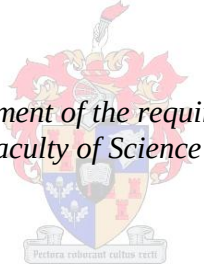
The effect of lactic acid bacteria and fungi on the malting of barley

by
Melanie Hattingh



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Supervisor: Prof. Leon Milner Theodore Dicks

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Summary

Barley malt is the predominant raw material for beer brewing world-wide. To meet consumer demand, a constant high quality malt product is required. Malt quality is determined by the degree of substrate hydrolysis during germination and mashing which serves as fermentable substrates for alcoholic fermentation during brewing. It is often difficult to sustain malt of high quality due to inconsistent malt batches and poor germination capacities of dormant barley. External additives such as chemicals and gibberellic acid have been used to overcome these difficulties but are unwanted in the beverage industry. Maltsters are consequently always in search of alternative solutions.

Microbes produce diverse enzymes which can contribute to substrate hydrolysis during germination. The development of such starter cultures might provide a natural and economically feasible alternative to augment barley germination. Starter culture technology has been employed in the malting industry, although the main focus has been to improve the microbial stability of malt. The exploitation of cultures with hydrolytic capabilities to augment barley germination is consequently largely unexplored.

The aim of this study was to develop a starter culture which can contribute to the enzymatic degradation of barley polymers. *Geotrichum* spp. and *Lactobacillus plantarum* were isolated from substrates rich in polymers present in barley and screened for enzymatic capabilities. *Geotrichum* spp. produced cellulase, xylanase, protease and β-glucanase activities, while *L. plantarum* harboured cell-bound and extracellular α-amylase activities. These cultures were added in different combinations during the malting of Erica and SSG 564 cultivars, but did not enhance germination significantly. Improved malt parameters did not correlate with microbial enzyme activities and the data were not repeatable. Preliminary plate assays could thus not be used to predict enzyme production in a malting environment.

Cell-free supernatants with known enzyme activities of *Aspergillus* sp., *Trichoderma reesei* and *Rhizopus* sp. significantly enhanced malt quality. To our knowledge, the use of fungal supernatant to augment malt modification is a novel concept. Supernatant is more convenient than starter cultures and will aid to deliver more constant malt products than live cultures, as known enzyme levels are added.

Opsomming

Garsmout is wêreldwyd die oorheersende roumateriaal vir bier brou. Om die aanvraag van verbruikers te bevredig, word 'n konstante hoë gehalte mout produk vereis. Die kwailiteit van mout word bepaal deur die graad van substraathidrolise gedurende ontkieming, wat dien as fermenteerbare substraat vir alkoholiese fermentasie tydens verbrouing. Dit is dikwels moeilik om ’n konstante, hoë gehalte, moutproduk te lewer as gevolg van variasie in mout en die swak ontkiemingsvermoë van dormante gars. Hierdie probleem kan oorbrug word met eksterne toevoegings soos chemikalieë en gibberelliensuur, maar dit is nie ’n gewensde praktyk in die broubedryf nie. Vermouters is gevolglik gedurig op soek na alternatiewe oplossings.

Mikroörganismes produseer diverse ensieme wat kan bydra tot substraathidrolise gedurende ontkieming. Die ontwikkeling van sodanige suurselkulture is moontlik 'n natuurlike en ekonomies praktiese alternatief om die ontkieming van gars te stimuleer. Suurselkulture is reeds in die moutindustrie gebruik, alhoewel die fokus hoofsaaklik was om die mikrobiese stabiliteit van mout te verbeter. Die konsep om kulture met hidrolitiese vermoëns te gebruik om garsontkieming aan te vul is gevolglik grootliks onverken.

Die doel van hierdie studie was om 'n suurselkultuur te ontwikkel wat kan bydra tot 'n ensiematiese afbraak van die polimere in gars. *Geotrichum* spp. en *Lactobacillus plantarum* is uit substrate ryk aan polimere teenwoordig in gars geïsoleer en vir hul ensiem aktiwiteite getoets. *Geotrichum* spp. het sellulase, xylanase, protease en β-glukanase aktiwiteit getoon, terwyl *L. plantarum* sel-gebonde en ekstrasellulêre α-amilase aktiwiteit getoon het. Hierdie kulture is in verskillende kombinasies tydens die vermouting van Erica en SSG 564 kultivars bygevoeg, maar het nie tot ’n verbetering in die ontkieming van die gars gelei nie. Geen korrelasie is gevind tussen verbeterde mout parameters en mikrobiese ensiemaktiwiteit nie. Die resultate was ook nie herhaalbaar nie. Voorlopige plaattoetse kan dus nie as 'n maatstaf gebruik word om ensiem produksie deur suurselkulture in vermounting te voorspel nie.

Sel-vrye supernatante van *Aspergillus* sp., *Trichoderma reesei* en *Rhizopus* sp., met bekende ensiem aktiwiteit, het die gehalte van mout aansienlik verbeter. Sover ons kennis strek is die gebruik van supernatante van fungi om die ontkieming van gars te stimuleer ’n nuwe konsep. Supernatant is meer gerieflik as suurselkulture en sal help om konstante mout produkte te lewer aangesien ensiemvlakke beter beheer kan word.

Biographical sketch

Melanie Hattingh was born in Cape Town, South Africa on the 31st of May, 1987. She matriculated at Bellville High School, South Africa, in 2005. In 2006 she enrolled as B.Sc. student in Molecular Biology and Biotechnology at the University of Stellenbosch and obtained the degree in 2009. In 2010 she obtained her B.Sc (Hons) in Microbiology, also at the University of Stellenbosch. In January 2011 she enrolled as M.Sc. student in Microbiology.

Preface

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CHAPTER 1

Introduction

Introduction

Barley malt is indispensable to the beverage industry as it is the predominant raw material in beer production world-wide (Ross et al., 2002). The main purpose of malt is to supply yeast with fermentable sugars, amino acids and vitamins required for growth and alcoholic fermentation during beer brewing (Laitila et al., 2007; Noots et al., 1999; Rimsten et al., 2002). Malt is also one of the main ingredients in the brewing process and has a major influence on the organoleptic quality of the final product. Sugars, amino acids and lipids contribute to colour and flavour compounds, while proteins and polysaccharides are accountable for foam formation and the body of beer (Ullrich, 2011).

Malting involves the exploitation of the natural barley germination process, whereby enzymes degrade the cellular structure to fermentable substrates. The starchy endosperm is the key component during this process and accounts for 70 % of the total kernel weight. It consists mainly of large cells, packed with starch grains embedded in a protein matrix. The cell walls consist of 75 % β-glucan and 20 % arabinoxylan (Fincher, 1975; Jamar et al., 2011). Degradation of the cell walls is essential to allow access of proteases and amylases to the starchy endosperm. Inadequate degradation is often associated with poor malt quality and it is thus considered a critical step in beer brewing (Briggs, 1981; Fincher and Stone, 1986; Hough, 1994).

Maltsters often struggle to deliver a constant malt product, as they are faced with inconsistent malt batches and poor germination capacities of dormant barley. Various companies apply gibberellic acid (GA_3) to the malting process to break dormancy and reduce germination time. The external addition of this hormone has several disadvantages, mainly due to its dose-dependent response. The use of GA_3 is also prohibited in various countries.

Malt is a food product and the addition of chemical additives to enhance germination is thus discouraged. Microorganisms indigenous to barley may contribute to malt modification through the production of proteases, amylases, and cell wall degrading enzymes (Biovin and Malanda, 1997; Foszczynska et al., 2004; Haikara et al., 1993, 1995; Noots et al., 1999). The exploitation of such isolates as starter cultures to augment the hydrolysis process is thus an attractive alternative to the use of chemicals. Little research has been done on the addition of starter cultures to enhance malt modification, although the use of microorganisms to increase the microbial stability of malt is well known. Lactic acid bacteria and *Geotrichum candidum* have been extensively studied for this purpose and can successfully restrict the growth of spoilage microorganisms naturally present in malt (Laitila et al., 2006; Laitila et al., 2007; Linko et al., 1998; Lowe et al., 2005). Only one group of researchers have used a *Rhizopus oligosporus* starter culture with cellulase and xylanase activity to stimulate barley germination (Noots et al., 1993, 1999, 2001).

In this study, a number of lactic acid bacteria and *Geotrichum* spp. were screened for characteristics that would enhance the germination of barley. Strains were screened for amylase, β-glucanase, cellulase, xylanase and protease production by using plate assays. Enzymatic activities were quantified and combinations of the most promising strains were tested on two South African barley cultivars. Various strategies to optimize starter culture performance were also investigated. In addition, the ability of *Aspergillus* sp., *Rhizopus* sp. and *Trichoderma reesei* with known hydrolytic capabilities to enhance malt modification was also examined.

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CHAPTER 2

Literature review

Literature review

1. Introduction

Beer is one of the oldest alcoholic beverages known to man. It is also enjoyed by almost every known culture, making it one of the most widespread drinks world-wide (Ross et al., 2002). Beer brewing was first practiced by the Egyptians around 4300 years ago without any appreciation of microbial and biochemical activities. It was considered a mysterious beverage, but today this ancient practice is on the forefront of biotechnology (Hough, 1985; Noots et al., 1998; Laitila et al., 2006).

The earliest beer was produced from malted barley, and it is still the predominant raw material for beer production (Noots et al., 1998). Barley malt supplies yeast with fermentable sugars, amino acids and vitamins essential for alcoholic fermentation during brewing. Malt is also known to improve the nutritional value of the final beer product as it contributes to valuable bioactive compounds. In addition, malt is used in the production of distilled spirits, different food products and animal feed. Approximately 136 million tonnes of barley is harvested annually, making it the fifth most-produced crop world-wide and integral to global economy (Ullrich, 2011).

2. Morphology of the barley kernel

Barley (*Hordeum vulgare* L.) is a member of the *Gramineae*, commonly known as the monocotyledonous grass family. Two main types of barley can be distinguished based on the number of kernels on the stalk of the plant, namely two-row and six-row barley (Fig. 1). Two-row barley is preferred by the maltster because of its lower protein content and higher starch levels, which ensures a greater malt extract yield. The thinner husk of the two-row barley is further more favourable as it has lower levels of polyphenols (tannins) and therefore lowers the bitter taste of beer (Goldhammer, 2000).

Barley grain is composed of seven main parts i.e. the husk, pericarp, testa, endosperm, embryo, aleurone layer and scutellum (Fig. 2). On average, the mature barley kernel consists of 65 % starch, 10 - 12 % protein, 6 % cellulose, 9 % pentosans and 3 % lipids (Ullrich, 2011).

The kernel is enclosed by the husk (lemma and palea) which consists mainly out of lignin and hemicellulose. Immediately beneath the husk is the pericarp that is closely attached to the testa that surrounds the embryo. The husk and pericarp provide mechanical protection for the barrier and ensure rapid distribution of water over the surface of the corn by capillarity. In addition, the husk

mediates primary leaf development during germination (the acrospires). The testa is selectively permeable and prevents the outward diffusion of sugars and amino acids from the inner grain. It also prevents the entry of microorganisms that are present on the husk and pericarp (Hough, 1985; Briggs et al., 1981).

The main foodstore of the grain is the non-respiring starchy endosperm. It is surrounded by the aleurone layer and separated from the embryo by the scutellum. The endosperm consists of large cells packed with starch grains and embedded in a protein matrix. The walls of these cells consist mainly of 75 % β-glucan and 20 % arabinoxylan. The scutellum secretes hydrolytic enzymes from the embryo into the starchy endosperm to provide soluble food in the form of sugars and amino acids that can diffuse back into the embryo for sustained growth (Hough, 1985; Fincher and Stone, 1986).

The aleurone layer is comprised of two or three cell layers. Each is enclosed by thick cell-walls that consist of 26 % β-glucan and 67 % arabinoxylan. The aleurone layer is the main centre of enzymatic production during germination, but is restricted to carbohydrase and amylase during embryonic development (Hough, 1985; Briggs et al., 1981).

3. Malt Production

The mature barley kernel is a rich source of nutrients for yeast metabolism during brewing, although it is inaccessible in its biopolymeric state. The main objective during malting is to synthesize an array of enzymes that can partially degrade grain macromolecules into a soluble extract (Muller, 2003). Such an extract contains high levels of fermentable sugars, amino acids and vitamins that are accessible for yeast fermentation (Laitila et al., 2007; Noots et al., 1998).

Beer characteristics are greatly dependent on malt as it is one of the major ingredients during beer fermentation. Components such as sugars, lipids, amino acids and phenolics contribute to colour and flavour compounds, while malt proteins are largely accountable for beer foam. Non-fermentable dextrins and cell wall polysaccharides are further responsible for the body of beer (Ullrich, 2011).

The malting process traditionally consists of four stages: Grain cleaning and grading, steeping, germination and kilning (Booysen, 2001; Noots et al., 1998; Vaughan et al., 2005). Each stage in the process is carefully monitored to ensure good malt quality.

3.1 Grain cleaning and grading

Barley kernels undergo a series of pre-cleaning and grading processes before malting can commence. Initial cleaning involves the passing of grains through several sieves to remove dust and foreign objects such as stones and straw. This is followed by grading, which includes the separation of

kernels based on size. Grading is necessary to prevent inconsistent malt products, since kernel size have an impact on nitrogen content, water uptake and modification rates during the malting process. Broken or damaged grains are further rich in polysaccharide-producing microbes such as *Pseudomonas* spp. and *Flavobacterium* spp. that may cause filtering problems during brewing (Follstad and Christensen, 1962). As much as 20 % of the grain can be removed during this process. Grading usually separates kernels on sieves of 2.5 mm in diameter. The plumper fractions are malted, while the thin grains are sold as animal feed (Ullrich, 2011; Briggs et al., 1981).

3.2 Steeping

The main objective of the steeping process is to rapidly induce barley germination without losing viability. This is accomplished by the immersion of kernels in water which allows an increase in moisture content from 12 % to 42 – 48 %. Germination begins as the moisture content reaches 35 %, but is increased to ensure uniform distribution of moisture and diffusion of enzymes throughout the endosperm. Temperature is maintained between 14 - 18 °C and the process usually takes 40 – 48 h to complete.

As steeping proceeds, water uptake slows down and dissolved oxygen is rapidly depleted in the steep water. This is due to increased metabolic activity of the grain and the microbial population on the surface tissue. To maintain germination vigour, the immersion phase (wet stand) is aerated and alternated with an air rest. Normally two to three alternating cycles are conducted throughout steeping (Booysen, 2001; Vaughan et al., 2005). This technique replenishes dissolved oxygen and allows the removal of accumulated carbon dioxide and ethanol that can cause water damage (Ullrich, 2011).

Visual appearance of kernels at the end of steeping is an important quality parameter. The kernels (Fig. 3) are in the correct physiological state when the root sheath, also known as the chit, is present (Booysen, 2001; Ullrich, 2011).

3.3 Germination

During this phase the maltster exploits the natural germination process, whereby enzymes degrade the endosperm cellular structure. Germination is allowed to proceed only as far as necessary to ensure maximum fermentable products required by yeast during brewing. This is accomplished by carefully monitoring temperature, oxygen and carbon dioxide levels throughout the process (Ullrich, 2011).

When germination is initiated, the kernel undergoes extensive physiological and biochemical changes. The embryo secretes a plant hormone gibberellin, which triggers the scutellum and aleurone layer to produce hydrolytic enzymes (Palmer, 1989; Linko et al., 1998). These enzymes are deposited into the starchy endosperm where it attacks starch, cell wall polysaccharides and proteins (Jamar et al., 2011).

The biochemical degradation and physical weakening of the endosperm is generally referred to as modification. The hydrolyzed products diffuse back to the embryo to sustain its growth, although it is largely prevented during malting to avoid the depletion of nutrients that are essential for yeast fermentation.

Kernels are allowed to germinate for 4 - 6 days (Bamforth, 2000). Temperature is critical during this phase and is kept low (14 – 18 °C) to retard germination and ensure high nutrient levels for maximum yeast fermentation. At elevated temperatures germination is rapid and enzymes are produced at a premature stage. This in turn causes a greater loss in endosperm components due to sugar consumption by the embryo, and thus a reduced malt yield. As germination proceeds, the embryo withdraws moisture from the endosperm to sustain its growth. Approximately 0.5 % moisture is lost per day and humidified conditions are employed to prevent kernels from drying out. Moisture content may also be retained by spraying kernels with water (Ullrich, 2011). Gibberellic acid (GA_3) is applied by some maltsters as part of the water addition step.

Fresh air is blown through the grain bed to maintain high oxygen availability to the embryo. In some plants, however, air within the vessel is also re-circulated at the end of the process to create a carbon dioxide-rich environment that aids in reducing respiration and modification. The grain bed is turned twice daily to maintain even aeration, prevent temperature gradients and to separate barley rootlets (Lailtila, 2007; Ullrich, 2011).

Germinating barley is referred to by the maltster as green malt and is ready to be kilned when acrospires reach 75 % of the kernel length (Fig. 3). It is crucial to prevent further acrospires elongation as this is a main factor that contributes to malt losses and are referred to as over-modification (Hough, 1985; Briggs et al., 1981).

3.4 Kilning

The malting process is finally terminated by kilning, during which the moisture level of green malt is reduced from 45 to 4 % (Booysen, 2001; Wolf-Hall, 2007). The main objective of this phase is to arrest botanical growth and to preserve the majority hydrolytic enzymes required for further degradation of carbohydrates during mashing. Kilning also ensures microbial stability of malt and contributes to an assortment of colour and flavour compounds, which is mainly due to chemical changes during Maillard reactions (Noots et al., 1998; Bamforth and Martin, 1983).

The kilning process takes approximately 21 h during which temperature is gradually increased in a stepwise manner from about 50 °C to 85 °C with a reduction in airflow (Laitila, 2007). After kilning is completed, rootlets are removed and the malt product is stored in silos (Booysen, 2011).

4. Beer production

There is a vast array of beer types, each demanding its own equipment and processing. Lager or pilsner style beers are the most popular world-wide and therefore the discussion will focus on this process (Fig. 4). Brewing can broadly be divided into brew house operations and fermentation (Ullrich, 2011).

4.1 Brew house operations

The objective of brew house operations is to convert malt into a hydrolyzed sweet and hopped extract that can be fermented by brewer's yeast.

4.1.1 Milling

The first operation at the brew house is to crush malt into grist. The husk is kept intact to serve as a filter during lautering, while the endosperm is ground to particles. Malt extract is directly proportional to the particle size as fine milling will favour higher malt extract and in turn brew house yield. One of three methods can be employed, depending on the separation process used, namely roller, wet and hammer milling (Lewis and Young, 1995; Kunze, 1999). Roller milling, also known as dry milling, is the most common method and involves the crushing of malt between pairs of rollers to keep the husk intact while crushing the endosperm to fine grist. Wet milling entails the immersion of malt in water for up to 10 minutes to toughen the husk, before rollers tear open the grains. This method was traditionally developed to minimize damage to the husk and to maximize endosperm reduction. In modern breweries the lauter tun is replaced by a mash filter and therefore the integrity of the husk is not essential for separation. In such cases a hammer mill is employed.

4.1.2 Mashing and separation

The grist product is transferred to a mash tun where it is mixed with water to initiate the mashing process. The hydration of grist at 40 - 50 °C rejuvenates the majority of enzymatic activity that was produced during germination. Maximum extraction and enzymatic hydrolysis of partially degraded malt reserves is allowed through a stepwise temperature increase, as each class of enzymes functions optimally at different temperatures (Wolf-Hall, 2007; Briggs et al., 1981). The main focus is the hydrolysis of starch and proteins to fermentable sugars and amino acids respectively, as these substrates are essential for yeast fermentation. The breakdown of β-glucan is also required, as it significantly contributes to the viscosity of the medium. The mashing phase is completed when temperature is increased to 78 °C, which destroys all enzyme activities. At this stage the majority reserves have been degraded, leaving only a minor proportion of insoluble material, known as spent grains (Ullrich, 2011). Mash is filtered to separate the watery mixture, known as wort, from spent grains. Separation is normally conducted in a lauter tun, where spent grains serve as a filter, although

some modern practices utilize an automated mash filter. Spent grains are collected and sold as animal feed.

4.1.3 Wort boiling

Wort is boiled in a brew kettle for up to 2 h in the presence of hops during which complex reactions occur. This process is critical to the brew master as it allows sterilization, coagulation of proteins and tannins, distillation of unwanted volatile materials, wort concentration by the evaporation of water, caramelization of sugars that allows colour formation, and also the extraction and conversion of hop compounds. Hops resins ensure the characteristic bitter aroma of beer, while its essential oils contribute to flavour (Smith and Simpson, 1992; Hough et al., 1982). Wort is clarified after the boiling process to remove spent hops and any coagulated proteins.

