EVALUATION OF RECOMBINANT YEAST STRAINS EXPRESSING A XYLANASE, AMYLASE OR AN ENDO-GLUCANASE IN BREWING

by

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[redacted]

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DECLARATION

I hereby declare that the dissertation submitted to the University of Limpopo for the degree of Master of Science in Microbiology has not previously been submitted by me for any degree at this or any other University and that it is my own work and all the material contained therein has been properly acknowledged.

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Date: 15/01/2018

DEDICATIONS

I would like to dedicate this work to my wife Mamodumo Debora Makuru, my son Sehubetshwane Frans Teballo Makuru and my Mother Mmathapelo Christinah Makuru.

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ABSTRACT

Beer is one of the most widely consumed alcoholic beverages in the world. The brewing process is based on natural enzymatic activities that take place during the malting of barley grain, mashing of grist and fermentation of wort. Insufficient malt enzyme activity during the mashing process leads to high levels of barley β-glucan, arabinoxylan (AX) and dextrins in the wort as well as in the final beer. It was reported that high levels of β-glucan and AX increase wort and beer viscosity which lower the rate of beer filtration and this negatively affect the production rate in the brewery. During beer fermentation, brewing yeast catalyses the conversion of wort sugars to ethanol, carbon dioxide and other metabolic products. However, non-fermentable carbohydrates i.e., limit dextrins remain in the wort and final beer. These non-fermentable carbohydrates are known to contribute to the caloric value of beer which might lead to weight gain in consumers.

The objectives of this study were to evaluate the effect of recombinant yeast strains expressing an endo-β-1,4-glucanase or an endo-β-1,4-xylanase on beer viscosity (as an indicator of filterability) and an α-amylase on residual sugars levels. The effect of the above mentioned enzymes on the aroma, appearance, flavour, mouth-feel and overall quality of the beer was also determined. Wort was produced in the University of Limpopo micro-brewery and the wort was pitched with different recombinant strains. The wild-type strain served as control. The results obtained showed that the xylanase expressing strain produced a measurable decrease in viscosity over the course of the fermentation, but endo-glucanase did not have any effect on the beer viscosity. The α-amylase producing strain, did not show a measurable reduction of residual sugars in the final beer probably as a result of very low activity on α-1,6 glycosidic bonds in dextrins during fermentation. The xylanase and α-amylase producing strain fermented effectively with good attenuation (decrease in wort specific gravity). The beer produced by the α-amylase and control strains were preferred in terms of taste and had similar qualities. The secreted amylolytic activity was not sufficient to significantly reduce residual sugar in the final beer. Although the xylanase secreting strain produced a beer with lower viscosity, the enzyme had a negative impact on the taste of the beer.

Key words: Brewer's yeast, beer fermentation, low calorie beer, amylase, xylanase, endo-glucanase.

CHAPTER 1: INTRODUCTION

1.1 Background information

Beer is one of the most popular alcoholic beverages that people consume in large quantities in almost every country in the world (Nakao *et al.*, 2009). The brewing of beer is a traditional process that has been taking place for thousands of years and is now a huge global industry. The brewing process is sometimes referred to as old biotechnology, because of its long history (Ferreira *et al.*, 2010).

Previously, brewers produced beer without knowledge or an understanding of the involvement and importance of enzymes in the brewing process. The brewing of beer is based on natural enzymatic activities taking place during malting of barley grain, mashing of grist and fermentation of wort which is the sweet liquid extracted from grain (Bamforth, 2009). Barley, *Hordeum vulgare* L, is the fourth largest cereal crop produced worldwide and is mostly used in the food industry for only two purposes *i.e.* feed and malt production (Limberger-Bayer *et al.*, 2014).

The malting of barley is key in the production of enzymes for the brewing process and involves steeping, germination and kilning. During germination, starch converting enzymes or malt enzymes *i.e.*, α-amylases, β-amylases limit dextrinases and α-glucosidases as well as endo-β-glucanases, endo-β-xylanases are synthesized (Scheffler and Bamforth, 2005). The latter two enzymes hydrolyse barley cell wall components (β-glucan and arabinoxylan) and the starch endosperm (amylose and amylopectin). Many of the malt enzymes such as endo-β-glucanase and endo-β-xylanase are heat sensitive. These heat labile enzymes are inactivated during the kilning process where malt is roasted at a specific temperature depending on the type of malt needed.

The brewing enzymes are also reported to loose activity during mashing as a result of incubation at high temperatures (typically 63°C) (Jonkova and Surleva, 2013). The low activity of cell wall hydrolysing malt enzymes during mashing leads to high levels of barley β-glucan and arabinoxylan (AX) in the wort as well as beer. The presence of high molecular weight β-glucan and AX was reported to cause severe wort separation

problems such as increased viscosity, which reduces beer filtration rate in the brew house (Li *et al.*, 2005).

The increasing demand for low calorie beer due to consumer health concerns is gaining popularity world-wide. High concentrations of non-fermentable carbohydrates contribute to the calorie content of beer, which causes weight gain in consumers (Wang *et al.*, 2010). The caloric value of beer results from ethanol and from non-fermentable carbohydrates in the form of dextrins (Ragot *et al.*, 1989).

Therefore, the reduction of residual dextrins could reduce the caloric value of beer. Low calorie beer has been in the market for several years now. Its production is often troublesome requiring specialised mashing schedules since the brewing yeast, *Saccharomyces cerevisiae,* lacks the ability to produce amylolytic enzymes for efficient degradation of starch molecules to fermentable sugars (Zhang *et al.*, 2008b). Therefore, the production of low calorie beer in breweries remains a challenge in the brew-house.

An alternative approach would be the addition of exogenous enzymes such as α-amylase, glucoamylase and dextrinase of microbial origin during the mashing process in order to reduce non-fermentable carbohydrates in beer (Jonkova and Surleva, 2013). However, the cost of preparation of food grade commercial enzymes limits the feasibility of this strategy (Wang *et al.*, 2012). A study by Liu *et al.* (2004) reported the introduction of a *Saccharomycopsis fibuligera* α-amylase into a *S. cerevisiae* brewing yeast in order to hydrolyse dextrins and eliminate the need of adding expensive commercial enzymes during beer fermentation. This strain did not hydrolyse dextrins due to low activity on α-1, 6-glycosidic bonds.

The focal point of this study was to evaluate the effect of recombinant yeasts expressing an α-amylase, endo-β-1,4-glucanase or an endo-β-xylanase on beer viscosity, residual sugars, aroma, appearance, flavour, mouth-feel and overall quality. The genes for the recombinant enzymes used in this study, were isolated from various sources. The α-amylase was isolated from *Lipomyces kononenkoae*, the endo-β-xylanase from *Trichoderma reesei,* and the endo-β-1,4-glucanase from *Butyrivibrio fibrisolvens.*

1.2 Research Problem

The demand for low calorie beverages has increased in recent years as consumers are becoming more health conscious (Liu *et al.*, 2004). The brewing yeast *S. cerevisiae* lacks the enzymes needed to hydrolyse polysaccharides in beer. Incomplete hydrolysis of starch results in dextrins in beer which increase the calorific value of beer, leading to weight gain in consumers, a major health concern. The presence of AX and β-glucan in beer, on the other hand leads to an increase wort viscosity which decrease beer filtration rates.

1.3 Motivation of the study

Brewing starts when ground malted barley is heated in a controlled process called mashing. During the mashing process, enzymes (i.e. β-amylase and α-amylase) hydrolyse starch components (amylose and amylopectin) and produce fermentable carbohydrates (glucose, maltose) as well as non-fermentable carbohydrates (limit dextrins) while the husks lead to glucan and xylan in wort (Harrison, 2009). Hence, the recombinant expression of the appropriate hydrolases in the brewing yeast may remove unwanted polysaccharides with subsequent decrease in wort viscosity and reduced caloric levels in beer.

1.4 Aim

In a study at the University of Stellenbosch, three recombinant brewing yeast strains have been constructed. These strains express either an α-amylase, endo-β-1,4-glucanase or an endo-β-1,4-xylanase (La Grange, 1999). This study will evaluate enzyme production by these recombinant brewing strains and their effects on beer residual sugar and viscosity under micro-brewery conditions. The resulting beers will also be evaluated on aroma, flavour, mouth feel, appearance, and overall quality.

1.5 Objectives

i. Determine the enzyme activity of xylanase, endo-glucanase and an α-amylase produced by the respective recombinant yeast strains.

ii. Determine the effect of the recombinant α-amylase from *Lipomyces kononenkoae* on beer residual sugar and ethanol concentration.

iii. Determine the effect of the recombinant endo-β-1,4-glucanase from *Butyrivibrio fibrisolvens* and endo-β-xylanase from *Trichoderma reesei* on beer viscosity.

iv. Determine the effect of α-amylase, glucanase, and xylanase on the aroma, appearance, flavour, mouth-feel and overall quality of the beer.

CHAPTER 2: LITERATURE REVIEW

2.1 Brewing

The principal aim in beer brewing is to hydrolyse the starch molecules in malted barley into a sweet liquid, “wort” containing fermentable sugars (maltose and glucose) (Liao *et al.*, 2010). The simple sugars are then consumed by the brewer’s yeast *saccharomyces cerevisiae* to produce ethanol, CO_2 and other flavouring metabolic by-products (Tang and Li, 2017). The main biological changes occurring in the brewing process are cause by natural enzymes from barley during the process of malting (Bamforth, 2009).

The brewing process consists of six steps: 1. malting of barley (steeping, germination and kilning), 2. Mashing of grist, 3. Wort lautering (separation), 4. Boiling, 5. Fermentation and 6. Post fermentation processing (Figure 2.1) Waites *et al.*, 2001. Fermentable sugars are produced during mashing and this step involves the use of barley enzymes to break down starch component into fermentable sugars and proteins into amino acids which are used as nutrients by yeast for growth and fermentation, respectively (Epinosa-Ramirez *et al*., 2014).

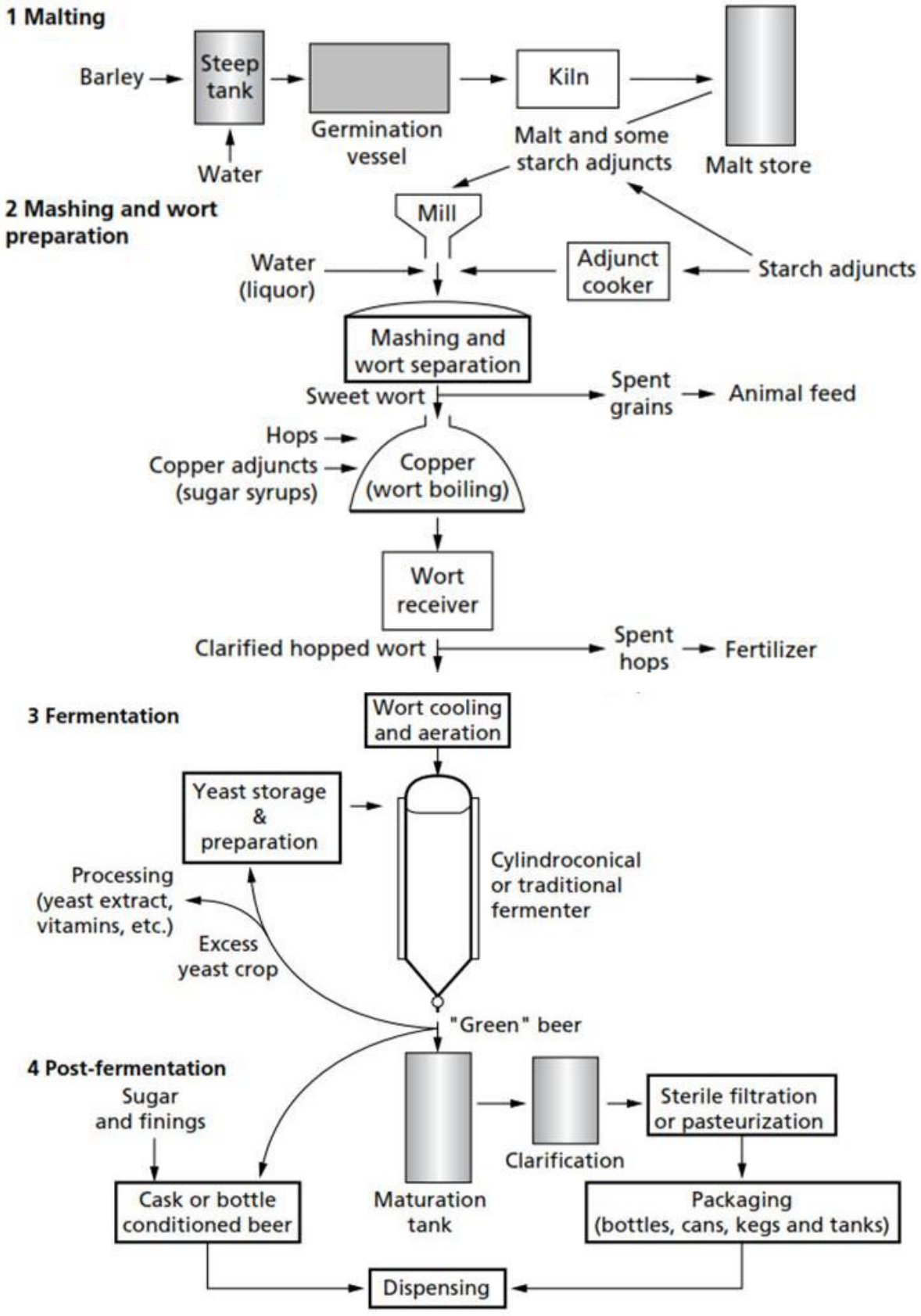

Figure 2.1. A schematic outline of the brewing process (Waites *et al.*, 2001).

