Triticale grain fermentation for production of bio ethanol and animal feed

by

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Abstract

High quantity of starch and relative high protein content, compared to other cereal grains, make triticale ideal as a feedstock for production of bioethanol and animal feed. It is mostly planted as feed for livestock and ground cover, therefore (if planted on marginal lands) no competition exists with the human food industry, as is the case when maize or wheat is used for bioethanol production. The conventionally applied warm conversion process of starch to ethanol, requires high heat energy inputs, thus increasing the cost of production. The lesser used cold conversion process requires less heat energy, but a higher enzyme dosage is required to achieve similar conversion efficiencies and ethanol yields. Therefore, reduction in enzyme dosage in conjunction with lower energy requirements will decrease operation costs for the cold conversion process, possibly increasing profitability. The main aim of this study was to optimise four process configurations, using whole-milled and debranned-milled triticale grains as feedstock for the conventional warm and cold conversion processes, in an effort to reduce the enzyme dosage required to achieve industry standards for fermentation performance (above 90% of the theoretical maximum yield). The next step was to scale up all process configurations and determine which configuration yields the best quality distiller's dried grains with solubles (in terms of protein and fibre content) while maintaining industry standards for fermentation performance. A central composite design (CCD), with enzyme dosage and hydrolysis time as independent variables, was used and experiments were carried out in 250 mL flasks. Fermentation performance was measured in terms of ethanol concentration, ethanol yield and ethanol productivity. Statistical models, relating independent variables and optimal performance measures, were developed. The models were validated in 5 L and 100 L scale-up experiments. The quality of distiller's dried grains with solubles (DDGS) produced from the scaled-up experiments were measured an compared across all process configurations and to canola oil cake and soy protein. Of the 100 L scaled-up experiments, the warm debranned configuration performed best with a final ethanol yield (as a % of the theoretical maximum) of 94.2%. The cold debranned configuration and warm whole-milled configuration came second and third, with 92.1% and 90.3% respectively. The cold whole-milled configuration did not reach the

benchmark of 90% yield. Productivities for warm whole-milled and debranned grains were 1.7 and 2.5 g/L/h respectively, while cold whole-milled and debranned configurations achieved 1.4 and 2.2 g/L/h. Debranning of grains had a significant ($p<0.05$) positive effect on both fermentation performance and quality of DDGS of the warm and cold processes. Moreover, DDGS produced from the cold debranned configuration had the highest quality. With a 44% crude protein and 13.85% acid detergent fibre content, it is ideal as a high-protein animal feed for monogastric animals.

Opsomming

Met 'n hoë hoeveelheid stysel en 'n relatiewe hoë proteïeninhoud, in vergelyking met ander grane, is korog ideaal as voermateriaal vir die produksie van bioëtanol en diervoer. Dit word meestal geplant vir veevoer en grondbedekking, en is daarom (as dit op marginale lande geplant word) geen kompetisie vir die menslike kos industrie nie, soos die geval is wanneer mielies of koring gebruik word vir die produksie van bioëtanol. Die konvensioneel toegepaste warm-omskakelingsproses van stysel na etanol benodig hoë hitte-energie insette, en verhoog dus die koste van produksie. Die minder gebruikte koue-omskakelingsproses vereis minder hitte, maar 'n hoër ensiem dosis om soortgelyke omskakelingsdoeltreffendheid en etanolopbrengste te bereik. Daarom sal vermindering in ensiem dosis saam met laer energie vereistes operasionele kostes vir die koue-omskakelingsproses verminder, en moontlik winsgewendheid verhoog. Die hoofdoel van hierdie studie was om vier proseskonfigurasies te optimeer deur gebruik te maak van heel-gemaalde en ontsemelde-gemaalde koroggraan as voermateriaal vir die konvensionele warm en nuwer koue omskakelingsprosesse, om die nodige ensiem dosering te verminder maar steeds bedryfstandaarde vir fermentasie prestasie te behaal (bo 90% van die teoretiese maksimum opbrengs). Die volgende stap was om alle proseskonfigurasies op te skaleer en te bepaal watter opset die beste gehalte distilleerders droë korrels lewer (in terme van proteïen- en veselinhoud), terwyl industriële standaarde vir fermentasie prestasie behou word. 'N sentrale saamgestelde ontwerp (SSO), met ensiem dosering en hidrolise tyd as onafhanklike veranderlikes, is gebruik en eksperimente is uitgevoer in 250 ml flesse. Fermentasie prestasie is gemeet in terme van etanol konsentrasie, etanol opbrengs en etanol produktiwiteit. Statistiese modelle, met betrekking tot onafhanklike veranderlikes en optimale prestasiemaatreëls, is ontwikkel. Die modelle is gevalideer in 5 L en 100 L opgeskaleerde eksperimente. Die gehalte van distilleerders droë korrels en oplosbares (DDKO), wat uit die opgeskaleerde eksperimente geproduseer is, is gemeet en vergelyk tussen proseskonfigurasies en met kanola oliekoek en soja-proteïen. Van die 100 L eksperimente het die warm ontsemelde konfigurasie die beste werkverrigting gehad met 'n finale etanol opbrengs (as 'n persentasie van die teoretiese maksimum) van 94.2%. Die koue ontsemelde en

warm heel-gemaalde konfigurasies het tweede en derde gekom, met 92.1% en 90.3% onderskeidelik. Die

koue heel-gemaalde konfigurasie het nie die doel van 90% opbrengs behaal nie. Produktiwiteit vir warm

heel-gemaalde en ontsemelde graan was 1.7 g/L/h en 2.5 g/L/h onderskeidelik, terwyl koue gemaalde en

ontsemelde konfigurasies 1.4 g/L/h en 2.2 g/L/h bereik het. Ontsemeling van korog het 'n beduidende (p

<0.05) positiewe effek op beide fermentasieprestasie en gehalte van DDKO van die warm en koue prosesse

gehad. Verder het DDKO geproduseer uit die koue ontsemelde konfigurasie die hoogste gehalte gehad .

Met 'n ru-proteïen konsentrasie van 44% en 13.85% suurvasmiddel-veselinhoud, is dit ideaal as 'n hoë-

proteïen voer vir monogastriese diere.

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1 Introduction

Burning fossil fuels release greenhouse gasses and aromatic compounds that contribute to global warming and negatively affect the environment (Delucchi, 2010). Also, the fossil fuel reserves available are becoming rapidly depleted due to current high consumption, because of evolving energy-intensive technologies and population growth. Alternative, renewable fuel sources are thus required to sustain the environment and the economy (BP statistical review of world energy, 2015). Based on current global markets and usage, bioethanol is presently the alternative liquid fuel of choice especially for the transport sector (Sánchez and Cardona, 2008). The biggest disadvantage associated with bioethanol production from food crops, such as maize or wheat, is the negative impact on food prices and availability. Bioethanol produced from small grains not used as staples for human consumption such as triticale, can easily mitigate this effect especially when these grains are planted on land not economically viable for food production (Glen Meyers, 2012).

For centuries ethanol has been used as solvent, preservative, antiseptic and fuel amongst others ("Ethanol History", 2011). Development of biofuels, such as ethanol, will alleviate the detrimental effects of global warming caused by burning of fossil fuels. The higher octane number of ethanol compared to most other petroleum fuels, enables it to burn cleaner and more controllable, preventing early ignition and causing less cylinder knocking (Biofuels association of Australia, 2014). Furthermore, when ethanol is blended with petroleum, the elevated oxygen content (Bothast and Schlicher, 2005) of the fuel promotes complete combustion to occur and consequently more carbon dioxide and less toxic bi-products such as carbon monoxide and aromatic compounds are generated and released into the atmosphere.

The industrial production of bioethanol relies mostly on a fermentation process based on carbohydrate (sugar) rich feedstocks. These feedstocks are mainly sugar-containing feedstocks such as sugarcane and sugar beet, starch-containing cereal grains and tubers and lignocellulosic biomass. All of the predominantly used starch and sucrose-containing feedstocks, such as maize and sugar cane, play a significant role in the

human food industry (Pradhan and Mbohwa, 2014). The use of sucrose and starch-containing crops as feedstock for ethanol production increases the demand and the cost of these crops due to competition with the food industry. Triticale, a hybrid between wheat and rye, has many advantages as a feedstock for the production of bioethanol, not only does it not compete with the human food industry, it is also resistant to drought, fungi and insects and requires very little nutrient inputs (Tsupko, 2009). On marginal farm lands, triticale can achieve acceptable yields where yields for food grains will be very low (Table 2.1). Thus, lands not suitable for food production can be utilised and competition with food can be avoided (Melamu, 2015). Triticale is mostly planted for animal feed and ground cover and the cost of grains is relatively low compared to other cereals (Tsupko, 2009). These benefits all contribute to the reduction in the price of raw grains, ultimately reducing the operation cost of bioethanol production from these grains.

In industry, the conventional process used to convert starch-containing cereal grains to ethanol, includes a gelatinisation step where milled grains are mixed with water and heated to about 90˚C to disrupt the crystalline structure of starch (Sánchez and Cardona, 2008). After gelatinisation, high temperature α-amylase enzymes reduce starch molecules to shorter dextrin chains which result in the liquefaction of starch. Glucoamylase enzyme and yeast are added to the resultant liquid slurry in the fermenter vessel for the conversion of dextrin chains to glucose sugars and for the conversion of glucose to ethanol respectively, in a process known as simultaneous saccharification and fermentation (Kim et al., 2004).

The energy required for the high temperatures applied during the liquefaction process, can account for up to 20 % of total energy value of the ethanol that is produced (Robertson et al., 2006). The search for alternative ways to break down starch and decrease energy cost has led to the discovery and isolation of enzymes capable of degrading raw uncooked starch at temperatures lower than 50˚C, substituting the commonly used warm conversion process with an improved cold conversion process requiring much less heat input. (Sun et al., 2010). Where enzymes with the ability to degrade raw starch are commercially available, these enzymes and associated processes are not yet widely used during industrial starch to ethanol processing (Genencor, 2010). A contributing factor is the increased enzyme dosage required for

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the use of cold enzymes and the cost associated with this (Pieters, 2016). Some research is therefore focussed on developing genetically engineered yeast with the ability to express these enzymes resulting in low temperature liquefaction, saccharification and fermentation occurring simultaneously in one vessel, known as consolidated bioprocessing (Görgens et al., 2015).

Ethanol fermentation plants battle to make a profit by relying solely on bioethanol produced for income, this is mainly due to its own cost challenges and direct competition with petroleum. Bio-refinery processes should thus be optimised to decrease overhead expenses and increase income by adding value to waste products. Dried Distiller's grains with solubles (DDGS), that are left after fermentation and distillation, could be sold as high-quality animal feed when nutritional requirements are reached. The current project will investigate various process configurations (warm and cold) in combination with milled triticale grains with and without debranning (removing the outer hull and germ fractions) and the effects of these process configurations on the DDGS produced, in favour of reduced energy input, lowest required enzyme dosage and highest quality DDGS, to keep operation costs to a minimum and maximise profit. Debranning of grains before the fermentation process has shown to increases the initial starch loading as well as decrease non-digestible fibres in resultant DDGS, yielding high quality animal feed.

2 Literature review

2.1 Historical overview of ethanol

Before 1850, ethanol was mainly used as an alcoholic beverage and only after the decline in whale oil around this time did ethanol gain popularity as a lighting fuel (Abebe, 2008). The first internal combustion engine created by Samuel Moray in 1824-1826, used a mixture of ethanol and turpentine as fuel. The Quadricycle, Henry Ford's first automobile, was built in 1896 and designed to use only pure ethanol as a fuel source. From 1908 to 1927 the model T Ford, also designed by Henry Ford, was produced with a modified hybrid engine adapted to run on ethanol as well as gasoline and kerosene ("Ethanol History", 2011).

Oil restrictions on America, from major oil producing countries during 1970, brought about a renewed interest in the production of bioethanol. In order to lessen America's dependence on imported oil and also encourage agricultural growth, tax incentives were offered for the production of bioethanol (Bothast and Schlicher, 2005). With the addition of bioethanol as oxygenate, to reduce carbon monoxide emissions, production of ethanol grew during 1988. A major amendment to the Clean Air Act in 1990 enforced the removal oxygenates such as benzene, toluene and xylene from gasoline to reduce toxic air pollution. In 2000, the ban of another oxygenate, Methyl tert-butyl ether, due to contamination of ground water, magnified the need for greener additives that burn with cleaner emissions. The Energy Policy Act of 1992 granted tax rebates for the purchase of vehicles that utilise alternative fuel such as E85 (Fuel blend consisting of at least 85% ethanol). These acts raised the demand and production of ethanol in the United States ("Ethanol History", 2011). Until 2005, Brazil was unsurpassed in terms of ethanol production, thereafter, America became the leading producer of ethanol (Balat et al., 2008).

2.2 Bioethanol as fuel source

Presently, fossil fuels such as oil, coal and gas are the predominant energy supply for commercial and household purposes which power the world economy (Beretta, 2007). The BP statistical review of world energy (2015) stated that the total proved oil reserves has the capacity to sustain global production for 52 more years. Moreover, the burning of these fossil fuels causes the release of greenhouse gasses, such as carbon dioxide and carbon monoxide, into the atmosphere. The amount of carbon dioxide that is released when bioethanol, produced from biomass, is burned, is equivalent to the amount of carbon dioxide consumed during the growth cycle of the plants used as feedstock for the production of bioethanol, thus the process is renewable and more sustainable (Balat and Balat, 2009). Greenhouse gasses contaminate water sources and contribute greatly to global warming and its consequent negative effects (Delucchi, 2010). Additionally, 79.4% of the world's oil reserves are localised in eight countries, 76.9% of coal reserves in six countries and 73.9% of natural gas reserves are located in eight countries only (BP statistical review of world energy, 2015). The confinement of these valuable resources to certain regions of the world (Organization of Petroleum Exporting Countries – OPEC) and the political unrest of these areas, greatly affect the energy security of countries dependent on these fuels.

If biomass to be used as feedstock for ethanol production is grown locally, energy security for producing countries would be greatly improved. National governments, to encourage the implementation of systems using renewable energy sources, are providing financial incentives for the production of biofuel. These incentives enable producers of biofuel to sell their product at a price competitive with that of current petroleum fuel products. Other countries encouraged consumers to buy cars that run on biofuel by offering reduced road taxes and registration fees on these vehicles (Mandil and Shihab-Eldin, 2010).

2.3 Feedstocks for bioethanol production

The feedstock used for bioethanol production determines whether it is first, second or third generation. First generation refers to the use of starch and sucrose-containing food crops, second generation to the use of lignocellulosic biomass like bagasse and cereal straw and third generation to the use of algae as feedstock for bioethanol production. The most widely used types are starch and sucrose-containing feedstocks and lignocellulosic biomass (Sims and Taylor, 2008)

Feedstocks primarily consisting of starch such as wheat, rye, barley, triticale, cassava, sorghum and maize are suitable for commercial bioethanol production. Maize is currently the most widely used starch-based feedstock for production of bioethanol (Balat et al., 2008). A main disadvantage of maize as a crop is the high requirement for fertilizer and pesticides, adding to the feedstock cost. Maize has a dry grinding liquefaction temperature of 90°C. The higher the liquefaction temperature, the greater the energy requirement and subsequent operation cost of the process. Table 2.1 (below) outlines the beneficial and undesirable properties of the most widely used sucrose and starch containing feedstocks, in terms of bioethanol production.

Maize and other cereals such as wheat, barley and sorghum play a significant role in the food industry and is a staple food for many people in South Africa. This drives the fuel versus food debate and makes these plants less suitable as feedstocks for bioethanol. Also, sorghum varieties with tannin have reduced hydrolysis due to the inhibitory action of tannin (Wang et al., 2008) Barley and rye can be grown in less desirable conditions but the lower starch contents and higher mash viscosity increases operation cost in terms of energy required for mixing, pumping and fermenting and additional enzymes necessary to mitigate the effect of viscosity (Hicks et al., 2004) (Wang et al., 1997).

Triticale is a hybrid between wheat and rye (Tsupko, 2009). This hybrid grain is highly drought resistant and requires low nutrient inputs, thus being resistant to climate change (Kučerová, 2007). High protein (7-12%) and starch (± 66%) content makes the grain a suitable feedstock for animal feed and bio-ethanol

production (Pejin et al., 2009), while simultaneously assisting in controlling soil erosion. Moreover, triticale can tolerate higher levels of acidity in soil and is more resistant to diseases and pests (Kučerová, 2007), making it suitable for planting on marginal lands. When triticale, mostly used for animal feed or ground cover, is planted on economically unproductive lands no competition exists with the food industry for human consumption (Tsupko, 2009).

The benefits associated with triticale makes it an ideal crop for bioethanol production. Low agricultural requirements and high-quality animal feed co-produced with bioethanol makes triticale profitable as a feedstock. Different grinding, hydrolysis and fermentation technologies need to be evaluated to further optimise the process and reduce operation cost of bioethanol production from triticale grains.

Table 2.1: Properties and requirements of sucrose and starch containing feedstocks for bioethanol production. Review of factors that contribute to the operation cost and overall profitability of ethanol production for some of the most widely used sucrose and starch containing feedstocks.

Feedstock (with moisture content)	Starch or sucrose content	Ethanol yield (Litre ethanol/ton of crop)	Gelatinisation temperature (in case of starch)	Crop yield under unfvourable conditions	Rainfall requirement	Fertilizer requirement	Other requirements/characteristics	Human consumption	References
Sugar cane (65%)	±16% sucrose (www.sugarcanecrops.com)	90 (Barcelos, C.A.et al.2011)	—	Poor	High	High	Tropical or subtropical climate. High pesticide requirement.	Yes	(www.sugarcanecrops.com)
Sugar beet (75%)	12-21% sucrose (Bowen, 2010)	92 (Almodares et al., 2009)	—	Average	Medium	Medium	Requires less water than sugar cane. Has a high herbacide and pesticide requirement.	Yes	(Kašičková et al., 2013)
Corn (15%)	65% starch (Gago et al.,2013)	350-400 (Kreith et al. 2010)	90˚C (Gago et al.,2013)	Poor	High	High	High pesticide requirement.	Yes	(Du Plessis, 2003)
Wheat (13%)	±65% starch (Gago et al.,2013)	409-432 (Carver, B.F. 2009)	65˚C (Gago et al.,2013)	Poor	Medium	High	High pesticide and herbacide requirement.	Yes	(Bowden et al., 2008)
Barley (14%)	50-55% strach (Hicks et al., 2005)	402 (Nghiem et al., 2010)	60˚C (Aldén, 2008)	Poor	Medium	High	High pesticide and herbacide requirement.	Yes	(Cook, 2013)
Sorgum grain (10%)	64-74% starch (Wang et al., 2008)	380-390 (Sheorain,V. et al 2000) 450 (Barcelos, C.A. et al 2011)	86˚C (Zhao et al., 2009)	Average	Medium	Medium	More drought resistant and less sensitive than corn.	Yes	(Hammer and Muchow, 1994)
Rye (12%)	60-62% starch (Wang et al., 1997)	350-360 (Wang, S. et al 1998)	70˚C (Aldén, 2008)	Good	Medium	Low	High drought tolerence and performs well in infertile soil.	Yes	(White et al., 2006)
Triticale (12%)	±66% starch (Pejin et al., 2009)	345-388 (Markovic, M. et al 2011) 470 (Amigun, B et al. 2012)	60˚C (Pejin et al., 2009)	Very good	Low	Low	Highly resistant to drought and climate change. Requires low inputs.	No	(Eudes, 2015)

2.4 Production technologies for starch conversion to bioethanol

2.4.1 Structure of starch

Starch is a homopolymer molecule made up of thousands of glucose monomers (Balat et al., 2008). The two predominant components forming starch are helical amylose and branched amylopectin. Amylose (depicted in Figure 2.1) is a linear polymer consisting of tightly packed glucose units (about 1000) bound by α-1,4 glycosidic linkages (R. F. Tester et al., 2004). This semi-crystalline framework makes amylose more resistant to hydrolysis agents such as enzymes or acid. Amylopectin (depicted in Figure 2.2) in comparison is comprised of long α-1,4 glucan chains branched from one another via α-1,6 glycosidic linkages after each 10 to 12th glucose units (Stevnebø et al., 2006). Typically, 20-30% of starch is composed of amylose and 70-80% is amylopectin. Generally, feedstocks with a high starch content generate high ethanol yields following fermentation.

α, 1-4 glycosidic linkage
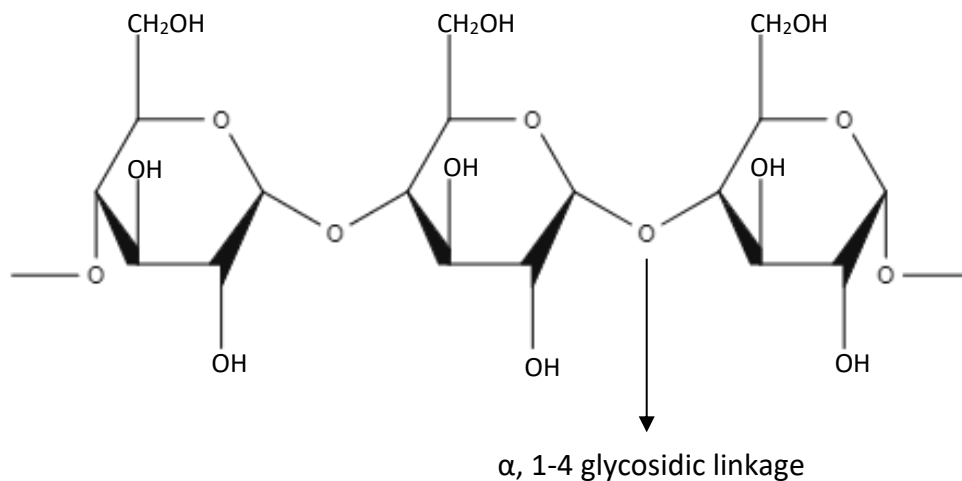
Figure 2.1 : Indicating the linear arrangement of glucose molecules connected by α, 1-4 glycosidic linkages in the amylose starch structure.

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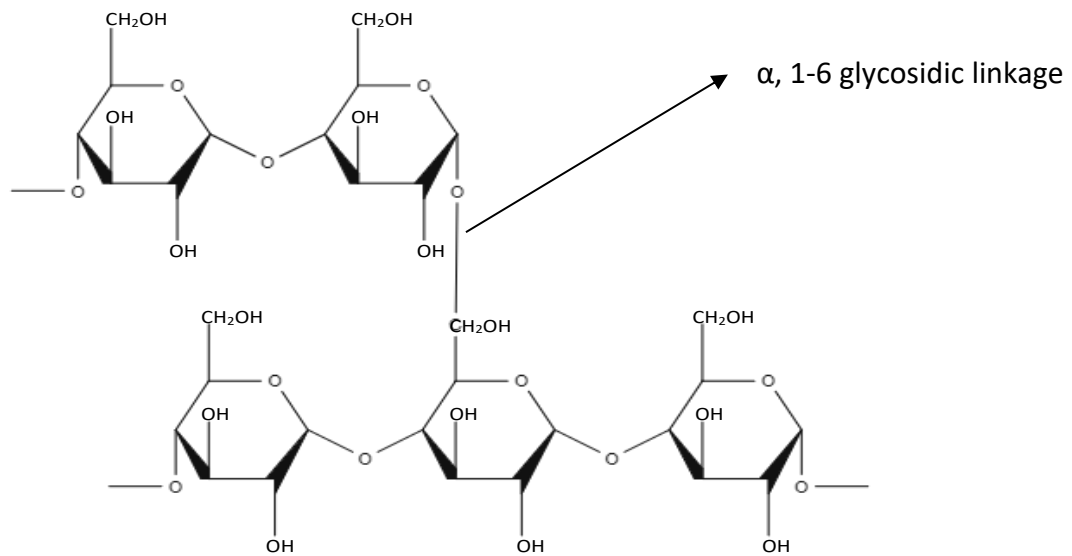
Figure 2.1: Indicating the branched arrangement of glucose molecules connected by α, 1-6 glycosidic linkages in the amylopectin starch structure.

2.4.2 Hydrolysis and fermentation

Different configurations of hydrolysis and fermentation may be utilised for the conversion of starch to ethanol. Before fermentation by yeast, hydrolysis usually occurs via two stages, i.e. liquefaction followed by saccharification. The liquefaction step is performed by an α-amylase enzyme while the saccharification step is carried out by a glucoamylase enzyme (Kim et al., 2004). Saccharification can be performed prior to or simultaneously with fermentation. The two steps of hydrolysis and fermentation are discussed in this section, as well as some of the most common configurations of these processes as shown in Figures 2.3 - 2.7. These processes can be applied to any small grain cereals used as feedstock for bioethanol production.

2.4.2.1 Liquefaction

Uncooked or raw starch molecules are arranged in a semi-crystalline structure with the inner most molecules sequestered from enzymes. During liquefaction (first step of conventional warm process), the slurry is heated to above 90˚C, causing the hydrogen bonds holding the structure in place to break due to increased heat energy in the presence of water, known as the gelatinization of starch (R. f. Tester et al., 2004). This cooking step (usually carried out at temperatures ranging between 90 and 110˚C) allows starch inside the grain to swell (Sánchez and Cardona, 2008). During this gelatinisation of starch, the slurry becomes considerably more viscous (Pieters, 2016). This increase in viscosity demands greater energy inputs for continuous and effective mixing and limits the starch loading that can be accomplished for liquefaction and subsequent saccharification and fermentation.

Thermostable α- amylase enzymes, usually procured from thermophilic bacteria such as *Bacillus licheniformis* or genetically engineered microbes expressing these enzymes, are added to the slurry during the liquefaction step (Sánchez and Cardona, 2008). These endo-enzymes hydrolyse the internal α-1,4 glycosidic linkages of amylose and amylopectin components. This cleavage yields shortened glucose chains of about 10 – 20 units, also called dextrins (van Zyl et al., 2012). Liquefaction results when the molecular weight of the starch molecules decrease due to the cleavage activity of α-amylases, thus causing a major reduction in viscosity of the slurry (Pieters, 2016). Additionally, under the same enzyme dosage, peak viscosity has been shown to decrease up to 3 fold when the grain coat of other cereal grains, such as sorghum, are removed before the liquefaction process (Wu et al., 2007). This shows potential for reduction in operation cost as well as increasing the nutritive value of co-produced DDGS as animal feed for monogastric animals, by removing indigestible fibre content, when bran is removed from triticale prior to the liquefaction process.