At this stage the hopped wort is an excellent substrate for yeast fermentation as it is rich in sugars, amino acids and other nitrogenous materials, mineral salts and vitamins which are essential for yeast metabolism. Sugars can be utilized as carbon source for energy production and biosynthesis, while salts and vitamins are metabolically fundamental (Hough et al., 1982).

4.2 Fermentation

Beer fermentation is technically a straightforward procedure although the production of premium beer with balanced flavour and consistent quality is rather complex.

The fermentation process traditionally consists of two main stages, namely primary fermentation and secondary fermentation (lagering). The main feature of primary fermentation is the conversion of sugars to ethanol and carbon dioxide. The majority of flavour compounds are produced during this stage as numerous by-products are released by yeast. There are relatively few changes that occur during secondary fermentation although this stage significantly contributes to beer quality (Linko et al., 1998).

The by-products produced during main fermentation that contributes to flavour/aroma compounds include organic and fatty acids, aldehydes, carbonyls, alcohols, esters and sulphur compounds. The production of these compounds is dependent on a complex array of factors which mainly includes temperature, oxygen and yeast genetics (Ullrich, 2011; Hough et al., 1982).

Fermentation is initiated when yeast (*Saccharomyces cerevisiae*) is added to aerated wort. The duration of traditional bottom fermentation (lager) is generally 8 - 10 days at low temperatures of 7 - 15 °C. The most popular modern vessels available are closed cylindro-conical fermenters with a steep angled cone at the base. This cone structured base is convenient as it allows efficient sedimentation of yeast, leaving the majority of the vessel relatively free thereof. In this manner yeast can effortless

be removed upon completion of fermentation without the need for centrifugation. Post-fermentation treatments can also be conducted in the same vessel (Hough et al., 1982).

As fermentation starts, yeast enters an initial lag phase during which oxygen is assimilated and components essential for growth are synthesized. Alcoholic fermentation commences as the exponential phase is reached during which glucose and fructose are rapidly consumed, followed by maltose and maltotriose. When available sugars become limited, a stationary phase is reached where after yeast flocculate and settle in the bottom of the tank (Ullrich, 2011).

The beer product after completion of primary fermentation is known as 'green' beer, contains little CO_2, and has an inferior taste and aroma to mature beer. Unwanted yeast and colloidal material are also present. Lagering is usually done at low temperatures of -1 °C to 4 °C for two weeks (Hough et al., 1982).

Diacetyl is the main concern during lagering. It is produced during primary fermentation and its synthesis is closely connected to amino acid metabolism. This compound is known for its butter flavour but is largely unwanted in lager beer. Its taste threshold is extremely low, 0.05 mg/L or less, and is above the threshold after primary fermentation. During secondary fermentation yeast reabsorbs diacetyl and reduces it to butanediol which has a much higher flavour threshold (Linko et al., 1998; Kunze, 1999).

After maturation, beer is clarified by filtration and/or centrifugation and stabilized with various reagents. This product may be stored at low temperatures for several weeks and is carbonated and pasteurized immediately prior to packaging (Ullrich, 2011).

5. Biochemical changes during malting and mashing

Biochemically, malting and mashing are considered controlled processes of endosperm mobilization. These processes are initiated with steeping, reach maximal activity during germination and are terminated by the high temperatures and reduction in moisture content during kilning. Many of these processes are reinitiated when malt grist is rehydrated during mashing.

Modification is used to define the overall physical and biochemical changes that occur in the barley endosperm during malting. Well-modified malt is friable and consequently easily crushed, whereas the opposite is true for poorly modified malt. This physical change is caused by the degradation of cell walls and proteins within the endosperm (Jamar et al., 2011).

The extent of protein and cell wall degradation during malting is crucial to the maltster, as it determines the accessibility of starch to amylases, and consequently the extract yield during brewing.

Incomplete modification leads to poor extract availability, while over-modification results in reduced malt extract yield as glucose liberated by starch degradation is consumed by embryo respiration (Ullrich, 2011; Muller, 2003).

Germination is initiated as water enters the embryo during steeping. Gibberellic acid (GA_3) is synthesized in the embryo and initiates the synthesis and secretion of proteases, α-amylases and cell wall degrading enzymes. β-Amylase which is already present in bound form in the endosperm is also activated. The majority hydrolytic enzymes increase during the 4 - 5 day germination period and continue through the early stages of kilning. Activity is eventually halted and the amount of activity retained for mashing depends on the enzyme type and manner in which kilning is conducted (Ullrich, 2011).

5.1 Cell wall degradation

The endosperm cell wall mainly consists of 75 % (1,3;1,4)-β-D-glucans and 20 % arabinoxylan. The degradation of these polysaccharides is regarded as the most important event during malting and mashing, since it serves as a physical barrier between hydrolytic enzymes and their substrates contained in the starchy endosperm. Inadequate degradation thereof results in poor malt quality traits such as reduced malt extract, high viscosity and poor lautering performance (Fincher and Stone, 1986; Ullrich, 2011). It is important to note that β-glucan and arabinoxylan are not fully degraded to monomers (or fermentable products) during malting and mashing. The main concern is that these polymers are hydrolyzed to a point where it is soluble in cold and warm water so that no precipitation occurs in the beer product.

The (1,3;1,4)-β-D-glucans are linear homopolymers of β-D-glucopyranosyl monomers (glucose) polymerized through (1,3)- and (1,4)-linkages in the ratio of 2.2 - 2.6:1 (Fig. 5). Mature grains do not actively produce β-glucanase, but it is synthesized in vast quantities by the aleurone layer and scutellum during germination (Balance et al., 1986; Hrmova and Fincher, 2001). Four classes of β-glucanases are responsible for the conversion of β-glucan to a mixture of oligosaccharides and glucose. These include (1→4)-β-glucan 4-glucanohydrolase (cellulase), (1→3)(1→4)-β-glucan 4-glucanohydrolase (lichenase or barley β-glucanase), (1→3)-β-glucan glucanohydrolase [endo-(1→3)-glucanase] and β-glucosidase (Leah et al., 1995; Jin et al., 2004). Barley β-glucanase is considered the most important of these classes since it represents the most abundant class during germination. It hydrolyzes (1→4)-β-glucosyl linkages in β-glucan adjacent to (1→3)-β-glucosyl linkages. The major hydrolysis products are (1,3;1,4)-β-D-tri- and tetrasaccharides, although oligosaccharides of up to 10 units can be produced. These oligosaccharides are further hydrolyzed by the other three enzyme classes.

The optimum temperatures for β-glucanase enzymes are 40 – 45 °C. It is critical that the majority of the substrate is converted during malting since these enzymes are rapidly inactivated at mash-in temperatures of 50 °C (Jin et al., 2004). Optimal β-glucan degradation is also assured during kilning where drying starts at mild temperatures of 40 - 50 °C (Bamforth, 1994).

Xylan is a minor constituent of the entire grain as its content ranges between 4 – 9 % (Ullrich, 2011). This hemicellulose is integral to grain structure because it acts as cement between cellulose fibres and contributes to the skeletal framework of cell walls (Fincher and Stone, 1986).

Arabinoxylans consist of a (1,4)-β-D-xylan backbone to which α-L-arabinofuranosyl units are substituted at C(O)2 and/or C(O)3 of the xylosyl units (Fincher, 1975). The complete degradation of this polysaccharide requires the combined action of (1,4)-β-D-xylan endohydrolases, α-L-arabinofuranosidases and β-D-xylosidases (Fig. 6). The (1,4)-β-D-xylan endohydrolases catalyze the hydrolysis of the arabinoxylan backbone by cleaving β-(1,4) xylosidic linkages while α-L-arabinofuranosidases and β-xylosidases are responsible for further degradation of oligosaccharides. Arabinofuranosidases are mainly responsible for the breakdown of C(O)2 and C(O)3 linked arabinofuranose units, while β-xylosidases catalyze the hydrolysis of β-(1,4) xylosidic linkages within the oligosaccharides (Hrmova et al., 1997; Slade et al., 1989; Taiz and Honigman, 1976).

Xylanase enzymes are synthesized during germination although its activity is only detected several days after (1,3;1,4)-β-D-glucanases (Banik et al., 1997; Martien et al., 2001). Xylanases are more heat stable than β-glucanases and therefore degradation also occurs during mashing.

5.2 Protein degradation

The total dry weight of a mature barley kernel consists out of 10 - 12 % protein and is a minor grain component compared to carbohydrates. Its degradation during malting has been extensively studied as it greatly influences the quality of malt. One of the most critical parameters is the liberation of sufficient amino acids during malting to sustain yeast metabolism and fermentation during brewing.

Barley proteins are largely classified based on their solubility in different solvents. Four major groups are recognized, namely albumins (soluble in water); globulins (salt solutions); prolamins (alcohol); and glutelins (alkali). Albumins and globulins represent the enzymatic fraction of barley, while glutelins are known as structural proteins. Prolamins, better known as hordeins, are the major endosperm storage proteins and comprise 35 – 50 % of the total grain proteins (Shewry, 1993). These proteins are largely present as a matrix in which starch granules are embedded in the endosperm. The breakdown of this matrix is essential during malting so that starch granules can be released and effectively solubilised during mashing. This matrix is further a key parameter by which malt modification can be evaluated (MacGregor, 1996; Jones, 2005).

Insoluble storage proteins are hydrolyzed to soluble proteins, peptides and amino acids during malting. Globulins and albumins might also be degraded, although its degradation is not as imperative as for hordeins (Jones, 2005; Baxter, 1981). It has been illustrated by Baxter (1981) that well-modified malt contains less than 50 % of hordeins present in unmalted barley. The mature grain has very little proteolytic activity and the majority of all proteinases are synthesized during germination (Burger and Prentice, 1970; Jones et al., 2000). Barley protein degradation is largely catalyzed by endo- and carboxypeptidases where endopeptidases initially hydrolyze reserve proteins to oligopeptide substrates for carboxypeptidases (Sopanen et al., 1978). Over 40 different endoproteases have been isolated from green malt, although the nature, amount, and substrates of these proteinases are still largely unknown. It has been concluded that cysteine proteases are the most active enzymes during malting (Zhang and Jones, 1995; Ullrich, 2011).

Barley proteases are inactivated at 55 °C, although some activity is retained during the high kilning temperatures of 78 °C. This is largely due to protection provided by the kernel itself. All protease activity is destroyed during mashing, and is attributable to the loss of protection caused by milling of malt, as well as the submersion of enzymes in water (Jones et al., 2000).

Protein degradation is carefully monitored during malting to achieve a fine balance between solubilized proteins and amino acids. High protein levels are unwanted as it causes haze formation in beer products during storage. Complete hydrolysis of barley proteins is also undesirable since proteins assist in the general characteristics of beer (Jones, 2005). These characteristics include components responsible for the formation of beer foam and the distinctive mouthfeel of beer (Hough et al., 1982).

The majority of brewing yeast strains do not possess extracellular proteolytic enzymes and are unable to utilize proteins or polypeptides. Amino acids are therefore an essential source of nitrogen for growth (Hough et al., 1982). Wort generally contains about 17 different amino acids that are liberated from protein degradation during malting. Of these amino acids, proline, which is the most abundant (37.5 % of total amino acids) is not utilized by yeast during beer fermentation and is the major amino acid present in the final beer product (Hough et al., 1985; Jones and Pierce, 1964; Jones, 2005).

5.3 Starch degradation

Insoluble starch granules in the endosperm are the major constituent of the barley grain and contribute to 64 % of its total dry weight. Two forms of starch can be identified, namely amylopectin which represents 75 % of all starch, and amylose which makes up the other 25 %. Amylopectin is a branched glucose polymer, consisting out of α-(1,4) and α-(1,6) bonds, while amylose is unbranched and linked through only α-(1,4) bonds (Fig. 7).

Amylose and amylopectin can be degraded by α-amylase, β-amylase, debranching enzymes (also known as limit dextrinase) and α-glycosidase to glucose and small oligosaccharides. α-Amylase is an endo-enzyme which cleaves the internal (1,4)-α-glucosyl linkages of amylose and amylopectin in a random fashion, yielding free branched- and unbranched dextrins. β-Amylase is an exo-enzyme which cleaves molecules at their non-reducing termini, generating maltose disaccharides and a small amount of maltotriose (Dunn, 1974). α-Amylase secretion by the aleurone layer and scutellum is initiated by GA_3-activation during germination, while β-amylase differs from most starch mobilizing enzymes in that it is exclusively synthesized in the starchy endosperm (MacGregor, 1977; Grime and Briggs, 1996). β-Amylase is also synthesized during grain development and may account for 1 – 2 % of the total protein of mature grains. These proteins are mainly attached to the surface of starch granules and therefore in a non-functional state. As germination proceeds, bound β-amylase is released and activated by proteolytic enzymes. These activated enzymes are unable to degrade starch granules and α-amylase is cardinal for its functioning because it yields free non-reducing termini which can be degraded by β-amylase.

Debranching enzymes hydrolyze branched (1-6)-α-linkages in amylopectin or (1,4;1,6)-α-oligoglucosides generated by α-amylase. These enzymes play an important role in the complete hydrolysis of starch to glucose as it generates debranched oligosaccharides that can be attacked by α- and β-amylases. Small amounts of debranching enzymes are isolated from non-germinated barley, but the activity is largely evident during germination since its synthesis and secretion is stimulated by GA_3-activation from the aleurone layer. α-Glucosidases are responsible for the complete degradation of maltose and other small dextrins to glucose. This enzyme is present in the pericarp, embryo and aleurone of ungerminated barley and its activity rapidly increases during germination by GA_3-induced synthesis by the aleurone and embryo.

A limited amount of 15 – 18 % starch is hydrolyzed during malting, and 12 % of this fraction is assimilated by the embryo. Malting is thus essential for the activation and synthesis of starch degrading enzymes, while the primary objective of mashing is to degrade starch to fermentable sugars. α-Amylase and β-amylase are the key enzymes during mashing and are rapidly activated as milled malt is mixed with water. β-Amylase is more heat liable than α-amylase, and are inactivated at 65 °C, as opposed to 75 °C for α-amylase. The time and temperature schedule during mashing is crucial to the brewer and determines the degree of starch hydrolysis and thus the composition of the final wort.

The total carbohydrate content of beer generally includes approximately 46 % glucose, 9 % maltose, 15 % maltotriose and 30 % dextrins. Brewer's yeast is not able to degrade polysaccharides and flourishes in the presence of monosaccharides such as D-glucose, D-fructose, D-mannose and D-

galactose as carbon source. Some disaccharides and trisaccharides can also be used, although higher glucose polymers such as maltotetraose and dextrins are not metabolized (Hough et al., 1982).

6. Microbial community

Malt quality is not only dependent on the inherent physiological performance of barley, but also the activity and composition of its indigenous microbial community (Noots et al., 1998). This community is extremely diverse and its structure changes drastically with each stage of the production chain. For simplistic reasons the three major ecological niches are categorized into field, storage and malting.

In general, barley microbiota is predominantly psychotrophic and mesophilic, consisting of Gram-negative and –positive bacteria, filamentous fungi, and yeast. Low levels of viruses, slime moulds and protozoa may also be present (Noots et al., 1998; Flannigan, 2003).

6.1 Field

Field microbiota largely consists of parasitic and saprophytic organisms and its community structure is influenced by agricultural practices such as crop protective agents and fungicides, climate, soil type, and plant variety (Noots et al., 1998). Climate has the greatest influence, since barley that is cultivated in different regions has dissimilar microbial communities (Etchevers et al., 1977).

Microbial colonization can be detected soon after ear emergence from the leaf-sheaths, and proliferation proceeds throughout the growing season. The community structure diversifies as microbes from air, soil, rain, bird droppings etc. colonize the kernel structure. The microbial community is restricted to the external parts of the developing grain as the testa serves as a protective barrier against microbial attack of the internal endosperm region (Flannigan, 2003; Petters et al., 1988).

Field microbiota is dominated by Gram-negative bacteria, with the most abundant species being *Erwinia herbicola (*Angelino and Bol, 1990; Haikara et al., 1977). Yeast is the second most abundant group, although filamentous fungi may exceed their numbers during later stages of ripening. The most widespread field fungal genera include *Alternaria, Cladosporium, Epicoccum, Fusarium, Cochliobolus, Drechslera* and *Pyrenophora* (Table 1) (Ackermann, 1998; Flannigan, 2003; Noots et al., 1998).

6.2 Harvest and storage

Barley is normally stored after harvest from 2 months to two years, to overcome seed dormancy before malting (Pyler and Thomas, 2000). The microbial population is significantly altered during this stage and its composition is influenced by the inclusion of chaff, broken grains, insects, and most importantly storage conditions such as aeration and temperature (Armolik et al., 1956). Under ideal storage conditions bacteria, yeast and field fungi are not metabolically active. This is attributed to low water activity (a_w) (< 0.90), and consequently storage microflora is dominated by saprophytic and xerophilic microorganisms (Haikara et al., 1977; Lutey and Christensen, 1963).

Barley quality is influenced by storage fungi, of which species of *Aspergillus*, *Eurotium*, *Micropolyspora*, *Penicillium*, *Rhizomucor*, *Thermoactinomyces* and *Thermomyces* usually dominate (Flannigan and Healy, 1983; Kaur, 2009; Pitt and Hocking, 1997). These species are absent from freshly harvested barley and are introduced as contamination by dust and air in the storage environment, harvesters, elevators and grain silos. Under poor storage conditions the metabolic activity of these species release water by respiration which causes elevated temperatures in the grain mass. If aeration is insufficient, it may result in grain spoilage through germ damage, discoloration, reduction in percentage germination and off aromas and flavours (Noots et al., 1998).

6.3 Evolution of microbes during malting

Malt production is regarded as the most critical phase for microbial proliferation during brewing as it provides particularly favourable conditions in terms of available nutrients, temperature and moisture content (Noots et al., 1998).

The initial wet stand period of steeping increases the moisture content of barley to 42 % which rapidly activates dormant microbes present on grains. Microbial proliferation is also augmented by steep aeration and the leakage of nutrients into steep water (Kelly and Briggs, 1992). Vegetative bacteria, yeasts and moulds start to multiply shortly after the onset of the first wet stand, while spores are only activated and start to grow after a certain lag period (Briggs and McGuinnes, 1993). A large number of microbes are washed away during steep water draining, although the microbes present at the end of steeping is significantly higher than in stored grains (Petters et al., 1988; O'Sullivan et al., 1999).

Maximal viable microbial counts are reached during germination and are attributed to extensive degradation of the grain structure to metabolizable components (Petters et al., 1988; Haikara et al., 1977). The high temperature of kilning reduces the microbial load, although it is generally higher in malt than in native barley (Noots et al., 1998).

The microbial composition is not only determined by the initial microbial load of stored barley, but also specific process conditions and additives. A given malting plant will have its own specific

microflora (in-house microflora), although major microbial groups in the malting ecosystem have been identified (Table 2) (Etchevers et al., 1977; Noots et al., 1998; O'Sullivan et al., 1999). Numerous studies have concluded that *Pseudomonas* spp. and *Enterobacter* spp. are amongst the predominant bacteria throughout the malting process (Douglas and Flannigan, 1988; Haikara et al., 1977; Noots et al., 1998; O'Sullivan et al., 1999; Petters et al., 1988). Lactic acid bacteria (LAB) are barely present in native barley, although substantial proliferation has been recorded during steeping (Booysen et al., 2002; O'Sullivan et al., 1999; Petters et al., 1988). Yeast and fungal communities are very much dependant on different malting practises although *Aspergillus, Penicillium, Rhizopus, Fusarium* and *Mucor* are examples of the most frequent fungi encountered (Douglas and Flannigan, 1988; O'Sullivan et al., 1999; Petters et al., 1988; Noots et al., 1998).

The active interaction of these communities with the barley grain throughout the production chain significantly influences the technological, nutritional and organoleptic qualities of the final malt and beer product. These impacts may either be deleterious or beneficial, depending on the nature and amount of microbes present (Laitila et al., 2007; O'Sullivan et al., 1999).

7. Microbiological impact on malt quality

7.1 Negative effects of microflora

The indigenous microbial community of barley is often associated with unwanted trends such as variability in malt batches, grain dormancy, undesirable aromas and interference with barley respiration (Doran and Biggs, 1993). These phenotypes may cause inconsistent brew house performance and are not only caused by physical impedance of grain germinative functions by these cultures, but also microbial metabolism during malting (Noots et al., 1998). Malt microbial contamination is of minor concern at the brew house as the majority can effectively be removed during the high temperatures of mashing and wort boiling. Metabolites produced by these organisms are however regarded as hazardous at this stage since it can survive such harsh conditions and contribute to poor beer quality (Lowe, 2005a; Van Nierop et al., 2004; Laitila et al., 2007; O'Sullivan et al., 1999). It is therefore evident that microbial contamination needs to be controlled early in the malting process, even prior to harvesting, to prevent production losses during brewing.