2.1.1 Malting

The process of malting is important for the production of enzymes that degrade starch, proteins, and other grain components. Malted barley is the cereal most often used in beer production (Mathias *et al*, .2014). The barley kernel consists of a husk covering an embryo inside. Part of the kernel is the endosperm, which consist of large dead starch filled cells encapsulated by a thick cell wall composed of mainly β-glucan (a polymer of glucose molecules linked by β-1,4-glycosidic bonds) and pentosan (an arabinoxylan polymer) as well as a small amount of protein (Figure 2.2). (Aastrup *et al.*, 2004).

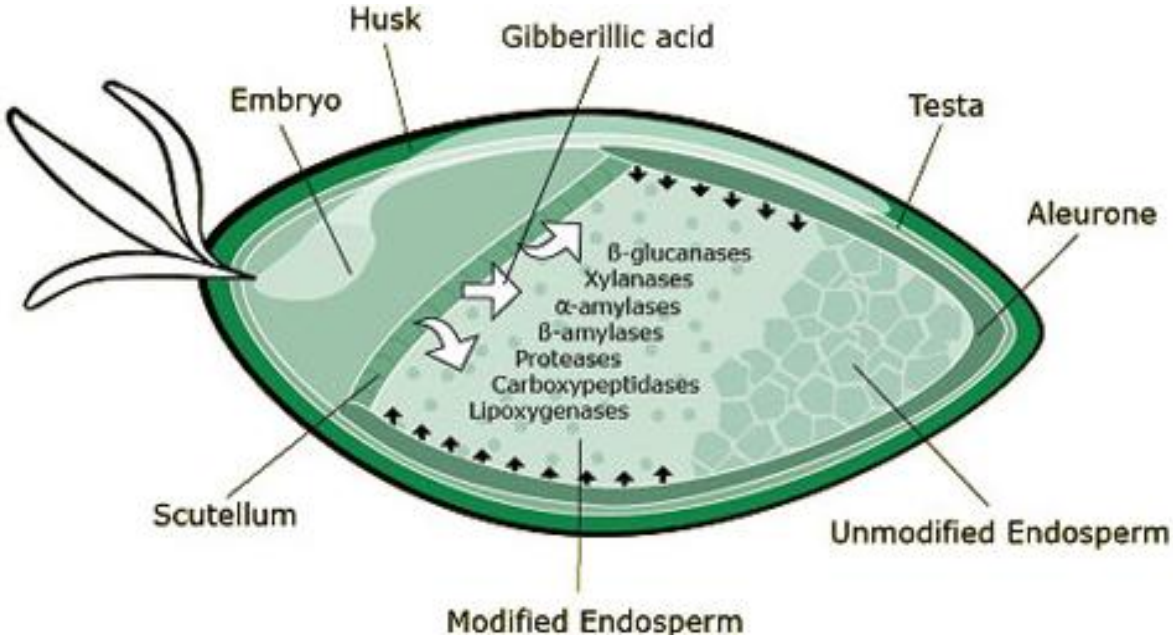

Figure 2.2. Schematic representation of the structure of a barley kernel (Aastrup *et al.*, 2004).

The malting process relies on the steeping, germination and kilning of barley grain (Limure and Sato, 2013). After harvest, the barley is immersed in 10 - 15°C aerated water for four to six days in a process called “steeping”. During this period the germination cycle is stimulated and the water content of barley increases to 38 - 45%. In modern breweries the steeping process is under strict control to ensure extracts with high enzyme activities (Linko *et al.*, 1998). After germination the barley grains are heated / roasted at 45 to 150°C in a process called kilning to suspend all the biochemical activities in the barley and also to facilitate the development of colour (Jin

et al., 2013). The kilning temperatures normally depend on the desired malt (Table 2.1). The main purpose of kilning is to halt germination at the desired endpoint (Schmitt *et al.*, 2013).

There are various kilning temperatures used in brewing, depending on the type of malt needed (Table 2.1). Some malts are roasted completely hence high temperatures are required while others, like lager malt only requires mild kilning at up to 85°C.

Table 2.1. Kilning temperatures of five different types of malt used for wort production (Moreira *et al.*, 2013).

	Malt type	Kilning Temperature (°C)
Light Malts	Pilsner/ lager	85
	Melano	130
	Carared	160
Dark Malt	Chocolate	220
	Black	230

The kilning process provides barley malt with specific characteristics which gives the final beer a special colour, aroma and taste (Hucker *et al.*, 2012). The malt is stored in a dry, dark and well ventilated room at a temperature of 10°C after kilning. Most of the endogenous cell wall degrading enzymes such as glucanase and xylanase responsible for the hydrolysis of β-glucans and arabinoxylan, respectively are heat inactivated, and high non-polysaccharide β-glucans remaining may cause problems such as high viscosity of brewers mash as well as turbidity (Bai *et al.*, 2010).

2.1.2 Mashing

Mashing has been referred to as the engine of beer brewing (Durand *et al.*, 2009). This process involves the use of various temperatures depending on the type and style of the desired beer (Figure 2.3). Normally, during mashing, the ground malted barley is mixed with hot water at 45°C, for degradation of β-glucan by β-glucanase and 52°C. For proteolysis (protein rest) proteins are modified and degraded to amino acid by malt

proteases to serve as nitrogen source for the brewing yeast (Limure and Sato, 2013). The temperature of the mixture is then increased and maintained at 63°C for β-amylase to hydrolyse starch to maltose.

The temperature is increased to 72°C to enhance α-amylase activity for the stage known as the saccharification rest. After saccharification rest, the temperature is further increased to 80°C to ensure the inactivation of all enzymes in the mash.

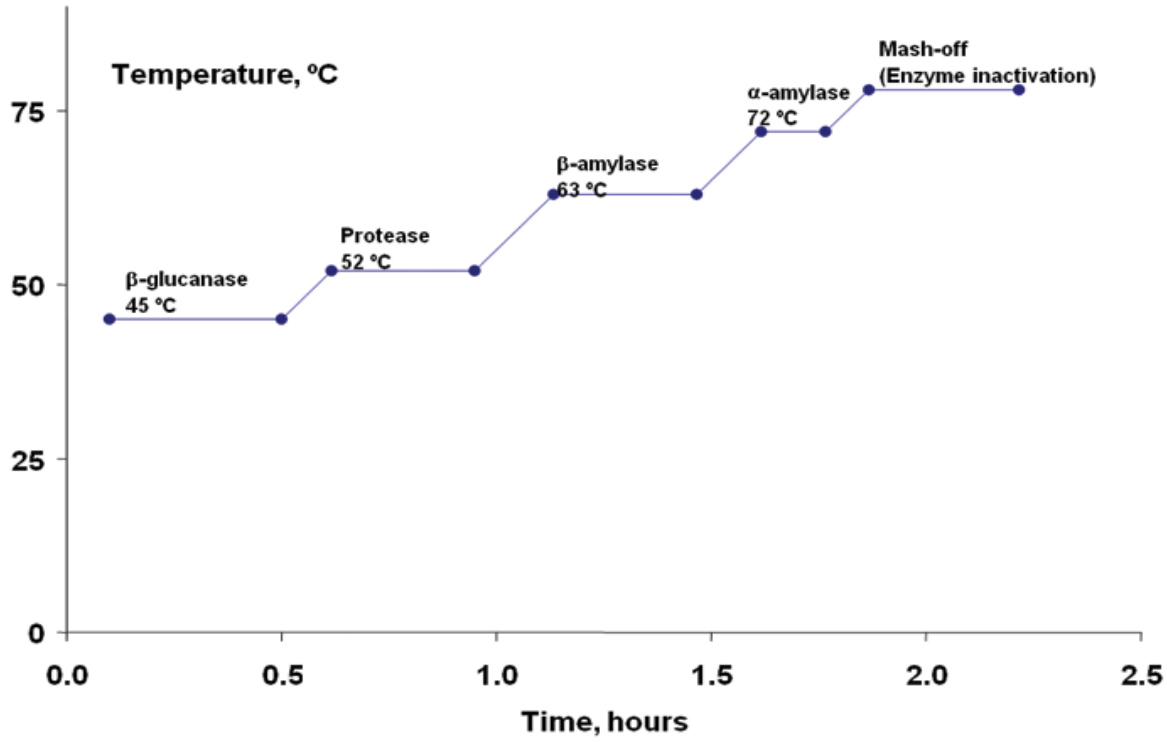

Figure 2.3. The traditional mashing temperature profile is determined by the temperature optima for the various malt enzymes (Aastrup *et al.*, 2004).

The main purpose of mashing is to ensure the hydrolysis of barley starch components, to produce a wort with suitable amounts of fermentable sugars and various nutrients for the growth of the brewing yeast (Montanari *et al.*, 2005).

Starch consist of amylose and amylopectin (Derde *et al*., 2012). The α-amylases (EC. 3.2.1.1) are endo-amylases which randomly hydrolyse the internal α-1,4 linkages in the glucose polymers to produce oligosaccharides along with α-D-maltose and dextrins, while β-amylases (EC.3.2.1.2) are exo-amylases, which catalyse the

successive removal of maltose from non-reducing ends of glucose polymers producing β-maltose from the amylose portion of starch.

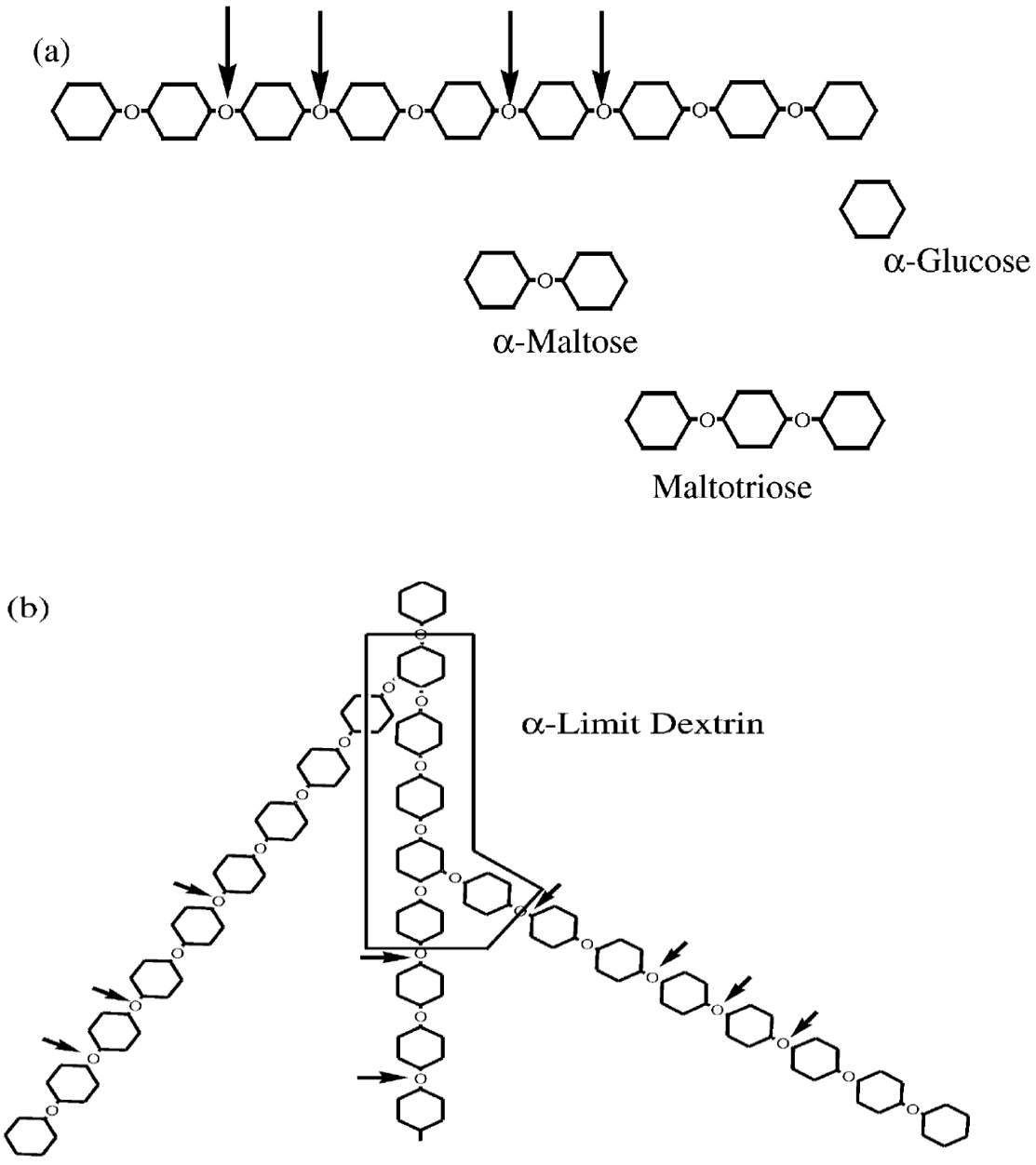

Figure 2.4. The reaction of α-amylase on (a) amylose and (b) amylopectin (Muralikrishna and Nirmala, 2005).

Other enzymes (i.e. glucanase, xylanase, proteases, and dextrinases) are also synthesised during malting. However, most of these enzymes are inactivated during mashing due to their temperature sensitivity (Table 2.2), resulting in high non-starch

polysaccharides (β-glucans, arabinoxylan) in the mash, which subsequently increases the viscosity of the wort (Bai *et al.*, 2010).

Table 2.2. Properties of starch and cell wall degrading enzymes in brewing (Willaert, 2007).

