The effect of sorghum grain decortication on bioethanol production technologies

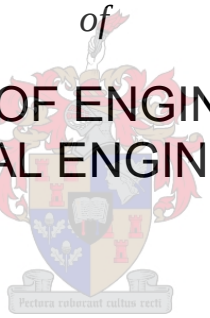
By

Edouard Yves Nkomba

Thesis presented in partial fulfilment
of the requirements for the Degree

of

MASTER OF ENGINEERING
(CHEMICAL ENGINEERING)



in the Faculty of Engineering
at Stellenbosch University

Supervisors

Prof. Johann Gorgens
Dr. Annie Chimphango

December 2015

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Abstract

Due to issues surrounding the burning of fossil fuels such as the effect of greenhouses gases on the climate and the threat energy security poses to non-producing nations, biofuels are being promoted for their potential local availability and carbon neutrality. Depending on the materials used, biofuels can be qualified as first (edible) or second (non-edible) generation. Whereas second generation technologies are still not economically viable, first generations biofuels (such as bioethanol from starch) will hold a major share of renewable liquids fuels in the short to medium term. The recent commercialization of enzymes with marked activity towards non-gelatinized or raw starch (cold processing), and their subsequent expressions by genetically modified organisms (Consolidated bioprocessing) could potentially cut the costs and energy requirements of the conventional high temperature processing, which involve cooking or gelatinizing starch. Hence, alternatives such as low temperature cold processing are being investigated for industrial application, while processes to improve the performance of the consolidated bioprocessing are being explored. Furthermore given that biofuels production is continuously increasing, the availability of the main co-product of the conversion process known as distillers dried grains with solubles (DDGS), is following the trend. It has been shown that sorghum grains decortication (removal of bran) prior entering the conversion process could significantly improves the DDGS quality, by reducing the fibre content thereof, hence increasing its market value. Furthermore, the bran components in grains have been shown to negatively affect starch hydrolysing enzymes. In this study, three bioethanol conversion processes (conventional warm, cold and consolidated bioprocessing) and the effect of decortication on key performance measures was assessed using sorghum grains. When using whole grains, the cold and conventional processing achieved similar ethanol concentration (130.4 and 132.1 g/L), productivity (1.55 and 1.51 $g.L^{-1}.h^{-1}$) and ethanol yield as a fraction of the theoretical maximum (89.7 % and 89.03 %). Although a slight decrease in the ethanol yield from consumed glucose was observed in slurries containing decorticated grains, performance of the cold processing was not significantly affected. However, the ethanol productivity of the conventional warm processing decreased with

decortication (1.25 $g.L^{-1}.h^{-1}$). The performance of the cold processing using decorticated grains could match the whole grains process, while using 11.7 wt% less enzymes. The DDGS obtained from decorticated grains had higher average protein content (26%) and lower crude fibre content (30.7 %), compared to DDGS from whole grains processing. The acid and neutral detergent fibres contents in DDGS from both types of grains were on average decreased by 17.6 and 26.7% respectively by the cold processing relatively to the conventional processing. The performance of the consolidated bioprocessing could not match the enzyme-based processing, mostly due to limited production of starch-hydrolysis enzymes. The low ethanol tolerance of the recombinant yeast (approximately 90 g/L) prevented consumption of all of the glucose released in the very high gravity slurry. Furthermore, the CBP yeast inoculum size did not have a significant effect on the rate of starch hydrolysis and ethanol productivity, despite design of a fermentation process with high yeast biomass and yeast-produced enzyme concentrations in the starch slurry. Further improvements to the inoculum production, to increase biomass and enzyme concentrations, can be considered, although CBP yeast still lacks sufficient amylase production to achieve efficient starch grains conversion without supplementation with enzymes.

Uittreksel

Verskeie kwessies rondom die verbranding van fossielbrandstowwe, soos die effek van groenhuisgasse op die klimaat en energie-onsekerheid in nie-olie-produserende lande, promoveer biobrandstof as potensiële alternatiewe energiebron, weens koolstof neutraliteit en plaaslike beskikbaarheid van roumateriale. Afhangend van die aard van die roumateriaal kan biobrandstof in twee kategorieë verdeel word, nl. eerste (eetbare) en tweede (nie-eetbare) generasie biobrandstof. Gegewe dat tweede generasie biobrandstof nog nie ekonomies lewensvatbaar is nie, is die vooruitsig dat eerste generasie biobrandstof steeds die grootste aandeel van vloeibare, hernubare brandstofmark in die kort- tot mediumtermyn sal beslaan. Die onlangse kommersialisering van ensieme wat beduidende aktiwiteit tot ongegelatiniseerde, oftewel rou stysel, toon (koue prosessering), en die uitdrukking van hierdie ensieme deur geneties gemodifiseerde mikro-organismes (gekonsolideerde bioprosessering), het die weg gebaan om kostes en energiebehoeftes aansienlik te besnoei, vergeleke met konvensionele hoë-temperatuurprosesse waar stysel eers gekook en gegelatiniseer moet word om die amilose vir ensiemvertering toeganklik te maak. Derhalwe geniet alternatiewe prosesse soos lae temperatuurprosessering vir industriële toepassing baie aandag, terwyl die werkverrigting van gekonsolideerde bioprosessering in diepte ondersoek word. In pas met die toenemende produksie van biobrandstof, volg die produksie van ’n hoof byproduk, nl. distilleerders droë korrels en oplosbares (DDKO), ’n soortgelyke tendens. Daar is verder bewys dat ’n sorghumgraan semelverwyderingstap, wat die stysel na etanol omskakelingsproses voorafgaan, ’n beduidende verhoging in die kwaliteit van die DDKO teweeg kan bring, omdat die verlaging in veselinhoud die markwaarde van die finale produk verhoog. In hierdie studie is drie bio-etanol produksieprosesse, nl. die konvensionele warm proses, die koue proses, asook gekonsolideerde bioprosessering, ondersoek. Die invloed van semelverwydering van die sorghumgraan is ook op sleutel prestasie maatstawwe bepaal. Heel graan (graan waarvan semels nie afgeskil is nie) het onderskeidelik tot soortgelyke etanol konsentrasies (130.4 en 132.1 g/L) in die koue en konvensionele warm prosesse gelei, waar soortgelyke klein verskille ook in die

produktiwiteit (1.55 en 1.51 $g.L^{-1}.h^{-1}$) en opbrengs as persentasie van die teoretiese maksimum (89.7% en 89.03%) waargeneem is. Alhoewel 'n klein afname in die etanol opbrengs van suspensies met semel-vrye graan bespeur was, het hierdie prosesstap geen beduidende invloed op die werkverrigting van die koue proses gehad nie. Semelverwydering het wel tot 'n afname in die produktiwiteit (1.25 $g.L^{-1}.h^{-1}$) van die warm proses gelei, maar die werkverrigting van die koue proses kon dié van die warm proses ewenaar deur 11.7 massa% minder ensiem te gebruik. Daar is bevind dat die gemiddelde proteïeninhoud in die DDKO van die semel-vrye graan 26% hoër en die kru veselinhoud 30.7% laer was as dié van graan waarvan die semels nie afgeskil is nie. Daar is ook bevind dat relatief tot die warm proses, die suur-gewaste en neutraal-gewaste vesel in die DDKO van beide tipes graan onderskeidelik 17.6 en 26.7% laer in die koue proses was. Die werkverrigting van die gekonsolideerde bioprosessering benadering was aansienlik laer as dié van prosesse waar kommersiële ensieme gebruik is (warm en kou prosesbenaderings), hoofsaaklik weens beperkings in die produksie van ensieme wat die rou stysel kon hidroliseer. Daarbenewens is ook bevind dat die geneties gemodifiseerde gis 'n laer etanol toleransie (ongeveer 90 g etanol/L) gehad het wat die opname van alle beskikbare glukose in hoë-gravitasie suspensies verhoed het. Die grootte van die inokulum van die geneties gemodifiseerde gis het geen beduidende invloed op die tempo van stysel hidrolise of etanol produktiwiteit gehad nie, ten spyte van eksperimente wat vir hoë biomassa- en ensiemkonsentrasies ontwerp is. Verdere ontwikkelingswerk vir inokulum voorbereiding ten einde die biomassa- en ensiemkonsentrasie van die rekombinante gis te verhoog is aangedui. Die rekombinante gis se amilase produksievermoëns bly egter vir effektiewe stysel omskakeling onvoldoende, wat daarop dui dat eksterne ensiem byvoeging steeds benodig word.

Acknowledgments

I would like to thank the following people for their assistance in the process of completing this work:

- My supervisors Prof Johann Gorgens and Dr Annie Chimphango for the opportunity given to me to conduct this work and the constant guidance.
- Dr Eugene Van Rensburg for the invaluable assistance and advices throughout this work.
- Dr Maria Garcia Aparicio for the assistance when required.
- Mr Henry Solomon for the assistance and knowledge I received in the handling of the grains.
- Mrs Manda Rossouw, Mrs Levine Simmers and Mr Jaco Van Rooyen for the assistance with HPLC analysis.
- Kim O'Kennedy from Pionnerfoods Pty (Ltd) for the assistance with sorghum grains decortication and allowing me to use their facility.
- Lisa Warburg from the Department of Microbiology (Stellenbosch University) for performing the amylase assay.
- Dr E. Pieterse from the Department of Animal Sciences (Stellenbosch University) for the analysis of the distillers' dried grains with solubles.
- My parents for the support throughout my studies.

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Nomenclature

ADF	Acid detergent fibre
ANOVA	Analysis of variance
CBP	Consolidated bioprocess
CCD	Central composite design
CDS	Condensed distillers solubles
DDGS	Distillers' dried grains with solubles
DOE	Department of Energy
FAN	Free amino nitrogen
HG	High gravity
HPLC	High performance liquid chromatography
LTE	Low temperature enzymes
NDF	Neutral detergent fibre
OD	Optical density
RSHE	Raw starch hydrolyzing enzymes
SSF	Simultaneous saccharification and fermentation
VHG	Very high gravity
WDG	Wet distillers' grains
WDGS	Wet distillers' grains with solubles

1 Introduction

Although it is known that fermentation was used in ancient Egypt in a brewing process, details of the process are not well known. Reports of the utilization of fermentation technology on a large scale only date back to the early 1700s, in the production of beer (alcoholic fermentation), using wild type yeast microorganisms (Stanbury, et al., 1995). In modern age, through the development of techniques for improvement of microorganisms, such as induced mutagenesis and genetic engineering, the conversion efficiency of various fermentation processes have been significantly improved. A wide variety of value added products such as metabolites, biopharmaceuticals and enzymes among others, are now produced using this technology in several industries worldwide (Stanbury, et al., 1995).

Of all metabolic by-products from fermentation using yeast, ethanol has the largest market share by volume: 73% is used for fuel, 17% for beverages and 10% for industrial uses (Sanchez & Cardona, 2008). Raw materials used for alcoholic fermentation are commonly classified into three groups: sugars, starch and lignocellulosic materials. To date the majority of industrial ethanol plants use either sugars or starches as raw materials (Lin & Tanaka, 2005). Fermentable sugars (specifically sucrose, glucose, mannose and fructose) available in fruits, molasses and crops such as sugarcane or sugar beet can be metabolized directly by microorganisms and converted into ethanol. Starches, on the others hand, are polymers of sugars and cannot be used directly by relevant microorganisms such as *Saccharomyces cerevisiae*; they must undergo some pre-treatment and hydrolysis to convert them into fermentable sugars (Lin & Tanaka, 2005).

Starches are natural plants reserves and are present in cereals grains and tubers, amongst others, making them raw materials of choice for alcoholic fermentation (Sanchez & Cardona, 2008). Grains used in alcoholic fermentation include corn, oats, wheat, barley and sorghum among others; their starch content varies between 55 and 75% (wt/wt) on a dry basis (Preiss, 2009). Tubers, such as

cassava and potatoes have been reported to be used in industrial ethanol production (Lin & Tanaka, 2005) with starch content of up to 90 % (wt/wt) on a dry basis (Sanchez & Cardona, 2008).

Within the South African context, the Department of Energy has identified sorghum and sugarcane among the potential feedstocks for commercial bio-ethanol production for use as biofuel. Although maize is widely used for biofuel (ethanol) production abroad, its use for this purpose in South Africa was prohibited, due to maize being a staple food (Department of Energy). Commercial developers of bio-ethanol production plants have favoured grain sorghum for local ethanol production, due to potential for cultivation in a large part of the country, drought resistance and lower water use requirements. As a result, grain sorghum has a larger potential for agricultural expansion compared to sugarcane cultivation.

The current conventional technologies for starch conversion to ethanol include separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF), with the latter preferred by industrial users. The conventional processes convert dry-milled starch grains to ethanol, starting with a gelatinization step where the starch/water mixture is heated to temperature around 90^{o}C or above, to disrupt the crystalline structure of the starch. The exposed starch molecules are simultaneously converted to short-chain dextrins via the action of high temperature enzymes (HTEs), i.e. α-amylases, resulting in liquefaction. The liquefied slurry is fed to the fermenter together with yeast and glucoamylases, for simultaneous saccharification (conversion of dextrins to fermentable sugars) and fermentation (Bothast & Schlicher, 2005).

Due to the high energy costs associated with the high temperature required for gelatinization and liquefaction, which account for 10-20 % of the energy value of the ethanol produced (Robertson, et al., 2006), alternatives were sought to hydrolyse raw uncooked starch. This quest led to the discovery and isolation of low temperature enzymes (LTEs) capable of hydrolysing raw starch at lower temperature (< 50^{o}C; Genencor, 2010). These developments have led to intensive researches aiming at replacing the conventional warm process with an improved cold process, without the need

to gelatinize starch before hydrolysis (Van Zyl, et al., 2012). Such LTEs are already commercially available such as Stargen 002 from Genencor. POET, one of the largest corn-ethanol producers in the USA, has exclusive rights to the LTE enzymes produced by Novozymes. Further research are currently done to develop a consolidated bioprocessing (CBP), where genetically engineered microorganism strains expressing LTEs could eventually perform liquefaction, saccharification and fermentation of raw (uncooked) starch to ethanol simultaneously, in a single reactor vessel (Gorgens, et al., 2014).

2 Literature review

2.1 Biofuels driving forces

The majority of energy currently used for industrial and domestic purposes, and which drive the world economies, originates from fossils fuels such as coal, oil and gas (Beretta, 2007). However, several factors are making these energy sources unsustainable in the long term. For instance, 89% of the coal is localized in eight countries, 81% of oil in eight countries and 70% of natural gas reserves in six countries (Sayigh, 1999). The localization of these important resources to specific parts of the world and the political instability of some of these regions affects the energy security of non-producing nations. Furthermore, the burning of these fossil fuels causes a net increase in the release of greenhouses gases such as carbon dioxide into the atmosphere, contributing to global warming and its resulting negative effects (Delucci, 2010). Also fossil fuels are non-renewable and the reserves are depleting as a consequence of current high consumption. It is believed that the current proved oil reserves can sustain the world for 46 more years (BP, 2011). For all these reasons alternatives renewable and sustainable energy sources are preferred.

Currently, bioethanol is the preferred alternative liquid fuel for road transport, based on present global sales and utilisation (Sanchez & Cardona, 2008). Its production from biomass makes it a renewable source with potential for sustainability, since it is based on the natural cycle of carbon: Ethanol is burned releasing carbon dioxide into the atmosphere, which is sequestered back into plant biomass through photosynthesis and converted to ethanol again (Peterson & Hustrulid, 1998). If a biofuel production system is implemented in an efficient way, such that the biomass to be used is produced locally, it will improve local energy security for producing nations. In a bid to promote the implementation of technologies using renewable sources, national governments are implementing different policies for biofuels production. For example, South Africa aims for biofuels to account for 2% of total liquid fuels (Department of Minerals & Energy, 2007), hence providing financial incentives to allow producers to sell biofuels at prices competitive with the present petroleum fuel products. Other countries such as Sweden placed the focus on consumers by

reducing the registration fees and road taxes to cars running on biofuels (Mandil & Shihab-Eldin, 2010).

2.2 Desirable characteristics of sorghum grain as a feedstock for bioethanol production

The average chemical composition of sorghum grain (table 2.1) shows that starch content is between 65 and 75 %; similar to the average starch content of 72 % for corn (Eckhoff & Watson, 2009). Both type grains have the highest starch content among cereals. Furthermore, the average agricultural yields of sorghum and corn in South Africa are very similar at 2.87 and 2.97 ton/hectare, respectively (National Agricultural Marketing Council, 2007). However, sorghum can achieve yields comparable to corn while using up to 33% less water (Rooney, et al., 2007).

In South Africa, sorghum has other advantages compare to other potential feedstocks such as sugar cane and sugar beet. For instance the potential of expansion of sugar cane is limited due to its high water requirements. The disadvantage of sugar beet is the scarcity of reliable data related to its cultivation in South Africa, hence hampering the construction of accurate economic model, unlike sorghum which used to be cultivated extensively in South Africa (Department of Energy).

Table 2-1: Average chemical composition of sorghum grain

Component	Content (%)
Starch	65 – 76
Protein	8 – 15
Lipids	2 – 5.5
Fibres	1 – 5
Tannins	2 – 7
Ash	1 – 2.5

(Wu X et al. 2007; Corredor D et al. 2006; Udachan, et al., 2012)

2.3 Grain sorghum chemistry and properties

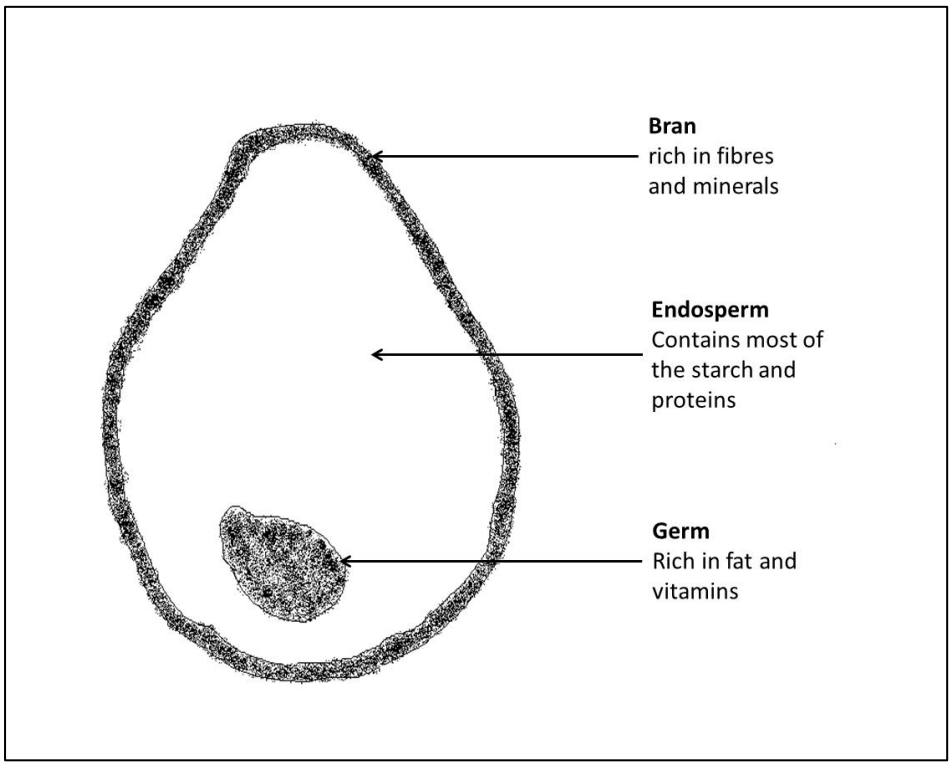

Figure 2-1: Schematic representation of structure of sorghum grains

The starch content of grain sorghum is localized in the endosperm (Koehler & Wieser, 2013; Fig 2-1). Starch is a molecule made up of 2 components, amylose and amylopectin. Amylose is a linear and unbranched polymer of approximately 1000 glucose units linked together with an α-1,4 glycosidic bond (Tester, et al., 2004). Chemically, amylopectin is similar to amylose; the difference being that in amylopectin the long glucose chains are branched to other α-1,4 glucan chains via an α-1,6 glycosidic bond after every 10-12 glucose molecules (Stevnebo, et al., 2006). These two components are arranged in a way that results in two distinct regions in raw starch: The amorphous region that mostly contains amylopectin and is easily accessible to hydrolysis agents such as acids or enzymes, and the less accessible semi-crystalline regions, richer in amylose molecules (Daniel, et al., 2000). The proportion of amylose and amylopectin in the endosperm depends greatly on the type of sorghum.

Sorghum grains can be classified in several ways, one of them which the most popular, is based on the colour of the grain and pigmentation of the testa, giving 4 different groups: white, yellow, brown and mixed sorghum. However, classifications relevant to the fermentation industry, includes the ones based on tannins levels in the kernel, known as type 1, 2 and 3. Type 1 refers to no tannins, type 2 and type 3 to low and high levels respectively (Price & Butler, 1977). Tannins are polyphenolic compounds which have no metabolic function, but rather are part of the plant's defence system against insects, fungi or herbivores. Starch in grain sorghum can also be classified as waxy, heterowaxy and non-waxy, differing in their proportions of amylose/amylopectin. Waxy endosperms are made up of almost only amylopectin, heterowaxy ones contains less than 20% amylose and around 25% for non-waxy ones (Wu, et al., 2006). Another important component of sorghum grain is the protein content, which acts as a nitrogen sources for microorganisms during fermentation. The amylose: amylopectin ratio, tannins, proteins content and their significance to bioethanol production process are discussed later.

2.4 Bioethanol production technology using cereals grains

The three bioethanol production technologies discussed below have upstream and downstream processes common to all of the them. These processes are first discussed. The particularities of each technologies are then discussed later. The processes describle here apply to any cereals grains, including grain sorghum.

2.4.1 Decortication

Decortication is the removal of the outer part of the sorghum grains, consisting mainly of non-fermentable materials such as fibers (Koehler & Wieser, 2013). The conventional ethanol production from other cereals such as corn and wheat do not involve degermination or dehulling of grains, which are similar processes to decortication in sorghum grains. However, in the case the sorghum grains have high level of tannins (>1%;w/w) and flavonoids, which can inhibit amylases (Awika & Rooney; Sales, et al., 2012), this step might be required to achieve the desired ethanol yield and productivity from grain sorghum, in comparison corn, given that sorghum starch has intrinsically

lower digestibily compare to corn (Wu, et al., 2007; Ai, et al., 2011). Ways in which tannins can affect ethanol production from starch are discussed in more detailed later.

Furthermore, Alvarez et al. (2010) have shown that starch hydrolysis to glucose can be improved by sorghum decortication. At the end of saccharification, glucose concentration in mashes with decorticated sorghum was 13% higher compared to their counterpart with non-decorticated grains. One reason for this improvement was attributed to the removal of fibers during decortication, which acted as a physical barrier hindering access to amylases. A potential negative effect of decortication is the loss of nutrients (minerals and proteins) located in the outer part of the grains, removed during decortication, which can decrease performance of the fermenting microorganism (Wang, et al., 1999).

Information on the effect of sorghum grain decortication on the ethanol production from starch are only available for the conventional warm process. No literature was found describing the use of decorticaticated sorghum grains for ethanol production using the cold processing and consolidated bioprocessing.

2.4.2 Milling

Grinding is a mechanical treatment aimed at reducing the particle size of the cereal grains before hydrolysis and fermentation (Kelsall & Lyons, 2003). During this process grains are broken into finer particle size to the increase of the overall surface area, exposing regions at the core of the grain to enzymes. The digestion of starch is believed to proceed by diffusion and is inversely proportional to the average size of the particles (Mahasukhonthachat, et al., 2010). Experiments by Wang et al. (2008) have shown that the conversion efficiency of coarsely ground samples were approximately 5% lower than that of finely ground samples, when using the conventional process.

Previous studies on ethanol production using sorghum grains have used different particle sizes, although always less than 2 mm (Corredor, et al., 2006; Wu, et al., 2008; Zhao, et al., 2008). However, a detailed study by Rausch, et al. (2005) of nine corn dry grind ethanol plants using the

conventional process found that the average particle size following milling was 0.94 mm, with no significant variation between plants. Data from industrial ethanol plants using sorghum grains are not available; hence corn as being the most used cereal grain for bioethanol production is the reference.

In comparison with the conventional process, the conversion of raw starch to ethanol by the cold processing and consolidated bioprocess have the disadvantage of requiring finely ground particles. Genencor recommend a maximum of 5 % particles with a diameter larger than 0.6 mm (Genencor, 2010). In literature, authors usually mill the grains into flour to pass through a 0.5 mm screen (Begea, et al., 2010). Finer particles increase the energy demand during milling, adding up to the production costs.

2.4.3 Distillers Dried grains with solubles (DDGS) production

Distillers' Dried Grains with Solubes (DDGS) is the main co-product of bioethanol production from cereal grains. Significant revenue is generated from the sales of DDGS as an animal feed ingredient (Bothast & Schlicher, 2005). The schematic diagram of a DDGS production process is illustrated in figure 2-2. Following distillation, the resulting mixture made up of nonvolatile compounds, is separated into a liquid and solid fractions. The liquid fraction is concentrated by removing water through evaporation. The concentrated liquid is known as condensed distillers solubles (CDS), and then combined again with the solid fraction (known as wet distillers' grains, WDG) becoming the wet distillers' grains with solubles (WDGS). The WDGS are then dried to produced DDGS (US Grains Council, 2013). Hence throughout the production process all the initial nonvolatile nurients that entered the process (including yeast) are concentrated in the resulting DDGS. The concentration of nutrients present initially in grains can increase up to 3 fold in the DDGS (Liu, 2011). Among nutrients present in DDGS, proteins are of particular interest for the animal production industry, while there is a preference for low-fibre DDGS. The continuous increase over the years of bioethanol production from cereal grains has resulted in the availablility of increasingly large amount the DDGS, hence becoming a commodity in the USA and elsewhere (Liu, 2011).

Addition of a decortication process step before dry milling of grains is a potential option to improve the nutritional quality of DDGS as animal feed. The outer part of the grains removed during decortication consist mainly of fibers. As the initial amount of fibers is removed from the grain, thus not entering the conversion process, the concentration of other nutrients, of which proteins are the most relevance in our context, is increased in the DDGS. Corredor, et al. (2006) found that depending of the degree of decorticatication, protein content can increase by up to 11.7% while fibers content decreasing by up to 4.5 %.