The presence of aerobic microbes in and on the outer layers of barley grains is a high risk to successful malting. These organisms inhibit barley germination by competing with the embryo for oxygen, or by physically prohibiting the entry thereof (Doran and Briggs, 1992; Lowe, 2005b; Noots et al., 1998; Kelly and Briggs, 1992; Laitila et al., 2007). Such interference often leads to a phenomenon known as water sensitivity during which kernels are unable to germinate in an excess of

water. Steeping relies on wet stand periods, and therefore it may adversely affect the final malt product.

Water sensitivity is also caused by microbial biofilms which are usually associated with extracellular polysaccharide (EPS) production. Such a slime covering may disrupt root emergence and gas exchange of kernels, ultimately inhibiting germination. Microbial EPS is responsible for mash filtration and wort separation difficulties (Raulio et al., 2009; Haikara and Home, 1991). The organisms that are of main concern are *Pseudomonas* spp., LAB, and members of *Enterobacteriaceae* (Laitila et al., 2007).

Filamentous fungi are largely unwanted during malting due to their production of mycotoxins. These toxins are products of secondary metabolism in response to stress conditions, and are known as the most toxigenic metabolites of plant natural origin (Medina et al., 2006). The extremely stable nature of mycotoxins allows its survival in the final beer product and may pose serious health hazards (Medina et al., 2006; Macdonald, 1997). The most prevalent toxigenic fungi associated with barley belong to the genera *Penicillium*, *Fusarium* and *Aspergillus.*

Fusarium species are regarded as the most deleterious plant pathogens world-wide and barley quality is immensely affected by the plant disease Fusarium head blight (FHB). The infection of cereal heads by these species do not only reduce harvest yield, but also result in micotoxigenic contamination (McMullen et al., 1997; Ioos et al., 2004). These species produce a vast array of mycotoxins of which the most important include trichothecenes: T-2 toxin, deoxynivalenol (DON), diacetoxyscirpenol (DAS), nivalenol (NIV), fusarenon-X and an estrogenic toxin known as zearalenone (ZER). Deoxynivalenol has been reported to be the most predominant mycotoxin in infested barley and is predominantly produced by *F. graminearum* in warm regions, and *F. culmorum* in cold regions (Laitila et al., 1997; Lowe and Arendt, 2004; Wolf-Hall, 2007; Schwarz et al., 1995a). The majority mycotoxins are water-soluble and levels are greatly reduced during the soaking and aeration steps of steeping. Numerous authors have demonstrated an increase in *Fusarium* species and its mycotoxin levels during subsequent germination, leading to even higher toxin levels than in raw barley (Lancova et al., 2008; Schwarz et al., 1995b).

Some mycotoxins, especially trichothecenes, interfere with barley germination through the inhibition of α-amylase and protease synthesis (Noots et al., 1998). Fermentation efficiency is also affected by mycotoxins, and it has been demonstrated that *Fusarium* toxins can decrease the fermentation rate of *S. cerevisiae* by 50 – 80 % (Wolf-Hall, 2007). It has been suggested that these toxins cause slower oxygen utilization by yeast due to their inhibition of mitochondrial functions, ultimately causing slower growth rates. Premature yeast flocculation (PYF) is also associated with mycotoxins. This results in incomplete beer fermentation and unwanted aroma profiles as yeast prematurely dies off and settles at the bottom of the fermentation tank. Fungal enzymes have the ability to degrade husk

arabinoxylans which leads to the formation of PYF inducing factors (Van Nierop et al., 2004; Wolf-Hall, 2007).

Another quality defect during beer production related to fungal infection is known as primary gushing (Lowe and Arendt, 2004; Sarlin et al., 2007; Prentice and Sloey, 1960). It is defined as the spontaneous over-foaming of beer immediately after opening of the packaged product, and may cause major economic losses of beer brands (Laitila et al., 2002; Schwarz et al., 1996). The compounds responsible for this phenomenon still remain largely unknown due to their complex nature. Various attempts to solve this problem have indicated a correlation between mycotoxins and the gushing potential of beer, with *Fusarium* toxins recognized as the main gushing inducers (Noots et al., 1998; Lowe and Arendt, 2004; Wolf-Hall, 2007). It has also been suggested that various other compounds including hydrophobins and non-specific lipid transfer proteins (ns-LTPs) may be responsible for this phenomenon (Sarlin et al., 2007). Hydrophobins are small fungal proteins which may be secreted by various fungi in the field or during the malting process, and is a communicative tool of fungi with the environment. In contrast, ns-LPTs are synthesized by kernels in response to fungal infection.

7.2 Positive effects of microflora

Despite these negative impacts of barley microflora, some have the potential to improve malt properties and brew house performance.

The most noteworthy contribution of beneficial microbes is enhanced polymer degradation due to their secretion of hydrolytic enzymes. These enzymes mainly include amylolytic, proteolytic and cell wall degrading enzymes (Yin et al., 1989; Hoy et al., 1989). Of the cell wall degrading enzymes, β-glucanase is of utmost importance as it breaks down β-glucan cell walls that physically impede the entry of other essential hydrolytic enzymes into the endosperm (Bamfort, 1994; Raulio et al., 2009). Angelina and Bol (1990) demonstrated that microbes contribute to 50 – 80 % of the barley β-glucanase pool, ultimately improving malt characteristics. It has also been found that the indigenous microbial community significantly contributes to the xylanolytic activity of malt. Van Camperhout (2000) demonstrated that 75 % of malt xylanase activity was from microbial origin, while only 25 % was derived from the grain itself. Microbes may further enhance malt protease activity through the secretion of extracellular protease during malting and by stimulating the release of a bound form of grain protease through the secretion of phytohormones (Angelino and Bol, 1990; Prentice and Sloey, 1960).

Germinating barley requires a fine balance in plant hormones, of which gibberellic acid (GA_3), indole-3-acetic acid (IAA) and abscisic acid (ABA) are most essential (Noots et al., 1998). Gibberellic acid stimulates the production of hydrolytic enzymes from the aleurone layers and is the most widely applied external additive in the malting industry. Indole-3-acetic acid supports the action of GA_3,

although ABA has been reported to suppress GA_3 inducible enzymes. Abscisic acid is therefore important to limit endosperm degradation in response to unfavourable environmental conditions, and have been externally applied to reduce malt losses. It is well known that microbes have the ability to produce all of these hormones and can significantly contribute to good malt quality by stimulating grain germination (Prentice and Sloey, 1960; Flannigan, 2003). While a limited amount of barley microflora has been found to produce GA_3 and ABA, IAA production seems to be a common trait (Tuomi et al., 1995).

Barley microflora has the ability to increase the nutritional value of malt by enhancing the bioavailability of minerals and vitamins, and by digesting anti-nutritive compounds (Laitila et al., 2007; Hammes et al., 2005; Steinkraus, 1998). The communities are also known to compete with each other through the production of various antimicrobial factors (Van Nierop et al., 2004; Laitila et al., 2007; Lowe and Arendt, 2004).

8. Optimization of malt quality through externally applied factors

Breweries are constantly in search of novel approaches in which to optimize production time and microbial stability. Malt is regarded as a food product, and therefore it is always necessary to consider the safety for human consumption. The majority of attempts have focussed on eliminating harmful microbes during the malting process, and not the direct enhancement of malt modification. These attempts can broadly be classified into chemical, physical and biological treatments.

8.1 Chemical and physical treatments

Optimization of malt production and quality essentially demands that the natural microbial community is controlled. Inorganic acids such as sulphuric acid, phosphoric acid and hypochloride have been demonstrated to successfully reduce the microbial population of barley and consequently improve germinative capacity (Doran and Briggs, 1993; Gaber and Roberts, 1969; Briggs and McGuinness, 1993). These chemicals are usually only effective at high concentrations and may adversely affect seed germination and therefore reduce malt quality and brew house performance. Such chemicals are even more unacceptable to brewers and consumers when retained in the final beer product (Wolf-Hall, 2007). Precautions must be taken as sublethal doses of some chemicals may stimulate the production of detrimental metabolites such as mycotoxins and gushing factors (Noots et al., 1998; Laitila et al., 2007).

Gamma-irradiation has also been proposed as a non-chemical means to improve the microbial stability and quality of malt. This treatment has the advantage in not only eliminating detrimental microbes but also insects, the principal microbial vectors in plant ecosystems. Previous investigations

have illustrated that irradiation can significantly reduce mycotoxin production and increase malt yields, although it may lead to decreased amylase activity (Noots et al., 1998; Wolf-Hall, 2007). The malting of partially sterilized grains may result in the recontamination of strains that are more resistant to gamma-irradiation, especially *Fusarium* species (Laitila et al., 2007).

8.2 Biological treatments

8.2.1 Gibberellic acid

Gibberellins are fundamental during germination since it stimulates the secretion of hydrolytic enzymes by the aleurone layer to degrade the starchy endosperm. Various maltsters apply a similar plant hormone, gibberellic acid (GA_3), during the malting process to augment the actions of the kernel's gibberellins. Gibberellic acid was originally isolated from the fungus *Gibberella fujikuroi*, and is also industrially isolated from the culture filtrate of this organism (Hough, 1985).

Gibberellic acid does not only aid the production of greater enzyme quantities, but may also be applied to break dormancy and reduce germination time. The external addition of this hormone does have several disadvantages, which are mainly due its dose-dependent response. Excessive GA_3 levels facilitate extensive rootlet and acrospires growth, which leads to over-modification and consequently major malt losses. High sugar and soluble nitrogen levels can also be obtained which is detrimental to pale malts as it increases colour development. Such unwanted effects are diminished by spraying a restricted dosage of 0.1 - 0.5 ppm onto the kernels as they enter the germination vessels (Hough et al., 1982; MacGregor and Bhatty, 1993).

8.2.2 Starter cultures

The exploitation of microflora as starter cultures during malting offers a natural and economical feasible alternative to improve product quality as opposed to unwanted chemical and physical treatments. The use of starter cultures to enhance the microbial stability of malt is well-known, although very few authors have added microbes to augment barley germination (Table 3). The demand for such starter cultures is growing, as the addition of GA_3 has been prohibited in various countries.

8.2.2.1 Bacterial starter cultures

Most studies regarding bacterial starter cultures have focussed on the addition of LAB to improve the microbial stability of malt. *Pseudomonas herbicola* has also been added to the malting process and it was found that this bacterium could significantly reduce germination by 24 h (Pekhtereva et al., 1981).

Lactic acid bacteria

Lactic acid bacteria have been successfully employed in the food industry for centuries due to their generally regarded as safe (GRAS) status and broad range of antimicrobial properties. In addition, these cultures contribute to the flavour, nutritional value and structure of products (Lewis, 1998; Vaughan et al., 2005). The application of LAB starter cultures during malting has been extensively studied. Most research has focussed on the improvement of microbial stability, although additional beneficial effects such as reduced viscosity and β-glucan content of wort, and increased malt yield have been reported (Vaughan et al., 2005; Noots et al., 1998; Lowe et al., 2005a; Haikara et al., 1993).

LAB strains have proven to be successful in restricting the growth of both fungi and bacteria during the malting process. *Lactobacillus plantarum* and *Pediococcus pentosaceus* strains have been employed to control the growth of harmful EPS-producing bacteria when applied to the steep water at a final viable count of approximately 10^7cells/g barley (Laitila et al., 2006). These cultures furthermore restricted the growth and DON production by *Fusarium* contaminants (Laitila et al., 2007; Laitila et al., 2002). The reduction of aflatoxin producing moulds and aerobic heterotrophic bacteria by LAB starter cultures have also been reported (Vaughan et al., 2005; Karunaratne et al., 1990; Gourama and Bullerman, 1995; Doran and Briggs, 1993).

The antagonistic action of LAB is largely based on the production of bacteriocins, hydrogen peroxide, diacetyl and other low molecular weight antimicrobial compounds. These microbes can also enhance microbial stability by the production of various organic acids such as acetic acid and lactic acid that cause a decrease in pH (Lowe et al., 2004; Vaughan et al., 2005). The exact antimicrobial mechanisms of LAB in the malting environment are difficult to explain due to the complex interactive nature of the various compounds (Lowe et al., 2004; Noots et al., 1998; Laitila et al., 2007).

Lactic acid production by LAB is not only effective in controlling microbial proliferation, but has also been exploited by various breweries to produce acid malt which can facilitate enhanced malt and beer quality (Vaughan et al., 2005; Lewis, 1998; Laitila et al., 2006). The main objective in biological acidification is to produce mash and ultimately beer with a defined pH which cannot be achieved by the brewer without the addition of an external acid source. The pH of mash is normally 5.7 - 5.75, which is higher than the optimum pH of enzymes essential for mashing. The addition of LAB to malt allows a decrease in the pH, resulting in higher enzymatic potential. Besides a substantial increase in enzyme activity, other benefits of biological acidification include improved filterability and organoleptic qualities of beer, faster beer fermentation rates and increased beer foam stability. There are various methods in which LAB may be utilized to produce acid malt. Germinating barley may, for instance, be held under anaerobic conditions for 24 h to acidify it by the action of omnipresent LAB. Green malt may further be sprayed with LAB before kilning, especially thermophilic LAB such as *L. delbrueckii* and *L. amylovorous* (Lowe et al., 2004). Alternatively, kilned malt can be

steeped at 45 - 50 °C until 1 % lactic acid has been produced (Vaughan et al., 2005). The addition of LAB at the start of the malting process have however also been shown to reduce malt pH and increase enzyme potential (Laitila et al., 2006).

LAB, in general, do not produce enzymes that can enhance malt modification. These organisms are, however, good protease producers and it has been demonstrated that proteolytic strains can increase the nitrogenous content of malt (Haikara et al., 1993; Lowe et al., 2005a). Amylase-producing *L. amylovorus* and *L. amylolyticus* have also been applied to the malting process, but could not significantly enhance starch degradation (Lowe et al., 2005b). Laitila et al. (2006) observed increased xylanase and β-glucanase activity in malt produced with *L. plantarum*. These authors concluded that *L. plantarum* stimulated the growth of yeast with these hydrolytic capabilities, and therefore contributed indirectly to enhanced malt modification.

9.2.2.2 Fungal starter cultures

Barley-associated fungi are not only known for their antimicrobial properties, but also their ability to produce various hydrolytic enzymes which can contribute to malt modification (Hoy et al., 1981; Sarlin et al., 2005; Yin and MacGregor, 1989). In contrast to LAB, the use of fungi as starter cultures has not been extensively studied. The majority of researchers have added fungi to improve the microbial stability of malt, and the direct enhancement of malt modification by such cultures is still a relatively unexplored field.

Precautions should be taken when choosing a potential candidate, as various fungi are known to produce toxigenic substances. In addition, the spores of certain fungi might be allergens or cause lung disease in workers of malt houses, especially immune-compromised individuals.

Geotrichum candidum is a recognized starter culture in the production of various cheeses, and not known to pose any risk to human health (Pottier et al., 2008). Numerous authors have investigated the potential of this fungus as a starter culture for malting. Biovin and Malanda (1997) demonstrated how *G. candidum* could effectively restrict undesirable fungi and mycotoxin production when added during the malting process. It was also found in this study that *G. candidum* have the potential to effectively enhance malt modification. The addition of this starter culture has further been shown to increase malt yield, lower wort viscosity and increase the enzymatic potential of malt (Foszczynska et al., 2004; Dziuba et al., 2000).

Laitila et al. (2011) added *Wickerhamomyces anomalus* (synonym *Pichia anomala*) with antagonistic activity against moulds, to the malting process. This yeast restricted hydrophobin production during malting, as well as beer gushing. To broaden the antimicrobial spectrum and improve malt brew

house performance, the combination of this yeast with *L. plantarum* resulted in improved germination and faster wort filtration.

Rhizopus oligosporus was the only fungus found in the literature which was employed in malting with the particular aim to contribute to malt modification. This fungus produced β-glucanases, xylanases and proteases, although it could not degrade starch. These enzymes were active during mashing and notably improved lautering performance. The success of this culture depended on culture activation prior to malt addition, as dormant spores could not enhance modification (Noots et al., 2001a, 2001b).

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Table 1. Microflora (genera) present on barley after harvesting (Noots et al., 1998)

Bacteria		Fungi/yeasts			
Gram$^-$	**Gram$^+$**	**Ascomycota**	**Basidomycota**	**Zygomycota**	**Mitosporic fungi**
Clavibacterium	*Actinomyces*	*Alternaria*	*Cryptococcus*	*Absidia*	*Acremoniella*
Enterobacter	*Arthrobacter*	*Arthrinium*	*Rhizoctonia*	*Mucor*	*Acremonium*
Erwinia	*Bacillus*	*Aspergillus*		*Rhizopus*	*Arthrobotrys*
Escherichia	*Corynebacterium*	*Botrytis*		*Syncephalastrum*	*Aureobasidium*
Flavobacterium	*Lactobacillus*	*Candida*		*Thamnidium*	*Cephalosporium*
Pseudomonas	*Leuconostoc*	*Chaetomium*			*Doratomyces*
Xanthomonas	*Micrococcus*	*Cladosporium*			*Epicoccum*
	Pediococcus	*Cochliobolus*			*Harzia*
	Streptomyces	*Curvularia*			*Helminthosporium*
	Thermoactinomyces	*Didymella*			*Hormodendrum*
		Drechslera			*Monilia*
		Eupenicillium			*Papiulaspora*
		Fusarium			*Rhodotorula*
		Geotrichum			*Sclerotium*
		Gonatobotrys			*Septonema*
		Hansenula			*Spicaria*
		Hyphopichia			*Sporobolomyces*
		Hypocrea			*Thermomyces*
		Microdochium			*Thielaviopsis*
		Neocosmospora			*Torula*
		Nigrospora			*Trichothecium*
		Paecilomyces			*Ulocladium*
		Papularia			*Verticillium*
		Penicillium			*Wallemia*
		Phoma			
		Pithomyces			
		Pyrenophora			
		Saccharomyces			
		Scopulariopsis			
		Septoria			
		Sordaria			
		Stemphylium			
		Talaromyces			
		Thermoascus			
		Thielavia			
		Torulopsis			
		Trichoderma			
		Williopsis			

Table 2. Microflora (genera) present on malt (Noots et al., 1998)

Bacteria		**Fungi/yeasts**		
Gram$^-$	**Gram$^+$**	**Ascomycota**	**Zygomycota**	**Mitosporic fungi**
Clavibacterium	*Actinomyces*	*Alternaria*	*Absidia*	*Cephalosporium*
Enterobacter	*Arthrobacter*	*Aspergillus*	*Mucor*	*Epicoccum*
Erwinia	*Bacillus*	*Botrytis*	*Rhizopus*	*Helminthosporium*
Escherichia	*Lactobacillus*	*Candida*		*Rhodotorula*
Flavobacterium	*Leuconostoc*	*Cladosporium*		*Trichothecium*
Pseudomonas	*Micrococcus*	*Pyrenophora*		*Ulocladium*
	Pediococcus	*Geotrichum*		*Verticillium*
		Penicillium		
		Phoma		
		Fusarium		

Table 3. Microbes exploited for starter cultures in malting, including strain numbers, their purpose and reference

Microbes	Strain	Purpose	Reference
1. Bacteria			
A) LAB			
Lactobacillus amylolyticus	TMW1.268	Biological acidification (BA)	Lowe et al., 2005b
L. amylovorus	FST 1.1	BA	Lowe et al., 2005a
L. delbrueckii		BA	Van Waesberghe, 1991
L. plantarum	TMW 1.460	BA	Lowe et al., 2005a
	VTT E-78076	Restrict *Fusarium* and Gram-negative bacteria	Haikara et al., 1993, 1994, 1995, 2001
		Enhancement of malt processability	Laitila et al., 1997, 1999, 2002
Pediococcus pentosaceus	VTT E-90390	Restrict *Fusarium* and Gram-negative bacteria	Haikara et al., 1993, 1994, 1995, 2001
		Enhancement of malt processability	Van Campenhout, 2000
	L7230	Bacteriocin production	Van Campenhout, 2000
B) Gram-negative			
Pseudomonas herbicola		Reduced germination time	Pekhtereva et al., 1981
2. Fungi			
A) Filamentous fungi			
Rhizopus oligosporus		Enhancement of malt modification	Coppens et al., 1996; Noots et al., 2003; Dufait and Coppens, 2002
B) Yeast-like fungi			
Geotrichum candidum	IFBM	Inhibition of toxigenic fungi	Biovin and Malanda, 1996, 1997; Biovin, 2002
	S1	Extract yield and improvement of wort filtration	Dziuba and Fosczynska, 2001
C) Yeast			
Wickerhamomyces anomalus		Restrict hydrophobin production and beer gushing	Laitila et al., 2011