Enzymes	pH Optimum	Temperature °C		Hydrolysis Reaction
		Optimum	Inactivation	
Starch hydrolysis				
α-amylase (EC. 3.2.1.1)	5.5 – 5.8	70 – 75	80	α-1, 4 bonds
β-amylase (EC.3.2.1.2)	5.4 – 5.6	60 – 65	70	α-1, 4 at reducing end
Limit dextrinases	5.1– 5.5	55 – 60	65 – 70	α-1, 6 bonds in starch
Saccharase	5.5	50	55 – 67	Saccharose
Cell wall degradation				
Endo-β-1, 4-glucanase (EC 3.2.1.4)	4.5 – 5.0	40 – 45	50 – 55	β-1, 4 bonds
Endo-xylanase (EC.3.2.1.8)	5.0	45		Pentosan, xylan chain

In morden breweries, exogenous enzymes are often added during mashing to ensure the complete hydrolysis of non-starch polysaccharides (Jonkova and Surleva, 2013). Due to the cost of commercial enzymes preparations, recombinant yeast strains that secrete amylolytic enzymes with the ability to hydrolyse complex polysaccharides, have been developed (Pretorius, 2000).

2.1.3 Wort lautering (separation)

Lautering is the process where the sweet wort is separated from the spent grain with the grain husks acting as a filter in the mash tun (Figure 2.5) (Wunderlich and Back, 2009). The grain bed is sparged (rinsed) with hot water (80°C) to extract the maximum amount of sugars without extracting tannins from the grain husks. Temperature is critical at this stage, because temperatures above 80°C could increase the viscosity

and that will results in poor lautering, because the wort will contain particles. At this stage most enzymes are inactivated (Wunderlich and Back, 2009). During lautering wort is transferred to a vessel known as the wort kettle for boiling.

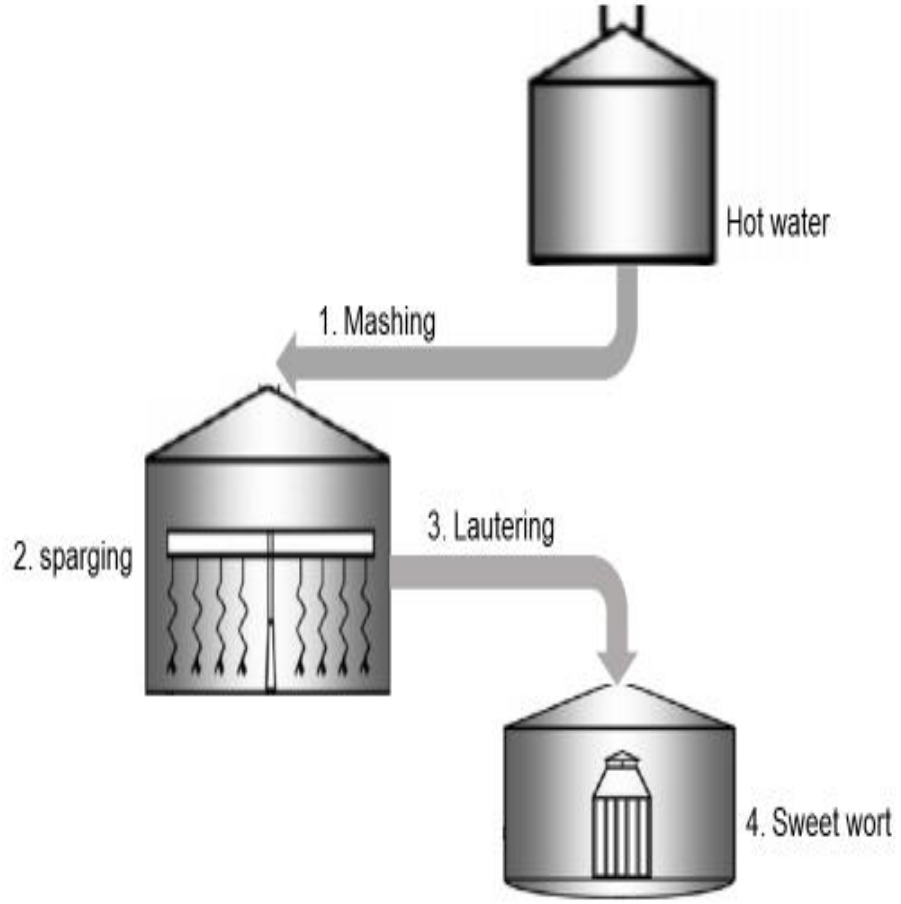

Figure 2.5. Diagram depicting the lautering process used to separate the sweet wort from the spent grain. Adapted from (Wunderlich and Back, 2009).

2.1.4 Wort boiling

The purpose of wort boiling is to ensure maximum extraction of the hop components; sterilisation of the wort, colour development, removal of undesirable volatiles, coagulation of proteins, inactivation of all remaining malt enzymes and removal of dimethyl sulphide through evaporation (Limure and Sato, 2013).

The wort is normally boiled for one to two hours. During boiling, hops (*Humulus lupulus*) are added. The hops flowers contain pinhead-sized glands of lupulin. The lupulin carries bitter acids and essential oils which gives beer its bitter taste, aroma, flavour, colour and helps in preserving the beer (Ferreira *et al.*, 2010).

At the beginning of fermentation, yeast cells are known to contain insufficient amounts of sterols and unsaturated fatty acids and need oxygen to synthesise these essential membrane components (Akada, 2002). Therefore, after boiling, the wort is cooled to about 13 - 16°C and aerated to ensure efficient yeast sterol production. The wort is then pumped into a fermentation vessel and “pitched” (inoculated) with the appropriate yeast strain to initiate fermentation.

The strain of yeast used depends on the type of beer desired. Normally, lager beers are produced using bottom fermenting yeast, which ferment at a temperature range of 6 - 15°C with the fermentation process taking 2 - 7 days, while ale beers are produced by top-fermenting yeast at the temperature of 18 - 27°C and takes 5 - 7 days (Harrison, 2009).

2.1.5 Fermentation

Fermentation is broadly defined as the metabolism of simple sugars (glucose, maltose, maltotriose) by microorganism to produce ethanol and CO_2 as well as various metabolic by-products including aldehydes, esters and organic acids (Bai *et al.*, 2008). The most commonly used microorganism in alcoholic fermentation is *S. cerevisiae*. Alcoholic fermentation, particularly beer, has two separate stages namely primary fermentation and maturation (Montanari *et al.*, 2005).

During primary fermentation, wort sugars are converted to ethanol and carbon dioxide under anaerobic condition (Bamforth, 2009). Secondary fermentation (maturation) occurs only once all the sugars have been consumed and the green beer is stored. During this process a priming sugar may be added to stimulate carbonation as well as to boost yeast metabolism and aid in the conditioning process (Ferreira, 2009). Fermentation of beer occurs at various temperatures, depending on the type of beer

produced (Table 2.3) and also the type of yeast used. During the fermentation process some of the yeast proteins are released into the beer (Limure and Sato, 2013).

Table 2.3. Beer fermentation temperatures (Saerens *et al.*, 2010)

Type of beer	Temperature (°C)
Lager	8 - 15
Ale	15 - 26
Stout	17- 25
Guinness	18 - 25

2.2 Polysaccharides in beer

2.2.1 Starch

Starch is the major carbon reserve in most plants and a biopolymer of considerable significance to humans (Hii et al., 2012). It is considered a macro-constituent in many food products and has many applications in industry (Wang *et al.*, 2011). It is synthesised by plants in the presence of sunlight and water via photosynthesis. The structure of starch has an impact on its degradation rate, since starches of different origin have different ratios in terms of amylose and amylopectin. The representative percentages of amylose and amylopectin in barley starch have been found to be 25 - 28% and 72 - 75%, respectively (Sharma and Satyanarayana, 2013).

In the sweetener industry, the conversion of starch to sugars used to be carried out through acid hydrolysis. This method has drawbacks and acid has been replaced by starch hydrolysing enzymes for the production of glucose from starch. These enzymes are also used in other industrial applications such as the baking and detergent industries (Bamforth, 2009). The hydrolysis of amylose is simpler due to its linear chain of glucose molecules whereas amylopectin is highly branched in nature complicating enzymatic hydrolysis (Nielsen *et al.*, 2012).

Amylose is a linear polymer of glucose made up of α-1,4-D-glucopyranosyl residues containing less than 1% α-1,6 branching points (Figure 2.6) (Derde *et al.*, 2012). The

molecular weight and the degree of polymerisation (DP) of amylose varies depending on the source and the origin of the starch (Hii *et al.*, 2012). A linear chain of amylose is completely degraded by α-amylase and β-amylase yielding fermentable carbohydrates (glucose and maltose) (Naguleswaran *et al.*, 2014).

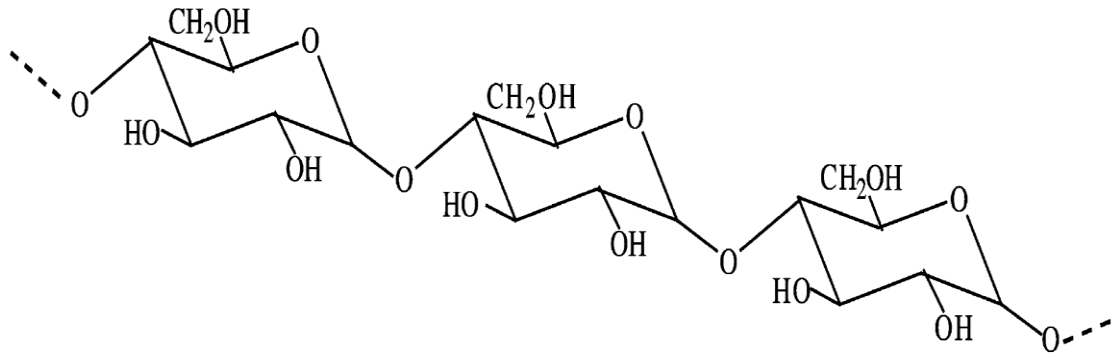

Figure 2.6. Structure of amylose (Muralikrishna and Nirmala, 2005).

Amylopectin is a highly branched polysaccharide consisting of a chain of both α-(1,4) linked D-glucopyranosyl units with α-(1,6) branch point (Figure 2.7). Both α-amylase and β-amylase act on the hydrolysis of amylopectin to yield soluble sugars (glucose, maltose), and branched low molecular weight limited dextrins (Naguleswaran *et al.*, 2014). The malt enzymes cannot hydrolyse α-1,6 bonds in amylopectin during mashing (most are heat inactivated), and hence the limit dextrins remain in the wort and final beer.

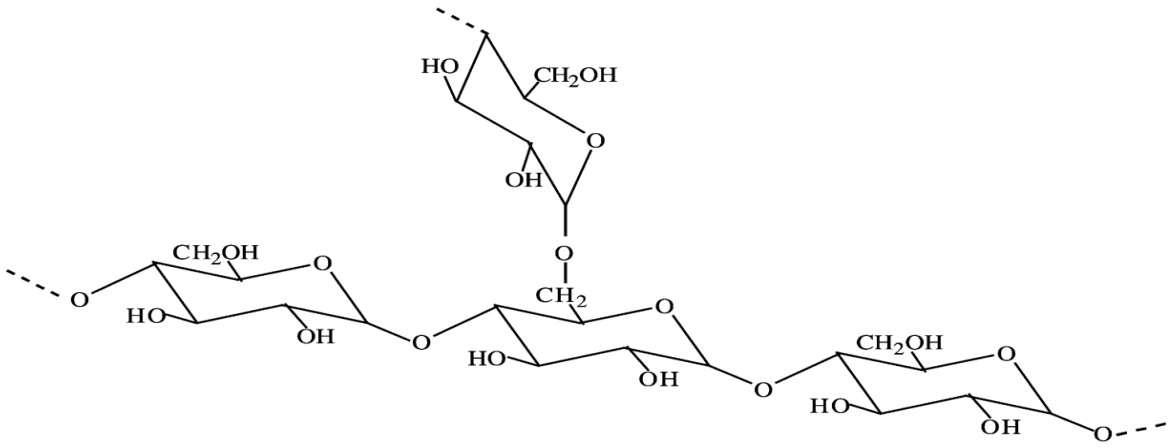

Figure 2.7. Structure of amylopectin (Muralikrishna and Nirmala, 2005).

2.2.2 Arabinoxylan

Arabinoxylans (AX) are non-starch polysaccharides located in the cell wall of the aleurone layers and endosperm of barley grains (Fox, 2009). Structurally AX found in barley is made up of linear chains of D-xylopyranose residues joined by β-(1,4)-glycosidic bonds with units such as L-arabinofuranose attached as branches at β-(1,2) or β-(1,3) linkages (Figure 2.8) (Han, 2000).

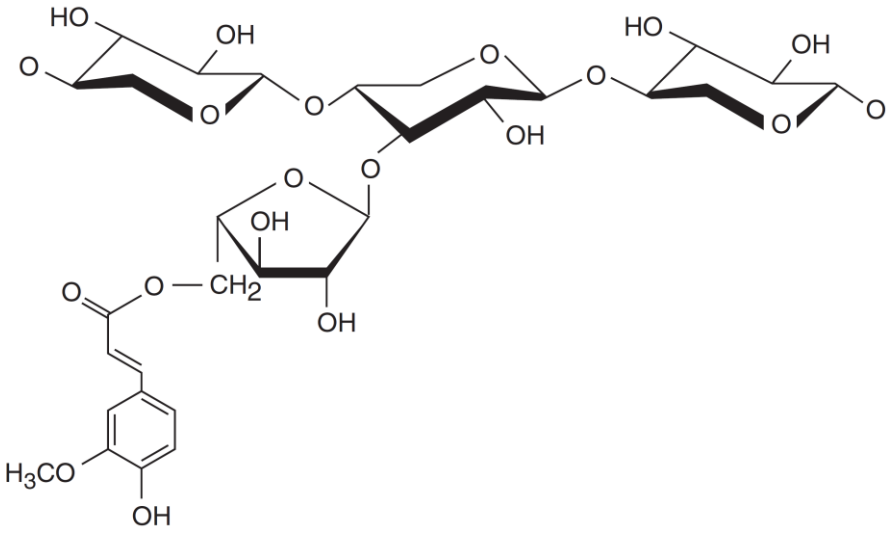

Figure 2.8. Structure of arabinoxylan (AX) (Fox, 2009).