Furthermore, in an effort to optimise liquefaction for sorghum starch, Aggarwal et al., (2001) demonstrated that amylase enzyme dosage could be reduced by one third of the manufacturer's

recommended dosage, by adding 200mg of calcium chloride per litre to the slurry. The positively charged calcium ions act as co-factors, binding to the negatively charged amino groups of the enzyme and thus stabilizing the enzyme-starch complex (Bush et al., 1989). As a result, the 3-dimensional shape and hence the enzymatic activity is maintained for longer when calcium is present, which equates to a reduced enzymes dosage necessary to achieve the same degree of hydrolysis.

2.4.2.2 Saccharification

Saccharification is the final stage of hydrolysis where single glucose units are cleaved from the dextrin chains liberated during the initial liquefaction stage, thus completing hydrolysis. This cleavage action is accomplished by a glucoamylase enzyme, mostly procured from *Rhizopus* and *Aspergillus* species (Sánchez and Cardona, 2008). These exo-enzymes cleave the α-1,4 glycosidic linkage of the glucopyranosyl unit positioned at the non-reducing end of the dextrin chain, liberating glucose units into slurry (van Zyl et al., 2012). Even though the activity of glucoamylases are to cleave the α-1,4 glycosidic linkages, some of them have a cleavage action which includes α-1,6 glycosidic linkages when the adjacent bond is a α-1,4 linkage (Fierobe et al., 1998). The cleavage of both these linkages leads to the complete hydrolysis of starch, which can be measured according to the percentage of glycosidic linkages cleaved, a measurement referred to as dextrose equivalent (DE). The optimal working temperature of glucoamylases ranges between 30 and 70˚C (Sánchez and Cardona, 2008), for this reason liquefaction (performed 90 – 110˚C) and saccharification are always performed separately.

2.4.2.3 Fermentation

During fermentation, the glucose molecules yielded during hydrolysis are converted to ethanol by yeast. For industrial fermentation, *S. cerevisiae* is preferred above other yeasts, due to its tolerance for high ethanol concentrations. Even though bacteria, such as *Zymomonas mobilis* have been shown to achieve higher ethanol efficiency and productivity (Bai et al., 2008), these organisms are not commonly accepted in animal feed and decrease overall profitability of the process because costs associated with waste disposal

(Höfer, 2009). *S. cerevisiae* thus provides the benefit of higher ethanol tolerance and value-added animal feed that can be co-produced.

The incomplete breakdown of glucose (during fermentation) yields carbon dioxide and ethanol and takes place under anoxic conditions or can be caused by an excess of sugar in the mixture. Based on this metabolic pathway, the theoretical maximum yield for each gram of glucose is 0.49 grams of carbon dioxide and 0.51 grams of ethanol (Borglum, 1980). This theoretical maximum yield is however, not achieved due to other biological processes (occurring simultaneously) which produce by-products that prohibit either glucose, or other intermediate products of this fermentation pathway, from the formation of ethanol (Bai et al., 2008).

The industry standard for high gravity fermentation used to be a solids loading of 20 % (w/w) or higher, which would yield an ethanol concentration of about 7 – 10% v/v (Serna-Saldívar et al., 2012). Lately, due to its process advantages, a solids loading of 30 % (w/w) or higher, known as very high gravity (VHG) fermentation, has been suggested as a preferred alternative. Added benefits of VHG fermentation include a reduction in the amount of water required as well as improving the throughput of the process (Sánchez and Cardona, 2008). A final ethanol concentration of about 15 – 18% v/v are typically achieved with VHG fermentation and when distillation costs are compared to that of high gravity fermentation, a higher starch loading provides a significant reduction in the final distillation cost (Serna-Saldívar et al., 2012), thus its preference for industrial production of bioethanol.

2.4.3 Configurations of hydrolysis and fermentation stages

After liquefaction the starchy mixture is cooled, from there saccharification and fermentation can occur separately (SHF, discussed in section 2.4.3.1) or simultaneously (SSF, discussed in section 2.4.3.2). Both SHF and SSF are usually supplemented with additional nitrogen in the form of urea or ammonium sulfate to enhance the rate of ethanol production (Bothast and Schlicher, 2005).

2.4.3.1 Separate hydrolysis and fermentation (SHF)

Separate hydrolysis and fermentation (SHF) occur when hydrolysis and fermentation take place independently and in separate reactors as depicted in Figure 2.3. Here both these processes can proceed under optimum conditions (Balat et al., 2008). The enzymes required for saccharification when using triticale, function optimally at 60˚C, whereas yeast performs best at 35 ˚C (Pejin et al., 2009). As starch is converted to glucose, the sugar concentration increases and inhibits hydrolytic enzyme activity. Also of concern is the available sugar that can be utilized for growth by other microorganisms, which significantly increases the risk of contamination (Savić et al., 2009). Some of the handicaps associated with SHF can be overcome with simultaneous saccharification and fermentation.
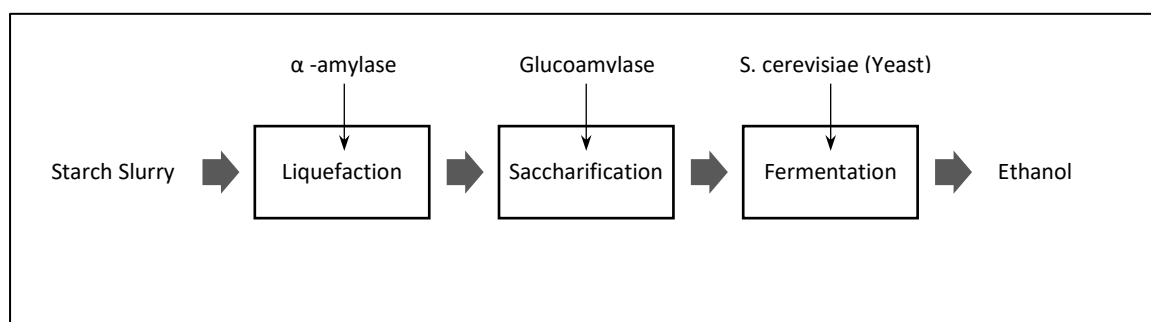
Figure **2.3**: Separate hydrolysis and fermentation (SHF).

2.4.3.2 Simultaneous saccharification and fermentation (SSF)

Simultaneous saccharification and fermentation (SSF) occurs when both saccharification (the final step of hydrolysis) and fermentation are carried out in the same reactor, as shown in Figure 2.4. Glucoamylase and yeast are added together to one reactor and as the enzymes liberate glucose molecules, the yeast converts it to ethanol. The process conditions for this reactor are usually at a pH of 4.8 and a temperature range of 30 - 35˚C (Balcerek and Pielech-Przybylska, 2013). A benefit of this configuration is that glucose gets used by yeast as it is produced, thereby no glucose inhibition occurs due to limited glucose build up. The lower

temperature of the process increases risk of contamination (Savić et al., 2009), this is lessened by the

limited amount of glucose present in the solution due to the presence of yeast in the fermentation

solution. Additionally, as the ethanol produced by the yeast increases, the risk of microbial contamination

decreases as a result of the killing effect ethanol has on most microbes (UCSB Science Line, 2015). A

drawback of SSF is the fact that the hydrolytic glucoamylases do not function at their optimal temperature

(60˚C) thus decreasing the rate of glucose production (Cardona and Sánchez, 2007). Even though these

glucoamylases are handicapped by sub-optimal conditions, (Savić et al., 2009) reported a greater ethanol

yield for SSF than for SHF. This is mainly due to enzymatic inhibition caused by high glucose concentrations

during SHF. Moreover, for SSF only one reactor is required where SHF requires two reactors, this decreases

the capital cost for SSF. For these reasons SSF is preferred for industrial scale production of bioethanol

(Savić et al., 2009). Current research focuses on creating fermenting organisms that can tolerate higher

temperatures, this will improve the rate of glucose production of the hydrolytic enzymes for this process

(Cardona and Sánchez, 2007).
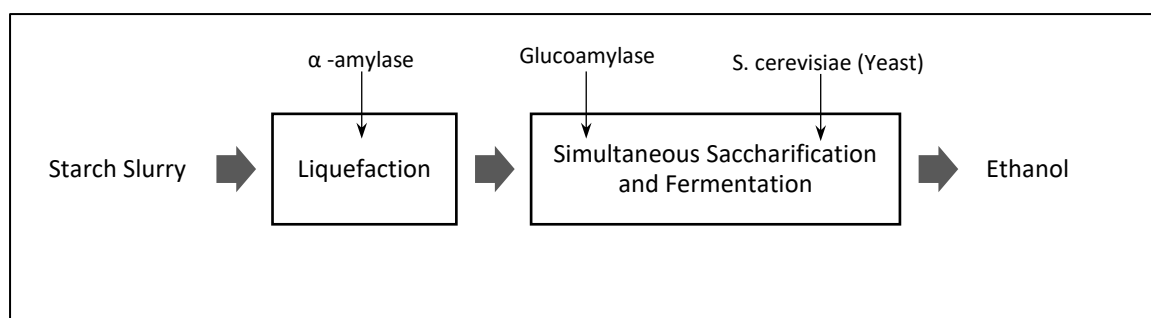
Figure 2.4: Simultaneous saccharification and fermentation (SSF).

2.4.3.3 Consolidated bioprocessing (CBP)

During CBP, depicted in Figure 2.5, both hydrolysis and fermentation occur together in one reactor, like

with SSF, except no external hydrolytic enzymes are added to the fermentation broth (Vohra et al., 2014b).

Genetically engineered yeast can produce either α-amylase or glucoamylase enzymes or both (Görgens et

al., 2015), thus reducing the cost of buying additional hydrolytic enzymes. Some *S. cerevisiae* strains are modified to express raw starch digesting enzymes (discussed in section 2.4.4.2) which entirely hydrolyses raw starch without the requirement of added heat. This process could lead to a one step conversion process from raw starch to bioethanol without the addition of extra heat energy or external enzymes, thus increasing profitability. Also, because no heat is applied, the starch does not gelatinize and therefore less mechanical energy is required for stirring or pumping of the mixture, further reducing operation cost (van Zyl et al., 2012). Major drawbacks with CBP is that fermentation requires more time to complete and ethanol yields are lower. This is due to the small amount of yeast used for inoculation and therefore only few enzymes are produced at the start of the process (van Zyl et al., 2012). Less enzymes means less available glucose for yeast to use which limits the conversion rate of this process. In some cases, the amount of enzymes produced is not sufficient to hydrolyse all the starch and therefore ethanol yield is lower. To overcome this limitation, additional amylolytic enzymes can be added to the fermentation broth at the beginning of the process or a biomass production step for yeast can be added prior to fermentation (Nkomba, 2015). This process shows great potential to reduce both the operation and initial setup costs.
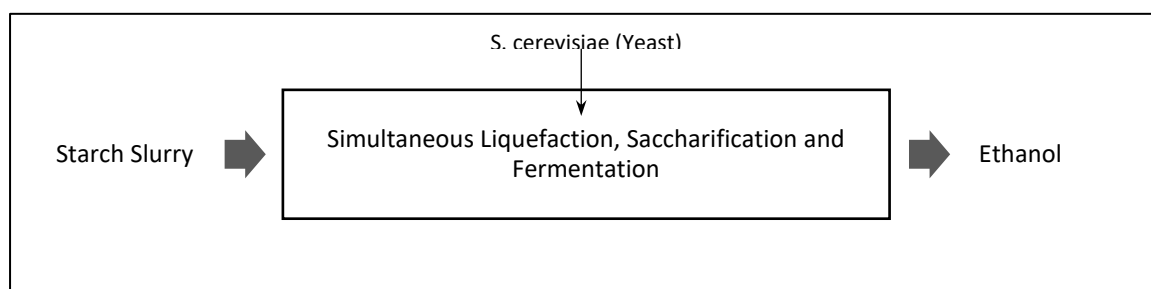
Figure 2.5: Consolidated bioprocessing (CBP).

All methods that include a liquefaction step above the specific starch gelatinisation temperature form part of the conventional warm process of starch conversion to ethanol. Because of the energy required for heating during liquefaction, production costs are increased significantly for this process. The constant quest for more economical methods for production of bioethanol has led to the development of a cold process

for starch conversion that completely bypass the need for such high temperatures. Both these processes used with triticale as feedstock will be discussed in section 2.4.4.

2.4.4 Starch conversion processes for triticale grain

2.4.4.1 Conventional warm SSF process

In industry, the conventional warm SSF process (shown in Figure 2.6) is applied in conjunction with the dry grinding process. The liquefaction step is carried out at temperatures above the gelatinization temperature of starch. Compared to maize (90˚C) and wheat (65˚C), triticale (60˚C) has the lowest liquefaction temperature (Pejin et al., 2009). Milled triticale grain (discussed in section 2.4.5.2) is mixed with water to obtain a slurry for liquefaction. The slurry is then heated to 60˚C and thermostable α-amylase enzymes are added (Pejin et al., 2009). Glucoamylase enzymes are only added to the mixture during SSF which is performed at 30˚C (Wang et al., 1998). The solids loading of triticale is 33 % (w/w), the pH is regulated at 5.5. and the liquefaction process is carried out for about 65 minutes. For SSF, when the slurry is supplemented with calcium and magnesium ions as well as urea, the final ethanol yield of this process is improved (Pejin J.D et al., 2015). According to Tsupko, (2009), the South African triticale cultivars that perform best in terms of ethanol yield are D1, D2 and H1 in the Swartland area and H1 and G2 in the Overberg area.

The peak viscosity reached by the triticale slurry is considerably lower compared to that of other grains, such as maize or wheat, and hence requires less mechanical energy to mix or pump (Pejin et al., 2009). The lower viscosity and liquefaction temperature of triticale contribute to the reduction in operation cost for the conventional warm process.

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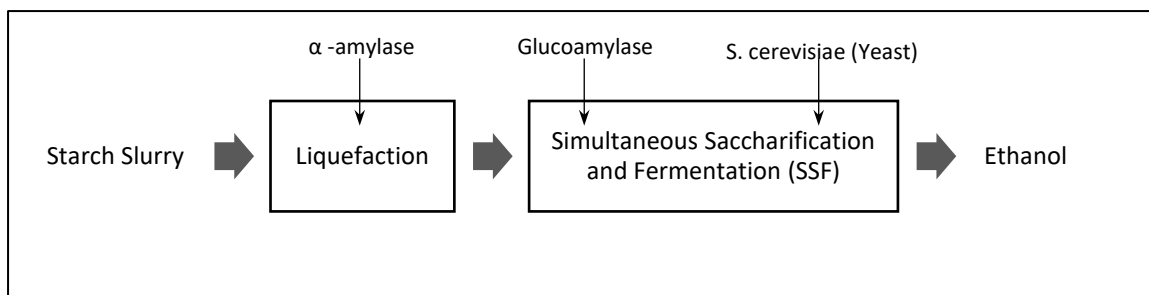
Figure 2.6: Hydrolysis and fermentation configuration for the conventional warm starch conversion process.

2.4.4.2 Cold process

During the cold conversion process, as illustrated in Figure 2.7, the pre-saccharification (hydrolysis) step is carried out at temperatures below the gelatinisation temperature of starch by making use of enzymes able to degrade raw starch. These raw starch hydrolysing enzymes (RSHE) occur naturally in microorganisms such as bacteria, yeast or fungi that decompose starchy matter (Sun et al., 2010). Raw starch hydrolysing enzymes used in industry are produced by recombinant organisms. The heat energy input for the liquefaction and saccharification steps carried out during the conventional warm process accounts for 10 - 20 % of the energy value of the total ethanol produced with this process (Robertson et al., 2006). By utilizing RSHE, temperatures remain below the gelatinization temperature of starch hence slurry viscosity remains much lower than in the warm conversion process, therefore less energy is required for mixing and pumping these slurries. Decreased viscosity has the added benefit of allowing higher initial solids loading, which would positively impact overall productivity, process yield and distillation cost (Nkomba, 2015; Pieters, 2016). By working at lower temperatures, a better conversion efficiency could be expected due to less undesirable reactions, such as the Maillard reaction, occurring at higher temperatures applied during liquefaction in the warm process (Cinelli et al., 2015). On the other hand, at lower temperatures the cold process is more vulnerable to contamination by microorganisms, whereas during the warm process these microbes are killed by higher temperatures associated with liquefaction. Most RSHE require a pre-saccharification step, carried out at a temperature below the gelatinisation temperature of these starch-containing feedstocks, which can assist in controlling the growth of microbes. To further restrict the

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propagation of these microbes, the pre-saccharification step can be carried out at a lower pH, combined with acid tolerant enzymes. The manufacturer's recommended temperature for the pre-saccharification step varies for each of the cereal grains, from 49-51˚C for rye, 56-57˚C for triticale and 62-63˚C for maize (Genencor, 2010). This mild heat treatment also serves as a catalyst to increase the rate of hydrolysis and the final ethanol concentration (Genencor, 2010). Options for controlling contamination in the cold hydrolyses process include the use of antibiotics, such as Ampicillin, or gamma radiation of the feedstock before hydrolysis (Robertson et al., 2006).
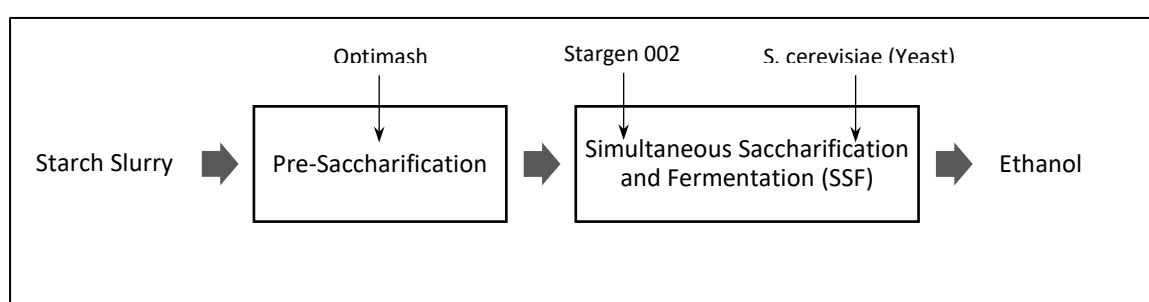
Figure 2.7: Hydrolysis and fermentation configuration for the cold starch conversion process.

When using maize at 25% solids loading , Wang et al., (2007) observed similar performance outputs for both the warm and cold hydrolysis processes. He reported similar values for fermentation rate (> 94% of maximum), ethanol conversion rate (88% of maximum) and ethanol yield (14.1% v/v) for both processes in a linear comparison. For both the warm and cold conversion processes Wang et al.,(2007) used enzyme dosages of at 2.86 mL/g of grains on a dry weight basis (dwb), which is in excess of the manufacturers recommended dosage of 2 mL/g of grains (dwb). Other studies however concluded that the warm process had a better efficiency for the conversion of starch to glucose and obtained higher final ethanol concentrations and ethanol yields than the cold conversion process (Pejin J.D et al., 2015);(Pieters, 2016); (Wang et al., 2007).

Stargen 002 is an enzyme cocktail commonly used for the hydrolyses of starch-containing feedstocks during the cold conversion process (Tsupko, 2009; Nkomba et al., 2016; Pieters, 2016). The cocktail, consisting of

acid α-amylases and glucoamylases originally procured from two species, *Aspergillus niger* and *Aspergillus kawachii,* are produced by recombinant strains by Genencor International (Genencor, 2010). The glucoamylase creates cavities which allow the α-amylase to penetrate the starch molecule and hydrolyse it from the inside out. Starch-containing feedstocks with a higher fraction of amylopectin is hydrolysed more efficiently by the Stargen enzyme cocktail than feedstock with a higher amylose fraction (Tsupko, 2009).

Furthermore, the required enzyme dosage can be significantly decreased by removing the bran from the grain before milling. By making use of the cold conversion process and initial solids loadings of more than 30%, a 11.7% decrease in enzyme dosage was reported for using decorticated sorghum instead of whole-milled sorghum (Nkomba et al., 2016). According to our knowledge no articles attempting to reduce the enzyme dosage by using debranned instead of whole-milled triticale were found for the cold conversion process. However, according to Schill, (2008), the enzyme dosage for the cold process could be decreased by as much as 35 % when parameters like glucose concentration and by-product formation are dynamically controlled throughout the process so that glucose concentration stays within the optimal range, avoiding osmotic stress in yeast when the glucose concentration is too high or a deficiency for when it is too low (Cinelli et al., 2015). Seeing that the cost of enzymes contribute significantly to the overall production cost of bioethanol from starch (Robertson et al., 2006),reducing the dosage of these enzymes could boost production profitability.

Different pre- and post-treatment processes can be performed on triticale. Before liquefaction, triticale grains can be debranned and milled to facilitate complete hydrolysis of starch, and as a value-added co-product, the Wet Distillers Grains (WDG) and thin stillage, collected after fermentation and distillation can be mixed, dried and sold as animal feed. Debranning, milling, distillation and the co-production of Distillers Dried Grains with Solubles (DDGS) will be discussed next.

2.4.5 Pre- and post-treatment processes for triticale

2.4.5.1 Debranning

Debranning, also known as dehulling, pearling or decortication, is the mechanical process by which the outer layer, containing mostly non-fermentable fibres, of the triticale grain is removed by either crushing or friction and abrasion (Eudes, 2015). By using friction and abrasion methods such as pearling, the grain kernel usually remains unbroken, while with the crushing method the grain is, as the name implies, crushed and broken to separate the outer bran layer from the inner endosperm fraction.

Because the amount of non-fermentable fibre is reduced, the starch-rich solids is increased and results in higher levels of final ethanol concentration and yield. Removing fibre allows more starch to be loaded into the 35% solids loading maximum of the VHG process, thus increasing the final ethanol concentration. On the other hand, removing fibre can also increase accessibility of the starch to the enzymes, thereby increasing hydrolysis efficiency and ethanol yield. Wang et al., (1999) stated that debranning of triticale prior to SSF can increase the starch loading by 8% and coupled with VHG fermentation with a solids loading of 33%, achieved final ethanol concentrations of up to 15.7% (v/v). They used a SATAKE laboratory abrasive mill for the debranning process and removed 12% of the dry grain mass after 3 abrasive cycles of 30 seconds each. Total starch losses for triticale grains were reported to be 4.3% following the three cycle abrasion process (Wang et al., 1999). Furthermore, with the addition of urea at 16 mM, total yeast cell numbers increased 5- to 7-fold in the first 48 hours (due to more sugar being converted to biomass), increasing the catalytic activity and therefore fermentation rates and fermentation efficiencies decreased (from 94.3% to 86% of maximum). Also, the removal of bran may cause deficiencies in minerals, vitamins and amino acids aiding the fermentation process which would also have a negative effect on the fermentation efficiency (Wang et al., 1999; Nkomba, 2015)

SATAKE mills are regarded as sophisticated machinery for the debranning of cereal grains such as triticale (Sosulski et al., 1997); (Wang et al., 1999). The outer bran layer of the grain is removed during successive

abrasion cycles before milling takes place. During the flour milling process, residual bran in the crease of grains such as triticale and wheat are split from the white flour.

The crushing process is performed with a roller mill. Grain moisture content is increased to 16% to soften the interior part of the grain and toughen the outer bran layer (Campbell, 2007). It is this contrast in physical traits of these two parts of the grain on which the functioning of the roller mill is based. During the first stage of milling, the grain is crushed by the rollers, this causes the softer endosperm to crumble into smaller fragments while the tougher bran layer tears into larger fragments (Campbell, 2007). The consistency and hardness of the grains play the most important role during this process. As the hardness of the grain increase, the effectiveness of the debranning process and hence the flour yield decrease, these factors are therefore directly related (Campbell, 2007). The softer the endosperm of the grain, the more easily it crumbles while the bran layer only tears and remains mostly intact. Grains with a harder endosperm will resist breaking until they eventually crumble together with the bran layer, thus offering less effective separation. After the first crushing phase, the smallest endosperm fragments are sieved out, while the larger endosperm fragments together with the bran continues on to the next phase where air purifies the stream by blowing and filtering out smaller bran fractions. The fractions that remain move to the next phase and the process continues on like this until all milling phases are completed. The end product consists of various fractions of white flour, a fraction of relatively pure bran and a pollard fraction which is a mixture smaller bran fragments attached to endosperm (Campbell, 2007). Bran finishing is the process by which the pollard stream is processed again by more milling phases on smaller rollers in an attempt to further fractionate the bran from the endosperm ("Impact Bran Finisher MKLA," accessed October 2018.).

2.4.5.2 Milling

Milling or grinding is the physical method by which grain fragment size is reduced prior to hydrolysis and fermentation (Naidu et al., 2007). This process, also called dry-grinding and usually done with a hammer

mill, crushes the endosperm into smaller fragments thus increasing the total surface area and allowing enzymes access to core regions of the grain. Seeing that hydrolysis of starch occurs via diffusion, the size of the particles is inversely proportional to the hydrolysis efficiency (Mahasukhonthachat et al., 2010). Using the warm process, larger particles obtained from coarser grounds showed a 5% drop in hydrolysis efficiency when compared to finer ground sorghum grains (Wang et al., 2008).

Prior research on the production of ethanol from triticale grains, utilizing the conventional warm process, all used different milling sizes, none exceeding a 2mm sieve hole diameter (Wang et al., 1999) (Pejin et al., 2009), (Tsupko, 2009), (Pejin J.D et al., 2015). The cold process, by which raw starch is converted to ethanol, has a disadvantage in requiring even finer milled particles. Genencor, (2010) recommend that 95% of particles be smaller than 0.6 mm in diameter for maize starch. Finer milled particles require more energy which ultimately increase the production cost of ethanol.

2.4.5.3 Distillation and the production of Distillers Dried Grains with Solubles

Distillation is the principal means and most established technique for the purification of bioethanol in industry. This process makes use of the varying volatilities of different constituents contained in a mixture (Onuki et al., 2008). The main mechanism of action is that volatile components with a low boiling point vaporize first and when condensation takes place, these vapours concentrate in the condensate liquid phase. Distillation is a very effective separation technique, however, it does have some disadvantages. Impurities in the mixture, with similar boiling points to ethanol, will vaporize at the same time and deposit in the ethanol condensate. Also, the energy requirement for cyclical vaporization and condensation is high and contributes significantly towards the operation cost for production (Onuki et al., 2008).

