - while SSF Time is the shortest incubation time required to reach the Max EtOH Conc.

$$Ethanol\ yield\ as\ a\ fraction\ (\%)\ of\ theoretical\ maximum\ =\ M_{corn}\ \times Starch\ \% \ \times \frac{180}{162} \times 0.51$$

Where M_{corn} is the amount of corn added to the mash in grams, $^{180}/_{162}$ is the maximum theoretical conversion of starch to glucose and 0.51 is the maximum theoretical conversion of glucose to ethanol using fermenting yeast (Megazyme 2014).

3.4.2. Statistical Design and Analysis

The impact of STARGEN™ 002 enzyme dosage and solids loading on final ethanol concentration, ethanol productivity and ethanol yield as fraction (%) of theoretical maximum were investigated using a face-centred central composite design (FCCCD), which is a variation of a central composite design (CCD). When considering Figure 3-2, the eight CCD star points (on the green circle) are all the same distance (α) from the centre point. In contrast, the eight star points for the FCCCD have an axial spacing of 1.0, where α > 1. The FCCCD was chosen over the CCD, due to its ability to overcome factor level constraints that were encountered during solids loading optimisation. Solids loading

above 40% showed inconsistent mixing. STATISTICA version 12 (StatSoft, Inc., 2015) was used to plot a surface responses.
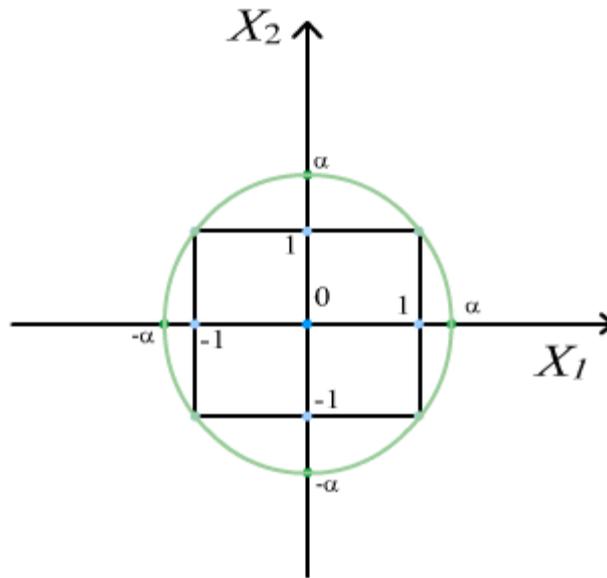

Figure 3-2: Generation of a Central Composite Design (CCD) and Face-Centred Central Composite Design (FCCCD) for two factors (X1 and X2).

3.5.   Process Simulation

The CSH ethanol production process at the James Sedgwick distillery has a production capacity of 950 L/h ethanol at a purity of 94.5%. This industrial-scale process starts with the HT pre-treatment (cooking) of the corn slurry, using two pre-treatment vessels, together with two shell and tube heat exchangers relying on low pressure steam and cooling water for heating and cooling, respectively. A typical pre-treatment from start to finish can be achieved within seven hours, with a large amount of that time (90 minutes) allocated to the heating of the slurry from 55 ^{o}C to 90 ^{o}C. The pre-treated corn slurry is subsequently batch fermented for 72 hours in one of several jacketed fermentation vessels, while using cooling water to keep the fermentation at a constant temperature. After fermentation the fermented slurry is continuously washed and distilled at a rate of 10 m^3 per hour to obtain the ethanol product at the desired purity. The washing and distillation section consists of an 18 sieve tray wash column followed by a 58 sieve tray rectifier column using high pressure and medium pressure steam, respectively. The final ethanol product is taken from tray 43 to 45 in the rectifier column at a temperature of 79 ^{o}C. A simplified process flow diagram (PFD) of the ethanol CSH production process at the James Sedgwick distillery is shown in Figure 3-3.
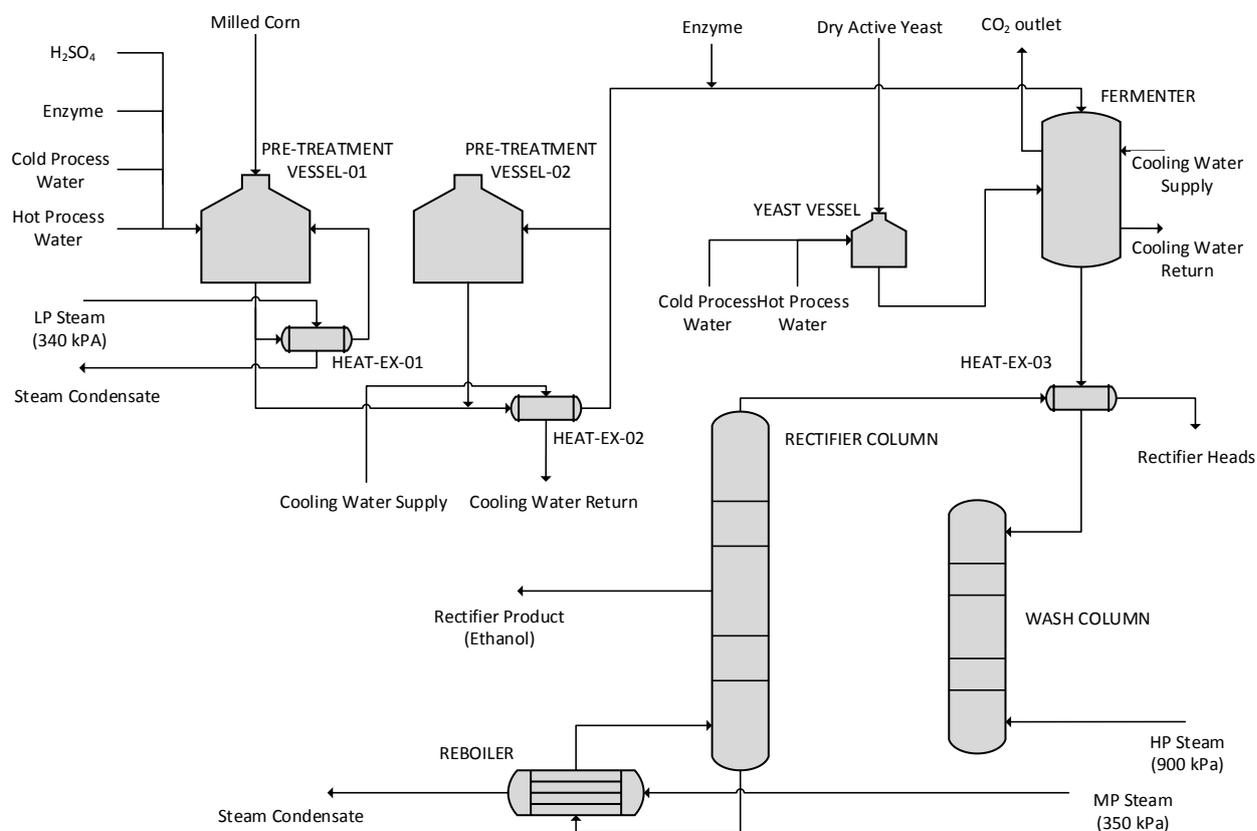

Figure 3-3: Schematic representation showing the ethanol production process from corn pre-treatment through to ethanol distillation at the James Sedgwick distillery

3.5.1. Methodology

In order to quantify the energy benefits associated with the RSH process, a process simulation of the James Sedgwick distillery (CSH process) was developed, where after the simulation was modified accordingly to mimic the RSH process. All process simulations were done using Aspen Plus® software, Version 7.3.2 (Aspen Technologies Inc., Cambridge, MA, USA). Once the energy usage of the RSH process was known, it was included in an associated cost model that was developed based on the operating costs (raw material and utility costs) of the process, using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). This economic model was subsequently used to calculate the cost per litre ethanol produced for each process. The methodology behind the calculation of the cost per litre ethanol produced can be summarised by the following steps:

i.   Develop an Aspen Plus® simulation of the industrial-scale CSH process at the James Sedgwick distillery, which is defined as the CSH-Aspen Plus® simulation, using the PFD presented in Figure 3-3.

ii. Compare the performance of the industrial-scale CSH process at the James Sedgwick distillery with that of the newly developed CSH-Aspen Plus® simulation. Focusing specifically on energy usage during HT pre-treatment and ethanol distillation, as well as the amount of ethanol produced (at 94.5% purity) per batch.

iii. Use performance figures of the CSH-Aspen Plus® simulation to calculate the total operational costs per batch, while using raw material and utility costs per unit supplied by the James Sedgwick distillery (Table 3-1).

iv. Use the CSH-Aspen Plus® simulation, together with experimental performance of the RSH process to develop a RSH-Aspen Plus® simulation.

v.   Use the performance newly developed RSH-Aspen Plus® simulation to determine the energy usage during LT pre-treatment and ethanol distillation, as well as the amount of ethanol produced (at 94.5% purity) per batch.

vi. Use performance figures of the RSH-Aspen Plus® simulation to calculate the total operational costs per batch using raw material and utility costs per unit supplied by the James Sedgwick distillery (Table 3-1), as well as the reported STARGEN™ 002 price.

The raw material and utility costs for both the CSH and RSH processes that were used in the cost models can be seen in Table 3-1. The different costs were supplied by the James Sedgwick distillery (J Green 2013, personal communication, 13 August). Additionally, the price for the STARGEN™ 002 is the reported price for this RSHE in South Africa (M Garcia 2015, personal communication, 30 January).

Table 3-1: Cost of raw material and utility per unit for both the CSH and RSH processes

Raw Material/Utility	Cost
Corn (R/kg)	R 2.90
Water (R/kg)	R 0.13
CSH & RSH Pre-treatment Enzyme (R/kg)	R 65.00
CSH SSF Enzyme (R/kg)	R 95.00
RSH SSF Enzyme (STARGEN™ 002; R/kg)	R 100.00
Yeast (R/kg)	R 79.00
Steam (R/kg)	R 0.18

4. RESULTS

4.1. Process comparisons with small-scale fermentation

The results to follow were a comparison of the current James Sedgwick process (CSH process) with the RSH process that utilized STARGEN^{TM} 002 as a RSHE. This comparison was done based on the ethanol production performance of 30 different whole corn cultivars as substrate.

4.1.1. Fermentation substrate

The resistant starch (RS) portion of the total starch (TS) present in each whole corn cultivar before pre-treatment is shown in Table 4-1. RS portions for the 30 cultivars varied between 0.2% and 4.7%, with a value of 1.2% for the control mixture. Low levels RS (< 1%) were seen in 40% of the cultivars, while elevated levels (> 3%) were detected in 37% of the cultivars. Low RS levels correspond to low starch amylose content (0-10%), while elevated RS levels correspond to normal levels of amylose in dent corn (±30%), due to a strong positive correlation between starch amylose content and RS (Sajilata et al., 2006).

Table 4-1: Resistant Starch values of 30 cultivars and control mixture

Cultivar #	Resistant Starch %	Cultivar #	Resistant Starch %	Cultivar #	Resistant Starch %
Control	1.2±0.5	M11	4.0±0.8	M22	0.3±0.2
M1	3.8±1.1	M12	3.1±0.9	M23	0.3±0.1
M2	3.5±2.1	M13	0.9±0.2	M24	0.2±0.1
M3	3.3±1.0	M14	0.4±0.3	M25	0.2±0.1
M4	3.2±1.2	M15	4.7±1.2	M26	1.2±0.3
M5	0.5±0.2	M16	2.3±0.4	M27	1.3±0.6
M6	3.8±1.3	M17	0.6±0.4	M28	0.4±0.2
M7	3.4±0.9	M18	2.3±0.8	M29	0.5±0.4
M8	0.4±0.2	M19	1.5±0.9	M30	2.1±0.3
M9	3.8±1.5	M20	0.6±0.1	-	-
M10	1.8±0.2	M21	3.2±0.4	-	-

4.1.2. Viscosity behaviour during pre-treatment

Given the desire to increase the amount of fermentable starch per batch (substrate), which in turn will increase the final ethanol concentration, the viscosity behaviour of LT pre-treatment mash at different solids loadings (30%, 35% and 40%) was compared to that of HT pre-treatment (30% solids). This comparison was used to maximise the solids loading for subsequent optimisation of the STARGEN^{TM} 002 enzyme. Slurry temperatures for HT and LT pre-treatment were respectively increased from 30 to 90 ^{o}C and from 30 to 48 ^{o}C at a heating rate of 4 ^{o}C per minute and maintained at the maximum temperature for 60 minutes (Figure 4-1).
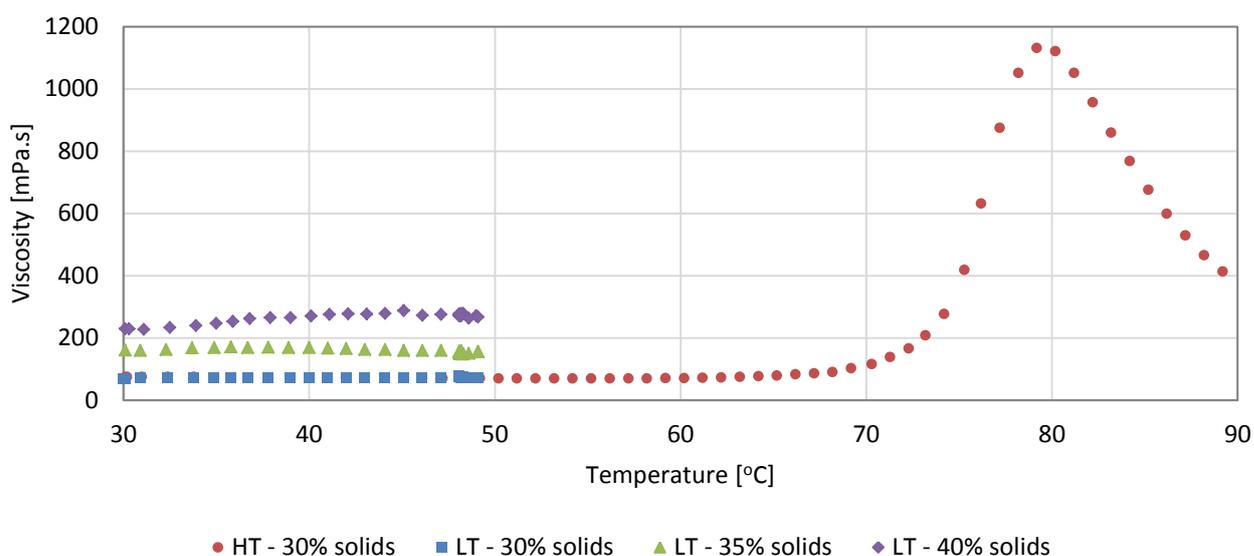
Figure 4-1: Slurry viscosity profiles with an increase in temperature (4 ^{o}C/min) during HT and LT pre-treatment at different solids loading, with a measurement taken every 15 seconds.