Arabinoxylan can be extracted with hot water from the cell walls of the starchy endosperm. About 50% of AX in barley is found in the husk. In the brewing industry, the barley husks contribute to the filter bed formed in the mash tun during the mashing process for smooth lautering. Several enzymes (1,4-β-endoxylanases, β-D-xylosidases, α-L-arabinofuranosidases and esterases) are involved in the hydrolysis of AX (Fox, 2009). These enzymes are synthesized during the steeping and germination processes. Endoxylanase is the key enzyme which breaks down the xylan backbone into short xylooligosaccharides (Zhao *et al.*, 2013).

However, most of the barley 1,4-β-endoxylanases are sensitive to high temperatures and they lose activity during kilning and mashing (Li *et al.*, 2005). The incomplete

degradation of AX and β-glucans during malting and mashing are known to cause problems such as high wort viscosity, poor filtration rate as well as beer haze (Fox, 2009). Therefore, brewers are continuously looking for ways to improve filtration technologies, while on the other hand barley breeders are trying to produce barley cultivars with lower levels of AX and β-glucans.

2.2.3 β-glucans

Beta-glucans are the main constituent of the endosperm cell wall in barley and it is a high-molecular weight carbohydrate consisting of chains of glucose units, linked by mixed β-1,3 and β-1,4-glycosidic bonds (Figure 2.9) (Vis and Lorenz, 1997). Based on the type of glycosidic linkages, β-glucans are grouped into four categories namely, β-1,3-1,4-glucans (lichenan), β-1,4-glucan (cellulose), β-1,3-glucan (curdlan) and β-1,3-1,6-glucan (laminarin). Barley β-glucans has been reported to reduce the filterability of worts and the cause haze in beer (Lu *et al.*, 2009).

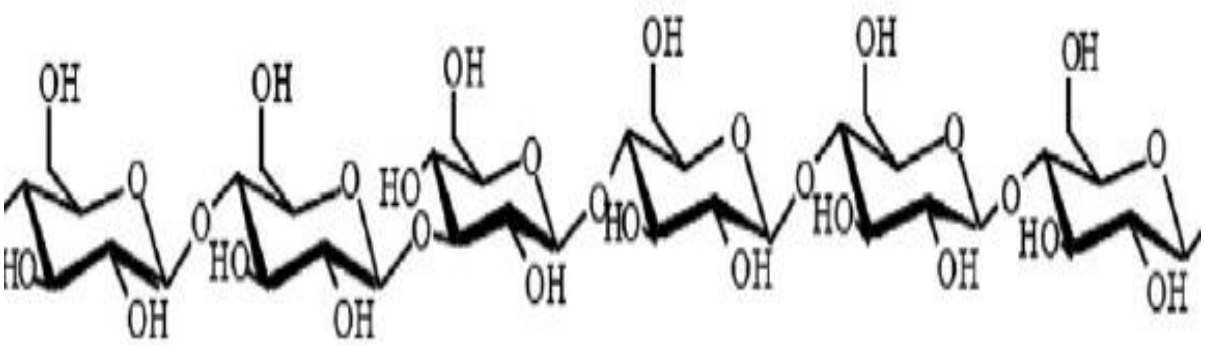

Figure 2.9. Structure of β-1,3-1,4 glucan from barley (Zhu *et al.*, 2016).

Endo-β-glucanases are enzymes responsible for the breakdown of barley β-glucan during the malting process (Petersen, *et al.*, 2013). The degradation of β-glucan depends on the amount of cell wall degrading enzymes and the cell wall structure. These enzymes are only present in small quantities in the wort. Due to their temperature sensitivity, they are unable to efficiently degrade β-glucan during mashing, resulting in β-glucan in wort and also beer (Davydenko *et al.*, 2010).

2.3 Role and effect of enzymes in beer production

Beer is a fermented alcoholic beverage that is made from malted cereal grain containing carbohydrates, amino acid, minerals, vitamins and compounds rich in polyphenols (Nogueira *et al*., 2005). The most common raw materials used for beer production are malted barley, adjuncts, water, yeast and hops (Epinosa-Ramirez *et al.*, 2014). Barley contains natural enzymes which play a very important role in beer production.

Limure and Sato (2013) reported that enzymes such as α-amylases, β-glucanases, proteases, limit dextrinases and α-glucosidases are synthesized during the malting process and are activated during the mashing process. During the brewing process, enzymes are responsible for the hydrolysis of starch in barley to fermentable sugars (glucose, maltose and maltotriose), which are subsequently fermented to ethanol by the yeast (Figure 2.10). The mechanism and dynamics of starch hydrolysis are known to be affected by various factors, such as the size and structure of starch grains and the proportionality of amylose and amylopectin and the type of grain used as the source of starch (MacGregor *et al*., 2002). Amylose is completely hydrolysed into glucose, maltose, and maltotriose while amylopectin is hydrolysed to glucose and limited dextrins (Bamforth, 2009). The sugars released from starch hydrolysis are used by the yeast as a carbon and energy source in the production of ethanol. However, not all sugars released during hydrolysis are fermentable, because brewing yeasts lack amylolytic enzymes to hydrolyse limit dextrins in wort (Wang *et al.*, 2012). In many breweries, exogenous amylolytic enzymes are used for efficient hydrolysis of starch to fermentable sugars (Limure and Sato, 2013). However, the commercial enzymes were reported to be prohibitively expensive and causes allergenic-related symptoms (Wang *et al*., 2010).

Enzymes also play an important role in the degradation of arabinoxylan (AX) and β-glucan during malting and mashing (Davydenko *et al.*, 2010). Enzymes such as xylanase and β-1,4-glucanase are produced during malting and are responsible for the hydrolysis of AX and β-glucan, respectively. However, both these enzymes are heat labile and are inactivated during kilning and mashing (Jonkova and Sureleva,

2013). High levels of AX and β-glucan remain in the wort after mashing, leading to an increase in viscosity (Davydenko *et al.*, 2010).

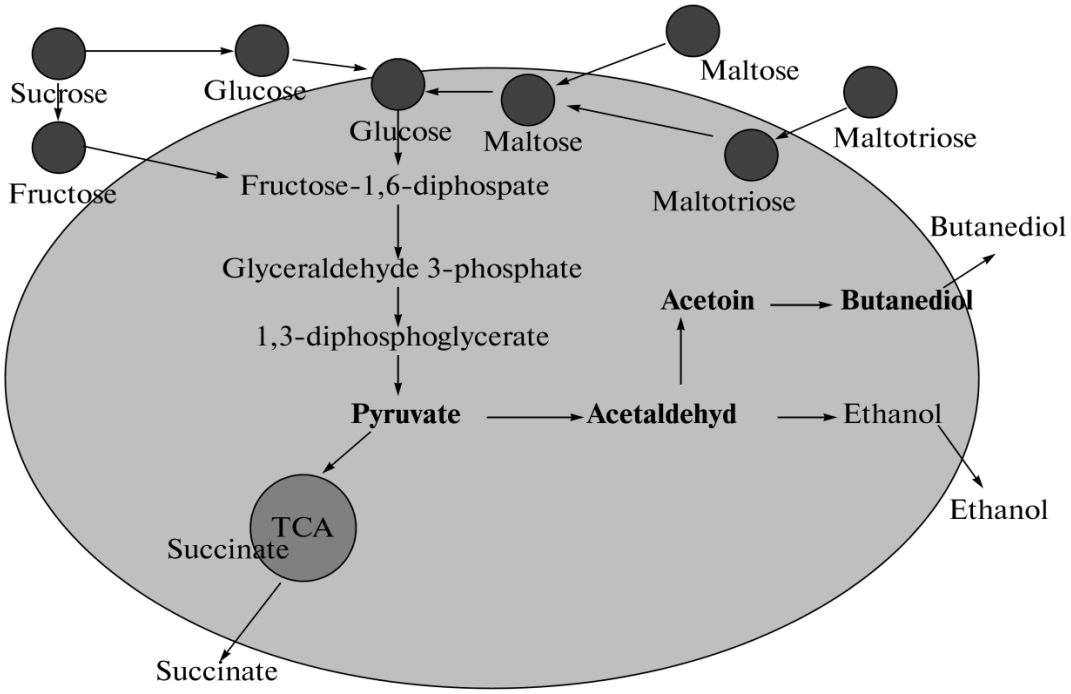

Figure 2.10. Sugar metabolism by the yeast *Saccharomyces cerevisiae* (Davydenko *et al.*, 2010).

2.4 Reduction of dextrins in beer

Dextrins are mixtures of D-glucose oligomers made of linear α-1,4 glycosidic and branched α-1,6 glycosidic linkages (Saerens *et al.*, 2010). It is a low molecular weight oligosaccharides resulting from the hydrolysis of amylopectin in starch (Wang *et al.*, 2012). Limited dextrins together with glucans and pentosans are the major non-fermentable sugars found in beer and account for about 20 - 25% of total wort carbohydrates. These non-fermentable carbohydrates remain in the wort during the brewing process and they contribute to the caloric value of beer, mainly due to the lack of amylolytic activity in the brewing yeast (Wang *et al.*, 2010). Therefore, in order to

produce low caloric beer, the carbohydrate concentration or residual saccharides, mainly dextrins should be reduced or removed completely from the wort (Park *et al.*, 2014).

The addition of exogenous enzymes (α-amylase, glucoamylase, and dextrinases) to wort prior to fermentation to degrade dextrins or excessive saccharides to fermentable sugars provide a solution to the problem. However, due to the cost of enzyme preparations high levels of dextrins is still a challenge to brewers. Therefore, an alternative approach of producing beer with low level of dextrins would be to use genetically modified brewing yeast strains capable of producing the required amylolytic enzymes to hydrolyse dextrins to fermentable sugars.

2.5 Low calorie beer

The market for low calorie beer or low carbohydrate beer is growing as consumers are becoming more health conscious (Zhang *et al*., 2008b). A beer with high caloric content is known to contribute to consumer's body weight. There are many conflicting opinions that calories from ethanol contributes to the weight gain of consumers, but most people who drink alcoholic beverages such as spirits are generally not overweight (Wang *et al.*, 2010).

When beer is consumed, ethanol is normally absorbed by the gastrointestinal tract through diffusion and is swiftly distributed in the blood, before it goes into the tissues. Ethanol is metabolised in the stomach and liver to toxic acetaldehyde (Rajendram and Preedy, 2009). Ethanol has a caloric value of 7.1 Kcal/g, compared to carbohydrates (dextrins) 3.9 Kcal/g. However, it appears as though calories in ethanol cannot be used effectively by humans, hence are sometime referred to as "empty calories". It was reported that alcohol can replace some nutrients that humans consume (carbohydrates or proteins) which then leads to malnutrition (Figure 2.11) (Lieber, 1991).

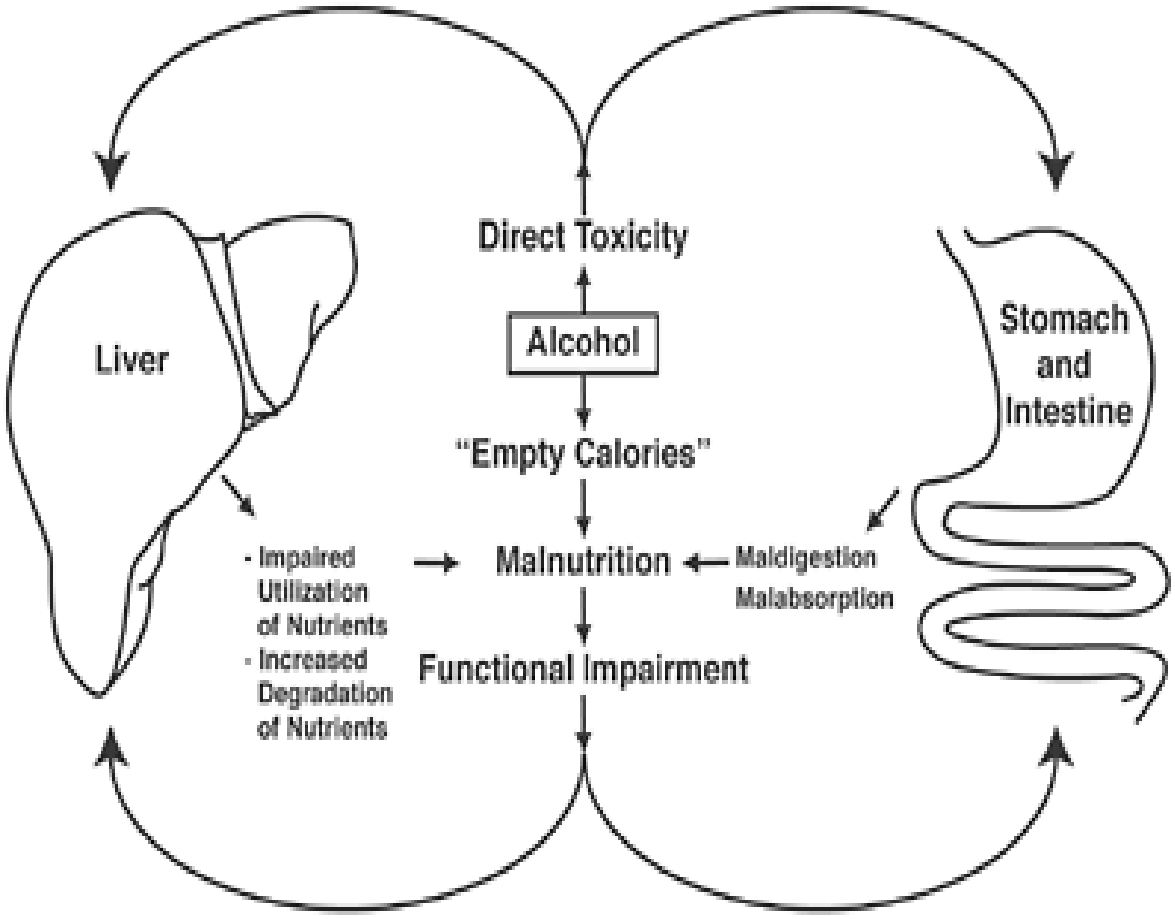

Figure 2.11. Representation of alcohol utilization in the human body (Lieber, 1991).