The liquid mixture obtained after fermentation, is transferred into distillation columns where the ethanol is separated from water and other solids. A 95% pure ethanol solution is obtained after distillation ("ICM INC - Ethanol Production Process," 2012). Further purification of ethanol is done by molecular sieves containing specialised beads that absorb water molecules and lets ethanol through. A purity of 99% can be achieved

with this technique ("ICM INC - Ethanol Production Process," 2012). After distillation, a denaturing chemical, such as methanol is added to the ethanol, this renders the ethanol unfit for human consumption, and it is either stored or sold directly ("ICM INC - Ethanol Production Process," 2012).

After the first distillation column, water and silage collected at the bottom, is transferred to a centrifuge where solids are separated from liquids. The liquid portion is further concentrated by evaporation of water and the syrup-like liquid that remains is called condensed distillers solubles (CDS). The solid portion, referred to as wet distiller's grains (WDG) is mixed with the CDS and this combination is known as wet distiller's grains with solubles (WDGS). The moisture content of WDGS is further decreased by drying in a rotary drum. Distiller's dried grains with solubles has a final moisture content of about 10% -12% and can be sold as quality animal feed (US Grain Council, 2013).

The nutrients contained within triticale residues, as well the enzymes and yeast added during hydrolysis and fermentation, represent the constituents of the DDGS. The amount of nutrients (such as protein, fat, fibre, vitamins and minerals) present initially in the dry grain can be concentrated three to five times in the DDGS, due to selective removal of starch-components by hydrolysis-fermentation (Gibreel et al., 2011). Protein is of particular interest in the animal feed industry, specifically for non-ruminant animals such as chickens or pigs. A high protein concentration and low fibre content is preferable for DDGS. Gibreel et al., (2011) reported that, by using whole-milled triticale for both the cold and warm starch conversion processes, the cold conversion process performed best with a protein content of 49% in the resulting DDGS. Gibreel et al., (2011) also reported that the protein present in DDGS of the cold process is of higher quality than that found in DDGS from the warm conversion process. This relates to the amino acid composition, the digestibility and the increased concentration of minerals and soluble dietary fibres of triticale compared to wheat and other grains (Gibreel et al., 2011). Additionally, the debranning of triticale can increase the protein content to between 60% – 65% in DDGS because most of the fibre is removed before the grains are milled (García-Aparicio et al., 2011). This increased protein content, in DDGS obtained

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as a by-product of bioethanol production, will result in a higher value animal feed product which would contribute significantly to the economic viability of such a production plant (Gibreel et al., 2011).

2.4.5.4 Nutritional requirements for DDGS as animal feed

To optimise production goals, DDGS has to meet nutritional requirements to serve as animal feed for monogastric animals like pigs and poultry. Feed quality is determined by nutritional factors such as energy, protein content, fat, digestibility of starch, fibre content, vitamins and minerals which are required by all animals for growth and maintenance functions (Hertom, 2013). The daily energy requirements can range between 12.1 and 28.8 MJ/day for growing pigs (Rehutaulukot, 2010).

Growing animals also require amino acids for protein synthesis, most of which can be synthesized by the animal itself (non-essential) as well as some that can only be acquired through feed (essential). The essential amino acids required by most animals include Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Threonine, Tryptophan and Valine (Novak et al., 2014). A high protein content in the grains used as feedstock for ethanol production will result in DDGS with a substantial protein amount which could be sold at a higher price, adding value to the product (Gibreel et al., 2011). Protein is increased approximately 3-fold during the ethanol fermentation process, i.e. wheat with 11% protein yield DDGS with 33% protein (Barrico, 2010).

Monogastric, or single-chambered stomach animal require feed with a higher protein content and a lower fibre content. Fibre can be classified as two different types, namely Acid Detergent Fibre (ADF) and Neutral Detergent Fibre (NDF). The difference between ADF and NDF is hemicellulose: ADF consists of cellulose, lignin, silica, cutins and tannins, and NDF consists of all these components plus hemicellulose. NDF can be digested somewhat by rumens and is usually a close estimate of the total fibre content of a material while ADF on the other hand is the least digestible fibre present in material. High quality animal feed will thus contain low levels of ADF and high levels of protein, to keep digestible energy content as high as possible ("US Grains council, 2016).

2.5 Key performance measures for production of ethanol from triticale

For any type of conversion process of starch to ethanol, a few key measures are used to assess the performance of these processes. These measures include:

- Total solids loading - initial weight of the grains loaded expressed as a percentage of the total weight of the slurry. A higher solids loading contributes to a higher throughput for the process. During VHG fermentations, high solids loading increases viscosity which could negatively impact ethanol yield and volumetric ethanol productivity.

- Total starch loading – percentage of starch present in the initial solids loading. The starch content present in the feedstock as well as the solids loading affects the starch loading. For the current study, the starch loading will be kept constant between debranned and whole-milled processes to enable comparable ethanol concentrations and ethanol productivities between different process configurations.

- Final ethanol concentration – measured after completion of fermentation. To achieve a high final ethanol concentration, a high initial starch loading, near-complete starch hydrolysis and high yield of ethanol on sugars from hydrolysis is required. A higher final ethanol concentration increases the profitability of the process.

- Volumetric ethanol productivity – final ethanol concentration divided by the fermentation time needed to achieve that concentration. A higher volumetric productivity increases the efficiency of equipment and capital investment. For the current study, the ethanol productivity was taken at the time point where the ethanol concentration did not increase by more than 5%.

- Hydrolysis conversion efficiency – the percentage of starch hydrolysed to glucose. The higher the percentage of starch hydrolysed, the better the conversion efficiency of raw starch to glucose.

- Yield of ethanol on glucose – (fermentation conversion efficiency) the percentage of available glucose that was converted to ethanol.

- Ethanol yield as a percentage of the theoretical maximum – (combined hydrolysis and fermentation efficiency) final ethanol yield obtained is expressed as a percentage of the maximum theoretical yield of

ethanol that can be achieved. A high hydrolysis conversion efficiency and a high fermentation conversion efficiency is needed to obtain a high final ethanol yield on starch.

The hydrolysis and fermentation conversion efficiencies are usually combined into one conversion efficiency for the combined hydrolysis-fermentation process and is expressed as a percentage of the theoretical maximum yield. For the ethanol production process to be profitable, these important performance measures must match present industry standards. Currently, wheat is more readily used as feedstock for ethanol production than triticale and because triticale is a hybrid with wheat, this is used as an industrial reference standard for the above-mentioned parameters. Prior research reporting these performance indicators for using either whole-milled or debranned triticale as feedstock for ethanol production using the conventional warm or cold conversion processes are compared in Table 2.2. The conventional warm process is preferred for the industrial production of bioethanol from whole-milled wheat, hence its inclusion as a baseline for comparison in Table 2.2.

Table 2.2 : Performance parameters achieved when using triticale as feedstock for bioethanol production.

			References					
Feedstock	Performance measures		Thomas and Ingledew, (1990)[c]	Pejin et al., (2009)				
		Solids loading (%)	33	33				
	Whole wheat grains	Ethanol concentration (g/L)	135.00	108.30				
		Ethanol productivity (g/L/h)	1.88	—				
		Conversion efficiency (%)	—	—				
		Starch hydrolysis (%)	—	—				
			Wang et al., (1998)[a]	Wang et al., (1998)[b]	Pejin et al., (2009)	Pejin et al., (2015)	Wang et al., (1997)[d]	Wang et al., (1997)[e]
CONVENTIONAL WARM CONVERSION PROCESS	Whole triticale grains	Solids loading (% or disoved sugar g/L)	285 g/L	285 g/L	33	33	33	33
		Ethanol concentration (g/L)	119.1	116	101.3	119.8	79.1	76.3
		Ethanol productivity (g/L/h)	0.83	1.21	—	1.66	1.1	1.59
		Conversion efficiency (%)	93	89.4	—	92.2	91.4	90.2
		Starch hydrolysis (%)	—	—	—	—	—	—
			Wang et al., (1999)[a]	Wang et al., (1999)[b]				
	Debranned triticale grains	Solids loading (%)	33	33				
		Ethanol concentration (g/L)	127.3	124				
		Ethanol productivity (g/L/h)	0.76	1.3				
		Conversion efficiency (%)	94.3	86				
		Starch hydrolysis (%)	—	—				
			Gibreel et al., (2009)	Gibreel et al., (2009)				
	Whole wheat grains	Solids loading (%)	20	20				
		Ethanol concentration (g/L)	133	125				
		Ethanol productivity (g/L/h)	1.85	1.74				
		Conversion efficiency (%)	85.2	90.2				
COLD CONVERSION PROCESS		Starch hydrolysis (%)	—	—				
			Gibreel et al., (2009)	Gibreel et al., (2009)				
	Debranned wheat grains	Solids loading (%)	20	20				
		Ethanol concentration (g/L)	129	142				
		Ethanol productivity (g/L/h)	2.48	1.97				
		Conversion efficiency (%)	79.9	93.7				
		Starch hydrolysis (%)	—	—				

[a] No urea added dring fermentation - fermentation time = 168 hour

[b] 16mM urea added during fermentation - fermentation time = 96 hours

[c] Supplemented with yeast extract

[d] No urea added during fermentation - fermentation time = 72 hours

[e] 8nM urea added during fermentation - fermentation time = 48 hours

Most of the studies mentioned in Table 2.2 used the conventional warm conversion process combined with VHG slurries with about 33% initial solids loading and none provided information on the percentage starch hydrolysis (residual solids should be measured to calculate percentage starch hydrolysis). Ethanol concentrations achieved were between 10% – 15% v/v when using whole-milled triticale compared to 16% v/v when using debranned triticale. Only one article by (Wang et al., 1999) describing ethanol production from debranned triticale, was found. When whole-milled wheat grains were used as feedstock, an ethanol concentration of 17.1% v/v was achieved and the fermentation time was 72 hours with a volumetric ethanol productivity of 1.88 g/L/h when supplemented with yeast extract (Thomas and Ingledew, 1990). Fermentation times varied between studies, but most were between 72 and 96 hours with volumetric ethanol productivities of 0.76 and 1.66 g/L/h, depending mostly on supplementation with an additional nitrogen source, which doubled the ethanol productivity (Wang et al., 1997), (Wang et al., 1998), (Wang et al., 1999), (Pejin et al., 2009), Pejin et al., 2015). The industrial threshold for the efficiency for ethanol conversion from starch is considered 90%, but higher conversion efficiencies are usually achieved in practice.

With a solids loading of 33%, the highest ethanol concentration achieved with whole-milled triticale was 15.2% v/v with a fermentation time of 72 hours when 160 mg/L of magnesium ions were added and the final ethanol yield of 92.1% was achieved by Pejin et al., (2015). They also illustrated that when 160 mg/L of calcium ions were added, a 69.31% increase in glucose concentration after hydrolysis was observed compared to no calcium supplementation before hydrolysis. They achieved a volumetric ethanol productivity of 1.66 g/L/h, the highest for whole-milled grain triticale and closest to that achieved with whole-milled wheat (Pejin et al., 2015).

 (Wang et al., 1998) reported that, at a dissolved sugar concentration of 285 g/L, when supplementing fermentation with 16 mM urea, which provide free amino nitrogen (FAN), fermentation time was decreased from 144 to 96 hours and volumetric ethanol productivity increased from 0.83 to 1.21 g/L/h when compared to no supplementation. However, the final ethanol concentration decreased by 3g/L and the conversion efficiency also decreased from 93% to 89.4%. They suggested future experiments with lower urea concentrations supplementation for fermentation (Wang et al., 1998). (Wang et al., 1999) supplemented with 8 mM of urea and

although 2.8g/L lower ethanol concentrations were achieved, volumetric ethanol productivity increased from 1.1 to 1.59 g/L/h and the conversion efficiency remained above 90% (Wang et al., 1999). Thus, indicating that a smaller trade off in efficiency for productivity is required when lower urea concentrations are added.

Wang et al., 1999 investigated the effect of urea supplementation at 16mM concentration during fermentation on the production of ethanol from debranned triticale. At 33% solids loading, they observed a decrease in fermentation time from 168 hours to 96 hours which equates to a 0.54 g/L/h increase in volumetric ethanol productivity. Yet, correlating with (Wang et al., 1998), the addition of urea at 16 mM decreased the final ethanol concentration by 3.3g/L and the conversion efficiency by 8.3%. The decrease in final ethanol concentration and conversion efficiency is due to more glucose being used for biomass generation by the yeast due to the availability of FAN (Wang et al., 1999).

Gibreel et al., (2009) used hulled and dehulled wheat and barley in conjunction with the cold conversion process at a solids loading of 20%. He reported higher ethanol concentrations were for debranned fermentations, but higher ethanol yields for whole wheat fermentations. The productivities were also higher for debranned compared to whole wheat fermentations.

No information regarding the use of the cold conversion process for ethanol production with debranned triticale as feedstock was found. Therefore, further investigation is required to assess the performance measures for the cold conversion process using debranned triticale as feedstock and the subsequent effect on the profitability of such a production process for industrial bioethanol.

2.6 Factors that influence processing rate and efficiency

2.6.1 Process variables

This section discusses the variables which can be altered by the operator, increasing or decreasing the performance measures mentioned in section 2.5, and ultimately determining the overall performance of the production process.

2.6.1.1 Enzyme dosage

Enzymes usually account for about 7% of the total cost of operation for the production of bioethanol (Eidman, 2007), therefore industrial ethanol plants will strive to achieve the lowest possible enzyme dosage for hydrolysis which still deliver a desirable performance (parameters mentioned in Table 2.2). During the warm conversion process, liquefaction and saccharification steps involving α- and glucoamylase enzymes respectively, are performed individually due to large difference in optimum temperature requirements for these enzymes. Seeing that these enzymes work cooperatively, the optimal dose of each is required to achieve complete hydrolysis of starch. Table 2.3 describes the enzyme dosages for the warm and cold conversion processes recommended by the manufacturer, these dosages will also be utilized in the current project. Grain properties such as starch and protein content, amongst others discussed in section 2.6.2, significantly influence the conversion efficiency of starch to ethanol, hence the broad dosage range of 0.25 – 0.5 kg α-amylase and 0.45 – 0.75 kg glucoamylase per ton of grains (Novozymes) The lowest optimal dosage for each type of cereal grain needs to be experimentally determined.

The cold conversion process, in contrast to the warm conversion process, does not involve such a high temperature treatment as required during liquefaction. The Stargen 002 cocktail of α- and glucoamylase enzymes are added together during SSF but requires even higher enzyme dosages (1 – 3 kg of enzyme per ton of grains) for efficient hydrolysis of starch, compared to the warm process. Also, a pre-saccharification step, required by most RSHE, need an acid α-amylase enzyme dosage of 0.13 – 0.16 kg per ton of grains used (Genencor, 2008).

Table 2.3: Enzyme dosages for this project, as recommended by the manufacturers.

	Enzymes	Dosage in kg/ton grains		Enzymes	Dosage in kg/ton grains
Warm conversion process	α-amylase (Termamyl SC)	0.25 - 0.5	**Cold conversion process**	Acid α-amylase (GC626)	0.13 - 0.16
	Glucoamylase (Saczyme)	0.45 - 0.75		α- and glucoamylase cocktail (Stargen 002)	1.0 - 3.0

2.6.1.2. Temperature of treatments required

For the warm conversion process, a temperature of about 65˚C is required for the gelatinization and liquefaction of triticale starch. The residence time spent at that temperature is of equal importance for gelatinization and hydrolysis, Novozymes recommends a range between 90 and 150 minutes. The optimal residence time for each type of feedstock needs to be determined experimentally seeing that grain properties of a specific feedstock, starch loading and specific enzyme dosages used, all influence this liquefaction time. For the complete gelatinisation and hydrolysis of starch the optimal residence time is crucial, whilst also keeping side reactions resulting in nutrient losses, to a minimum (Galvez, 2005).

The cold conversion process involves a pre-saccharification step which also requires a mild heat treatment at an optimal temperature obtained through experimental optimisation. The residence time for pre-saccharification recommended by Genencor ranges from 40 to 120 minutes, usually just below the gelatinisation temperature of starch for a specific feedstock (Genencor, 2008).

2.6.1.3. Supplementation with nitrogen or addition of protease enzymes

Additional nitrogen sources are required by yeast to reach optimal biomass in order to obtain the highest possible

efficiency for fermentation, especially in the case where debranned triticale grains are used, resulting in a higher

starch loading during VHG fermentations (Wang et al., 1999; Nkomba, 2015). Nitrogen can be added to the

fermentation mixture in an organic form, such as free amino nitrogen (FAN), or in an inorganic form like urea or

ammonium sulfate, both would improve the conversion of glucose to ethanol (Bothast and Schlicher, 2005).

Ethanol Red is an *S. cerevisiae* strain specifically developed for the industrial production of bioethanol and will be

used for fermentation during the current project. The manufacturer of Ethanol Red recommends at least a 300

ppm (preferably more) supplementation of FAN in the fermentation mixture for optimal performance. Instead of

adding nitrogen, another option is to add proteases to hydrolyse endogenous proteins in the grain and avail

inherent nitrogen which yeast can utilise (Pérez-Carrillo et al., 2008). These methods can be used separately or in

conjunction with one another to obtain optimal ethanol productivity (Johnston and McAloon, 2014a).

2.6.1.4. Quantifying the effect of process parameters on performance indicators

Each variable involved in the process would affect the performance outcome in some way, therefore the separate

and cumulative effects of these variables on the outcome need to be known. One suitable technique widely used

for the quantification of process parameters, called the response surface methodology (RSM), makes use of

statistical and mathematical modelling to determine the cumulative effect of a range of process variables on the

outcome. The modelling equation that relates the values of these explanatory variables to a response variable (or

performance indicator) is written as: $Y = b_o + \sum_{i=1} b_i X_i + \sum_{i=1} b_{ij} X_i X_j + \sum_{i=1} b_{ii} X_i^2$

Y represents the predicted response, being either ethanol yield, concentration or volumetric productivity, b_o is

the constant coefficient, b_{ij} the interaction coefficient and b_{ii} is the quadratic coefficient.

Moreover, using this RSM technique will enable the operator to assess the effect of altering specific process

parameters, such as enzyme dosage and liquefaction and pre-saccharification times for this project, on the overall

performance of the process by means of model equations generated. This will allow the operator to achieve the optimal process performance. RSM has been widely used in the field of bioethanol production from several feedstocks, with great success (Romaní et al., 2012), , (Yingling and Zhengfang, 2013), (Nkomba et al., 2016).

2.6.2 Grain properties

2.6.2.1 Phytic acid content

Most cereal grains contain phytic acid, which binds and form complexes with metal ions such as calcium, iron, zinc, copper and more, as well as other macromolecules such as proteins and starch. These phytic acid complexes are stable over a broad pH range and results in decreased amounts of available starch, by making starch molecules resistant to degradation by α-amylase enzymes when complexed to phytic acid (Pejin et al., 2009). Co-factors such as calcium, iron and zinc necessary for optimal metabolic reactions in yeast, are rendered inaccessible to yeast when complexed with phytic acid. Also, phytic acid-protein complexes reduce the amount of FAN available for use by yeast. Even though this is the case, (Pejin et al., 2009) observed that the bioethanol yields obtained from different wheat and triticale cultivars were not influenced by the phytic acid content of the grains.

2.6.2.2 Starch content and amylose to amylopectin ratio

Various authors have illustrated the linear relationship that exists between the starch content and the ethanol yield per unit biomass obtained from the grains (Wu et al., 2006), (Zhan et al., 2003). Yet, studies using different sorghum cultivars with similar starch contents have shown that yields differ between cultivars. Between waxy, hetero-waxy and non-waxy starches, waxy varieties had the highest ethanol yield while no-waxy varieties had the lowest (Wu et al., 2007). Wang et al., (2008) demonstrated that final ethanol concentration differed by up to 7.4% between 70 sorghum grain cultivars with similar starch contents. They concluded that the amylose to amylopectin ratio was responsible for this variance. As the amylose content increased the bioethanol yield decreased and vice versa, therefore a lower amylose content is preferred for grains used in the production of bioethanol.

Similar results were obtained from studies with maize as feedstock, using the warm and cold conversion processes, ethanol yields for both processes increased as amylose content decreased (Sharma et al., 2010). The effect of the amylose to amylopectin ratio was more noticeable for the cold process with a final bioethanol yield of 5.2% compared to 6.3% obtained from the warm process when using high amylose maize as feedstock for both processes (Sharma et al., 2010). This correlates with findings from Van Hung et al., (2005), indicating that starches with a high amylose content had increased resistance to the amylolytic hydrolysis, yielding less glucose which results in reduced ethanol yields (Van Hung et al., 2005).

2.6.2.3 Protein content

Starch, followed by protein, are the two most abundant molecules making up the triticale grain (Tsupko, 2009). Therefore, as the protein content increases the starch content decrease, ultimately reducing the amount of ethanol obtainable from that grain. Yet again, some studies indicate that grains with similar protein and starch concentrations, displayed different conversion efficiencies. These differences were attributed to difference in digestibility of the proteins during fermentation, even though no proteases were added during fermentation. A strong linear correlation was observed between the extent of protein digestibility and the conversion efficiency for ethanol (Wang et al., 2008). In contrast to this, Shuping (2011) observed no correlation between the digestibility of the proteins and the efficiency of conversion, the variation in yield was ascribed to no exo-protease enzymes being produced by yeast. The digestibility of protein will influence the amount of FAN that is available for yeast growth, thus it should be taken into consideration.

Another factor influencing ethanol conversion efficiency and yield could be that, following the cooking step, denatured and cross-linked proteins form web-like structures that ensnare smaller starch molecules, making them inaccessible to amylose enzymes. This theory is supported by data reported by Wang, et al. (2008) showing that the ethanol conversion efficiency decreased as the degree of protein crosslinking increased, irrespective of the process conditions used.

2.7 Conclusion

Triticale, being a hybrid between wheat and rye has the best of both worlds. A high starch and protein content, resistance to fungi and pests, as well as requiring minimal water and nutrient inputs, making triticale ideal as a feedstock for production of bioethanol and animal feed. Critical assessment of the performance achieved by present production technologies is required to make the most profitable choice. Utilising the cold conversion process has its advantages over the conventional warm process, mainly being a higher quality animal feed produced and the reduction in required heat energy. On the other hand, the cold process requires a higher enzyme dosage and additional measures to control contamination which could add to operation costs. Moreover, available performance data for the cold conversion of raw, debranned triticale to ethanol is lacking. Even though debranning has been shown to improve starch hydrolysis, ethanol yield and resultant DDGS quality, only one study was found investigating the effect of decortication of sorghum grains on performance outputs, enzyme dosage requirement and resultant DDGS quality for the cold conversion process. No data was acquired on the performance of debranned triticale as feedstock for the cold conversion of starch to ethanol. The availability of sufficient data is necessary for the evaluation of present production technologies using triticale grain as feedstock.

3 Hypotheses, objectives, research questions and deliverables

By considering data from previous studies, it was hypothesized that:

• The use of debranned triticale grains in conjunction with the cold conversion process of starch to ethanol, could significantly decrease the RSHE dosage required while maintaining industry standards for ethanol concentration, ethanol yield and ethanol productivity.

• The use of debranned triticale grains in conjunction with the cold conversion process of starch to ethanol, could significantly increase quality of DDGS produced, while maintaining industry standards for ethanol concentration, ethanol yield and ethanol productivity.

• Process configurations will produce industry acceptable standards for ethanol concentration, yield and productivity when scaled up to 5 l and 100 L bio-reactors.

The main aim of this study was to optimise all process configurations in an effort to reduce the enzyme dosage required to achieve industry standards for ethanol concentration, ethanol yield and ethanol productivity. The secondary aim was to scale up all process configurations and determine which configuration yields the best quality DDGS (in terms of protein and fibre content) while maintaining industry standards for ethanol concentration, yield and productivity. This was achieved by meeting the following objectives:

- Determination of the lowest enzyme dosage and liquefaction time required by the warm conversion process of starch to ethanol, when using whole-milled and debranned-milled triticale grains, to match industry standards for ethanol concentration, ethanol yield and ethanol productivity.

- Determination of the lowest enzyme dosage and pre-saccharification time required by the cold conversion process of starch to ethanol, when using whole-milled and debranned-milled triticale grains, to match industry standards for ethanol concentration, ethanol yield and ethanol productivity.

- Successful scaling up of all process configurations, using whole-milled and debranned-milled grains, from bench (5 L) to pilot (100 L) scale bioreactors.

- Determination of the effect of the warm and cold conversion processes on the composition of resultant DDGS (focussing on protein and fibre content) when using whole-milled and debranned-milled triticale grains.

- Determination of the effect of debranning on the resultant DDGS (focussing on protein and fibre content) when using the conventional warm process and newer cold conversion process.

Research questions that will be addressed during this study are:

- When using whole-milled and debranned-milled triticale grains as feedstock for bioethanol production, to what extend does the performance of the cold conversion process match that of the conventionally used warm conversion process?

- How does debranning of triticale grains affect the performance of the cold and warm conversion processes of starch to ethanol?

- How does process configuration and debranning of grains influence the chemical composition of dried distiller's grains with solubles (DDGS) produced from the conversion of triticale to ethanol?

Deliverables for this study are as follows:

- ➤ Master's thesis (this report)

- ➤ Article submitted to a scientific journal

- ➤ Optimised cold conversion process data of starch to ethanol, using whole-milled or debranned-milled triticale grains as feedstock, in 5 L and 100 L bioreactors.

- ➤ Optimised warm conversion process data of starch to ethanol, using whole-milled or debranned-milled triticale grains as feedstock, in 5 L and 100 L bioreactors.

- ➤ Pilot scale (100 L) production data illustrating the effect of debranning of triticale grains on the chemical composition of DDGS as a value-added co-product in the production of ethanol from triticale for warm and cold conversion processes.

➢ Hundred and sixty kilograms of high protein animal feed (DDGS) to be used in an animal trail to determine the feasibility of using this co-product as feed for mono-gastric animals in SA.