Fig. 1. Difference between two-row and six-row barley

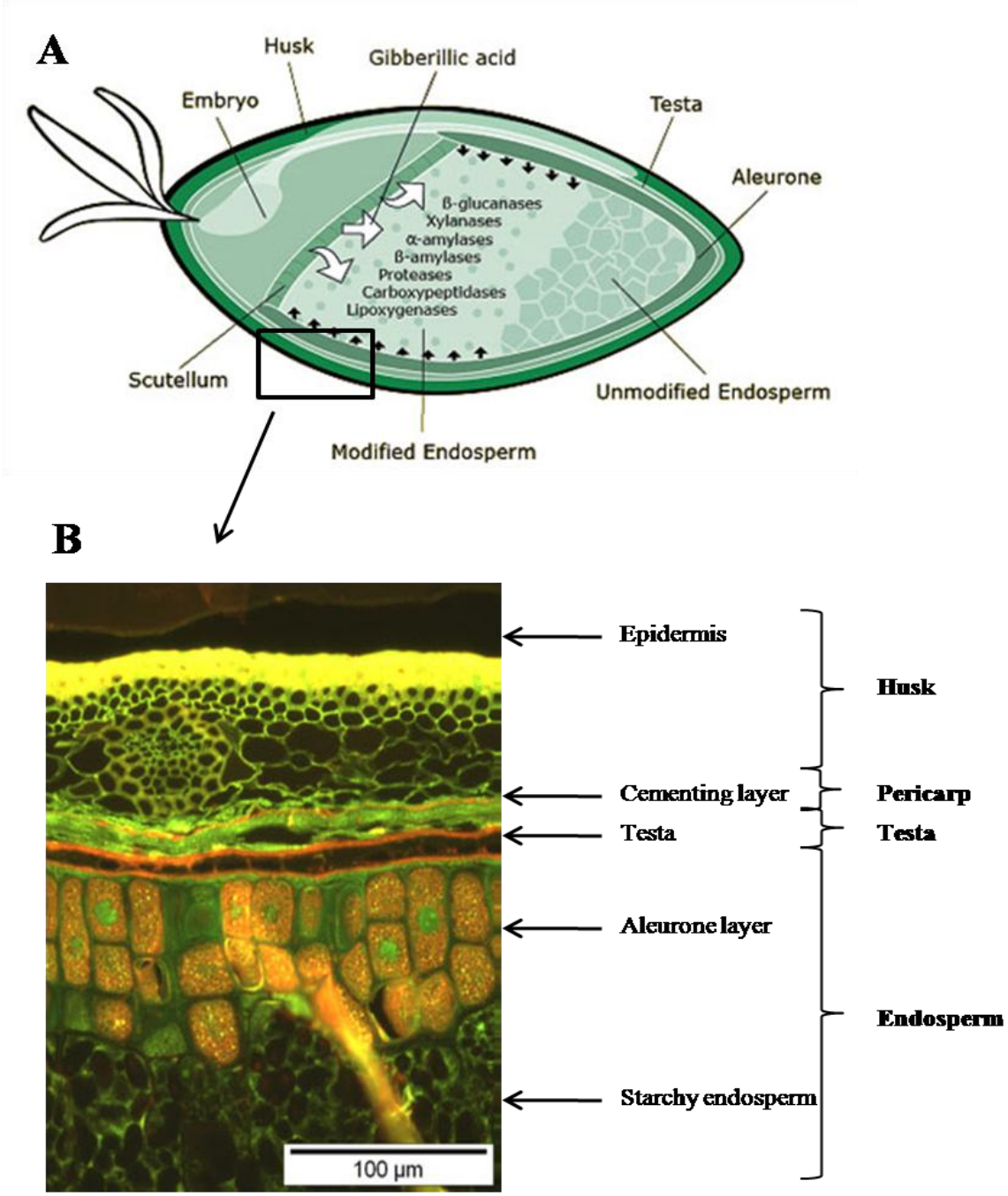

Fig. 2. A) Structure of the outer layers of mature barley grain, including enzymes secreted by the scutellum; B) Barley grain structure (adapted from Olkku et al., 2005)

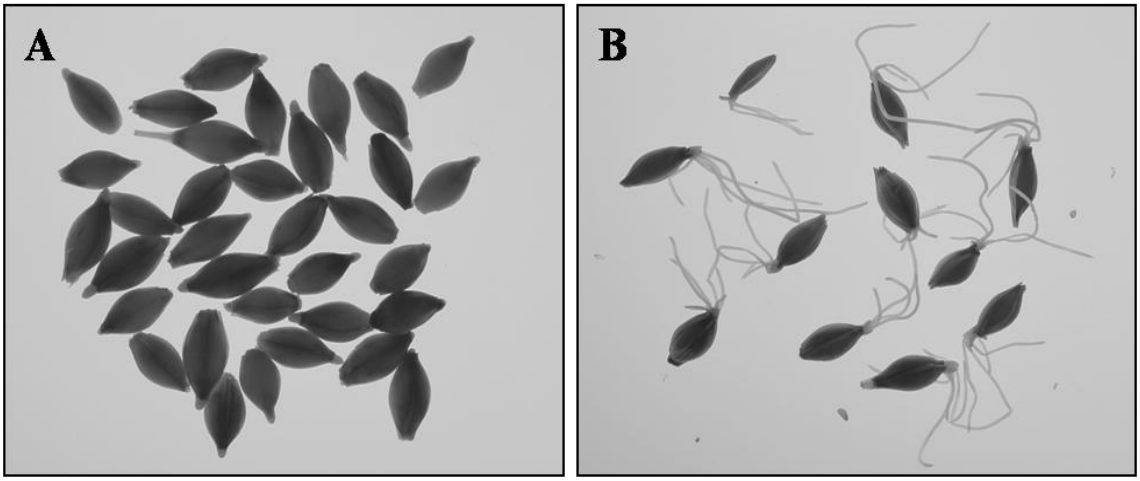

Fig. 3. A) Steeped kernels with chits visible; B) Barley kernels after 84 h germination. The acrospires is approximately 75 % the kernel length at this stage

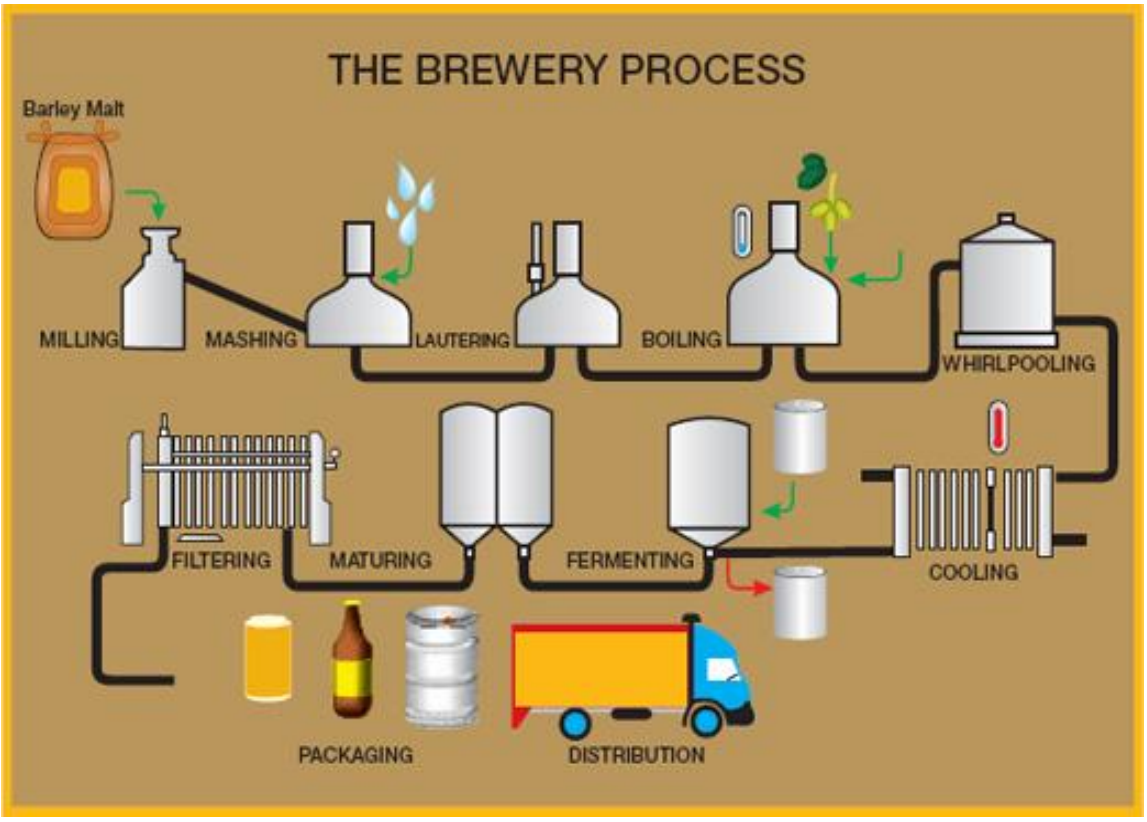

Fig. 4. Stages in the brewing process

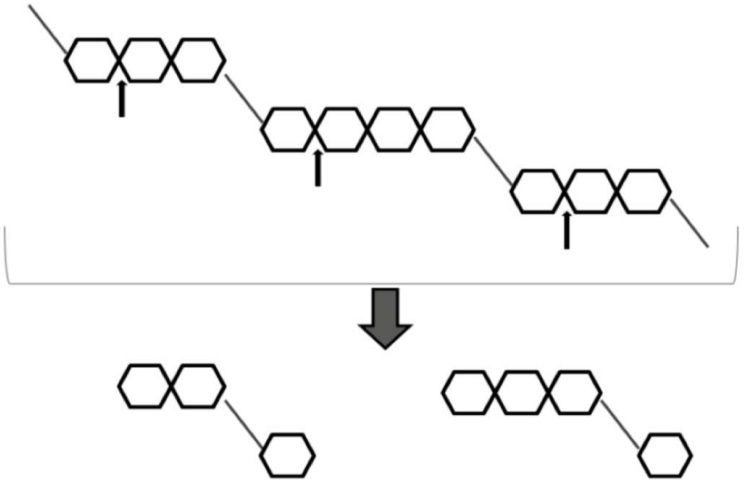

Fig. 5. Mode of action of (1,3;1,4)-β-glucanase on (1,3;1,4)-β-glucan, arrows indicate hydrolysis site of enzyme. Horizontally aligned hexagons: represents (1,4)-β-linked D-glucopyranosyl units, while oblique lines represents (1,3)-β-linkages (adapted from Jamar et al., 2011)

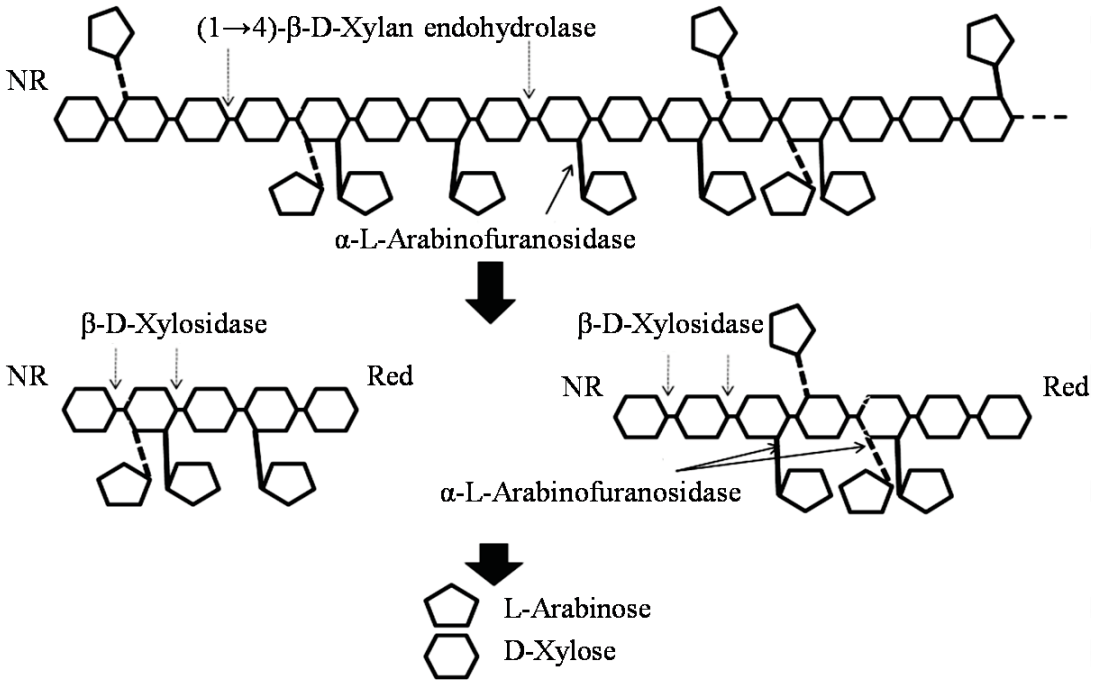

Fig. 6. Arabinoxylan structure and enzymes involved in hydrolysis (adapted from Jamar et al., 2011). Horizontally aligned hexagons represents the (1,4)-β-D-xylosyl residues of the (1,4)-β-D-xylan back-bone. Pentagons with solid attachment lines represents α-L-arabinofuranosyl substituents, which occur at C(O)2 of the xylosyl units, while pentagons with striped attachments represent α-L-arabinofuranosyl substituents, which occur at C(O)3 of the xylosyl units

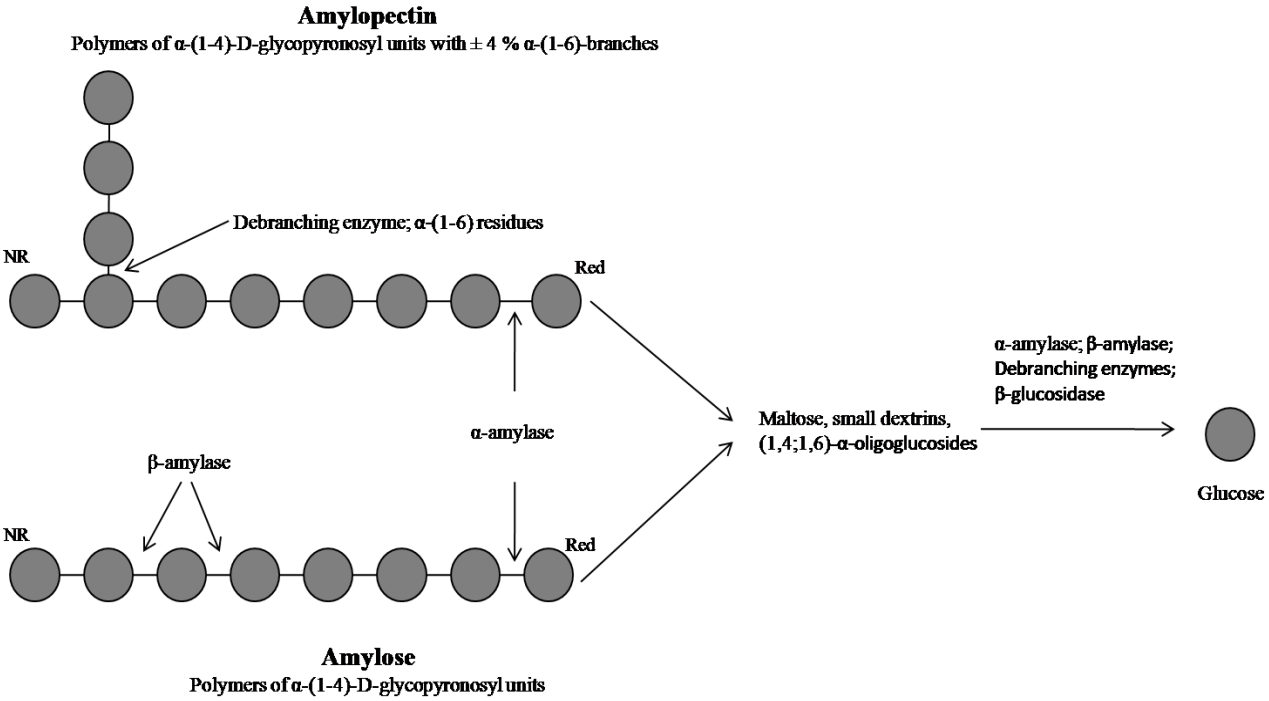

Fig. 7. Structures of amylopectin and amylose, including cleaving sites of hydrolyzing enzymes and their products

CHAPTER 3

Determination of enzyme activities produced by strains of *Geotrichum candidum*

Determination of enzyme activities produced by strains of *Geotrichum candidum*

Abstract

Twelve strains of *Geotrichum* spp. were isolated from maize, sorghum malt, ground nuts, barley malt and bananas. Analysis of the D1/D2 domains of the large-subunit rRNA gene sequences revealed that all isolates belonged to *Geotrichum candidum.* The ability of the strains to degrade proteins, cellulose, β-glucan, xylan and starch was determined by using plate assays. All twelve strains displayed β-glucanase and cellulase activity, whilst none degraded starch. Nine strains displayed extracellular protease activity and only three strains degraded xylan. All strains produced β-glucanase (0.2 - 1.0 nkats/mL) and cellulase (0.2 - 2.5 nkats/mL), according to results obtained with Dinitrosalicyclic (DNS) assays. Xylanase production was, however, poor and ranged between 0.6 - 0.8 nkats/mL. *Geotrichum* spp. 1173 and 1998 produced the largest variety of enzymes and were considered the most promising starter cultures for malting.

Introduction

The Genus *Geotrichum* is an unusual member of the fungal kingdom, as it is taxonomically positioned on the border of typical yeasts and moulds. *Geotrichum candidum* is the most extensively studied species in this group due to its application in different biotechnological processes. This ascomycetous fungus was first described in 1809 and is the anamorph of *Galactomyces candidus,* formerly *Gal. geotrichum* (Berger et al., 1999; Butler and Petersen, 1972; Gente et al., 2006).

Geotrichum candidum has been isolated from water, soil, air, plant tissue, foodstuffs and silage (O'Brien et al., 2005). Strains within the species typically produce a number of different proteases and aromatic compounds and are thus used as starter cultures in the production of cheese (Boutrou and Gueguen, 2005; Gente et al., 2006; Potts et al., 2000). The species is a constituent of the natural microbiota of the human digestive tract, and does not appear on the official list of harmful biological agents published by the Advisory Committee on Dangerous pathogens (2004) (Gente et al., 2006; Thornton et al., 2010; Pottier et al., 2008). Only 100 cases of *G. candidum* infections have been reported between 1842 and 2006, with most of the patients classified as immune compromised (Robertson and Patel, 1989; Thornton et al., 2010).

Geotrichum candidum grows rapidly, colonizes barley, has antimicrobial properties and produce extracellular enzymes (Dziuba et al., 2000; Foszcynska et al., 2004; Piegza et al., 2005). Based on these characteristics, the species is an attractive candidate for the development of a malt starter culture. Several studies have shown that *G. candidum* has the ability to degrade polymers in barley (Piegza et al., 2005; Witkowska and Piegza, 2006).

The barley kernel is mainly composed of 65 % starch, 11 % non-starchy polysaccharides (xylan and β-glucan), 5 % cellulose and 12 % proteins (Ullrich, 2011). Modification of barley involves the degradation of these polymers. In this study, 12 strains of *Geotrichum* spp. were screened for the production of enzymes that would degrade the polymers and enhance malt quality.

Materials and methods

1. Isolation and identification of fungal isolates

Samples of maize, sorghum malt, ground nuts, barley malt and banana were homogenised in sterile saline, serially diluted, streaked onto Yeast Peptone Dextrose (YPD) (Biolab, Gauteng, South Africa) medium and incubated at 26 °C for 72 h. Colonies that resembled typical *G. candidum* morphology were randomly selected and their DNA isolated using the ZR Fungal/Bacterial DNA kit (Zymo Research Corporation, Orange, California, USA). The D1/D2 region of the 26S rDNA (of rRNA gene) was amplified using forward primer F63 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and reverse primer LR3 (5'-GGTCCGTGTTTCAAGACGG-3'). PCR conditions were as follows: initial denaturation at 94 °C for 4 min, then 35 cycles of 30 s at 94 °C, 30 s at 63 °C and 1 min at 72 °C, followed by a final extension at 72 °C for 7 min. PCR products were kept at 4 °C. The amplicons were purified using the QIAquick PCR purification kit (Qiagen Inc., Valencia, California, USA) and sequenced at the DNA sequencing unit, Central Analytical Facility, University of Stellenbosch. Data from forward and reverse sequencing primers were aligned with ClustalW. Sequences were compared with DNA sequences in the NCBI databank, using the basic local alignment search tool (BLAST) program (Altschul et al., 1990). The closest match was considered the most likely correct identification and identity scores > 98 % were regard as significant (Hall et al., 2003). All isolates were stored at – 80 °C in sterile YPD broth supplemented with glycerol (40 %, v/v, final concentration).