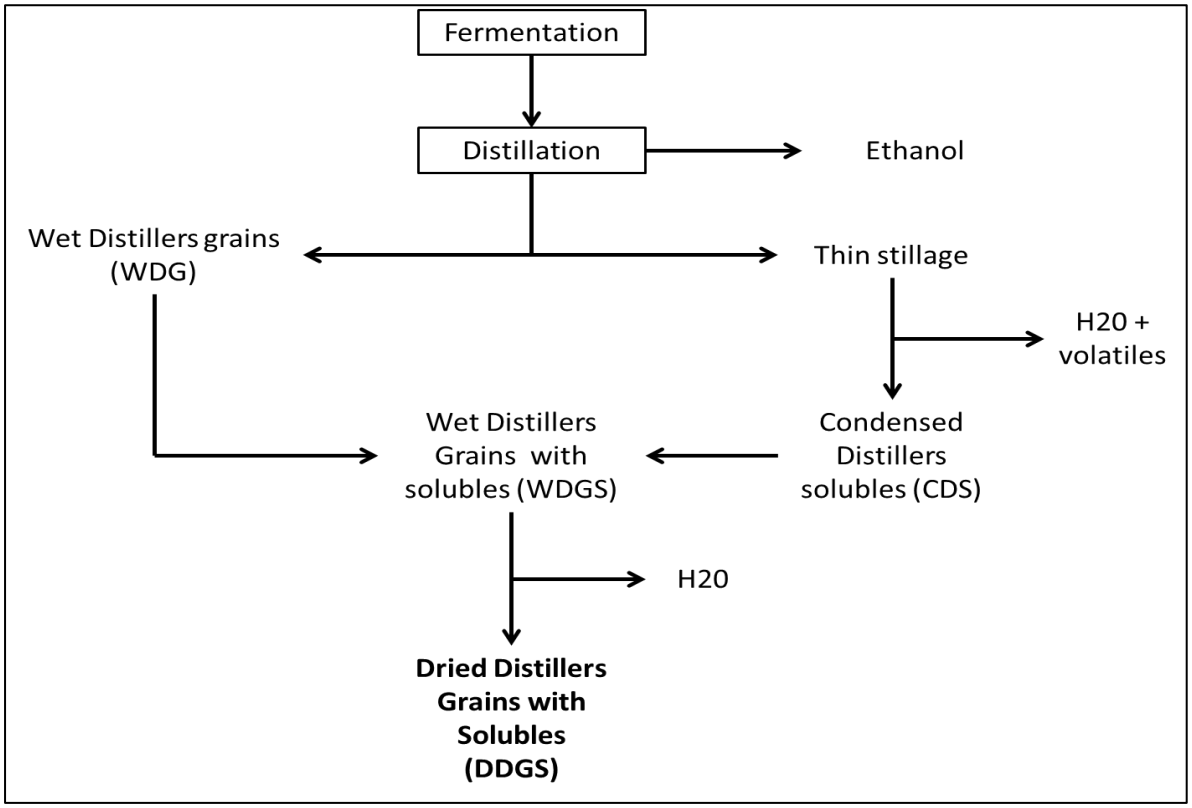

Figure 2-2: Schematic diagram of a DDGS production process

2.4.4 Conventional process for starch conversion to ethanol

Conventionally, the preparation of cereal grains for ethanol production can be achieved through two kind of processes: the wet mill and the dry mill process. The dry mill process is responsible for 67% of fuel ethanol production from cereal (Bothast & Schlicher, 2005). It is preferred to the wet mill process, as it is less capital and energy intensive. It is foreseen that most of the ethanol growth

would be through the dry mill process (Bothast & Schlicher, 2005). The dry mill process involve several steps: grinding (milling), liquefaction, saccharification, fermentation and distillation (Fig 2-3).

The wet mill process is designed to make use of all the components of the grain. During this process, the grain is first steeped in water, followed by separation to isolate the fibers, germ and gluten, which can be further processed and sold separately. For example, plant oil can be can extracted from the germ and sold as a separate product, while the gluten rich fraction mixed with the fibers can be sold as high protein feed for animals (Bothast & Schlicher, 2005). Then the starch-rich fraction can be converted to ethanol in a way similar to the dry mill process (as described below), starting from liquefaction.

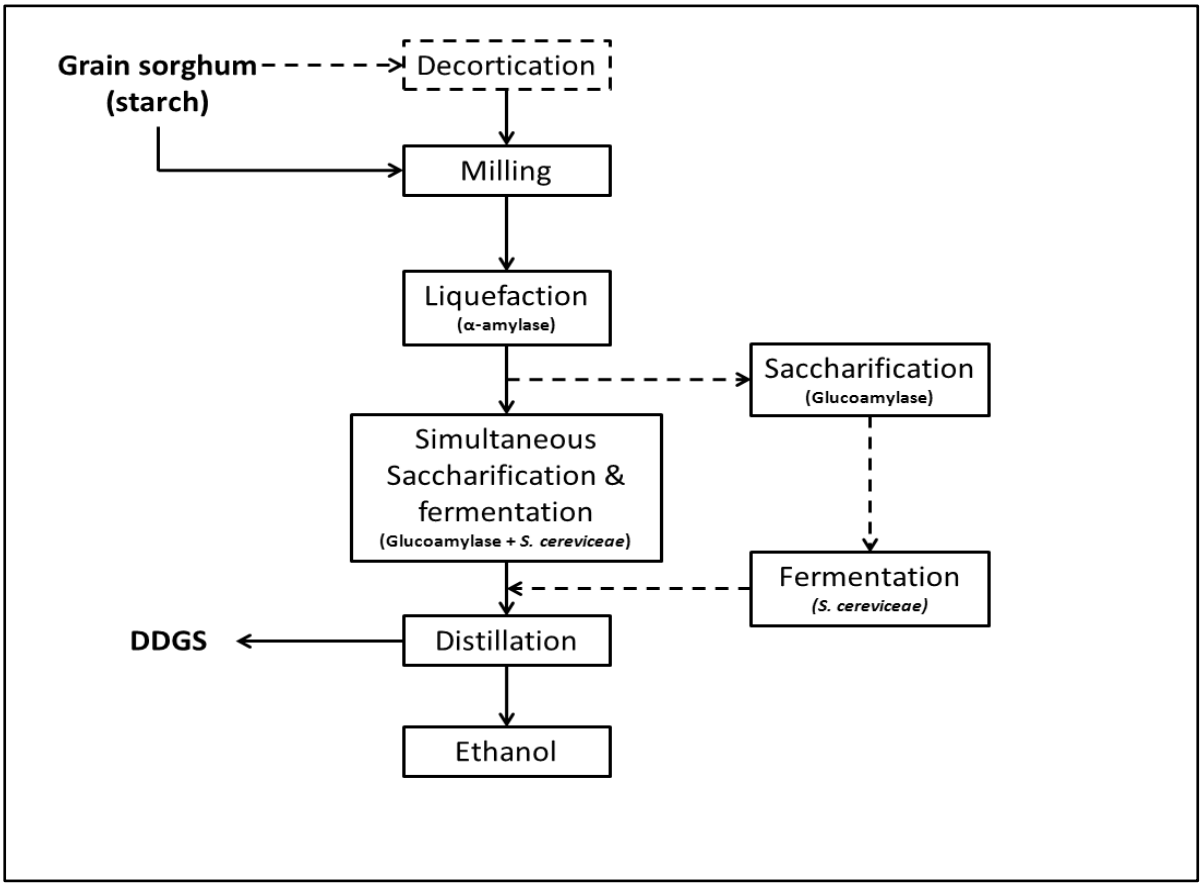

Figure 2-3: Schematic diagram of a conventional ethanol dry grind production process

2.4.4.1 Gelatinization & Liquefaction

Raw starch molecules have a naturally semi-crystalline structure, which prevent enzymes from accessing the inner molecules. During the gelatinization and liquefaction steps, usually performed a temperature above 86^{o}C , the hydrogen bonds integral to forming the crystalline structure are disrupted, due to the high temperatures in the presence of water (Tester, et al., 2004). The starch structure swells as water is absorbed, leading to the disruption of the crystaline matrix. The gelatinization temperature of sorghum starch is variety dependent and usually starts between 64-67 ^{o}C (Ai, et al., 2011; Udachan, et al., 2012). The cooking step is usually performed at temperatures between 90-110 ^{o}C, to allow for the starch kernels to be broken (Sanchez & Cardona, 2008). The viscosity of the slurry increases sharply during gelatinization, which lead to increased energy input for continuous efficient mixing, and limits the solids loading that can be achieved in liquefaction and subsequent saccharification and fermentation.

During the cooking process, thermostable α-amylase, obtained from thermophilic microorganisms such as *Bacillus licheniformis* or any engineered microorganisms expressing such enzymes, is added to the slurry (Sanchez & Cardona, 2008). The α-amylases are endo-type enzymes hydrolysing the internal α-1,4 glycosidic bonds of both amylose and amylopectin molecules. Their activity results in the liberation of short glucose chains of approximately 10 to 20 units known as dextrins (Van Zyl, et al., 2012). The decrease in the average molecular weight of the starch molecules cause the viscosity of the slurry to decrease as the consequence of the α-amylases activity, resulting in liquefaction. Furthermore the viscosity associated with liquefaction is greatly reduced when the sorghum grains have been decorticated. Wu et al. (2007) found that the peak viscosity of the slurry containing whole grains during liquefaction at normal amylase dosage can be approximately 3 fold higher than the peak viscosity for the slurry containing decorticated grains at the same amylase dosage.

In their optimization of the liquefaction of sorghum starch powder, with the response being the detection of starch via the iodine test, Aggarwal, et al. (2001) found the optimum solid loading to be 25% at 105 ^{o}C for 45 minutes under steam pressure. More importantly, they found that the amount

of amylase enzymes used (Biotempase) could be lowered by 33% compared to the recommended dosage from the manufacturer, by the addition of $CaCl_2$ to a concentration of 200 mg/l, without affecting the degree of liquefaction. The mechanism responsible for the reduced enzymes requirement is the interaction between amylases and calcium ions. The negatively charged amino acids in the enzymes interact with the positively charge ions, which stabilizes its structure (Bush, et al., 1989). As a consequence the 3D structure of the enzyme and its activity is maintained for longer period in a presence of calcium ions, compare to when they are absent. This results in lower enzyme dosages required to achieve similar extent of hydrolysis.

2.4.4.2 Saccharification

Saccharification is the step leading to the complete hydrolysis of starch via the release of glucose molecules from the dextrins released during the liquefaction step. This action is performed by glucoamylases that are isolated mainly from *Aspergillus* or *Rhizopus* species (Sanchez & Cardona, 2008). They are exo-type enzymes hydrolysing the α-1,4 glycosidic bond of the glucopyranosyl unit located at the non-reducing end of the dextrins, thus releasing glucose molecules in the slurry (Van Zyl, et al., 2012). Although glucoamylases can specifically hydrolyze α-1,4 glycosidic bonds, their activities can also be extended to α-1,6 glycosidic bonds when the next bond is a α-1,4 one (Fierobe, et al., 1998). Hence the activity of the glucoamylases on both chemical bonds lead to the complete hydrolysis of starch to glucose, which can be quantified in term of dextrose equivalent (DE): the fraction of hydrolysed glycosidic bonds. Glucoamylases work optimally at significantly lower temperature compare to α-amylase, in the range of 60-70 ^{o}C (Sanchez & Cardona, 2008), which is the main reason these two steps are often performed separately. Industrial conversion of cereals grains to ethanol is usually performed by simultaneous saccharification and fermentation (SSF) of liquefied slurries.

2.4.4.3 Fermentation

During the fermentation step, the glucose molecules present in the slurry are used by microorganisms and converted to ethanol. The yeast *Saccharomyces cereviseae* is the

microorgananism of choice for industrial ethanol fermentation. Although the bacterium *Zymomonas mobilis* can achieve higher ethanol productivity and efficiency (Bai, et al., 2008). *S. cereviseae* has the main advantage over *Z. mobilis* of higher tolerance for final ethanol concentrations, while being acceptable as animal feed, hence decreasing the costs associated with wastes disposals (Bai, et al., 2008).

The metabolic pathway of glucose conversion to ethanol by *S. cereviseae* is shown in figure 2-4. This pathway occurs under anaerobic condition or can be induced by the availability of surplus sugars, leading to the incomplete breakdown of glucose to ethanol and CO_2. The theoretical maximum conversion, according to this pathway, is when every gram of glucose molecule is converted to 0.51 g of ethanol and 0.49 g of CO_2. However, other cellular processes and the production of byproducts, divert the glucose and other intermediates products from ethanol formation resulting in fermentation yields that are lower than the theoretical maximum (Bai, et al., 2008).

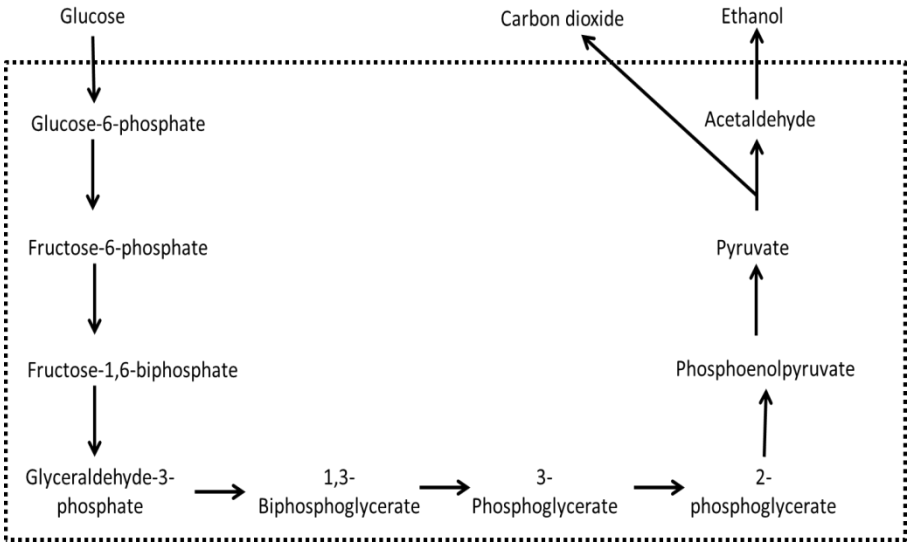

Figure 2-4: Metabolic pathway involve in the conversion of glucose to ethanol by *S. cerevisiae*.

After the cooling of the mashes (fermentable starchy mixture) following liquefaction, fermentation can be performed separately from saccharification or both can be combined and occur

simultaneously (SSF; Fig 2-3). Temperature of fermentation (or SSF) is usually around 30 ^{o}C and the medium is often supplemented with external nitrogen sources such as ammonium sulfate or urea to improve the conversion rate (Bothast & Schlicher, 2005; Chang, et al., 2011; discussed later). High gravity fermentation with slurry containing solids contents of 20% or above used to be the standard in the industry, resulting in ethanol concentration between 7-10 % (Serna-Saldivar, et al., 2012). More recently, the fermentation of mashes with solids loadings of 30% (w/w) or above, termed as very high gravity (VHG) fermentation, has been proposed as a better alternative due to processing advantages: It reduces the water requirement, while increasing the throughput of the ethanol plant (Sanchez & Cardona, 2008). VHG fermentation often have final ethanol concentrations between 15-18% or more, providing significant decreases in the cost of distillation, compared to high gravity fermentation (Serna-Saldivar, et al., 2012). Currently most industrial bioethanol production is performed with VHG slurry.

When comparing the older separate hydrolysis and fermentation with more recent SSF configuration, combining saccharification and fermentation has the advanges of reducing the risks of contamination, which could occur during the transfer of the substrate to the fermentation vessel, lowering the osmotic stress of cells exposed to the high sugar concentration mashes following saccharification (under SHF), and is generally more energy-efficient than separate hydrolysis and fermentation (Bothast & Schlicher, 2005). Chang, et al., (2011) compared the performance of SHF and SSF processes using sorghum grains at VHG (40%), with and without supplementation of free amino nitrogen (FAN) to the mashes. The fermentation rate for the SSF was higher than SHF for the same FAN supplementation. The final ethanol concentration achieved for SSF was not significantly affected by the supplementation of FAN, reaching approximately 18.5 % (v/v), as opposed to the fermentation rate which was improved by FAN supplementation. Without FAN the SSF took 156 hours to complete while only 48 hours was required when FAN was supplemented. The final ethanol concentration in SHF were approximately 17.7% and 15.8%, with and without FAN respectively. The SHF fermentation time of 185 hours was reduced to 64 hours with FAN

supplementation. SSF is currently the process of choice for industrial ethanol production, and is this also preferred for grain sorghum in the present study.

2.4.5 Cold process for starch conversion to ethanol

Some enzymes are capable of degrading raw starch, with activities evident in the microorganisms responsible for the rotting of starchy materials. Such raw starch degrading enzymes (RSDE) have been reported in yeasts, bacteria and fungi (Sun, et al., 2010). The utilisation of these enzymes in the conversion process of starch to ethanol could reduce the production costs, specifically the energy requirement for heating during the liquefaction and saccharification steps, which is equivalent to 10-20% of the energy value of the ethanol produced (Robertson, et al., 2006). The successful development and implementation of such RSDE would thus lead to significant energy savings in the conversion of raw starch to ethanol, due to the low temperature treatment and the reduced energy required to mix the lower viscosity slurries at such temperatures. The lower viscosity of the cold process also allows higher solids loadings of the hydrolysis-fermentation slurries, which benefits the final ethanol concentration and decreases the cost of subsequent distillation. The overall conversion efficiency might also be improved at lower temperature because of the limited extent of side reactions occuring at higher temperature during liquefaction (Galvez, 2005). However, a major disadvantage associated with the cold process is its vulnerability to microbial contamination, which is mitigated in the conventional process by the high temperature used during liquefaction. To control and limit the propagation of contaminants, the cold conversion process can be performed at low pH (4.2) with the addition of a pre-saccharification step at a temperature below the geletanization point of the starchy material (Fig 2-5). The recommended temperature can vary widely , from 49^{o}C for rye to 63^{o}C for corn (Genencor, 2010). Apart from being a control measure against contamination, the mild heat treatment also results in higher fermentation rates and ethanol yields (Genencor, 2010). Other measures to control contaminants could included the used of antibiotics (Ai, et al., 2011) or disinfectants such as chlorine dioxide (Genencor, 2010).

In terms of performance, the cold process has been shown to be able to achieve similar output as the conventional process when using corn. A direct comparison between the conventional and the cold processing, at 25% solid loading, using corn found that the conversion efficiency, final ethanol concentrations and fermentation rate were similar for both processes (Wang, et al., 2007). However, in this study no attempt was made to reduce the dosage of the cold enzyme.

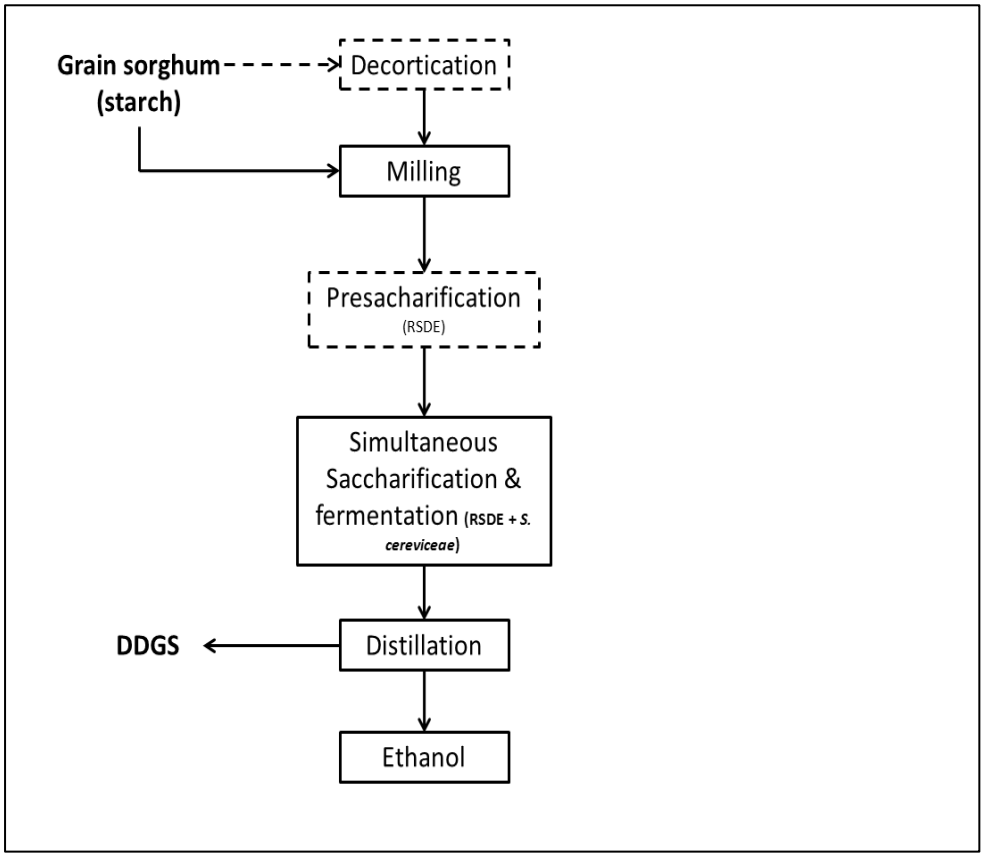

Figure 2-5: schematic diagram of the cold conversion process. (Process steps in dotted lines can be omitted, depending on particular feedstock or other considerations)

2.4.6 Consolidated bioprocess of starch conversion to ethanol

Several researchs groups have genetically engineered microorganisms for expression of either α-amylase or glucoamylase or both (Gorgens, et al., 2014). Some strains of *S. cereviseae* were engineered for RSDE expression to allow for complete conversion of raw starch to ethanol, without

addition of exogenous enzymes to the slurry. The implementation of such genetically engineered strains could lead to a one step (including starch hydrolysis and fermentation) conversion of raw starch to ethanol (Fig 2-6). Such consolidated bioprocessing (CBP) could further make the conversion process more profitable by reducing the cost associated with enzymes and energy required for pumping and stirring the slurry during the liquefaction or pressachafication steps (Van Zyl, et al., 2012).

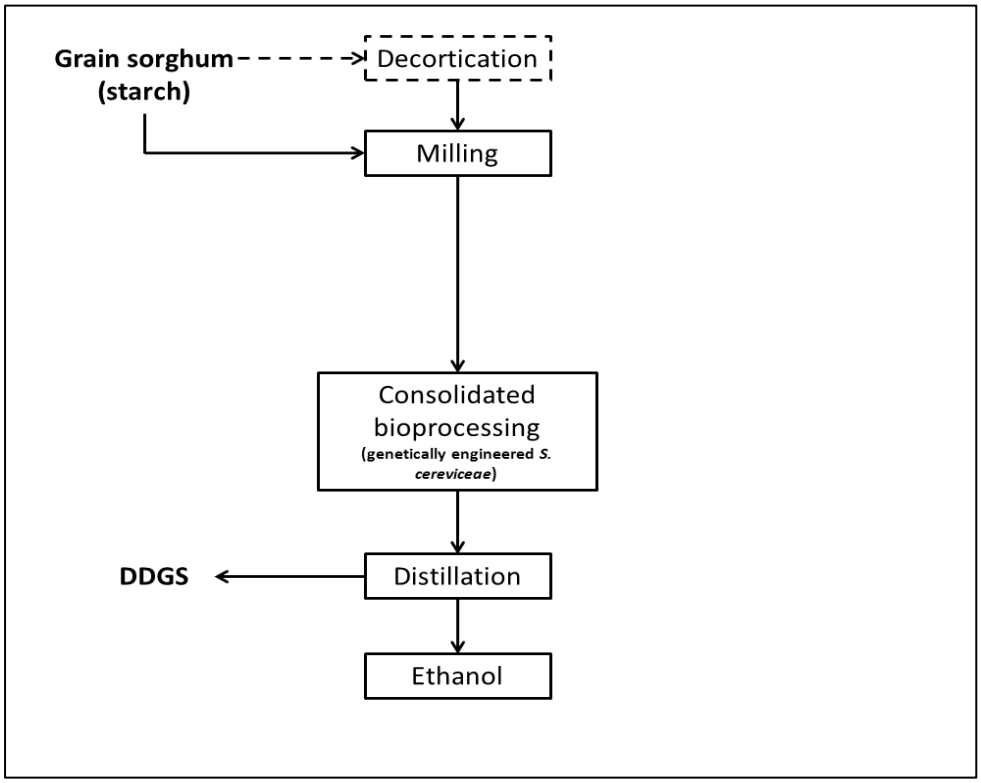

Figure 2-6: Schematic diagram of a consolidated bioprocess. Liquefaction, saccharification & fermentation are occurring simultaneously

In an example of CBP yeast and process performance, up to 10.3 % (v/v) ethanol could be achieved in a 6 days fermentation at 20% solid loading (Kim, et al., 2011). The long time required for the completion of fermentation was attributed to the initial low concentration of enzymes; below the levels required for starch liquefaction and saccharification to proceed effectively. Thus, the low initial enzymes loading appear to be one of the major disadvantages of CBP processes, in the

absence of supplementation with exogenous amylolytic enzymes (Gorgens, et al., 2014; Van Zyl, et al., 2012; Sun, et al., 2010; Robertson, et al., 2006). To achieve faster fermentation rates, several authors have included an initial yeast biomass production phase prior to fermentation. At the end of this phase, the biomass is harvested to inoculate the starch slurry resulting in significantly higher inoculum size. The two strategies employed have been to use the whole fermentation broth at the end of the biomass production phase to inoculate the slurry (Yamada, et al., 2011) or the collection of the biomass by centrifugation of the broth, only to inoculate the starch slurry with cell pellet (Shigechi, et al., 2004). The potential advantage of the first strategy is the addition of amylases produced during the biomass production to be added to the starch slurry, hence increasing the initial enzyme titer compare to the latter strategy. Using these strategies initial biomass concentration of up to 15 g/L have been used; significantly higher than the 0.5 g/L recommended for the conventional processing when using the industrial *S. cerevisiae* strain Ethanol Red (Phibro, 2012).

Khaw et al. (2006) used a fedbatch strategy to achieve a high biomass concentration in the inoculum prepared by yeast cultivation. This phase consisted on a predetermined feeding regime in which the feeding rate of the growth limiting nutrient is increased exponentially to keep the growing cells at a predetermined growth rate. At the end of the biomass production the broth was centrifuged and the cell pellet used to inoculate. Different strategies used in the consolidated bioprocess found in literature are summarized in figure 2-7. It appears that a biomass production phase using the fedbatch regime followed by inoculation using the whole broth has not been employed. Using this strategy, the initial concentration of both biomass and amylases in the starch slurry could be higher and improve hydrolysis and fermentation rates.