Depending on the type, beer is an alcoholic beverages rich in nutrients such as carbohydrates (Table 2.4), vitamins, minerals, amino acids and polyphenols (phenolic acids, flavonoids etc.). Beer wort contains monosaccharides (glucose, fructose), disaccharides (maltose, sucrose) as well as oligosaccharides (maltotriose). Maltose is known to be the major sugar in beer and incomplete fermentation of this sugar by yeast results in low ethanol and high residual sugar which increase the caloric value of beer (Saerens *et al.*, 2010).

Table 2.4. Carbohydrate, alcohol and calorie content of different beer styles (Bamforth, 2005).

Beer Style	Alcohol (%v/v)	Carbohydrate (g/12 fl oz.)	Calories (per 12fl oz.)
Lager	5.0	10.6	143
Pale ale	5.6	12.3	200
wheat beer	5.4	12.9	171
Light lager	4.2	6.6	110
ice beer	5.5	8.9	148
Barley wine	9.5	24.6	285
Low alcohol beer	0.4	5.8	96
Stout	4.0	10.0	125
Low carb lager	4.2	2.6	95

Fluid ounce (fl oz) is a unit of volume approximately equivalent to 30 millilitres (mL).

2.6 Beer filtration

Filtration is one of the most important and common processes used in the production of fermented beverages, particularly beer (Stewart *et al*., 1998). This process is crucial; at the end of beer fermentation, the green beer normally has a high degree of solids, barley residues, yeast cell and proteins which settle at the bottom of the fermenter. These components remain in the final beer and affect the value and quality of the beer and shorten its shelf life if not removed (Buttrick, 2007).

Breweries use various techniques such as centrifugation, cross-flow filtration, and powder filtration with diatomaceous earth (DE) or Keiselguhr as filtration aids. The most commonly used filters in breweries are frame and plate filters, although some breweries use candle filters (Cimini and Moresi, 2014). Keiselguhr is a filter aid deposited on porous membrane filters and also mixed with beer as it is fed into the filter. The beer is then forced through the membrane and solid particles are removed. In the cross-flow technique, unfiltered beer circulates across a polymeric 0.45–0.6 micron membrane. A cross-flow rate of approximately 1.2 m/s provides more pressure on the unfiltered side than the filtered beer side. This is known as trans-membrane differential pressure, which provides the force to push the beer through the membrane to take out any particles (Buttrick, 2007).

Although the techniques used are fairly reliable, some of the solid particles pass through into the final beer and affect the quality of the beer. Due to the fact that brewer's yeast *S. cerevisiae* is unable to produce hydrolytic enzymes which can efficiently degrade polysaccharides, brewers often add β-glucanases and/or xylanases to the wort to reduce filtration problems caused by β-glucans and arabinoxylan. The addition of β-glucanase during mashing decrease the viscosity and increase the filtration rate of wort (Celestino *et al.*, 2006). The addition of β-1,4 glucanase from *Phialaphora sp*.G5 was reported to show potential for the reduction of wort viscosity and improve the filtration rate (Zhao *et al.*, 2012).

CHAPTER 3: MATERIALS AND METHODS

Three recombinant Hoegaerden brewing yeast strains were generously provided by Prof W.H. van Zyl from the Department of Microbiology at the University of Stellenbosch. These strains express either the endo-β-1,4-xylanase from *Trichoderma reesei* (La Grange *et al.*, 1996), the endo-β-1,4-glucanase from *Butyrivibrio fibrosolvens* (Van Rensburg *et al.*, 1996) or the α-amylase from *Lipomyces kononenkoae* (Steyn and Pretorius, 1995). A non-recombinant Hoegaerden strain (wild type) was used as negative control.

3.1 Microbial strains

The sources and relevant genotypes of the yeast strains used (La Grange, 1999) are summarized in table 3.1.

Table 3.1. Microbial strains used in this study.

Yeast strain	Genotype	Source
Hoegaerden	Control strain	(La Grange, 1999)
Hoegaerden pDLG 21	bla *SMR1*-410 *PGK1*$_P$-*XYN2*-*PGK1*$_T$	(La Grange, 1999)
Hoegaerden pDLG 30	*bla SMR*1-410 *ADC1*$_P$-*MFα1*$_S$-*END1*-*PGK1*$_T$	(La Grange, 1999)
Hoegaerden pDLG 31	*bla SMR*1-410 *PGK1*$_P$-*LKA1*-*PGK1*$_T$	(La Grange, 1999)

3.2 Confirmation of enzyme production

Endo-β-1,4-xylanase production by Hoegaerden pDLG21 strain was confirmed on SC medium containing 0.2% 4-*O*-methyl-D-glucorono-D-xylan-remazol brilliant blue R (RBB-xylan) prepared according to the method described by Bailey *et al*., (1992). Hoegaerden pDLG21 was grown at 30°C for 24 hours. A clear zone around colonies on RBB-xylan plates indicate the presence of an active xylanase enzyme (La Grange *et al.*, 1996).

The endo-β-1,4-glucanase producing strain, Hoegaerden pDLG 30, was screened on Yeast potato dextrose (YPD) containing 1% yeast extract, 2% peptone, 2% glucose (Merck) and 1% carboxymethylcellulose (CMC) (Sigma). Plates were incubated at 30°C for 48 hours. After incubation the colonies were rinsed off the plates with Tris-ethylenediaminetetraacetic acid (Tris-EDTA) buffer (10mM Tris HCl, 1mM EDTA, pH 7) before staining with 0.1% Congo-red for 30 minutes. Plates were distained with 1M NaCl (Sigma) (Van Rensburg *et al.*, 1994). Colonies with glucanase activity show clear zone around the inoculum.

Alpha-amylase producing strains were confirmed on starch plates containing 1,5 g yeast extract, 2.0 g soluble starch, 0.5 g peptone, 1.5 g NaCl and 15.0 g bacteriological agar. Plates were incubated at 30°C for 48 hours before flooding with Gram's iodine solution (0.33% I_2, 0.66% KI and dH_20). Clear zone around colonies on a dark background indicate α-amylase activity.

3.3 Brewing

3.3.1. Raw materials

Grounded pale malt and pre-isomerised hops (PIH) pellets with an alpha acid content of 12%, were generously provided by South African Breweries, Polokwane. Munich malt and Vienna malt were purchase from The Beer Keg (Pretoria).

3.3.2. Small scale mashing

Nine kilograms of pale malt, 1 kg of Munich malt and 479.3 g of Vienna malt were added to a 50 L mash tun filled with 26 L of hot water (58°C) while stirring with a wooden spoon. The temperature and total volume changed to 45°C and 34 L, respectively after addition of the malts. The mash was stirred continually for 30 minutes at 45°C (protein rest). After 30 minutes, the temperature was increased and maintained at 63°C for 60 minutes (saccharification rest) and then increased further to 80°C for 5 minutes to inactive the malt enzymes.

3.3.3. Lautering

After five minutes of enzyme inactivation, the sweet wort was passed through the grain bed into the wort kettle. The wort was sparged with hot water (80°C) while monitoring the specific gravity of the wort with a hydrometer. Once a target gravity of 1.064 was reached, a total volume of 36 L of wort was collected and boiled for 60 min with the addition of 40 g of PIH hops to allow for a 50 min boil and another 20 g was added 10 min before the end of the boil. The gravity of the wort was adjusted from 1.070 to 1.064 by the addition of 3 L of 80°C water. The wort was then quickly cooled with a counter current heat exchanger to 20°C.

The three recombinant strains, Hoegaerden pDLG31 (α-amylase), Hoegaerden pDLG30 (endo-β-1,4-glucanase) and Hoegaerden pDLG21 (endo-β-1,4-xylanase) as well as the Hoegaerden wild type strain were routinely cultured in liquid YPD and incubated at 30°C in a rotary shaker (Exella E25 Advanced solutions) at 200 rpm. Five millilitres of the YPD cultures were used to inoculate 250 mL wort (gravity: 1.064) in 500 mL conical flasks and stirred overnight at room temperature. Three litres of the prepared wort was added to four 5 L Erlenmeyer flasks and pitched with the different yeast strains.

3.3.4. Fermentation

Fermentations in the 5 L Erlenmeyer flasks were carried out at 13°C in a circulating water bath for 10 days. Samples were taken on a daily basis and the degree of attenuation (measure of the degree to which sugar in wort has been fermented to alcohol) and viscosity were measured using hydrometer and viscometer, respectively. At the completion of fermentation, the beers were bottled and stored at 4°C for 10 days before sending to South African Breweries (SAB), Polokwane for tasting. The tasting panel examined the beer samples based on the aroma, appearance, flavour, mouth-feel and overall quality.

3.4. Beer viscosity

The viscosity of beer samples was measured in triplicates using a Cannon-Fenske capillary viscometer tube (Sigma) immersed in a 20°C water bath. Samples of beer were collected aseptically at 1 day intervals. A 15 ml sample of each beer was degassed and centrifuged (Heraeus Megafuge16R centrifuge, Thermo scientific) for 5 minutes at 5000 rpm. A 5 ml sample was transferred to the arm of the capillary viscometer and the flow rate of each beer sample was determined.

3.5. Alcohol concentration measurement

The concentration of alcohol was derived from the gravity using hydrometer (S.G 1.000-1.200, 300 MM, Lasec). The gravity measured before addition of the yeast, original gravity (OG) and during fermentation, final gravity (FG) was determined for each beer. Alcohol by volume (ABV) were calculated from alcohol by weight (ABW) as stated in the formula below:

- ABW = 76.08 (OG – FG) / (1.775 – OG)
- Therefore, Alcohol by volume (ABV)
- ABV = ABW (FG / 0.794)

3.6. Quantification of residual sugar

The analysis of glucose, fructose, sucrose and maltose from hydrolysis of dextrins were determined by High Performance Liquid Chromatography (HPLC) (Shimadzi, Tyoko, Japan) connected to a refractive index detector (RID 10A). Beer samples were filtered using 0.22 µm nylon syringe filters and transferred to HPLC vials. Separation was performed on a Rezex RCM-Monosaccharide Ca^{+2} (8%) column (300×7.80mm, Phenomenex, USA) using an isocratic flow (0.6 mL/min) of deionised water as eluent. The injection volume was 20 µL. The column temperature was maintained at 85°C. The carbohydrates in the beers were identified by comparison with retention times of their respective standard solutions. Standard solutions with different concentrations

were used (0.1 to 5 g/L) for each sugar (Ferreira, 2009). Chromatographic data were collected and processed using LC solutions (Shimadzu with LC 10AT and SPD-M10 AVP).

3.7. Beer taste profile

3.7.1. The tasting conditions.

The beer tasting occurred at room temperature in a tasting room in individual booths with minimised lighting. The room was quiet, free from distractions and ventilated with odour-free air allowing tasting to be performed in a controlled environment.

3.7.2. Tasting panel

Six professional testers were provided with palate cleansers (plain biscuits and carbonated water) before commencing with the test. All the beer samples were presented in a 500 mL glass containing 20 mL beer per glass covered with a glass top. A global score sheet (Table A1, Appendix 1) was use as a reference for recording the score of each beer sample.

CHAPTER 4: RESULTS

The brewing process involves various natural enzymatic activities, particularly during the mashing process. Most of these enzymes are inactivated at high temperatures (Jonkova and Surleva, 2013). Commercial brewer's yeasts are unable to hydrolyse residual sugar (i.e. dextrins) which contribute up to 25% of wort sugars and significantly increase the caloric content of beer. These can be removed by exogenous enzymes but this will increase the cost of beer production (Wang *et al.*, 2010). In this study, Hoegaerden brewing yeast strains expressing xylanase, glucanase and α-amylase were evaluated for their effect on beer viscosity, residual sugars levels and the overall beer quality.

4.1 Endo-β-1,4-xylanase production by Hoegaerden DLG21

The endo-β-1,4-xylanase production by Hoegaerden DLG21 was confirmed by plating on RBB-xylan medium. Clear zones around Hoegaerden DLG21 colonies are an indication of xylanase activity (Figure 4.1) (La Grange *et al*., 1996). The difference in size of the zones around colony 2 (24 hrs incubation) and colony 3 (48 hrs incubation) indicate that xylanase activity increased with an increase in incubation time.

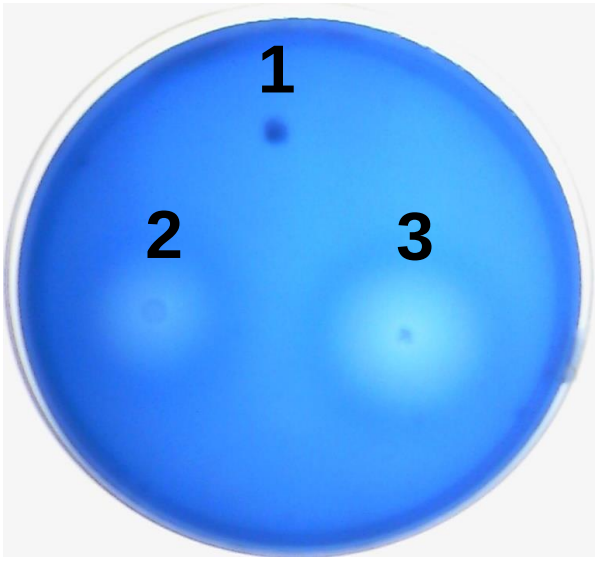

Figure 4.1. Confirmation of endo-xylanase production by Hoegaerden DLG21 after 48 hrs incubation at 30°C. (1) Hoegaerden control strain, (2 and 3) Hoegaerden DLG21 at 24 and 48-hours incubation at 30°C, respectively.