2. Screening for enzymatic activity

Protease, cellulase, β-glucanase, xylanase and amylase activity were tested by inoculating specific growth media with 5 µL of an 8-h-old culture. The plates were incubated at 26 °C for 7 days and

treated as described below to visualize substrate hydrolysis. Clear zones surrounding the colonies were recorded as a positive result.

Protease activity was determined on 1.4 % skimmed milk agar (Difco, Becton, Dickinson and Company). Cellulase production was determined on 0.67 % (w/v) YNB (Yeast Nitrogen Base) Agar (Sigma-Aldrich, St. Louis, Missouri), supplemented with 1.0 % (w/v) carboxymethylcellulose (CMC, Sigma). Colonies were flooded with 0.1 % (w/v) Congo red (Sigma) for 15 min. Excess dye was rinsed off with 1M NaCl. β-Glucanase activity was determined on YPD plates containing 0.1 % (w/v) β-glucan (Difco), as described by Strauss et al. (2001). Xylanase activity was determined on Potato Dextrose agar (PDA) (Biolab, Biolab Diagnostics, Midrand, South Africa), supplemented with 0.5 % beechwood xylan (Carl Roth GmbH, Karlsrube, Germany). β-Glucanase and xylanase activity was recorded by flooding the colonies with 0.1 % (w/v) Congo red. Xylanase activity was enhanced by destaining plates with 1 M HCL, as described by Ruijssenaars and Hartmans (2001). Amylase activity was determined on Nutrient agar (Biolab) and YNB agar (0.67 %), each supplemented with 0.2 % (w/v) soluble starch (Merck, Darmstadt, Germany), as described by Buzzini and Martini (2002). Plates were flooded with a KI solution (0.6 %, w/v, K and 0.3 %, w/v, I crystals, dissolved in distilled water).

3. Quantification of enzyme activities

Isolates with β-glucanase, cellulase and xylanase activities were grown in Erlenmeyer flasks with 50 mL SC-medium (YNB without amino acids 1.7 g/L; glucose 10 g/L; amino acid mix 0.72 g/L; $(NH_4)_2SO_4$ 5 g/L; pH 5.5 - 6.0), supplemented with either 0.1 % (w/v) barley β-glucan, 1.0 % (w/v) low viscosity CMC, or 0.5 % (w/v) xylan. Incubation was aerobic, on a rotary shaker (250 rpm), at 26 °C for 5 days. Cells were harvested (10 000 x*g*, 15 min, 4 °C) and enzyme activities measured in the cell-free supernatant, using the Dinitrosalicyclic (DNS) assay described by Miller et al. (1960).

To determine β-glucanase and cellulase activity secreted by the isolates, 30 µL cell-free supernatant was added to 20 µL 0.1 % (w/v) barley β-glucan and CMC suspended in 0.05 M citrate buffer, pH 4.8, respectively (Miller et al., 1960). Reactions were performed directly in a 96-well microtitre plate, pre-incubated for 5 min at 50 °C. The plate was incubated at 50 °C for 30 min and the enzymatic reaction stopped by the addition of 100 µL DNS solution [1 % (v/v) 3,5-dinitrosalicyclic acid (Sigma), 20 % (w/v) potassium sodium tartrate, 1.0 % (w/v) NaOH, 0.2 % (v/v) phenol and 0.05 % (w/v) Na_2SO_3], followed by boiling for 15 min. The samples were allowed to cool to room temperature and readings were taken at 540 nm (Bio Rad, Smartspec Plus). The same method was used to determine xylanase activity, except that 6 µL cell-free supernatant was added to 54 µL substrate (1.0 % beechwood xylan). The samples were incubated for 60 min and the reaction stopped by the addition of 90 µL DNS solution. Enzymatic activity was expressed as nkat units/mL.

Results and Discussions

All strains of *G. candidum* included in this study are theoretically excellent candidates to degrade barley polymers, since the majority were isolated from substrates rich in proteins, cellulose, β-glucan, pentosans and starch. Strains 2894 and 2211 were the only two isolated from barley malt (Table 1).

Sequencing of the D1/D2 region have proofed valuable in the classification of yeast on genus and species level (Garner et al., 2010; Putignani et al., 2008). DNA fragments amplified from the D1/D2 region of the 26S rDNA of all 12 strains shared 99 – 100 % similarity with DNA sequences of *G. candidum* and are thus regarded as members of this species (Table 1).

All twelve strains had β-glucanase and cellulase activity (Table 2). Nine strains displayed extracellular protease activity and only three degraded xylan (Fig. 1). *Geotrichum* spp. 1173 and 1998 produced protease, cellulase, β-glucanase and xylanase, and are considered the most promising strains to be developed into starter cultures. *Geotrichum* spp. 2241 and 4964, on the other hand, displayed only β-glucanase and cellulase activity. None of the 12 strains displayed amylase activity, which is an unexpected finding as all strains were isolated from starch-rich sources. Amylase production is, however, not characteristic of *Geotrichum* spp. Arotupin (2007) isolated a *G. candidum* strain from Cassava waste water that hydrolyzed starch and Falih (1998) described two amylase-producing *G. candidum* strains isolated from soil. Of the 10 *Geotrichum klebahnii* strains that Buzzini and Martini (2002) isolated, only one strain with amylase activity was described. None of the *Geotrichum* isolates studied by Laitila et al. (2006) or Subash et al. (2005) degraded starch.

Beta-glucanase activity of all strains ranged between 0.2 – 1.0 nkats/mL after 7 days of incubation with barley β-glucan as carbon source (Fig. 2). Strain 2590 displayed the highest activity, while strain 2211 was the weakest producer. Corresponding to the results found in this study, Piegza and co-workers (2005) found β-glucanase activity of *G. candidum* strains on barley β-glucan to range between 0.3 and 1.0 nkat/mL. The authors recorded maximal β-glucanase activity, between 2.0 and 6.0 nkats/mL, when lichenin was used as carbon source. Higher β-glucanase activity might thus have been observed in this study if lichenin was used as substrate. Lichenin is, however, not present in barley and β-glucan was the most suitable substrate to evaluate the β-glucanase activity of cultures during malting.

According to plate assays with CMC as carbon source, all strains had the ability to degrade cellulose. With spectrophotometric assays the cellulase activity of all strains ranged between 0.2 and 2.5 nkats/mL after 7 days of incubation (Fig. 3). Isolate 2211 displayed the highest activity, while isolate 2241 was the weakest producer. Similar results were reported by Piegza and co-workers (2005). The *Geotrichum* spp. in their study displayed cellulase activity (0.5 – 3.3 nkats/mL) on CMC as substrate

after 5 days of incubation. Concluded from our findings and that reported in literature, the degradation of cellulose appears to be a general characteristic of the genus.

Xylan is a minor constituent of grain, with content ranging between 4 and 9 % (Ullrich, 2011). It acts as cement between cellulose fibers and its degradation is therefore crucial to ensure good malt modification (Fincher and Stone, 1986). Numerous studies have proven the xylan degrading abilities of *Geotrichum* spp. (Piegza et al., 2005; Rodionova et al., 2000; Witkowska and Piegza, 2006; Yaoi and Mitsuishi, 2004). Only strains 1998, 2894 and 1173 harboured xylanase activity with preliminary plate assays. Based on spectrophotometric assays, these isolates are poor xylanase producers, as maximal activity ranged between 0.6 and 0.8 nkats/mL after 7 days (Fig. 4). Piegza and co-workers (2005), however, illustrated that xylanase activity of *G. candidum* depends on the carbon source. The authors found CMC to be the best xylanase inducer, with maximal activity of 5 nkats/mL after 5 days. In the present study, xylan served as carbon source for xylanase induction. Strains with higher xylanase activity could have been isolated if CMC was used as carbon source.

These results clearly demonstrated the ability of *G. candidum* to degrade substrates essential in delivering a high quality malt product. It is nevertheless important to note that preliminary identification of enzyme-producing strains does not guarantee that these cultures will secrete enzymes during malting. Different parameters such as low pH, low temperature, microbial competition, low water activity and poor oxygen and substrate availability may impede cell growth and enzyme production. To achieve a more complete evaluation of starter culture potential, future screening studies should also take such parameters into account.

Valuable information will be gathered if a medium that reflects the components in barley are used. Milled malt and milled barley were investigated by Foszczynska et al. (2005) to determine the potential of *G. candidum* as starter cultures for malting. The authors found high enzyme activities in milled malt (1 – 9 nkat/mL), while noticeably lower activities (less than 1 nkat/mL) were observed in milled barley. This is not surprising, since malt contains more hydrolyzed polysaccharides than barley.

The only way to evaluate enzyme production by the strains selected in this study is to add them as starter cultures during malting. Foszczynska and co-workers (2005) added *G. candidum* isolates with proven hydrolytic activity on milled malt to the malting process and found that the enzymatic potential of malt could be increased. Higher β-glucanase activities (24 U/kg) and α-amylase (20 U/g) were observed compared to control malts. Dziuba et al. (2000), on the other hand, selected random *G. candidum* strains as starter cultures which contributed to a greater yield in malt extract. It was however noted that protein modification and viscosity was adversely affected. These results clearly indicated that preliminary selection of *G. candidum* isolates based on enzyme activities is useful to assess their potential as starter cultures.

Conclusion

This study illustrated the diverse hydrolytic capabilities of *Geotrichum* spp., making them excellent starter culture candidates for malting. With the aid of plate- and spectrophotometric assays, isolates 1173 and 1998 were identified as the most promising strains. It is however not known how these cultures will perform in a malting environment because their activities were not tested under similar conditions. It is also not known how it will react to our South African barley, as the effect of starter cultures is highly dependent on barley variety (Dziuba et al., 2000). For future studies it would therefore be fundamental to also take these parameters into account when searching for potential starter cultures.

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Table 1. Strains of *Geotrichum candidum* screened for enzymatic activity

Strain Number	Substrate	Genotypic identification by D1/D2 rDNA sequencing[a]			
		Species	Accession number	Query coverage (%)	Maximum identity (%)
0866	Maize kernels	*G. candidum*	JF262181	100	99
1173	Sorghum malt	*G. candidum*	JQ713185	100	100
1998	Sorghum malt	*G. candidum*	EF536924	100	99
2241	Sorghum malt	*G. candidum*	JF262192	100	99
2589	Ground nuts	*G. candidum*	JN974268	100	100
2590	Ground nuts	*G. candidum*	JN974266	100	100
2591	Ground nuts	*G. candidum*	JF757237	100	99
2592	Ground nuts	*G. candidum*	EU753717	100	100
2894	Barley malt	*G. candidum*	AB438128	100	100
B	Unknown[b]	*G. candidum*	HM754450	100	100
4964	Banana	*G. candidum*	EU708992	100	100
2211	Barley malt	*G. candidum*	JN974269	100	99

[a]The closest species was retrieved by BLASTN sequence homology searches in the NCBI database. Species, accession numbers, query coverage and maximum identities are reported

[b]Obtained from The Department of Microbiology, University of Stellenbosch

Table 2. Enzyme activity of *Geotrichum* strains, according to preliminary plate assays

Strain	Presence or absence of enzyme activity*				
	α-amylase	β-glucanase	cellulase	protease	xylanase
0866	-	+	+	+	-
1173	-	+	+	+	+
1998	-	+	+	+	+
2241	-	+	+	-	-
2589	-	+	+	+	-
2590	-	+	+	+	-
2591	-	+	+	+	-
2592	-	+	+	+	-
2894	-	+	+	-	+
B	-	+	+	+	-
4964	-	+	+	-	-
2211	-	+	+	+	-

* Positive and negative activity is indicated with a + or -, respectively

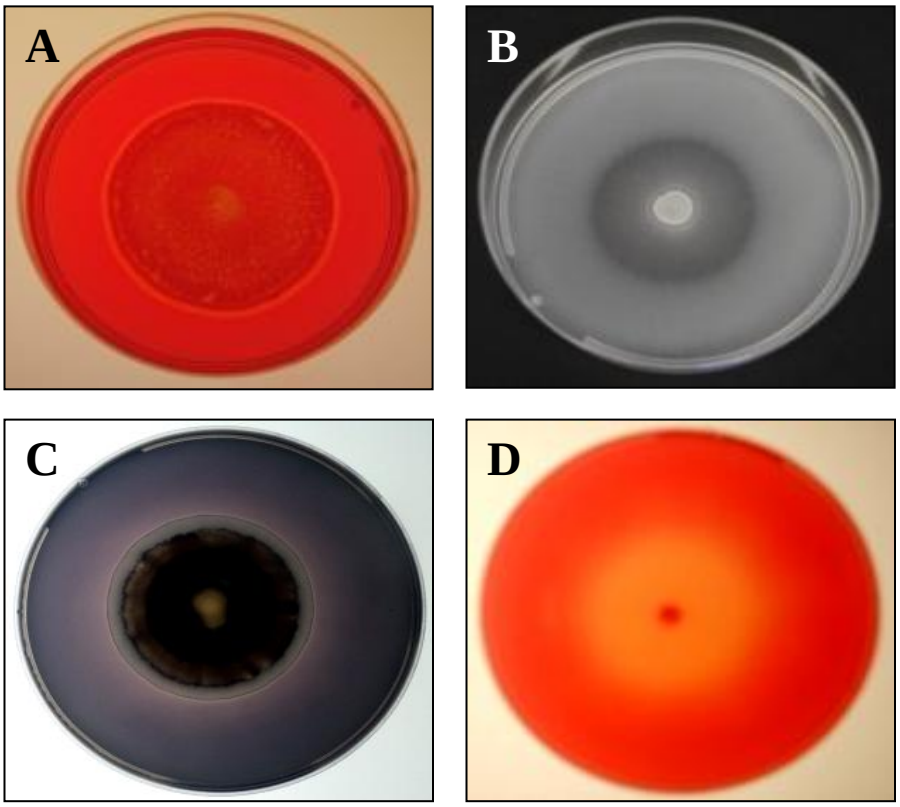

Fig. 1. Substrate hydrolysis by *Geotrichum* strains after 7 days of incubation at 26 °C. (A) Beta-glucanase activity on YPD + 0.1 % β-glucan; (B) Protease activity on 1.4 % skim milk; (C) Xylanase activity on PDA + 0.5 % xylan; (D) Cellulase activity on YNB + 1 % CMC

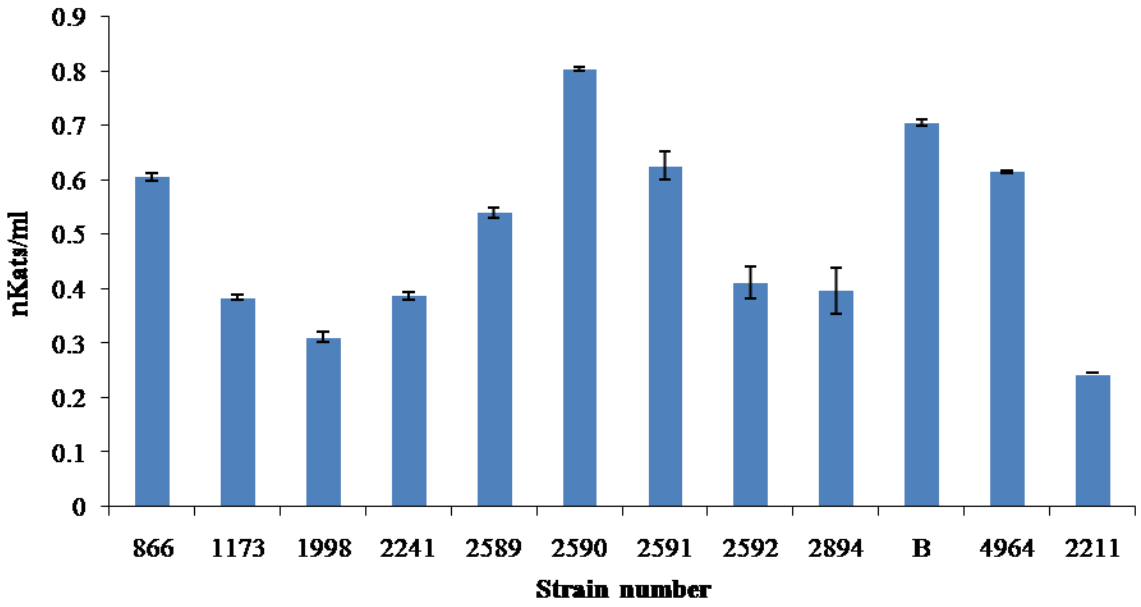

Fig. 2. Beta-glucanase activity of 12 *Geotrichum* strains, quantified after 7 days with a spectrophotometric DNS assay

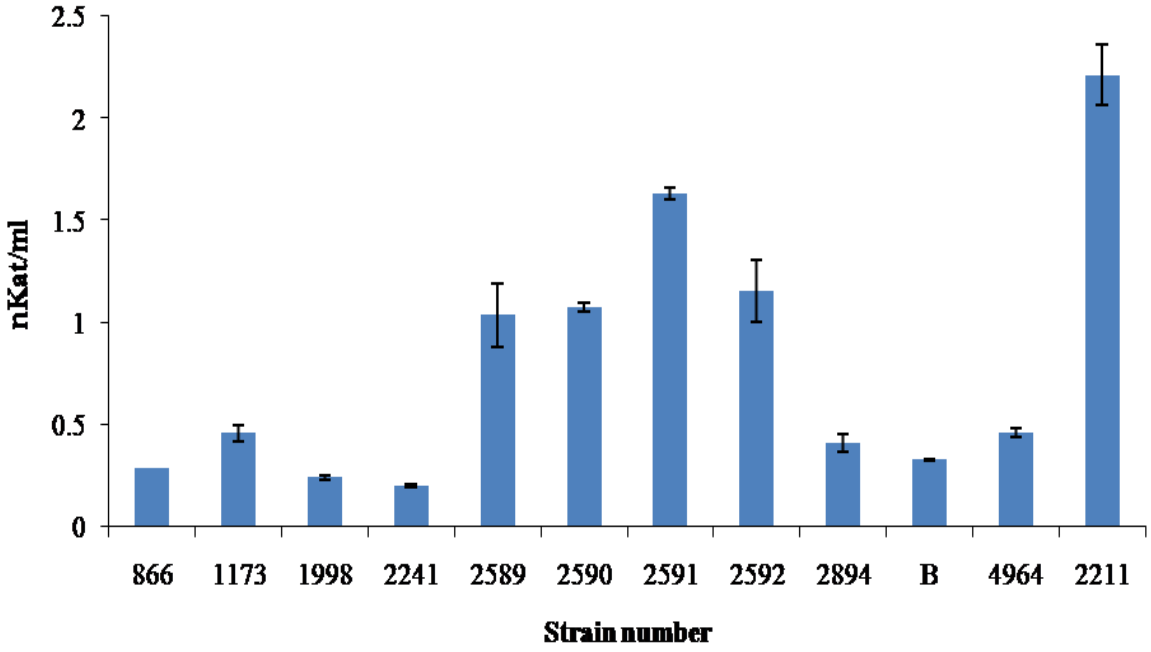

Fig. 3. Cellulase activity of 12 *Geotrichum* strains, quantified after 7 days with a spectrophotometric DNS assay

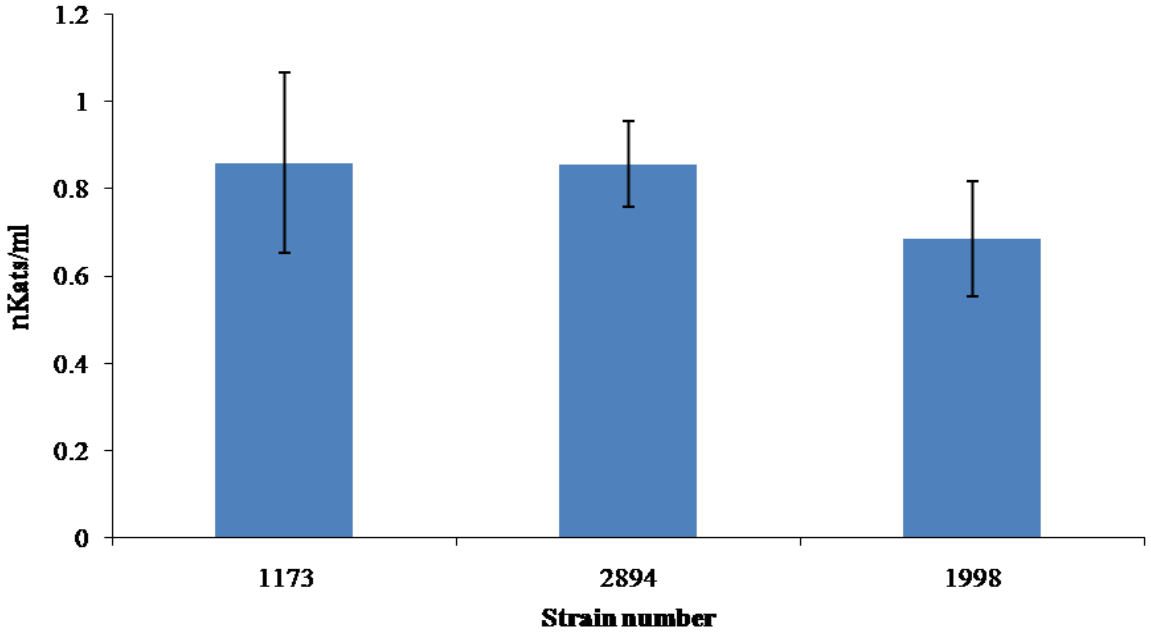

Fig. 4. Xylanase activity of three *Geotrichum* strains, quantified after 7 days with a spectrophotometric DNS assay

CHAPTER 4

Amylolytic *Lactobacillus plantarum* B.S1.6 and A.S1.2 isolated from barley

Amylolytic *Lactobacillus plantarum* B.S1.6 and A.S1.2 isolated from barley

Abstract

Two amylolytic lactic acid bacteria (ALAB) were isolated from South African barley cultivar SSG 564. Sequence analysis of the 16s rDNA and *rec*A genes identified both strains as *Lactobacillus plantarum*. Enzyme activities were characterized to evaluate the potential of these cultures to degrade starch during malting. Both strains displayed extracellular and cell-bound amylase, although enzyme production was poor at low malting temperatures. Enzyme production was subjected to catabolic repression by sugars present in malt, although high activity levels were recorded in low Malt Extract concentrations. Extracellular amylase from both cultures was unique compared to known amylolytic *L. plantarum* strains, and resembled the characteristics of other ALAB species. This ability was most likely acquired though plasmid exchange or horizontal gene transfers.