4 Methodology and materials

4.1 Raw materials

Dried triticale grains were obtained from Overberg Agri in Bredasdorp, vacuum packed and stored at room temperature. Some process configurations required debranned grains, this was performed by dry grinding using a roller mill with agitating sieves to separate triticale into three fractions – bran, germ and endosperm. The debranning process removed 27.2% solids of which 20.9% was bran and 6.3% was germ. Whole and debranned grains were milled using a hammer mill to pass through a desired screen size (2mm for whole and 0.6 mm for debranned grains). Different triticale cultivars from one harvest were mixed together to obtain a homogenous mixture before debranning and milling of grains.

4.2 Reagents, yeast and enzymes

For the conventional warm conversion process, thermostable α-amylase enzymes (Termamyl SC from Novozymes, Denmark), from *Bacillus lichenformis* was utilised. This Termamyl SC enzyme has a declared activity of 120 KNU/g. KNU (kilo novo units α-amylases), refers to the amount of enzyme capable of breaking down 5.26 grams of starch per hour at a temperature of 37˚C, a pH of 5.6, a Ca^{2+} ion concentration of 0.0043 M and a reaction time of 7 to 20 minutes. The glucoamylase enzyme for the warm process, Saczyme from Novozymes, has a declared activity of 750 AGU/g, where AGU (amyloglucosidase units) refers to the amount of enzyme capable of catalysing the conversion of one μmol maltose per minute at a substrate concentration of 10 mg/ml, a temperature of 37˚C, a pH of 5.0 and an incubation time of 30 minutes. For the cold conversion process, an acid stable α-amylase enzyme (GC626 from Genencor, USA) and an enzyme cocktail (Stargen 002 from Genencor, USA) was used. The Stargen 002 blend of α- and glucoamylase enzymes have a declared activity of 570 GAU/g, GAU (glucoamylase unit) translates to the amount of enzyme that releases one gram of glucose per hour at a temperature of 60˚C and a pH of 4.2, from soluble starch.

For fermentation, Ethanol Red dry yeast produced from LEAF Technologies in France, was used, with a fresh inoculum prepared for each fermentation batch. Two and a half grams of yeast was rehydrated in a 50 mL, 2% glucose solution at a temperature of 33˚C for 25 minutes in an incubator shaking at 100rpm. One mL of this rehydrated yeast broth was added as the inoculum to each flask. To supplement fermentation, calcium chloride and urea procured from Sigma-Aldrich, USA was added to each flask.

4.3 Mash preparation

Erlenmeyer flasks (250 mL) were weighed before performing experiments. For the warm process (see Figure 4.2) a final slurry mass of 100 grams included 35 grams of whole-milled or 25.44 grams of debranned-milled triticale flour (to keep the starch loading the same between whole-milled and debranned-milled experiments), 5 mg of calcium chloride and water (for whole-milled experiments) or bran and germ hydrolysate (for debranned experiments – see Table 4.1 and Figure 4.1) to make up the rest of the mass. Sulfuric acid at a three molar concentration was used to adjust the pH to 5.8 before the addition of a pre-determined amount of Termamyl SC α-amylase (see Table 4.2). The slurry was then be heated in a shaking incubator at 65˚C at 200 rpm for the adequate liquefaction time (see Table 4.2). After liquefaction, the flasks containing the mash were placed in water at room temperature to cool down to 30˚C. The slurry mass of each flask was readjusted to 100 grams to accommodate for water lost during liquefaction. The appropriate amount of Saczyme glucoamylase (see Table 4.2), 0.07% urea and one mL of the yeast inoculum were added to each cooled flask. One hundred microlitres of an Ampicillin stock solution (100 mg/ 10 mL) was also added to each flask to prevent contamination. SSF was then performed in a shaking incubator, at 30˚C at 200 rpm for 120 hours. Samples were collected every 12 hours.

For the cold conversion process (see Figure 4.3), a final slurry mass of 100 grams included 35 grams of whole-milled or 25.44 grams of debranned-milled triticale flour (to keep the starch loading the same between whole-milled and debranned-milled experiments), 5 mg of calcium chloride and water (for whole-milled experiments) or bran and germ hydrolysate (for debranned experiments – see Table 4.1 and Figure 4.1) to make up the rest of the mass. Sulfuric acid at a three molar concentration was used to adjust the pH to 4.2 before the addition of GC626

acid stable amylase at 14 µl/100 g of grains. Instead of a liquefaction step, a pre-saccharification step at 50˚C at 200 rpm for the adequate pre-saccharification time (see Table 4.3) was performed. After pre-saccharification, the flasks containing the mash were placed in water at room temperature to cool down to 30˚C. The slurry mass of each flask was readjusted to 100 grams to accommodate for water lost during the pre-saccharification step. The appropriate amount of Stargen 002 (see Table 4.3), 0.07% urea and one mL of the yeast inoculum were added to each cooled flask. One hundred microlitres of an Ampicillin stock solution (100 mg/ 10 mL) was also added to each flask to prevent contamination. SSF was then performed in a shaking incubator, at 30˚C at 200 rpm for 120 hours. Samples were collected every 12 hours.

Validation experiments were performed in 5 L jacketed bioreactors produced by Sartorius in Germany. These bioreactors were equipped with a propeller-like mixer and, to prevent ethanol or water loss through evaporation, an exhaust cooling system at 4˚C . Before every experiment was performed, the reactor was washed and autoclaved at 121˚C for 15 minutes, to sterilise it. Liquefaction and pre-saccharification steps were, however not performed aseptically, to better imitate industrial conditions. Each 12-hour sampling point was tested for lactic and acetic acid to determine if any contamination occurred during fermentation. For the 5 L reactors, the working mass of the slurry was 3 kg. Starch loading was kept constant between whole-milled and debranned-milled experiments for direct comparison.

 For the liquefaction step of the warm process, 1.050 kg of whole-milled grains or 0.763 kg of debranned grains and water (for whole-milled experiments) or bran and germ hydrolysate (for debranned-milled experiments – see Table 4.1 and Figure 4.1) were added to the bioreactor. One hundred and fifty milligrams of calcium chloride were added and the pH was adjusted to 5.8. Termamyl SC enzyme was added to the slurry at a dosage of 165.25 µl/100 g grains (lowest optimum dosage as predicted by statistical models). Liquefaction was performed for 120 min for whole-milled and 170 min for debranned-milled experiments (lowest optimum times as predicted by statistical models). After liquefaction, the mash was cooled down to 30˚C before adding Saczyme enzyme at a dosage of 323 µl/100 g grains (lowest optimum dosage as predicted by statistical models) for both whole-milled and debranned, 0.07% urea and 15 mL of rehydrated yeast inoculum. Fermentations were carried out for 120 hours

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while samples for HPLC analysis were taken every 12 hours. The pH was adjusted to 5.8 every 12 hours with three molar potassium hydroxide solution .

For the pre-saccharification step of the cold process, 1.050 kg of whole-milled grains or 0.763 kg of debranned grains and water (for whole-milled experiments) or bran and germ hydrolysate (for debranned-milled experiments – see Table 4.1 and Figure 4.1) were added to the bioreactor. One hundred and fifty milligrams of calcium chloride were added and the pH was adjusted to 4.2. GC626 enzyme (14 µl/100 g of grains) was added to the slurry and pre-saccharification was performed for 162 min for whole-milled and debranned-milled experiments (lowest optimum times as predicted by statistical models). After pre-saccharification, the mash was cooled down to 30˚C before adding Stargen 002 enzyme cocktail at a dosage of 437 µl/100 g grains for whole-milled and 346.5 µl/100 g grains for debranned-milled experiments (lowest optimum dosage as predicted by statistical models), 0.07% urea and 15 mL of rehydrated yeast inoculum. Fermentations were carried out for 120 hours while samples for HPLC analysis were taken every 12 hours. The pH was adjusted to 4.2 every 12 hours with three molar potassium hydroxide solution.

Table 4.1: Masses for debranned flour, germ, bran and whole-milled flour used for small (250 mL), bench (5 L) and pilot (100 L) scale experiments.

	Debranned flour	Germ	Bran	Whole-milled flour
Mass used in **small** scale experiments (250 mL flasks)	25.44 g	2.20 g	7.30 g	35.00 g
Mass used in **bench** scale validation experiments (5 L bioreactors)	0.7632 kg	0.0660 kg	0.219 kg	1.050 kg
Mass used in **pilot** scale validation experiments (100 L bioreactors)	25.44 kg	2.20 kg	7.30 kg	35.00 kg

Further scale-up experiments were performed in 100 L jacketed bioreactors. The methods were similar to that performed in the 5 L bioreactors, except the working mass of the slurry would be 100 kg, which equates to 35 kg of whole-milled grains or 25.44 kg of debranned grains, 5 g of calcium chloride, 70 g of urea, lowest optimum dosage of warm or cold conversion enzymes and 500 mL of rehydrated yeast inoculum. Liquefaction and pre-saccharification steps were carried out at lowest optimum time as determined by the statistical models.

Fermentations were carried out for 120 hours while samples for HPLC analysis were taken every 12 hours. The pH was adjusted to pH 5.8 for the warm and pH 4.2 for the cold process every 12 hours with three molar potassium hydroxide or three molar sulfuric acid solution as needed.

For all debranned experiments, the corresponding ratio of bran and germ to the amount of debranned flour (see Table 4.1), was incubated with 15 µl/g grains of an endo-protease enzyme, Alkalase 2.5L, at 55°C at 200rpm for 24 hours. A pH of 8 was maintained throughout the incubation period by the addition of three molar potassium hydroxide solution as required. After 24 hours, the slurry was sieved to remove solid residues and the hydrolysate was used as the liquid fraction during debranned fermentation experiments .
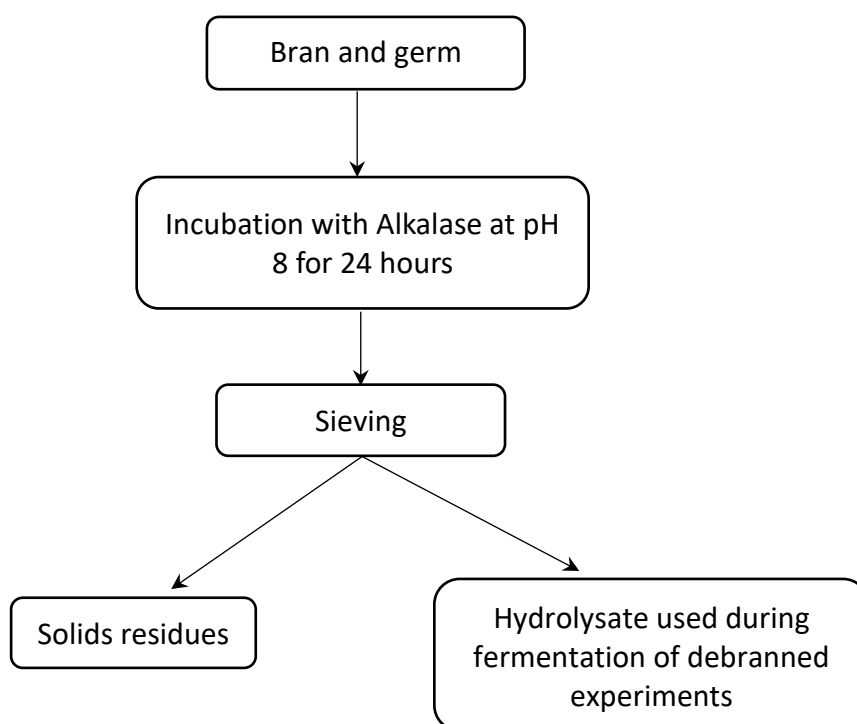
Figure 4.1: Incubation of bran and germ with Alkalase enzyme. Hydrolysate obtained after sieving was mixed with debranned-milled flour before fermentations for both warm and cold debranned experiments.

4.4 Workflow diagrams

Process configurations are demonstrated as workflow diagrams (Figures 4.2 and 4.3)
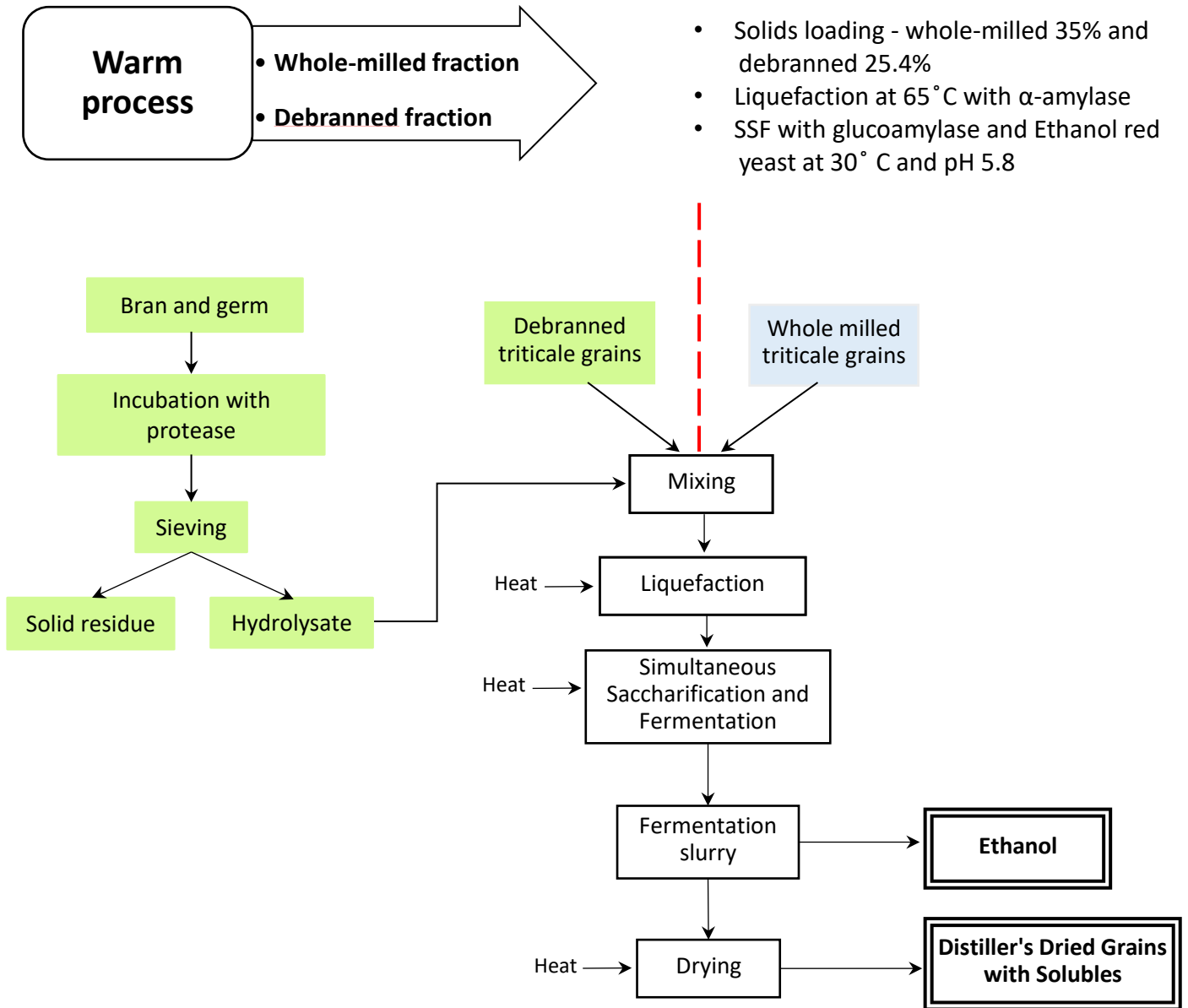
Figure 4.2: Conventional warm process using whole-milled or debranned triticale grains as feedstock for production of bioethanol and DDGS.
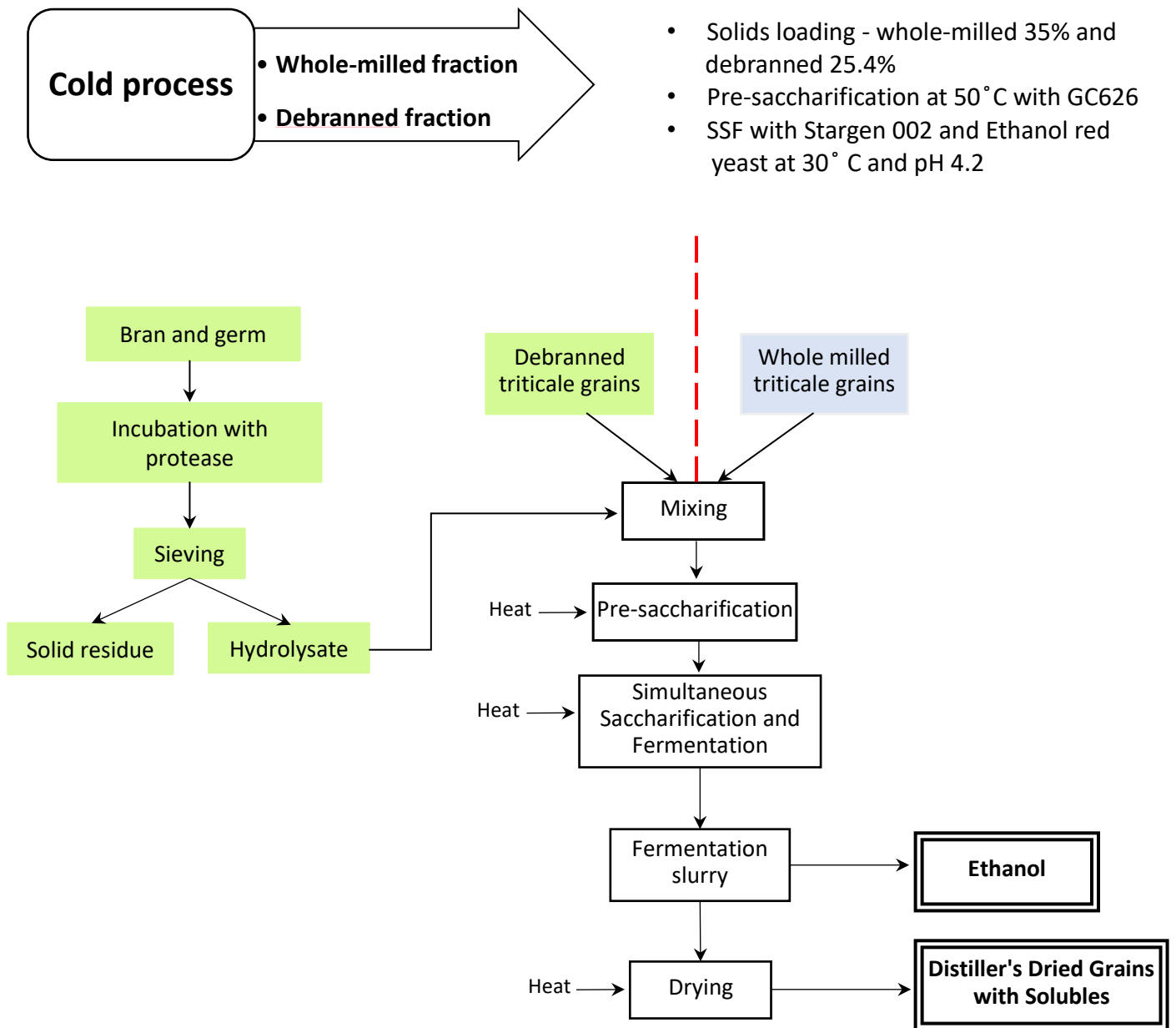
Figure 4.3: The cold conversion process using whole-milled and debranned triticale grains as feedstock for production of bioethanol and DDGS.

4.5 Statistical design and analysis of data

A central composite design (CCD) provides information on the individual and combined effects of independent (input) variables on specific response (output) variables of a process. By investigating prior studies done on ethanol conversion from starch grains, low, centre and high values were selected for each factor/independent variable (see Table 4.2 and 4.3) within the ranges of the manufactures' guidelines for the chosen enzymes (see Table 2.3). Centre points were done in triplicate and high and low points were done in duplicate. Using data obtained from these experimental runs, a response surface methodology was used to create surface plots to illustrate the effect of each process configuration on ethanol concentration, yield and productivity. The desirability plots predicted the optimum values for the independent variables to achieve ethanol concentrations, yields and productivities matching that currently achieved in industry. The statistical software used for analysis was Design Expert 7 from Stat-Ease Inc, USA. The time point at which the ethanol productivity was determined for each experiment, was taken as the time beyond which the ethanol concentration did not increase by more than 5%.

The following equations were used to calculate the response variables:

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

(1)

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

$$Ethanol\ productivity\ (g/L/h) = \frac{Ethanol\ concentration\ (\frac{g}{L})}{Fermentation\ time\ (hrs)} \tag{3}$$

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

The responses obtained from the set of experiments, that were carried out according to the CCD, are related to the independent variables examined, by the following empirical model:

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

Y represents the predicted response, being either ethanol yield, concentration or volumetric productivity, b_o is the constant coefficient, b_{ij} the interaction coefficient and b_{ii} is the quadratic coefficient.

Table 4.2: Factors and their levels used in the central composite design for the conventional warm conversion process.

Factors	Levels		
	Low value	Centre point	High value
Liquefaction time (min)	90	120	150
α-amylase dosage (μl/100g starch)	58	116	174
Glucosidase dosage (μl/100g starch)	114	192	270

Table 4.3: Factors and their levels used in the central composite design for the cold conversion process.

Factors	Levels		
	Low value	Centre point	High value
Pre-saccharification time (min)	60	120	180
Stargen 002 dosage (μl/100g starch)	128	256	384

4.6 Optimization and validation experiments

Statistical optimisation was done by fitting empirical models to experimentally obtained data to establish the lowest enzyme dosages and hydrolysis and fermentation times needed to obtain at least 90% of the theoretical maximum ethanol yield within 72-96 hours for all process configurations. Desirability plots were used to depict targeted responses, indicating the area of the design space where these responses are met. Aiming to achieve the lowest enzyme dosage (for both warm and cold processes) and least amount of time for the liquefaction (warm process) and pre-saccharification (cold process) steps, values will be chosen for the validation experiments. Validation experiments in 5 L bioreactors was performed in triplicate and 100 L scaled-up experiments was performed in duplicate.

4.7 Production of DDGS

After fermentation, the slurries from the 5 L and 100 L validation experiments were dried in a fan oven at 70˚C for 48 hours and then at 90˚ C for another 48 hours to simulate temperatures used during bioethanol distillation.

4.8 Analytical methods

The starch content of the triticale grains and DDGS was confirmed by utilising an enzymatic starch assay kit produced by Megazyme, Ireland, according to the AACC Method 76 – 13. The moisture content of the grains was determined by placing the sample in a convection oven at 105˚C and drying for 24 hours until a constant weight was reached. Samples collected from the fermentation slurries were centrifuged for 5 minutes at 11300 g, the supernatant was then diluted twenty times and filtered through a 0.22 µm pore size membrane from Anatech, SA, prior to HPLC HPLC (high performance liquid chromatography) analyses. Glucose and ethanol concentrations in the supernatant was established by HPLC fitted with a BioRad guard column and RI detector. The ash content for whole-milled, debranned-milled and all DDGS samples produced were determined by placing the sample in a furnace for six hours at 600˚C. The crude fat content was established by ether extraction according to the AACC approved methods 30 -25. Nitrogen content was analysed by the Dumas Method, using a LECO nitrogen

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instrument (TruSpec N produced in Michigan, USA). The crude protein content was calculated by multiplying the

nitrogen by a factor of 6.20, according to the AACC approve methods 46 – 30. The crude fibre content, including

NDF (neutral detergent fibre) and ADF (acid detergent fibre) were established using an ANKOM 200 Fibre

Analyser produce by Macedon, NY. Amino-acid profiling was done by mass spectrometry using the molecular

weight of each amino acid for determination.

5 Results

The results are presented in five sections. In section 5.1 the effect of the input variables (liquefaction time, α-amylase and glucoamylase dosage) on the response variables (ethanol concentration, percentage yield and productivity) of the warm process was evaluated, with a focus on differences between milled grains with and without prior debranning. The cold process with two input variables, pre-saccharification time and Stargen 002 dosage, are discussed in section 5.2. Regression models describing the responses were developed for all process configurations. Data for the first two sections were obtained from shake flasks experiments. Optimum conditions that were determined by the models developed from the shake flask experiments in sections 5.1 and 5.2 were validated in bench scale (5 L) and pilot scale (100 L) bioreactors. The performances from all process configurations (warm and cold using milled grains with and without debranning) were compared and the results are presented in section 5.3. Significance was determined by using student t-tests and analysis of variance. The effect of debranning and the effect of different processing methods (warm vs cold) on the quality of the DDGS are shown in section 5.4 and 5.5.

5.1 Effect of debranning on response variables during warm starch processing.

Table 5.1 shows the experimental treatment and values of response variables based on a central composite design for the warm process, using whole-milled and debranned-milled grains. For each experiment a sample was taken at the zero-hour time point and analysed for starch, glucose an ethanol. Starch concentrations were 18.39 ± 1.8 g/L starch for milled grain experiments and 17.88 ± 1.2 g/L for debranned grain experiments. Figure 5.1 depicts ethanol profiles of selected fermentation experiments. These experiments (Table 5.1 exp no. 4 and 5) were chosen to best illustrate the effect of an increased glucoamylase dosage on fermentation performance when α-amylase dosages and liquefaction times are kept the same. When the glucoamylase dosage was increased from 114 µl/100g_{starch} to 270 µl/100g_{starch} for whole-milled grains, the final ethanol concentrations increased significantly ($p < 0.05$) from 117 g/L to 136 g/L as shown in Table 5.1 (exp no. 4 and 5 respectively). For these experiments the liquefaction time was 90 minutes and the α-amylase dosage 174 µl/100g_{starch}. The maximum

ethanol concentrations for both cases were reached at 72 hours, indicating a higher ethanol volumetric productivity at an increased glucoamylase dosage. For the debranned grains (shown in Figure 5.1), the final ethanol concentration also increased significantly ($p < 0.05$) from 134.54 g/L to 141.63 g/L when the glucoamylase dosage was increased. The maximum ethanol concentration in both debranned cases were reached at 62 hours, indicating that an increase in glucoamylase dosage also resulted in a higher ethanol volumetric productivity of debranned grains. The effect of changing the glucoamylase dosage on the ethanol concentration, was more pronounced in the milled grain (19.1 g/L) vs debranned grain fermentations (7.1 g/L).