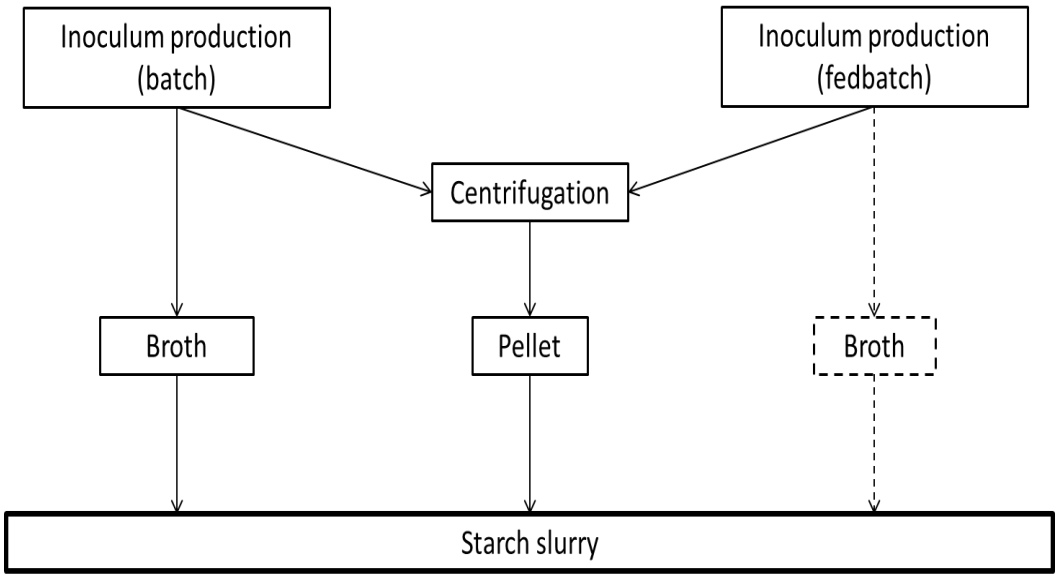

Figure 2-7: Different strategies employed to achieve high initial biomass concentration in starch slurries

To date all reports in literature using the CBP were performed at solid loading of 20% or less, achieving ethanol concentrations around 90 g/L at most (Yamakawa, et al., 2012). Data on the performance of a CBP yeast under VHG conditions are currently unavailable, but is necessary for comparison of CBP to other bioethanol production technologies.

2.5 Performance of ethanol production technologies using sorghum grains as raw materials

The performance of processes for the conversion of starchy materials to ethanol is commonly assessed by several measures. Keys ones include the initial and total solids loading (expressed as the mass percentage of the initial amount of grains to the slurry), the final ethanol concentration, the ethanol volumetric productivity (the final ethanol concentration divide the time required to achieve that concentration), the ethanol yield, expressed as a fraction (percentage) of the theoretical maximum yield, and the fraction of starch hydrolysed. The ethanol yield and fraction of starch hydrolysed are often combined into a single conversion efficiency, expressed as a fraction of the theoretical maximum for the combined hydrolysis-fermentation process. To be economically sound, the abovementioned key performance indicators for the conversion of sorghum grain to ethanol

must meet the current industry standard. To date, ethanol from starch is mainly produced from corn using the conventional process, making it the reference in the industry. Key performances indicators of ethanol production technologies using whole and decorticated sorghum grains found in literature are shown in table 2-2. Although dedicated to sorghum grains, the first entry in the table relates to corn and is only included as a reference for comparison.

Table 2-2: Keys performances measures achieved when using sorghum grains for ethanol production

			References				
		KPM	Devantier et al. (2005)				
High temperature (conventional) process	Whole corn grains	Solids loading (%)	35				
		Ethanol concentration (g/L)	(>118) 130				
		Ethanol productivity (g/Lh)	(>1.7) 1.9				
		Conversion efficiency (%)	(>90) 92				
		Starch hydrolysis (%)	~97				
	Whole sorghum grains		Corredor et al. (2006)	Wu el al. (2008)	Chang et al. (2011)	Wu et al. (2010)	Wu et al. (2007)
		Solid loading (%)	35	30	40	30	30
		Ethanol concentration (g/L)	121.6	95-117	145	-	99 - 112
		Ethanol productivity (g/Lh)	1.7	1.3 - 1.6	3	-	1.3 - 1.5
		Conversion efficiency (%)	-	88 - 93	94	84 - 91.8	85.2 - 90.2
		Starch hydrolysis (%)	-	-	-	-	-
	Decorticated sorghum grains		Corredor et al. (2006)	Perez-Carrillo et al. (2011)	Perez-Carrillo et al. (2008)	Alvarez et al. (2010)	
		Solid (%)/sugars concentration (g/L)	35	20 ^{o}P (~200 g/L)	13 ^{o}P (~130 g/L)	-	
		Ethanol concentration (g/L)	136.5	94.6	67	87	
		Ethanol productivity (g/Lh)	1.89	1.3	4.5	-	
		Conversion efficiency (%)	-	-	86.5	86	
		Starch hydrolysis (%)	-	-	98.3	-	
Cold process	Whole sorghum grains		Ai et al. (2011)				
		Solid loading (%)	35				
		Ethanol concentration (g/L)	-				
		Ethanol productivity (g/Lh)	-				
		Conversion efficiency (%)	78.3 - 80.9				
		Starch hydrolysis (%)	-				

	Decorticated sorghum grains	N/A
Consolidated bioprocess (CBP)	Whole sorghum grains	N/A
	Decorticated sorghum grains	N/A

N/A: No available data

2.5.1 Performance using cooked starch

The conventional warm process using VHG slurry typically achieves a final ethanol concentration of at least 118 g/L (15 %v/v) within 72 hours, corresponding to a volumetric ethanol productivity of at least 1.7 $g.L^{-1}.h^{-1}$. A hydrolysis-fermentation conversion efficiency of at least 90 % is considered as the threshold for an efficient conversion process. However in practice, better performances are usually achieved. Key performance measures achieved at current bioethanol plants using corn (Table 2-2; Wu, et al., 2008) include conversion efficiency and final ethanol concentration around 92% and 130 g/L (16.5%; v/v), respectively, with ethanol productivity around 2 $g.L^{-1}.h^{-1}$ (Devantier, et al., 2005).

Currently few plants use sorghum grains as raw material for biethanol production. When using an initial solids loading of 30%, a final ethanol concentration of up to 117 g/L (14.8 %; v/v) and conversion efficiencies up to 93% could be achieved (Table 2-2). Although ethanol volumetric productivities of 1.7 $g.l^{-1}.h^{-1}$ were not achieved, the maximum ethanol concentrations were achieved within 72 hours (Wu, et al., 2007; Wu, et al., 2008; Wu, et al., 2010). At initial solids loading of 35%, a final ethanol concentration of 121 g/L (15.3 %; v/v) was achieved in 72 hours, corresponding to an ethanol volumetric productivity of 1.7 $g.L^{-1}h^{-1}$ (Corredor, et al., 2006). Fermentation at initial solids loading up to 40% were performed by Chang, et al. (2011), achieving a final ethanol concentration of 145 g/L and a volumetric ethanol productivity of 3 $g.L^{-1}.h^{-1}$. The conversion efficiency was 94%, significantly above the threshold of 90% for efficient industrial processes.

The conventional process using decorticated sorghum grains in VHG slurries has not been investigated as extensively as whole grain slurries. Only one author was found to have investigated such configuration (Corredor, et al., 2006), using an initial solid loading of 35% achieving an ethanol concentration of 136 g/L and volumetric productivity of 1.9 $g.L^{-1}.h^{-1}$. All other authors have performed their fermentation at initial solid loading lower than 30% (w/w). Some studies using decorticated grains for bioehanol production have included a filtration step after liquefaction and before saccharification, to remove some soilds. At a sugars concentration adjusted to 20 ^{o}P (~200

g/L) Perez-Carrillo, et al. (2011) achieved a final ethanol concentration of 94 g/L with an ethanol productivity of 1.3 $g.L^{-1}.h^{-1}$. The conversion efficiency was not mentioned and could not be calculated, since the starch content of the solids retained was not determined. In another study, a conversion efficiency of 86% was obtained when an initial saccharide concentration was adjusted to 13 ^{o}P (~130 g/L), with the ethanol concentration reaching 67 g/L (Perez-Carrillo, et al., 2008). A similar conversion efficiency was achieved by Alvarez, et al. (2010), with a final ethanol concentration of 87 g/L.

2.5.2 Performance using raw starch

Only one author (Ai, et al., 2011) was found to have reported the conversion of raw sorghum starch to bioethanol production, where sorghum grains from 5 varieties were compared. The initial solids loadings were 35% and the highest conversion efficiency was 80.9% (Ai, et al., 2011). The final ethanol concentration and extent of starch hydrolysis were not reported.

2.6 Factors affecting efficient conversion of starch to ethanol

2.6.1 Process parameters (variables) and quantification of their effect

Process parameters affecting the conversion process refers to variables that can be manipulated by operators, resulting in increased or decreased process performance, according to aforementioned KPMs. Process parameters described earlier, including solids loading, decortication and milling, are not included in this section.

2.6.1.1 Enzyme dosages

As enzymes account for about 5% to 7% of the operating costs of the conventional process (Eidman, 2007; McAloon, et al., 2000)), industrial processes for grain-ethanol production will aim to use the lowest possible enzyme dosage, to achieve the desired hydrolysis-fermentation performance – as per the KPMs mentioned earlier. Alpha-amylase and glucoamylase have to be added separately in the conventional process, because of the large temperature difference between liquefaction and SSF. As the two types of amylases work synergistically, the correct dosage of each is necessary to achieve efficient and complete starch hydrolysis. The manufacturer's recommended dosage for the

enzymes used in conventional process were applied in the present study, as shown in table 2-3. Because the inherent cereal grain properties can affect the conversion efficiency (e.g. starch content and others, discussed later), the recommended dosage range of 0.25-0.5 kg/ton grains for α-amylase and 0.45-0.75 kg/ton grains for glucoamylase is large. Previous reports have applied optimisation methods to determine the correct (minimum) dosages for a particular feedstock (Yingling, et al., 2013; Zhang, et al., 2013).

As opposed to the conversional warm process, the cold process does not require a high temperature treatment. Both types of amylases are added simultaneously to the cold process, although, similar to the conventional process, the high recommended dosages (1 – 3 kg/ ton grains) requires optimization/minimisation for efficient starch hydrolysis. Combining the recommended dosages for enzymes in the cold process results in a much larger amount of enzyme added, compared to the conventional warm process. For reasons discussed previously a pre-saccharification step can be include in the cold process, during which only the acid α-amylases GC626 is added to the slurry (Table 2-3).

Table 2-3: Recommended dosage for enzymes used in this study

	Conventional process		Cold process	
	Termamyl SC (α-amylase)	Saczyme (glucoamylase	GC 626 (Acid α-amylase)	Stargen 002 (cocktail of α- & glucoamylase)
Dosage (Kg/ ton grains)	0.25 - 0.5	0.45 - 0.75	0.13 - 0.16	1.0 - 3.0

2.6.1.2 Temperature treatment (liquefaction and pre-saccharification)

Temperatures around 90 ^{o}C are required for liquefaction for the conventional process. Equally important is the duration the slurry stays at the liquefaction temperature. Novozymes recommends minimum and maximum residence times of 90 and 150 minutes, respectively. The correct residence time for each feedstock have to be determined experimentally through optimization, as it depends on other factors such as solid loading, enzymes dosage and other properties of a particular

feedstock. Finding the required residence time is important to achieve complete gelatinization and hydrolysis of starch and also limits the extent of side reactions causing nutrient losses (Galvez, 2005). Both of which could results in lower conversion efficiencies.

In the case pre-saccharification is used for the conversion of raw starch (cold process and CBP), the determination of the correct heat treatment through optimization experiments is necessary to achieve the aim of this step. Genencor recommends pre-saccharification times between 40 and 90 minutes.

2.6.1.3 Nitrogen supplementation and protease addition

The supplementation of fermentation slurries with nitrogen is often required for yeast to achieve optimum fermentation performance, particularly for VHG slurries. The medium may be supplemented with organic (e.g. free amino nitrogen, FAN) or inorganic (typically ammonium sulfate or urea) nitrogen sources to improve the glucose conversion to ethanol (Bothast & Schlicher, 2005). The manufacturer of Ethanol Red, one of the preferred microorganism for industrial bioethanol fuel production, recommended supplementation to achieve at least 300 ppm of FAN in the fermentation slurry, and to avoid stuck fermentations. Another way to meet the nutritional requirement of the yeast without adding nitrogen, is to hydrolyze the inherent proteins of the grains into FAN through the addition of proteases (Perez-Carrillo, et al., 2008). Both methods or their combination can be used to achieve higher ethanol productivity (Johnston & McAloon, 2014).

2.6.1.4 Quantification of the effect of process parameters

To achieve optimum ethanol production performance, it is necessary to understand the effect of each variables involve in the process. An adequate technique commonly used to achieve it is known as response surface methodology (RSM). It is a modelling technique based mathematical and statistical tools used to monitor the effect of several process variables simultaneously. The model equation relating values of the process variables to a process response (or KPM) is expressed as:

$$\mathrm{Y} = b_o + \quad_{i=1} b_i X_i + \quad_{i=1} b_{ij} X_i X_j + \quad_{i=1} b_{ii} X_i^2$$

Where Y is the predicted response (ethanol concentration, ethanol yield or ethanol productivity), b_0 the constant coefficient, b_i the linear coefficient, b_{ij} the interaction coefficient and b_{ii} the quadratic coefficient.

Furhermore, as the performance of the ethanol production process is assessed on several KPM, generating model equations for desired process responses, enable the operator to predict the effect of variation in specific process parameters on the overall process performance and hence achieve desired process performances. This technique has been successfully used by several authors in bioethanol production from starch and other raw materials (Yingling, et al., 2013; Zhang, et al., 2013; Romani, et al., 2012; Yingling, et al., 2011).

2.6.2 Grain properties

2.6.2.1 Tannins content

The presence of tannins in sorghum grains is generally known to have a negative effect on the ethanol production from sorghum grains by interacting with components in the slurry. It is believed that at least four types of interactions can possibly occur between the proteins and tannins: (i) hydrogen bonding between hydroxyl and receptor groups in proteins such as –NH, SH and –OH groups (Van Buren & Robison, 1969), (ii) ionic bonding between anionic and cationic groups in tannins and proteins respectively, (iii) hydrophobic interaction and (iv) covalent bonding between both compounds (Butler, et al., 1984). Furthermore, there is also evidence that tannins can interact with polysaccharides (Rooney & Pflugfelder, 1986).

Tannins may adversely affect the conversion of starch to ethanol through these interactions with inherent (polysaccharides and proteins) or exogenous (enzymes) components in the process. Wu et al. (2007) studied the effects of the chemical composition of seventy genotypes of grain sorghum on ethanol production, and identified tannins content as one of the factors negatively affecting the process. The conversion efficiency of high tannins level grains were significantly lower ($P < 0.05$) than their low tannins' level counterpart. Grain with high tannins content were more difficult to liquefy,

requiring a longer liquefaction time, while the viscosities of the mashes were also higher. Furthermore, Zhao et al. (2008) showed that there is a strong linear relationship between tannin content and final viscosity of the mashes (R^2 = 0.91, P <0. 0001). High viscosity is not a desirable characteristic for mashes, when aiming to achieve the industrial standard of at least 15 % (v/v) final ethanol concentration on the fermentation broth, which will require VHG fermentation with solids loading above 30 % (w/w).

Several grain pretreatment steps have been found to reduce tannin's levels or lower their negative effects on later stages. Mechanical pretreatment such as dehulling and decortication can be very effective. These methods rely on the fact that tannins present in sorghum grains are located in the outer layers of the grain known as pericarp and sometimes in the (pigmented) testa. By decorticating of grains beforehand, their levels in subsequent fermentation can be reduced significantly, thus improving the process efficiency (Chibber, et al., 1980; Perez-Carrillo, et al., 2008). Zhao et al. (2008) have shown that decortication can removed up to 96% of total tannins, and following that treatment sorghum grains with initially high and no tannins levels had similar viscosities. Similar results were found by Wu et al. (2007) who found that the viscosity profile of decorticated and non-decorticated sorghum grain with high levels of tannins were completely different, and that the fermentation effciency of the latter was improved. Corredor et al. (2006) found that the decorticated grains had 12% less protein, 89% less fibre and 16% greater starch content, compared to the original grains. The resulting increase in final ethanol concentration up to 18% (v/v) was attributed to avoiding the negative effects of tannins and achieving higher starch loadings with decorticated grains (Corredor, et al., 2006).

Sorghum grains can also be treated chemically to deactivate the tannins. Soaking the grains in dilute solutions of formaldehyde was very effective (Daiber, 1975). It is believed that a polymerisation reaction takes place during the process, similar to the ones resulting in phenol-formaldehyde resins (Morrison & Boyd, 1983). However, toxicity of formaldehyde to humans and risks of residual content

make this treatment particularly unpopular for the food industry. Although not as effective as the formaldehyde treatment, using dilute aqueous sodium hydroxide and ammonia solutions are also known to be effective (Okolo & Ezeogu, 1996; Price & Butler, 1979).

2.6.2.2 Starch content and amylose:amylopectin ratio

Several authors have demonstrated a linear relationship between starch content and the amount of ethanol produced per unit biomass (Zhan et al., 2003; Wu et al., 2007). However at similar starch contents, all grain sorghum varieties do not have similar yield. Waxy and heterowaxy varieties were shown to result in improved ethanol yields compared to grains containing non-waxy starches. Generally, a lower amylose content appears to be desirable, to improve conversion efficiency, which could be of importance for sorghum crop development and selection of varieties. Wang et al. (2008) compared grains for 70 genotypes of sorghum using the conventional warm process and found a difference of up to 7.4% in final ethanol concentration for grains with similar starch contents, but differing in their amylose/amylopectin ratios; the ethanol concentration was higher for grains with low amylose content. Similar observations were made by Wu et al. (2007.

Sharma, et al. (2007) studied the effect of different amylose:amylopectin ratios on ethanol production from maize (corn) using both conventional and raw starch hydrolyzing enzymes. For both the conventional and cold processes, the final ethanol concentration decreased as the amylose content increased. However, the detrimental effect of higher amylose ratios were more pronounced for the cold process.The highest ethanol concentrations achieved when using conventional and raw starch hydrolyzing enzymes on high amylose maize were 6.3 % (v/v; 49.7 g/L) and 5.2 % (v/v; 41g/L) respectively.

2.6.2.3 Protein content

Proteins are the second most abundant component in grain sorghum, with their content varying between 8 – 14 wt%. Thus, the ethanol yield per ton of grain usually decreases as the protein content thereof increases, as a consequence of lower starch content associated with the latter (Wang, et al., 2008; Wu, et al., 2007; Zhan, et al., 2003). However, when the protein and starch

contents were similar, the conversion process of some grain types was more efficient compare to others. Some reports attributed the improvement in conversion efficiency to better protein digestibility during fermentation, even though the experiments were performed without the addition of proteases. Wang et al. (2008) found that there was a strong linear correlation (R^2 = 0.91) between protein digestibility and ethanol conversion efficiency, with variation of up to 8%. Wu et al. (2007) also observed that samples with high protein disgestibility had improved ethanol yields and conversion efficiencies, although no correlation between the two was reported. On the other hand, similar experiments performed by Shuping (2011) did not agree with the authors above, as no correlation between protein digestibility and conversion efficiency was found – attributed to the absence of exoprotease production by yeast cells. Protein digestibility should be of importance for starch conversion to ethanol, as the inherent proteins of the grains are often used to meet the assimilable nitrogen requirement for yeast, through protein hydrolysis by proteases.

Another possible mechanism responsible for low ethanol yield and conversion efficiency is the entrapment of small starch granules in the protein matrix making them inaccessable to enzymes, particularly following the cooking step where proteins denature and cross-link which each other, forming web-like matrix. This hypothesis is supported by the observation that sorghum grains exhibit a reduced conversion efficiency when they have a high degree of protein crosslinking, independently of the process conditions used (Wang, et al., 2008).

2.7 Conclusion of literature review

Due to its desirable characteristics, sorghum grains is a promising energy crop for bioethanol production in South Africa. As the biofuels industry is to be developed, the choice of the technology to be used should rely on critical assesmment of the performance of current production technologies. The production of bioethanol from raw starch has the advantages over the conventional process of requiring less energy and could decrease costs; but this is counteracted by the increased enzyme dosage and the need to implement mechanisms to control contamination. Furthermore, there is a lack of available performance data for the conversion raw starch sorghum to ethanol. Although decortication has been shown to improve sorghum starch hydrolysis, no study was found investigating how the cold and consolidated bioprocessing would be affected or whether enzymes dosage requirements can be reduced by decortication. The availability of such data is required to provide a way to better compare the current bioethanol production technologies using sorghum grains.

2.8 Research questions and strategy

The questions to be answered in this work and the strategy employed are as follow:

- Can the performance of the cold processing and the consolidated bioprocessing match the performance of the conventional processing when using sorghum grains?

Keys performance indicators relevant to the ethanol fuel production (ethanol concentration, ethanol yield, ethanol productivity and yield from consumed glucose) achieved when using each processing methods were determined and compared, under conditions of minimum required enzyme dosages for acceptable fermentation process performance.

- How is the performance of the conventional, cold and consolidated bioprocessing affected by decortication?

Experiments where performed at different values of the independent variables using whole and decorticated grains. Variations in the values achieved for the key performance indicators for each processing methods were compared under different grains configurations. Furthermore all configurations were optimized to achieve pre-determined key performance indicators values while minimizing the enzyme requirements. The amount of enzymes required to achieve the desirable indicators were then compared.

- What are the benefits of decortication and the processing methods on the chemical composition of Distillers dried grains with solubles (DDGS).

DDGS were produced from whole and decorticated grains obtained from two different sorghum varieties using different processing methods. The average variation in the nutrient contents between grains configurations and processing methods in the resulting DDGS were compared.

2.9 Research chapters layout

These strategies are developed in 2 research chapters as follow:

- Effect of sorghum decortication on ethanol production using the conventional and cold processing and on the chemical composition of the distillers' dried grains with solubles (Chapter 3):

In this chapter the conventional and cold processing are compared by investigating the effects of the investigated variables on the keys performance indicators as affected by decortication. The chemical composition of the DDGS obtained from all the investigated processing configurations is also compared. An unabridged version of this chapter is to be submitted for publication.

- Bioethanol production from sorghum grain using a consolidated bioprocessing yeast producing raw starch hydrolysing enzymes (Chapter 4):

In this chapter the performance of a CBP yeast in a consolidated bioprocess was compared to the performance of the conventional grain-ethanol process.

3 Effect of sorghum decortication on ethanol production using the conventional and cold processing and on the quality of the dried distillers grains with solubles

3.1 Abstract

The availability of commercial enzymes capable of hydrolysing raw starch (cold processing) has the potential of decreasing the energy associated costs of grain-ethanol production. However, the higher dosages of RSHE for complete starch hydrolysis, compared to cooked starch (conventional) processing method, may eradicate this advantage. It has been reported that sorghum grain decortication can improve the kinetics of starch hydrolysis, and thereby could decrease the require dosage of RSHE. In this study the conventional and cold processing using sorghum grains were compared in terms of minimum enzyme dosages, and the effect of decortication on key performance indicators (ethanol concentration, productivity, yields, and starch hydrolysis) was assessed, as well as the quality of the distillers' dried grains with solubles (DDGS). The conventional and cold processing achieved similar performance when using whole grains. The ethanol yield on glucose was slightly decreased (not significant $p > 0.05$) for both processing methods using decorticated grains, but the performance of the cold process using decorticated grains still matched its whole grains configuration. Decortication had a significant negative effect on the conventional processing, as the ethanol productivity was decreased by 19%. Decortication significantly benefited the cold processing, as the required enzyme dosage could be reduced by 11.7%, while achieving a performance similar to the whole grains configuration. DDGS from decorticated grains had their average crude protein content increased by 26%, with a decrease in average crude fibre content of 30.7 %. However, the acid detergent fibre (ADF) and neutral detergent fibre (NDF) contents of the DDGS were not affected. Cold processing decreased the average ADF and NDF by 17.6 % and 26.7 % respectively when using both type of grains, compared to the conventional processing. The mineral composition was largely unaffected. Hence DDGS from the cold processing using decorticated grains

would be preferable because of higher protein content and the lower ADF content making it more suitable for non-ruminants. No previous reports were found investigating enzymes requirements on the performance of the conventional and cold processing as affected by decortication in very high gravity slurries using sorghum grains, nor on the effect of these processes on DDGS composition.

3.2 Introduction

The burning of primarily non-renewables fossil fuels is required to meet the energy demands of the world's growing populations and economies (Beretta, 2007). Apart from their environmentally damaging conversion process, such as air pollution and global warming (Delucci, 2010), fossil fuels are finite (BP, 2011) and unequal geographical distribution pose serious threat to the energy security of non-producing nations (Sayigh, 1999). As a consequence, the usage of locally available renewable energy sources is being promoted.

Bioethanol, produced by bioconversion of biomass to ethanol, is presently the most important biofuel, based on global production and utilisation (BP, 2015). As bioethanol production using primary food crops such as sugar cane and maize raises ethical issues about competing applications, the use of dedicated non-food energy crops is believed to be a promising alternative (Sanchez & Cardona, 2008). Sorghum grain has been identified as a good candidate for such purpose in water scare countries since it is drought resistant and has a high yield, while being of limited importance in human diets (Rooney, et al., 2007).

The conventional (warm) process for conversion of starches in cereal grains to ethanol involves the liquefaction, saccharification and fermentation of the starch slurry (Bothast & Schlicher, 2005). Liquefaction is performed at temperatures above 90 ^{o}C and is a very energy consuming step, requiring the equivalent of 10-20 % of the energy content of ethanol produced (Robertson, et al., 2006; Gray, et al., 2006). During the liquefaction step, the starch present in the grain is gelatinized and loses its crystalline structure, making it more accessible to the α-amylases and gluco-amylases responsible for its complete hydrolysis (Bothast & Schlicher, 2005). Attempts to reduce both bioethanol production costs and carbon emissions have led to implementation of very high gravity (VHG) technology (>30% solids), to reduce the energy required for heating during liquefaction (>90 ^{0}C), and thus achieving final ethanol concentrations above 15% (v/v), hence also reducing subsequent distillation costs (Puligundla, et al., 2011).