Introduction

The lactic acid bacteria (LAB) group is a member of the *Clostridium-Bacillus* subdivision of Gram-positive Eubacteria (Holzapfel and Wood, 1995). LAB is Gram-positive, non-spore forming cocci, coccobacilli or rods. These bacteria are non-motile, catalase-negative, and produce lactic acid as the major product of carbohydrate fermentation (Vandamme et al., 1996).

This genus have acquired generally regarded as safe (GRAS) status due to their typical association with food. Lactic acid bacteria produce a variety of antimicrobial substances such as organic acids, hydrogen peroxide, carbon dioxide and bacteriocins. These substances can inhibit pathogenic and food spoilage bacteria and consequently LAB are well-known starter cultures in the production of various food products (Laitila et al., 2006; Lowe and Arendt, 2004; Vaughan et al., 2005).

LAB are generally not classified as amylase producing bacteria due to their fastidious nutrient requirements for carbohydrates, amino acids, peptides, nucleotide bases, vitamins, minerals and fatty acids (Aguirre and Collins, 1993). Amylolytic lactic acid bacteria (ALAB) have nevertheless been isolated from different amylaceous fermented foods such as cereals and cassava (Agati et al., 1998; Olympia et al., 1995; Sanni et al., 2002; Johansson et al., 1995). Their role is still unclear, since mono- and disaccharides such as glucose are readily available for lactic acid fermentation in these

substrates (Reddy et al., 2008). The main amylase producing strains have been identified as *Streptococcus bovis*, *S. equines*, *L. manihotivorus*, *L. fermentum*, *L. amylovorus* and *L. amylophilus* (Giraud, 1994; Kandler and Weiss, 1986; Nakamura, 1981).

The aim of this study was to screen barley for ALAB which could be used as starter cultures to enhance starch degradation during malting. This is the first study to our knowledge to investigate the presence of ALAB in barley, and the search thereof was justified by the high starch content of its endosperm.

Materials and methods

1. Culture isolation and detection of amylase producing strains

Indigenous LAB were isolated from eight barley samples (SSG 564 cultivar; SA Maltsters, Caledon) by incubating 10 g of each sample in 100 mL sterile distilled water at 30 °C for 48 h.

The spot-on-lawn method was used to screen LAB for amylase activities. Incubated barley samples were serially diluted and surface spread onto modified De Man, Rogosa and Sharpe (MRS) - starch agar [dextrose replaced with 2 % (w/v) soluble starch as carbon source] (Biolab Diagnostics, Midrand, South Africa), supplemented with 200 µg/mL Delvocid. After incubation at 30 °C for 48 h, starch hydrolysis zones were visualized by flooding plates with a KI solution (0.6 %, w/v, K and 0.3 %, w/v, I crystals, dissolved in distilled water). Positive isolates were restreaked onto fresh MRS-starch agar plates to ensure pure colonies. All isolates were stored at - 80 °C in sterile MRS broth supplemented with glycerol (40 %, v/v, final concentration).

2. Phenotypic and biochemical characterization

Cell morphology, mobility, and the presence of spores were observed by phase contrast microscopy. Gram reaction and catalase activity were determined to confirm that the isolates are LAB. The sugar fermentation profile of isolates was tested with the API 50 CHL system (BioMerieux, Maray 1'Etoile, France), according to the manufacturer's instructions.

3. Random Amplified Polymorphic DNA (RAPD)-PCR of ALAB

RAPD-PCR analysis was performed on four ALAB as described by Van Reenen and Dicks (1996) with slight modifications. Genomic DNA was extracted from overnight MRS broth cultures using the ZR Fungal/Bacterial DNA kit (Zymo Research Corporation, Orange, California, USA). Polymerase chain reactions (PCR) were performed in triplicate. Each reaction of 25 µL contained 40 ng genomic DNA, 1.25 mM of each dNTP, 1 µM of a single 10 base primer and 2.5 units Taq Polymerase. Three

single primers [5'-CCAGCAGCTT-3' (OPL-03), 5'-GGGCGGTACT-3' (OPL-12), 5'-AGGTTGC AGG-3' (OPL-16)] were used. The cycling program included an initial cycle of 94 °C for 4 min, followed by 45 cycles of 94 °C (1 min), 36 °C (1 min), and 72 °C (1 min). Final incubation was at 72 °C for 5 min and ended by cooling to 4 °C.

Amplification products were resolved by electrophoresis in 1.4 % (w/v) agarose gels with TAE buffer, stained with ethidium-bromide and photographed. Lambda DNA, digested with *Hind*III and *Eco*R1 (Boehringer Mannheim), served as molecular weight marker.

4. Identification of isolates

Total DNA extraction was done using the ZR Fungal/Bacterial DNA kit (Zymo Research Corporation, Orange, California, USA). PCR was used to amplify the 16S rDNA gene using primers 8F (5'-CACGGATCCAGACTTTGATYMTGGCTCAG-3') and 1512R (5'-GTGAAGCTTACGGY TAGCTTGTTACGACTT-3') as described by Felske et al. (1997). The *rec*A gene was amplified using primers AmpF (5'-GCCCTAAAAAARATYGAAAAGAAHTTYGGTAAAGG-3') and AmpR (5'-AATGGTGGCGCYACYTTGTTTTTHACAACTTT-3'), as described by Endo and Okada (2008). PCR products of the 16S rRNA gene (1500 bp) and *rec*A (600 bp) were purified with a QIAquick PCR purification kit (Qiagen Inc., Valencia, California, USA), cloned into pGEM-T Easy vector (Promega Corporation, Madison, Wisconsin, USA) and transformed into *Escherichia coli* DH5*α*.

Plasmids were isolated using the Qiagen plasmid miniprep kit (Qiagen, Hilden, Germany) and sequenced (DNA sequencing unit, Central Analytical Facility, University of Stellenbosch). Blast analysis was conducted on the 16S rDNA and *rec*A sequences and aligned with ClustalW. Phylogenetic trees were constructed with sequences of approximately 1500 bp 16S rDNA and 600 bp *rec*A. Phylogenetic analysis was done using the neighbour-joining method, maximum-likelihood (Cavalli-Sforza and Edwards, 1967) and maximum parsimony algorithms (Kluge and Farris, 1969). Bootstrapping was performed as described by Felsenstein (1985).

5. Quantification of LAB amylase activity

Extracellular and cell-bound α-amylase activity was determined with the Cerelpha method (Megazyme International, Ireland, Ltd, County Wicklow, Ireland). Results were expressed in Cerelpha Units (CU)/mL. One unit of activity (Cerelpha Unit) is defined as the amount of enzyme required to release one micromole p-nitrophenol from a blocked p-nitrophenyl maltoheptaoside oligosaccharide substrate (BPNPG7) per minute under defined assay conditions. Malt flour was used as positive control while a *L. plantarum* which showed no amylase activity on plate assays served as negative control.

Starch fermentation was monitored in 250 mL Erlenmeyer flasks containing 200 mL MRS-starch broth with an initial pH of 7. All studies were conducted in triplicate without pH control. Each flask was inoculated with 4 % (v/v) of an overnight culture propagated in MRS-starch broth and incubated at 30 °C for 40 h. Culture medium was gently shaken at 120 rpm to maintain homogeneity. To determine extracellular α-amylase activity, samples were drawn every 2 - 3 h and centrifuged at 10 000 x*g* for 15 min. Cell-bound activity was measured after 24 h on cells that were washed twice in sterile saline (8.5 g/L), centrifuged at 10 000 x*g* for 15 min, and resuspended in extraction buffer (1 M sodium malate, 1 M sodium chloride, 40 mM calcium chloride; pH 5.4).

Cell growth was determined by serially diluting samples at each interval and plating it out onto MRS-starch agar. Plates were incubated at 30 °C for 48 h. Residual starch was determined by measuring the iodine complexing ability of starch as described by Nakamura (1981).

6. Enzyme characterization

6.1 Temperature and pH optimum

Characterization studies were performed in triplicate on extracellular α-amylase after 24 h incubation at 30 °C in MRS-starch broth. Standard assay conditions were applied as described previously, except for the desired variation in pH and temperature.

Temperature activity profiles were determined between 20 and 70 °C. The pH stability of enzymes was measured in a pH range of 3 - 9. Citrate phosphate buffer (0.1 mol/L) was used for pH 3 - 7; phosphate buffer (0.1 mol/L) for pH 7 - 8; and Tris buffer (0.1 mol/L) for pH 8 - 9.

6.2 Substrate specificity

Growth and substrate specificity of extracellular amylase was measured in different carbon sources. In each case, (2 %, w/v) dextrose in MRS-broth was replaced with the respective carbon source. Starch (Merck, Darmstadt, Germany), maltose (Sigma-Aldrich, St. Louis, Missouri), cellobiose (Sigma), Malt Extract (Muntons, Suffolk, England), including 4 % (w/v) Malt Extract, 6 % (w/v) Malt Extract, 2 % (w/v) glucose (Merck) + 2 % (w/v) maltose and 2 % (w/v) Malt Extract + 2 % (w/v) maltose were evaluated. Cultures (4 %, v/v) were incubated at 30 °C for 24 h. Cell growth was determined by serial dilutions on MRS agar, while extracellular α-amylase activity was measured on cell-free supernatant as described previously.

Results and discussions

1. Isolation and characterization of ALAB

The spot-on-lawn method identified 90 barley isolates with amylase activity based on clear halos around colonies. The hydrolytic activity of these isolates was confirmed by measuring the starch content in MRS-starch broth after 24 h incubation. Only four isolates, namely A.S1.2, A.S4.10, A.S8.10 and B.S1.6, displayed significant starch hydrolysis (Fig. 1). The other 86 isolates were regarded as false-positive. It has been reported that colony growth can inhibit iodine binding to starch in solid media, and may as a result resemble substrate degradation (Ruijssenaars and Hartmans, 2001). The high occurrence of false-positives can be attributed to the inability to discriminate between hydrolysis zones and dye blockage.

Biochemical characterization revealed the four isolates to be catalase-negative and Gram-positive. Morphologically, cells were classified as short rods, non-motile and non-spore forming. Based on these results all isolates were assigned to the LAB group.

Numerous studies have identified ALAB as common inhabitants of cereal fermented food (Agati et al., 1998; Diaz-Ruiz et al., 2003; Tou et al., 2006; Songré-Ouattra et al., 2008). The low incidence of ALAB on barley in this study might be explained by the low-throughput method used to isolate cultures. LAB are further rarely detected in native barley and only starts to proliferate once metabolizable nutrients become available during malting (Booysen et al., 2002; Bujalance et al., 2006; Laitila et al., 2006; Raulio et al., 2009). More ALAB would have been detected if it was isolated from steeped -, germinated - or kilned malt.

RAPD-analysis was conducted to discriminate between the four positive isolates since starch hydrolysis in MRS-starch broth was similar for A.S1.2, A.S4.10 and A.S8.10. The banding pattern of these isolates was identical for all three primers and differed significantly from B.S1.6 (Fig. 2). As a result, isolates A.S1.2, A.S4.10 and A.S8.10 were recognized as a single strain, and only two different ALAB strains were thus isolated from barley. To avoid confusion, isolate A.S1.2 was used in further studies. API 50 CHL analysis of A.S1.2 and B.S1.2 confirmed their individuality as isolates differed in the consumption of five carbon sources (arabinose, rhamnose, α-Méthyl-D-Mannoside, lactose and inuline) (Table 1).

Sequence analysis of the 16s rDNA and *rec*A genes identified both strains as *Lactobacillus plantarum* (Fig. 3 + 4). This species rarely has the ability to produce amylase, although such isolates have been identified. Giraud et al. (1991) isolated the first amylolytic *L. plantarum* from cassava roots. Thereafter, three separate studies identified amylolytic *L. plantarum* strains from fermented foods (Olympia et al., 1995; Songré-Ouattara et al., 2008; Sanni et al., 2002). To the best of our knowledge,

no author has previously described an ALAB from barley. Lowe and Arendt (2004) screened barley *L. plantarum* strains for amylase activity but none was found.

2. Enzyme quantification

Fermentation studies were conducted with *L. plantarum* A.S1.2 and B.S1.6 in MRS-starch broth. The behaviour of a *L. plantarum* strain without starch degrading properties was also investigated. Fig. 5 illustrates the evolution of extracellular α-amylase activity, pH, cell growth and starch degradation over a 40 h period.

L. plantarum A.S1.2 and B.S1.6 demonstrated similar patterns of starch utilization and pH reduction, although different growth patterns and extracellular α-amylase production was observed.

The three phases of a traditional growth curve was evident in A.S1.2. A short lag phase was followed by exponential growth, and ended with a stationary phase at 4 X 10^8 cfu/mL after 22 h. This phase was unstable, and a gradual decrease in cell numbers was observed. *L. plantarum* B.S1.6, in contrast, demonstrated a typical di-auxic pattern, with two exponential growth phases. At the end of the second stationary phase, low cell counts of 2 X 10^8 cfu/mL were reached. Although B.S1.6 did not grow as well as A.S1.2 in MRS-starch broth, cell counts remained stable until the end of fermentation. The di-auxic pattern has been demonstrated for an amylolytic *L. manihotivorans* strain and is due to its preference for glucose (Guyot and Morlon-Guyot, 2001). It can be speculated that B.S1.6 consumed glucose during the first exponential phase, after which transcriptional machinery was prepared for starch degradation during the second lag phase. It is also evident as the second exponential phase was accompanied with rapid starch hydrolysis. Similar growth patterns were expected for these isolates as they are both *L. plantarum* strains and therefore closely related. The difference might be due to variation in amylase genes, although further studies will have to be conducted to justify this statement.

In both ALAB strains no starch hydrolysis occurred during the first 7 h of incubation. Rapid starch consumption followed until it reached 1.89 g/L and 2.1 g/L for A.S1.2 and B.S1.6, respectively, at ± 22 h, after which no further degradation was observed. The negative control was unable to degrade starch throughout fermentation, illustrating the absence of amylase activity.

Growth of *L. plantarum* A.S1.2 and B.S1.6 resulted in a decrease in pH from 7.0 to 3.7 and 3.8, respectively, while a minor decrease to 5.9 was observed in the negative control. In general, LAB decreases the pH of media because of the conversion of fermentable sugars to lactic acid (Reddy et al., 2008). Starch degradation by ALAB isolates generated high glucose levels which could be metabolized to lactic acid, resulting in the large pH reduction observed. The negative control did not harbour this ability and consumed glucose present in the basal media. Glucose levels were, however,

low and therefore low cell counts of 4 X 10^8 cfu/mL was observed at stationary phase, accompanied by poor pH reduction.

Amylase activity was detected in A.S1.2 after 10 h of fermentation and correlated with starch degradation. Activity levels accompanied the growth curve and reached maximum levels at 20 h during stationary phase, after which it decreased. It has been demonstrated that this decrease in activity might be due to the accumulation of lactic acid (Calderon et al., 2001; Fossi et al., 2001). In B.S1.6, however, amylase activity was only observed after the stationary phase was reached at 13.5 h, and did not correlate with starch consumption. Activity increased exponentially until 29 h, and no decrease was observed as for A.S1.2. It is thus possible that the extracellular α-amylase of B.S1.6 is more stable at high lactic acid levels than A.S1.2.

The occurrence of amylase activity only once stationary phase is reached is common in *L. plantarum*, and authors have demonstrated that this species only produces amylase once all glucose in the medium has been consumed (Giraud et al., 1994). Amylase activity in *L. plantarum* A.S1.2 was unusual in that it resembled the pattern of *L. fermentum* amylase. In this organism amylase activity follows the growth cycle and decreases after stationary phase, resembling a tight association of amylase production with cell growth (Calderon et al., 2001; Fossi et al., 2001).

The ability of the ALAB to produce cell-bound amylase was also investigated after 24 h of growth (Fig. 6). *L. plantarum* A.S1.2 and B.S1.2 displayed levels of 3.2 and 1.8 CU/mL, respectively. These levels were > 5 times higher than the extracellular activity present at 24 h and indicated that both ALAB produced greater quantities of cell-bound amylase. The cell-bound activity of *L. plantarum* A.S1.2 and B.S1.6 was higher than isolates previously described. Songré-Ouattara et al. (2008) reported *L. plantarum* A6 to have cell-bound activity of 0.4 CU/mL after 24 h, while *L. plantarum* 6.1 and 4.4 displayed levels of < 0.1 CU/mL.

Literature regarding cell-bound and extracellular amylase activities of *L. plantarum* is poor. Most studies on amylolytic *L. plantarum* have focussed on extracellular amylase activity and therefore the incidence of cell-bound amylase in these cultures is not known (Giraud et al., 1991; Olympia et al., 1995; Sanni et al., 2002). Cell-bound amylase has been demonstrated in two *L. plantarum* strains, although these isolates did not produce extracellular enzymes (Songré-Ouattara et al., 2008). Only one *L. plantarum* was found in literature which demonstrated both cell-bound and extracellular amylase activity (Giraud et al., 1991). It is therefore not known whether the presence of both enzyme types in isolates from this study is significant.

3. Characterization of extracellular amylase

Extracellular amylase activity of the two isolates was characterised in terms of pH and temperature optimums (Fig. 7). The temperature profile was similar for both isolates and displayed optimum activity at 50 °C, although A.S1.2 was more stable at 70 °C than B.S1.6. These results correlate with the extracellular amylase from the *L. plantarum* strain isolated by Giraud et al. (1991). Olympia et al. (1995) demonstrated, however, that the amylase from *L. plantarum* is extremely thermoliable, with no activity at 50 °C.

The extracellular amylase of *L. plantarum* A.S1.2 and B.S1.6 demonstrated optimum activity at pH 8 and 7, respectively. The amylase of B.S1.6 was stable over a wider pH range than A.S1.2, although A.S1.2 still harboured 65 % activity at pH 9 compared to only 8 % in B.S1.6.

The majority of ALAB, including amylolytic *L. plantarum* strains, have an optimum pH of 4 – 5 (Giraud et al., 1991; Olympia et al., 1995; Agati et al., 1998; Aguilar et al., 2000; Champ et al., 1983). ALAB that function in alkaline environments have been identified and isolates from this study closely resemble *S. equines* and *Leuconostoc dextranicum* with optimum pH of 6.5 - 9 and 5 - 8, respectively (Dunican and Seeley, 1962; Lindgren and Refai, 1984).