4.2. Endo-β-1,4-glucanase production by Hoegaerden DLG30

Endo-β-1,4-glucanase production by the Hoegaerden DLG30 strain was determined by plating on SC medium containing 1% CMC. Very faint clearing zones were observed around Hoegaerden DLG30 colonies after flooding with Congo-red (Figure 4.2). The faint zones indicate that very low levels of glucanase activity is produced by the recombinant strain.

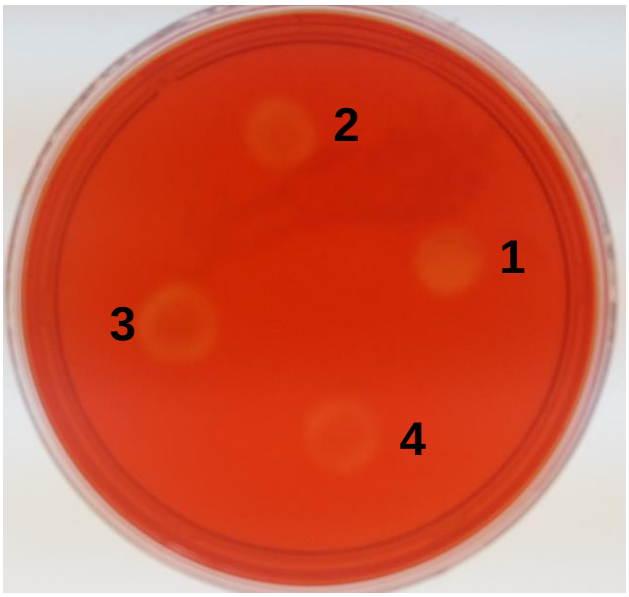

Figure 4.2. Endo-β-1,4-glucanase activity produced by Hoegaerden DLG30 after three days of incubation at 30°C. (1) Hoegaerden control strain, (2, 3 and 4) Hoegaerden DLG30.

4.3 α-Amylase production by Hoegaerden DLG31

The α-amylase production by Hoegaerden DLG31 strain was confirmed by plating on SC medium containing soluble starch. Clear zones were observed around Hoegaerden DLG31 colonies after flooding with Gram's iodine indicate the secretion of an active α-amylase (Figure 4.3).

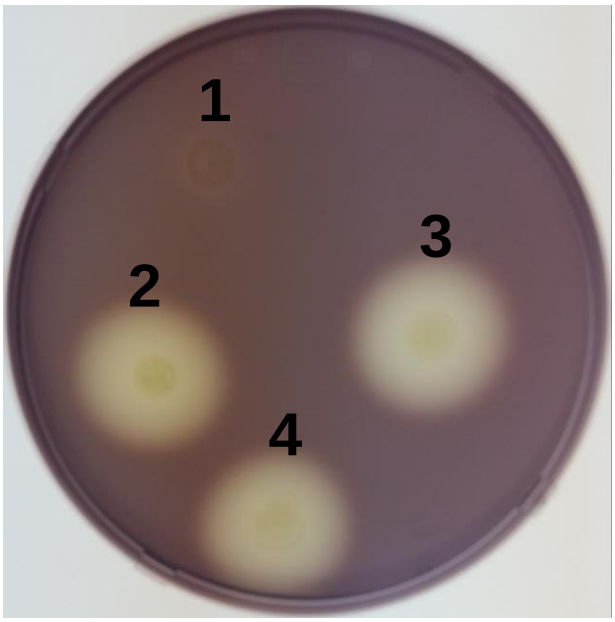

Figure 4.3. Confirmation of α-amylase production by Hoegaerden DLG31 after three days of incubation at 30°C. (1) Hoegaerden control strain (2, 3 and 4) Hoegaerden DLG31.

4.4. Wort fermentation

During fermentation the specific gravity of the wort decreased rapidly with increase in fermentation time, in Hoegaerden pDLG21 (xylanase), Hoegaerden pDLG31 (amylase) and in the Hoegaerden wild type (Figure 4.4). Hoegaerden pDLG30 (endo-glucanase) fermented very poorly resulting in a slight decrease in gravity. The original gravity (OG) of wort was 1.064 and upon completion of fermentation, the final gravity (FG) of the wort fermented by xylanase, glucanase, and amylase producing strain were 1.016, 1.060, and 1.014 respectively. The final gravity of the control strain was 1.014.

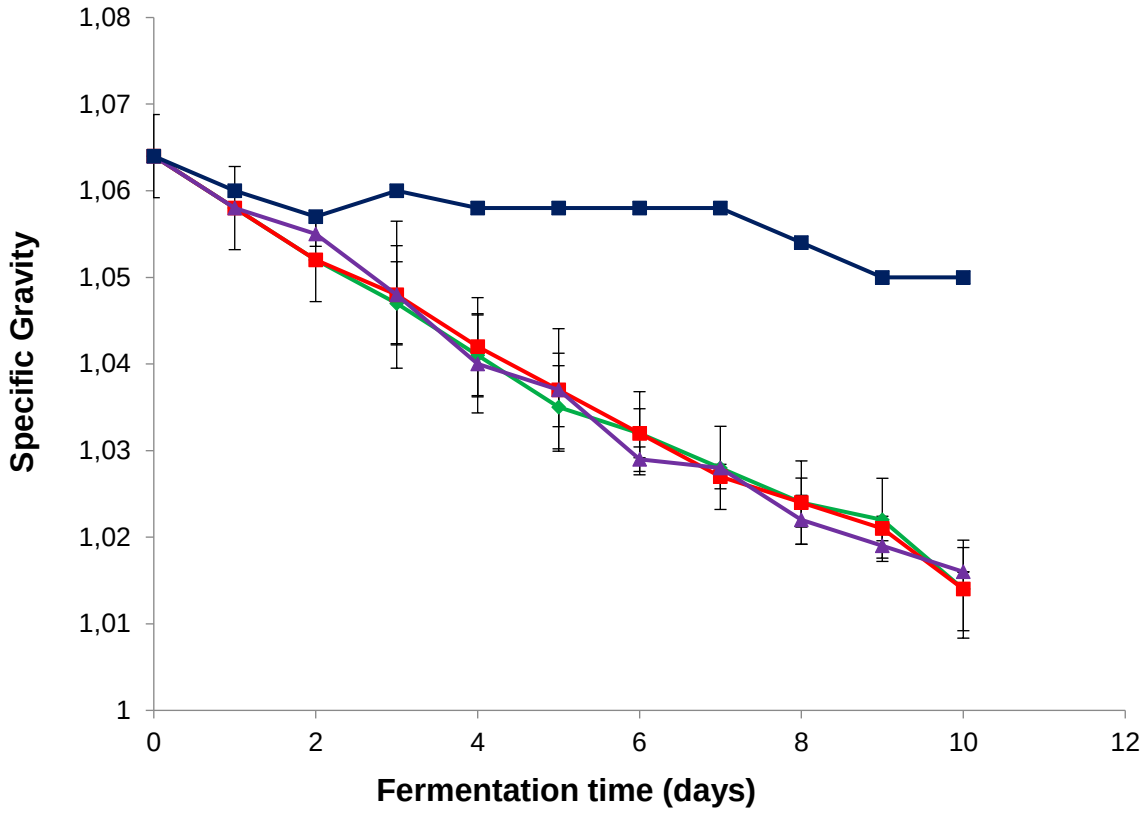

Figure 4.4. Beer specific gravity during fermentation with different recombinant Hoegaerden brewing yeast. (▲) Hoegaerden DLG21, (■) Hoegaerden DLG30, (■) Hoegaerden DLG31 and (♦) Hoegaerden control strain. Values are represented as standard deviation (SD) of means of independent replicates.

4.5. Ethanol production during fermentation

Ethanol increased with increase in fermentation time (Figure 4.5). The xylanase producing strain produced 6.57 % ethanol while the glucanase producing strain only produced 1.98% ethanol. The amylase and control strains both produced 6.83% at the end of fermentation.

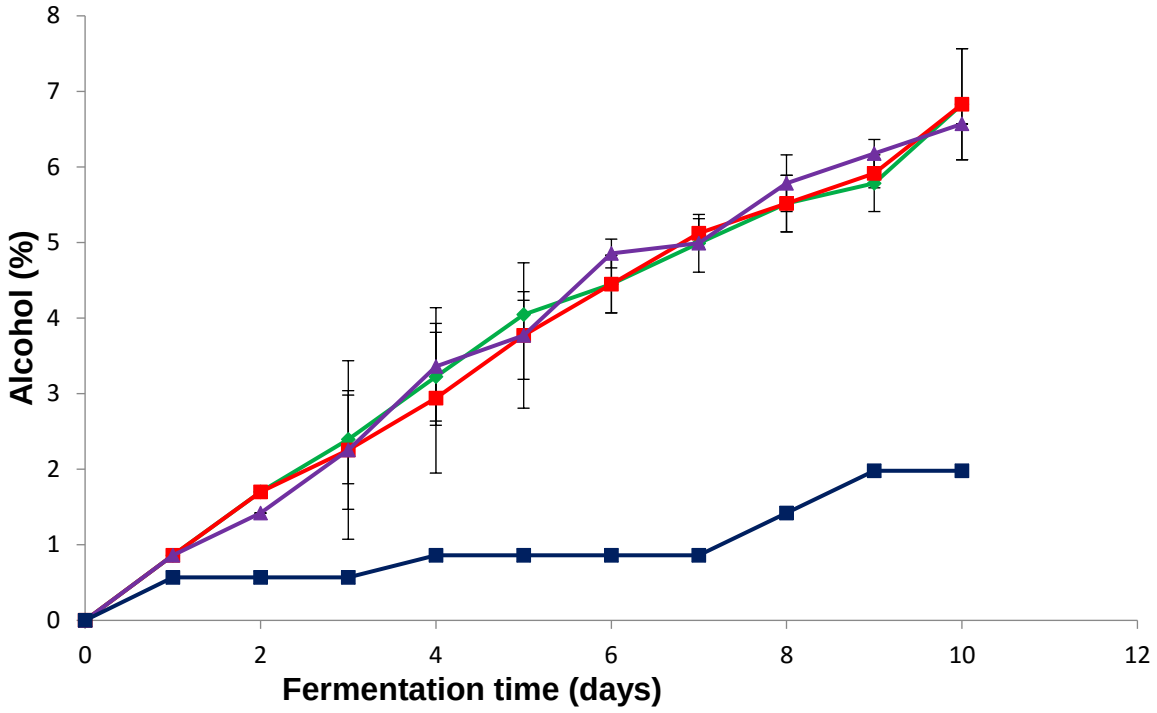

Figure 4.5. Ethanol production during wort fermentation by recombinant brewing yeast strains. (▲) Hoegaerden DLG21, (■) Hoegaerden DLG30, (■) Hoegaerden DLG31 and (♦) Hoegaerden control strain. Values are represented as SD of means of independent replicates.

4.6. Beer viscosity

Barley β-glucan and AX increase wort viscosity during mashing and cause problems during wort separation and often also impact on beer filtration rate. The Hoegaerden strains producing a glucanase and xylanase were evaluated for their ability to reduce beer viscosity. The results indicate that the xylanase producing strain was able to reduce beer viscosity and but no reduction in viscosity was observed with glucanase producing strain (Figure 4.6).

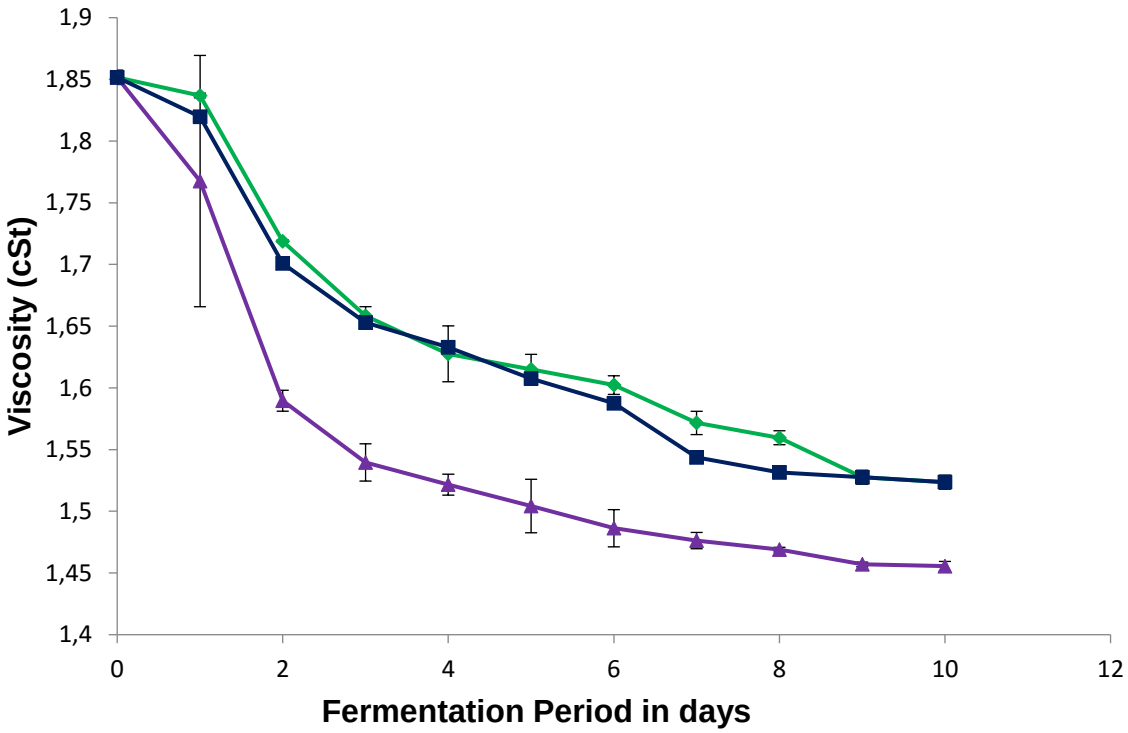

Figure 4.6. The effect of recombinant yeast strains on beer viscosity during fermentation. (▲) Hoegaerden DLG21, (■) Hoegaerden DLG30 and the (♦) Hoegaerden control strain. Values are represented as SD of means of independent replicates.