During the HT pre-treatment a viscosity spike, due to gelatinization, was clearly noticeable, which started at 73 ^{o}C and peaked at 80 ^{o}C, with a maximum value of 1130 mPa.s (Figure 4-1) corresponding to previous reports (Li et al., 2012; Uthumporn et al., 2013). This viscosity spike is the leading cause that prohibits the SSF with CSH process to increase solids loading per batch. Since the LT pre-treatment was carried out at sub-gelatinization temperatures no viscosity spikes were noticeable during an increase in temperature, although an exponential increase in mash viscosity was evident with an increase of solids loading from 30% to 35% and 40%.

4.1.3. Fermentation profiles

Fermentation profiles depicting residual glucose concentration and ethanol production during both production methods, using the control corn mixture as substrate, are compared in Figure 4-2. These curves can be regarded as representative of all subsequent fermentation experiments conducted using the 30 cultivars. The most prominent difference between the two methods was the initial glucose concentrations at the onset of the fermentations. The RSH process delivered a 20 g/L initial glucose concentration, while the CSH process resulted in a 50% higher initial glucose concentration of 30 g/L. This higher glucose concentration could probably be attributed to the amylase enzymes having a greater degree of accessibility to the starch during HT pre-treatment (Cinelli et al., 2015). After 24 hours the residual glucose concentration in the CSH process decreased, indicating a possible decrease in the rate of glucose release by enzymatic hydrolysis, in comparison to the rate of glucose consumption by yeast, possibly due to a decrease in the amylase enzyme activity and/or the enzymes encountering starch that was increasingly resistant to attack (Sharma et al., 2010). The residual glucose of the RSH process decreases after eight hours. This residual glucose turning point is possibly when the yeast reached its exponential growth phase. At this point the glucose consumption for the conversion to ethanol is higher than the glucose release rate from enzymatic hydrolysis.

On average, the CSH process reached a final ethanol concentration of 9.82 ± 0.13% (v/v) within 84 hours, while the RSH process achieved 9.63 ± 0.19% (v/v) within 72 hours, which corresponded to average ethanol productivities of 0.92 ± 0.01 g/L.h and 1.06 ± 0.02 g/L.h, respectively (Figure 4-2). This higher productivity for the RSH process is due to an increase in ethanol production rate after a fermentation period of 6 hours; however this high rate was only sustained up until the depletion of residual glucose, which occurred at 36 hours. Fermentation profiles depicting glycerol production concentration during both production methods, using the control corn mixture as substrate, are compared in Figure 4-3. For both the CSH and RSH processes the glycerol concentrations were similar during the first 36 hours, where after the RSH glycerol concentration stabilized at 6.54 g/L, while the CSH glycerol increased until 84 hours where it stabilized at 8.00 g/L. These stabilization points for both glycerol concentration curves corresponded with the time of residual glucose depletion in the fermentation slurry.

Negligible levels (< 0.5 g/L) of lactic acid were detected in all cultures grown using both CSH and RSH processes, indicating no increase in bacterial contamination when using the RSH process.
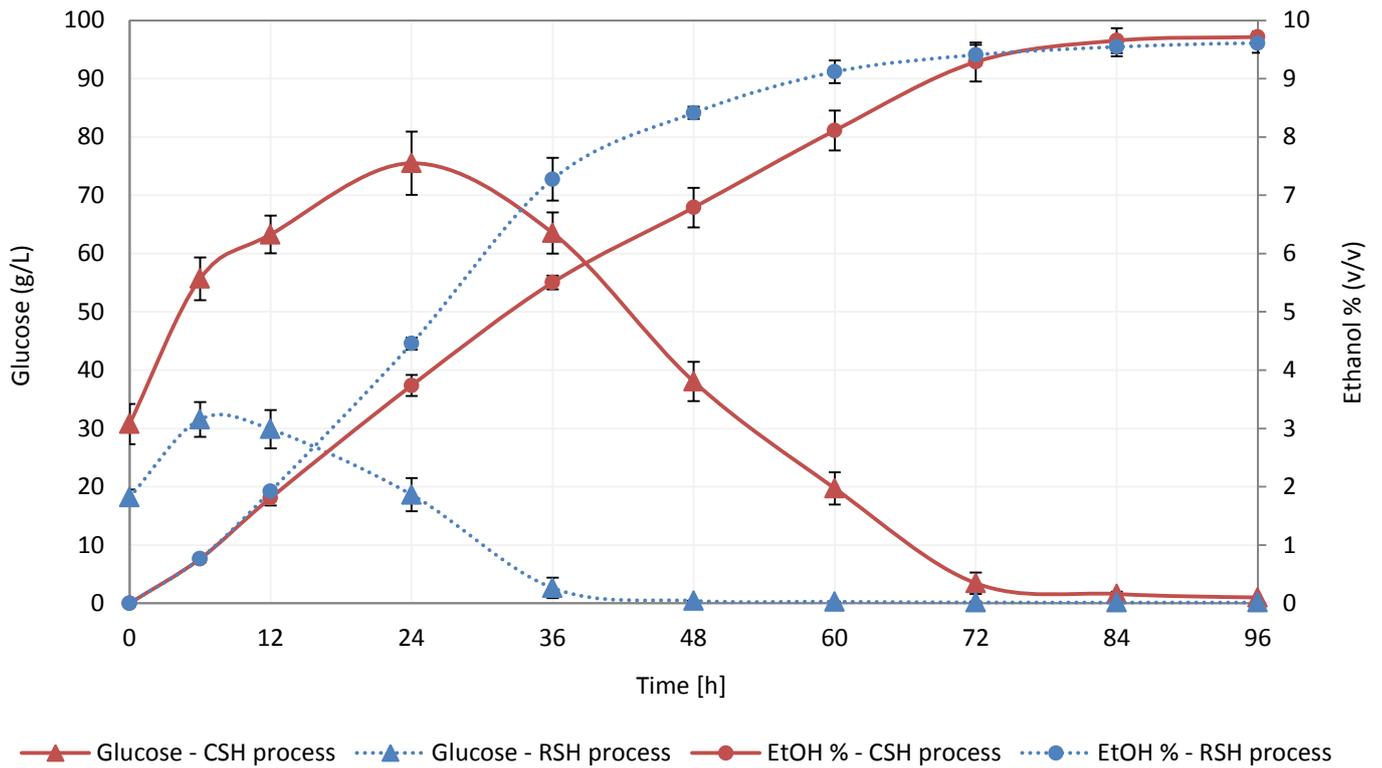
Figure 4-2: Glucose and ethanol profiles, using the control mixture as substrate, during a 96 hour fermentation period for both ethanol production methods.
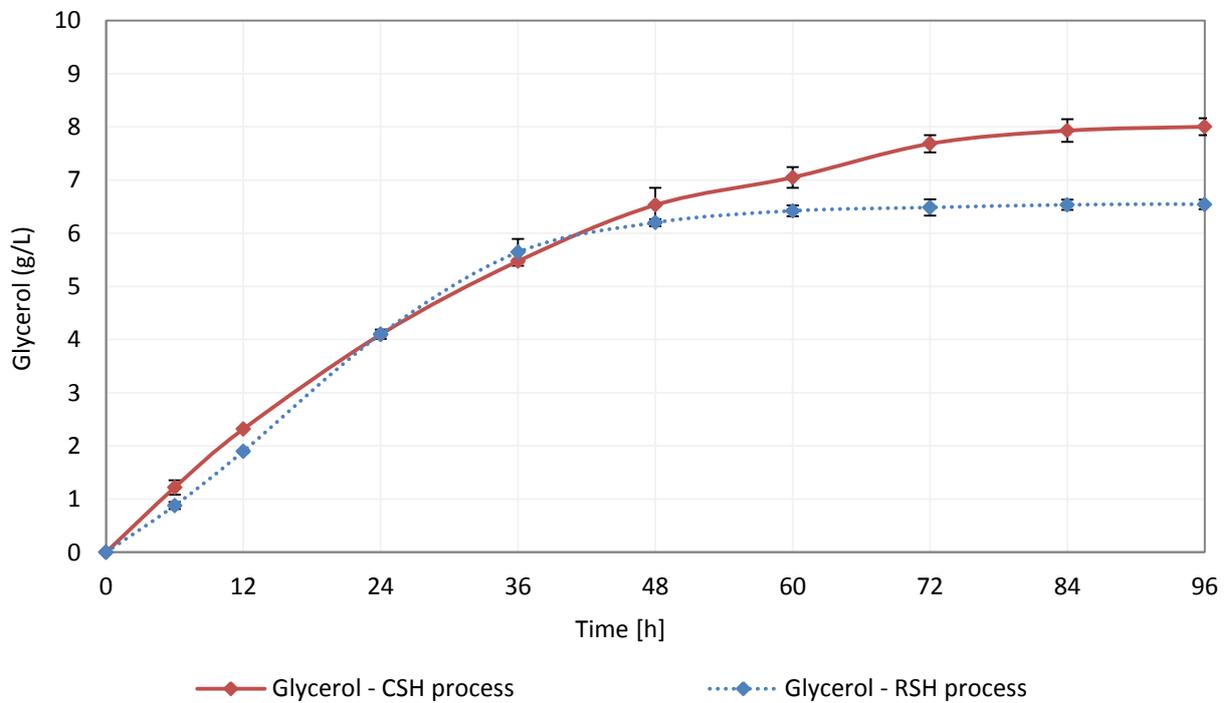

Figure 4-3: Glycerol profiles, using the control mixture as substrate, during a 96 hour fermentation period for both ethanol production methods.

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4.1.4.   Key Performance Indicators (KPIs) for both ethanol production processes

The three key performance indicators (KPIs) used to determine the success of the two ethanol production methods, namely final ethanol concentration (% v/v), ethanol productivity and ethanol yield as fraction (%) of theoretical maximum are shown in Figure 4-4, Figure 4-5 and Figure 4-6, respectively. Within each of the three KPIs a process comparison was done between the HT and RSH processes for each cultivar, using analysis of variance (ANOVA). In Figure 4-4, Figure 4-5 and Figure 4-6, a green triangle was used to indicate when the process comparison yielded a statistically significant difference ($p < 0.05$) between CSH and RSH processes for that specific cultivar.

The average values for the final ethanol concentrations (% v/v) for the 30 cultivars, using both the CSH and RSH production methods, are shown inFigure 4-4. In all cases the maximum (highest, final) ethanol concentrations were recorded at the end of the exponential growth phase, with no further accumulation of ethanol at significant levels. For the CSH process the final ethanol concentrations ranged from 8.71% to 9.91% (v/v), with cultivars M16, M8 and M5 giving the highest concentrations of 9.84%, 9.90% and 9.91% (v/v), respectively. For the RSH process the final ethanol concentrations ranged from 8.86% to 9.92% (v/v), with cultivars M16, M5 and M24 giving the highest concentrations of 9.88%, 9.90% and 9.92% (v/v), respectively. The control mixture of corn obtained final ethanol concentrations of 9.82% and 9.63% (v/v) for CSH and RSH processes, respectively. A comparison between the two methods, based on the final ethanol concentrations of all 30 corn cultivars, is shown in Figure 4-4 where a green triangle indicates a significant difference ($p < 0.05$) between methods for that specific cultivar. Twenty-five of the 30 cultivars showed no significant difference between methods, four cultivars (M6, M7, M11 and M15) showed significant higher final ethanol concentrations with the RSH process, while only one cultivar (M26) showed significant higher final ethanol concentration with the CSH process.

The second key performance indicator, productivity, is shown in Figure 4-5 for all cultivars using both the CSH and RSH production methods. For the CSH process the ethanol productivities ranged from 0.818 to 1.086 g/L.h, with cultivars M28, M24 and M5 providing the highest productivities with values of 1.076, 1.077 and 1.086 g/L.h, respectively. For the RSH process the ethanol productivities ranged from 0.906 to 1.302 g/L.h, with cultivars M19, M28 and M5 providing the highest productivities with values of 1.290, 1.299 and 1.302 g/L.h, respectively. The control corn mixture provided productivities of 0.92 and 1.05 g/L.h ethanol for CSH and RSH processes, respectively. A comparison between the two methods, based on the ethanol productivity of all 30 corn cultivars, is shown in Figure 4-5 where a green triangle indicates a significant difference ($p < 0.05$) between methods for that specific cultivar. Only one of the 30 cultivars (M24) showed no significant

difference between methods, while twenty-nine cultivars showed significant higher ethanol productivities with the RSH process.