Malting is usually conducted at neutral pH, between 14 – 18 °C. Both strains demonstrated poor activity at 20 °C, although > 50 % activity was observed at pH 6. Consequently, these enzymes will not function optimally during malting, but high cfu/g barley might compensate for low activity levels.

It is evident from these results that *L. plantarum* A.S1.2 and B.S1.6 differ from the characteristics of known amylolytic *L. plantarum* strains. Poor correlation was also found between amylase activities of different *L. plantarum* strains from literature, and isolates from this study resembled features of other ALAB. This ability was most likely acquired through horizontal gene transfer or plasmid exchange. To support this statement, *L. plantarum* rarely have the ability to degrade starch. Amylase genes have also been found on both the genome and plasmids of this genus (Giraud and Cuney, 1997; Olympia et al., 1995). Amplification of the respective genes, or plasmid curing, might provide more insight regarding the origin and function of *L. plantarum* A.S1.2 and B.S1.6 amylase activity in future studies.

The malting environment is comprised of diverse carbon sources such as glucose, maltose, starch and cellobiose. Certain substrates can inhibit the synthesis of carbohydrate degrading enzymes in bacterial species through catabolic repression (CR) (Teodoro and Martins, 2000). The effect of different substrates on amylase activity of *L. plantarum* A.S1.2 and B.S1.6 was investigated to evaluate how they will function in a malting environment.

Similar activity profiles were observed in both strains and were greatly dependent on carbon source (Fig. 8). In both cases maltose generated the highest amylase activity, followed by 2 % Malt Extract and 2 % starch. Comparable substrate specificities have been identified in ALAB, although no study was found regarding the specificity of these substrates in *L. plantarum* (Fossi et al., 2001; Guyot and Morlon-Guyot, 2001; Pompeyo et al., 1993). Various authors have also found ALAB to be more effective on maltose than starch. It has been speculated that the efficacy of starch degradation may be limited by the accumulation of limiting dextrins which cannot be fermented by ALAB, thereby restricting its growth and amylase synthesis (Calderon et al., 2001).

For both isolates, cellobiose and glucose generated the poorest amylase activity, even in combination with maltose. An increase in Malt Extract also resulted in decreased activity. These results suggest that certain substrates, especially cellobiose and glucose, inhibit extracellular amylase production in both isolates. This inhibition is most probably due to CR, and was also observed during the fermentation studies in *L. plantarum* B.S1.6. CR of LAB in relation to amylase expression has not been studied yet, although putative catabolic responsive element (cre) which mediates CR has been identified in *amy*A genes from different ALAB (Rodriguez et al., 2000; Hueck and Hillen, 1995). Due to CR, it was hypothesized that both strains would only produce sufficient amylase at the start of germination. As germination commences the mobilization of sugars might inhibit activity.

An inverse correlation existed between cell numbers and amylase activity, as the highest activity was observed in substrates that resulted in the lowest cfu/mL. Growth of B.S1.6 was very poor in starch compared to A.S1.2 and correlated with results from the fermentation study.

Conclusion

The incidence of ALAB in barley was low compared to other fermentable cereals reported in literature and may be attributed to the low-throughput screening method used. Barley itself was also not an ideal substrate for ALAB isolation since the majority LAB are normally present during the malting process. To our knowledge this is the first study in which amylolytic LAB were isolated from barley. The identification of these isolates as *L. plantarum* was further significant as this species does not usually harbour amylase activity.

Both isolates were attractive starter cultures because they contained cell-bound and extracellular amylase activity. Extracellular amylase production was accompanied by a decrease in pH due to the production of organic acids. Such a pH reduction in the malt environment will not only increase the microbial stability of malt, but will also assist in greater enzyme potential as the majority enzymes functions at low pH. Amylase activity in both strains was only 20 % functional at 20 °C and therefore

low malting temperatures might result in poor amylase activity. High cfu/g barley might compensate for the low production levels. Fermentable sugars such as glucose and cellobiose in the malting environment will also reduce amylase production through CR. Amylase secretion might therefore only occur during the initial stages of germination, when minor mobilization of the starchy endosperm has occurred.

The extracellular amylase from both cultures was unique compared to known amylolytic *L. plantarum* strains, and resembled characteristics of other ALAB species. This ability was most likely acquired though plasmid exchange or horizontal gene transfers. Valuable insight regarding the activity and regulation of amylase synthesis will be gained in future studies once the position and sequence of the respective genes have been identified.

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Table 1. Differential carbohydrate fermentation reactions between A.S1.2 and B.S1.6

Isolate	L-arabinose*	Rhamnose	α-Méthyl-D-Mannoside	Lactose	Inuline
A.S1.2	-	-	+	-	+
B.S1.2	+	+	-	+	-

***Positive and negative reactions indicated by + and -, respectively

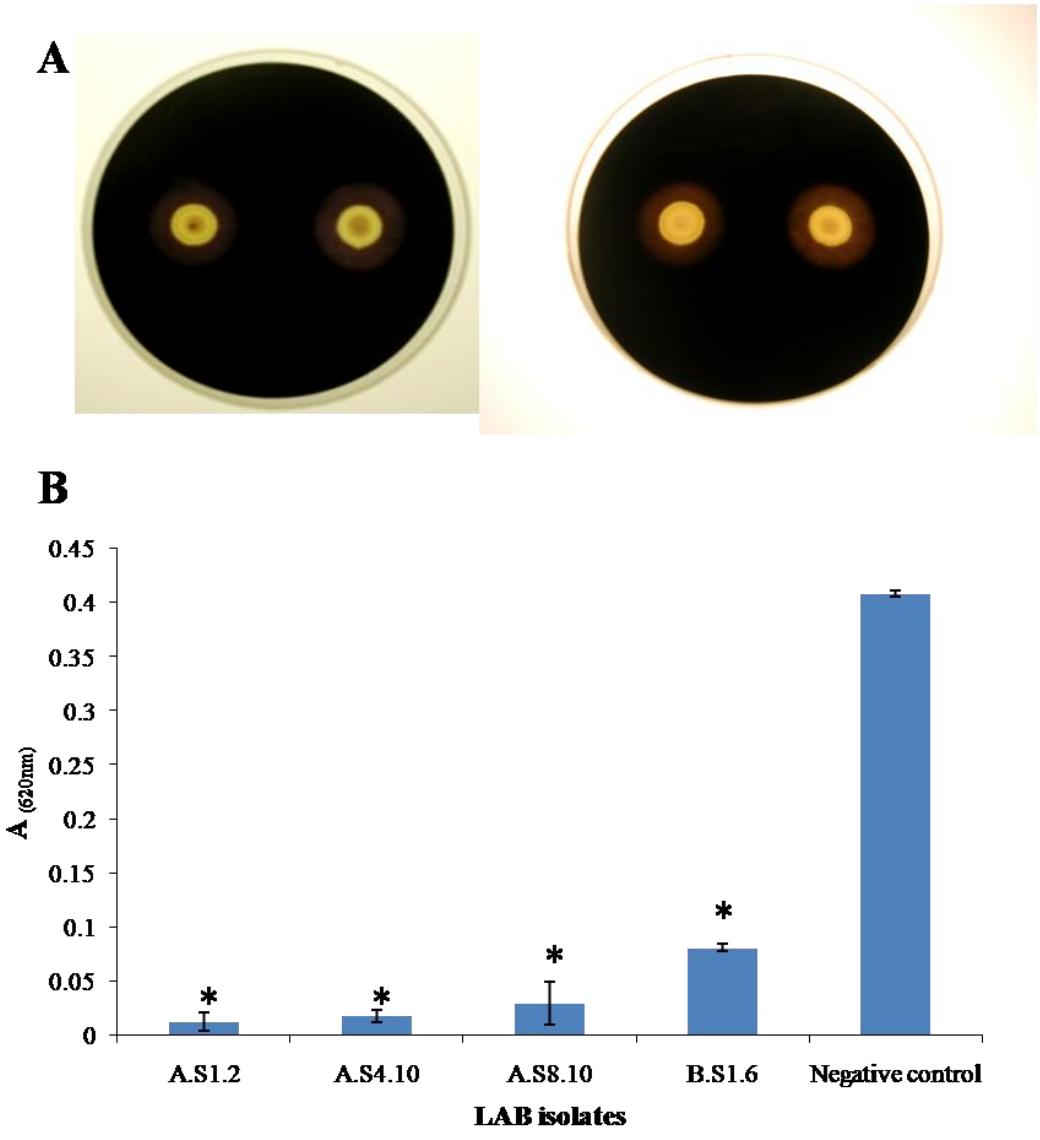

Fig. 1. (A) Preliminary identification of four ALAB by the spot-on-lawn method. Clear zones around colonies illustrate substrate hydrolysis. From left to right: A.S1.2, A.S4.10, A.S8.10, B.S1.6; (B) Starch degradation determined spectrophotometrically. Significant levels in the paired t-test: *$p < 0.05$

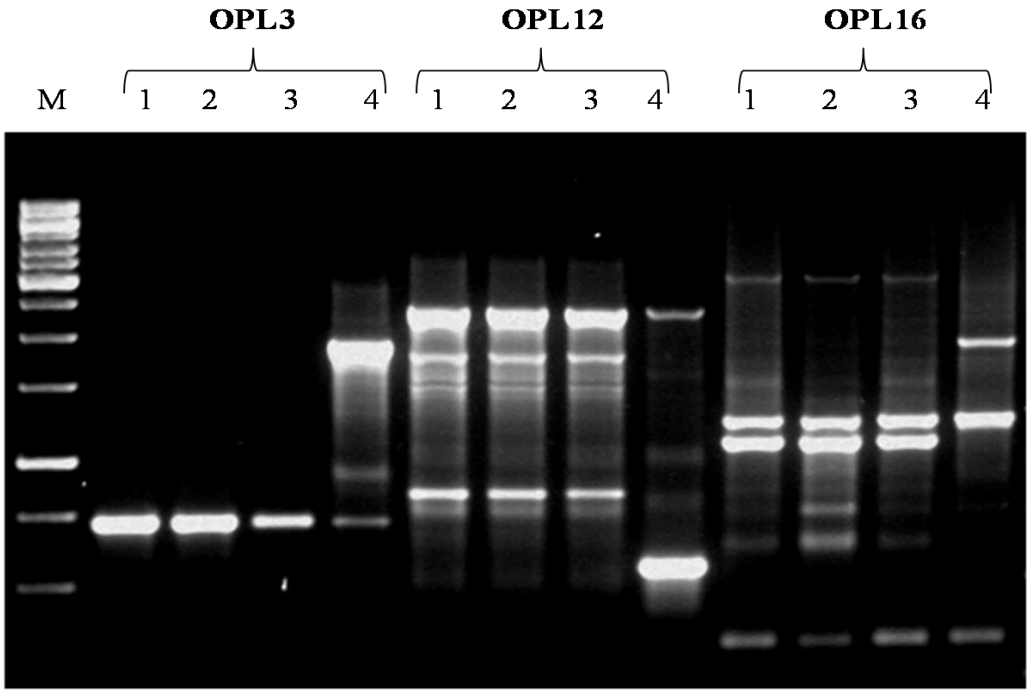

Fig. 2. DNA fragments obtained after RAPD-PCR of the genomic DNA of the four ALAB isolates. 1, A.S1.2; 2, A.S4.10; 3, A.S8.10; 4, B.S1.6; M, Molecular marker

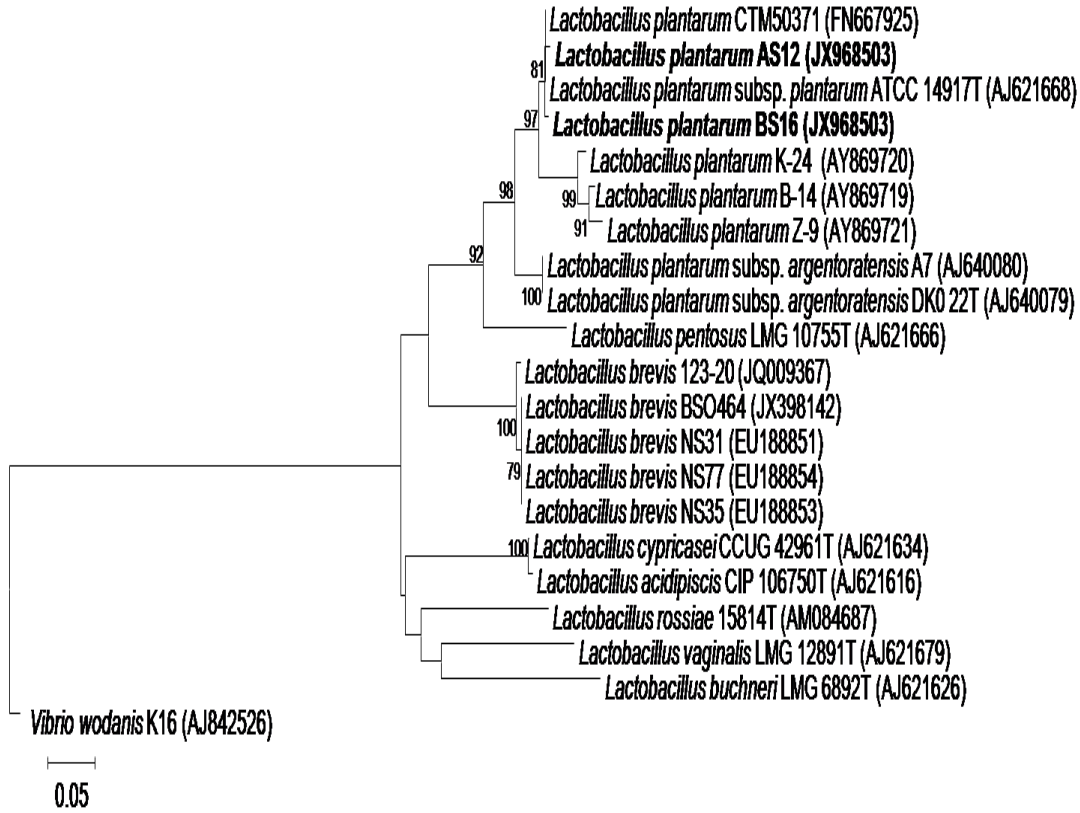

Fig. 3. Phylogenetic relatedness of *L. plantarum* A.S1.2 and B.S1.6 based on *rec*A sequences. The maximum-likelihood method was used to construct the phylogenetic tree. *Vibrio wodanis* was used as an outgroup

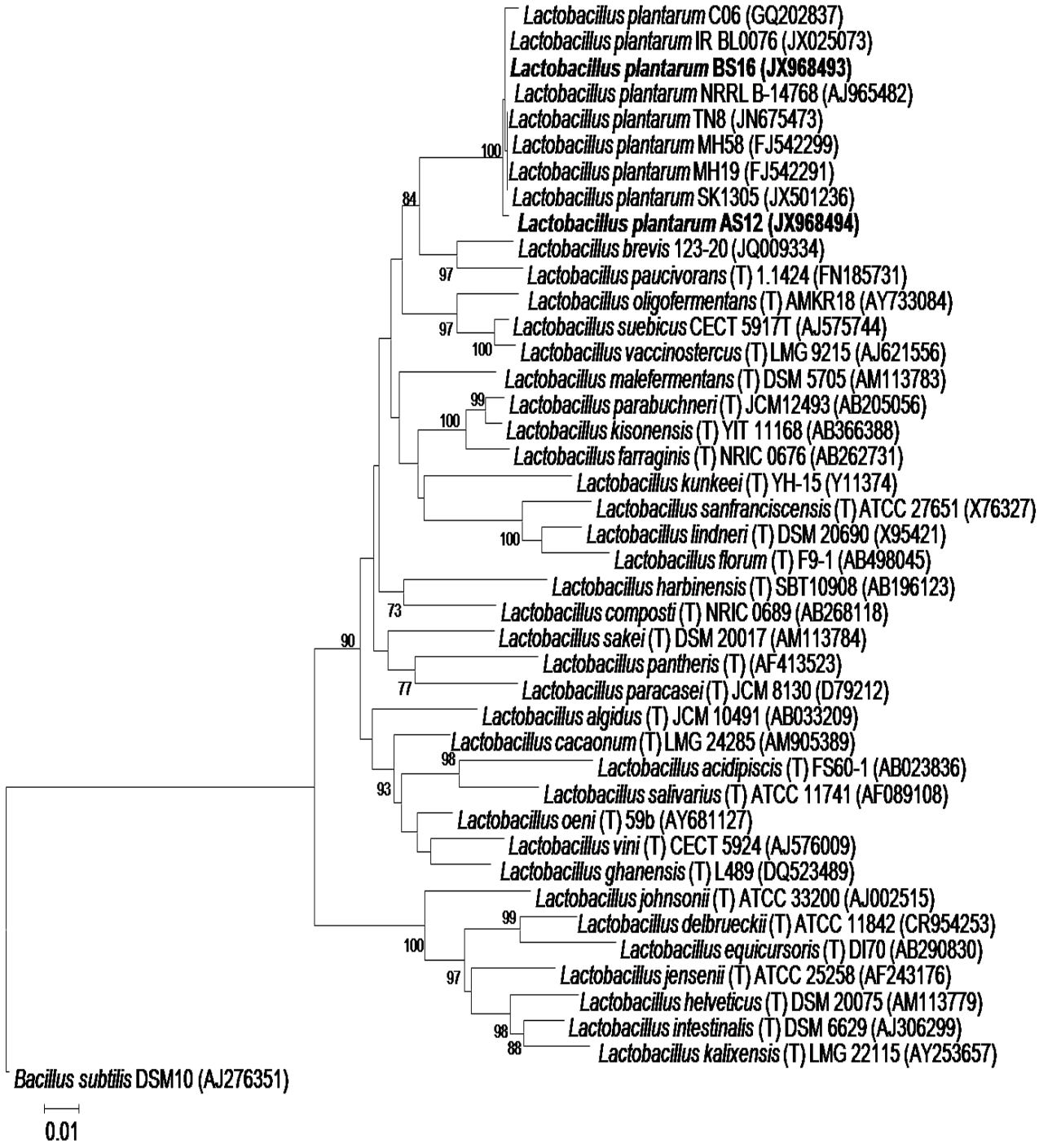

Fig. 4. Phylogenetic relatedness of *L. plantarum* A.S1.2 and B.S1.6 based on partial 16S rRNA sequences. The maximum-likelihood method was used to construct the phylogenetic tree. *Bacillus subtilis* was used as an outgroup

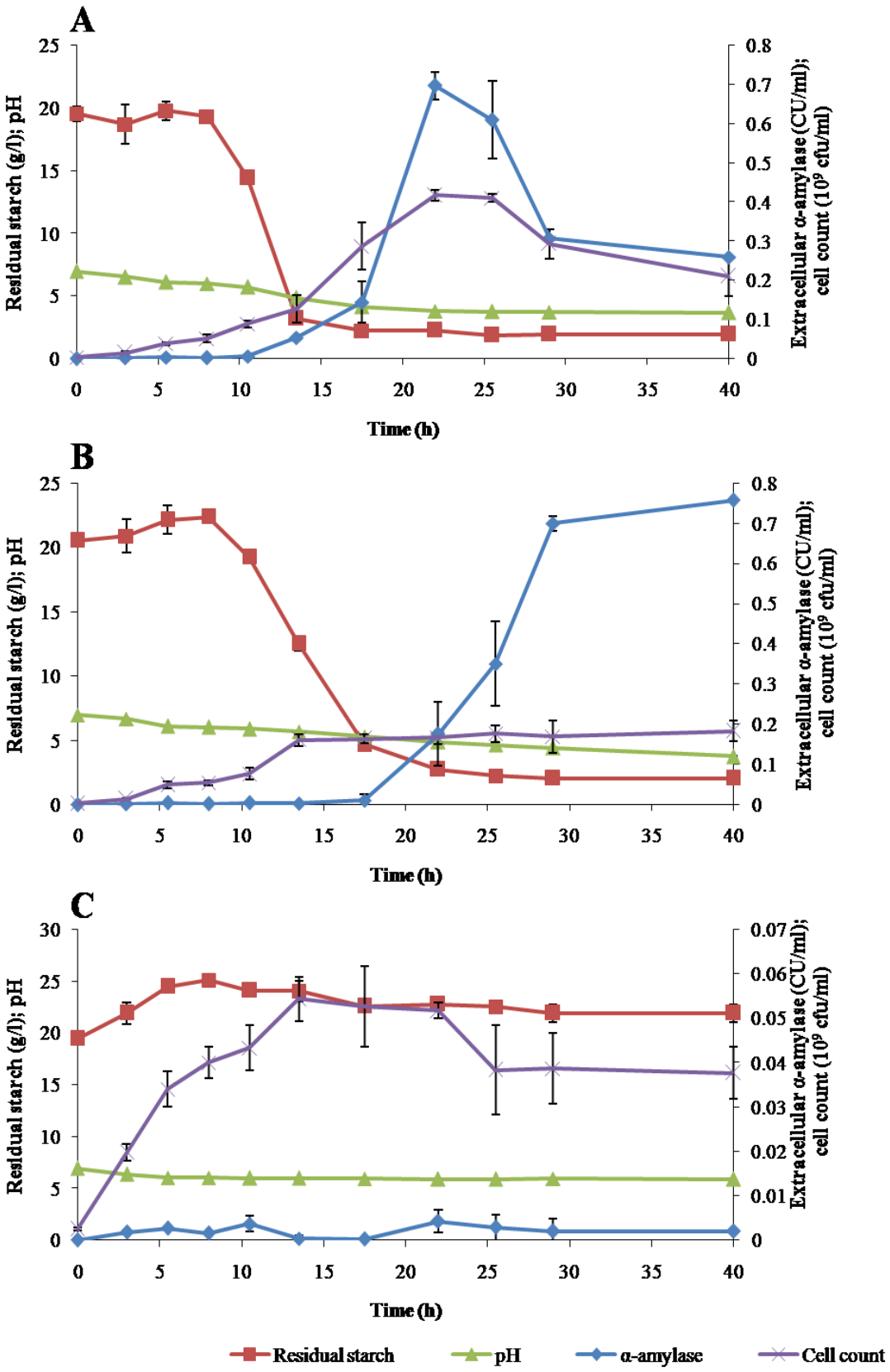

Fig. 5. Fermentation of *L. plantarum* A.S1.2 (A); B.S1.6 (B); negative control (C) in MRS-starch broth at 30 °C without pH control