4.7. Amylase activity during fermentation

The presence of an active *Lipomyces kononenkoae* α-amylase in Hoegaerden DLG31 was confirmed by spotting 5 µl of beer samples taken daily on a starch plate containing sodium azide to prevent yeast growth (Figure 4.7). The results after 48 hour incubation indicate that the Hoegaerden DLG31 produced an active α-amylase throughout the fermentation period.

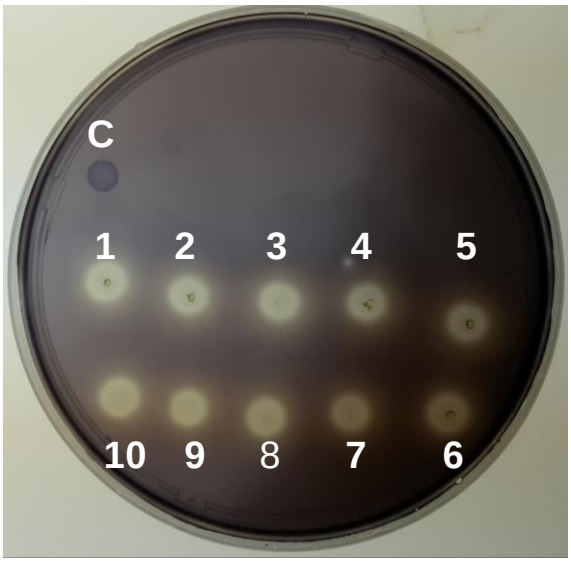

Figure 4.7. Alpha-amylase activity produced by Hoegaerden DLG31 during beer fermentation. (1 -10) beer samples taken daily for 10 days (C) wild type strain sampled on day 5.

4.8. Residual sugars

The brewing yeast *S. cerevisiae* normally utilize sugars in an ordered sequence during wort fermentation. Glucose is utilised first, followed by fructose and complex sugars (Maneses, 2002). Glucose and fructose hydrolysis was reported to occur simultaneously in brewing strains of *S. cerevisiae.* Both these monosaccharides are transported across yeast cell membrane by a common carrier membrane protein (Cason and Reid, 1987). Sucrose is hydrolysed extracellularly by a secreted invertase (EC 3.2.1.26) resulting in glucose and fructose, while maltose and maltotriose is transported into the cell and hydrolysed to glucose intracellularly. Maltose is the predominant sugar in the wort and it is typically assimilated by yeast after glucose uptake. In this study, the recombinant yeast strain consumed glucose and fructose during the first 2 days of fermentation. Figure 4.8 shows maltose utilization by the control and recombinant strain expressing an α-amylase. Maltose levels in the wort was high in day 0 and after inoculation with yeast, a decrease in maltose was observed as fermentation time increase.

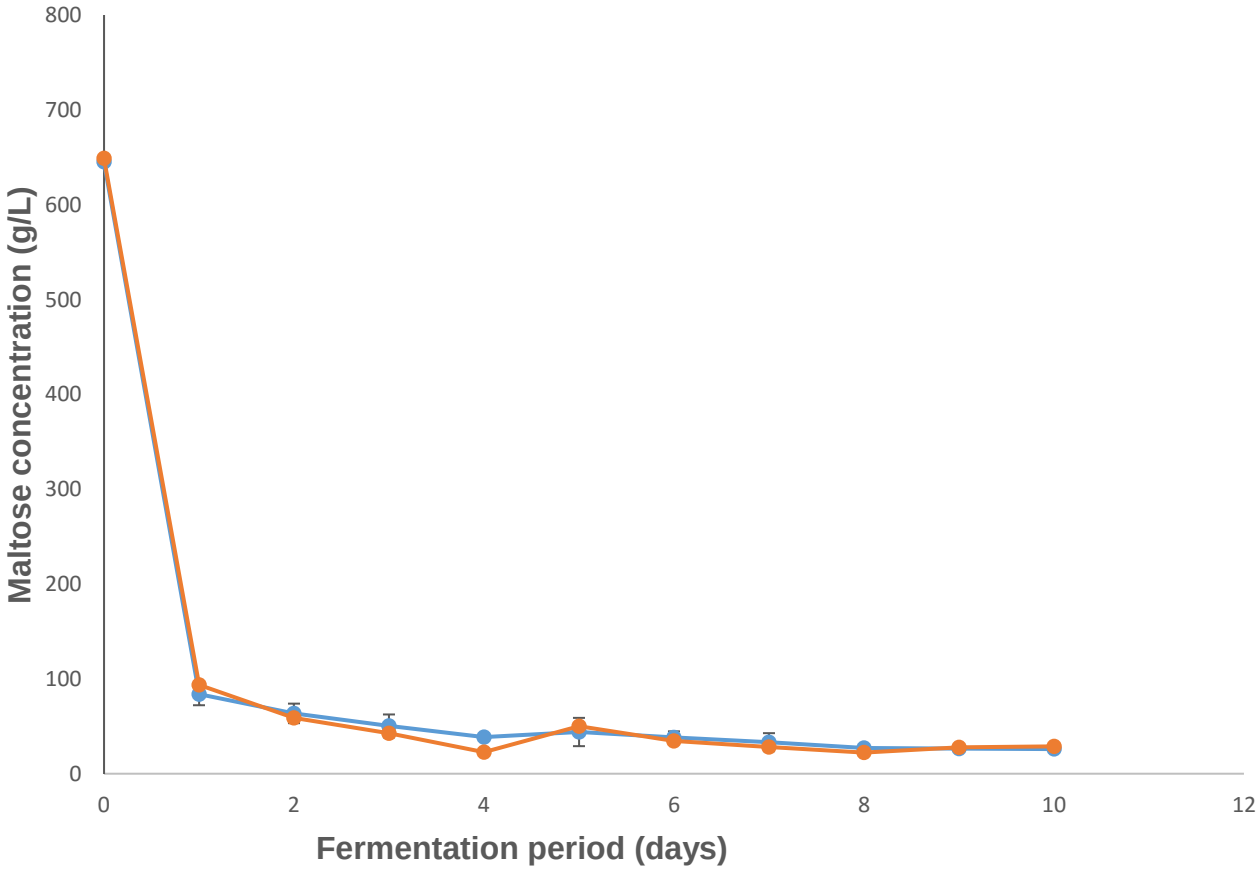

Figure 4.8 Maltose utilization during beer fermentation by the (●) Hoegaerden control strain and (●) Hoegaerden DLG 31 strain expressing an α-amylase. Values are represented as SD of means of independent replicates.

4.9. Beer taste profile

The sweetness, bitterness, body, astringents, sulphitic and maltiness of the beer were determined during beer tasting. Table 4.1 shows the values flavours obtained for the beers brewed in this study. The sweetness of the beers in this study (3-4) compares well with that of a typical lager (2.9±0.6) (Table A1, Appendix 1). The sweet taste in the beer is often associated with the beer constituents (i.e. glucose, fructose, maltose, etc.). The bitterness of the beer ranged from 6-7 which made these beers more biter than typical commercial lager beers which have an average bitterness score of 5.1±1.1 (Table A1, Appendix 1). The bitterness in the beer resulted from the iso-α-acids in the hops.

The body (measures the fullness of the flavour and mouthfeel) of the beer shows that it ranges between 4-5 which is also comparable to some of the commercial beers shown in (Table A1, Appendix 1). A good body of the beer ranges between 5 and 6 depending on the type of beer (Table A1, Appendix 1). Therefore, the body of the beer produced with recombinant yeast strains had a well-rounded flavour and mouthfeel.

Esters like aliphatic esters are produced by yeast during fermentation and are mostly noted by a fruity or spicy odour in beer. These compounds are normally detected at very low levels. The results show that in terms of esters, the beers (3-4) in this study compare well with a typical lager (2.5±0.64). The levels of the hops were found to range between 5-6 which was high when compared to the standard level of the commercial lagers (2.9±0.8). This also correlates well with the high bitterness level obtained for the beers in this study.

Astringency indicates the sensation of dryness and/or puckering of the lining of the mouth produced by aqueous solutions of various substances (i.e. tannins). The level of astringents in the beer produced ranged between 3-4, which is slightly higher when compared to standard level of lager beers (Table A1, Appendix 1). Sulphitic flavour compounds ranging up to 1.8 were detected in the beers produced in the study. Sulphitics are detected as a sensation of sulphuric dioxide or sulphurous-SO_2 during tasting. According to the reference standards, the study beers are high in sulphutic compounds (Table A1, Appendix 1). The maltiness of the beers were reported as being well rounded ranging between 1-2. The maltiness is often as a result of toasted malt which contributes to the flavour of the beer.

Table 4.1. Analysis of bottled beers produced with the control strain as well as the recombinant yeast strains expressing a xylanase and an amylase.

Analysis	Yeast strain used								
	Control	pDLG 21	pDLG31	Control	pDLG21	pDLG31	Control	pDLG21	pDLG31
Core flavours	Batch one			Batch two			Batch three		
Sweet	2.8	3.0	3.0	3.3	3.6	3.1	3.4	3.6	3.0
Bitter	7.4	6.6	6.0	7.7	6.3	6.2	6.6	6.4	6.2
Body	4.6	4.8	5.0	5.0	5.7	5.2	5.0	5.2	4.8
Estery	3.5	3.5	3.2	4.7	4.0	4.0	4.0	4.1	4.1
Hoppy	5	5	5.8	4.5	6.2	6.9	5.5	5.3	5.0
Brand flavours									
Astrigent	3.6	3.0	3.5	3.7	3.3	3.7	3.8	3.4	3.5
Sulphitic	1.7	1.0	1.0	1.7	2.0	1.3	1.6	1.8	1.8
Malty	2.0	2.0	2.0	1.0	1.0	1.0	1.0	0.7	0.7

Keys: score range: 1-10 (The score range differs from brand to bran

CHAPTER 5 DISCUSSION

Wort with high levels of AX and β-glucans result in beer with increased viscosity resulting in a decrease in filtration rate. Incomplete hydrolysis of starch leads to high levels of limited dextrins in beer, resulting in beer with a high calorie content. Both these problems can be solved through the addition of the appropriate enzyme to degrade the polysaccharide responsible. In previous studies, genes encoding amylolytic enzymes were introduced into the brewing yeast *S. cerevisiae* for degradation of residual polysaccharides during fermentation (Wang *et al*., 2012, Park *et al*., 2014). A comparative laboratory brewing test in this study was performed on the Hoegaerden pDGL21 recombinant strain expressing a xylanase, Hoegaerden pDGL30 expressing a glucanase, and Hoegaerden pDGL31 expressing an amylase.

5.1 Endo-β-1, 4-xylanase production by Hoegaerden DLG21

Trichoderma reesei is a well-known filamentous mesophilic fungus with cellulolytic and xylanolytic enzymatic activities (Penttila *et al.*, 1990). The *T. reesei* endo-β-1,4-xylanases catalyse the hydrolysis of the xylan backbone producing xylo-oligosaccharides which are further degraded to xylose by β-D-xylosidases. The *S. cerevisiae* strain used in this study only express a xylanase and can therefore only degrade xylan to xylo-oligosaccharides (La Grange *et al*., 1996). The Hoegaerden DLG21 strain is able to produce an active endo-β-1, 4-xylanase which was confirmed on RBB-xylan plates (Figure 4.1).

5.2 Endo-β-1, 4-glucanase production by Hoegarden DLG30

Endo-β-1,4-glucanases hydrolyse the internal β-1, 4-linkages in glucan (van Rensburg *et al*., 1997). Hoegaerden DLG30 was able to produce an active endo-β-1,3- 4-glucanase. This was confirmed on YPD plates with CMC. The results obtained show that Hoegaerden DLG30 secretes an active endo-β-1,3-4-glucanase, though at very low concentrations (Figure 4.2). Similar zones were reported by Guo *et*

al., (2010) as well as, Van Rensburg *et al*., (1994) when testing the activity of *B. fibrisolvens* endoglucanase coding gene (end1) in *S. cerevisiae* transformants.

5.3 Alpha-amylase production by Hoegarden DLG31

The α-amylase hydrolyse the internal α-1, 4-glucosidic linkages in starch and this enzyme is known for its wide range of applications in various industries such starch processing, baking, brewing etc. (Liu *et al*., 2010). The Hoegaerden DLG31 strain produce an active α-amylase and this is confirmed by clearing zones on starch medium (Figure 4.3).

5.4 Specific gravity and ethanol production

The results in figure 4.4 show the degree of attenuation of beer produced with three recombinant Hoegaerden strains and the unmodified control strain. A reduction of specific gravity (SG) of the wort fermented by Hoegaerden DLG21, DLG31 and control strain was observed.

The glucanase expressing strain showed very poor growth and fermentation ability. Hence a very poor reduction in wort specific gravity was observed. According to literature a number of different endo-β-1,4-glucanase genes have been expressed successfully in *S. cerevisiae* (Penttila *et al*., 1987, Suihko *et al*., 1991, Van Rensburg *et al*., 1997, Lu *et al*.,2009 and Zhang, *et al*., 2008a). However, the activities of the endo-β-1,4-glucanase were very low in the recombinant. Therefore, it might be possible that the *B. fibrosolvens* endo-β-1,4-glucanase gene used in this study could be attacking the host cell wall, hence a low activity of the enzyme.