Ethanol yields (as a percentage of the theoretical maximum) as illustrated in Figure 5.2, followed similar trends as the ethanol concentrations. When using milled grains at a high glucoamylase dosage, the ethanol yield was significantly higher ($p < 0.05$) at 90.6% than when using a low dosage yielding 77.6%. The debranned fermentations had an ethanol yield of 93.4% at a high glucoamylase dosage vs a lower ($p < 0.05$) yield of 88.7 % at the lower dose. Similar to the ethanol concentrations, the differences in ethanol yields were larger for the milled grains (13.0%) than for debranned grains (4.7%) at high and low glucoamylase dosages.

Table 5.1: Experimental conditions used and values of response variables achieved from experiments carried out based on the central composite design for the warm process, using whole-milled (**M**) and debranned (**D**) grains.

Experiment number	α-amylase dosage (µl/100 g starch)	Glucoamylase dosage (µl/100 g starch)	Liquefaction Time (min)	Ethanol concentration (g/L)		Ethanol yield (% of theoretical max)		Ethanol productivity (g/L/h)	
				M	D	M	D	M	D
1	116	192	70	130.70	129.73	87.5	86.1	1.82	2.09
2	58	114	90	127.58	134.28	85.3	87.9	1.77	2.16
3	58	270	90	131.34	135.34	87.1	89.2	1.83	2.18
4	174	114	90	116.96	134.54	77.6	88.7	1.63	2.17
5	174	270	90	136.03	141.63	90.6	93.4	1.89*	2.29
6	19	192	120	126.97	128.03	84.4	83.8	1.17	2.07
7	116	61	120	118.80	133.72	79.8	88.8	1.48	2.03
8	116	323	120	138.30	148.97*	92.5	97.6*	1.65	2.40
9	116	192	120	131.75	139.98	88.5	91.7	1.83	2.81
10	116	192	120	134.57	143.21	90.0	93.8	1.87	2.30
11	116	192	120	127.52	140.52	85.3	91.4	1.52	2.26
12	116	192	120	132.65	145.46	88.6	94.6	1.58	2.33
13	116	192	120	134.35	140.31	89.8	91.9	1.60	2.81
14	116	192	120	127.82	146.55	86.1	95.3	1.78	2.36
15	214	192	120	134.79	145.24	91.5	95.8	1.61	2.34
16	58	114	150	127.98	143.65	87.0	95.4	1.33	2.87
17	58	270	150	140.35	144.02	93.9	95.0	1.46	2.32
18	174	270	150	142.21*	145.02	94.4*	95.6	1.69	2.90*
19	174	114	150	134.17	135.11	89.5	89.7	1.59	2.70
20	116	192	170	135.51	144.88	90.1	95.5	1.41	2.77

*Highest values for ethanol concentration, ethanol yield and ethanol productivity for whole-milled and debranned experiments are indicated with an asterisks (*).
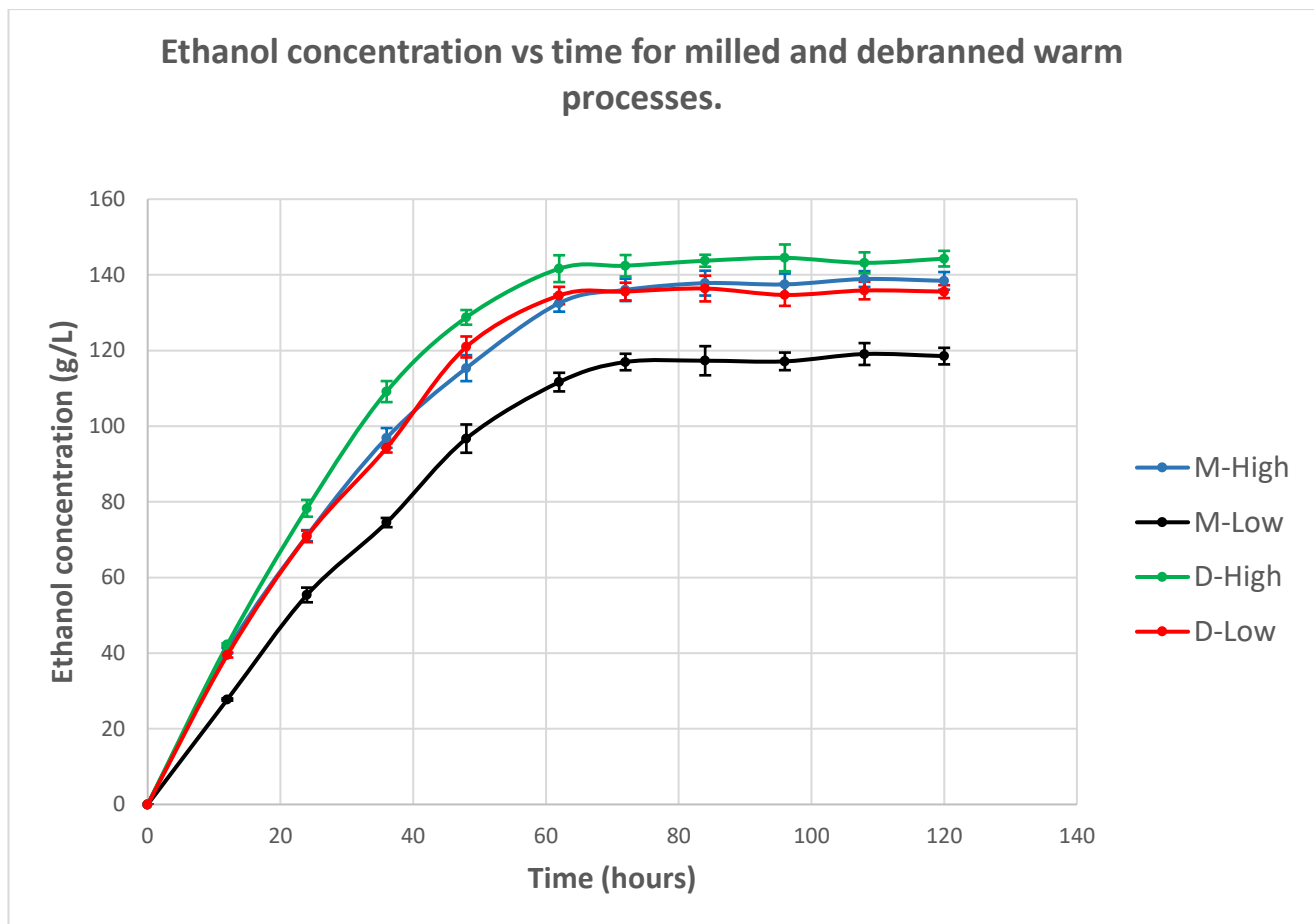
Figure 5.1: Ethanol profiles of fermentations carried out using milled (M) and debranned (D) grains at low glucoamylase dosage of 114 µl/100g$_{starch}$ and a high dosage of 270 µl/100g$_{starch}$. For these experiments the liquefaction time was 90 minutes and the α-amylase dosage were 174 µl/100g$_{starch}$. Error bars represent standard deviation of duplicate experiments.
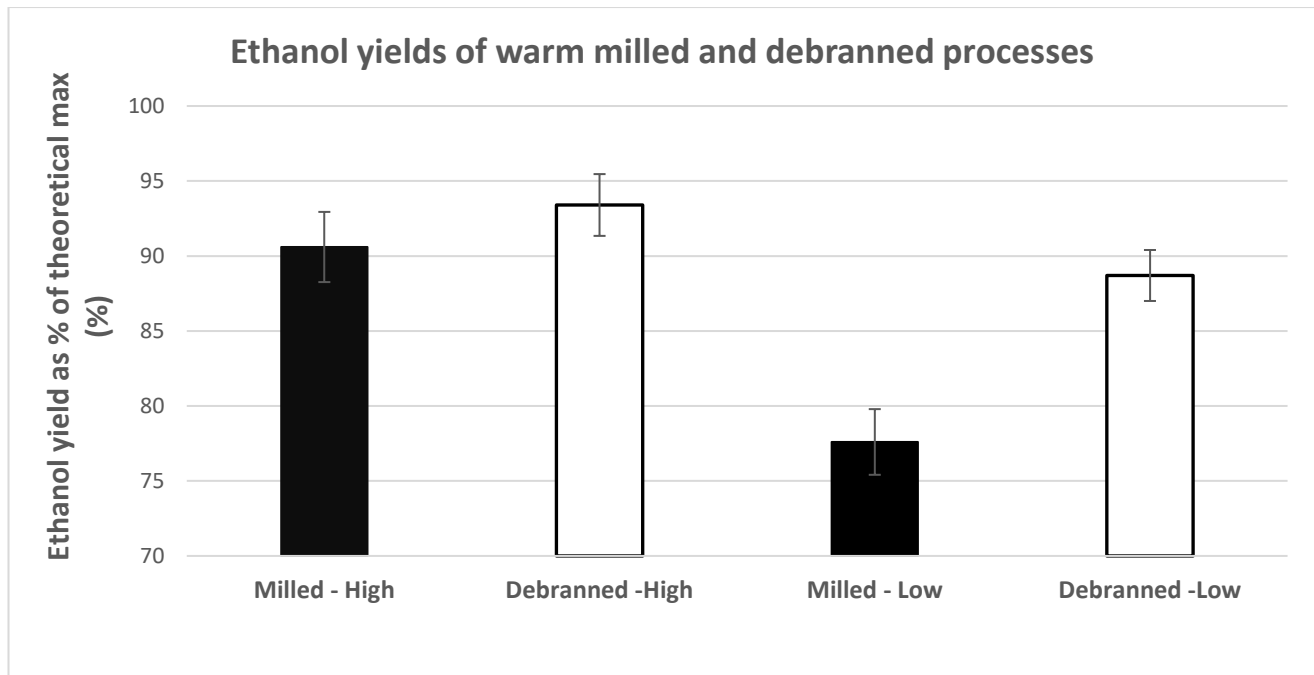
Figure 5.2: Ethanol yields (as a % of the theoretical maximum) of experiments performed using milled and debranned grains at a **low** glucoamylase dosage of 114 µl/100g$_{starch}$ and a **high** dosage of 270 µl/100g$_{starch}$. The liquefaction time was 90 minutes and the α-amylase dosage were 174 µl/100g$_{starch}$. Error bars represent standard deviation of duplicate experiments.

5.2 Mathematical modelling of response variables as functions of the input variables for the conventional warm process.

Mathematical models describe a system by a set of variables. The data obtained from the fermentation experiments were used to develop mathematical regression equations which could be used to predict ethanol concentration, ethanol yield and ethanol productivity. The equations that related the input variables to the response variables are shown in Equation 5, 6 and 7 for the whole-milled grains and Equation 8, 9 and 10 for debranned grains:

Ethanol concentration = 130.8537 - 0.1645x_a + 0.0995x_b - 0.2246x_c - 0.0001x_b^2 - 0.0010x_c^2 　　　　　　(5)

Ethanol yield (as a percentage of the theoretical maximum) = 81.87475 − 0.10359x_a + 0.06968x_b − 0.06423x_c

+ 0.00003x_a^2 − 0.0009x_b^2 + 0.00044x_c^2 　　　　　　(6)

Ethanol productivity = $2.029097 + 0.001133x_a + 0.001932x_b - 0.008219x_c - 0.000024x_a^2 - 0.000003x_b^2$

$+ 0.000001x_c^2$ (7)

Ethanol concentration = $78.53191 + 0.20510x_a - 0.00693x_b + 0.66413x_c - 0.00056x_a^2 - 0.00004x_b^2 - 0.00188x_c^2$ (8)

Ethanol yield (as a percentage of the theoretical maximum) = $57.44015 + 0.13072x_a - 0.01475x_b + 0.34796x_c$

$- 0.0003x_a^2 + 0.0003x_b^2 - 0.00075x_c^2$ (9)

Ethanol productivity = $1.309776 - 0.001670x_a + 0.004316x_b + 0.004572x_c - 0.000018x_a^2 - 0.000009x_b^2$

$+ 0.000024x_c^2$ (10)

Where x_a is the α-amylase dosage, x_b is the glucoamylase dosage and x_c is the liquefaction time.

To simultaneously study the effect of several process variables (α-amylase dosage, glucoamylase dosage and liquefaction time) on the process, a response surface methodology was used to model the response variables (final ethanol concentration, ethanol yield and ethanol productivity). An analysis of variance (appendix A) was used to choose the models that best fit the relationship between the input and response variables. The models that were chosen were all significant with a p value < 0.05. The R^2 values for final ethanol concentration, ethanol yield and ethanol productivity when using whole-milled grains were 0.82, 0.78 and 0.72 respectively. For the debranned grains R^2 values for final ethanol concentration, ethanol yield and ethanol productivity were 0.75, 0.75 and 0.65 respectively. These R^2 values indicate that the models were well fitted to the experimental data which implied relatively low unexplained error and hence, high reproducibility.

The analysis of variance (Appendix A) of the treatment effects when using whole-milled grains indicated that the glucoamylase dosage and liquefaction time had a significant ($p < 0.05$) effect on ethanol concentration and yield, where glucoamylase dosage had the most significant main effect as evident from the lowest P-value. On the other hand, only liquefaction time had a significant effect on productivity ($p < 0.05$). For the debranned grains, the analysis of variance indicated that all three input variables had a significant effect ($p < 0.05$) on the ethanol concentration and yield, with liquefaction time having the most pronounced effect. Similar to what was observed

for the ethanol productivity of the milled grains, liquefaction time was the only variable that had a significant effect on the ethanol productivity of the debranned grains.

Response surface plots of the quadratic models developed from the central composite design were created to highlight the effect that the input (independent) variables have on the response variables. Ethanol concentration was closely correlated to ethanol yield. Therefore, only surface plots for ethanol yield (A,B) and ethanol productivity (C,D) are shown. The response surface plot shown in Figure 5.3 A revealed a distinct positive relationship between both treatments and ethanol yield, which implied that lower glucoamylase dosages are required at longer liquefaction times to reach an ethanol yield of 95% of the theoretical maximum, or above. At a liquefaction time of 120 minutes, an ethanol yield of 95% can be achieved with a glucoamylase dosage of 270 µl/100g$_{starch}$. For the debranned grains the response surface plot for ethanol yield (Figure 5.3 B) indicated that a liquefaction time of above 120 minutes and a glucoamylase dosage of above 200 µl/100g$_{starch}$ are required to achieve a maximum ethanol yield of 95%. An optimum can be seen at 140 minutes liquefaction time, where 210 µl/100g$_{starch}$ is required to achieve an ethanol yield of 95% and above. The response surface plot of ethanol productivity for the whole-milled grains (Figure 5.3 C) showed that productivity increase as liquefaction time decrease and glucoamylase dosage increase. At a glucoamylase dosage of 270 µl/100g$_{starch}$ a liquefaction time of 120 min is required to achieve an ethanol productivity of 1.8 and above. For the debranned grains, the response surface plot for ethanol productivity (Figure 5.3 D) demonstrated an increase in productivity as both liquefaction time and glucoamylase dosage increased. An optimum productivity of 2.5 and above can be seen at a glucoamylase dosage of 200 µl/100g$_{starch}$ and a liquefaction time of 120 minutes.
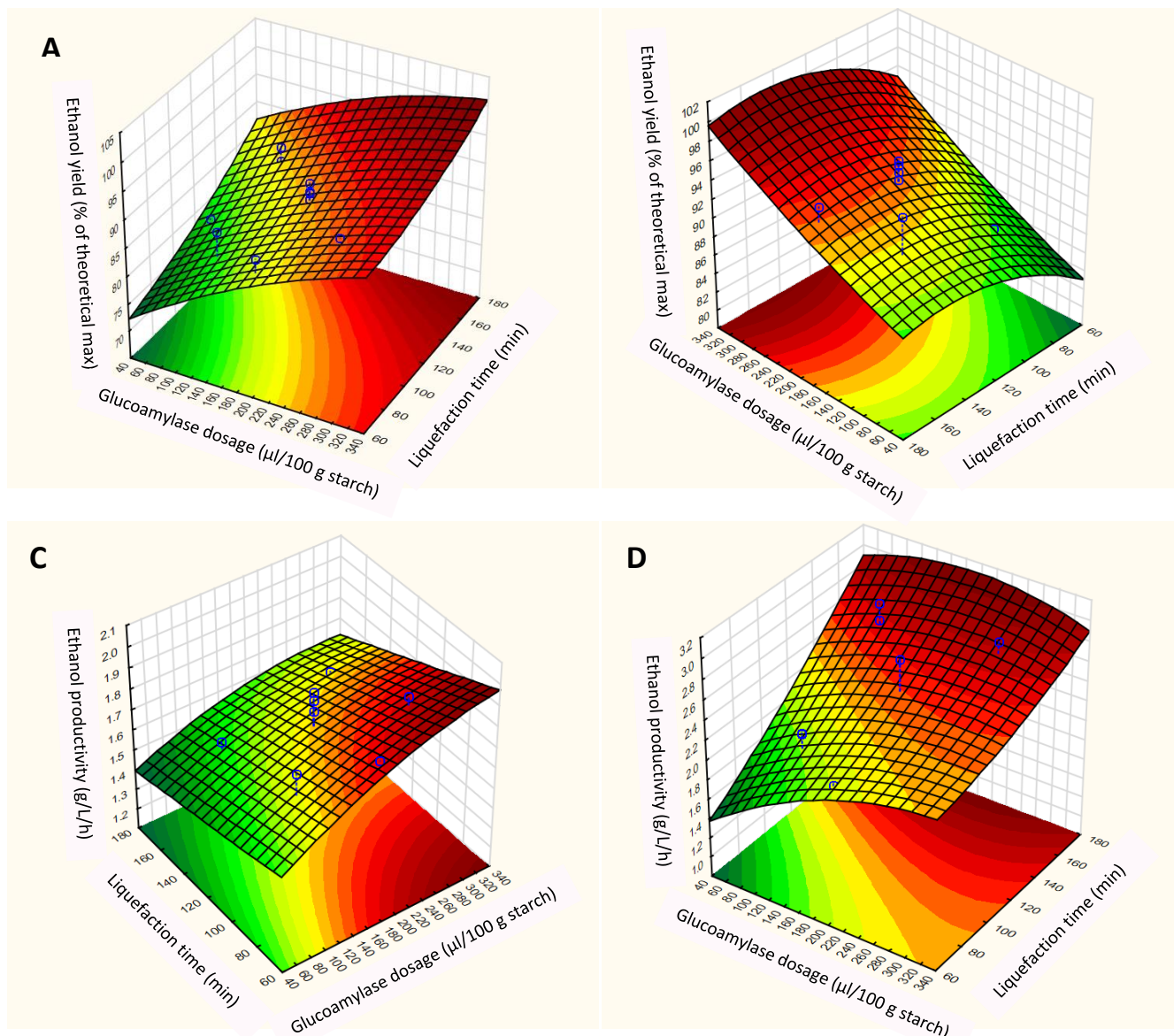
Figure 5.3: Response surface plots for conventional warm process using whole milled and debranned grains, α-amylase dosage 165 µl/100g$_{starch}$ (A-D). **A:** Ethanol yield as a percentage of theoretical maximum for **whole-milled** grains. **B:** Ethanol yield as a percentage of theoretical maximum for **debranned grains**. **C:** Ethanol productivity for **whole-milled grains**. **D:** Ethanol productivity for **debranned grains**. Axes were chosen to best represent the effect.

5.3 Effect of debranning on response variables when using the cold processing method

The experimental conditions used and values obtained for the response variables, when using cold processing for whole-milled and debranned grains, are shown in Table 5.2. For each experiment a sample was taken at the zero-hour time point and analysed for starch, glucose an ethanol. Starch concentrations were 18.12 ± 2.0 g/L for milled grain experiments and 17.53 ± 1.6 g/L for debranned grain experiments. The ethanol profiles of chosen fermentation experiments (exp no. 2 and 3) for both milled (M) and debranned (D) are shown in Figure 5.4. These experiments were chosen to best illustrate the effect of an increased Stargen dosage on fermentation performance when pre-saccharification times are kept constant. Low and high Stargen doses with a pre-saccharification time of 60 minutes were used for these experiments. For both milled and debranned grains a higher ethanol concentration ($p<0.05$) was achieved at a high Stargen dosage of 384 µl/100g$_{starch}$ (135.09 g/L and 137.89 g/L respectively) than at a low Stargen dosage of 128 µl/100g$_{starch}$ (116.56 g/L and 121.98 g/L respectively). Using debranned grains resulted in a more rapid conversion, decreasing fermentation from 84 to 70 hours. Figure 5.5 shows the ethanol yields (as a percentage of the theoretical maximum) of the fermentations depicted in Figure 5.4. Significantly higher ($p<0.05$) ethanol yields were achieved for milled grains when using a high Stargen dosage (86.6%) than when using a low dosage (76.3%). The same was observed for the debranned grains at an ethanol yield of 91.6% when using a high Stargen dose vs a yield of 82.1% when using a low dose.

For fermentations with milled grains, increasing the Stargen dosage from low to high, decreased the required time to reach maximum ethanol concentrations from 120 to 84 hours (Figure 5.4). As a result, the ethanol productivity was significantly lower ($p<0.05$) at a low Stargen dosage (0.97 g/L/h) than at a high dosage (1.82 g/L/h). Similar variations in ethanol productivity was seen with debranned grains, with a maximum ethanol concentration achieved at 70 hours for the high Stargen dose and at 96 hours for the low dose, corresponding to significantly different ($p<0.05$) volumetric ethanol productivities of 2.30 g/L/h and 1.27 g/L/h respectively.

Table 5.2: Experimental conditions used and values of response variables achieved from experiments carried out based on the central composite design for the cold process, using whole-milled (**M**) and debranned (**D**) grains.

Experiment number	Pre-saccharification time (min)	Stargen dosage (µl/100 g starch)	Ethanol concentration (g/L)		Ethanol yield (% of theoretical max)		Ethanol productivity (g/L/h)	
			M	**D**	**M**	**D**	**M**	**D**
1	35	256	129.33	129.17	85.9	85.2	1.37	1.61
2	60	128	116.56	121.98	76.3	82.1	0.97	1.27
3	60	384	135.09	137.89	86.6	91.6	1.6	1.97
4	120	75	105.46	113.11	68.8	76.1	0.85	1.18
5	120	256	129.46	135.83	85.4	91.4	1.71	1.89
6	120	256	132.65	138.32	87.5	91.2	1.72	1.92
7	120	256	127.87	133.04	84.3	87.7	1.67	2.22
8	120	256	127.02	140.15	83.8	92.4	1.67	1.95
9	120	256	128.48	140.73	84.7	92.8	1.67	1.95
10	120	437	136.21	142.65*	90.4	94.7*	1.92*	2.38*
11	180	128	113.69	130.45	75.5	86.6	0.93	1.09
12	180	384	138.46*	138.89	91.3*	92.2	1.87	2.31
13	205	256	121.83	139.50	80.9	92.6	1.38	2.32