An alternative to reduce the energy demand of the current process is the cold hydrolysis process. It involves the application of no or a mild heat treatment, below the gelatinization temperature of the starchy material, in combination with the utilization of raw starch hydrolysing enzymes (RSHE) capable of hydrolysing non-gelatinized starch (Van Zyl, et al., 2012). Apart from the reduced energy requirement, other benefits include the reduced viscosity of the starch slurry, which can allow higher solids loadings and final ethanol concentrations, and the prevention of nutrient degradation and the formation of undesirable by-products at high temperature through the Maillard reactions (Galvez, 2005). A side by side performance comparison has shown that the two conventional and cold hydrolysis strategy can achieve similar fermentation process outputs when using corn (Wang, et al., 2007).

The bran of sorghum grain is known to contain components such as tannins and flavonoids that are inhibitory to amylases (Awika & Rooney, 2004; Sales, et al., 2012). Their presence or increased levels in whole grains have been shown to negatively affect the hydrolysis, hence increasing the required enzyme dosages for acceptable hydrolysis. As they are located in the bran, their removal from the grain through a decortication process can significantly reduce the flavonoids content of grains (Awika, et al., 2005), with possible benefits in terms of the required enzyme dosages. Furthermore, a conversion process with an initial decortication step prior to milling will benefit from the lower viscosities and peak viscosities of the mashes (Wu, et al., 2007), and higher protein contents of the distillers' dried grains with solubles (DDGS), the main co-product of cereal-ethanol plants (Corredor, et al., 2006). Another advantage of decortication is that the bran removed has a long shelf life and has a high antioxidant activity, which could be valuable for the food and health industries (Awika, et al., 2005). In the field of cereal sciences, the removal of the fibrous seed coat of cereal grains, known as debranning, dehulling or decortication, depending on the type of grains, has been studied extensively (Sikwese & Duodu, 2007; Wang, et al., 1999). The effect of bran components on starch hydrolysis by amylases has also been described (Sales, et al., 2012; Alvarez, et al., 2010). However, very few studies have described the effect of debranning/decortication on the required enzyme

dosages and performance of the ethanol production, particularly in VHG slurries. The relationship between debranning and the final ethanol concentration in fermentation has been investigated (Corredor, et al., 2006; Perez-Carrillo, et al., 2008), although VHG fermentations, cold processing and ethanol productivities were not considered.

The main co-product of cereal-ethanol plants, the distillers' dried grains with solubles (DDGS) is the dry residue obtained after the removal of ethanol, water and other volatile compounds from the fermentation broth (slurry). Except for starch, which is hydrolysed and converted to ethanol during fermentation, other nutrients such as proteins, fats, minerals and fibres present in the initial grains, are concentrated by approximately three-fold in the DDGS (Liu, 2011). DDGS has become a feed ingredient of choice in ruminant production systems because it contains proteins (in adequate amounts for ruminants), energy and important ions (phosphorus, calcium) (Singh, et al., 2005). Furthermore, the increased interest in DDGS as animal feed has been due to the increase in prices of alternative feed ingredients (corn, soybean meal) required to meet the dietary requirement of industrially reared animals (US Grains Council, 2013).

The continuous increase in first generation bioethanol production from cereals grains is expected to result in increasingly larger amount of DDGS being available (Liu, 2011). As further increases in the production of bioethanol are likely to be from the dry grind process, this could lead to an overproduction of DDGS. Hence diversifying the market for DDGS has become an important issue. In the US where DDGS is mainly obtained from corn, variation in DDGS production methods have resulted in the availability of several quality DDGS such as higher protein, lower fibre or lower fat content to cater for different animal production systems (Christen, et al., 2010).

Sorghum decortication and cold processing as means to improve the DDGS quality have not been studied, nor the quality of the DDGS obtained by these processes compared to corn DDGS. Sorghum DDGS is known to have a higher protein content than corn DDGS (US Grains Council, 2013). This protein content can be further increased by decortication before hydrolysis-fermentation, which

removes the bran consisting of mainly fibres, and is thus expected to decrease fibre content in DDGS. Corredor, et al. (2006) have shown that 10% decortication of sorghum grains can increase the protein content of DDGS by up to 8 %, while the concentrations of the minerals assessed (only phosphorus and calcium) were not significantly altered. The proportion of other important fibres such as neutral detergent fibre (NDF) and acid detergent fibre (ADF), which are good indicators of the amount of forage for ruminant and fibre digestibility for non-ruminants respectively, were not determined in that study. In animal nutrition systems, lower ADF content are preferred, particularly for non-ruminants, since they relate to the amount of least digestible fibre. NDF are of particular importance for ruminants, since it include ADF as well as fibres that are digestible to them. Furthermore studies with corn have shown that DDGS produced using the cold conversion process have a lower amount of NDF and ADF than the one obtained by conventional processing, hence making them more digestible for non-ruminant species (Kelzer, et al., 2010; Robinson, et al., 2008). Such data were not found in literature for sorghum DDGS.

In this work, the effect of decortication on ethanol concentration, ethanol volumetric productivity and ethanol yield is assessed when using the conventional high temperature process and the cold conversion process. A response surface methodology was used to model the effect of variables on the desired responses, with experimental validation of the predicted optimum parameters, allowing comparison of different process configurations. The composition of the DDGS as affected by decortication and enzymatic hydrolysis methods (conventional and cold) was also assessed.

3.3 Materials and Methods

Raw materials

White sorghum grain was obtained from Agricol (Pty) Ltd (Brakenfell, Cape Town, South Africa). The grains were air-dried for 3 days, vacuum packed and stored at room temperature until needed. The moisture content of the stored grains was 8% (w/w). Before usage, the grains were milled using a Retsch mill (SM 100, Haan, Germany) to pass through a desired screen size. Sorghum grains were decorticated using a modified rice miller tester 65 (Grainman Corp, Miami, Florida, USA). Before

decortication the grains were conditioned at room temperature to 16 % moisture for 10 min. Grains were decorticated in batches of 500 g for 20 seconds. Starch content of decorticated grains was 73 %.

For DDGS production, two sorghum varieties were used in this study. PAN 8816 obtained from Pannar Seed (Pty) Ltd (Greytown, South Africa) referred to as Sorghum 01 in this work. The second variety, referred to as Sorghum 02 in this work, was the one initially described.

Enzymes, microorganisms and reagents

Enzymes used for the conventional (high temperature) process were thermo-stable α-amylase from *Bacillus licheniformis*, Termamyl SC (Novozymes, Bagsvaerd, Denmark) with a declared enzyme activity of 120 KNU/g (KNU, kilo novo units α-amylases – the amount of enzymes which breaks down 5.26 g of starch per hour at 37^{o} C, pH 5.6, 0.0043 M Ca^{2+}, reaction time 7-20 minutes) and glucoamylase Saczyme (Novozymes) with declared activity of 750 AGU/g (AGU, amyloglucosidase units – the amount of enzyme that catalyses the conversion of one µmol of maltose per minute at assay conditions of 37 ^{o} C, pH 5.0, substrate concentration 10 mg/ml, incubation time 30 min). For the cold process, acid stable α-amylase GC626 (Genencor, California, USA) and enzyme cocktail Stargen 002 (Genencor) were used. Stargen 002 is a blend of alpha- and gluco-amylases with declared activity 570 GAU/g (GAU, glucoamylase unit – the amount of enzyme that liberates one gram of glucose per hour from soluble starch at 60 ^{o} C, pH 4.2).

Ethanol Red dry yeast (LEAF Technologies, Marcq-en-Baroeul, France) was used for the fermentation. The inoculum was freshly prepared for each fermentation. Ethanol Red dry yeast (2.5 g) was rehydrated in 50 mL of 2% glucose solution at 33 ^{o}C for 25 minutes in a shaking incubator at 100 rpm. One mL of the broth was added to each flask as inoculum. Urea and calcium chloride for fermentation supplementation were obtained from Sigma-Aldrich (St. Louis, Missouri, USA).

Mash preparation and SSF procedures

Experiments were performed in pre-weighed 250 mL flasks using sorghum grain milled to pass through a 2 mm screen (Fig 3-1). Thirty five grams of sorghum grain flour was mixed with water and calcium chloride (5 mg / 100 g slurry) to achieve final mass slurry of 100 g. The pH of the slurry was adjusted to 5.8 with 1M H_2SO_4, before the adequate amount of α-amylase (Termamyl SC) was added (Table 3-1). The slurry was heated to 88°C in a water bath for the required time (Table 3-1). An overhead stirrer was ensuring adequate mixing throughout liquefaction. At the end of liquefaction, the mash was cooled to 30°C by placing the flasks in water at ambient temperature, before the addition of the adequate amounts of glucoamylase (Saczyme), 0.07 % urea and the inoculum (Table 3-1). Due to water losses during liquefaction, the mass of the flask was readjusted 100 g. SSF was performed at 30°C in a shaker for 120 hours and samples were taken every 12 hours.

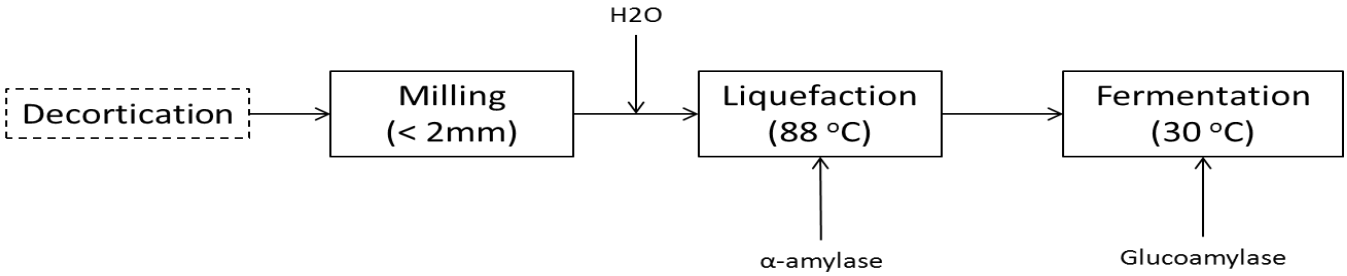

Figure 3-1: Schematic representation of the procedure used for the conventional processing.

For cold process (Fig 3-2), instead of liquefaction at 88°C, a mixing (presaccharification) step was performed at 65°C for the required time (Table 3-2) with the addition of acid stable amylase GC 626 to the slurry (14 µl/100 g grain). The pH was adjusted to pH 4.2. The mash was cooled to 30°C similarly to the conventional process before the addition of the required dosage of Stargen 002 (Table 3-2). The grains were milled to pass through a 0.5 mm screen.

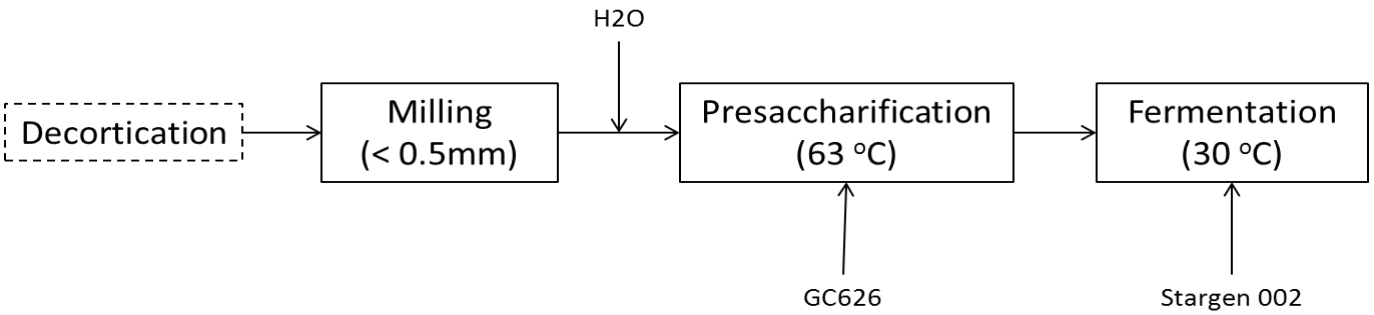

Figure 3-2: Schematic representation of the procedure used for the cold processing.

Scale-up experiments were performed in 5 L jacketed bioreactors (Sartorius, Goettinger, Germany) fitted with propeller type impeller and an exhaust cooler at 4°C to prevent ethanol and water losses through evaporation. Prior each experiment the reactor was washed, and sterilized in an autoclave at 121 °C for 15 minutes. However, liquefaction and pre-saccharification were not performed aseptically in order to simulate industrial conditions. The working mass of slurry in the reactor was 3 kg. For liquefaction, water (1.95 kg) was added and heated to 88 °C. Once the temperature was reached, the pH was first adjusted to 5.8 and the required dose of Termamyl SC added. Then the milled grains (1.05 kg) were added to the reactor, while the pH was constantly maintained at 5.8. At the end of the liquefaction, the slurry was cooled to the temperature setpoint of 30 °C, before the addition of the inoculum and the required dosages of urea and Saczyme. The fermentations (SSF) were run for 120 hours and samples taken every 12 hours for HPLC analysis. The pH was not controlled. For the cold process instead of liquefaction at 88°C, a pre-saccharification was performed at 65°C in a similar manner, with appropriate enzymes.

Statistical design and analysis

A central composite design was used to determine the effect of decortication on ethanol concentration, ethanol productivity and conversion efficiency. The values of the independent variables were chosen based on preliminary experiments. The factors and their levels are shown in Table 3-1 (conventional process) and Table 3-2 (cold process). The centre points were replicated five

times. The statistical software Design Expert 7 (Stat-Ease Inc., Minneapolis, USA) was used for analysis. For both configurations the responses studied were the final ethanol concentration, the ethanol yield as a fraction (percentage) of the theoretical maximum and ethanol volumetric productivity. The time at which the fermentation was completed, was defined as the time beyond which the ethanol concentration did not increase by more than 5%.

The responses were calculated as follow:

$$Ethanol\ yield\ as\ \%\ of\ theoretical\ max. = \frac{Volume\ liquid\ at\ fermentation\ end\ \ L\ \times ethanol\ concentration\ (\frac{g}{L})}{\text{Initial mass of starch (g)} \times 0.567} \times 100 \quad (1)$$

$$Percentage\ starch\ hydrolysis\ (\%) = \frac{Initial\ starch\ \ g\ - residual\ starch\ (g)}{\text{Initial starch (g)}} \times 100 \quad (2)$$

$$Ethanol\ productivity\ (g.L^{-1}.h^{-1}) = \frac{Ethanol\ concentration\ (\frac{g}{L})}{\text{Fermentation time (hrs)}} \quad (3)$$

$$Ethanol\ yield\ from\ consumed\ glucose\ (\%) = \frac{Percentage\ theoretical\ yield\ (\%)}{\text{Percentage starch hydrolysis (\%)}} \quad (4)$$

The responses (dependent variables) obtained by the experiments performed according the central composite design were related to the independent variables investigated by empirical models:

$$Y = b_o + \ _{i=1}\, b_i X_i + \ _{i=1}\, b_{ij} X_i X_j + \ _{i=1}\, b_{ii} X_i^2 \quad (5)$$

Where Y is the predicted response (ethanol concentration, ethanol yield or ethanol productivity), b_0 the constant coefficient, b_i the linear coefficient, b_{ij} the interaction coefficient and b_{ii} the quadratic coefficient.

Table 3-1: Factors used in the central composite design and their levels using the conventional process

Factors	Levels		
	-1	**0**	**1**
Liquefaction time (min)	90	120	150
α-amylase dosage (µl/100g starch)	29	58	87
G-amylase dosage (µl/100g starch)	57	96	135

Table 3-2: Factors used in the central composite design and their levels using the cold process

Factors	Levels		
	-1	**0**	**1**
Pre-saccharification time (min)	30	60	90
Stargen dosage (µl/100g starch)	128	256	384

Optimization and validation experiments

Statistical optimization was performed with the models fitted to experimental data, to determine the minimum amount of enzymes required to achieve similar starch hydrolysis and fermentation time for all the process configurations (conventional and cold using whole and decorticated grains). Given that the maximum performances (final ethanol concentration and ethanol yield) achieved by both conventional and cold processing using whole grains were higher than with decorticated grains, the targeted responses for optimization were not the same for all process configurations. The targeted responses for each configuration were set by the criteria of achieving at least 95% of maximum ethanol concentration achieved in each experimental design after 72 hours. The optimum conditions for the validation experiments were determined using the model equations relating the independent variables to the responses. The targeted responses were described as a desirability plot, showing area of the design space where these responses are met. The values used for

validation where chosen to minimize the independent variables for the conventional (Liquefaction time, α-amylase and glucoamylase dosage) and cold (pre-saccharification time and Stargen dosage) processing.

The validation experiments were performed in triplicate for whole grains and in duplicate for decorticated grains.

Distillers dried grains with solubles production procedures

The procedure used to produce DDGS in illustrated in figure 3-3. Experiments were performed in pre-weighed 1 L flasks using sorghum grain milled to pass through a 2 mm screen. One hundred and sixty seven g of sorghum grain flour was mixed with water to achieve final mass slurry of 500 g. Liquefaction and SSF were performed as previously described. Liquefaction was performed for 2 hours after the addition of 100 µl of α-amylase (Termamyl SC) and followed by the addition of 150 µl glucoamylase (Saczyme) prior to SSF. For the cold process, 430 µl of Stargen 002 was added, using grains milled to pass through a 0.5 mm screen. At the end of fermentation the solids (wet distillers' grains) were collected by centrifugation for 5 minutes at 10020 g. The supernatant (thin stillage) was heated on a hot plate (Boiling) until 20% of its initial volume was left (condensed distillers solubles). The wet distillers' grains and condensed distillers solubles were mixed and placed in an oven at 100 ^{o}C for 2 hours to obtain the DDGS.

To obtain the DDGS from decorticated grains, the bran removed during decortication was mixed with water (10% solids). The pH was adjusted to 4.2 followed by the addition of the proteases Fermgen (165 µl) and Stargen (85 µl). The mixture was incubated for 6 hours in a shaking incubator at 30^{o}C and 150 RPM. At the end of the incubation time, the liquid fraction was collected by centrifugation for 5 minutes at 10020 g and added to the milled decorticated grains. This was performed to maximize the protein concentration in the DDGS. The procedure then continued as described previously.

The DDGS production for each configuration (conventional and cold processing using whole and decorticated grains) was performed in triplicate. DDGS obtained from each triplicate experiment were mixed together and analysed once.

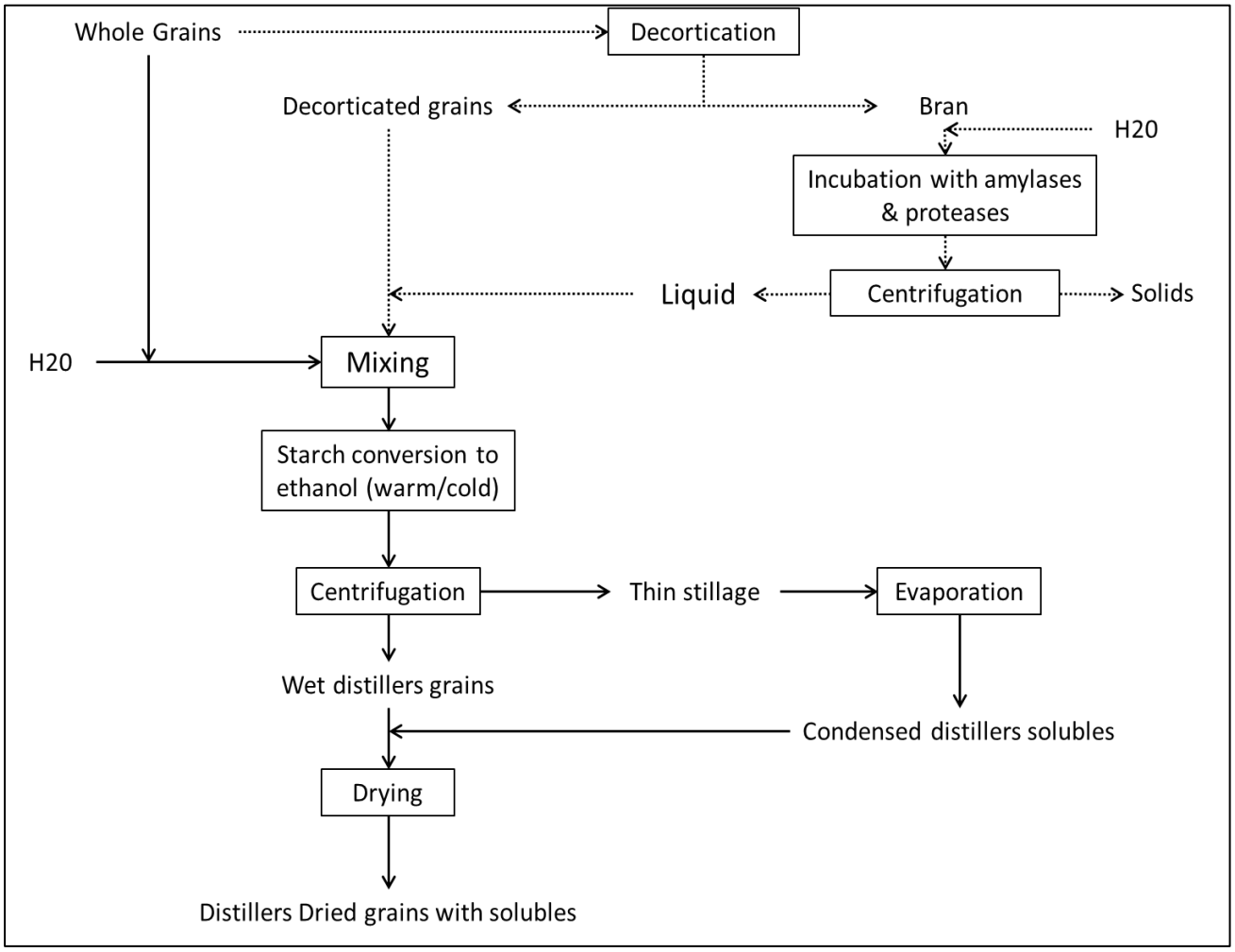

Figure 3-3: Schematic diagram of the DDGS production processes. Dotted lines indicate the steps specific for DDGS production from decorticated grains. A 500 g slurry was used per batch.

Analytical methods

The starch content of sorghum grains (68 %) was determined according to the AACC Method 76-13, using the enzymatic starch assay kit (Megazyme, Co. Wicklow Ireland). Starch content of decorticated grains was 73 %. Moisture content of the material was determined by drying samples in a convection oven adjusted to 105^{o}C until constant weight. Samples taken from the fermentation slurries were centrifuged at 11300 g for 5 min, the supernatants were

diluted 20 times and filtered through a membrane of 0.22 μm pore size (Anatech, Gauteng, South Africa), prior to analysis. Ethanol and glucose concentrations in the supernatant were determined by high performance liquid chromatography (HPLC) equipped with a BioRad guard column and an RI detector. The Aminex HPX-87H column was operated at 65 ^{o}C. The compounds for detection were eluted using 5 mM H_2SO_4 at a flow rate of 0.6 mL/min. The ash was determined by placing the samples in a furnace at 600^{o}C for 6 hours. Crude fat content was determined by ether extraction (AACC approved methods 30-25). The nitrogen content was determined by the Dumas Methods using a LECO nitrogen instrument (FP528, St Joseph, MI). The crude protein content was obtained by multiplying the nitrogen by a factor of 6.25 (AACC approved methods 46-30). The crude fibre, acid detergent fibre (ADF) and neutral detergent fibre (NDF) were determined using an ANKOM 200 Fiber Analyzers (Macedon, NY).

3.4 Results and Discussion

The results and discussions are divided into five sections. First the effect of liquefaction time, α-amylase and glucoamylase dosage (variables) on ethanol concentration, yield and productivity (responses) of the conventional process was assessed, highlighting differences between whole and decorticated grains. Relationships relating variables and responses were developed. The next section focuses on the cold processing, using presaccharification time and Stargen dosage as variables. Data from these two sections were obtained from shake flask experiments. In the third section, experiments were performed at optimum conditions determined using the models developed in the two previous sections. The experiments were performed in bioreactors for all processing configurations (conventional and cold using whole and decorticated grains) and their performances were compared. The effect of decortication and processing methods (conventional and cold) on the quality of the DDGS are then discussed in the fourth and fifth sections respectively.

Effect of decortication on responses when using the conventional processing

The responses of ethanol concentrations, yields and productivities from conventional (warm) processing of grains to ethanol, based on the central composite design using whole and decorticated grains, are shown in Table 3-3. Selected ethanol profiles of the fermentations experiments are depicted in figure 3.4A. The final ethanol concentration increased significantly ($P < 0.05$) from 111.5 g/L to 121.7 g/L, when increasing the glucoamylase dosage from low (57 µl/100g_{starch}; W_L) to high (135 µl/100g_{starch}; W_H), using whole grains (Exp. no 5 and Exp. no 4 respectively; Table 3-3). In both cases the maximum ethanol concentration was achieved at the same fermentation time (60 hrs), indicating that the ethanol volumetric productivity was also higher at higher glucoamylase dosage. The final ethanol concentration was also increased ($P<0.05$) for decorticated grains when increasing the glucoamylase dosage from low (D_L; 110.9 g/L; Fig 3.4A) to high (114.7 g/L; D_H). Both maximum ethanol concentrations were achieved at 84hr, indicating a similar effect of glucoamylase dosages on the volumetric ethanol productivity of decorticated grains. Changing the glucoamylase dosage had a more pronounced effect on ethanol concentrations obtained with whole grains (10.2 g/l), compared to decorticated grains (3.8 g/l).

The ethanol yields as a percentage of the theoretical maximum (Fig 3-4B) followed similar trends as the ethanol concentrations, due to the fixed solids loading applied in fermentations. The ethanol yield when using whole grains at the high glucoamylase dosage (78.8 %) was significantly higher ($P < 0.05$) than at the low dosage (71.4 %). For decorticated grains the ethanol yield at high dosage (74.6%) was decreased to 72.9 % ($P < 0.1$) as a result of lowering the glucoamylase dosage. As with the ethanol concentration, the differences in yields at low and high glucoamylase dosages were more significant for whole grains (7.4 %) than for decorticated grains (1.7 %).

Table 3-3: Experimental conditions and responses values achieved from experiments performed based on the central composite design for whole (W) and decorticated (D) grains using the conventional processing.