The amylase producing strain produced a beer comparable to the wild type strain. The α-amylase produced by Hoegaerden DLG31 did not lead to a significant reduction in the residual sugar (i.e. dextrins) during wort fermentation. Steyn and Pretorious, (1995) reported that the *Lipomyces kononenkoae* amylase has low α-1,6-activity. Alpha-amylases can only act on the α-1,4-glycosidic bonds and cannot hydrolyse α-1,6-glycosidic bonds or α-1,4-glycosidic bonds close to α-1,6-glycosidic bonds in

limit dextrins (Wang *et al*., 2012). Although the *L. kononenkoae* α-amylase express well in *S. cerevisiae,* the fact that it can only hydrolyse α-1,4-glycosidic bonds limits its effectiveness in removing limit dextrins from beer.

5.5 Beer Viscosity

The viscosity of the wort and beer have a great influence on the brewing process and production efficiency. High wort viscosity is known to lower the efficiency of wort separation from the grain, beer filtration as well as the quality of the final beer product (Jin *et al.*, 2004). An increase in beer viscosity is mostly caused by high levels AX and β-glucans in the wort (Lu and Li, 2006). This study evaluated the effect of endo-β-1,4-xylanase and endo-β-1,4-glucanase producing strains on beer viscosity. The results showed that the xylanase expressing strain was capable of decreasing viscosity of beer (Figure 4.6). The recombinant *T. reesei* endo-β-1,4-xylanase was reported to show a temperature optimum of 60°C (La Grange *et al*., 1996). Even though this is significantly higher than the temperature used during beer fermentation (13°C), the long reaction time (several days), allowed the enzyme to hydrolyse a sufficient amount of AX to significantly decrease viscosity.

The endo-β-1,4-glucanase expressing strain produced a very poor quality beer and it secreted insufficient quantities of glucanase to degrade β-glucans present in the wort, hence no reduction in beer viscosity with this strain. It has been reported that endo-β-1,4-glucanase exhibit the temperature range of 50-80°C (Dequin, 2001 and Panttila *et al.*,1987). In most cases, these enzymes show their maximum activity after a period of 24 hours, then after it loses activity.

5.6. Amylase activity during fermentation

The α-amylase breaks down the α-1, 4-glucosidic linkages of the starch molecules to produce simple sugars to serve as nutrients for yeast growth and fermentation. The Hoegaerden DLG31 produced active α-amylase throughout fermentation period.

However, the activity was not sufficient enough and this is confirmed by a very small clear zone on the starch medium (Figure 4.7).

5.7. Residual sugar concentration in beer

5.7.1. Non-fermentable sugars

Residual sugars are known to impart the sweetness, mouthfeel and body of the beer flavour. However, if properly matured, it is unlikely that there will be significant levels of fermentable sugars left in the beer after fermentation, but some sugars (i.e. dextrin) persist because they are non-fermentable. These non-fermentable sugar contribute up to 25% of the carbohydrates in the final beer (Saerens *et al*., 2010). The production of low calorie beer may be obtained by reducing the content of residual carbohydrates such as dextrins.

5.7.2. Fermentable sugars

High performance liquid chromatography (HPLC) was used to determine the amount of maltose present in the beer during fermentation. The HPLC analysis revealed that maltose was hydrolysed in the first 24 hours of fermentation in both the control strain and the recombinant amylase producing strain (Figure 4.8). The recombinant amylase producing strain did not make any differences to maltose levels when compared to the control strain.

Wang *et al.*, (2010) conducted a study were an α-amylase expressed in brewer's yeast was also unable to effectively degrade residual sugar (i.e. dextrins) to fermentable sugars, because the recombinant enzyme showed low activity on α-1, 6-glycosidic bonds. Consequently, the expression of an amylase with α-1, 6 glyosidic bond activity higher than that observed for the *L. kononenkoae* α-amylase is needed to overcome the problem of residual sugars in beer. The expression of a pullulanase would probably yield the best result.

5.8 Beer product evaluation

Taste is the single most important factor when it comes to food and beverage selection by consumers (Glanz *et al*., 1998). All beers produced in the study were bottled and stored at 4°C for 7 days before they were sent to South African Brewery (SAB) (Polokwane) for tasting. Several components (i.e. sweetness, bitterness, body, hoppy, esters, astringent, suphitic and maltyness) were evaluated during tasting. A panel of six professional tasters were involved in the beer tasting and Table A1, Appendix 1 were used for comparison and the evaluation report is presented in Table 4.1. The panel concluded that all the yeast strains produce a sweet beer with a bitter taste and well-rounded body. The panel indicated that the sweet taste in the beer was due to the presence of non-fermentable sugars (i.e. dextrins) which also adds to the “body”. It is well known that dextrin’s play important role in the body or mouth feel of beer (Saerens *et al*., 2010).

The bitterness in beer is derived mainly from hops which contain isomerised alpha acids (iso-α-acids), while oxidation products of β-acids formed during wort boiling can also add bitterness (Oladokun *et al*., 2017). The bitter taste in beer play an important role in the flavor attribute that consumer expect and enjoy in beer (De Keukeleire, 2000). Although the beers brewed in this study had higher bitterness levels than a typical lager, the panel concluded that this complements the flavour.

Esters are compounds produced during yeast metabolism (Figure A2, Appendix 2) and they have a great impact on beer flavour. The ester content in beer is normally low in good beer, since high levels has a very negative impact on beer flavour (Tang and Li, 2017). These components are mostly noted in the beer due to their fruity or spicy odour, which gives the beer a full body and harmonious taste. The panel revealed that the beers contain esters ranging from 3-4 which is in the acceptable range.

The panel also detected astringent and malty flavours in the beer. Astringency is known as a complex sensory property, normally characterized by drying, roughing, puckering and often an unpleasant tart vinegar-like taste in beer. Polyphenols from malt and hops are known to contribute several characteristics of beer flavour, notably astringency (Francois *et al.,* 2006). Hops also support astringency in beer. The

maltiness detected during tasting was derived from the Munich and Vienna malts, which contribute melanoidins and toasty flavours. The hop bitterness is generally high enough to support the malt flavours.

Sulphites were detected at levels higher than would be expected in a good beer. Sulphur compounds such as sulphite (SO_2) are only desirable at very low levels, because they have antiaging effects on the beer and help to sustain the flavour stability of the beer. Moreover, the presence of free SO_2 will react with acetaldehyde formed during fermentation and generate an antiseptic odour during the pasteurization process. Therefore, they prevent oxidative reactions (antioxidant) in bottled beer, which increases its shelf life (Saerens *et al*., 2010). These compounds mostly come from yeast metabolites during fermentation, and their synthesis are normally associated with the metabolism of amino acids in yeast (Tang and Li, 2017). Thus, when the rate of amino acid synthesis is more than the reduction of sulphate in the yeast, the level of sulphur dioxide increases. The brewer's yeasts are known to contain an active sulphite reductase which counteract the accumulation of sulphite in the beer (Saerens et al., 2010). Therefore, the accumulation of sulphites in the produced beer was probably due to lack of sulphite reductase production by the yeast strain. However, further research is needed to confirm this.

The overall impression of the beers produced in the study was that, there was a drastic change compared to normal lager beer, however the change was acceptable. There was not much difference between the beers produced with the yeast strain expressing α-amylase and control strain, but these two beers were more preferred in terms of flavour and aroma over the beer fermented with the strains producing the endo-β-1,4-xylanase and endo-glucanase. The panel also indicated that there was consistency in the results, and thus brewing conditions were the same throughout.

5.9 Conclusion

This study evaluated the effect of Hoegaerden recombinant yeasts expressing xylanase, endo-β-1, 4-glucanase and α-amylase on beer viscosity, residual sugar and overall beer quality. The yeast strain expressing xylanase resulted in a decrease in beer viscosity, but lead to an overall decrease in the quality and taste of the beer. The endoglucanase secreting strain produced a very poorly attenuated beer with no measurable decrease in beer viscosity. The expressed α-amylase did not show any effect on the sugar level when compared to control strain. However, the recombinant strain did produce better quality beer. Again this enzyme did not show any effect on the residual sugar (i.e. dextrins) because of low α-1, 6 debranching activities.

Future studies: Further investigation should entail the level of expression of the enzymes in the host cell. The gene dosage (single or multiple copy)**.**The genetic stability and activity/interaction of the recombinant strain during beer fermentation process. Sulphite reductase production by the recombinant yeast strain**.** The expression of a pullulanase (with α-1, 6 activity) together with an α-amylase in the brewer's yeast strain to improve the efficiency and the rate of hydrolysis of residual dextrins to produce a low calorie beer.

CHAPTER 6: REFERENCES

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CHAPTER 7: APPENDICES

Appendix 1

Table A1 The beer tasting profile for comparison

SAB The South African Breweries	THE SOUTH AFRICAN BREWERIES LIMITED POLOKWANE BREWERY TASTE BEER PROFILES	REF NO: E.QA.R.0034 PAGE: 1 OF 1 ISSUED: 25/10/2007 VERSION: AB REVISED: 11/09/2014 DEPARTMENT: QA

Beer Profiles	Castle Lager	Castle Draught	Castle Lite	Castle Milk Stout	Carling Black Label	Hansa Pilsener	Hansa Marzen Gold	Lion Lager	Ohlsson's Lager	Miller Genuine Draft	Peroni Nastro Azzurro	Pilsner Urquell	Grolsch	Amstel	Heineken
Core Flavour															
Sweet	3	3	2	4	3	2	3	3	2	3	3	4	3	3	3
Bitter	6	6	5	7	4	5	5	4	4	3	5	7	5	5	5
Body	5	5	4	6	5	5	5	5	4	3	5	6	5	5	5
Estery	3	3	2	2	4	2	3	3	2	2	3	2	2	2	3
Hoppy	3	3	3	2	2	3	4	3	2	2	3	5	3	3	3
Brand Flavour		=	Brand Flavour												
Astringent	3	3	3	3	3	3	3	3	2		2	3	2	3	3
Sulphitic (SO2) Max *	1	1	1	1	1	1	1	1	1		1		1	1	1
Caramel				3				1			1	2			
Burnt non specific				2											
Smoky				1											
Roasted												2			
Diacetyl				3								3			
Malty							1			3	3	4	3	1	1
Sour											1		1		
FCG														1	

Appendix 2

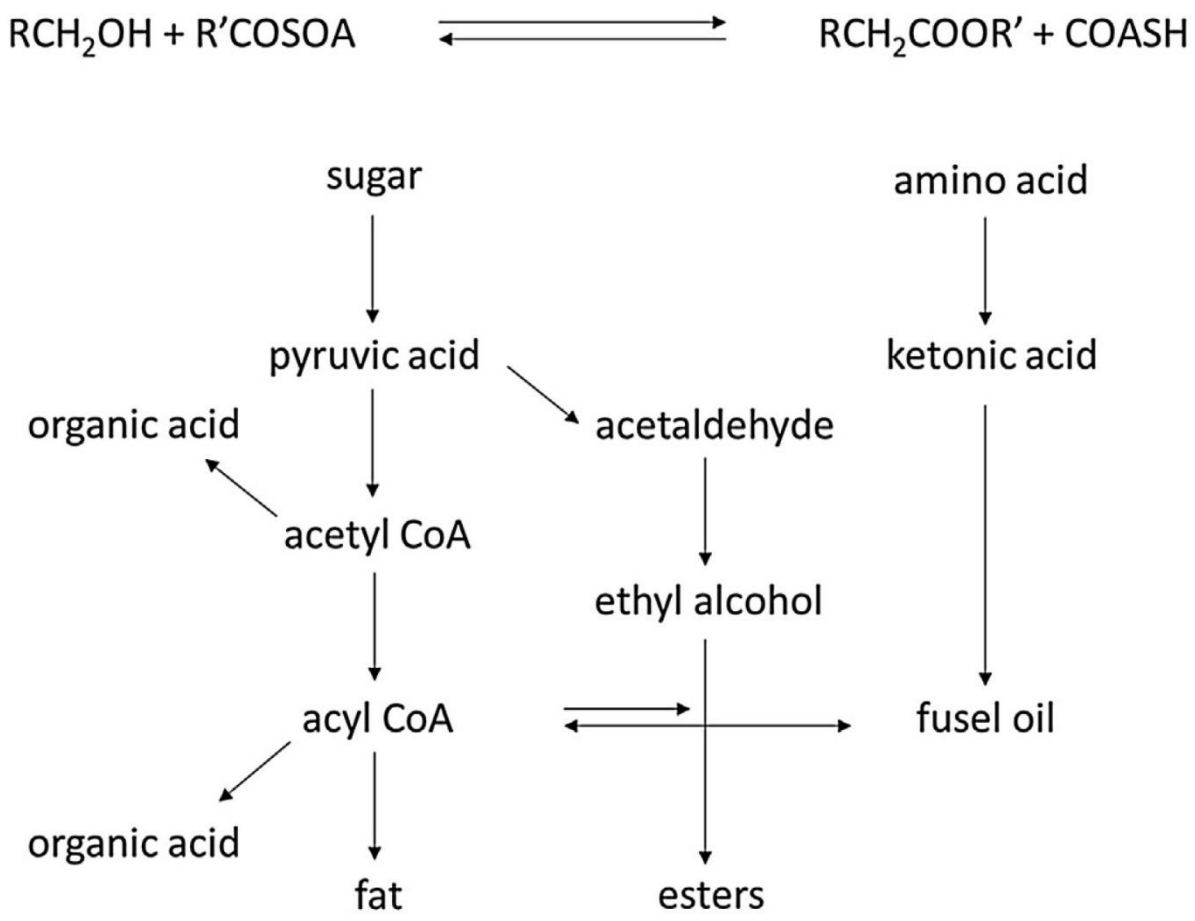

Figure A2: The pathway of esters formation in beer (Tang and Li, 2017).