The third performance indicator, ethanol yield as fraction (%) of theoretical maximum, is shown in Figure 4-6 for all cultivars, using both the CSH and RSH production methods. For the CSH process ethanol yield as fraction (%) of theoretical maximum ranged from 77.09% to 86.31%, where cultivars M17, M25 and M8 gave the highest performance with values of 85.38%, 86.02% and 86.31%, respectively. For the RSH process ethanol yield as fraction (%) of theoretical maximum ranged from 78.91% to 86.00%, where cultivars M28, M22 and M23 gave the highest performance with values of 85.59%, 85.74% and 86.00%, respectively. The control corn mixture had ethanol yields of 83.90% and 82.22% ethanol for CSH and RSH processes, respectively. A comparison between the two methods, based on the ethanol yield as fraction (%) of theoretical maximum of all 30 corn cultivars, is shown in Figure 4-6 where a green triangle indicates a significant difference ($p < 0.05$) between methods for that specific cultivar. Twenty-five of the 30 cultivars showed no significant difference between methods, four cultivars (M6, M7, M11 and M15) showed significant higher ethanol yields with the RSH process, while only one cultivar (M26) showed significant higher ethanol yields with the CSH process.
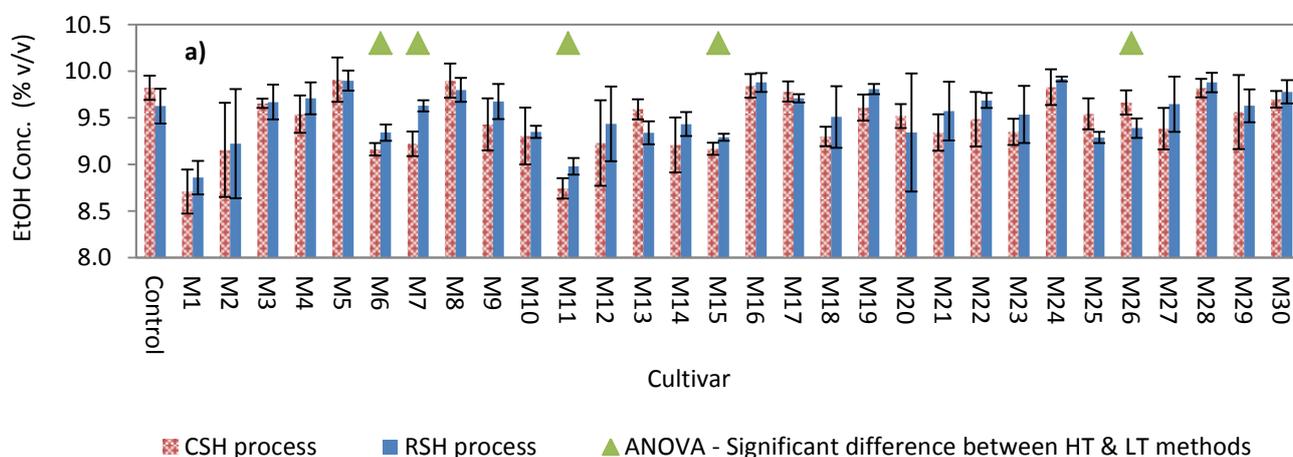
Figure 4-4: CSH and RSH process comparison with 30 cultivars as substrate based on final ethanol concentration expressed as % v/v. Green triangle indicates a significant difference between CSH and RSH process for that specific cultivar
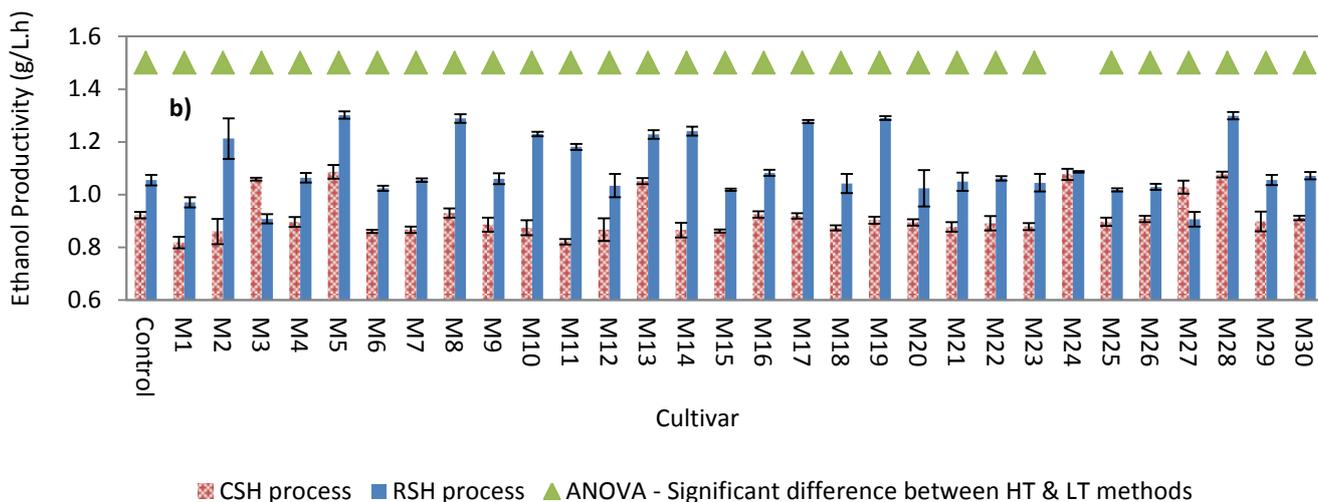
Figure 4-5: CSH and RSH process comparison with 30 cultivars as substrate based on ethanol productivity expressed as g/L.h. Green triangle indicates a significant difference between CSH and RSH process for that specific cultivar
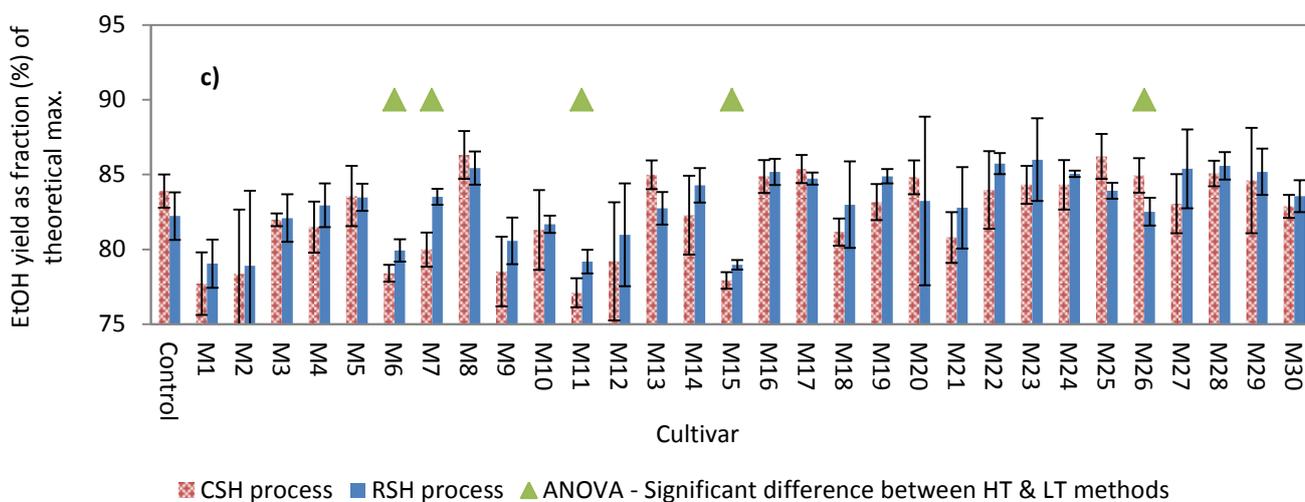


Figure 4-6: CSH and RSH process comparison with 30 cultivars as substrate based on ethanol yield as fraction (%) of theoretical maximum. Green triangle indicates a significant difference between CSH and RSH process for that specific cultivar

4.2.   RSH process optimisation

The fermentation results to follow are the optimisation of the RSH process. In these results the influences of process scale-up on ethanol production performance was also determined. The optimisation was achieved through small and medium-scale multi-response experimental designs. While scale-up influence investigation was done through a sequential increase in fermentation volume from 1 L Erlenmeyer flasks to 5 L bioreactors and finally a 150 L bioreactor. The CSH process was not optimised, since this process has already been implemented on industrial scale at the James Sedgwick distillery and has therefore already been optimised in terms of enzyme dosage and solids loading.

4.2.1.   Small-scale experimental design (1 L)

A face-centred central composite design (FCCCD) was used to optimise the RSH production method in 1 L Erlenmeyer flasks, by simultaneously varying both the solids loading and STARGEN^{TM} 002 enzyme dosage. The response variables for the design were the three KPIs, namely final ethanol concentration, ethanol productivity and ethanol yield as fraction (%) of theoretical maximum. The substrates used for the process optimisation were the control corn mixture plus the two best performing cultivars (M5 & M8) selected previously from screening experiments (section 4.1). The selection of the cultivars for process optimisation was done with a scoring system where the three KPIs where normalised to dimensionless values between zero and one. The normalisation of the three KPIs was necessary, in order for the KPIs to be comparable. See Appendix A for the table with the score of each cultivar for each KPI, as well as the final score for each cultivar.

Preliminary experiments were performed in order to determine the optimisation for both FCCCD independent variables. The boundaries of the first independent variable, namely solids loading, had an upper limit of 40% due to viscosity constraints. Viscosity profiles during pre-treatment (Figure 4-1), showed an exponential increase in viscosity with an increase in solids loading. Furthermore, a solids loading above 40% during pre-treatment resulted into a mash that could not be stirred homogenously, while a significant amount substrate got stuck to the Erlenmeyer flask wall. Additionally, pre-treatments with lower than 30% solids would not be preferred, as it would result in final ethanol concentrations that are lower than the CSH process. The boundaries of the second independent variable, namely enzyme dosage, were determined through performing RSH process runs with 35% solids at six different STARGEN^{TM} 002 enzyme dosages; 0.6, 0.8, 1.0, 1.2, 1.4 and 1.6 g/kg (Figure A. 1). Similar ethanol productivities and yields at 1.6 and 1.4 g/kg suggested the latter

dosage as the upper boundary, the lower limit of enzyme dosage was selected by considering the significant decrease of 23% in productivity between 1.0 g/kg and 0.8 g/kg. Such low productivity would result in fermentations times much longer than that of the CSH process and would not be beneficial to RSH process implementation. Therefore, FCCCD optimisation boundaries were 30% to 40% for solids loading and 1.0 to 1.4 g/kg for enzyme dosage, with a median of 35% and 1.2 g/kg respectively.

Data obtained from the optimisation experiments were used to develop models to predict the final ethanol concentrations, ethanol productivity and ethanol yield as fraction (%) of theoretical maximum when using the control corn mixture, cultivar M5 and cultivar M8 as substrates. Since models of all three substrates presented with similar trends, only the models for the best performing cultivar (M5) are shown and can be regarded as representative of all three small-scale experimental designs. The correlation between independent variables and response variables (using cultivar M5 as substrate) can be seen in Equation 1, Equation 2 and Equation 3 for final ethanol concentration, ethanol productivity and ethanol yield as fraction (%) of theoretical maximum, respectively.

$$Z_1 = -3.1909 + 0.2451X + 3.1526Y + 0.0034X^2 + 0.015XY - 1.2303Y^2 \qquad \text{Eq. 1}$$

$$Z_2 = -3.6489 + 0.2243X + 0.544Y - 0.0034X^2 + 0.0215XY - 0.3265Y^2 \qquad \text{Eq. 2}$$

$$Z_3 = 51.2143 + 0.9102X + 26.1244Y - 0.0139X^2 - 0.1379XY - 6.8342Y^2 \qquad \text{Eq. 3}$$

Where Z_1, Z_2 and Z_3 represents final ethanol concentration, ethanol productivity and ethanol yield as fraction (%) of theoretical maximum, respectively and where X and Y represent the solids loading and enzyme dosage respectively. R^2 values of 0.989, 0.725 and 0.845 were obtained for final ethanol concentration, ethanol productivity and ethanol yield as fraction (%) of theoretical maximum respectively, which indicated that the model fitted the data obtained from the runs.