Experiments No	Liquefaction Time (min)	α-amylase concentration (µl/100 g starch)	Glucoamylase concentration (µl/100 g starch)	Ethanol concentration (g/L)		Ethanol productivity (g/L.h)		Ethanol yield (% of theoretical max)	
				W	D	W	D	W	D
1	70	58	96	116.5	110.3	1.94	1.31	75.6	70.4
2	90	29	57	114.4	111.8	1.91	1.33	72.8	73.0
3	90	29	135	116.8	114.2	1.95	1.36	74.9	74.2
4	90	87	135	121.7	114.7	2.03	1.37	78.8	74.6
5	90	87	57	111.4	110.9	1.86	1.32	71.4	72.9
6	120	58	161	121.9	119.3	1.69	1.42	78.9	77.2
7	120	9	96	117.6	114.2	1.63	1.36	72.4	74.4
8	120	58	96	116.1	115.2	1.94	1.37	73.6	74.3
9	120	58	96	117.4	115.7	1.96	1.61	75.8	73.7
10	120	58	96	119.8	116.1	1.66	1.38	75.3	74.9
11	120	58	96	118.3	114.6	1.97	1.36	77.5	74.0
12	120	58	96	118.5	117.2	1.65	1.39	76.5	75.4
13	120	107	96	120.5	118.7	1.67	1.41	78.9	77.6
14	120	58	96	119.9	114.3	1.67	1.59	78.1	73.8
15	120	58	30	111.5	110.0	1.16	1.31	71.5	71.4
16	150	29	57	116.4	116.8	1.39	1.62	74.0	75.4
17	150	87	135	122.3	115.9	1.70	1.61	79.5	74.8
18	150	29	135	125.0	116.8	1.49	1.39	80.1	75.2
19	150	87	57	119.8	111.0	1.66	1.54	76.5	72.2
20	170	58	96	121.2	117.1	1.44	1.39	78.8	75.1

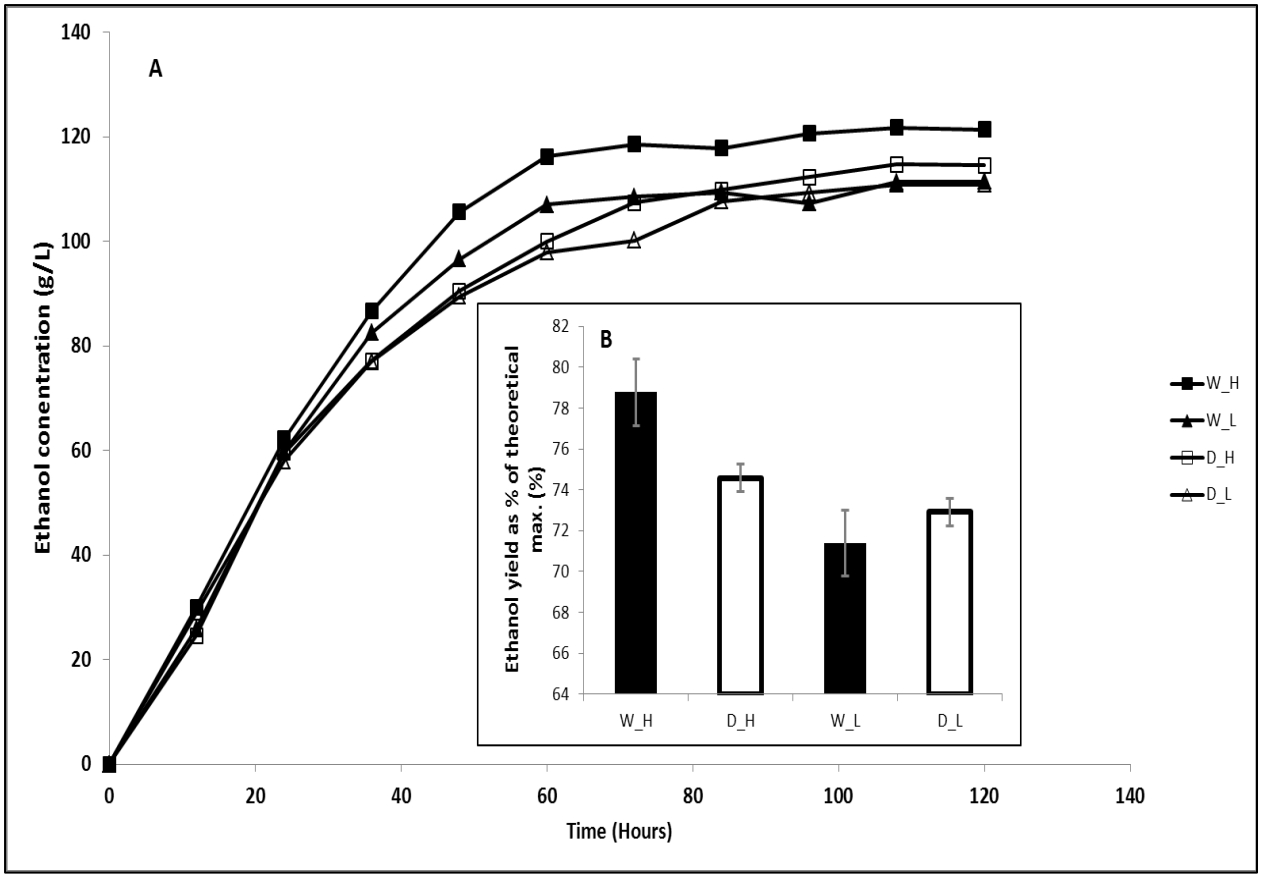

Figure 3-4: A: Ethanol profiles of experiments performed using whole (W) and decorticated (D) grains at low (L_;57 µl/100g_{starch}: -12) and high (H_; 135 µl/100g_{starch}; -11) glucoamylase dosage. α-amylase 88 µl/100g_{starch} and Liquefaction time 90 minutes. B: Ethanol yields as percentage of theoretical maximum, achieved by the experiments in A and at 150 min liquefaction time. Error bars represent ± standard deviation.

The increase in final ethanol concentration and ethanol yield at high dosages of glucoamylase (Fig 3-4A), was attributed to either increased starch hydrolysis, or improvement in the conversion rate of maltodextrins released from α-amylase activities into fermentable sugars at increasing glucoamylase activities in the slurry. Higher ethanol volumetric productivity in slurries with high glucoamylase dosage (Fig 3-4A) indicated that both the yields and kinetics of the hydrolysis reactions benefitted from increased glucoamylase activity, as reported previously by Devantier, et al (2005). Higher ethanol yields for whole grains compared to decorticated grains (Fig 3-4B) is similar to a report by Wang, et al. (1999) when comparing the performance of whole and debranned triticale grains. This decrease in ethanol yield was attributed to nutrient limitations in slurries with decorticated grains,

given that minerals and proteins in the bran are removed during decortication, which may negatively impact on fermentation vigour of the yeast (Pereira, et al., 2010; Wang, et al., 1999).

At similar conditions, the final ethanol concentrations were always achieved earlier when using whole grains (60 hours) compared to decorticated grains (84 hours) (Fig 3-4A), showing higher volumetric ethanol productivity with whole grains (1.39 – 2.03 $g.L^{-1}.h^{-1}$) compared to decorticated grains (1.31 – 1.62 $g.L^{-1}.h^{-1}$) (Table 3-3). Subsequent fermentations with 0.2% (w/w) urea supplementation showed that fermentation with decorticated grains could be completed within 72 hours (Appendix A), confirming the effect of changes in nutrient (nitrogen) content on the ethanol volumetric productivity of fermentations with decorticated grains. The reduction of nitrogen in slurries containing decorticated grains could be caused by nutrients (nitrogen and minerals) removed together with the bran during decortication. Changes in the concentration of nitrogen and minerals are known to affect the fermentation rate in very high gravity ethanol fermentations (Pereira, et al., 2010).

Mathematical modelling of the responses as functions of process variables when using the conventional processing

The process responses (ethanol concentration, ethanol productivity and ethanol yield) were modelled using a response surface methodology, to simultaneously monitor the effect of several variables (liquefaction time, α-amylase dosage and glucoamylase dosage). The models that best fit the relationship between the independent variables and the responses were chosen based on the analysis of variance (Appendix B). All models chosen were significant ($P < 0.05$). When using whole grains, the R^2 values for ethanol concentration, ethanol yield and ethanol productivity were 0.9, 0.89 and 0.65 respectively. For decorticated grains R^2 values for ethanol concentration and ethanol yield were 0.8 and 0.94 respectively. No model to predict the ethanol productivity from decorticated grains was chosen since α-amylase and glucoamylase dosages had no significant effect, and the model taking into account the only significantly factor (liquefaction time) had a very low R^2 value (0.29). Given that the solid loading was unchanged, variations in ethanol concentrations

corresponded to similar variations in the ethanol yields (as percentage of theoretical maximum). Hence surface plots of the ethanol concentration are not shown since they were similar to the ones depicting the effect of the independent variables on the ethanol yield. The equations relating independent variables to the responses are shown in Equation 6, 7 and 8 for whole grains and Equation 9 and 10 for decorticated grains:

Ethanol concentration = 118.35 + 1.99*A + 0.54*B + 3.02*C – 0.17*A*B – 0.2*A*C + 0.24*B*C – 1.75*A*B*C (6)

Ethanol productivity = $1.78 – 0.17*A + 0.091*C – 0.096*C^2$ (7)

Ethanol yield as percentage of theoretical maximum = $75.75 + 1.28*A + 1.94*B + 2.28*C – 0.054*A*B – 0.047*A*C + 0.27*B*C + 0.43*A^2 – 1.05*A*B*C – 1.40*A^2*B$ (8)

Ethanol conc. = $115.27 + 1.49*A + 1.34*B + 1.96*C – 0.79*A*B – 0.8*A^2 – 2.22*A^2*B$ (9)

Ethanol yield as percentage of theoretical maximum = $74.28 + 1.38*A + 0.96*B + 1.71*C – 0.51*A*B – 0.064*A*C + 0.41*B*C – 0.61*A^2 + 0.53*B^2 – 1.38*A^2*B – 1.06*A^2*C – 1.00*A*B^2$ (10)

Where A is the liquefaction time, B the α-amylase dosage and C the Glucoamylase dosage

The surface plots of ethanol yield as percentage of theoretical maximum obtained from whole (Fig 3-5A) and decorticated (Fig 3-5B) grains showed that for decorticated grains, an optimum liquefaction time of approx. 120mins was predicted, to reach a maximum ethanol yield of ~77 %. While for slurries containing whole grains the predicted maximum ethanol yield (~80 %) could be achieved at a much wider range of liquefaction time (90 – 150 min). The plots of the calculated values achieved for whole and decorticated grains (Fig 3-5C; Lliquefaction time 120 min) showed that the ethanol yield was higher for whole grains in most of the design space, as observed in Figure 3-4.

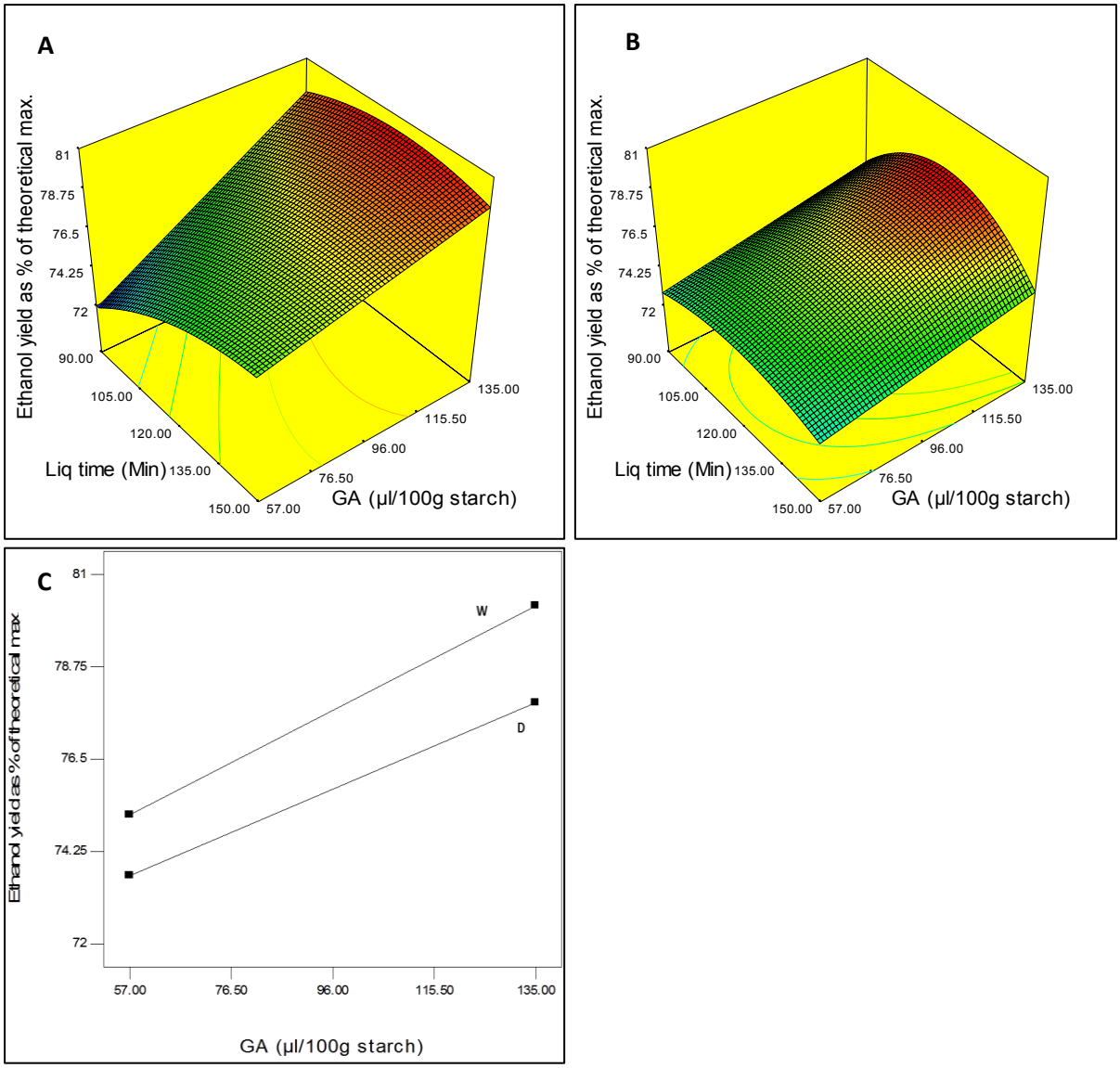

Figure 3-5: Response surface plot obtained when using the conventional processing, α-amylase dosage 87 µl/100g_{starch} (A, B). A: Ethanol yield as percentage of theoretical maximum whole grains. B: Ethanol yield as percentage of theoretical maximum decorticated grains. C: Comparison of ethanol yields achieved using whole (W) and decorticated (D) grains decorticated grains, α-amylase 87 µl/100g_{starch}, liquefaction time 120 min.

Effect of decortication on the responses when using the cold processing

The response values obtained when using the cold processing for whole and decorticated grains are shown in Table 3-4. Figure 3-6A shows the ethanol profiles of two experiments at high (384 µl/100g_{starch}; Exp. no 2) and low (128 µl/100g_{starch}; Exp. No 3) Stargen dosage using whole (W_H and W_L respectively) and decorticated grains (D_H and D_L respectively) performed at pre-saccharification time of 30 min. For fermentations with whole grains, a higher ($P<0.05$) ethanol concentration was achieved with the high Stargen dosage (W_H; 124.3 g/L) compared to the low dosage (W_L; 113.1 g/L). The ethanol yield as percentage of theoretical maximum achieved by the experiments with the ethanol profiles in Figure 3-6A are depicted in Figure 3-6B. Using whole grains, significantly higher ($P<0.05$) ethanol yield of 77% was obtained at high Stargen dosage, compare to a yield of 70 % at low Stargen dosage. For decorticated grains the differences in ethanol yields at high and low Stargen dosages (75 and 74%, respectively) were not significant ($P>0.05$). Thus, the increased ethanol concentration and ethanol yield at high enzyme dosage observed for the conventional processing, was thus replicated with Stargen enzymes and cold processing (Fig 3-6A), indicating the effect of enzyme dosage on starch hydrolysis and subsequent fermentation.

Table 3-4: Experimental conditions and responses values achieved from experiments performed based on the central composite design for whole (W) and decorticated (D) grains using the cold processing.

Experiments No	Pre-saccharification Time (min)	Stargen dosage (µl/100 g starch)	Ethanol concentration (g/L)		Ethanol productivity (g.L-1.h-1)		Ethanol yield (% of theoretical maximum)	
			W	D	W	D	W	D
1	18	256	123.6	115.4	1.72	1.92	76.5	74.6
2	30	384	124.3	117.0	1.73	1.95	77.0	75.1
3	30	128	113.1	114.4	0.94	1.19	70.4	73.8
4	60	256	124.2	117.0	1.72	1.62	77.1	76.0
5	60	256	123.6	114.3	1.72	1.59	77.0	74.0
6	60	75	86.4	105.7	0.72	1.10	51.8	67.3
7	60	256	122.5	115.7	1.70	1.93	76.4	74.7
8	60	437	124.4	122.2	1.73	2.04	76.9	78.8
9	60	256	122.6	119.7	1.70	1.66	76.5	77.1
10	60	256	122.1	117.9	1.70	1.64	75.7	75.7
11	90	384	126.5	117.0	1.76	1.95	78.8	75.4
12	90	128	105.6	116.8	0.88	0.97	64.5	75.2
13	102	256	119.8	117.0	1.43	1.95	74.6	74.9

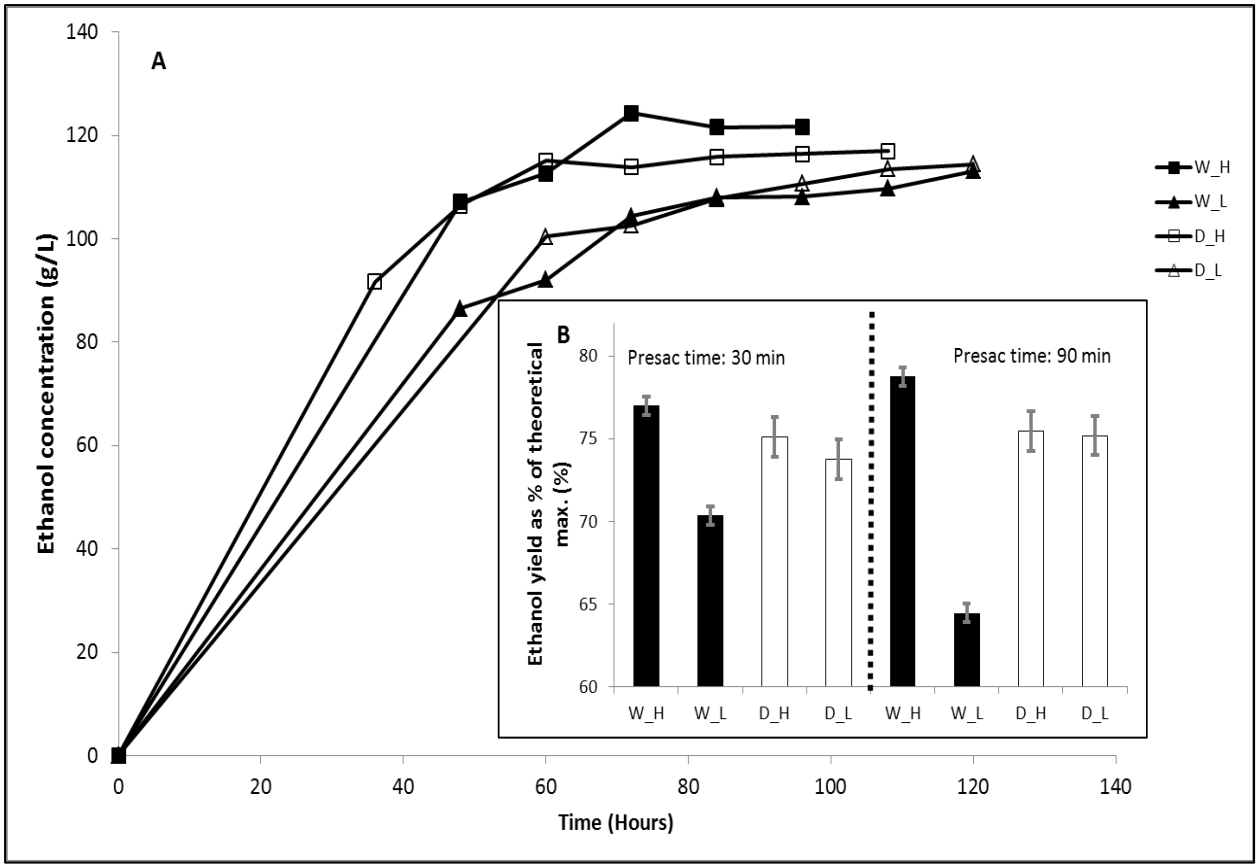

Figure 3-6: A: Ethanol profiles of experiments performed using whole (W) and decorticated (D) grains at low (L_; 128 µl/100g_{starch}: _10) and high (H_; 384 µl/100g_{starch}; _1) Stargen dosage. Pre-saccharification time 30 minutes. **B**: Ethanol yields as percentage of theoretical maximum achieved by the experiments in A and at 90 min pre-saccharification time. Error bars represent ± standard deviation.

Decreasing the Stargen dosage for fermentation with whole grains also increased the time required to reach the maximum ethanol concentration, from 72 hrs to 120 hrs (Fig 3.6A). As a consequence, the productivity at low Stargen dosage (0.94 $g.L^{-1}.h^{-1}$) was significantly lower ($P<0.05$) than at high dosage (1.73 $g.L^{-1}.h^{-1}$). Similar changes in the volumetric ethanol productivity was observed with decorticated grains, where the maximum ethanol concentration at high Stargen dosage (D_H ;117 g/L) was reached at 60 hrs, while at low dosage (D_L) the maximum ethanol concentration (114.4 g/L) was reached at 96 hrs (Fig 3-6A), corresponding to significantly different ($P<0.05$) volumetric ethanol productivities of 1.95 $g.L^{-1}.h^{-1}$ and 1.19 $g.L^{-1}.h^{-1}$ respectively. For cold processing, higher ethanol volumetric productivities could thus be achieved with decorticated grains, compared to

whole grains; this observation is the opposite to what was observed for the conventional (warm) processing when using decorticated grains. The major difference between the two processing configurations that could have led to a significant difference in the availability of nutrients in the slurries is the severity of the heat treatment. The more severe temperature treatment during liquefaction might have caused nitrogen present in the slurries to react with reducing sugars through Maillard reactions, making it unavailable for the yeasts (Galvez, 2005). Hence, decreased available nitrogen would explain the lower ethanol productivities (Pereira, et al., 2010) for the conventional (warm) processing compare to the cold processing when using decorticated grains. These observations highlight the benefits decortication can provide to the cold processing.

Since glucose concentration was low and no accumulation was observed during the fermentation in slurries containing whole and decorticated grains when using the cold processing (Appendix C), the increase in productivity was the consequence of faster starch hydrolysis in slurries containing decorticated grains as a consequence of the bran removal through decortication (Alvarez, et al., 2010; Perez-Carrillo, et al., 2008). Improved productivity could also be the result of some inhibition being lifted in decorticated grains slurries, as sorghum grain bran has been shown to contain compounds (Awika & Rooney, 2004) that negatively affect the activity of amylases (Sales, et al., 2012). Apparently these effects were not observed during the conventional (warm) processing with decorticated grains, because of the low ethanol productivities that could be achieved by yeast in low nutrient environment.

Mathematical modelling of the responses using the cold processing

The analysis of variance (Appendix D) of the chosen models showed that the effect of variables on the responses could be predicted accurately ($P < 0.05$ and $R^2 > 0.83$; Fig 3-7). For the reasons mentioned previously, only the surface plots of the ethanol yield and ethanol productivity are shown. The relations between independent variables and the responses are shown in Equations 11, 12 and 13 (whole grains) and Equations 14, 15 and 16 (Decorticated grains):

Ethanol concentration = 122.99 - 1.32*A + 13.44*B + 2.41*A*B + 0.31*A^2 – 7.84*B^2 – 5.42*A^2*B (11)

Ethanol productivity = 1.71 – 0.055*A + 0.39*B – 0.086*A^2 – 0.26*B^2 (12)

Ethanol yield as percentage of theoretical maximum = 76.54 – 0.86*A + 8.88*B + 1.92*A*B * 0.17*A^2 – 5.41*B^2 – 3.65*A^2*B (13)

Ethanol conc. = 116.1 + 0.59*A + 5.85*B – 0.59*A*B + 0.094*A^2 – 5.17*A^2*B (14)

Ethanol productivity = 1.73 + 0.38*B – 0.13*B^2 (15)

Ethanol yield as percentage of theoretical maximum = 75.49 + 0.27*A + 4.07*B – 0.28*A*B – 0.11*A^2 -0.98*B^2 – 3.66*A^2*B (16)

Where A is the pre-saccharification time and B the Stargen dosage

The plots of the ethanol yield as percentage of theoretical maximum for whole and decorticated grains predicted that at Stargen dosages below 235 µl/100 g_{starch} the ethanol yield from decorticated grains was higher than whole grains, while the opposite was observed for Stargen dosages above 235 µl/100 g_{starch} (Fig 3-7F). An increase in Stargen dosage thus had a more pronounced effect on whole grains, while decortication benefited the cold process most at lower Stargen dosages, possibly due to a nutrient effect (Puligundla, et al., 2011; Table 3-7).

Statistical models predicted that for whole grains the maximum ethanol productivity of 1.8 $g.L^{-1}.h^{-1}$ would be achieved with a Stargen dosage of approximately 280 µl/100_{starch}, while pre-saccharification time had little effect on the productivity (Figure 3-7A). However, for decorticated grains (Fig 3-7B) the productivity continued to increase in the range of Stargen dosages tested, with no apparent maximum achieved, while the pre-saccharification time also did not affect productivity. The productivity of fermentation using cold processing was higher for decorticated grains than for whole grains, when applying similar Stargen dosages and a pre-saccharification time 60 min (Fig 3-7C;).