*Highest values for ethanol concentration, ethanol yield and ethanol productivity for whole-milled and debranned experiments are indicated with an asterisks (*).
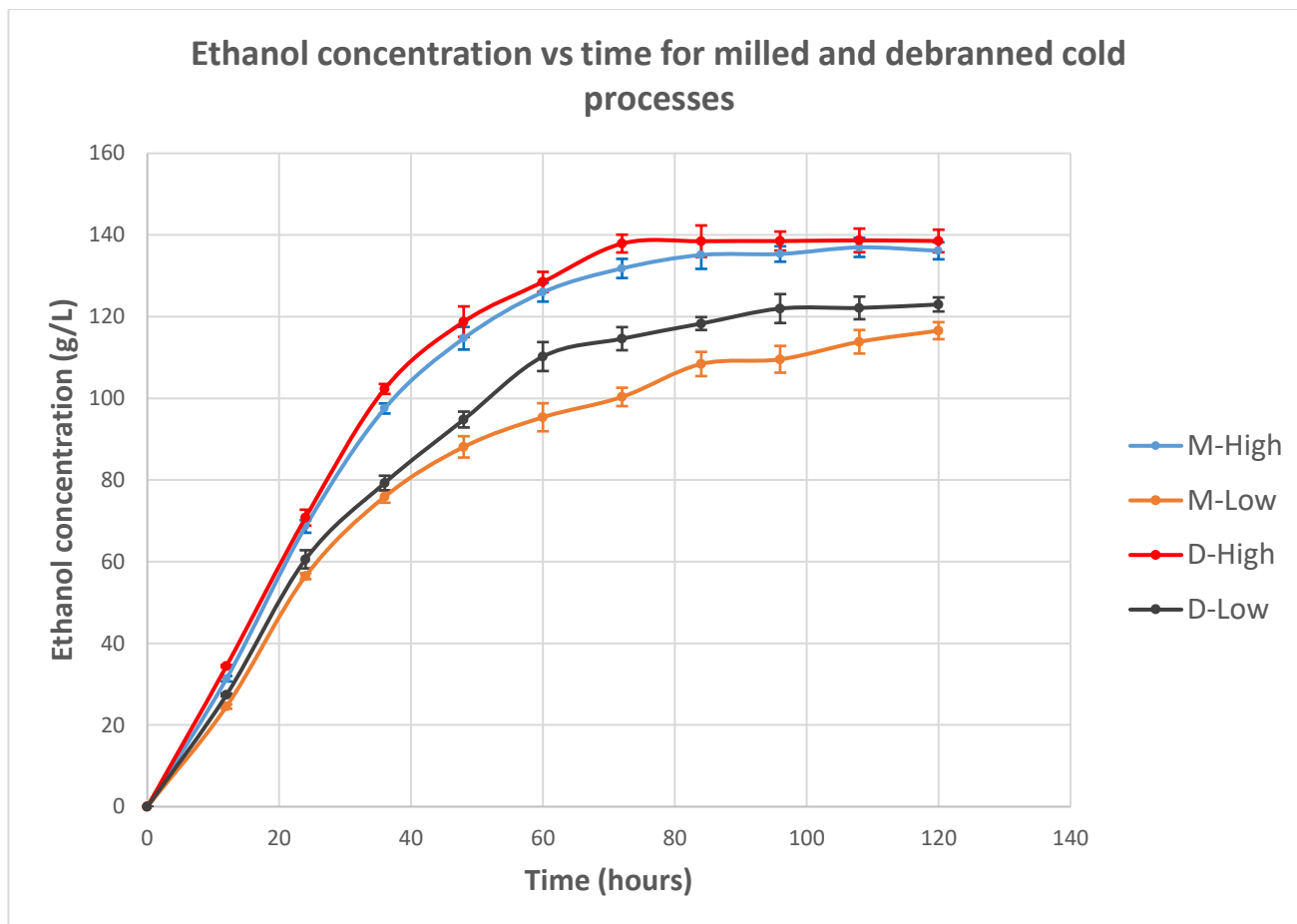
Figure 5.4: Ethanol profiles of fermentations carried out using milled (M) and debranned (D) grains at low Stargen dosage of 128 µl/100g$_{starch}$ and a high Stargen dosage of 384 µl/100g$_{starch}$. For these experiments the pre-saccharification time was 60 minutes. Error bars represent standard deviation of duplicate experiments.
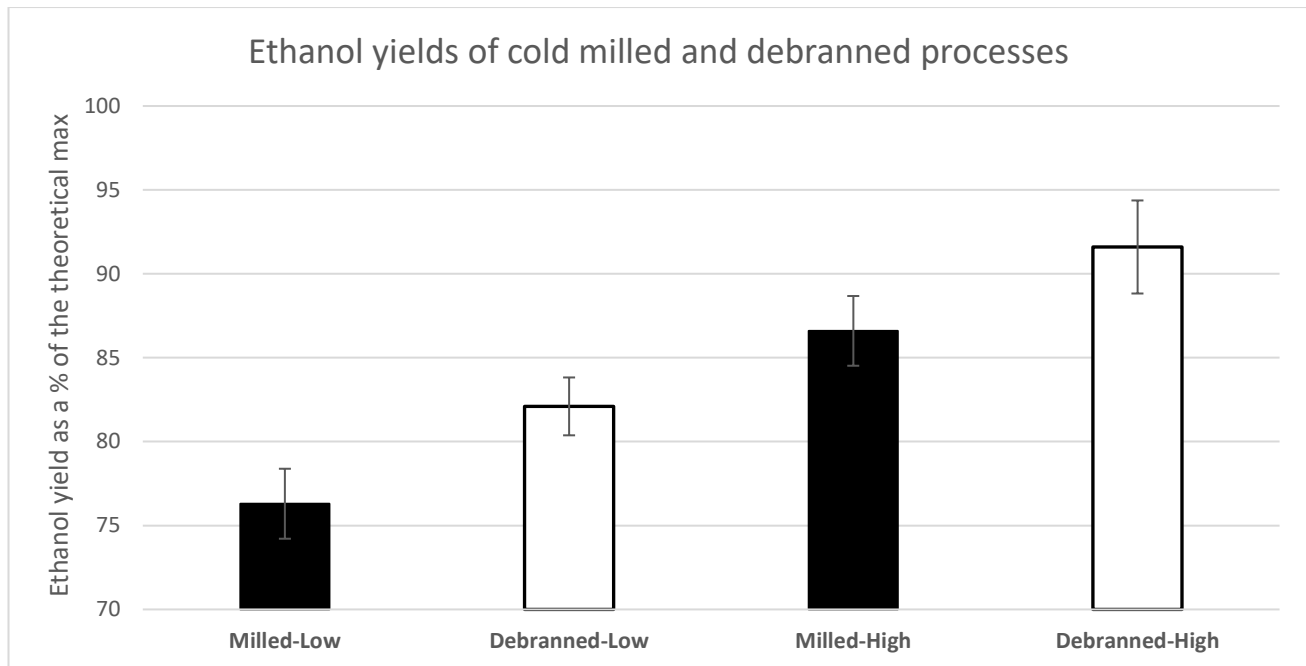
Figure 5.5: Ethanol yields (as a % of the theoretical maximum) of experiments performed using milled and debranned grains at a **low** Stargen dosage of 128 µl/100g$_{starch}$ and a **high** dosage of 384 µl/100g$_{starch}$. The pre-saccharification time was 60 minutes. Error bars represent standard deviation of duplicate experiments.

5.4 Mathematical modelling of response variables as functions of the input variables for the cold process.

Appendix B shows the analysis of variance performed on the models that were created to predict the effect of the input variables on the responses. R^2 values of the chosen models ($p<0.05$) for ethanol concentration, ethanol yield (as a percentage of theoretical maximum) and ethanol productivity were 0.95, 0.94 and 0.99 for the milled grains and 0.90, 0.89 and 0.89 for the debranned grains respectively. The relationships between each response variable and the input variables for the whole-milled grains are illustrated in Equations 11, 12 and 13 and for the debranned grains in Equations 14, 15 and 16:

Ethanol concentration = $98.08350 - 0.00151x_a + 0.16810x_b - 0.0003x_a^2 - 0.00021x_b^2$ (11)

Ethanol yield (as a percentage of the theoretical maximum) = $64.66816 - 0.00986x_a + 0.11229x_b - 0.00018x_a^2 - 0.00015x_b^2$ (12)

Ethanol productivity = $-0.131548 + 0.008836x_a + 0.006825x_b - 0.000046x_a^2 - 0.00001x_b^2$ (13)

Ethanol concentration = $83.94705 + 0.20185x_a + 0.23657x_b - 0.00037x_a^2 - 0.00028x_b^2$ (14)

Ethanol yield (as a percentage of the theoretical maximum) = $59.10166 + 0.1227x_a + 0.13691x_b - 0.00024x_a^2 - 0.00016x_b^2$ (15)

Ethanol productivity = $0.444234 + 0.002093x_a + 0.006381x_b - 0.000017x_a^2 - 0.000010x_b^2$ (16)

Where x_a is the Stargen dosage and x_b is the pre-saccharification time.

The analysis of variance (Appendix B) for the whole-milled grains when using the cold process indicated that Stargen dosage was the only variable that had a significant effect ($p<0.05$) on the ethanol concentration and ethanol yield. For the ethanol productivity on the other hand, both Stargen dosage and pre-saccharification time was significant ($p<0.05$), with Stargen dosage having the most significant impact. For the debranned grains, the same was observed in terms of ethanol concentration and yield, with Stargen dosage having a significant effect

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(p<0.05). Both Stargen dosage and pre-saccharification time showed a significant effect on the ethanol productivity of the debranned grains.

For reasons mentioned prior, only surface response plots for ethanol yield and ethanol productivity are shown. For whole-milled grains the response surface plot for ethanol yield (Figure 5.6 A) indicated that for a higher Stargen dosage, a wider range of pre-saccharification times (120 to 180 minutes) can be utilized to obtain 90% ethanol yield (% of the theoretical maximum). An optimum ethanol yield of above 90% can be seen at a Stargen dose of 450 µl/100g$_{starch}$ and 180 minutes pre-saccharification time. The response surface plot of ethanol yield for the debranned grains (Figure 5.6 B) showed a clear optimum of above 90% at 300 µl/100g$_{starch}$ Stargen dosage and 140 minutes pre-saccharification time. Thus, the Stargen dosage required to achieve above 90% ethanol yield is significantly less for debranned grains than for whole-milled grains when using the cold processing method. This corresponds to results obtained by Nkomba, (2015) where the decortication of sorghum grains (vs whole-milled grains) resulted in an 11.7% reduction of required enzyme dosage to achieve the same ethanol yield (% of theoretical maximum). The response surface plot of ethanol productivity for the milled grains (Figure 5.6 C) indicated a positive relationship between productivity, Stargen dosage and pre-saccharification time. reaching an optimum productivity of above 1.8 at a Stargen dose of 400 µl/100g$_{starch}$. For the debranned grains (Figure 5.6 D), an ethanol productivity of above 2.2 can be achieved at a Stargen dosage of 300 µl/100g$_{starch}$.
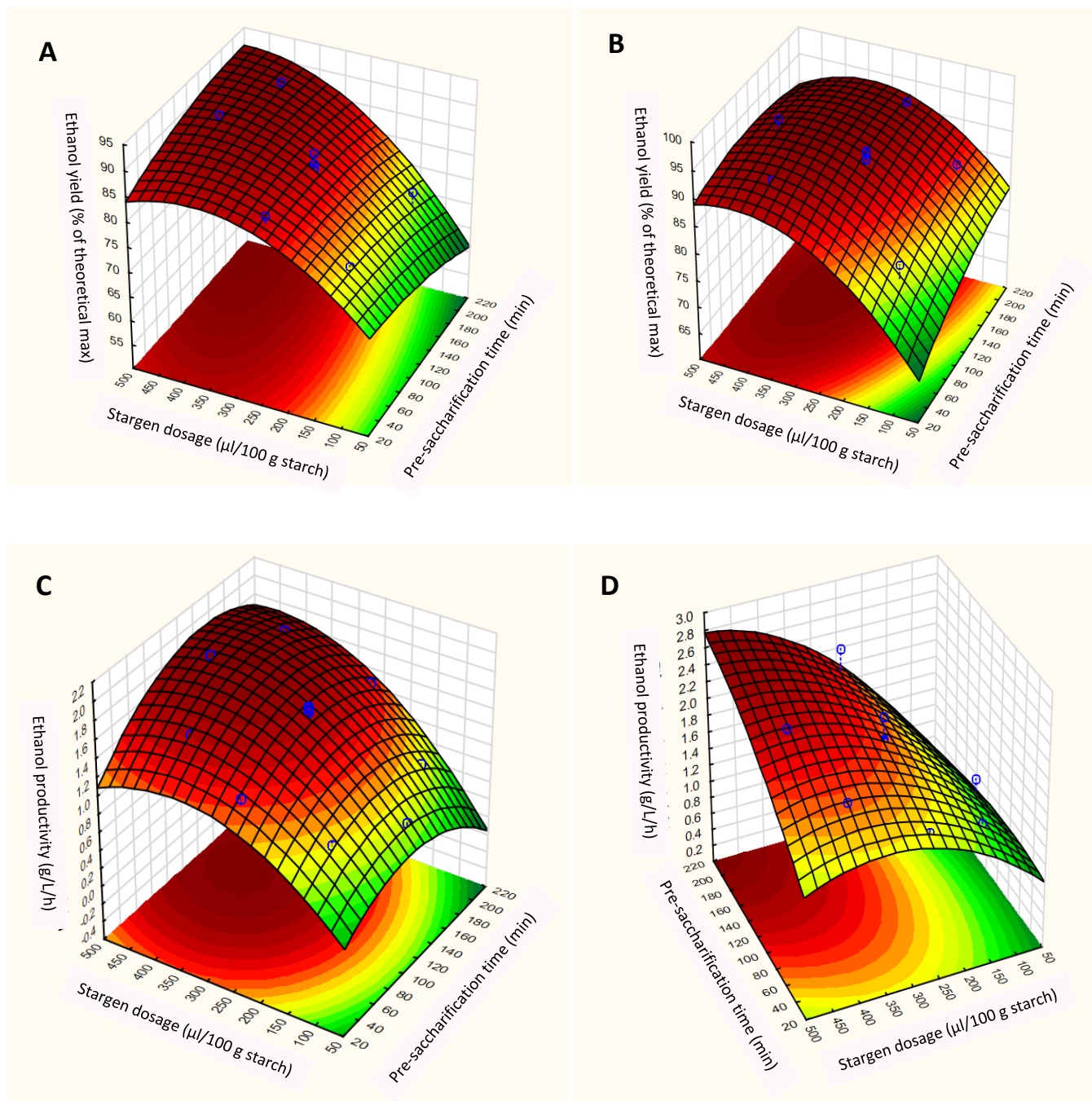
Figure 5.6: Response surface plots for cold process using whole milled and debranned grains (A-D). **A:** Ethanol yield as a percentage of theoretical maximum for whole-milled grains. **B:** Ethanol yield as a percentage of theoretical maximum for debranned grains. **C:** Ethanol productivity for whole-milled grains. **D:** Ethanol productivity for debranned grains. Axes were chosen to best represent the effect.

5.5 Optimization and validation experiments

Desirability plots (shown in Appendix C), for each process configuration, constructed from the statistical models, shows the optimized (to minimize enzyme dosage and maximise fermentation performance) values for the input variables and the predicted values for ethanol concentration, yield and productivity. Table 5.3 (warm) and 5.4 (cold) summarises the optimised values for all input variables and the predicted values for the response variables for all process configurations. Fermentation experiments to validate the predicted outputs from the statistical models were carried out (in duplicate) in both 5 L and 100 L fermenters. The results obtained from these validation experiments are also shown in Table 5.3 (warm) and 5.4 (cold).

Validation results for each process configuration did not differ significantly and followed similar trends between 5 L and 100 L fermentations, therefore only 100 L validation experiments will be discussed in detail. For the conventional warm process, ethanol concentrations of 139.74 and 147.73 g/L were recorded for whole-milled and debranned grains respectively. The cold process achieved 133.11 g/L and 140.65 g/L for the milled grains and debranned grains respectively. These results obtained in bioreactors closely match the prediction from the statistical model. The ethanol productivities achieved by all validation experiments were lower than predicted by the model, yielding 1.7 and 1.4 g/L/h for the milled grains and 2.5 and 2.2 g/L/h for the debranned grains when using warm and cold processing respectively.

Table 5.3: Predicted values for input and response variables used in validation experiments (obtained from desirability plots shown in Appendix C) and results obtained in 5 L and 100 L validation experiments for the warm conversion process.

Warm process								
PREDICTED			**OBSERVED**					
				5L Validation experiments		**100L Validation experiments**		
	Whole-milled grains	Debranned grains		Whole-milled grains	Debranned grains	Whole-milled grains	Debranned grains	
α-amylase dosage (µl/100 g starch)	165.25	165.25	α-amylase dosage (µl/100 g starch)	165.25	165.25	165.25	165.25	
Glucoamylase dosage (µl/100 g starch)	323	323	Glucoamylase dosage (µl/100 g starch)	323	323	323	323	
Liquefaction time (min)	120	170	Liquefaction time (min)	120	170	120	170	
Ethanol concentration (g/L)	142.21	148.79	Ethanol concentration (g/L)	141.85	146.94	139.74	147.73	
Ethanol yield (% of theoretical max)	94.4	97.6	Ethanol yield (% of theoretical max)	92.9	94.2	90.3	93.8	
Ethanol productivity (g/L/h)	1.9	2.9	Ethanol productivity (g/L/h)	1.8	2.4	1.7	2.5	
			Starch hydrolysed (%)	98.9	99	98.6	99.1	
			Ethanol yield on glucose (%)	93.9	95.2	91.6	94.6	
			Residual solids (%)	9	6.5	9.1	6.5	

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Table 5.4: Predicted values for input and response variables used in validation experiments (obtained from desirability plots shown in Appendix C) and results obtained in 5 L and 100 L validation experiments for the cold conversion process.

Cold process								
PREDICTED			OBSERVED					
					5L Validation experiments		100L Validation experiments	
	Whole-milled grains	Debranned grains		Whole-milled grains	Debranned grains		Whole-milled grains	Debranned grains
Stargen dosage (µl/100 g starch)	437	346.5	Stargen dosage (µl/100 g starch)	437	346.5		437	346.5
Pre-saccharification time (min)	162	162	Pre-saccharification time (min)	162	162		162	162
Ethanol concentration (g/L)	138.46	142.65	Ethanol concentration (g/L)	136.52	142.27		133.11	140.65
Ethanol yield (% of theoretical max)	91.3	94.7	Ethanol yield (% of theoretical max)	87.1	92.1		84.4	90.9
Ethanol productivity (g/L/h)	1.9	2.4	Ethanol productivity (g/L/h)	1.5	2.1		1.4	2.2
			Starch hydrolysed (%)	97.6	98.9		97.3	98.8
			Ethanol yield on glucose (%)	89.3	93.1		86.7	92
			Residual solids (%)	9.1	6.6		9.3	6.7

5.6 The effect of debranning on the chemical composition of DDGS

Table 5.5 shows the nutrient composition of the DDGS obtained from the different process configurations. Again, because of similar values and trends between 5 L and 100 L validation experiments, only 100 L experiments is discussed in detail. Resultant crude protein, ash, ADF and NDF % were significantly ($p<0.05$) affected by the debranning process. The crude protein increased from 31.41% for whole-milled grains to 44.05% for debranned grains in DDGS from the cold process and from 30.66% for whole-milled grains to 37.24% for debranned grains in DDGS from the warm process. Debranning also significantly affected ($p<0.05$) crude fibre content of DDGS, decreasing it from 6.23% to 2.57% for the warm process and from 6.73% to 3.08% for the cold process (Table 5.5). ADF and NDF contents were both significantly decreased (Table 5.5 and 5.6) by the debranning process

Table 5.6 illustrates the starch, protein, ADF and NDF weight percentages for the bran and germ fractions before and after incubation with Alkalase enzyme. Hydrolysate obtained after sieving, to remove solid residues, wil be used as the water fraction for cebranned experiments. Alkalase was effective in removing 86% of starch and 63% of protein of the initial combined amounts of the bran and germ fraction and concentrating it in the hydrolysate after incubation. Eighty five percent of ADF and 95% of NDF of the initial amount was concentrated in the solid residues that were removed during sieving. Table 5.6 indicates that the incubation of the bran and germ fraction with Alkalase had an overall positive effect on DDGS quality, in terms of recovering protein and starch and removing ADF and NDF from the initial bran and germ fractions and carrying it through to the resultant DDGS.

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Table 5.5: Chemical composition of DDGS produced from 5 L and 100 L validation experiment for all process configurations.

			Crude protein %	Crude fat %	Ash %	Crude fibre %	ADF %	NDF %
5L Validation experiments	**Warm process**	Whole-milled grains	30.28	4.14	7.28	6.99	17.85	43.1
		Debranned grains	35.21	4.83	10.88	3.32	12.28	17.46
	Cold Process	Whole-milled grains	29.05	4.95	5.42	7.54	16.21	34.79
		Debranned grains	42.6	4.59	13.16	2.46	5.53	12.81
100L Validation experiments	**Warm process**	Whole-milled grains	30.66	5.02	7.46	6.23	25.58	43.75
		Debranned grains	37.24	5.63	10.82	2.57	7.88	10.21
	Cold Process	Whole-milled grains	31.41	5.98	6.83	6.73	19.69	31.31
		Debranned grains	44.05	5.02	9.13	3.08	13.85	17.82

Table 5.6: Starch, protein, ADF and NDF weight percentages of combined bran and germ before and after incubation with Alkalase enzyme. Hydrolysate obtained after alkalse incubation and sieving to remove solid bran and germ residues, are used as the water fraction during warm and cold debranned validation experiments.

		Starch (wt %)			Crude protein (wt %)		
		Bran and germ before hydrolysis	Hydrolysate used in fermentation	Bran and germ after hydrolysis	Bran and germ before hydrolysis	Hydrolysate used in fermentation	Bran and germ after hydrolysis
Cold conversion process	5L validation experiments	23.95	20.55	2.98	14.26	9.02	4.97
	100L validation experiments	23.95	20.61	3.18	14.26	8.85	5.23
Warm conversion process	5L validation experiments	23.95	21.06	2.84	14.26	9.14	4.92
	100L validation experiments	23.95	20.52	3.02	14.26	8.91	5.14
		ADF (wt %)			NDF (wt %)		
		Bran and germ before hydrolysis	Hydrolysate used in fermentation	Bran and germ after hydrolysis	Bran and germ before hydrolysis	Hydrolysate used in fermentation	Bran and germ after hydrolysis
Cold conversion process	5L validation experiments	8.44	0.82	7.27	44.83	1.78	42.38
	100L validation experiments	8.44	0.94	7.08	44.83	1.89	42.15
Warm conversion process	5L validation experiments	8.44	0.84	7.25	44.83	1.83	42.31
	100L validation experiments	8.44	0.91	7.19	44.83	1.84	42.43

5.7 The effect of processing methods on the amino acid profile of DDGS.

Even though all 20 amino acids are required for growth and proper function, only 9 are considered essential. The nine essential amino acids (histidine, lysine, leucine, isoleucine, methionine, phenylalanine, valine, threonine and tryptophan) are not produced by mammals and should be acquired through diet (Novak, 2013). These amino acids are vital for protein synthesis, nutrient absorption and tissue repair. The amino acids shown in Table 5.7 are calculated on a weight % basis of the total of all the amino acids for the protein fraction for each process configuration. Soy protein (Rayaprolu et al., 2015) and canola oil cake ("Canola_meal_feed_industry_guide," 2015.) amino acid profiles are included for comparison.

Table 5.7 shows that for the warm and cold processes, the total percentage of essential amino acids did not differ significantly between milled and debranned configurations for both 5L and 100L experiments. The difference between the essential and non-essential amino acids were significant for each process configuration. The average split of the amino acids was about 40% essential and 60% non-essential for all process configurations. No significant difference can be observed for total essential and non-essential values between soy protein, canola oil cake and warm or cold process configurations.

Table 5.7: Essential and non-essential amino acids produced from 5L and 150L validation experiment for all process configurations.

Amino acid (wt%)	Warm process				Cold process				Other	
	5L Validation experiments		150L Validation experiments		5L Validation experiments		150L Validation experiments		From Industry	
	Whole-milled grains	Debranned grains	Whole-milled grains	Debranned grains	Whole-milled grains	Debranned grains	Whole-milled grains	Debranned grains	Soy protein	Canola oil cake
Essential amino acids										
Histidine	2.00	1.80	1.87	1.64	1.95	1.87	1.56	2.04	4.80	3.85
Lysine	5.43	1.15	4.83	4.99	4.81	5.31	4.80	5.47	8.89	6.73
Leucine	6.99	6.28	7.11	6.36	5.82	6.04	5.66	6.11	6.95	7.01
Isoleucine	3.19	3.27	3.15	3.37	2.83	3.21	3.22	3.33	2.86	3.95
Methionine	2.06	2.09	2.19	2.08	1.74	2.34	1.60	1.83	2.66	2.21
Phenylalanine	17.41	15.53	13.23	14.64	16.60	15.52	11.47	14.09	4.60	4.61
Valine	4.00	3.98	4.28	4.34	3.92	4.19	2.59	4.11	3.06	5.62
Threonine	2.51	2.48	2.90	2.05	2.79	2.27	2.59	3.09	2.35	4.87
Subtotal	**43.60**	**36.58**	**39.56**	**39.47**	**40.43**	**40.76**	**33.47**	**40.07**	**36.16**	**38.86**
Non-essential amino acids										
Arginine	4.36	4.21	4.80	3.42	3.55	4.06	4.12	4.24	6.23	7.52
Alanine	4.51	5.31	4.60	5.69	4.56	5.42	5.22	4.99	4.60	4.96
Glycine	4.15	4.16	4.18	4.10	4.64	4.19	4.08	4.62	4.60	5.59
Proline	8.90	10.08	8.59	9.09	9.17	9.86	9.32	9.00	4.29	6.79
Tyrosine	0.57	1.15	1.06	1.13	0.57	1.20	1.30	1.43	4.80	2.84
Asparagine	4.87	5.25	5.31	5.31	5.25	4.79	6.35	5.23	13.38	8.25
Glutamine	24.60	29.30	27.10	28.36	27.78	25.73	31.96	26.21	19.00	20.63
Serine	4.45	3.98	4.80	3.42	4.04	3.99	4.17	4.21	6.95	4.55
Subtotal	**56.40**	**63.42**	**60.44**	**60.53**	**59.57**	**59.24**	**66.53**	**59.93**	**63.84**	**61.14**

6 Discussion

6.1 Effect of debranning on response variables during starch processing.

Higher ethanol yields and final ethanol concentrations achieved at a higher glucoamylase dosages, for both milled and debranned grains, was ascribed to an improvement in the extent of maltodextrin chain conversion into fermentable sugar units when glucoamylase activity is increased in the slurry. Devantier et al., (2005) reported that both yield and kinetics of hydrolysis benefited from increased glucoamylase activity, achieving higher ethanol volumetric productivity, similar to what is observed in the present study . Higher ethanol yields were achieved for debranned grains compared to whole-milled grains (Figure 5.2). This is contradictory to a study by (Wang et al., 1999), who reported better performances for whole-milled triticale grains than for debranned grains. He attributed the decrease in yield to the nutrient limitations created when the bran is removed. The minerals and proteins contained in the bran fraction are required by yeast for optimal performance. The fermentation vigour of the yeast is negatively affected when these nutrients are absent from the slurry (Wang et al., 1999); Pereira et al., 2010). For the present study the bran and germ fractions removed during the debranning process was hydrolysed with a protease for 24 hours, filtered through a 2 mm sieve to remove remaining solids and added back into the vessel before liquefaction. During this hydrolysis step proteins, minerals and any residual starch still contained in these fractions are liberated and made available to the yeast. The nutrients (minerals and nitrogen) provided by the bran are crucial for optimal fermentation performance by the yeast (Pereira et al., 2010).

Higher ethanol yields and productivities were obtained when using debranned grains vs whole-milled grains for the warm and cold processes. The major difference in the process configuration between milled and debranned grains, is the 24-hour protease incubation step of the bran and germ fractions of debranned grains, before fermentation. The incubation of the bran and germ fractions with a protease would liberate FAN (free amino nitrogen) when peptide bonds are broken, providing more FAN in the slurry. This in turn would supplement yeast growth and increase fermentation performance allowing the final ethanol concentration to be reached sooner as reported by Chang et al., (2011) in a study with sorghum fermentations and the addition of FAN. He showed that

in slurries where FAN was added, productivity increased from 0.93 to 3.03 g/L/h. An additional factor contributing to increased productivity is the liberation of starch and glucose molecules when the protein networks inside the bran and germ fractions are broken down by a protease, thus acting as a form of pre-treatment making more glucose units available to the yeast at the start of fermentation and increasing the conversion rate to ethanol. This corresponds to what Johnston and McAloon, (2014) he reported the addition of a proteases significantly increased fermentation rates and yields. Starch is released faster in the presence of proteases, thus increasing the rate of fermentation and volumetric productivity.