Surface plots of the quadratic models predicting the three response variables, using cultivar M5 as substrate, were developed to visually illustrate the impact of solids loading and enzyme (Figure 4-7). An increase in the solids loading resulted in a linear increase in the final ethanol concentration, whereas an increase in enzyme dosage had no discernible effect on the final ethanol concentration (Figure 4-7a). This data suggested that the ranges for the experimental design were accurate and that the enzyme remained in excess. Figure 4-7c indicated that an increase in solids loading caused a drop in ethanol yield as fraction (%) of theoretical maximum, while an increase in enzyme dosage caused an improved ethanol yield as fraction (%) of theoretical maximum. Additionally, the ethanol

yield as fraction (%) of theoretical maximum decreased significantly at solid loadings in excess 36% and at enzyme dosage less than 1.2 g/kg. For productivity as a response variable (Figure 4-7b), a distinct optimum was evident corresponding to a solids loading of 37.5%, irrespective of enzyme dosage. Surface plots for the control mixture and cultivar M8 (data not shown) presented with a similar optimums with productivity as response variable (37.5% and 37.3% respectively).

a)

b)

c)
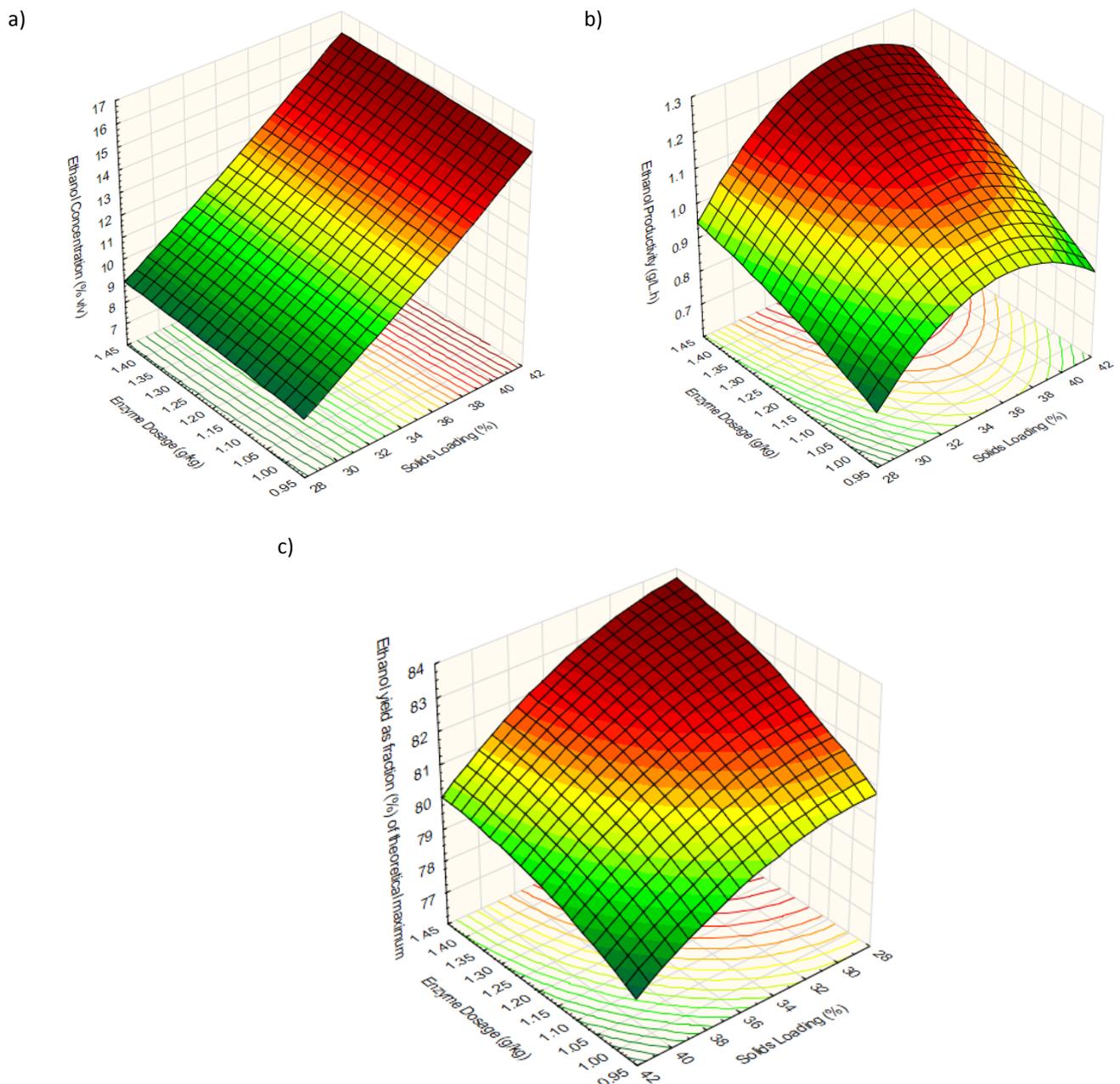
Figure 4-7: Surface plots of the quadratic models predicting the a) final ethanol concentration expressed as % v/v, b) ethanol productivity and c) ethanol yield as fraction (%) of theoretical maximum for small-scale optimisation when using cultivar M5 as substrate

To validate the quadratic models developed by the FCCCD (Equation 1 to 3), the optimum solids loading obtained from the optimisation (37.5%), together with maximum enzyme dosage (1.4 g/kg) were used. The maximum enzyme dosage was chosen, since there was no optimum for this variable in the three surface responses. Triplicate runs, in 1 L Erlenmeyer flasks, at this optimum solids loading and maximum enzyme dosage indicated an ethanol percentage 13.31 ± 0.22%, productivity of 1.29 ± 0.021 g/L.h and ethanol yield as % of theoretical max of 82.24 ± 2.01%. The quadratic models predicted an ethanol percentage of 13.57%, with a productivity of 1.23 g/L.h and an ethanol yield as % of theoretical max of 81.74%. This resulted into predicted values within 2.0%, 4.5% and 0.6% of the actual experimentally obtained values for ethanol percentage, productivity and ethanol yield as % of theoretical max respectively.

4.2.2.   Medium-scale experimental design (5 L)

Medium and pilot-scale RSH process experiments were carried out in bioreactors, in order to determine influence of process scale-up on ethanol production performance of the RSH process. The medium-scale experiments, carried out in a 5 L bioreactor with a 3 L final working volume, were a repetition of the small-scale optimisation experiments, which consisted out of a FCCCD with simultaneous variation in the solids loading and STARGEN^{TM} 002 enzyme dosage. Furthermore, the medium-scale optimisation experiments were performed with the same three substrates, namely the control mixture, cultivar M5 and cultivar M8.

Data obtained from the medium-scale optimisation experiments were used to develop models to predict the final ethanol concentration, ethanol productivity and ethanol yield as fraction (%) of theoretical maximum when using the control mixture, cultivar M5 and cultivar M8 as substrates. Since models of all three substrates presented with similar trends, only the models for the best performing cultivar (M5) are shown and can be regarded as representative of all three medium-scale experimental designs. The correlation between independent variables and response variables (using cultivar M5 as substrate) can be seen in Equation 4, Equation 5 and Equation 6 for ethanol concentration, ethanol productivity and ethanol yield as fraction (%) of theoretical maximum, respectively.

$$Z_1 = -5.8668 + 0.3962X + 3.5689Y + 0.0011X^2 + 0.015XY - 1.4211Y^2 \qquad \text{Eq. 4}$$

$$Z_2 = -3.8706 + 0.2361X + 0.6005Y - 0.0036X^2 + 0.0212Y - 0.3477Y^2 \qquad \text{Eq. 5}$$

$$Z_3 = 35.9893 + 1.7252X + 29.7782Y - 0.0265X^2 - 0.1303XY - 8.5844Y^2 \qquad \text{Eq. 6}$$

Where Z_1, Z_2 and Z_3 represents the ethanol concentration, ethanol productivity and ethanol yield as fraction (%) of theoretical maximum, respectively and where X and Y represent the solids loading and enzyme dosage respectively. R^2 values of 0.99, 0.70 and 0.88 were obtained for ethanol concentration, ethanol productivity and ethanol yield as fraction (%) of theoretical max respectively, which indicated that the models fitted the data obtained from the runs.

a)

b)

c)
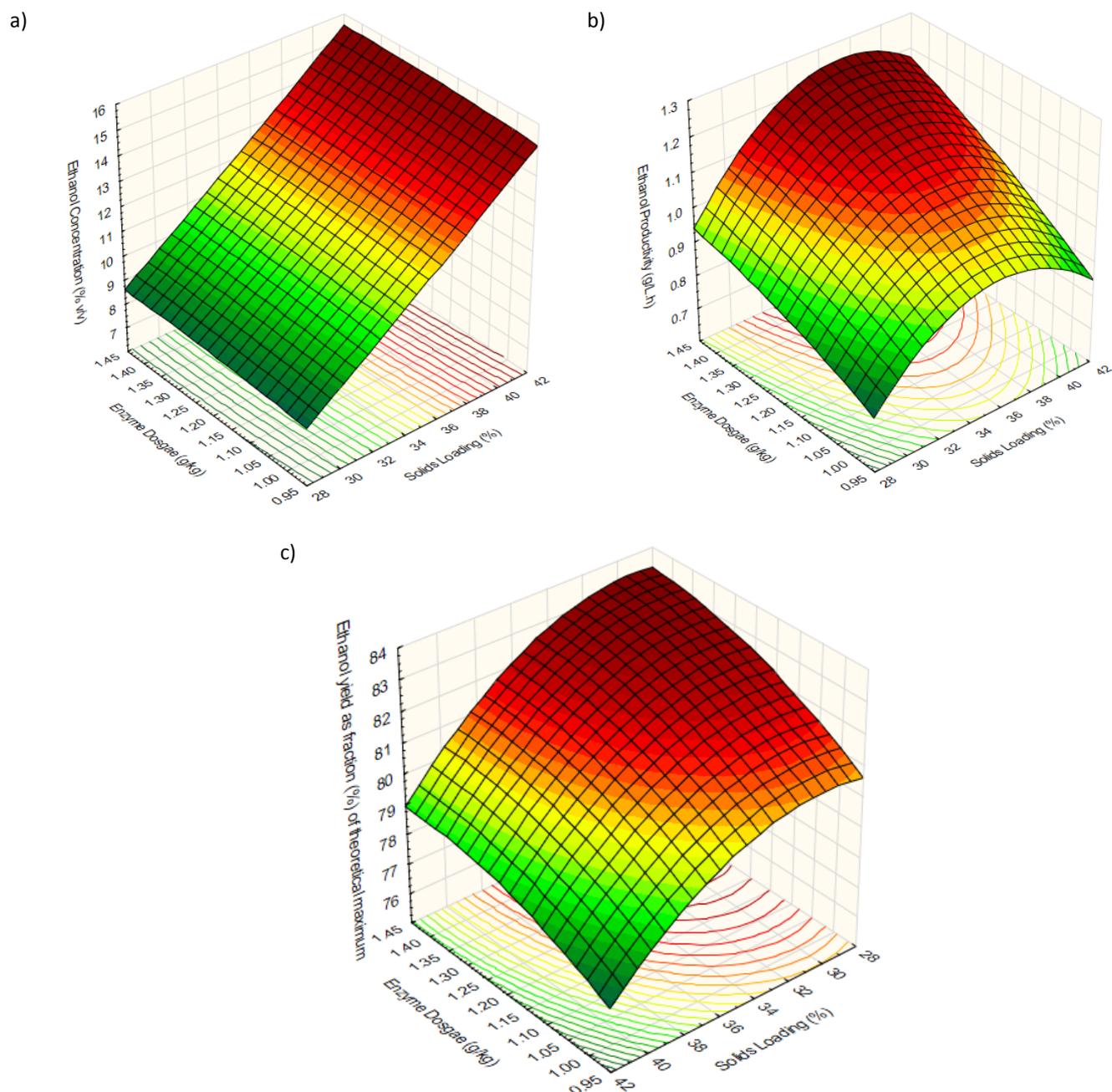
Figure 4-8: Medium-scale optimisation surface plots of the quadratic models predicting the a) final ethanol concentration expressed as % v/v, b) ethanol productivity and c) ethanol yield as fraction (%) of theoretical maximum for small-scale optimisation when using cultivar M5 as substrate

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Surface plots of the quadratic models predicting the three response variables during medium-scale optimisation, using cultivar M5 as substrate, were developed to visually illustrate the impact of solids loading and enzyme dosage (Figure 4-8). The three medium-scale surface plots were similar to that of the three surface plots during small-scale optimisation (Figure 4-7). Again an increase in solids loading caused a drop in ethanol yield as fraction (%) of theoretical maximum, while an increase in enzyme dosage caused an improved ethanol yield as fraction (%) of theoretical maximum. However, solids loading in excess of 36% during the medium-scale optimisation (Figure 4-8c) resulted in a greater decrease in ethanol yield as fraction (%) of theoretical maximum when compared to the small-scale optimisation (Figure 4-7c). This phenomenon may be attributed to lower mixing efficiencies at higher solids loading in 5 L bioreactors. Additionally, medium-scale surface plot with productivity as a response variable (Figure 4-8b) showed similar optimums compared to the small-scale optimisation. These productivity optimums were again in the region of at 37.5% solids loading irrespective of enzyme dosage for all three substrates (control mixture, cultivar M5 and cultivar M8).

Medium-scale quadratic model validation (Equation 4 to 6) was done through performing triplicate 5 L Bioreactor SSF runs with cultivar M5 as substrate. The values for the independent variables were again 37.5% for solids loading and 1.4 g/kg for the maximum enzyme dosage. The medium-scale triplicate validation runs (data not shown) indicated a final ethanol concentration 13.29 ± 0.56% (v/v), ethanol productivity of 1.25 ± 0.034 g/L.h and ethanol yield as fraction (%) of theoretical maximum of 80.31 ± 1.34%. The quadratic models predicted a final ethanol concentration of 13.54% (v/v), with an ethanol productivity of 1.19 g/L.h and an ethanol yield as fraction (%) of theoretical maximum of 81.44%. This resulted into predicted values within 1.9%, 4.6% and 1.4% of the actual experimentally obtained values for final ethanol concentration, ethanol productivity and ethanol yield as fraction (%) of theoretical maximum, respectively.