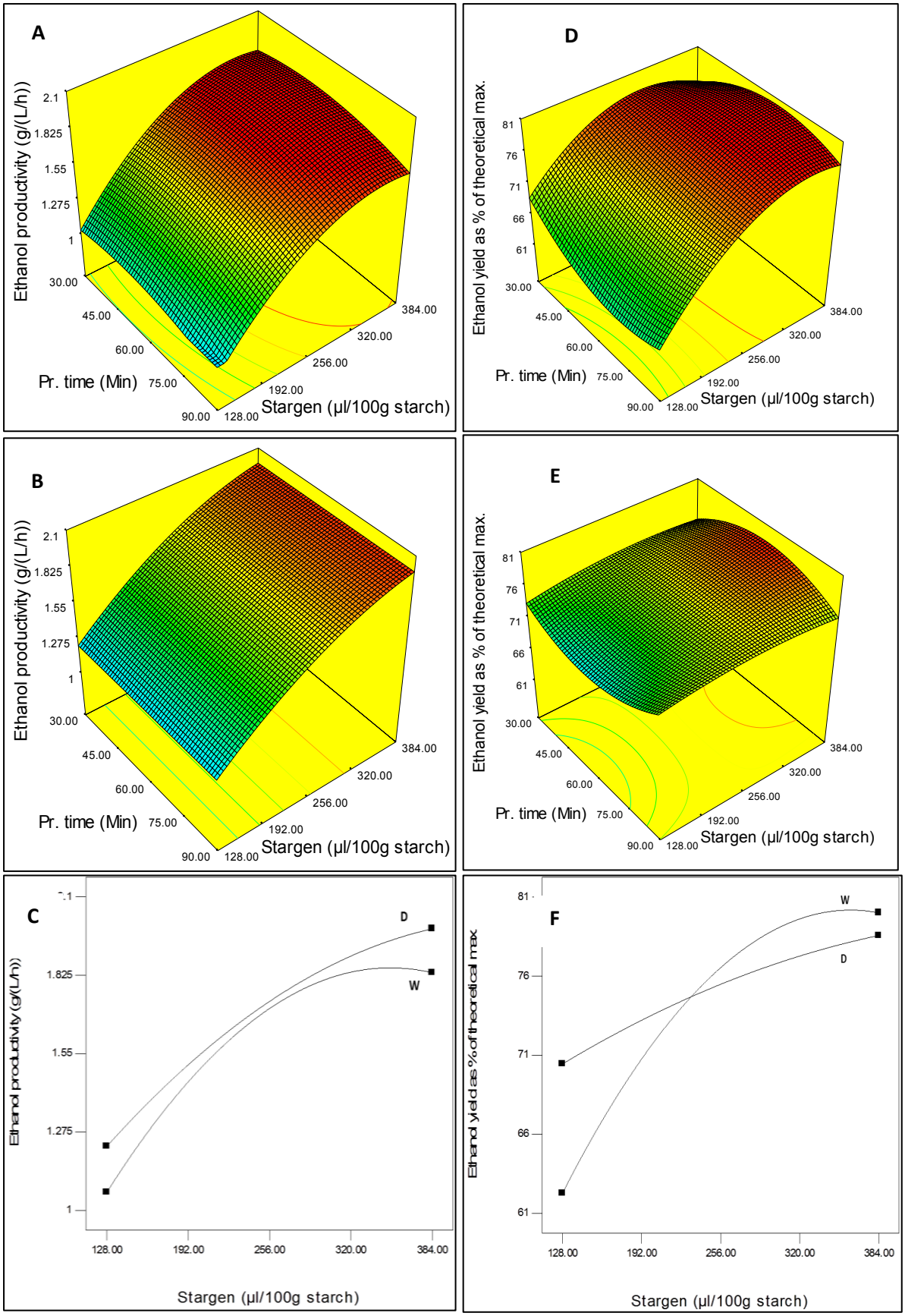

Figure 3-7: Response surface plots obtained when using the cold processing (A, B, D and E)). A: Ethanol productivity whole grains (R^2 0.97). B: Ethanol productivity decorticated grains (R^2 0.83). D:

Ethanol yield as percentage of theoretical maximum whole grains (0.98). E: Ethanol yield as percentage of theoretical maximum decorticated grains (0.9). C: Comparison of ethanol productivities using whole (W) and decorticated (D) grains, pre-saccharification time 60 min. F: Comparison of ethanol yield as percentage of theoretical maximum achieved using whole (W) and decorticated (D) grains decorticated grains, pre-saccharification time 60 min.

Optimization and validation experiments

The predicted values for ethanol concentrations and productivities, based on optimisation of input variables to achieve the desired key performance measures of grain-ethanol production, are shown in Table 3-5. Because of the low productivities achieved when using decorticated grains for the conventional processing, the key performance measure for fermentation productivity could not be met with this processing configuration. The predicted outputs from the statistical modelling were validated with experiments, using the corresponding input values from the models, as shown in Table 3-6. The models predicted that for the cold processing, decortication could provide significant benefits to the enzyme requirements, as the Stargen dosage of 250 µl/100 g $_{starch}$ for whole grains could be decreased to 221 µl/100 g_{starch} when using decorticated grains, while still achieving the desired fermentation performance. This corresponded to a decrease of 11.7% in Stargen requirement to achieve the same extent of starch hydrolysis and fermentation rate.

Table 3-5: Values of the responses used for optimization for the conventional and cold processing using whole and decorticated grains

	Conventional process		Cold process	
	Whole grains	**Decorticated grains**	**Whole grains**	**Decorticated grains**
Ethanol concentration (g/L)	119	113	120	116
Ethanol productivity	1.65	-	1.67	1.61

Table 3-6: Values of the independent variables used for validation experiments for the conventional and cold processing using whole and decorticated grains

		Whole grains	**Decorticated grains**
Conventional process	Liquefaction time (min)	125	150
	α-amylase dosage (µl/100 g starch)	63	34
	Glucoamylase dosage (µl/100 g starch)	88	71
Cold process	Pre-saccharification time (min)	50	85
	Stargen (µl/100 g starch)	250	221

The results obtained from the validation experiments are shown in table 3-7 (Ethanol profiles shown in appendix E). The ethanol concentrations obtained for the conventional processing were 130.37

g/L (whole grains) and 126 g/L (decorticated grains). The cold processing achieved ethanol concentrations of 132.12 g/L and 128.14 g/L for whole and decorticated grains respectively (Table 3-7). These values obtained in bioreactors are higher than the ones predicted by the statistical optimization, because the latter was based on shakeflask fermentations, which have poorer control of fermentation process conditions, such as exposure to oxygen and possible ethanol evaporation. Validation experiments did not achieve the predicted ethanol productivities for the conventional processing using whole grains (1.55 $g.L^{-1}.h^{-1}$) and the cold processing using whole (1.51 $g.L^{-1}.h^{-1}$) and decorticated grains (1.53 $g.L^{-1}.h^{-1}$), as fermentations were not completed after 72 hours but 84 hours instead. The reason for the longer fermentation time was likely due to the decreased growth rate by *S. cerevisiae* under strict anoxic conditions (Alfenore, et al., 2004). The percentage starch hydrolysed was similar for all the configurations (97.76 % – 98.34 %) indicating that most of the starch was consumed. The ethanol yields of 89.65 % and 89.03 % using whole grains for the conventional and cold processes, respectively, were higher than the values of 86.54 % and 87.39 % achieved when using decorticated grains (Table 3-7). This drop in ethanol yield was the consequence of lower ethanol yield on glucose consumed achieved in mashes containing decorticated grains compare to whole grains, given that similar extents of starch hydrolysis were achieved. There was a difference in the average ethanol yield of 3.1% for the conventional process between whole and decorticated grains while this difference was 1.64 % for the cold process. Although this difference was not significant ($P > 0.05$), a decrease in ethanol yield in a range similar to this study (1.8% - 3.4%) was also observed by Wang, et al. (1999) when comparing the performance of whole and debranned triticale and rye. These experiments confirmed that cold processing can match the performance of the conventional processing when using sorghum grains, similar to previous reports using sorghum grains (Ai, et al., 2011; Wu, et al., 2007; Corredor, et al., 2006) and corn (Wang, et al., 2007; Devantier, et al., 2005;)

The optimization using response surface methodology and the validation experiments have not been successful in achieving the targeted value for ethanol concentration, ethanol yield as percentage of

theoretical maximum and ethanol productivities for the reasons previously mentioned. However, the optimization succeeded in achieving similar performance (starch hydrolysis and ethanol productivities) between the different process configurations to enable meaningful comparison. In term of process performance, the conventional and cold processing using whole grains were the best configurations because of the highest percentage yields and ethanol productivities achieved. The slight decrease in ethanol yield using the cold processing with decorticated grains made it the next preferable configuration. The lower ethanol productivity and further decrease in ethanol yield when using the conventional processing with decorticated grains made it the worst configuration. However, when comparing the enzymes requirements to achieve similar starch hydrolysis and ethanol productivities, the cold processing using decorticated grains required 11.7 % less enzymes compare to the dosage required to achieved similar performance when using whole grains. This finding confirmed the beneficial effect of sorghum decortication on starch hydrolysis and the potential it has to decrease the enzymes associated cost while maintaining the same extent of starch hydrolysis and fermentation rate for the cold processing.

Table 3-7: Responses values obtained from the validation experiments using whole and decorticated grains for the conventional and cold process

	Conventional process		Cold process	
	Whole grains	**Decorticated grains**	**Whole grains**	**Decorticated grains**
Ethanol concentration (g/L)	130.37[a]	126.59[a]	132.12[a]	128.14[a]
Ethanol productivity ($g.L^{-1}.h^{-1}$)	1.55[a]	1.25[b]	1.51[b]	1.53[a]
Ethanol yield as percentage of theoretical maximum (%)	89.65[a]	86.54[a]	89.03[a]	87.39[a]
Starch hydrolysed (%)	98.34[a]	98.09[a]	97.76[a]	98.3[a]
Ethanol yield on glucose (%)	91.16[a]	88.22[a]	91.06[a]	88.91[a]
Residual solids (%)	9.1[a]	6.9[b]	9.2[a]	6.8[b]

[a] Averages followed by the same letter within the same row are not significantly different ($P < 0.05$).

The effect of decortication on chemical composition of distillers dried grains with solubles

The nutrient and mineral composition of DDGS obtained from the different configurations are shown in Table 3-8 and Table 3-9 respectively. The changes in DDGS composition caused by grains decortication are shown in Tables 3-8 (nutrients) and 3-9 (minerals). The DDGS content in crude protein, crude fibre and ash were significantly ($P<0.05$) affected by decortication. The average crude protein content increased from 37.7 % to 47.5%, an almost 10% difference between DDGS from whole and decorticated grains. A similar finding by Corredor, et al. (2006) recorded a difference of up to 8% in crude protein content between DDGS from whole and decorticated sorghum grains. The increase in protein content could be attributed to the facts that proteins are unevenly distributed throughout the grain. Protein concentration is lower in the bran removed during decortication (Koehler & Wieser, 2013). As a consequence its proportion in the decorticated grains increases with the removal of the bran. The protein content of DDGS is an important characteristic as they are used as protein sources in animal diets (US Grains Council, 2013). Higher protein concentration is generally desired in order to meet the dietary requirement of industrially grown animals. Compared to corn DDGS, having protein content varying between 25 and 33 % (Liu, 2011), the much higher protein content of sorghum DDGS from decorticated grains (up to 49%; Table 3-1) would make it more desirable as an animal feed ingredient.

Decortication also resulted in a significant decrease ($P<0.05$) in the crude fibre content from 8.8 to 6.1 % (Table 3-8), although the ADF and NDF contents were not significantly affected. The crude fibre concentration decreased in the DDGS from decorticated grains likely because of its high concentration in the bran (Koehler & Wieser, 2013). The uneven distribution of crude fibre in the grain resulted in its lower proportion in the DDGS from decorticated grains. The fact that the ADF and NDF were unchanged indicated that the quality of the fibres with regards to digestibility by non-ruminants and forage for ruminants was not affected. Hence animals will not increase their energy intake when consuming DDGS obtained from decorticated grains. The crude fat content was also not significantly ($P>0.05$) affected by decortication; a finding that is not in agreement with Corredor, et

al. (2006) who found an increase in the crude fat content with decortication. The ash content was significantly increased (P<0.05) from 5.2 % to 5.9 % in DDGS from decorticated grains. The increase in ash content could be consequence of the methods used to produce the DDGS from decorticated grains in this work, as the bran removed during decortication was washed following incubation with protease and amylase; a process during which the ash on the surface of the bran could have been transferred to the fermentation slurry. It is likely that most of the initial ash, removed from grains during decortication with the bran, was thus carried over to the fermentation broth and DDGS by the washing, resulting in higher ash content in the DDGS from decorticated grains. The mineral composition (Table 3-9) was not significantly affected by decortication.

Table 3-8: Chemical composition (dry weight) of DDGS obtained using two varieties of sorghum grains and its variations as affected by grains configurations and processing methods

			Ash %	Crude fat %	Crude protein %	Crude fibre %	ADF %	NDF %
Sorghum 01	Warm process	Whole grains	5.02	11.31	41.51	9.25	34.42	47.41
		Decorticated grains	5.75	11.14	49.17	7.22	36.85	38.39
	Cold Process	Whole grains	5.28	12.62	40.64	7.50	26.77	31.17
		Decorticated grains	5.98	8.45	49.97	5.17	-	30.28
Sorghum 02	Warm process	Whole grains	5.50	8.78	34.52	9.21	32.78	46.83
		Decorticated grains	5.83	6.62	43.51	6.77	38.33	43.53
	Cold Process	Whole grains	5.01	10.26	33.91	9.35	29.54	35.00
		Decorticated grains	5.89	6.45	47.24	5.35	31.65	32.66
Grains configuration	Average whole grains		5.20	10.74	37.65	8.82	30.88	40.10
	Average decorticated grains		5.86	8.17	47.47	6.12	35.61	36.22
	Difference		-0.66*	2.58	-9.83*	2.70*	-4.73	3.89
Processing methods	Average convent. processing		5.53	9.47	42.18	8.11	35.59	44.04
	Average cold processing		5.54	9.45	42.94	6.84	29.32	32.28
	Difference		-0.01	0.02	-0.76	1.27	6.27*	11.76*

*Differences followed by a star are significant (P < 0.05)

The effect of processing method on chemical composition

The contents of ash, crude fat, crude protein and crude fibre in the DDGS were not significantly affected by the processing method (P > 0.05; Table 3-8). Only the ADF and NDF were affected by the

processing method. Relative to the conventional processing, cold processing reduced the average ADF content from 35.6 % to 29.3 % and the NDF from 44 % to 32.3%. Variations in the ADF and NDF as the results of processing method were also reported by Kelzer, et al. (2010) and Robinson, et al. (2008). In their studies with corn DDGS, lower values of ADF and NDF were observed for DDGS obtained from the cold processing. The change in ADF content indicated DDGS from cold processing was more digestible for non-ruminants compared to DDGS obtained from the conventional processing, while for ruminants the amount of available forage will be lower in such DDGS because of the decreased NDF content.

The mineral composition of DDGS (Table 3-9) shows that only the difference in sodium content was statistically significant between the two processing methods. The modifications between the two processing methods that may have resulted in a significant difference in the sodium composition, is the addition of different type of enzymes. Cations such as sodium, calcium or zinc are known for their stabilizing effect on enzymes (Bush, et al., 1989) and may have been added in greater amount in one of the commercial enzymes formulation.

Table 3-9: Mineral composition (dry weight) of DDGS obtained using two varieties of sorghum grains and its variations as affected by grains configurations and processing methods

			P (%)	K (%)	Ca (%)	Mg (%)	Na (mg/kg)	Fe (mg/kg)	Cu (mg/kg)	Zn (mg/kg)	Mn (mg/kg)	B (mg/kg)	Al (mg/kg)
Sorghum 01	Warm process	Whole grains	1.13	1.06	0.08	0.54	353.00	118.14	63.69	89.45	44.04	4.78	57.75
		Decorticated grains	1.30	1.17	0.08	0.59	355.00	131.10	6.19	92.25	50.34	4.72	66.3
	Cold Process	Whole grains	1.27	1.17	0.11	0.62	268.00	136.82	5.16	90.99	48.75	5.18	100.8
		Decorticated grains	1.34	1.28	0.08	0.61	256.00	115.36	8.75	82.84	48.54	5.09	106
Sorghum 02	Warm process	Whole grains	1.19	1.55	0.07	0.52	296.00	99.01	3.27	65.23	42.62	6.15	33.28
		Decorticated grains	1.14	1.46	0.07	0.46	-	-	20.72	60.22	43.03	6.58	-
	Cold Process	Whole grains	1.07	1.39	0.06	0.45	76.00	103.41	4.97	70.09	39.23	5.31	49.06
		Decorticated grains	1.19	1.59	0.06	0.48	106.00	86.70	2.39	62.9	41.77	5.89	89
Grains configuration	Average whole grains		1.17	1.29	0.08	0.53	248.25	114.35	19.27	78.94	43.66	5.36	60.22
	Average decorticated grains		1.24	1.38	0.07	0.54	239.00	111.05	9.51	74.55	45.92	5.57	87.10
	Difference		-0.08	-0.08	0.01	0.00	9.25	3.29	9.76	4.39	-2.26	-0.22	-26.88
Processing methods	Average warm process		1.19	1.31	0.08	0.53	334.67	116.08	23.47	76.79	45.01	5.56	52.44
	Average cold process		1.22	1.36	0.08	0.54	176.50	110.57	5.32	76.71	44.57	5.37	86.22
	Difference		-0.03	-0.05	0.00	-0.01	158.17*	5.51	18.15	0.08	0.44	0.19	-33.77

*Differences followed by a star are statistically significant ($P < 0.05$)

3.5 Conclusion

Comparison between the conventional and cold processing using VHG whole sorghum grains slurries showed that both methods could achieve similar performance (ethanol concentration, yield and productivity). Decortication resulted in a slight decrease in ethanol yield compared to their respective whole grains configurations due to lower ethanol yield on glucose. The conventional processing was further negatively affected when using decorticated grains, by achieving significantly lower productivities, making it the worst configuration. However the cold processing benefited from decortication, because of improved hydrolysis as a result of bran removal, achieving the same performance (starch hydrolysis and ethanol productivity) as its whole grains configuration, while reducing the enzyme requirements by 11.7%. The use of decorticated grains increased the DDGS crude protein content from 37.7 % to 47.5 %, on average. Only the cold processing affected the ADF and NDF contents. Their values were decreased compare to the conventional processing from 35.6 % to 29.3 % (ADF) and 44 % to 32.3 % (NDF). Hence DDGS from the cold processing using decorticated sorghum grains would be preferable in animal feeds because of higher protein content and the lower ADF content making it more suitable for non-ruminants. Although the high protein content of decorticated sorghum DDGS (up to 49 %) make it preferable to corn DDGS, the lower ADF contents of corn DDGS (11.4 - 20.8 %) still make them more appropriate for non-ruminants.

4 Bioethanol production from sorghum grain using a consolidated bioprocessing (CBP) yeast producing raw starch hydrolysing enzymes

4.1 Abstract

The conversion of raw starch to bioethanol currently requires addition of a large amount of exogenous enzyme cocktails, which could be decreased when enzymes are produced *in situ* by an organism suitable for consolidated bioprocessing. Currently a major hurdle to the successful development of the CBP process is the low ethanol concentrations and productivities reported, which are below the industrial standards for fermentation process performance. The performance of a novel CBP yeast, producing raw starch hydrolysing enzymes, was assessed in this work. Included in the present study was the investigation of the effect of a modified inoculum production phase, aimed at increasing initial yeast biomass and enzymes concentrations in the slurry. However, despite achieving increases in the initial yeast biomass concentration (13.9 g/L), the amount of enzyme production in the inoculum and subsequent fermentation stages was insufficient to achieve the desired fermentation process performance. The lower ethanol tolerance (around 90 g/L) also prevented the utilization of all available glucose Further improvements to yeast genetics, to significantly enhance enzyme production and ethanol tolerance, as well as further development of the inoculum production strategy are required to improve the CBP process.

4.2 Introduction

The industrial fermentative microorganism *S. cerevisiae* has been engineered to express raw starch hydrolysing enzymes (RSHEs), used to hydrolyse uncooked starch (cold processing) (Viktor, et al., 2013; Yamada, et al., 2011). The capability of the resulting genetically modified microorganism (GMO) for the *in situ* production of both the RSHEs and ethanol has the potential to decrease the required dosage of exogenous enzymes, which could benefit operational costs by decreasing expenditure on costly commercial enzyme cocktails.

Experiments performed by several authors have shown that the use of a (consolidated bioprocess) CBP yeast for bioethanol production, with no addition of exogenous enzymes, has the major disadvantages of not achieving ethanol concentration comparable to the conventional processing (~120 g/L) and requiring a long fermentation time due to the very low initial amylases concentration in the slurry (Kim, et al., 2010; Shigechi, et al., 2004). This issue should be addressed before any commercial application is considered. Furthermore a review of the available literature show that due to this initial lag, the performance of the consolidated bioprocessing (ethanol concentration, ethanol productivity) cannot match the one of the cold and warm processes without the initial addition of some amount of commercial raw starch hydrolysing enzymes such as Stargen (Gorgens, et al., 2014).

In this chapter a consolidated bioprocessing (CBP) is performed using an *S. cerevisiae* strain engineered to express and secrete both α-amylases and glucoamylases into the medium. A modified inoculum production step was included, with the aim of increasing both the concentration of biomass and amylases in the fermentation broth, and thus mitigate the effect of the initial lag phase on ethanol yields and productivity. Hence, the aim of the inoculum strategy was to increase significantly the concentration of both yeast biomass and amylases in the inoculum, compared to conventional inoculum processes. The hypothesis is that the use of such inoculum would possibly results in increased starch hydrolysis rate and ethanol productivity in the fermentation slurry. The inoculum strategy involved the use of fed batch processing for inoculum production, to prevent the loss of carbon to ethanol through Crabtree effect, carbon which would then be used for biomass

production instead (Anane, et al., 2013). As the whole broth will be used for inoculation, an increase in the amylase concentration in the supernatant of the fed-batch culture would be transferred to the fermentation slurry, and be available to replace some of the process requirements for commercial raw starch hydrolysing enzymes – together with amylases produced *in situ* by the CBP yeast during fermentation. This hypothesis was investigated by using a response surface methodology technique (RSM) to determine the effect of both the inoculum size and the Stargen dosage on the amylases activity and fermentation performance in the slurry. Although the CBP yeast produces its own amylases, Stargen was included in the experimental design to determine if the requirement of commercial enzyme cocktail could be decreased by the use of a CBP yeast.

4.3 Materials and methods

Raw materials

White sorghum grain was obtained from Agricol (Pty) Ltd (Brakenfell, Cape Town, South Africa). The grains were air-dried for 3 days, vacuum packed and stored at room temperature until needed. The moisture content of the stored grains was 8% (w/w). Before usage, the grains were milled using a Retsch mill (SM 100, Haan, Germany) to pass through a 0.5 mm screen. The starch content of the ground sorghum was 68 %. The grains where decorticated as previously described (Chapter 3).

Microorganisms and enzymes

The yeast used was a *S. cerevisiae* strain MH1000, engineered to express both α-amylases and glucoamylases. This strain was obtained from Department of Microbiology, Stellenbosch University, South Africa. This strain was engineered to constitutively express and secrete α-amylase from *Saccharomycopsis fibuligera* and glucoamylase from *Thermomyces lanuginoses* under the control of phosphoglycerate kinase and enolase promoters respectively. The cultures were stored in 2 ml aliquots at -86 ^{o}C until needed. Stargen 002 (Genencor) a blend of alpha- and gluco-amylases with declared activity 570 GAU/g (GAU, glucoamylase unit – the amount of enzyme that liberate one gram of glucose per hour from soluble starch at 60 ^{o} C, pH 4.2) was used for hydrolysis.

Ethanol production process

The experimental layout used to achieve the same solids loading while varying the inoculum size is depicted in figure 4-1. In order to increase the amount of initial enzymes and biomass in the to the starch slurry, an inoculum production step was included before the starch fermentation.

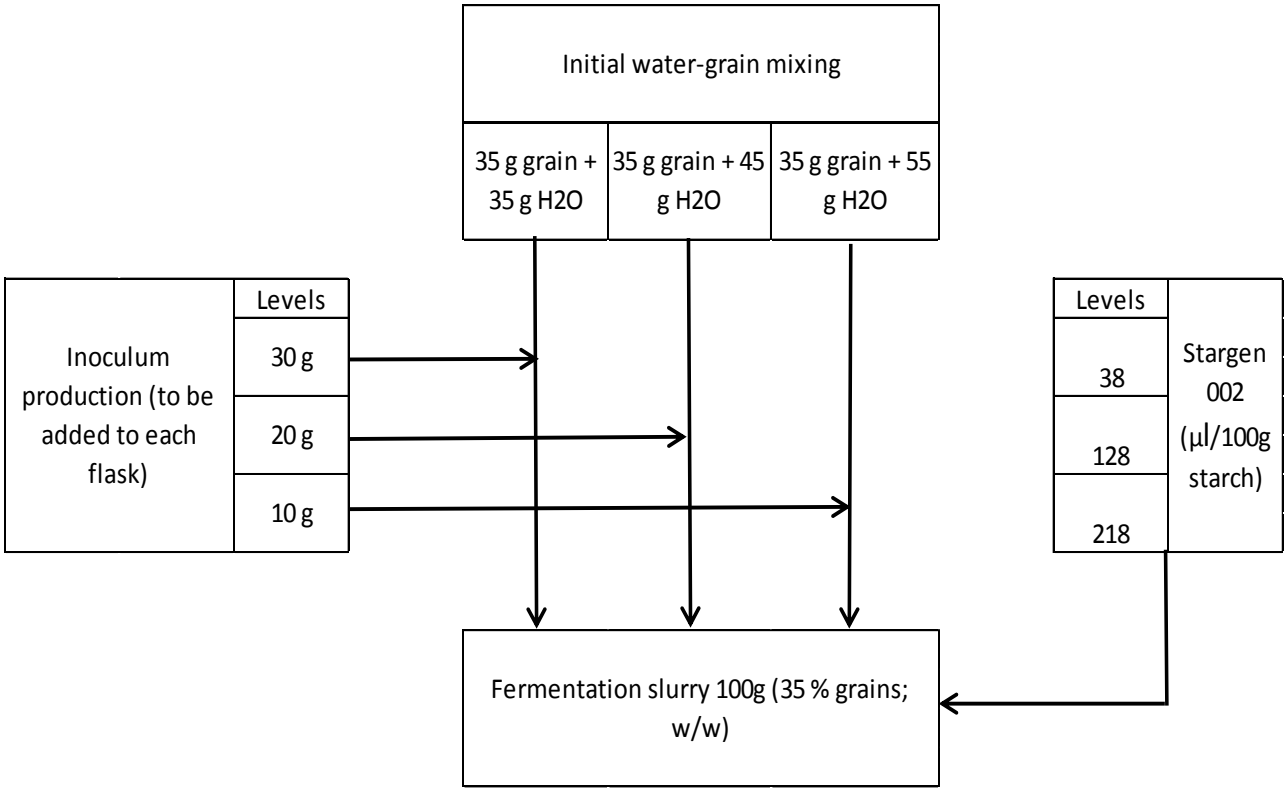

Figure 4-1: Strategy employed for bioethanol production using the consolidated bioprocess

Pre-inoculum preparation

Pre- inoculum was produced to inoculate the 5L bioreactor, where inoculum production for the ethanol fermentation from starch was performed (described later). Cultures stored at -86 ^{o}C were streaked on YPD (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 13 g/L agar) plates and incubated at 30 ^{o}C until clear colonies were formed. A single colony was picked to inoculate two 250 ml flasks containing each 100 mL medium consisting of (per litre): casein hydrolysate (10 g), $(NH_4)_2SO4$ (10 g), KH_2PO4 (2.4 g) and yeast nitrogen base without amino acids (6.7 g). A 300 g/L glucose solution was added to flask after autoclaving, to achieve a concentration of 20 g/L. The shake flasks were incubated at 30 ^{o}C for 18 hours on an orbital shaking incubator at 150 RPM. The

entire contents of two the flasks were used to inoculate one 5L bioreactor to make an initial volume of 1.5 L. The medium in the bioreactor was identical to the one in the flasks; except the initial glucose concentration that was reduced to 15 g/L in the bioreactor and the addition of antifoam 204 (Sigma-Aldrich, Missouri, USA) at an initial concentration of 300 µl/L.