The glucose concentration at the start of fermentation for the milled and debranned grains were 7.5 g/L and 14.8 g/L respectively. The higher initial glucose concentration observed for the debranned experiments could be attributed the selective removal of fibre, making the in starch more accessible to the enzymes. As observed by Brown and Johnson, (1970) an increase in glucose concentration at the start of fermentation will increase the conversion rate to ethanol, resulting in an increased volumetric ethanol productivity. The higher initial sugar concentration causes a repression in respiratory enzymes of yeast, shifting the metabolic pathway to favour ethanol production instead of biomass production (Polakis et al., 1965); (De Deken, 1966). It is documented that a glucose concentration of above 120 g/L would inhibit fermentation because of osmotic stress on yeast (Ivorra et al., 1999); (Liu et al., 2014); (Bafrncová et al., 1999).

Increased ethanol concentrations and yields are thus observed at higher enzyme dosages vs lower dosages, for both the conventional warm and cold process, using whole-milled and debranned grains. This highlights the effect of enzyme dosage on the hydrolysis of starch and subsequent fermentation. A study by Nkomba, (2015) using the same enzyme ranges with sorghum grains for ethanol production, reported an increase in ethanol yield of 7% for whole-milled and 1% for debranned sorghum grains. For the present study, a more pronounced increase (16%) was also observed for the whole-milled experiments of than for debranned experiments (9.6%) between low and high Stargen doses.

The removal of bran results in faster starch hydrolysis, positively affecting overall ethanol productivity as shown by Perez-Carrillo, et al., (2008) and Alvarez, et al., (2010). The bran fraction of triticale contains proteins that inhibit α-amylase activity (Täufel et al., 1997) as well as phytic acid that forms complexes with calcium ions. This results in less calcuim being available to function as a cofactor for amylase enzymes, decreasing the starch hydrolysis rate (Cawley and Mitchell, 1968). Removing the bran also removes the proteins and phytic acid able to inhibit amylase activity. Incubating the bran and germ fractions with a protease before starch hydrolysis and fermentation, regains most of the starch still attached to those fractions. Together, these factors contribute towards the increased ethanol productivity observed for debranned grains compared to whole-milled grains.

Difference in yield between whole-milled and debranned experiments is positive for both the warm and cold conversion processes. The yields achieved by debranned experiments are higher than the yields achieved by whole-milled experiments (see Figure 5.2 and 5.5). This is contrary to what was observed in a study on sorghum grains by Nkomba, (2015). He reported higher yields for whole-milled grains than for decorticated grains for both the warm and cold conversion processes. He stated the removal of the bran also removes minerals and nutrients (such as nitrogen) from the slurry, which negatively impacts fermentation performance. For the present study, the bran and germ fraction incubation with Alkalase recovers 86% of the starch and 63% of the protein contained within these fractions after debranning (see Table 5.6). The recovered starch and protein contained in the hydrolysate is added back into the slurry before fermentation, supplementing the slurry with more readily available nitrogen (FAN), minerals and starch and glucose units. This supplementation has a positive effect on fermentation performance, resulting in higher ethanol concentrations, yields and productivities for debranned experiments than for whole-milled experiments.

6.2 Optimization and validation experiments

The percentage of hydrolysed starch did not differ significantly between process configurations and ranged between 97.3% to 99.1%, indicating that almost all the starch was consumed. Higher ethanol yields were achieved when using debranned grains, 93.8% and 90.9%, than when using milled grains, 90.9% and 84.4%, for the warm

and cold process respectively (Table 5.3 and 5.4). As mentioned previously, the reason for this is likely due to the additional nutrients provided to the yeast by the protease incubation of the bran and germ fractions (Johnston and McAloon, 2014a) as well as the removal of amylase inhibitors contained in the bran fraction (Cawley and Mitchell, 1968). The ethanol yield on glucose consumed was lower for the cold process (86.7% for milled an 92% for debranned) than for the warm process (91.6% for milled and 94.6% for debranned).

The validation experiments were successful in achieving the predicted values for ethanol concentration and yield, but less so for the ethanol productivities of all process configurations (shown in Table 5.3 and 5.4). This could be attributed to scale up effects which results in less efficient mixing with larger slurry volumes, slowing down hydrolysis and fermentation. The optimization of input variables also succeeded in achieving similar responses between process variations to allow for meaningful comparison (shown in Table 5.3 and 5.4). In terms of performance, the debranned grains outperformed the milled grains for both the warm and cold process, with the debranned warm process configuration performing best overall by achieving the highest ethanol concentration, yield and productivity. More α-amylase enzyme was added for the warm process (Termamyl SC at 165.25 µl/100 g of grains) than for the cold process (GC626 at 14 µl/100 g of grains). Surprisingly, the debranned cold process performed second best overall, with a slightly higher ethanol concentration and productivity than the warm milled process, that came in third. This improved performance by the cold debranned process configuration could be ascribed to an increase in FAN and minerals provided by the bran and germ hydrolysate after incubation with Alkalase (Johnston and McAloon, 2014a). A study by Cinelli et al., (2015) also stated that higher yields were achieved with lower hydrolysis temperatures, associated with the cold conversion process. He ascribed this to the prevention of undesired side reactions, such as the Maillard reaction, which occur at higher temperatures associated with the warm process. The cold milled process configuration performed the worst, achieving the lowest ethanol concentration, yield and productivity. The cold debranned process configuration required 20.7% less enzyme and had a higher overall performance than the cold milled process configuration. The cold debranned process also matched the performance of the conventionally used warm milled process, requiring slightly less heat energy input to achieve similar process responses. This finding was also confirmed in studies with sorghum

grains and corn (Ai et al., 2011); (Corredor et al., 2006); (Devantier et al., 2005); (Nkomba et al., 2016); (Wang et al., 2007); (Wu et al., 2007). This result validates the positive outcome of bran removel on the hydrolysis of starch and its potential to reduce enzyme requirements while maintaining optimal process outputs for the cold process.

6.3 The effect of debranning on the chemical composition of DDGS

Debranning had a significant positive effect on the quality of DDGS produced,by significantly increasing the protein content and decreasing the fibre content. This is likely due to selective fibre removal during the debranning process, which automatically increases protein content and decreases fibre content in resultant DDGS (see Table 5.6). The protease incubation of the bran and germ fractions, recovers 63% of the proteins in those fractions, further concentrating the amino acids and peptides in the resultant DDGS (see Table 5.6). Similar findings were reported for studies using whole vs decorticated sorghum grains (Corredor et al., 2006); (Nkomba et al., 2016). Protein content is an important characteristic in DDGS as it is commonly used as a protein source in animal diets (Novak, 2013). A higher protein content is desired for DDGS especially when used as a feed source for monogastric animals, such as chikens and pigs (Novak, 2013). Compared to DDGS produced from corn, ranging in protein content between 25 to 33% (Liu, 2011), DDGS produced from triticale in the present study had a much higher protein content from debranned grains in conjunction with the cold conversion process (44%) as shown in Table 5.5.

The decrease in crude fibre of DDGS can be attributed to the removal of the bran, containing a highest concentration of fibres (Galanakis, 2018). The decrease in fibre content and increase in protein content would result in a more nutritious and calorie dense animal feed, especially for non-ruminant animals. The ash content increased significantly ($p<0.05$) with an average of 3% between milled and debranned grain configurations. The increase in ash content could be a result of the protease incubation of the bran, transferring ash located on the surface of the bran into the fermentation slurry and resulting DDGS. The crude fat content was not significantly affected by debranning and remained relatively constant between process configurations. Similar findings were reported by (Nkomba, 2015) for a study done on whole and decorticated sorghum grains.

The amino acid profiles of the DDGS produced from triticale grain fermentations do not differ significantly from that of soy protein or canola oil cake (Table 5.7). Values of limiting amino acids, lysine and methionine (Hudson, 2010), do not differ significantly in animal feed from soy protein, canola oil cake and DDGS from warm or cold triticale grain fermentations (see Table 5.7). Therefore, according to the amino acid profile, DDGS produced from triticale grain fermentations can be used as an animal feed for monogastric animals in similar ways as soy protein or canola oil cake.

7 Conclusion

The debranning of triticale grains had a significant positive effect on the performance of ethanol fermentations in terms of ethanol concentration, yield and productivity. Debranning of grains enabled the cold conversion process to compete with the warm conversion process by achieving similar fermentation performances, above 90% of the theoretical maximum. The cold process has added benefits such as less energy required during starch hydrolysis, lower viscosities and higher quality of DDGS produced as a value-added co-product of bio ethanol production. Debranning is the key to achieve high fermentation performances (above 90%) with raw starch digesting enzymes associated with the cold conversion process. Important to keep in mind is the nitrogen requirement of yeast. Debranned slurries require additional nitrogen supplementation, either with FAN in the form of urea or by recovering the nitrogen from the bran fraction with a protease. The DDGS produced by the cold process in conjunction with debranned triticale grains had the highest final crude protein percentage of 44%, making it ideal as an animal feed for monogastric animals.

8 Conclusions and recommendations

The current warm process conventionally used in industry, could profit from a reduction in enzyme and energy associated cost, if it could be successfully replaced by a raw starch digesting, cold process or consolidated bio-process. The motivation for this study was the lack of data that exists, especially for debranned triticale grains in combination with the cold conversion process. Based on the research questions previously stated, the following conclusions and recommendations have been made.

- **When using triticale as a feedstock, to what extend does the performance of the cold conversion process equal that of the warm conversion process for bioethanol production?**

Conclusion: The fermentation performance, in terms of ethanol concentration, yield and productivity, of the cold conversion process could match that of the conventional warm process in industry.

Recommendation: Cold processing with debranned triticale grains should be further scaled up to investigate whether what was observed in a 100 L bioreactor could be achieved on industrial scale.

- **How does debranning of grains affect the performance of the cold and warm conversion processes?**

Conclusion: Debranning positively affected the performance of both the warm and cold processing methods, increasing the ethanol concentration, yield and productivity. Furthermore, using debranned grains increased the rate of starch hydrolysis and ethanol productivity compared to whole-milled grains. This resulted in an enzyme dosage reduction of 20.7% while maintaining desired levels of performance output.

Recommendation: For debranned grain configurations, the effect of an increased solids loading (35%) on the fermentation outputs could be investigated. This would allow for a higher starch loading at the same solids loading, increasing the potential for higher yields in industry.

- **How does process configuration and debranning of grains influence the chemical composition of Distillers dried grains with solubles (DDGS)?**

Conclusion: Protein content of DDGS was significantly increased by debranning, making DDGS from debranned triticale grains a desired source of high protein animal feed. Cold processing in combination with debranned grains also decreased ADF (acid detergent fibres) and NDF (neutral detergent fibres), increasing the caloric density as an animal feed, especially for non-ruminant animals.

Recommendation: The DDGS produced from debranned grains in combination with warm and cold process configurations could be tested in an animal feed trial to investigated digestibility and uptake.

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Appendix A: Analysis of variance for best fit models of experimental data using the conventional warm process.

Whole-milled grains

Ethanol concentration:

Factor	ANOVA; Var.: **Ethanol concetration (g/L)** R-sqr=.81762; Adj:.65347 3 factors, 1 Blocks, 20 Runs; MS Pure Error=9.659187 DV: **Ethanol concetration (g/L)**				
	SS	df	MS	F	p
(1)α-amylase dosage (µl/100 g starch)(L)	17.0254	1	17.0254	1.76262	0.241681
α-amylase dosage (µl/100 g starch)(Q)	0.0748	1	0.0748	0.00774	0.933289
(2)Glucoamylsase dosage (µl/100 g starch)(L)	423.3069	1	423.3069	43.82428	0.001184
Glucoamylsase dosage (µl/100 g starch)(Q)	8.2367	1	8.2367	0.85273	0.398155
(3)Liquefaction time (min)(L)	122.9017	1	122.9017	12.72381	0.016094
Liquefaction time (min)(Q)	10.7185	1	10.7185	1.10967	0.340374
1L by 2L	15.0701	1	15.0701	1.56018	0.266928
1L by 3L	24.4301	1	24.4301	2.52920	0.172628
2L by 3L	0.7321	1	0.7321	0.07579	0.794103
Lack of Fit	91.0037	5	18.2007	1.88429	0.251815
Pure Error	48.2959	5	9.6592		
Total SS	763.7746	19			

Ethanol yield (as a percentage of the theoretical maximum):

Factor	ANOVA; Var.: **Ethanol yield (% of theoretical max)** R-sqr=.77624; Adj:.57485 3 factors, 1 Blocks, 20 Runs; MS Pure Error=3.747 DV: **Ethanol yield (% of theoretical max)**				
	SS	df	MS	F	p
(1)α-amylase dosage (µl/100 g starch)(L)	8.4183	1	8.4183	2.24669	0.194179
α-amylase dosage (µl/100 g starch)(Q)	0.1183	1	0.1183	0.03157	0.865958
(2)Glucoamylsase dosage (µl/100 g starch)(L)	168.4023	1	168.4023	44.94324	0.001118
Glucoamylsase dosage (µl/100 g starch)(Q)	4.3891	1	4.3891	1.17138	0.328531
(3)Liquefaction time (min)(L)	60.0603	1	60.0603	16.02891	0.010286
Liquefaction time (min)(Q)	2.1787	1	2.1787	0.58146	0.480147
1L by 2L	10.5800	1	10.5800	2.82359	0.153721
1L by 3L	6.4800	1	6.4800	1.72938	0.245569
2L by 3L	1.1250	1	1.1250	0.30024	0.607296
Lack of Fit	56.9282	5	11.3856	3.03860	0.123963
Pure Error	18.7350	5	3.7470		
Total SS	338.1375	19			

Ethanol productivity:

Factor	ANOVA; Var.: **Ethanol productivity (g/L/h)** R-sqr=.71962; Adj:.4672 3 factors, 1 Blocks, 20 Runs; MS Pure Error=.0217867 DV: **Ethanol productivity (g/L/h)**				
	SS	df	MS	F	p
(1)α-amylase dosage (μl/100 g starch)(L)	0.097893	1	0.097893	4.49323	0.087536
α-amylase dosage (μl/100 g starch)(Q)	0.091216	1	0.091216	4.18680	0.096111
(2)Glucoamylsase dosage (μl/100 g starch)(L)	0.051174	1	0.051174	2.34886	0.185948
Glucoamylsase dosage (μl/100 g starch)(Q)	0.004482	1	0.004482	0.20571	0.669159
(3)Liquefaction time (min)(L)	0.221639	1	0.221639	10.17316	0.024277
Liquefaction time (min)(Q)	0.000003	1	0.000003	0.00015	0.990620
1L by 2L	0.003613	1	0.003613	0.16581	0.700708
1L by 3L	0.040612	1	0.040612	1.86410	0.230372
2L by 3L	0.001013	1	0.001013	0.04647	0.837836
Lack of Fit	0.088989	5	0.017798	0.81691	0.585103
Pure Error	0.108933	5	0.021787		
Total SS	0.705895	19			

Debranned grains:

Ethanol concentration:

Factor	ANOVA; Var.: **Ethanol concentration (g/L)** R-sqr=.75329; Adj:.5312 3 factors, 1 Blocks, 20 Runs; MS Pure Error=8.111657 DV: **Ethanol concentration (g/L)**				
	SS	df	MS	F	p
(1)α-amylase dosage (μl/100 g starch)(L)	57.8934	1	57.8934	7.13706	0.044267
α-amylase dosage (μl/100 g starch)(Q)	51.7526	1	51.7526	6.38003	0.052795
(2)Glucoamylase dosage (μl/100 g starch)(L)	142.1936	1	142.1936	17.52954	0.008598
Glucoamylase dosage (μl/100 g starch)(Q)	0.8371	1	0.8371	0.10320	0.761019
(3)Liquefaction Time (min)(L)	164.7670	1	164.7670	20.31237	0.006359
Liquefaction Time (min)(Q)	40.3125	1	40.3125	4.96970	0.076234
1L by 2L	30.3031	1	30.3031	3.73575	0.111090
1L by 3L	24.8160	1	24.8160	3.05930	0.140688
2L by 3L	0.5671	1	0.5671	0.06991	0.802016
Lack of Fit	124.4445	5	24.8889	3.06829	0.121997
Pure Error	40.5583	5	8.1117		
Total SS	668.8063	19			

Ethanol yield (as a percentage of the theoretical maximum):

Factor	ANOVA; Var.:**Ethanol yield (% of theoretical max)** R-sqr=.75356; Adj:.53177 3 factors, 1 Blocks, 20 Runs; MS Pure Error=2.773667 DV: **Ethanol yield (% of theoretical max)**				
	SS	df	MS	F	p
(1)α-amylase dosage (µl/100 g starch)(L)	29.7782	1	29.77821	10.73605	0.022037
α-amylase dosage (µl/100 g starch)(Q)	14.6894	1	14.68944	5.29604	0.069658
(2)Glucoamylase dosage (µl/100 g starch)(L)	50.6986	1	50.69858	18.27854	0.007898
Glucoamylase dosage (µl/100 g starch)(Q)	0.4838	1	0.48380	0.17443	0.693536
(3)Liquefaction Time (min)(L)	76.3299	1	76.32992	27.51950	0.003338
Liquefaction Time (min)(Q)	6.3526	1	6.35258	2.29032	0.190599
1L by 2L	11.7613	1	11.76125	4.24033	0.094529
1L by 3L	12.7513	1	12.75125	4.59725	0.084874
2L by 3L	0.0313	1	0.03125	0.01127	0.919595
Lack of Fit	52.1772	5	10.43543	3.76232	0.086107
Pure Error	13.8683	5	2.77367		
Total SS	268.0009	19			

Ethanol productivity:

Factor	ANOVA; Var.:**Ethanol productivity (g/L/h)** R-sqr=.64933; Adj:.33372 3 factors, 1 Blocks, 20 Runs; MS Pure Error=.0670967 DV: **Ethanol productivity (g/L/h)**				
	SS	df	MS	F	p
(1)α-amylase dosage (µl/100 g starch)(L)	0.071486	1	0.071486	1.06542	0.349300
α-amylase dosage (µl/100 g starch)(Q)	0.050497	1	0.050497	0.75259	0.425324
(2)Glucoamylase dosage (µl/100 g starch)(L)	0.012717	1	0.012717	0.18954	0.681455
Glucoamylase dosage (µl/100 g starch)(Q)	0.045162	1	0.045162	0.67310	0.449304
(3)Liquefaction Time (min)(L)	0.719647	1	0.719647	10.72552	0.022077
Liquefaction Time (min)(Q)	0.006511	1	0.006511	0.09704	0.767985
1L by 2L	0.090313	1	0.090313	1.34601	0.298359
1L by 3L	0.010513	1	0.010513	0.15668	0.708560
2L by 3L	0.030013	1	0.030013	0.44730	0.533252
Lack of Fit	0.223501	5	0.044700	0.66621	0.666655
Pure Error	0.335483	5	0.067097		
Total SS	1.594024	19			

Appendix B: Analysis of variance for best fit models of experimental data using the cold process.

Whole-milled grains

Ethanol concentration:

Factor	ANOVA; Var.: **Ethanol concentration (g/L)** R-sqr=.95495; Adj:.92278 2 factors, 1 Blocks, 13 Runs; MS Pure Error=4.742849 DV: **Ethanol concentration (g/L)**				
	SS	df	MS	F	p
(1)**Pre-saccharification Time (min)**(L)	12.776	1	12.7763	2.6938	0.176083
Pre-saccharification Time (min)(Q)	7.999	1	7.9992	1.6866	0.263861
(2)**Stargen dosage (µl/100 g starch)**(L)	941.831	1	941.8314	198.5792	0.000147
Stargen dosage (µl/100 g starch)(Q)	82.553	1	82.5533	17.4058	0.014008
1L by 2L	9.709	1	9.7095	2.0472	0.225730
Lack of Fit	30.540	3	10.1800	2.1464	0.237115
Pure Error	18.971	4	4.7428		
Total SS	1099.130	12			

Ethanol yield (as a percentage of the theoretical maximum):

Factor	ANOVA; Var.: **Ethanol yield (% of theoretical maximum)** R-sqr=.94347; Adj:.90308 2 factors, 1 Blocks, 13 Runs; MS Pure Error=2.062498 DV: **Ethanol yield (% of theoretical maximum)**				
	SS	df	MS	F	p
(1)**Pre-saccharification Time (min)**(L)	1.2884	1	1.2884	0.6247	0.473528
Pre-saccharification Time (min)(Q)	2.8712	1	2.8712	1.3921	0.303429
(2)**Stargen dosage (µl/100 g starch)**(L)	401.2956	1	401.2956	194.5677	0.000153
Stargen dosage (µl/100 g starch)(Q)	43.9077	1	43.9077	21.2886	0.009926
1L by 2L	7.6177	1	7.6177	3.6934	0.127004
Lack of Fit	19.0029	3	6.3343	3.0712	0.153359
Pure Error	8.2500	4	2.0625		
Total SS	482.0641	12			

Ethanol productivity:

Factor	SS	df	MS	F	p
	ANOVA; Var.:**Ethanol productivity (g/L/h)** R-sqr=.99255; Adj:.9872				
	2 factors, 1 Blocks, 13 Runs; MS Pure Error=.0005782				
	DV: **Ethanol productivity (g/L/h)**				
(1)**Pre-saccharification Time (min)**(L)	0.007241	1	0.007241	12.523	0.024041
Pre-saccharification Time (min)(Q)	0.187180	1	0.187180	323.714	0.000056
(2)**Stargen dosage (µl/100 g starch)**(L)	1.186766	1	1.186766	2052.421	0.000001
Stargen dosage (µl/100 g starch)(Q)	0.180579	1	0.180579	312.297	0.000060
1L by 2L	0.024361	1	0.024361	42.130	0.002905
Lack of Fit	0.009279	3	0.003093	5.349	0.069482
Pure Error	0.002313	4	0.000578		
Total SS	1.555307	12			

Debranned grains

Ethanol concentration:

Factor	SS	df	MS	F	p
	ANOVA; Var.:**Ethanol concentration (g/L)** R-sqr=.90201; Adj:.83202				
	2 factors, 1 Blocks, 13 Runs; MS Pure Error=10.20451				
	DV: **Ethanol concentration (g/L)**				
(1)**Pre-saccharification Time (min)**(L)	72.4687	1	72.4687	7.10164	0.056104
Pre-saccharification Time (min)(Q)	12.5059	1	12.5059	1.22553	0.330368
(2)**Stargen dosage (µl/100 g starch)**(L)	546.6666	1	546.6666	53.57108	0.001854
Stargen dosage (µl/100 g starch)(Q)	145.1727	1	145.1727	14.22633	0.019574
1L by 2L	13.9726	1	13.9726	1.36926	0.306901
Lack of Fit	44.1538	3	14.7179	1.44230	0.355521
Pure Error	40.8180	4	10.2045		
Total SS	867.1823	12			

Ethanol yield (as a percentage of the theoretical maximum):

Factor	ANOVA; Var.:**Ethanol yield (% of theoretical maximum)**R-sqr=.8885; Adj:.80886 2 factors, 1 Blocks, 13 Runs; MS Pure Error=4.034853 DV: **Ethanol yield (% of theoretical maximum)**				
	SS	df	MS	F	p
(1)**Pre-saccharification Time (min)(L)**	30.8460	1	30.8460	7.64488	0.050593
Pre-saccharification Time (min)(Q)	5.2387	1	5.2387	1.29837	0.318114
(2)**Stargen dosage (µl/100 g starch)(L)**	213.6871	1	213.6871	52.96031	0.001894
Stargen dosage (µl/100 g starch)(Q)	47.2679	1	47.2679	11.71490	0.026714
1L by 2L	3.7143	1	3.7143	0.92055	0.391664
Lack of Fit	21.1933	3	7.0644	1.75085	0.294911
Pure Error	16.1394	4	4.0349		
Total SS	334.8203	12			

Ethanol productivity:

Factor	ANOVA; Var.:**Ethanol productivity (g/L/h)**R-sqr=.88945; Adj:.81048 2 factors, 1 Blocks, 13 Runs; MS Pure Error=.0175296 DV: **Ethanol productivity (g/L/h)**				
	SS	df	MS	F	p
(1)**Pre-saccharification Time (min)(L)**	0.169950	1	0.169950	9.69505	0.035744
Pre-saccharification Time (min)(Q)	0.025895	1	0.025895	1.47719	0.291042
(2)**Stargen dosage (µl/100 g starch)(L)**	1.640884	1	1.640884	93.60662	0.000639
Stargen dosage (µl/100 g starch)(Q)	0.171465	1	0.171465	9.78146	0.035269
1L by 2L	0.069818	1	0.069818	3.98285	0.116687
Lack of Fit	0.186394	3	0.062131	3.54437	0.126599
Pure Error	0.070118	4	0.017530		
Total SS	2.320260	12			