4.2.3.   Pilot-scale experiments

For pilot-scale RSH process experiments were performed in a 150 L bioreactor with a 90 L final working volume. Only two runs with preferred RSH process were completed at this scale, due to substrate availability and time constraints. These two experiments were done at the optimum solids loading (37.5%) and maximum enzyme dosage (1.4 g/kg) for the best performing corn cultivar as substrate (M5), as observed in small-scale and medium-scale optimisation.
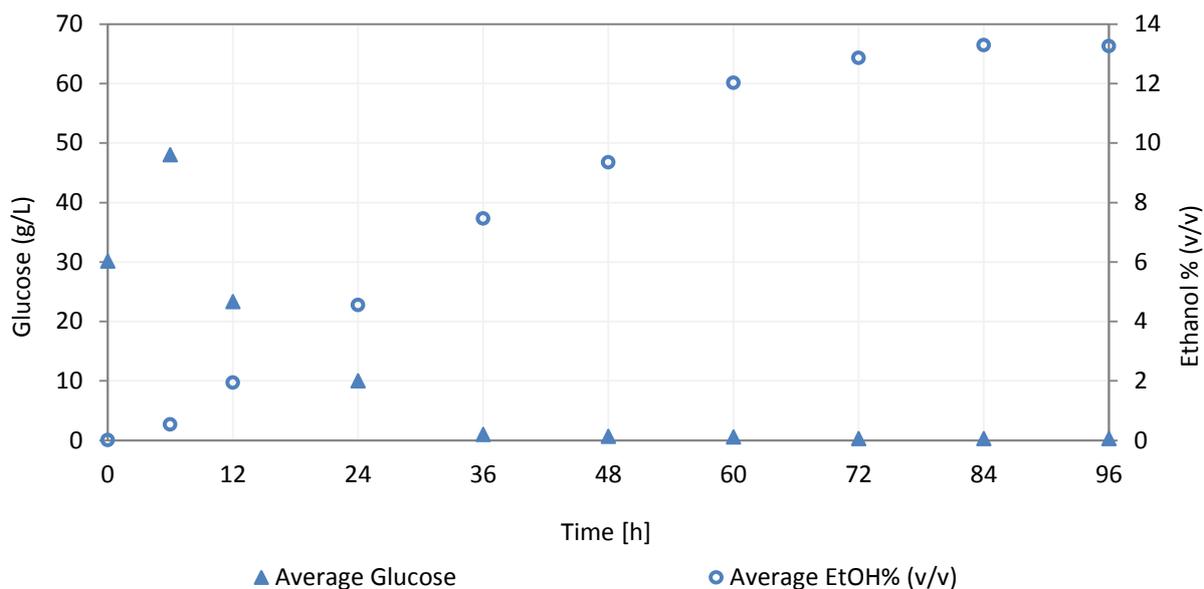
Figure 4-9: Average residual glucose and ethanol production profiles of two 150 L bioreactor fermentation runs, using cultivar M5 as substrate with the RSH process

Fermentation profiles depicting average residual glucose concentration and ethanol production of the two pilot-scale experiments, using the cultivar M5 as substrate, are showed in Figure 4-9. On average the maximum final ethanol concentration of 13.12% (v/v) was achieved within an incubation time of 84 hours at an ethanol yield of 78.4% theoretical maximum. A final comparison between small, medium and pilot-scale experiments is done in Table 4-2 when using cultivar M5 as substrate.

Table 4-2: Three KPIs for cultivar M5 when using the RSH process at different production scales

	Small-Scale (1 L)	Medium-Scale (5 L)	Pilot-Scale (150 L)
Final Ethanol Concentration (% v/v)	13.31%	13.29%	13.12%
Ethanol Productivity (g/L.h)	1.29	1.25	1.23
Ethanol yield as fraction (%) of theoretical maximum	82.24%	80.31%	78.40%

4.3.   Process simulation

4.3.1.   Aspen Plus® simulation

The pre-treatment section of the Aspen Plus® simulation developed for the industrial CSH ethanol production process at the James Sedgwick distillery is shown in Figure 4-10, while the fermentation and distillation sections of the simulation are shown in Figure 4-11. The industrial CSH ethanol production process at the James Sedgwick distillery combines batch pre-treatments and fermentations with continuous distillation. However, for the Aspen Plus® simulation, continuous pre-treatment and fermentation were chosen, where the rate per hour in the simulation was equal to the rate per batch of the industrial process. In the simulation corn/water slurry was created with milled corn (CORN), cold (WATER-1) water and hot water (WATER-2).The pre-treatment slurry was further heated with a heat exchanger (HEATX-1) using steam as a heat source, followed by a single stoichiometric reactor (MASH-R) that acted as the pre-treatment vessel in which starch was converted to glucose. Subsequent cooling of the slurry was achieved through the second heat exchanger (MASH-C) using cooling water before fermentation. The fermentation section was simulated through a combination of a single stoichiometric reactor (FERM-R) and flash drum (BEER-SEP). In the stoichiometric reactor the glucose was converted to ethanol, while the flash drum served as a vent for CO_2. Subsequent to fermentation, the fermented slurry was sent to the distillation section to be purified. The distillation section is a two-step process, consisting of a wash column (WC) and rectifier column (RC), which was simulated using 18 tray and 58 tray RadFrac distillation columns, respectively. The fermented slurry is firstly separated at the wash column into an effluent stream and a low quality ethanol stream (RC-FEED), where after the low quality ethanol stream is purified to a stream of 94.5% ethanol (ETOH).
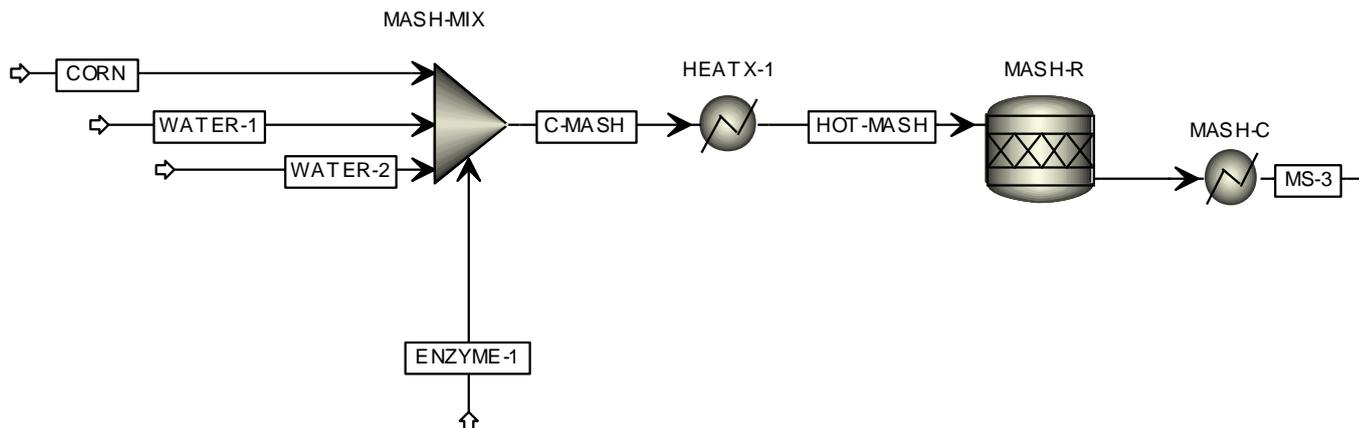
Figure 4-10: Pre-treatment section of the Aspen Plus® simulation developed for the industrial CSH ethanol production process at the James Sedgwick distillery
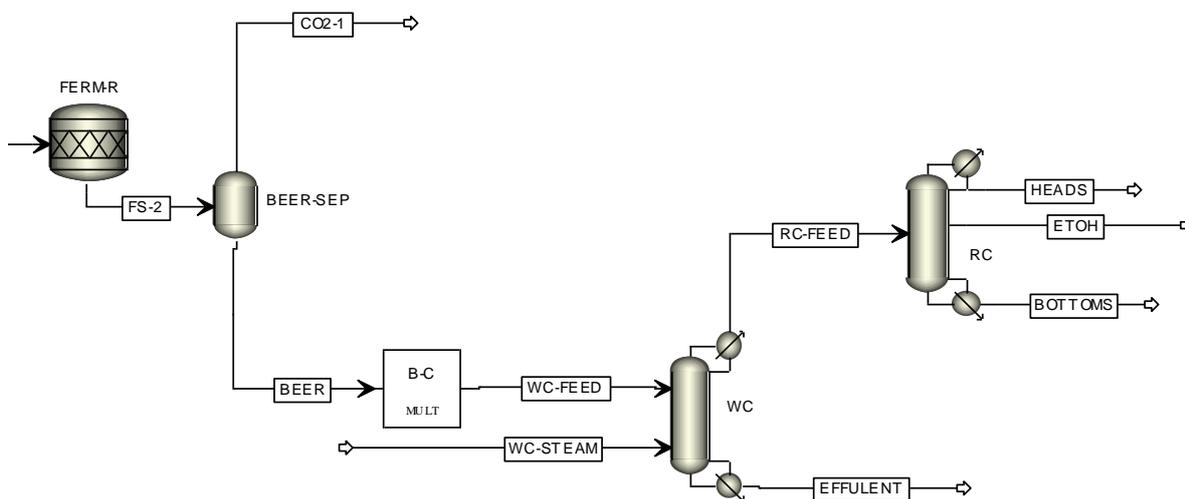
Figure 4-11: Fermentation and distillation sections of the Aspen Plus® simulation developed for the industrial CSH ethanol production process at the James Sedgwick distillery

For the Aspen Plus® simulation of the CSH process, a solids loading of 30% during pre-treatment (15 000 kg corn/batch) and final ethanol concentration of 9.6% (v/v) were chosen, to correspond to industrial practise of this method. The performance fixtures of both the CSH-Aspen Plus® simulation and industrial CSH process at the James Sedgwick distillery are shown Table 4-3. For the industrial application of the CSH process, energy demands of 2500 kg of steam per batch during pre-treatment and 12 671 kg of steam per batch during distillation were observed, while a final ethanol product of 6 365 litres per batch was achieved at 94.5% purity (J Green 2013, personal communication, 13 August). When considering the data of the CSH-Aspen Plus® simulation, pre-treatment and distillation steam requirements of 3 805 and 14 842 kg per batch were predicted. This gives an

overestimation by the CSH-Aspen Plus® simulation of 23% on the total steam requirement. Furthermore, the ethanol production for CSAH-Aspen Plus® simulation was predicted as 6 660 litres per batch, which was an overestimation of 4.6%.

Table 4-3: Performance specifications for the Actual industrial-scale CSH process compared to that of the HT-Aspen Plus® model and LT-Aspen Plus® model

Variable	Industrial CSH process	CSH-Aspen Plus® simulation	RSH-Aspen Plus® simulation
Corn (kg/batch)	15 000	15 000	18 748
Water (kg/batch)	52 594	52 594	31 247
Pre-treatment enzyme (kg/batch)	4.35	4.35	3
Solids during pre-treatment	30%	30%	37.5%
Pre-treatment steam for heating (kg/batch)	2 500	3 805	362
SSF enzyme (kg/batch)	4.80	4.80	26.25
Yeast (kg/batch)	67	67	67
Solids during SSF	22%	22%	28.5%
Final SSF volume (L)	67 000	67 000	67 000
Ethanol in beer (Wash Column feed)	9.6%	9.6%	13.3%
Steam for distillation (kg/batch)	12 671	14 842	17 471
Total steam requirement (kg/batch)	15 171	18 647	17 834
Ethanol product (L/batch)	6 365	6 660	9 061
Ethanol purity in product	94.5%	95%	95%
Steam per unit ethanol produced [kg/L]	2.38	2.80	1.97

For the Aspen Plus® simulation of the RSH process, the following parameters were chosen, based on bioreactor process optimisation; a solids loading of 37.5%, final ethanol concentration of 13.3% (v/v) and a corn batch size of 18 748 kg (compared to the 15 000 kg/batch of the CSH process). The performance fixtures of RSH-Aspen Plus® simulation are shown in Table 4-3. The first prominent difference between the predictions of the CSH- and RSH-Aspen Plus® simulations was the 40% decrease in water consumption for the RSH-Aspen Plus® simulation. The Aspen Plus® simulations predicted 52 594 kg water per batch requirement during HT pre-treatment of 30% solids, while the RSH process required only 31 247 kg water per batch to achieve the desired 37.5% solids during LT pre-treatment. Furthermore, predicted ethanol yield per batch increased by 36% with the RSH-Aspen Plus® simulation. Only 6 660 litre ethanol per batch were predicted by the CSH- Aspen Plus® simulation, compared with 9 061 litre ethanol per batch for the RSH-Aspen Plus® simulation. Another prominent difference between simulations was the required steam/energy per batch. The CSH-Aspen Plus® simulation predicted a total steam requirement of 18 647 kg per batch, which

corresponded to 2.80 kg steam per litre ethanol produced. The RSH-Aspen Plus® simulation predicted a total steam requirement of 17 834 kg per batch - corresponding to 1.97 kg steam per litre ethanol produced - which is a 30% decrease in energy requirement per unit ethanol produced.