Inoculum production

Inoculum production was performed in 5 L jacketed bioreactors (Sartorius, Goettinger, Germany) operated aseptically throughout the fermentation. The bioreactor was fitted with a Rushton turbine impeller and a condenser to prevent evaporation. The water circulating in the condenser was maintained at 4^{o}C. The air flow rate was kept constant at 1 L/min. The dissolve oxygen (DO) was maintained above 30 % of saturation by cascading DO with agitation. Throughout the fermentation the temperature was maintained at 30 ^{o}C and pH 5.5 by automatic titration using 3 M KOH.

The end of the batch phase was identified by a spike in DO levels. At that time a sample was taken to determine the biomass concentration and the total biomass in the bioreactor to determine the feeding profile to maintain a predetermined specific growth rate during the fed batch phase.

The growth rate was maintained by controlling the feeding rate of the growth-limiting nutrient glucose using a peristaltic pump. The following formula was used to determine the amount of glucose to be fed to maintain the growth rate:

Eq 1: $$S_m\ \mathrm{t}\ = \frac{x_0 V_0\ e^{\mu t} - 1}{Y_{x/s}} + S_0$$

S_m(t): mass of glucose added at time t (min)

x_0: concentration of biomass (g/L)

V_0: Volume of liquid in the reactor (L)

µ: predetermined growth rate

$Y_{x/s:}$ biomass yield coefficient on glucose

S_0: amount of glucose fed at t-1 (g)

Input values for μ (0.12 h^{-1}) and $Y_{x/s}$ (0.4 $g.g^{-1}$) used in this study were not determined experimentally; the values were carefully obtained from literature.

Since the feeding profile had to be entered manually into the pump controller, the feeding rate was updated every 10 minutes to reduce the time lag between end of the batch phase and the start of feeding.

Ethanol production from starch

Starch fermentations were performed as illustrated in figure 4-1. The water and milled sorghum grains were first mixed at the required ratio. Then the appropriate volume of the inoculum produced by fed-batch culture was added to achieve final mass slurry of 100 g corresponding to 35% solids. When necessary, the desired amount of Stargen 002 was added.

Determination of specific growth rate

The specific growth rate was calculated at the end of the fed batch, as the slope of the curve of natural logarithm of the total biomass in the bioreactor as a function of time from the start of fed batch. The volume of broth in the bioreactor at each sampling time was corrected for the volume of glucose fed into to reactor.

Experimental design

The experiments were performed using whole and decorticated grains. The effect of inoculum size and Stargen dosage on the enzymatic activity was investigated using a central composite design (table 4-1).

Table 4-1: Factors used in the central composite design and their levels using the consolidated bioprocessing

Factors	Levels		
	-1	**0**	**1**
Inoculum size (g)	10	20	30
Stargen dosage (µl/100g starch)	38	128	218

Analytical methods

The ethanol and glucose concentrations were determined by high pressure liquid chromatography as previously describes (Chapter 3). The biomass concentration was determined gravimetrically. Eight mL of fermentation broth was collected and centrifuge at 10020 g for 10 minutes. The cells pellet was washed twice with water and dry until constant weigh at 105 ^{o}C. A spectrophotometer was used to measure optical density at 600 nm of a sample taken simultaneously with the one for biomass concentration. The standard curve relating the biomass to the optical density was obtained by plotting the values obtained at different sampling time (Appendix F).

4.4 Results and Discussion

Inoculum production

The success of inoculum production phase was assessed by monitoring the growth of the culture and the enzymatic activity in the supernatant, given that the aim was to obtain a culture with high biomass and amylase concentrations. Figure 4-2 shows the plot of the natural logarithm of total biomass versus time during the fed batch phase. The growth of the yeast during this phase as indicated by the slope of the curve was 0.16 h^{-1}. The targeted growth rate was 0.12 h^{-1}, indicating that the growth rate was not successfully controlled by the methods used. However the strategy succeeded in increasing the biomass concentration in the fermentation broth which increased from 4.3 g/L (DCW) at the end of the batch phase to 13.9 g/L (DCW) at the end of the fed batch phase (Fig 6-3). The variations in amylase activities of the broth during the fed batch phase are depicted in figure 4-4. The activity of the amylases in the supernatant increased with fermentation time, thus indicating that the fed-batch strategy was successful in increasing the amylase concentration in the broth to 3.4 times the initial concentration. However, no variations of the amylases activity per biomass unit (Fig 4-4) were observed between the end of the batch and fed-batch phases as its value was maintained around 0.5 $u/g_{biomass}$, indicating that the amylase production by this strain was growth associated.

The observed increase in the biomass and amylases concentrations during the course of the fed batch phase is likely due to the controlled feeding of glucose at a rate that prevented ethanol production through the Crabtree effect. As a consequence the carbon that would have been converted to ethanol, should all the glucose was administered at the start (batch), is instead redirected to biomass and amylases production (Anane, et al., 2013). This was confirmed by the absence of ethanol accumulation during this phase (Appendix G). This fed batch strategy was also employed by Khaw, et al., (2006) to produce biomass prior inoculation to the starchy slurry for fermentation. Because the amylases were expressed constitutively, the activity per unit biomass remained constant throughout the inoculum production phase (Fig 4-4).

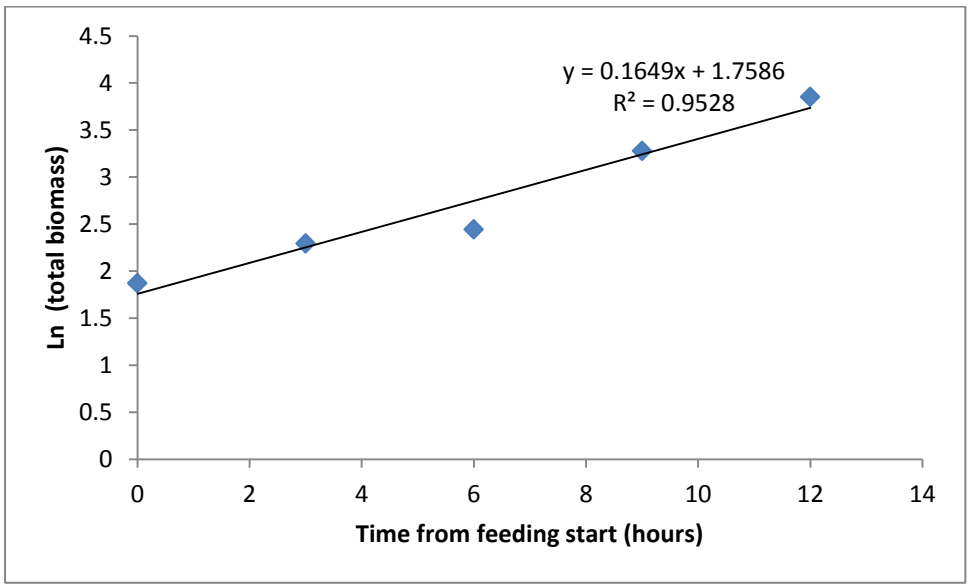

Figure 4-2: Plot of natural logarithm of the total biomass in the bioreactor during the inoculum production

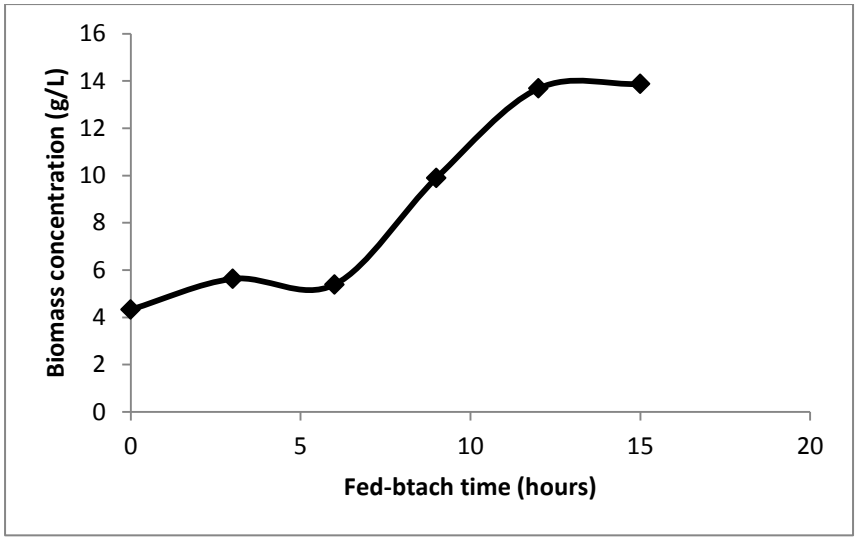

Figure 4-3: Variation of biomass concentration during fed-batch (inoculum production)

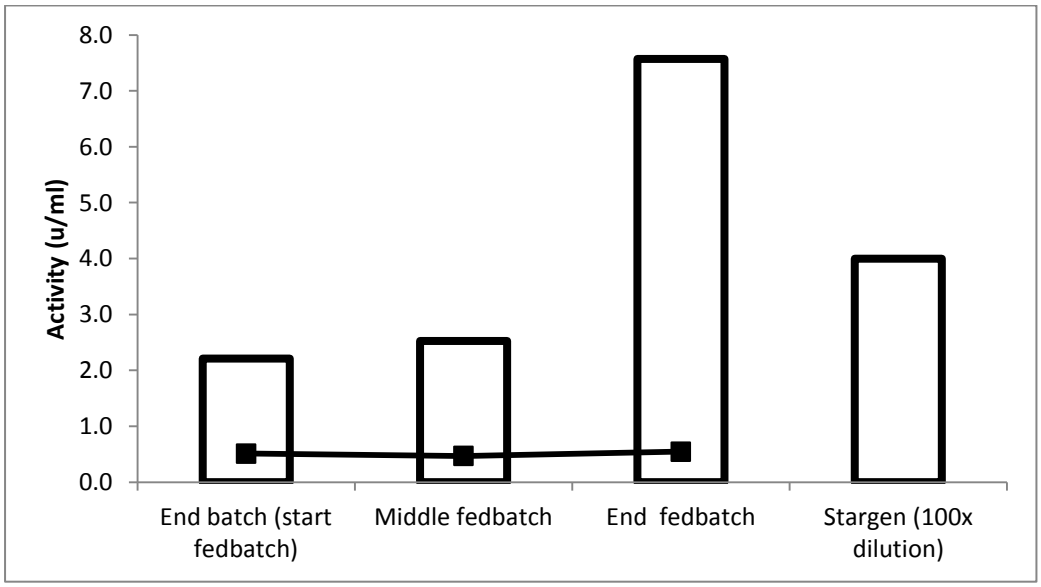

Figure 4-4: Amylase activity (u/ml) in the fermentation broth at various times during the fed batch compared to Stargen 002. The line represents the variations of activity per unit biomass (u/g $_{biomass}$).

Effect of inoculum size and Stargen dosage on ethanol productivity and amylase activity

The effect of the variables investigated on the responses were similar for whole and decorticated grains, hence only the results obtained from slurries containing one type of grains (decorticated) are

shown. The ethanol and glucose profiles obtained from the experiments are shown is figure 4-5. The yeast used in this study was inhibited at lower ethanol concentration (around 90 g/L) compared to Ethanol Red (Chapter 3). Once the inhibitory ethanol concentration was reached, glucose started accumulating in the slurry. The rate of glucose accumulation from that time was calculated at each sampling point as the slope of the curve. The average value was calculated and recorded as the relative amylase activity. The values achieved by each experimental run are shown in Table 4-2. The inoculum volume used corresponded to initial biomass concentrations (on DCW basis) of 1.4 g/L (10 ml), 2.8 g/L (20 ml) and 4.2 g/L (30 ml). The analysis of variance (appendix H) showed that only Stargen dosage had a significant effect ($P < 0.05$) on amylase activity in the fermentation broth, and not the CBP yeast inoculum. Hence the use of the CBP yeast in the conditions investigated, had no effect on the Stargen requirements to achieve complete starch hydrolysis.

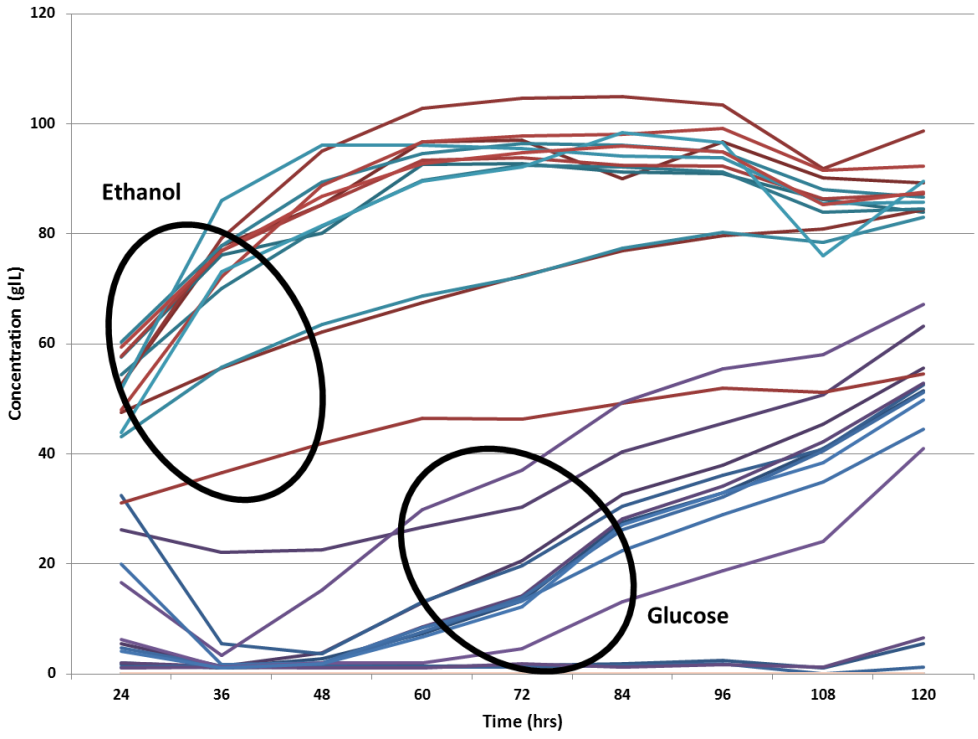

Figure 4-5: Ethanol and glucose profiles of fermentation performed according to the CCD using decorticated grains

Table 4-2: Experimental conditions and responses values achieved from experiments performed based on the central composite design for decorticated grains using the consolidated bioprocessing

No	Inoculum size (g)	Stargen dosage µl/100 g starch	Relative Amylase activity	Ethanol productivity (g/L.h)
12	6	128	0.7	1.17
4	10	218	0.5	1.54
8	10	38	0.1	0.86
1	20	128	0.6	1.62
2	20	128	0.6	1.54
6	20	128	0.6	1.61
7	20	0.72	0.0	0.45
9	20	128	0.6	1.56
10	20	255	0.8	2.0
13	20	128	0.6	1.6
3	30	38	0.1	0.7
5	30	218	0.7	1.75
11	34	128	0.5	1.65

The fact that increasing the inoculum size did not affect the rate of starch hydrolysis indicates that either higher amylases concentration are required to significantly affect hydrolysis rate or the activity of the amylases produced by the recombinant yeast is inhibited in the slurry. The possibility of the latter is strengthened by the fact that comparison of the amylase activity range (Table 4-3) investigated in this study (4.8×10^{-2} - 2.7×10^{-1} u/100 g_{slurry}) to the activity corresponding to Stargen at the manufacturer recommended dosage (3.7×10^{-2} u/100g_{slurry}) showed that based on the measured

amylases activities, enzymes present in the inoculum should have been able to completely hydrolyse the available starch. Slurries of cereals grains are known to contain compounds such as lipids, phytins, phenols or proteins which negatively affect amylases performance (Sales, et al., 2012) and can also be deficient in important metal ions (Yamada, et al., 2011). Such conditions could explain the poor performance of the amylases expressed by the CBP yeast. Several authors who used the consolidated bioprocessing and achieved higher ethanol productivity through faster starch hydrolysis without the addition of commercial raw starch hydrolysing enzymes did not use industrial slurries. The fermentation media used contained pure starch as carbon source, with various nutrient supplementations (Viktor, et al., 2013; Shigechi, et al., 2004). Hence amylases performance on analytical grade raw starch might significantly differ from grain slurries.

Table 4-3: Amylases activities corresponding to Stargen recommended dosage and range investigated in this study when using the CBP inoculum

	Activity (U / 100g slurry)
Activity corresponding to stargen recommended dosage	3.7×10^{-2}
Activity range corresponding to inoculum range investigated	4.8×10^{-2} - 2.7×10^{-1}

Alternatively, it is also possible that the CBP yeast did not produce sufficient amounts of amylases, either during inoculum growth or subsequent fermentation, to significantly affect the rate starch hydrolysis as measured in this study. Ethanol profile obtained from experiment No7 (Table 4-2), performed with no stargen addition (Fig 3-6) showed that the ethanol concentration increased slowly, reaching a concentration of 54 g/L at 120 hours corresponding to a volumetric ethanol productivity of 0.45 $g.L^{-1}.h^{-1}$. This low value was the consequence of slow starch hydrolysis as no glucose accumulation was observed. Comparison with ethanol profiles from experiments No 4 and 5 (Table 4-2) performed at high Stargen dosage (218 µl/100 g_{starch}) with lower (10 ml) and higher (30 ml) inoculum size respectively, showed that higher productivities could be achieved when using the CBP yeast (Fig 3-6), confirming that the slower starch hydrolysis rate of amylases produced by the CBP yeast was responsible for the low ethanol productivity achieved when using the CBP yeast only.

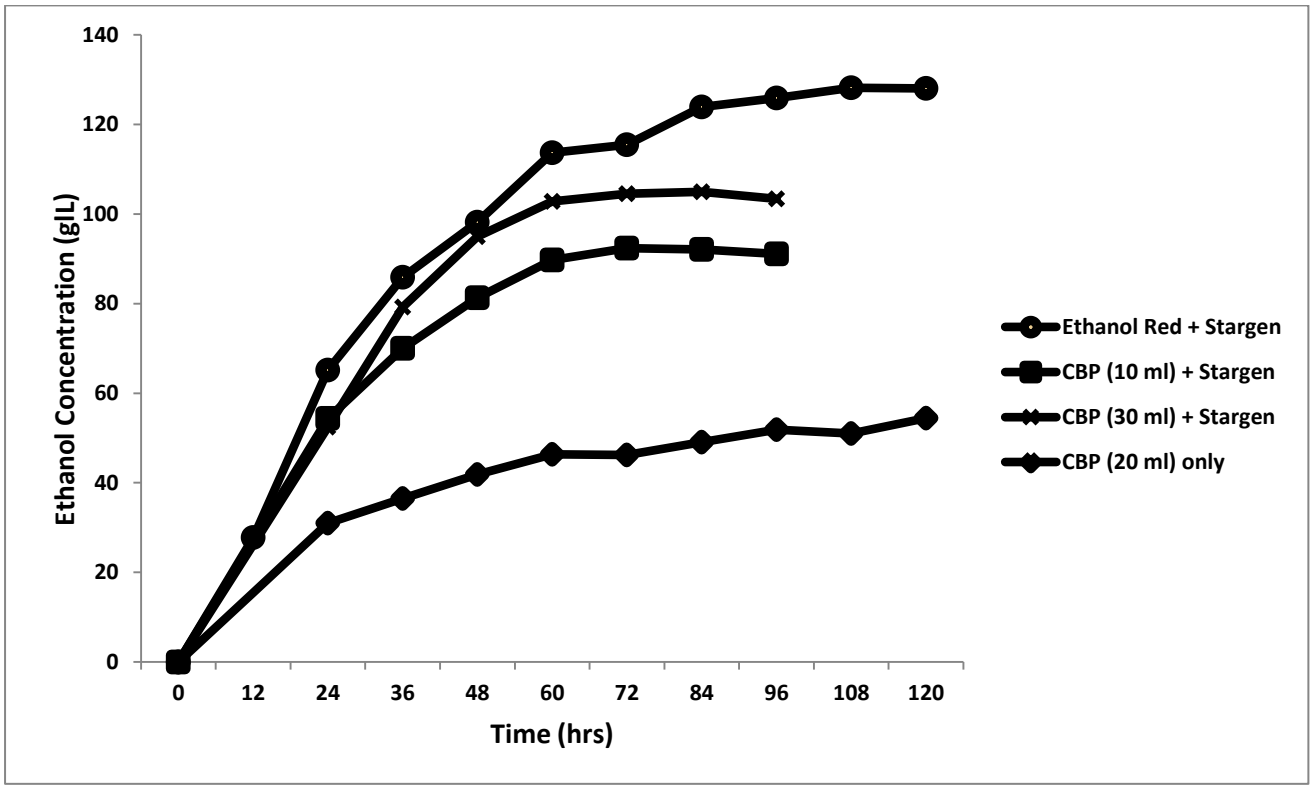

Figure 4-6: Ethanol profiles of fermentation performed using the CBP yeast only, the CBP yeast with Stargen (218 µl/100g_{starch}) and Ethanol red with Stargen (221 µl/100g_{starch}).

The observation that variations of Stargen dosage had a significant effect on the amylase activity while the variations of inoculum size did not, might be explained by the different sources from which both enzymes cocktail originates. Stargen is a cocktail of α-amylase and glucoamylase from *Aspergillus kawachi* and *Trichoderma reesei* respectively. While the genetically engineered yeast used in this study expresses α-amylases and glucoamylases from *Saccharomycopsis fibuligera* and *Thermomyces lanuginoses* respectively. It is known that enzymes can have different optimum conditions to achieved maximum activity (Illanes, 2008; Illanes, et al., 2008), hence explaining the significantly higher requirements for amylases expressed by the CBP yeast to achieve complete starch hydrolysis.

When considering the ethanol productivity, the analysis of variance (Appendix I) and surface plot obtained from the model developed ($P < 0.05$ and R^2 0.98) from values obtained (Fig 4-7), showed that the inoculum size did not affect the ethanol productivity as much as the Stargen dosage. The increase in inoculum size positively affected the ethanol productivity only at high Stargen dosage (> 100 µl/100g slurry), indicating that the higher ethanol productivity was the consequence of faster starch hydrolysis to glucose at higher Stargen dosage being converted to ethanol faster by increasing concentration of yeast cells and not because of higher amylase activity in larger inoculum size. This confirms previous observation that the use of the CBP yeast within the condition of this study did not significantly affect the rate of starch hydrolysis. Furthermore, comparison of the ethanol profiles obtained when using the CBP yeast at inoculum size 10 and 30 ml with the one obtained by yeast strain Ethanol Red (Chapter 3) at similar Stargen dosage (221 µl/100g_{starch}) (Fig 3-6) showed that before ethanol toxicity inhibited the growth the CBP yeast, the ethanol productivity achieved when using the CBP yeast at increasing inoculum size get closer to the one achieved by Ethanol Red. This comparison of the two yeast strains indicates that Ethanol Red has significantly higher fermentation capacity compare to the CBP yeast as only higher initial biomass concentration of the CBP yeast (4.2 g/L) could match the performance of Ethanol Red (0.5 g/L) at similar Stargen dosage.

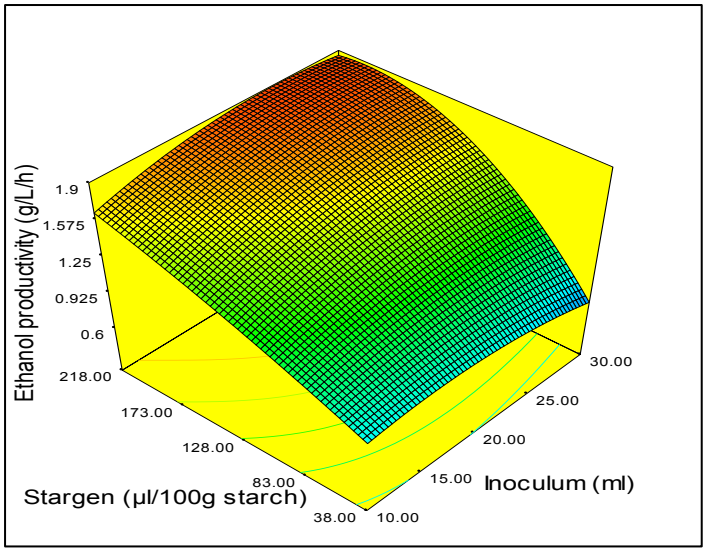

Figure 4-7: Surface plot of the effect of inoculum size and Stargen dosage of the ethanol productivity

4.5 Conclusion

The fed batch strategy employed in this work to increase the biomass and enzymes concentration has been successful. Biomass concentration and amylase activity in the inoculum increased 3.2 and 3.4 times respectively. When supplementing the slurry with high dosage of Stargen, the ethanol productivity achieved at high CBP inoculum size before growth ceased due to ethanol inhibition, could approach the one achieved by Ethanol Red. However, variations in the inoculum size (initial biomass and amylase loading) showed no significant effect on the rate of starch hydrolysis in the slurry. This was because of the need of even higher requirement for amylases in the slurry to achieve faster starch hydrolysis and ethanol productivities or the inhibition of enzymes produced by the CBP yeast in the slurry. As the inoculum size did not affect the performance of the ethanol conversion process, the use of the CBP yeast could not decrease the requirement of exogenous enzyme addition to the slurry. Further experiments are required to determine whether an enzymes inhibition or low enzymes dosage is responsible for the slow rate of starch hydrolysis. The inoculum production phase should also be optimized to achieve higher initial biomass and enzymes concentrations.