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Appendix C: Desirability plots for all process configurations
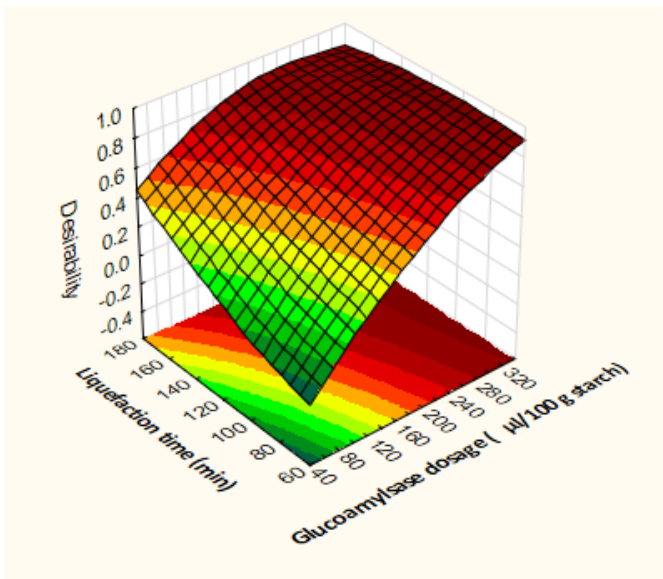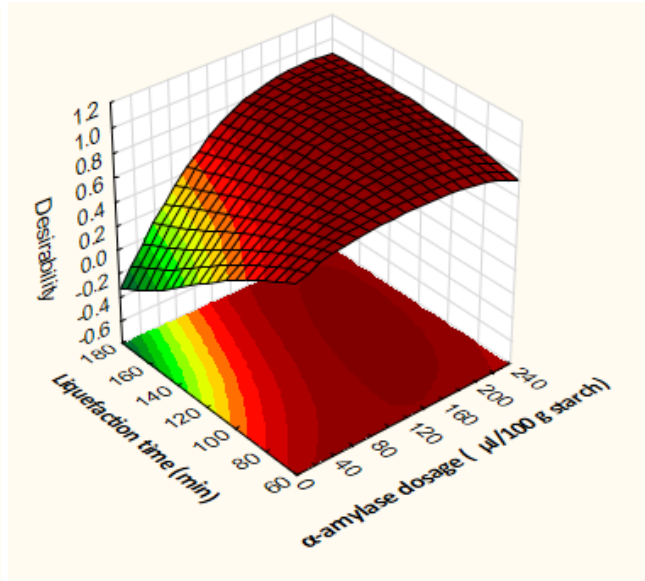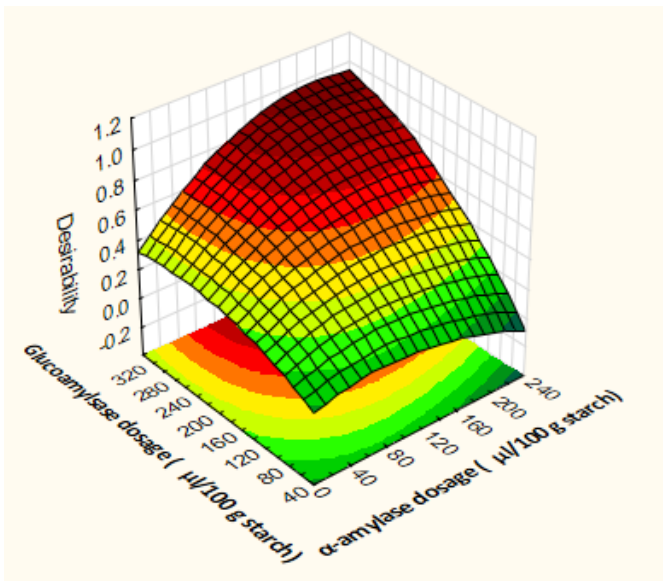
Desirability plots for conventional warm process for whole-milled grains:
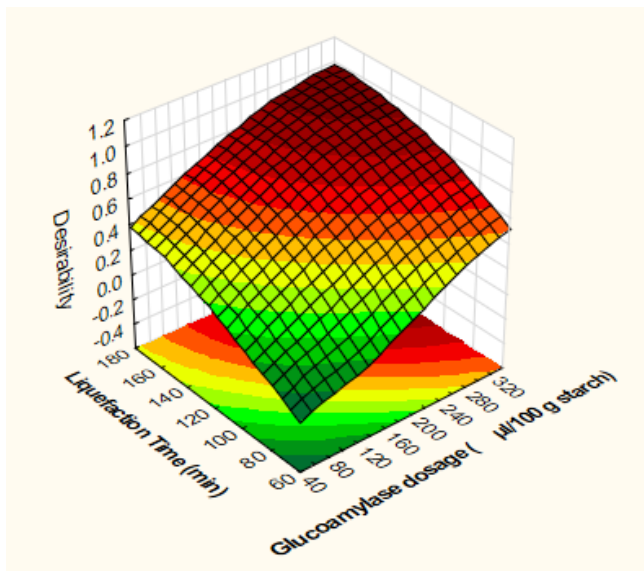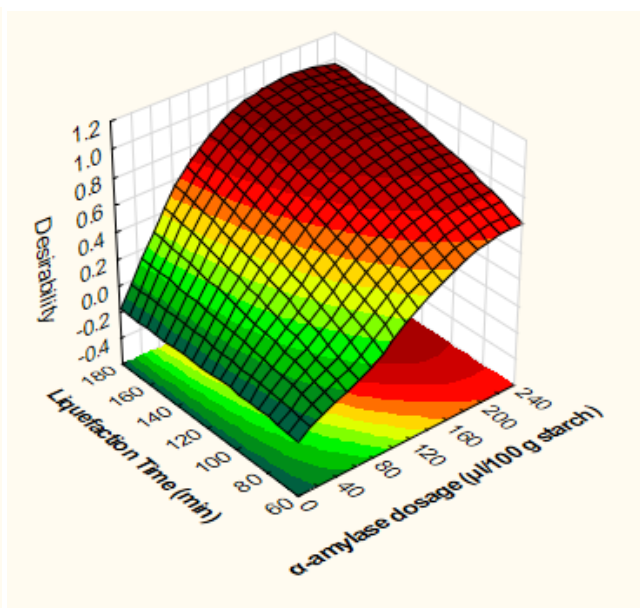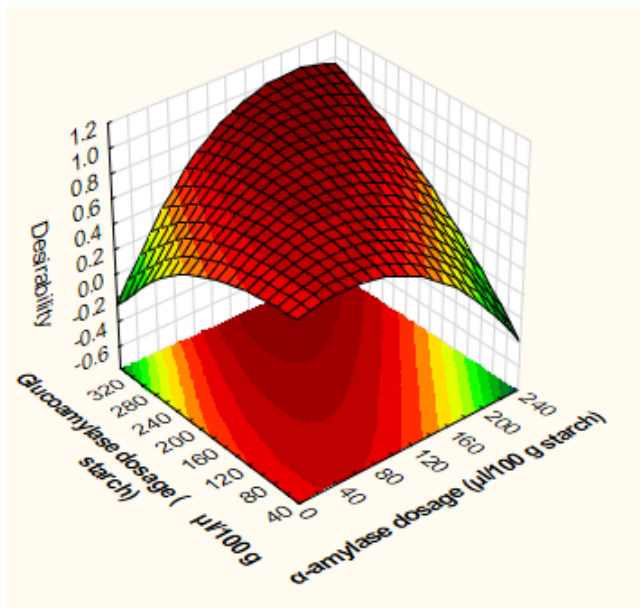
Desirability plots for conventional warm process for debranned grains:
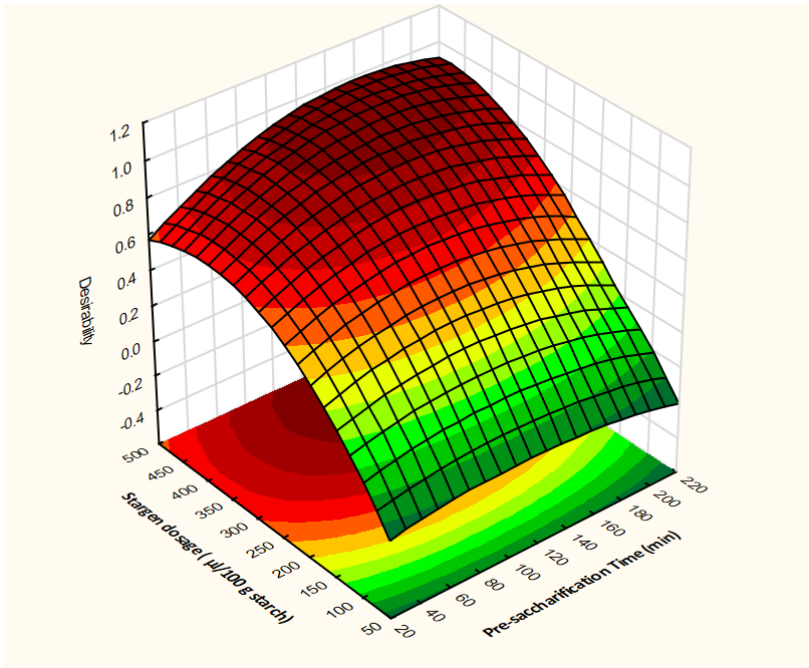
Desirability plots for conventional cold process for whole-milled grains:

Desirability plots for conventional cold process for debranned grains:

Appendix D: Regression coefficients for warm process configurations

Whole-milled grains

Ethanol concentration:

Factor	Regr. Coefficients; Var.**Ethanol concetration (g/L)** R-sqr=.81762; Adj:.65347 3 factors, 1 Blocks, 20 Runs; MS Pure Error=9.659187 DV: **Ethanol concetration (g/L)**					
	Regressn Coeff.	Std.Err. Pure Err	t(5)	p	-95.% Cnf.Limt	+95.% Cnf.Limt
Mean/Interc.	130.8537	21.23543	6.16205	0.001638	76.26632	185.4412
(1)**α-amylase dosage (μl/100 g starch)(**L)	-0.1645	0.10645	-1.54508	0.182986	-0.43813	0.1092
α-amylase dosage (μl/100 g starch)(Q)	0.0000	0.00024	0.08800	0.933289	-0.00060	0.0006
(2)**Glucoamylsase dosage (μl/100 g starch)(**L)	0.0995	0.08225	1.21020	0.280286	-0.11189	0.3110
Glucoamylsase dosage (μl/100 g starch)(Q)	-0.0001	0.00013	-0.92343	0.398155	-0.00047	0.0002
(3)**Liquefaction time (min)(**L)	-0.2246	0.25162	-0.89244	0.413043	-0.87137	0.4223
Liquefaction time (min)(Q)	0.0010	0.00092	1.05341	0.340374	-0.00140	0.0033
1L by 2L	0.0003	0.00024	1.24907	0.266928	-0.00032	0.0009
1L by 3L	0.0010	0.00063	1.59035	0.172628	-0.00062	0.0026
2L by 3L	-0.0001	0.00047	-0.27530	0.794103	-0.00134	0.0011

Ethanol yield:

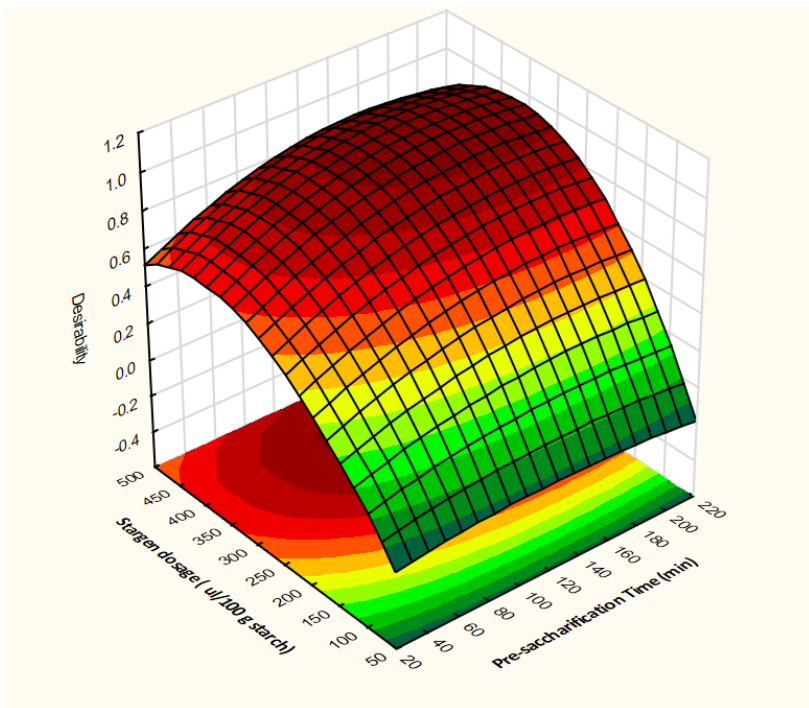| Factor	Regr. Coefficients; Var.**Ethanol yield (% of theoretical max)** R-sqr=.77624 3 factors, 1 Blocks, 20 Runs; MS Pure Error=3.747 DV: **Ethanol yield (% of theoretical max)**					
	Regressn Coeff.	Std.Err. Pure Err	t(5)	p	-95.% Cnf.Limt	+95.% Cnf.Limt
Mean/Interc.	81.87475	13.22613	6.19038	0.001605	47.87591	115.8736
(1)**α-amylase dosage (μl/100 g starch)(**L)	-0.10359	0.06630	-1.56228	0.178980	-0.27402	0.0669
α-amylase dosage (μl/100 g starch)(Q)	0.00003	0.00015	0.17767	0.865958	-0.00036	0.0004
(2)**Glucoamylsase dosage (μl/100 g starch)(**L)	0.06968	0.05123	1.36026	0.231863	-0.06200	0.2014
Glucoamylsase dosage (μl/100 g starch)(Q)	-0.00009	0.00008	-1.08230	0.328531	-0.00031	0.0001
(3)**Liquefaction time (min)(**L)	-0.06423	0.15672	-0.40982	0.698904	-0.46709	0.3386
Liquefaction time (min)(Q)	0.00044	0.00057	0.76254	0.480147	-0.00104	0.0019
1L by 2L	0.00025	0.00015	1.68035	0.153721	-0.00013	0.0006
1L by 3L	0.00052	0.00039	1.31506	0.245569	-0.00049	0.0015
2L by 3L	-0.00016	0.00029	-0.54794	0.607296	-0.00091	0.0006

Ethanol productivity:

	Regr. Coefficients; Var. **Ethanol productivity (g/L/h)** R-sqr=.71962; Adj:.46727 3 factors, 1 Blocks, 20 Runs; MS Pure Error=.0217867 DV: **Ethanol productivity (g/L/h)**					
Factor	Regressn Coeff.	Std.Err. Pure Err	t(5)	p	-95.% Cnf.Limt	+95.% Cnf.Limt
Mean/Interc.	2.029097	1.008524	2.01195	0.100396	-0.563396	4.621591
(1)α-amylase dosage (µl/100 g starch)(L)	0.001133	0.005056	0.22401	0.831617	-0.011864	0.014129
α-amylase dosage (µl/100 g starch)(Q)	-0.000024	0.000012	-2.04617	0.096111	-0.000053	0.000006
(2)Glucoamylsase dosage (µl/100 g starch)(L)	0.001932	0.003906	0.49467	0.641800	-0.008109	0.011974
Glucoamylsase dosage (µl/100 g starch)(Q)	-0.000003	0.000006	-0.45355	0.669159	-0.000019	0.000014
(3)Liquefaction time (min)(L)	-0.008219	0.011950	-0.68778	0.522188	-0.038938	0.022500
Liquefaction time (min)(Q)	0.000001	0.000044	0.01236	0.990620	-0.000112	0.000113
1L by 2L	0.000005	0.000012	0.40720	0.700708	-0.000025	0.000034
1L by 3L	0.000041	0.000030	1.36532	0.230372	-0.000036	0.000118
2L by 3L	-0.000005	0.000022	-0.21558	0.837836	-0.000062	0.000053

Debranned grains:

Ethanol concentration:

	Regr. Coefficients; Var. **Ethanol concentration (g/L)** R-sqr=.75329; Adj:.53125 3 factors, 1 Blocks, 20 Runs; MS Pure Error=8.111657 DV: **Ethanol concentration (g/L)**					
Factor	Regressn Coeff.	Std.Err. Pure Err	t(5)	p	-95.% Cnf.Limt	+95.% Cnf.Limt
Mean/Interc.	78.53191	19.46012	4.03553	0.009967	28.50807	128.5558
(1)α-amylase dosage (µl/100 g starch)(L)	0.20510	0.09756	2.10236	0.089485	-0.04568	0.4559
α-amylase dosage (µl/100 g starch)(Q)	-0.00056	0.00022	-2.52587	0.052795	-0.00114	0.0000
(2)Glucoamylase dosage (µl/100 g starch)(L)	-0.00693	0.07537	-0.09190	0.930346	-0.20068	0.1868
Glucoamylase dosage (µl/100 g starch)(Q)	-0.00004	0.00012	-0.32124	0.761019	-0.00036	0.0003
(3)Liquefaction Time (min)(L)	0.66413	0.23059	2.88016	0.034581	0.07139	1.2569
Liquefaction Time (min)(Q)	-0.00188	0.00085	-2.22928	0.076234	-0.00406	0.0003
1L by 2L	0.00043	0.00022	1.93281	0.111090	-0.00014	0.0010
1L by 3L	-0.00101	0.00058	-1.74909	0.140688	-0.00250	0.0005
2L by 3L	0.00011	0.00043	0.26441	0.802016	-0.00099	0.0012

Ethanol yield:

| Factor | Regr. Coefficients; Var.:**Ethanol yield (% of theoretical max)** R-sqr=.75356; Adj:.53177 3 factors, 1 Blocks, 20 Runs; MS Pure Error=2.773667 DV: **Ethanol yield (% of theoretical max)** | | | | | |
	Regressn Coeff.	Std.Err. Pure Err	t(5)	p	-95.% Cnf.Limt	+95.% Cnf.Limt
Mean/Interc.	57.44015	11.37936	5.04775	0.003941	28.18856	86.69173
(1)α-amylase dosage (µl/100 g starch)(L)	0.13072	0.05705	2.29150	0.070517	-0.01592	0.27736
α-amylase dosage (µl/100 g starch)(Q)	-0.00030	0.00013	-2.30131	0.069658	-0.00064	0.00004
(2)Glucoamylase dosage (µl/100 g starch)(L)	-0.01475	0.04407	-0.33474	0.751405	-0.12805	0.09854
Glucoamylase dosage (µl/100 g starch)(Q)	0.00003	0.00007	0.41764	0.693536	-0.00016	0.00022
(3)Liquefaction Time (min)(L)	0.34796	0.13484	2.58061	0.049396	0.00135	0.69457
Liquefaction Time (min)(Q)	-0.00075	0.00049	-1.51338	0.190599	-0.00202	0.00052
1L by 2L	0.00027	0.00013	2.05921	0.094529	-0.00007	0.00060
1L by 3L	-0.00073	0.00034	-2.14412	0.084874	-0.00160	0.00014
2L by 3L	-0.00003	0.00025	-0.10614	0.919595	-0.00067	0.00062

Ethanol productivity:

| Factor | Regr. Coefficients; Var.:**Ethanol productivity (g/L/h)** R-sqr=.64971; Adj:.33446 3 factors, 1 Blocks, 20 Runs; MS Pure Error=.0670967 DV: **Ethanol productivity (g/L/h)** | | | | | |
	Regressn Coeff.	Std.Err. Pure Err	t(5)	p	-95.% Cnf.Limt	+95.% Cnf.Limt
Mean/Interc.	1.309776	1.769870	0.740041	0.492538	-3.23982	5.859371
(1)α-amylase dosage (µl/100 g starch)(L)	-0.001670	0.008872	-0.188265	0.858072	-0.02448	0.021137
α-amylase dosage (µl/100 g starch)(Q)	-0.000018	0.000020	-0.869185	0.424496	-0.00007	0.000035
(2)Glucoamylase dosage (µl/100 g starch)(L)	0.004316	0.006855	0.629617	0.556591	-0.01331	0.021938
Glucoamylase dosage (µl/100 g starch)(Q)	-0.000009	0.000011	-0.814216	0.452539	-0.00004	0.000020
(3)Liquefaction Time (min)(L)	0.004572	0.020972	0.218006	0.836042	-0.04934	0.058481
Liquefaction Time (min)(Q)	0.000024	0.000077	0.309812	0.769200	-0.00017	0.000221
1L by 2L	0.000023	0.000020	1.160175	0.298359	-0.00003	0.000076
1L by 3L	0.000021	0.000053	0.395824	0.708560	-0.00011	0.000156
2L by 3L	-0.000026	0.000039	-0.668807	0.533252	-0.00013	0.000074

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Appendix E: Regression coefficients for cold process configurations

Whole-milled grains

Ethanol concentration:

| Factor | Regr. Coefficients; Var.**Ethanol concentration (g/L)** R-sqr=.95495; Adj:.92278
2 factors, 1 Blocks, 13 Runs; MS Pure Error=4.742849
DV: **Ethanol concentration (g/L)** | | | | | |
	Regressn Coeff.	Std.Err. Pure Err	t(4)	p	-95.% Cnf.Limt	+95.% Cnf.Limt
Mean/Interc.	98.08350	6.460616	15.18176	0.000110	80.14595	116.0210
(1)**Pre-saccharification Time (min)**(L)	-0.00151	0.067173	-0.02245	0.983168	-0.18801	0.18500
Pre-saccharification Time (min)(Q)	-0.00030	0.000229	-1.29868	0.263861	-0.00093	0.0003
(2)**Stargen dosage (µl/100 g starch)**(L)	0.16810	0.031491	5.33795	0.005933	0.08066	0.2555
Stargen dosage (µl/100 g starch)(Q)	-0.00021	0.000050	-4.17203	0.014008	-0.00035	-0.0001
1L by 2L	0.00020	0.000142	1.43080	0.225730	-0.00019	0.0006

Ethanol yield:

| Factor | Regr. Coefficients; Var.**Ethanol yield (% of theoretical maximum)** R-sqr=.94347; Adj:.90308
2 factors, 1 Blocks, 13 Runs; MS Pure Error=2.062498
DV: **Ethanol yield (% of theoretical maximum)** | | | | | |
	Regressn Coeff.	Std.Err. Pure Err	t(4)	p	-95.% Cnf.Limt	+95.% Cnf.Limt
Mean/Interc.	64.66818	4.260407	15.17887	0.000110	52.83937	76.49695
(1)**Pre-saccharification Time (min)**(L)	-0.00986	0.044297	-0.22258	0.834767	-0.13285	0.11313
Pre-saccharification Time (min)(Q)	-0.00018	0.000151	-1.17987	0.303429	-0.00060	0.00024
(2)**Stargen dosage (µl/100 g starch)**(L)	0.11229	0.020767	5.40749	0.005664	0.05464	0.16995
Stargen dosage (µl/100 g starch)(Q)	-0.00015	0.000033	-4.61396	0.009926	-0.00025	-0.00006
1L by 2L	0.00018	0.000093	1.92183	0.127004	-0.00008	0.00044

Ethanol productivity:

| Factor | Regr. Coefficients; Var.**Ethanol productivity (g/L/h)** R-sqr=.99255; Adj:.98722 (§
2 factors, 1 Blocks, 13 Runs; MS Pure Error=.0005782
DV: **Ethanol productivity (g/L/h)** | | | | | |
	Regressn Coeff.	Std.Err. Pure Err	t(4)	p	-95.% Cnf.Limt	+95.% Cnf.Limt
Mean/Interc.	-0.131548	0.071335	-1.8441	0.138939	-0.329606	0.066510
(1)**Pre-saccharification Time (min)**(L)	0.008836	0.000742	11.9127	0.000284	0.006776	0.010895
Pre-saccharification Time (min)(Q)	-0.000046	0.000003	-17.9921	0.000056	-0.000053	-0.000039
(2)**Stargen dosage (µl/100 g starch)**(L)	0.006825	0.000348	19.6298	0.000040	0.005860	0.007791
Stargen dosage (µl/100 g starch)(Q)	-0.000010	0.000001	-17.6719	0.000060	-0.000011	-0.000008
1L by 2L	0.000010	0.000002	6.4908	0.002905	0.000006	0.000015

Debranned grains:

Ethanol concentration:

Factor	Regressn Coeff.	Std.Err. Pure Err	t(4)	p	-95.% Cnf.Limt	+95.% Cnf.Limt
Regr. Coefficients; Var.**Ethanol concentration (g/L)** R-sqr=.90201; Adj:.83202 2 factors, 1 Blocks, 13 Runs; MS Pure Error=10.20451 DV: **Ethanol concentration (g/L)**						
Mean/Interc.	83.94705	9.476552	8.85840	0.000897	57.63593	110.2582
(1)**Pre-saccharification Time (min)(**L)	0.20185	0.098531	2.04857	0.109868	-0.07172	0.4754
Pre-saccharification Time (min)(Q)	-0.00037	0.000336	-1.10704	0.330368	-0.00131	0.0006
(2)**Stargen dosage (µl/100 g starch)(**L)	0.23657	0.046192	5.12143	0.006879	0.10832	0.3648
Stargen dosage (µl/100 g starch)(Q)	-0.00028	0.000074	-3.77178	0.019574	-0.00048	-0.0001
1L by 2L	-0.00024	0.000208	-1.17015	0.306901	-0.00082	0.0003

Ethanol yield:

Factor	Regressn Coeff.	Std.Err. Pure Err	t(4)	p	-95.% Cnf.Limt	+95.% Cnf.Limt
Regr. Coefficients; Var.**Ethanol yield (% of theoretical maximum)** R-sqr=.8885; Adj:.80886 2 factors, 1 Blocks, 13 Runs; MS Pure Error=4.034853 DV: **Ethanol yield (% of theoretical maximum)**						
Mean/Interc.	59.10166	5.958928	9.91817	0.000580	42.55702	75.64630
(1)**Pre-saccharification Time (min)(**L)	0.12270	0.061957	1.98040	0.118747	-0.04932	0.29472
Pre-saccharification Time (min)(Q)	-0.00024	0.000212	-1.13946	0.318114	-0.00083	0.00035
(2)**Stargen dosage (µl/100 g starch)(**L)	0.13691	0.029046	4.71352	0.009216	0.05626	0.21755
Stargen dosage (µl/100 g starch)(Q)	-0.00016	0.000046	-3.42270	0.026714	-0.00029	-0.00003
1L by 2L	-0.00013	0.000131	-0.95945	0.391664	-0.00049	0.00024

Ethanol productivity:

Factor	Regressn Coeff.	Std.Err. Pure Err	t(4)	p	-95.% Cnf.Limt	+95.% Cnf.Limt
Regr. Coefficients; Var.**Ethanol productivity (g/L/h)** R-sqr=.88945; Adj:.81048 2 factors, 1 Blocks, 13 Runs; MS Pure Error=.0175296 DV: **Ethanol productivity (g/L/h)**						
Mean/Interc.	0.444234	0.392771	1.13102	0.321261	-0.646274	1.534742
(1)**Pre-saccharification Time (min)(**L)	0.002093	0.004084	0.51245	0.635334	-0.009246	0.013431
Pre-saccharification Time (min)(Q)	-0.000017	0.000014	-1.21540	0.291042	-0.000056	0.000022
(2)**Stargen dosage (µl/100 g starch)(**L)	0.006381	0.001914	3.33302	0.029024	0.001066	0.011697
Stargen dosage (µl/100 g starch)(Q)	-0.000010	0.000003	-3.12753	0.035269	-0.000018	-0.000001
1L by 2L	0.000017	0.000009	1.99571	0.116687	-0.000007	0.000041