A cost model was developed to estimate the cost per litre ethanol produced for both the CSH and RSH processes. The data in the models were obtained through combining the raw material and utility cost per unit (Table 3-1 and Table 4-4). The resulting operational costs are shown in Table 4-4. For both processes the largest part of the operational cost was the corn per batch, which was 73% and 78% of the total cost for the CSH and RSH process, respectively. The second highest cost for each process was water consumption for the CSH process at 11% of the total costs and yeast usage for the RSH process at 8% of the total costs. The cost for steam during pre-treatment and distillation section for the CSH and RSH processes were at 6% and 5% of the total costs, respectively. The predicted total operating cost per litre ethanol produced for each processes are shown in Table 4-5. R 8.97 was required for the CSH process to produce one litre of ethanol, while the RSH process required R 7.70, which is 14% less.

Table 4-4: Costs of raw materials and utilities per batch for the CSH and RSH processes

Cost per batch	CSH-Aspen Plus® simulation		RSH-Aspen Plus® simulation	
	Value	% of total	Value	% of total
Corn	R 43 500	73%	R 54 369	78%
Water	R 6 837	11%	R 4062	6%
Pre-treatment Enzyme	R 282	< 1%	R 195	< 1%
SSF Enzyme	R 456	1%	R 2 625	4%
Yeast	R 5 293	9%	R 5 293	8%
Steam	R 3 357	6%	R 3 211	5%

Table 4-5: Costs per litre ethanol produced for the CSH and RSH processes

Variable	CSH-Aspen Plus® simulation	RSH-Aspen Plus® simulation
Cost per batch	R 59 725	R 69 755
Ethanol (L) per batch	6 660	9 061
Cost per litre ethanol	R 8.97	R 7.70

5. **DISCUSSION**

5.1. **Process comparison by screening with multiple cultivars**

Comparison of ethanol production performance by two production methods revealed how different pre-treatment temperatures and hydrolysing enzymes affected starch hydrolysis and consequently yeast fermentation ethanol. A comparison of three well-defined KPIs (final ethanol concentration, ethanol productivity and ethanol yield as fraction (%) of theoretical maximum), together with the screening using 30 different corn cultivars as substrates, provided the platform for a comprehensive comparison between the CSH and RSH production processes. This comparison also included corn composition variations, such as total and resistant starch content.

When considering the residual glucose concentration profiles (Figure 4-2), the RSH process had a 20 g/L glucose concentration at the onset of the fermentation compared to the 30 g/L of the CSH process, which was a 50% increase. This increase between processes is similar to previously observed values when comparing STARGEN™ 001 to other CSH enzymes (Sharma et al., 2010; Wang et al., 2007). This phenomenon can be attributed to α-amylase having a greater degree of accessibility to the starch during pre-treatment at temperatures above gelatinization, as done in the CSH process. During gelatinization of the starch molecules in the CSH process the granules became hydrated and start to swell, leading to the loss of crystalline structure, thus increasing enzyme accessibility to hydrolyse starch to dextrins and glucose (Zou et al., 2012; Naguleswaran et al., 2012; Cinelli et al., 2015). Figure 4-1 clearly shows the gelatinization phase (viscosity spike) during HT pre-treatment, while the gelatinization phase is absent in the LT pre-treatment.

The final ethanol concentrations and ethanol yield as fraction (%) of theoretical maximum results (Figure 4-4 and Figure 4-5, respectively) cannot show beyond any doubt that either the CSH or RSH processes gave higher final ethanol concentrations or yields, since 83% of the cultivars showed no significant difference between the two methods. Previous published work showed inconsistent conclusions on whether or not the methods utilizing STARGEN™ enzymes were able to produce higher final ethanol concentrations and ethanol yields compared to conventional CSH process with α- and glucoamylase enzymes. Two studies found no significant difference in the final ethanol concentrations, i.e. Sharma et al. (2010) studied effects of resistant starch on ethanol production from corn starch, and Wang et al. (2007) compared ethanol production from different enzymes cocktails on whole corn kernels. Data from a different study by Sharma et al. (2007) showed methods with conventional α- and glucoamylase enzymes achieved higher final ethanol

concentrations compared to STARGEN[TM] 001. While a review from Cinelli et al. (2015) stood in contrast to all the above studies, stating that lower pre-treatment temperatures (RSH process) lead to the exclusion of undersirable side reactions, such as the Maillard reaction, thus higher ethanol yield as fraction (%) of theoretical maximum.

To further analyse these results, the portion of RS for each cultivar was taken into consideration. The RS values for the 30 cultivars (0-5%) corresponded to corn starch with an amylose content of between 0 and 20% (Sajilata et al., 2006), which is comparable to that of waxy and regular dent corn respectively (Themeier et al., 2005). An inversely proportional correlation between RS and ethanol yield as fraction (%) of theoretical maximum was seen for both methods (Figure A. 2), which is due to the resistance of RS to enzymatic hydrolysis (Xie et al., 2006). When considering the four cultivars that showed significant higher final ethanol concentrations and ethanol yields as fraction (%) of theoretical maximum with the RSH process, all four indicated elevated levels of RS (RS > 3%). Additionally, seven cultivars with elevated RS levels showed no significant difference for final ethanol concentrations and ethanol yields as fraction (%) of theoretical maximum between the two methods. Thus, it cannot be showed beyond any doubt that RSH process was able to hydrolyse a larger portion of RS than the CSH process. However, it can be concluded that the RSH process can hydrolyse at least the same amount of RS compared to the CSH process. This is comparable to a previous report (Sharma et al., 2010).

Data for ethanol productivity (second KPI) showed that 29 of the 30 cultivars exhibited greater performance with the RSH process. This shows beyond any doubt that the RSH process was superior to the CSH process in terms of productivity. This increase in productivity for the RSH process, was mainly due to a 12 hour shorter incubation time (72 vs 84 hours), which in turn was due to a superior fermentation rate for the RSH process between 12 and 36 hours. High initial glucose concentration that caused osmotic stress on yeast cells during the CSH process may explain the slower ethanol production rate. However, when considering the glycerol production profiles (Figure 4-3), both processes had the same glycerol concentration between 12 and 36 hours. Thus, yeast cell osmotic stress, often revealed by increased glycerol production (Vijaikishore & Karanth, 1984), is unlikely to be the cause of a sluggish ethanol production rate for the CSH process, due to a lack of elevated glycerol concentrations during 12 to 36 hours (Wang et al., 2001). However, Lin et al. (2012) investigated the influence of substrate concentration on batch fermentation and found that initial glucose concentrations above a critical point (65 g/L) will prolong incubation time when using *S. Cerevisiae*. Even though this critical point of initial glucose concentration is higher than that of the CSH and RSH processes (20 and 40 g/L, respectively), it is believed that these trends are comparable. Thus, it is hypothesised that the mechanism behind high substrate concentrations inhibiting

fermentation rate is the occurrence the Crabtree effect during the first few hours of the fermentation where respiration (cell growth) is suppressed, which leads to low biomass production and consequently a low exponential grow phase (Lei et al., 2001).

For this study it was important to test the hypothesis that ethanol production with the RSH process will not be affected significantly by bacterial contamination. This hypothesis is supported by negligible levels (< 0.5 g/L) of lactic acid in all the fermentation runs performed under non-sterile conditions, together with the fact that 83% of the cultivars showed no significant difference in final ethanol concentration between the CSH and RSH processes. The absence of significant levels of bacterial contamination may be explained when considering the yeast inoculum size. Narendranath & Power 2004 found that bacterial contaminations (*Lactobacillus plantarum* and *Lactobacillus paracasei*) as high as 1×10^8 cells/mL mash did not lower final ethanol concentration of fermentation mashes as long as the yeast inoculum size was higher than 20×10^6 cells/mL mash. Since both the CSH and RSH processes fermentation runs had a yeast inoculum size of 18×10^6 cells/mL slurry, it can be concluded that the yeast inoculum size was one of the main contributors to the absence of any significant level bacterial contamination. Prolonged operation of the RSH process under industrial conditions at large scale has been demonstrated for fuel ethanol production at POET in the USA, although it is not known what measures were required to control the occurrence of bacterial contamination in these process plants (Schill, 2013; POET, 2015).

5.2.   Small-Scale Optimisation

Using the final ethanol concentration, ethanol productivity and ethanol yield as fraction (%) of theoretical maximum as the key response variables, the solids loading and enzyme dosage as independent variables were simultaneously optimised through a faced centred central composite design (FCCCD) at 1 L scale. A compromise between these two independent variables was required, since excessively high solids loading in the mash could lead to decreases in enzyme efficiency and hence, decreases in ethanol yield as fraction (%) of theoretical maximum, whereas unnecessarily high enzyme loadings would lead to increased process cost.

The increase in final ethanol concentration with an increase in solids loading (Figure 4-7a) was due to the increase in fermentable substrate (glucose) that could potentially be converted into ethanol. When considering Figure 4-7c, the drop in ethanol yield as fraction (%) of theoretical maximum at high solids loading suggested that not all available substrate was converted to ethanol, even though

a high final ethanol concentration was achieved. It is hypothesised that the unconverted substrate can be attributed to poor mass transfer properties of the high solids mash, which limited the enzyme efficiency (Rosgaard et al., 2007; Pietrzak & Kawa-Rygielska, 2015). The control mixture (with 73.4% total starch) delivered a final ethanol concentration of 12.02% (v/v) at 35% solids and 1.4 g/kg enzyme dosage, which corresponds to 434 litre ethanol per ton of corn. A similar study also untilizing STARGEN[TM] 002 (Gohel & Duan, 2011), found a yield of 449 litres of ethanol per ton of Indian broken rice with a enzyme dosage of 1.5 g/kg and 25% solids during fermentation. While Sharma et al. (2007) reported 9.10% (v/v) ethanol at 15% solids and a STARGEN[TM] 001 dosage of 2.8 g/kg.

A distinct optimum was evident with productivity as the response variable (Figure 4-7b), corresponding to a solids loading of 37.5%, irrespective of enzyme dosage. This data implied productivity could be maximised even at very low enzyme dosages, provided the 37.5% solids threshold is not exceeded. On the other hand, no distinct maximum enzyme dosage was evident, which is conceivable since large increases in the enzyme dosages would result in dramatically improved productivity, although such increases in enzyme could have serious cost implications for the RSH production process. Such increases in enzyme dosage might be possible if recombinant amylase-producing yeast is used. However yeast is limited in its capacity to produce amylases and might struggle to provide the same quantities of amylases as a standard STARGEN[TM] dosage would (Görgens et al., 2015). Therefore, in order to find an optimum STARGEN[TM] 002 dosage, a model incorporating process costs and earnings needs to be used.

It is recommended that other types of mixing techniques during pre-treatment are investigated in order to increase the potential higher solids loading, which will lead to higher ethanol yield per kg substrate. A study by Xu & Duan (2010) showed that 35% solids during SSF was possible when using STARGEN[TM] as a hydrolysing enzyme and a sorghum substrate. This high gravity fermenation corresponded with a ethanol production of 20% (v/v) within only 90 hours.

5.3. Scale-up to medium (5 L) and pilot (150 L) scale

Process scale-up was done to investigate if small-scale performance of the RSH process could be replicated at medium- and pilot-scale, which is an indication of potential industrial application. Furthermore, process scale-ups are also used to compare plant throughput of a specific method with another. Production throughput is highly sensitive to factors, such as material transfer times and heat-up rates, which are usually difficult to identity during small-scale experiments. This was the

case with an increase in bioreactor volume that resulted in increased heat-up and cool-down periods during pre-treatment. Process analysis of the CSH process at the James Sedgwick distillery showed that 22% of the total pre-treatment time was spend on slurry heat-up from 50 to 88°C. Since the LT pre-treatment is performed below 50°C, the heat-up time is not necessary. Furthermore, the RSH process has shown higher ethanol productivities compared to the CSH process. Therefore, a decrease of 22% in pre-treatment time, together with higher fermentation productivity, suggests that the RSH process will have a higher production throughput on industrial-scale compared to the CSH process.

RSH process optimisation was performed at medium-scale (5 L bioreactors; Figure 4-8) using a slurry volume six times larger than that of the small-scale optimisation (Figure 4-7). When comparing the small- and medium-scale surface plots (Figure 4-7 and Figure 4-8), a sharper drop in ethanol yields beyond 36% solids was seen for the larger fermentation volume. When comparing the pilot-scale experiments to that of small-scale (A final comparison between small, medium and pilot-scale experiments is done in Table 4-2 when using cultivar M5 as substrate.

Table 4-2), a 4% decrease in ethanol yield as fraction (%) of theoretical maximum was seen with scale-up from 1 L to 150 L. Mixing effects could thus be observed for both the 5 L and 150 L bioreactors, compared to the small-scale experiments. The pilot-scale mixing was less efficient compared to small-scale, especially during pre-treatment when it was difficult to achieve a homogenous corn-water mixture. During this period stationary corn "pockets" appeared behind mixing baffles, which limited the access of enzyme to these starch molecules. A pilot plant study (Chu-Ky et al., 2015) using STARGEN^{TM} 002 with a rice substrate reported a decrease of 3% in ethanol yield as fraction (%) of theoretical maximum when scale-up was done from small-scale to pilot-scale volume (25 L).

With a final ethanol concentration of 13.12% (v/v) and productivity of 1.23 g/L.h achieved at pilot-scale it can be concluded that the RSH process was able achieve high ethanol production performance at larger volumes. Therefore, it is recommended that the RSH process should be tested on an industrial-scale at solids loading not higher than 37.5% at an enzyme dosage of 1.4 g/kg.

5.4.   Process Simulation

The CSH process is a well-established ethanol production method in the industry, with typical energy requirements well known and published numerous times in literature. In contrast energy requirements for the RSH process has not yet been well defined (Robertson et al., 2006; Cinelli et al., 2015). Therefore, the development of a simulation to predict energy requirements for the RSH process was crucial for the comparison of the CSH and RSH processes, as well as for the calculation of maximum allowable STARGEN™ 002 cost.