5 Conclusions and Recommendations

The current (conventional) process used to convert cooked starch from cereal grains to ethanol could benefit from a reduction in energy- and enzymes- associated costs if it could be replaced by a successful raw starch (cold) conversion process or by a consolidated bioprocess. The lack of data for meaningful comparison of these conversion processes, particularly when using sorghum grains as starch source, was the motivation for this study. Based on the research questions initially stated, the following conclusions have been drawn. Recommendations for further work are also made.

- **Performance of the cold processing and consolidated bioprocessing relative to the conventional processing when using sorghum grains.**

Conclusions: The performance (ethanol concentration, yield and productivity) of the cold processing could match the performance of the conventional processing when using whole sorghum grains (Chapter 3). Because of the lower ethanol tolerance of the CBP yeast used and the very limited effect the enzymes it produced had on the rate of starch hydrolysis, the performance of the consolidated bioprocessing could not match the conventional and cold processing. The requirement for exogenous enzyme addition to maintain desired performance when converting raw starch to ethanol could also not be reduced when using the CBP yeast (Chapter 4).

Recommendations: The cold processing should be scale-up to determine whether the performance observed at 5L bioreactor scale can be achieved on industrial scale. For the consolidated bioprocessing, it must be determined whether increasing the initial dosage of enzymes produced by the CBP yeast can improve the process or if the poor performance of the process is due to their inhibition. The genetic capability of the yeast should also be improved to achieve higher ethanol tolerance.

- **Effect of decortication on the performance of conventional, cold and consolidated bioprocessing**

Conclusions: Decortication negatively affected the performance of the conventional and cold processing by slightly decreasing the ethanol yield, with the conventional processing being more affected. Furthermore, the ethanol productivity of the warm process was significantly decreased and could not match the one achieved when using whole grain without further nitrogen supplementation. However, the use of decorticated grains for the cold processing increased the starch hydrolysis and ethanol productivity compared to whole grains. As a result, the same performance (ethanol productivity and starch hydrolysis) could be achieved at enzyme dosage decreased by 11.7 % compare to whole grains (Chapter 3). The consolidated bioprocessing did not benefit from decortication (Chapter 4).

Recommendations: The slurries containing whole and decorticated grains when using the conventional processing should be supplemented with increased nitrogen (or protease addition) to ensure that ethanol productivity when using decorticated grains can match the whole grains one. Both configurations should then be optimized to determine whether the benefits from decortication observed for the cold processing are also observed when using cooked starch.

- **Benefits of decortication and processing (conventional and cold) methods on the composition of the distillers' dried grains with solubles (DDGS).**

Conclusions: Decortication significantly increased the protein content of the DDGS, making DDGS from decorticated grains a preferable source of protein in animal feed. Cold processing decreased the neutral detergent fibre (NDF) and neutral detergent fibre (NDF) of the DDGS relatively to the conventional processing. DDGS obtained from the cold processing are thus more valuable source of energy for non-ruminants (Chapter 3).

Recommendations: The NDF and ADF content of DDGS obtained by the cold processing using several varieties of sorghums grains and different processing conditions (such as drying time and temperature or ratio of wet distillers grains to condensed solubles) should be compared to

determine whether their respective content can be further decreased to values achieved by corn DDGS.

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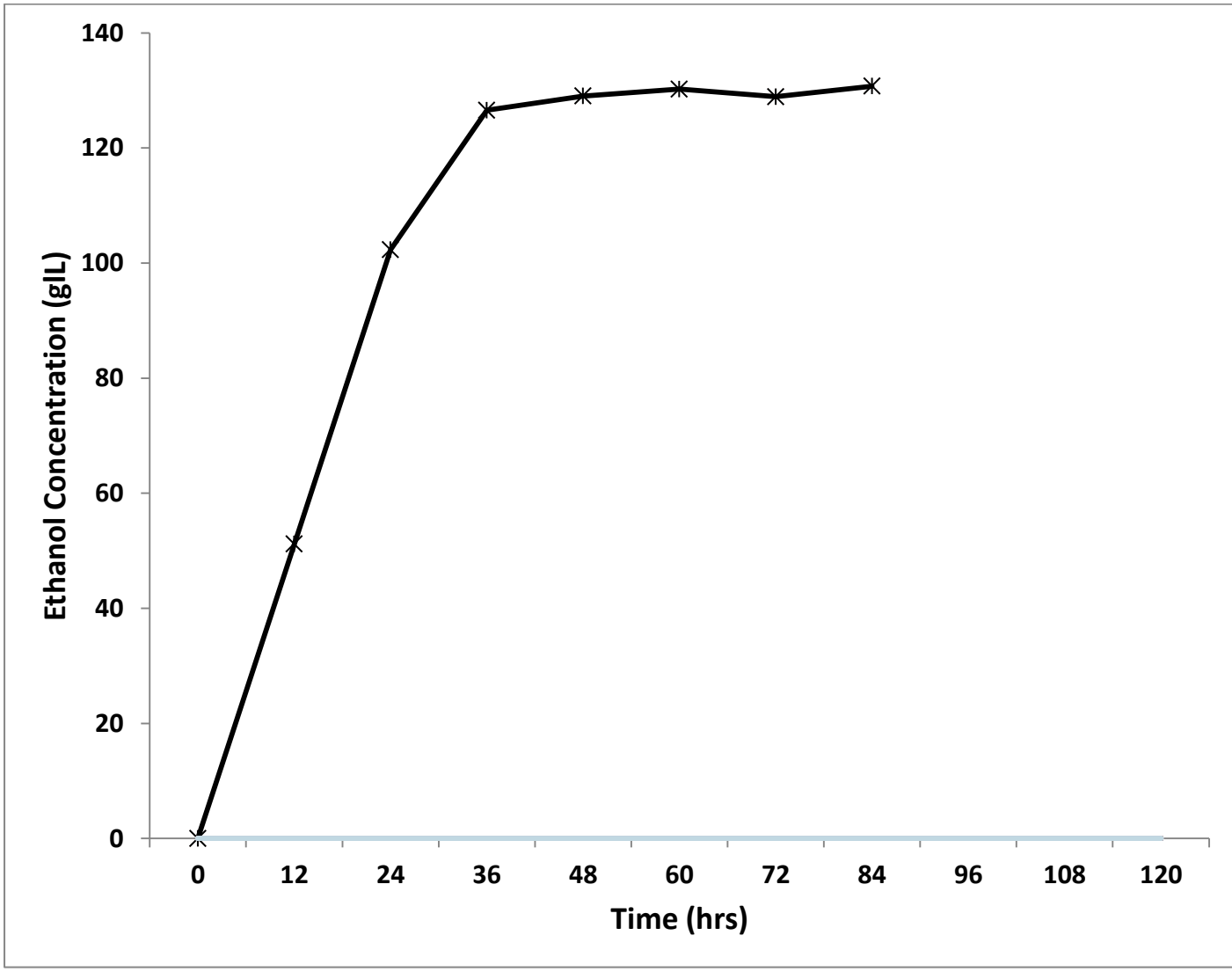
7 Appendices

7.1 Appendix A: Ethanol profile of fermentation performed with decorticated grains using the conventional processing at higher urea dosage (0.2 %; w/w slurry)

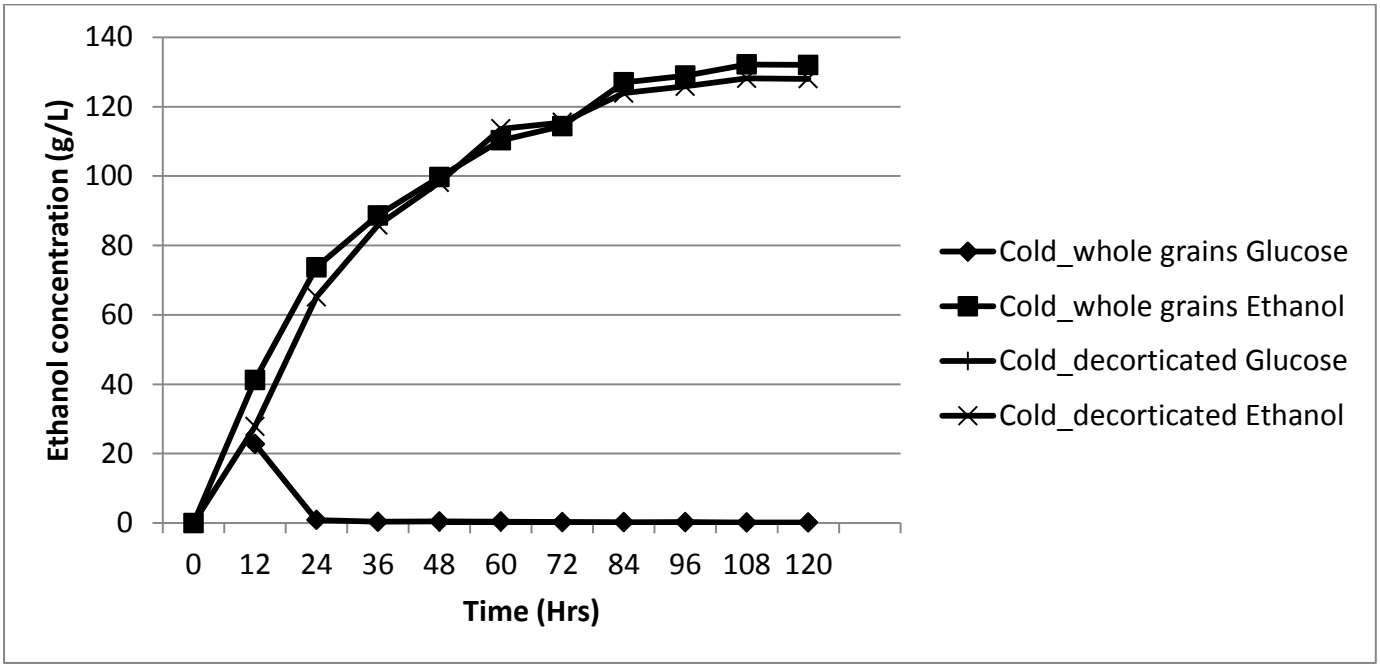


7.2 Appendix B: Analysis of variance for the model which best fit the experimental data using the conventional process

		Ethanol concentration (R^2 0.9)					Ethanol productivity (R^2 0.65)					Combined ethanol yield (R^2 89)				
	Source	Sum of squares	Degree of freedom	Mean square	F-value	p-value	Sum of squares	Degree of freedom	Mean square	F-value	p-value	Sum of squares	Degree of freedom	Mean square	F-value	p-value
Whole grains	Model	208.4407	7	29.77724	16.12089	< 0.0001	0.650804	3	0.216935	10.08691	0.0006	129.1717	9	14.35241	9.210711	0.0009
	A-Liq time	53.96756	1	53.96756	29.21711	0.0002	0.401163	1	0.401163	18.65307	0.0005	22.40276	1	22.40276	14.37705	0.0035
	B-A-amylase	3.992702	1	3.992702	2.16158	0.1672						21.23522	1	21.23522	13.62777	0.0042
	C-G-amylase	124.8518	1	124.8518	67.59261	< 0.0001	0.113057	1	0.113057	5.25685	0.0357	71.00346	1	71.00346	45.56671	< 0.0001
	AB	0.229165	1	0.229165	0.124066	0.7308						0.023302	1	0.023302	0.014954	0.9051
	AC	0.316012	1	0.316012	0.171084	0.6864						0.017308	1	0.017308	0.011107	0.9181
	BC	0.450301	1	0.450301	0.243785	0.6304						0.569744	1	0.569744	0.365635	0.5589
	A^2											2.717505	1	2.717505	1.743968	0.2161
	C^2						0.136584	1	0.136584	6.35081	0.0227					
	ABC	24.63318	1	24.63318	13.33598	0.0033						8.85237	1	8.85237	5.681039	0.0384
	A^2B											6.453196	1	6.453196	4.141361	0.0692
	Residual	22.16546	12	1.847122			0.344105	16	0.021507			15.58231	10	1.558231		
	Lack of Fit	11.63964	7	1.662806	0.78987	0.6261	0.211792	11	0.019254	0.727583	0.6941	3.004973	5	0.600995	0.23892	0.9289
	Pure Error	10.52582	5	2.105164			0.132313	5	0.026463			12.57734	5	2.515467		
	Cor Total	230.6062	19				0.994909	19				144.754	19			

		Ethanol concentration (R^2 0.80)					Combined ethanol yield (R^2 0.94)				
	Model	113.641	6	18.94017	8.757604	0.0006	52.17955	11	4.743596	11.18865	0.0011
	A-Liq time	30.3557	1	30.3557	14.03595	0.0024	10.7186	1	10.7186	25.2818	0.0010
	B-A-amylase	10.08903	1	10.08903	4.664993	0.0500	5.209118	1	5.209118	12.28667	0.0080
	C-G-amylase	52.70057	1	52.70057	24.36783	0.0003	16.45302	1	16.45302	38.8075	0.0003
	AB	4.948658	1	4.948658	2.288173	0.1543	2.0642	1	2.0642	4.868796	0.0584
	AC						0.03302	1	0.03302	0.077883	0.7873
	BC						1.313782	1	1.313782	3.098798	0.1164
Decorticated grains	A^2	9.302482	1	9.302482	4.301306	0.0585	5.50403	1	5.50403	12.98227	0.0070
	B^2						4.089491	1	4.089491	9.645821	0.0145
	A^2B	16.31627	1	16.31627	7.54436	0.0166	6.293044	1	6.293044	14.84331	0.0049
	A^2C						3.718441	1	3.718441	8.770631	0.0181
	AB^2						3.339865	1	3.339865	7.877688	0.0230
	Residual	28.11524	13	2.162711			3.391721	8	0.423965		
	Lack of Fit	22.63456	8	2.82932	2.581174	0.1556	1.087983	3	0.362661	0.787114	0.5506
	Pure Error	5.480685	5	1.096137			2.303738	5	0.460748		
	Cor Total	141.7563	19				55.57127	19			

7.3 Appendix C: Ethanol and glucose profile of cold processing experiments performed in bioreactors using whole and decorticated grains

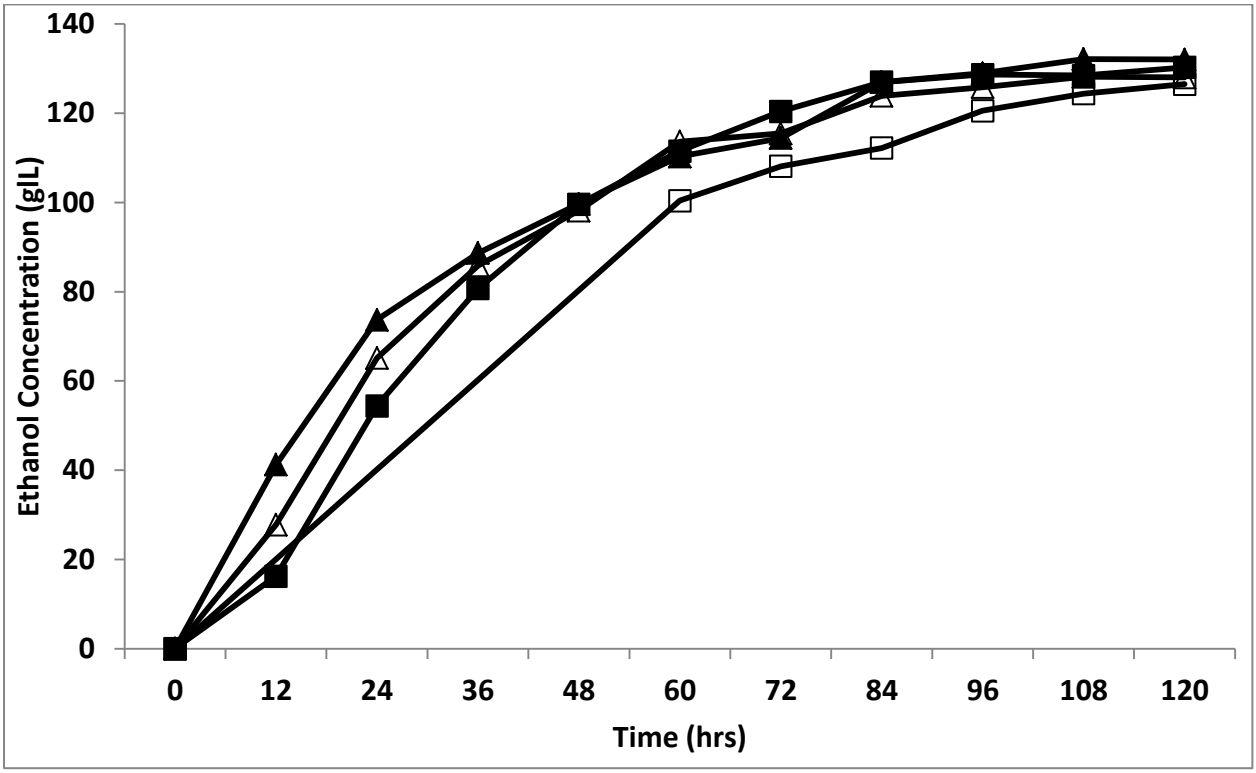


7.4 Appendix D: Analysis of variance for the model which best fit the experimental data using the cold process

		Ethanol concentration (R^2 0.98)					Ethanol productivity (R^2 0.98)					Conversion efficiency (R^2 98)				
	Source	Sum of squares	Degree of freedom	Mean square	F-value	p-value	Sum of squares	Degree of freedom	Mean square	F-value	p-value	Sum of squares	Degree of freedom	Mean square	F-value	p-value
Whole grains	Model	1457.67	6	242.95	45.18	< 0.0001	1.70	4	0.43	90.14	< 0.0001	654.123449	6	109.0206	41.03015	0.0001
	A-Presac. time	14.02	1	14.02	2.61	0.1575	0.02	1	0.02	5.17	0.0527	5.89653754	1	5.896538	2.219176	0.1869
	B-Stargen	722.46	1	722.46	134.35	< 0.0001	1.19	1	1.19	251.83	< 0.0001	315.290966	1	315.291	118.6605	< 0.0001
	AB	23.30	1	23.30	4.33	0.0826						14.7212559	1	14.72126	5.54038	0.0568
	A^2	0.66	1	0.66	0.12	0.7380	0.05	1	0.05	10.94	0.0107	0.20459359	1	0.204594	0.076999	0.7907
	B^2	427.85	1	427.85	79.56	0.0001	0.47	1	0.47	99.47	< 0.0001	203.280157	1	203.2802	76.50497	0.0001
	A^2B	58.66	1	58.66	10.91	0.0164						26.58934	1	26.58934	10.00696	0.0195
	Residual	32.26	6	5.38			0.04	8	0.00			15.9425055	6	2.657084		
	Lack of Fit	29.37	2	14.69	20.32	0.0080	0.04	4	0.01	66.80	0.0006	14.6151329	2	7.307566	22.02115	0.0069
	Pure Error	2.89	4	0.72			0.00	4	0.00			1.32737265	4	0.331843		
	Cor Total	1489.94	12				1.74	12				670.065955	12			

		Ethanol concentration (R^2 0.83)					Ethanol productivity (R^2 0.83)					Combined ethanol yield (R^2 0.90)				
Decorticated grains	Model	142.97	5	28.59	6.83	0.0127	1.28329768	2	0.641649	24.49489	0.0001	74.3802028	6	12.3967	9.4505	0.0076
	A-Presac. time	2.79	1	2.79	0.67	0.4409						0.59103541	1	0.591035	0.45057	0.5270
	B-Stargen	136.89	1	136.89	32.72	0.0007	1.16879759	1	1.168798	44.61874	< 0.0001	66.1676513	1	66.16765	50.44224	0.0004
	B^2						0.11450009	1	0.1145	4.37103	0.0631	6.65438445	1	6.654384	5.072903	0.0652
	AB	1.38	1	1.38	0.33	0.5836						0.31021381	1	0.310214	0.236488	0.6440
	A^2	0.06	1	0.06	0.02	0.9059						0.08248788	1	0.082488	0.062884	0.8104
	A^2B	53.45	1	53.45	12.77	0.0090						26.8305225	1	26.83052	20.45398	0.0040
	Residual	29.29	7	4.18			0.26195216	10	0.026195			7.87050455	6	1.311751		
	Lack of Fit	12.51	3	4.17	0.99	0.4811	0.18685203	6	0.031142	1.658692	0.3249	2.0728679	2	1.036434	0.715073	0.5426
	Pure Error	16.78	4	4.19			0.07510014	4	0.018775			5.79763665	4	1.449409		
	Cor Total	172.26	12				1.54524984	12				82.2507073	12			

7.5 Appendix E: Ethanol profiles of validation experiments obtained from the conventional and cold processing using whole and decorticated grains



Each point represents average of at least duplicate experiments

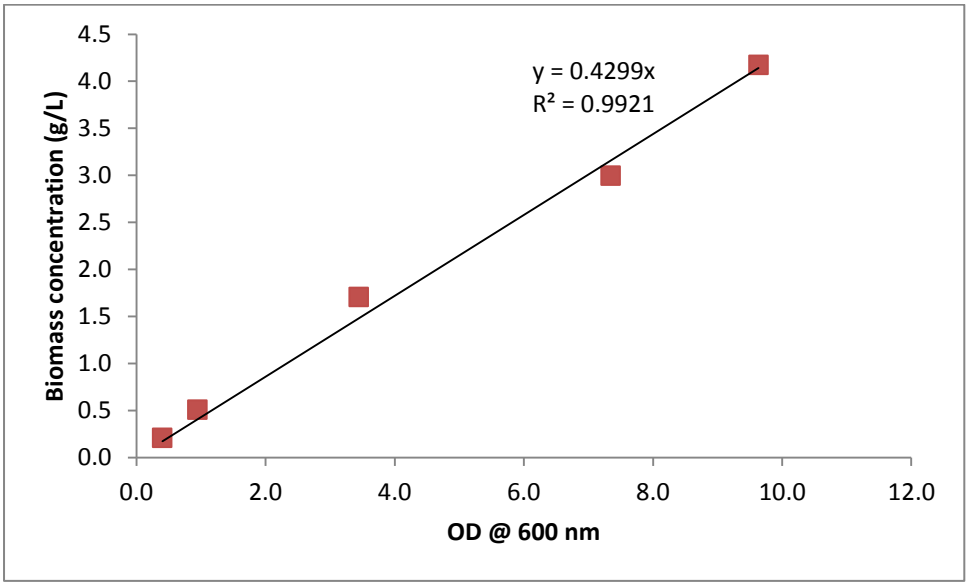
■: Conventional processing using whole grains

□: Conventional processing using decorticated grains

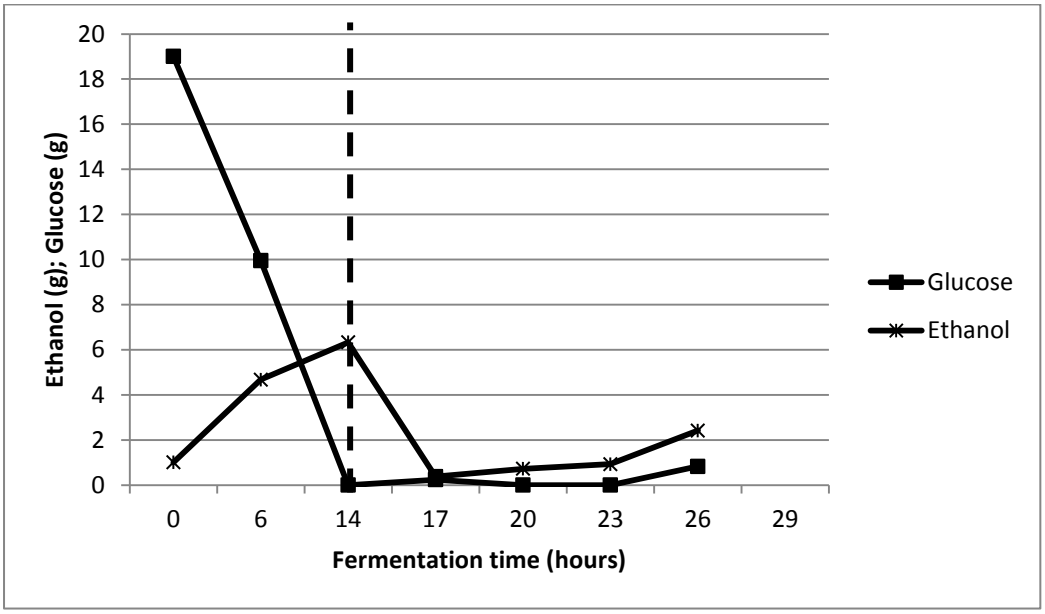
▲: Cold processing using whole grains

△: Cold processing using decorticated grains

7.6 Appendix F: Standard curve relating the biomass concentration to optical density



7.7 Appendix G: Total glucose and ethanol in the bioreactor during the inoculum production.



The vertical line indicate the start of fed batch

7.8 Appendix H: Analysis of variance of the model relating the inoculum size and Stargen dosage to the observed amylase activity

	Amylase activity				
Source	**Sum of squares**	**Degree of freedom**	**Mean square**	**F-value**	**p-value**
Model	0.74	5	0.15	15.49	0.0012
A-Inoculum size	0.00	1	0.00	0.01	0.9134
B-Stargen dosage	0.56	1	0.56	58.64	0.0001
AB	0.01	1	0.01	1.21	0.3080
A^2	0.02	1	0.02	2.03	0.1975
B^2	0.16	1	0.16	16.75	0.0046
Residual	0.07	7	0.01		
Lack of Fit	0.06	3	0.02	35.28	0.0025
Pure Error	0.00	4	0.00		
Cor Total	0.81	12			

7.9 Appendix I: Analysis of variance of the best model relating the inoculum size and Stargen dosage to the ethanol productivity

	Ethanol productivity (R^2 0.98)				
Source	**Sum of squares**	**Degree of freedom**	**Mean square**	**F-value**	**p-value**
Model	2.41	6	0.40	46.40	< 0.0001
A-Inoculum size	0.12	1	0.12	13.39	0.0106
B-Stargen dosage	1.91	1	1.91	220.78	< 0.0001
AB	0.03	1	0.03	3.96	0.0937
A^2	0.09	1	0.09	10.43	0.0179
B^2	0.29	1	0.29	33.84	0.0011
AB^2	0.05	1	0.05	5.85	0.0519
Residual	0.05	6	0.01		
Lack of Fit	0.05	2	0.02	25.71	0.0052
Pure Error	0.00	4	0.00		
Cor Total	2.46	12			