A performance analysis of the CSH-Aspen Plus® simulation and a comparison with the industrial CSH process (Table 4-3) showed that the CSH-Aspen Plus® simulation overestimated the total steam requirements and ethanol production by 23% and 4.6%, respectively. The CSH-Aspen Plus® simulation was therefore only used for comparisons between CSH and RSH processes, taking into consideration the inherent inaccuracies of the energy balance calculations predicted by Aspen Plus® software.

When considering the results of both the CSH- and RSH-Aspen Plus® simulations (Table 4-3), the RSH process had multiple advantageous over the CSH process; such as a 40% reduction in water consumption and a 36% higher ethanol yield per batch. These two advantageous were made possible by the utilization of LT pre-treatment instead of HT pre-treatment during the RSH process. Sub-gelatinization temperatures during LT pre-treatment resulted in the avoidance of a starch gelatinization phase, which meant that the RSH process could be carried out at a high solids loading of 37.5%, compared to 30% solids during the CSH process. Since the slurry volumes for both processes were the same, the higher solids loading for the RSH process resulted in lower water consumption, as well as more fermentable substrate to be converted to ethanol. The reductions in water consumption and increase in ethanol yield per batch are comparable to results found in a study by Kollaras et al. (2011), which investigated the techno-economic implications of the high gravity corn mash fermentations.

In terms of energy usage the RSH process also outperformed the CSH process, with 30% less steam required per litre ethanol produced. This reduction in steam requirement by the RSH process was due to the less water that had to be separated from the ethanol during distillation, as well as a lower pre-treatment temperature. The LT pre-treatment for the RSH process had a temperature of 48 ^{o}C, compared to 90 ^{o}C for the HT pre-treatment during the CSH process.

Closer inspection of the total costs for each process (Table 4-4) showed that the total enzyme cost for the RSH process was higher, compared to the CSH process. These high enzyme cost for the RSH process was due to a combination of high STARGEN^{TM} 002 required dosages and enzyme cost. The STARGEN^{TM} 002 required dosages was still relatively high even after enzyme dosage optimisation, which means that the high energy dosages are an inherent drawback of the RSH process. However, even with these high STARGEN^{TM} 002 dosage requirements and enzyme cost, the RSH process still had a 14% lower cost per litre ethanol produced, compared to the CSH process (Table 4-5). It can be concluded that the energy saving and higher ethanol yield per batch for the RSH process outweighed the large enzyme dosage requirements and cost.

Based on the prediction that the RSH process is more energy efficient than the CSH process, together with 14% lower production cost per litre ethanol for the RSH process, it is recommended that this process be tested on industrial-scale with the intention of industrial implementation.

6. **CONCLUSION & RECOMMENDATIONS**

The aim of this study was to compare the cooked starch hydrolysis (CSH) and raw starch hydrolysis (RSH) production methods, using small-scale simultaneous saccharification and fermentation (SSF) experiments with 30 different corn cultivars as substrate. The information obtained from the small-scale experimental work was used to optimise the RSH process and determine whether the process performance can be replicated in pilot-plant scale with optimal process parameters, such as solids loading and enzyme dosage. An Aspen Plus® simulation was developed, based on the industrial ethanol production process at the James Sedgwick distillery, to assist in the prediction of energy requirements for the RSH process, as well as to predict total cost per unit ethanol produced.

A thorough process comparison between the CSH and RSH processes showed that no significant performance difference could be established between methods when based on either final ethanol concentration or ethanol yield as fraction (%) of theoretical maximum. However, the comparison showed beyond any doubt that the RSH process was able to produce ethanol at a higher productivity, which was mainly due to a higher fermentation rate during 12 to 36 hours of the fermentation period. Thus, allowing a greater annual throughput for industrial facilities at higher solids loadings, incurring significant cost savings relative to the CSH process.

Negligible levels (< 0.5 g/L) of lactic acid in all the fermentation runs performed under non-sterile conditions indicate that ethanol production with the RSH process was not affected significantly by bacterial contamination. This absence of bacterial contamination, even with non-sterile conditions, can be attributed to the large yeast inoculum size of 18×10^6 cells/mL fermentation slurry.

The absence starch gelatinization during the RSH process was favourable and led to the opportunity for higher gravity fermentation compared to the CSH process. The increase in solids loading resulted in an exponential increase in viscosity, which caused mixing and mass transfer complications at solids loadings higher than 40%. This upper limit of solids loading for the RSH process has not yet - to the knowledge of this author - been described in literature. Multi-response optimisation was successfully used to find an optimum for solids loading at 37.5% for the RSH process. However, no optimum for the enzyme dosage was found.

Pilot-scale experiments resulted in final ethanol concentration of 13.12% (v/v) at a productivity of 1.23 g/L.h. These results are within 5% of ethanol performance fixtures produced in 1 L Erlenmeyer flask experiments, which leads to the conclusion that the RSH process was able achieve to replicate small-scale performance on pilot-scale (150 L). It is recommended that the RSH process should be

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tested on an industrial-scale at solids loading not higher than 37.5% at an enzyme dosage of 1.4 g/kg.

With the help of an Aspen Plus® simulation the energy requirements for the RSH process were successfully predicted. The RSH process used 1.97 kg steam per litre ethanol produced, compared to the 2.80 kg steam per litre ethanol of the CSH process, which was mainly due to the absence of high pre-treatment temperatures during LT pre-treatment. The simulation predicted a total cost of R 7.70 per litre ethanol produced for the RSH process, which was 14% less (R 8.97) than the cost for the CSH process. These fixtures quantify the energy usage and costs of a RSH process when using STARGEN^{TM} 002 as RSHE when based on an existing industrial production process. This information was limited in literature and will therefore add to the development and implementation of RSHEs in the ethanol industry.

RSHEs, such as STARGEN^{TM} 002, are ready to be implemented at an industrial-scale ethanol production process, such as the James Sedgwick distillery. The advantages of the RSH process outweigh the drawbacks such as high enzyme dosage requirements. The RSH process, which is a more energy efficient when compared to the CSH process, is definitely an attractive option in a future filled with high energy costs and possible carbon taxation.

In light of the work of this study, the following recommendations are made:

- The numerous advantages of the RSH process over the CSH process are due to the possibility of the RSH process to carry out high gravity fermentations. The main constraint to further increase solids loading of the RSH porcess is the inibility to mixing homogenously above 40% during pre-treatment. Therefore, other types of mixing techniques during pre-treatment for the RSH process need to be investigated in order to furhter increase the potential higher solids loading, which will lead to higher ethanol yield per kg substrate, together with less water and energy consumption.

- The cost model for the RSH process is based on an Aspen Plus® simulation that only determines the ethanol yield per batch. The main advantage of the RSH process is a higher productivity and is thus not included in a model that only considers one ethanol batch. The cost model should be done over a time period, e.g. one year, which will incorporate the productivity advantage of the RSH process.

REFERENCES

Adams, J.M. et al., 2011. Scanning Electron Microscopy and Fermentation Studies on Selected Known Maize Starch Mutants Using STARGEN™ Enzyme Blends. *Bioenergy Research*.

Berry, C.S., 1986. Resistant starch: formation and measurement of starch that survives exhaustive digestion and amylolytic enzymesduring the determination of dietary fiber. *Journal of Cereal Science*, 4, pp.301-14.

Broda, M. & Grajek, W., 2009. Ammonia Disinfection of Grains Intended for Ethanol Fermetnation. *ACTA Scientiarum Polonorum Technologia Alimentaria*, 8, pp.33-38.

Chu-Ky, S., Pham, T.H., Bui, K.L. & Nguyen, T.T., 2015. Simultaneous Liquefaction, Saccharification and Fermentation at Very High Gravity of Rice at Pilot Scale for Potable Ethanol Production and Distillers Dried Grains Composition. *Food and Bioproducts Processing*, http;//dx.doi.org/10.1016/j.fbp.2015.10.003.

Cinelli, B.A., Castilho, L.R., Freire, D.M. & Castro, A.M., 2015. A brief review on the emerging technology of ethanol production by cold hydrolysis of raw starch. *Fuel*, 150, pp.721-29.

Curá, J.A., Jansson, P.E. & Krisman, C.R., 1995. Amylose is not strictly linear. *Starch*, 47, pp.207-09.

Evans, A. & Thompson, D.B., 2004. Resistance to alpha-amylase digestion in four native high-amylose corn starches. *Cereal Chemistry*, 81, pp.31-37.

Genencor, 2009. *Granular Starch Hydrolyzing Enzyme for Ethanol Production,* New York: Danisco US Inc.

Gohel, V. & Duan, G., 2011. No-Cook Process for Ethanol Production Using Indian Broken Rice and Pearl Millet. *International Journal of Microbiology*.

Gohel, V. & Duan, G., 2011. No-Cook Process for Ethanol Production Using Indian Broken Rice and Pearl Millet. *International Journal of Microbiology*, 2012, pp.1-9.

Görgens, J.F., Bressler, D.C. & van Rensburg, J., 2015. Engineering Saccharomyces cerevisiae for direct conversion of raw, uncooked or granular starch to ethanol. *Critical Reviews in Biotechnology*, 35(3), pp.369-91.

Haralampu, S.G., 2000. Resistant starch: a review of physical properties and biological impact of RS3. *Carbohydrate Polymers*, 41, pp.285-92.

Jacques, K.A., Lyons, T.P. & Kelsall, D.R., 2003. *The Alcohol Textbook*. 4th ed. Nottingham: Nottingham University Press.

Kimura, A. & Robyt, J.F., 1995. Reaction of enzymes with starch granules: kinetics and products of the reaction with glucoamlase. *Carbohydrate Research*, 277, pp.87-107.

Kollaras , A. et al., 2011. Techno-economic implications of improved high gravity corn mash fermentation. *Bioresource Technology*, 102, pp.7521-25.

Kwiatkowski, J.R., McAloon, A.J., Taylor, F. & Johnston, D.B., 2006. Modeling the process and costs of fuel ethanol production by the corn dry-grind process. *Industrial Crops and Products*, 23, pp.288-96.

Lei, F., Rotboll, M. & Jorgensen, S.B., 2001. A biochemically structured model for Saccharomyces cereisiae. *Biotechnology*, 88, pp.205-21.

Lin, Y. et al., 2012. Factors affecting ethanol fermentation using Saccharomyces cerevisiae BY4742. *Biomass and Bioenergy*, 47, pp.395-401.

Li, J., Vasanthan, T. & Bressler, D.C., 2012. Improved cold starch hydrolysis with urea addition and heat treatment at subgelatinization temperature. *Carbohydrate Polymers*, 87, pp.1646-56.

Megazyme, 2014. *Resistant Starch Assay Procedure,* Wicklow: Megazyme Internationaln Ireland.

Naguleswaran, S. et al., 2012. Amylolysis of large and small granules of native triticale, wheat and corn starches using a mixture of alpha-amylase and glucoamylase. *Carbohydrate Polymers*, 88, pp.864-74.

Narendranath, N.V., Hynes, S.H., Thomas, K.C. & Ingledew, W.M., 1997. Effects of Lactobacilli on Yeast-Catalyzed Ethanol Fermentations. *Applied and Enviromental Microbiology*, 63, pp.4158-63.

Narendranath, N.V. & Power, R., 2004. Effect of yeast inoculation rate on the metabolism of contaminating lactobacilli during fermentation of corn mash. *Journal of Industrial Microbiology and Biotechnology*, 31, pp.581-84.

Narendranath, N.V., Thomas, K.C. & Ingledew, W.M., 2001. Effects of acetic acid and lactic acid on the growth of Saccharomyces cerevisiae. *Journal of Industrial Microbiology & Biotechnology*, 26, pp.171-77.

Nevoigt, E., 2008. Progress in Metabolic Engineering of Saccharomyces cerevisiae. *Microbiology and Molecular Biology Reviews*, 72(2), pp.379-412.

Nigam, P. & Singh, D., 1995. Enzyme and microbial systems involved in starch processing. *Enzyme and Microbial Technology*, 17, pp.770-78.

Olmstead, J., 2012. *Bugs in the System: How the FDA fails to Regulate Antibiotics in Ethanol Production*. Insitiue for Agriculture and Trade Policy.

Pietrzak, W. & Kawa-Rygielska, J., 2015. Simultaneous saccharification and ethanol fermentation of waste wheat–rye bread at very high solids loading: Effect of enzymatic liquefaction conditions. *Fuel*, 147, pp.236-42.

POET, 2015. *POET's Technology Advantage*. [Online] Available at: http://www.poet.com/advantage [Accessed 30 October 2015].

Puligundla, P., Smogrovicova, D., Obulam, V.S. & Ko, S., 2011. Very high gravity (VHG) ethanolic brewing and fermentation: a research update. *Journal of Indrustrial Microbiology and Biotechnology*, 38, pp.1133-44.

Robertson, G.H. et al., 2006. Native or Raw Digestion: A Key Step in Energy Efficient Biorefining of Grain. *Journal of Agricultural and Food Chemistry*, 54, pp.353-56.

Rosgaard, L. et al., 2007. Effects of Substrate Loading on Enzymatic Hydrolysis and Viscosity of Pretreated Barley Straw. *Applied Biochemistry and Biotechnology*, 143, pp.27-40.

Saha, B.C., Nichols, N.N., Qureshi, N. & Cotta, M.A., 2011. Comparison of separate hydrolysis and fermentation and simultaneous saccharification and fermentation processes for ethanol production from wheat straw. *Applied Microbiology and Biotecnology*, 92, pp.865-74.

Sajilata, M.G., Singhal, R.S. & Kulkarni, R., 2006. Resistant Starch - A Review. *Comprehensive Reviews in Food Science and Food Safety*, 5(1), pp.1-17.

Schill, S.R., 2013. *Ethanol Producer Magazine*. [Online] Available at: http://www.ethanolproducer.com/articles/10033/getting-it-done [Accessed 30 October 2015].

Sharma, V. et al., 2010. Effect of Resistant Starch on Hydrolysis and Fermentation of Corn Starch for Ethanol. *Application of Biochemical Biotechnology*, 160, pp.800-11.

Sharma, V., Rausch, K.D., Tumbleson, M.E. & Singh, V., 2007. Comparison Between Granular Starch Hydrolyzing Enzyme and Conventional Enzymes for Ethanol Production from Maize Starch with Different Amylose: Amylopectin Ratios. *Starch*, 59, pp.549-56.

Themeier, H., Hollmann, J., Neese, U. & Lindhauer, M.G., 2005. Structural and morphological factors influencing the quantification of resistant starch II in starches of different botanical origin. *Carbohydrate Polymers*, 61(1), pp.72-79.

Uthumporn, U., Karim, A.A. & Fazilah, A., 2013. Defatting improves the hydrolysis of granular starch using a mixture of fungal amylolytic enzymes. *Industrial Crops and Products*, 43, pp.441-49.

Uthumporn, U., Zaidul, I.S. & Karim, A.A., 2010. Hydrolysis of granular starch at sub-gelatinization temperature using a mixture of amylolytic enzymes. *Food and Bioproducts Processing*, 88, pp.47-54.

Vijaikishore, P. & Karanth, N.G., 1984. Glycerol production by fermetnation. *Applied Biochemistry and Biotechnology*, 9, pp.243-54.

Wang, W.J., Powell, A.D. & Oates, C.G., 1996. Sago starch as a biomass source- Raw sago starch hydrolysis by commercial enzymes. *Bioresource Technology*, 55, pp.55-61.

Wang, P. et al., 2007. Comparison of Raw Starch Hydrolyzing Enzyme with Conventional Liquefaction and Saccharification Enzymes in Dry-Grind Corn Proccesing. *Cereal Chemistry* , 84(1), pp.10-14.

Wang, P. et al., 2005. Comparison of Enzymatic (E-Mill) and Conventional Dry-Grind Corn Processes Using a Granular Starch Hydrolyzing Enzyme. *Cereal Chemistry* , 82(6), pp.738-38.

Wang, Z., Zhuge, J., Fang, H. & Prior, B.A., 2001. Glycerol production by microbial fermentation: A review. *Biotechnology Advances*, 19., pp.201-23.

Wrenn, B.A., 2008. *Summary Report for Pursuit Dynamics*. Edwardsville: National Corn to Ethanol Research Center.

Xie, X., Liu, Q. & Cui, S.W., 2006. Studies on granular structure of resistant starches from normal, high amylose and waxy corn starch citrates. *Food Reserach International*, 39, pp.332-41.

Xu, H. & Duan, G., 2010. Effect of temperature on the no cook, very high gravity ethanol fermentation process. *Chinese Journal of Biotechnology*, 26, pp.330-34.

Zou, W. et al., 2012. Effects of amylose/amylopectin ratio on starch-based superabsorbent polymers. *Carbohydrate Polymers*, 87, pp.1583-88.

APPENDIX A

Table A. 1: Normalised scoring system for each cultivar when using both the CSH and RSH methods.

| Cultivar #	CSH				RSH			
	Normalised EtOH %	Normalised Productivity	Normalised EtOH Yield	Rating Score	Normalised EtOH %	Normalised Productivity	Normalised EtOH Yield	Rating Score
M1	0	0	0	0	0.06	0	0.25	0.31
M2	0.37	0.16	0.02	0.55	0.39	0.73	0.13	1.24
M3	0.8	0.34	0.73	1.87	0.77	0.26	0.68	1.72
M4	0.69	0.29	0.58	1.56	0.82	0.28	0.83	1.94
M5	1	1	0.39	2.39	0.99	1	0.36	2.35
M6	0.53	0.22	0.41	1.16	0.31	0.09	0.05	0.45
M7	0.43	0.18	0.34	0.95	0.75	0.25	1	2
M8	0.91	0.38	0.72	2.01	0.99	1	0.85	2.84
M9	0.88	0.37	0.66	1.91	0.79	0.27	0.49	1.55
M10	0.53	0.22	0.31	1.06	0.46	0.76	0.22	1.44
M11	0.23	0.09	0.34	0.66	0	0.56	0	0.56
M12	0.43	0.18	0.13	0.74	0.58	0.19	0.45	1.22
M13	0.79	0.9	0.85	2.54	0.46	0.77	0.25	1.47
M14	0.42	0.18	0.55	1.14	0.57	0.81	0.9	2.29
M15	0.71	0.3	0.87	1.88	0.36	0.11	0.22	0.69
M16	0.98	0.41	0.67	2.06	0.94	0.33	0.58	1.85
M17	0.89	0.38	0.76	2.03	0.82	0.92	0.61	2.36
M18	0.67	0.28	1	1.95	0.45	0.15	0.61	1.21
M19	0.92	0.39	0.87	2.17	0.73	0.88	0.52	2.13
M20	0.68	0.28	0.64	1.6	0.49	0.16	0.32	0.97
M21	0.53	0.22	0.45	1.2	0.55	0.18	0.54	1.27
M22	0.65	0.27	0.42	1.34	0.8	0.27	0.73	1.8
M23	0.53	0.22	0.49	1.25	0.67	0.22	0.78	1.67
M24	0.93	0.97	0.46	2.36	1	0.35	0.56	1.91
M25	0.69	0.29	0.82	1.81	0.39	0.12	0.27	0.78
M26	0.58	0.25	0.39	1.21	0.53	0.18	0.33	1.04
M27	0.78	0.33	0.92	2.03	0.53	0.17	0.45	1.16
M28	0.93	0.96	0.64	2.53	0.97	0.99	0.71	2.67
M29	0.71	0.3	0.57	1.59	0.75	0.25	0.66	1.66
M30	0.89	0.38	0.73	2	0.81	0.28	0.57	1.66
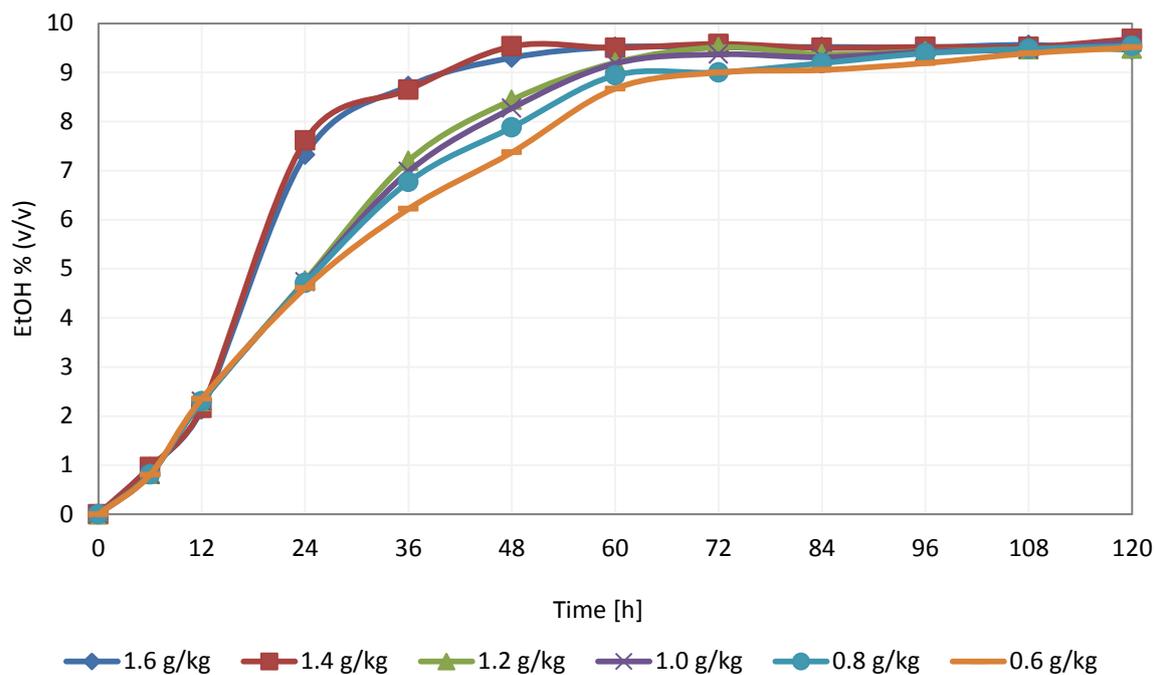
Figure A. 1: Preliminary RSH experiments used to determine the optimum boundaries of the enzyme dosage in the FCCCD
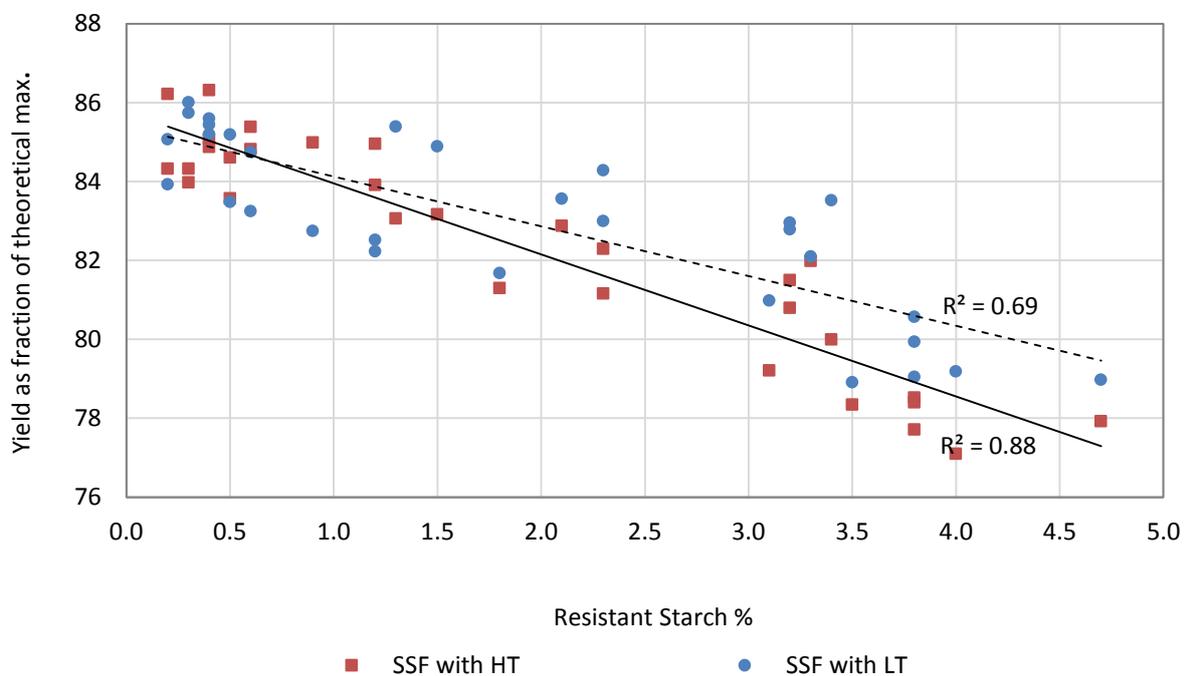

Figure A. 2: Correlation between yield as fraction of theoretical maximum and the resistant starch for each cultivar when using both the CSH (SSF with HT) and RSH (SSF with LT) methods

APPENDIX B

Sample calculations to follow are for the determination of the optimum KPI values, using the predicted quadratic models.

Ethanol Concentration is calculated using the following formula:

$$Z_1 = -3.1909 + 0.2451X + 3.1526Y + 0.0034X^2 + 0.015XY - 1.2303Y^2$$

Where Z_1 is the ethanol concentration, X is the solids loading and Y is the enzyme dosage

$$Z_1 = -3.1909 + 0.2451(37.5) + 3.1526(1.4) + 0.0034(37.5)^2 + 0.015(37.5)(1.4) - 1.2303(1.4)^2$$

$$Z_1 = 13.57\% \ (v/v)$$

Ethanol Productivity is calculated using the following formula:

$$Z_2 = -3.6489 + 0.2243X + 0.544Y - 0.0034X^2 + 0.0215XY - 0.3265Y^2$$

Where Z_2 is the ethanol productivity, X is the solids loading and Y is the enzyme dosage

$$Z_2 = -3.6489 + 0.2243(37.5) + 0.544(1.4) - 0.0034(37.5)^2 + 0.0215(37.5)(1.4) - 0.3265(1.4)^2$$

$$Z_2 = 1.23 \ g/L.h$$

Ethanol Yield is calculated using the following formula:

$$Z_3 = 51.2143 + 0.9102X + 26.1244Y - 0.0139X^2 - 0.1379XY - 6.8342Y^2$$

Where Z_2 is the ethanol productivity, X is the solids loading and Y is the enzyme dosage

$$Z_3 = 51.2143 + 0.9102(37.5) + 26.1244(1.4) - 0.0139(37.5)^2 - 0.1379(37.5)(1.4) - 6.8342(1.4)^2$$

$$Z_3 = 81.74 \ \%$$