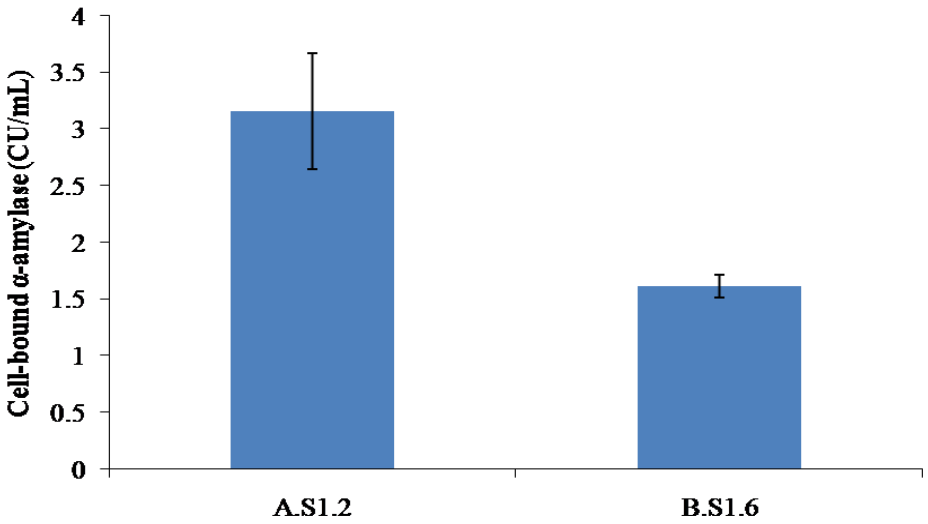

Fig. 6. Cell-bound α-amylase activity of *L. plantarum* A.S1.2 and B.S1.6 after 24 h fermentation at 30 °C

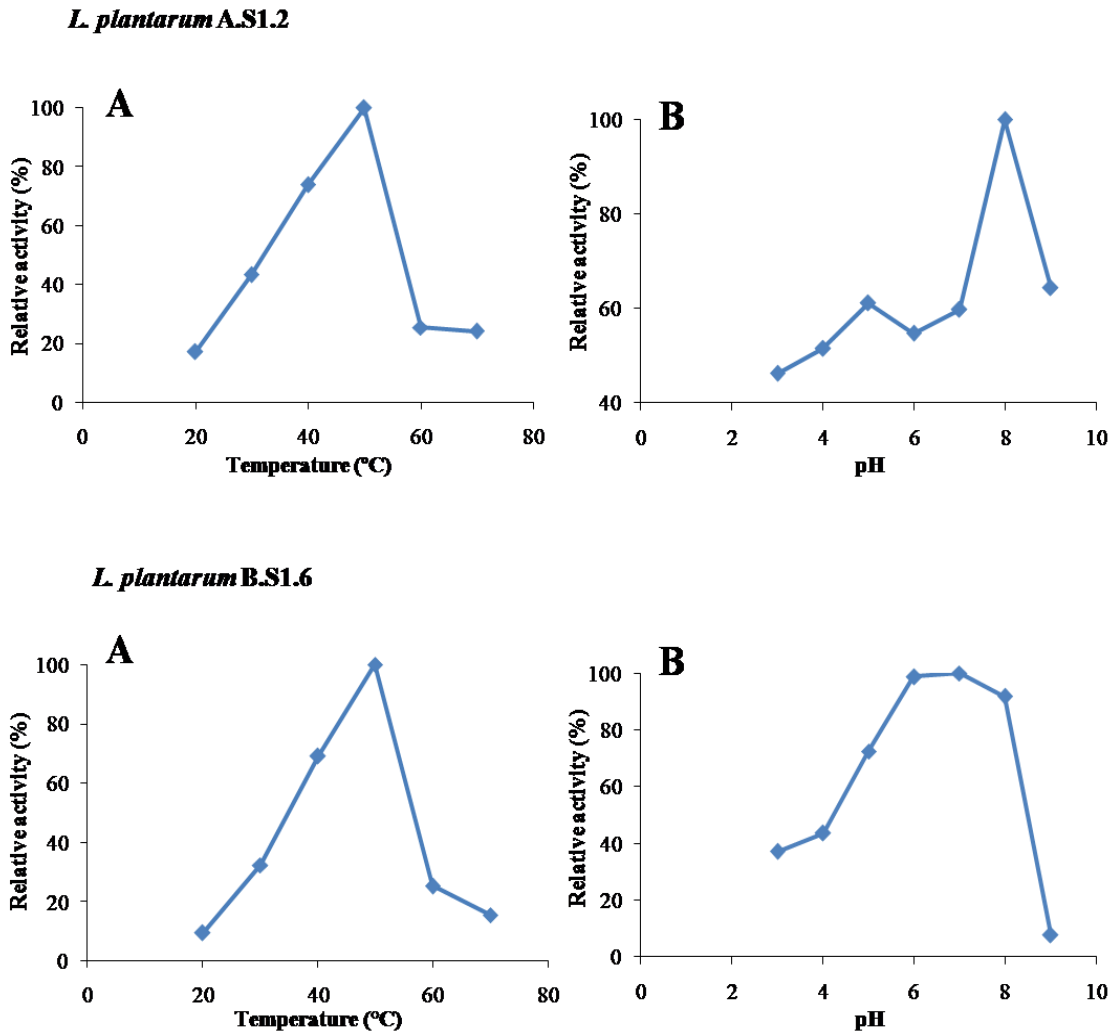

Fig. 7. Temperature (A) and pH (B) optimum of *L. plantarum* A.S1.2 and B.S1.6 extracellular α-amylase

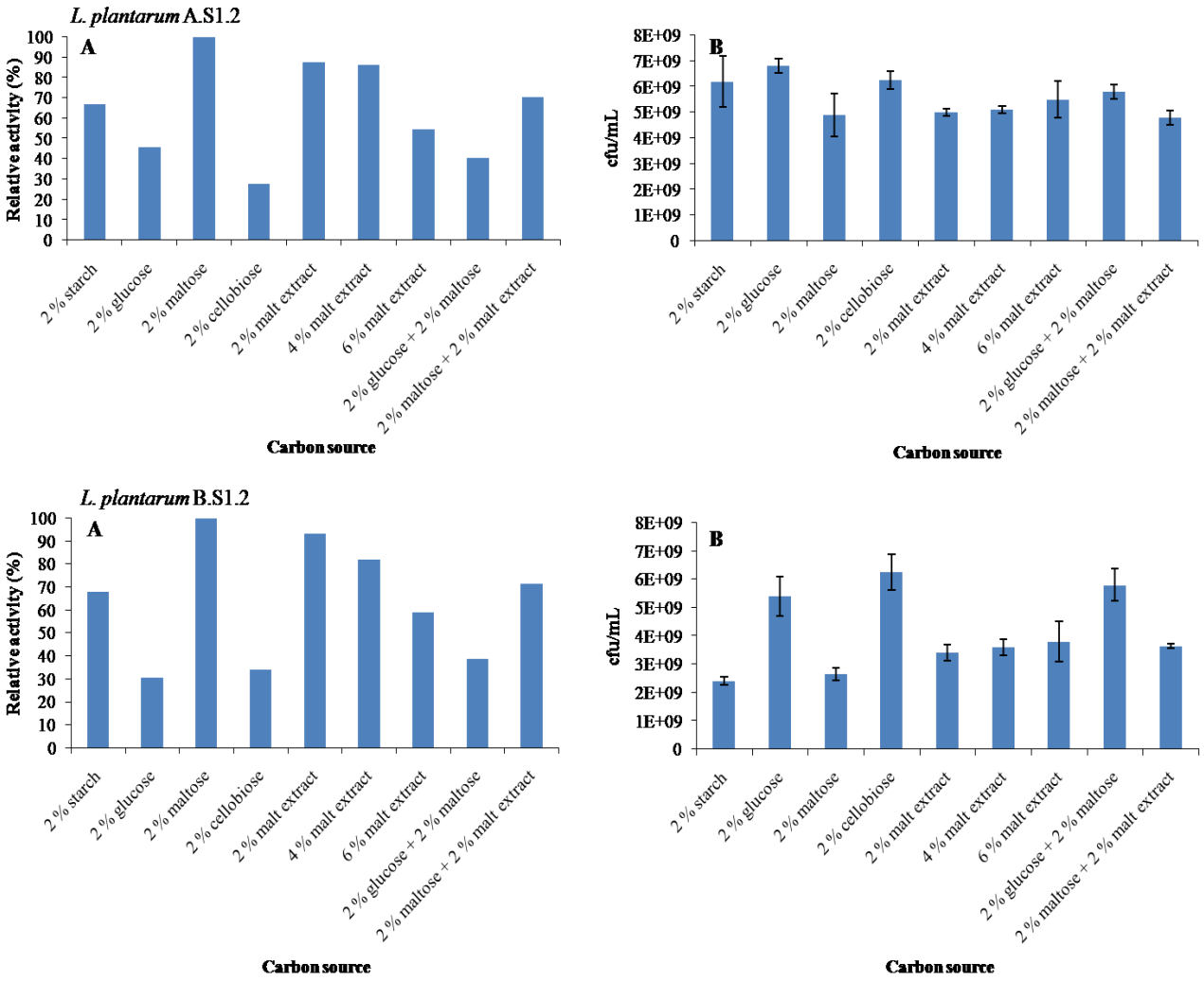

Fig. 8. Substrate specificity of *L. plantarum* A.S1.2 and B.S1.6 extracellular α-amylase. (A) Relative activity (%) on different carbon sources; (B) Growth (cfu/mL) on respective carbon sources

CHAPTER 5

Evaluation of starter cultures to improve the malting of barley for beer brewing

Evaluation of starter cultures to improve the malting of barley for beer brewing

Abstract

Maltsters very often struggle to deliver a constant malt product as they are faced with inconsistent malt batches and poor germination capacities of dormant barley. Various companies apply gibberellic acid (GA_3) to the malting process to augment the hydrolytic capabilities of the kernel's gibberellins (Noots et al., 1999). New legislation has, however, prohibited the addition of GA_3 to South African barley. In this study strains of *Geotrichum candidum*, *Aspergillus* sp., *Rhizopus* sp., *Trichoderma reesei* and *Lactobacillus plantarum* with known hydrolytic capabilities were added as starter cultures during malting to enhance germination. Various optimization strategies for starter cultures were investigated, including addition time, physiological state of spores, barley variety, and malting method. *G. candidum* and *L. plantarum* could not contribute to malt modification or microbial stability, while *Aspergillus* sp., *Rhizopus* sp., and *T. reesei* significantly enhanced malt quality. Enzymes in the cell-free supernatants of strains from the latter three species produced better results than viable cells. This is the first report on the use of cell-free culture supernatants to improve malt modification. This is not only more convenient than starter cultures, but is more controlled, since enzymes of known activity can be added to the malting process.

Introduction

Barley malt is the predominant raw material for beer production world-wide (Haraldsson et al., 2004). The main purpose of malt in brewing is to supply yeast with fermentable sugars, amino acids and vitamins required for alcoholic fermentation (Laitila et al., 2007; Noots et al., 1999; Rimsten et al., 2002).

During malting kernels are induced to sprout and initiate a sequence of three events. Steeping involves the soaking of kernels in water to increase moisture content to 42 - 44 % (Noots et al., 1999). This is followed by germination, during which the starchy endosperm is degraded to provide nutrition for the growing embryo. Germination is initiated when hormones, mainly gibberellins from the scutellum, stimulates the aleurone layer to synthesize hydrolytic enzymes. The process is finally terminated by kilning, whereby a gradual increase in temperature halts all metabolic processes and enzymatic activities (Rimsten et al., 2002). The dried malt product is rich in preserved enzymes and

partially modified polymers (Linko et al., 1998). To function as a fermentable substrate, malt biopolymers are further degraded at the brew house during mashing. This process involves the rehydration of malt at increasing temperatures, during which its own preserved enzymes allow substrate hydrolysis. Optimal enzyme synthesis during germination is thus integral to malt quality.

The starchy endosperm accounts for 70 % of the total weight of a barley kernel and consists of large cells packed with starch grains, embedded in a protein matrix (Laitila et al., 2006b). The walls of these cells consist of 75 % β-glucan and 20 % arabinoxylan (Fincher, 1975). Protease and amylase can only enter and hydrolyze the starchy endosperm if the cell walls are adequately degraded. Production of β-glucanase and xylanase during germination is thus critical in malt quality (Briggs, 1981; Fincher and Stone, 1986; Hough, 1994).

Maltsters very often struggle to deliver a constant malt product as they are faced with inconsistent malt batches and poor germination capacities of dormant barley. Brewers often add gibberellic acid (GA_3) to the malting process to overcome these difficulties (Noots et al., 1999). Gibberellic acid does not only aid in the production of greater enzyme quantities, but may also be applied to break dormancy and reduce germination time. The external addition of this hormone does, however, have several disadvantages, which are mainly due to its dose-dependent response. Excessive GA_3 levels facilitate extensive rootlet and acrospires growth, leading to over-modification and major malt losses. If not controlled, high sugar and soluble nitrogen levels are produced, which in turn increases unwanted colour development (Fox et al., 2003). In South Africa, new legislation has prohibited the use of GA_3. To produce the same malt quality, maltsters are searching for alternative methods to enhance germination.

Some microorganisms indigenous to barley produce proteases, amylases, and cell wall-degrading enzymes (Biovin and Malanda, 1997; Foszczynska et al., 2004; Haikara et al., 1993; Haikara et al., 1995; Noots et al., 1999) and may thus be explored as an alternative to the addition of GA_3. Knowledge regarding this concept is poor as the majority of research has focussed on the addition of starter cultures to enhance the microbial stability of malt. Lactic acid bacteria (LAB) and *Geotrichum candidum* have been the most extensively studied and can successfully enhance malt quality by restricting the growth of unwanted contaminants (Laitila et al., 2006a, 2007; Linko et al., 1998; Lowe et al., 2005). Only one author applied a *Rhizopus oligosporus* starter culture to the malting process with the specific aim to enhance malt modification (Noots et al., 1993; Noots et al., 1999; Noots et al., 2001).

The main objective of this study was to evaluate if *G. candidum* (isolates 1173, 4964 and 2590) and *Lactobacillus plantarum* B.S1.6 with known hydrolytic activities can contribute to malt modification. The concept of using starter cultures was tested on two South African barley cultivars, i.e. Erica and SSG 564. Various strategies were investigated to optimize conditions for starter culture performance.

The influence of *Aspergillus* sp., *Rhizopus* sp. and *Trichoderma reesei* on malt modification was also studied.

Materials and methods

1. Strains and culture conditions

1.1 Fungi

1.1.1 Non-activated spores

Geotrichum candidum were grown on Yeast Peptone Dextrose (YPD) (Biolab, Biolab Diagnostics, Midrand, South Africa) agar for 48 h at 26 °C. *Rhizopus* sp. and *Aspergillus* sp. were grown on Potato Dextrose Agar (PDA) (Biolab) and *T. reesei* on Malt Extract Agar (MEA) (Biolab) for 7 days at 26 °C. In all cases spores were washed from plates with 5 mL sterile physiological saline and diluted to yield a spore count of 1 X 10^6 spores/g barley. Spores were counted using a haemocytometer (Paul Marienfeld GmbH & Co, Germany).

1.1.2 Activated spores

Geotrichum candidum spores were harvested as described previously, diluted to 2 X 10^6 spores/mL and inoculated into 100 mL 2 % (w/v) Malt Extract broth (Biolab), 2 % (w/v) molasses (Voermol, Maidstone, South Africa) and sterile distilled water, respectively. Flasks were incubated at 30 °C on a rotary shaker for 6 h. Changes in pH and spore germination was monitored every 30 min. Each spore sample was stained with lacto phenol cotton blue to inhibit germination and to enhance visualization under the microscope. Germination (P) was expressed as a percentage value, calculated as P (%) = ($N_{\text{germinated spores}}/N_{\text{total spores}}$) X 100. Spores were considered germinated when the germ tube was longer or equal to the largest dimension of the swollen spore (Dantigny et al., 2006). At the end of germination cultures were centrifuged (10 000 x*g*, 10 min, 26 °C) and resuspended in sterile saline. The number of germinated spores was determined with a haemocytometer and adjusted to 1 X 10^6 spores/g barley before adding to the malting process.

1.1.3 Cell-free culture supernatant

Aspergillus sp., *Rhizopus* sp. and *T. reesei* were grown on plates and their spores harvested as described before. The number of spores was adjusted to 1 X 10^6 spores/mL.

Three different induction media types were evaluated, namely 2 % (w/v) Malt Extract (Biolab), 2 % (w/v) molasses, and Yeast Nitrogen Base (YNB) (0.67 g/L) (Sigma-Aldrich, St. Louis, Missouri) + 0.5 % (w/v) Malt Extract (Biolab) + 1 % (w/v) low viscosity carboxymethylcellulose (CMC) (Sigma).

Enzyme production of each fungal isolate was monitored over 8 days in 250 mL Erlenmeyer flasks, containing 100 mL of each induction media. Flasks were incubated at 26 °C on a rotary shaker (125 rpm). Cell-free supernatant was obtained by filtration through a 0.22 µm nitrocellulose membrane.

Amylase activity was measured using the Cerelpha method (Megazyme, International Ireland, Ltd, County Wicklow, Ireland). The Dinitrosalicyclic assay (DNS) method was used to quantify xylanase, β-glucanase and cellulase activities with 1 % (w/v) beechwood xylan (Carl Roth GmbH, Karlsrube, Germany), 1 % (w/v) CMC and 0.1 % (w/v) barley β-glucan (Difco, Becton, Dickinson and Company), respectively, as substrate in 0.05 M citrate buffer (pH = 4.8) (Miller et al., 1960).

In the case of cellulase and β-glucanase activity, 20 µL substrate was added to a 96-well microtitre plate with a multi-channel pipette and pre-incubated at 50 °C for 5 min. Thirty µL crude supernatants were added and incubated at 50 °C for 30 min. The reaction was stopped by the addition of 100 µL DNS solution [1 % (w/v) 3,5-dinitrosalicyclic acid (Sigma); 20 % (w/v) potassium sodium tartrate; 1 % (w/w) NaOH; 0.2 % (v/v) phenol; 0.05 % (w/v) Na_2SO_3] and boiled for 15 min. Samples were cooled down and absorbency values determined at 540 nm (Bio Rad, Smartspec Plus). The same method was used for xylanase activity, but in this case 54 µL substrate, 6 µL enzyme and 92 µL DNS was used. Activity units were defined as nkat units/mL.

A 30 mL cell-free supernatant of each fungal isolate grown in YNB (0.67 g/L) + 0.5 % (w/v) Malt Extract + 1 % (w/v) CMC flasks was freeze-dried for 24 h. The enzyme activity of dried samples was determined by diluting each in 30 mL sterile distilled water.

1.2 *Lactobacillus plantarum* B.S1.6

Lactobacillus plantarum was propagated in De Man, Rogosa and Sharpe (MRS) broth (Biolab). Cultures were centrifuged (10 000 x*g*, 26 °C), washed twice in sterile saline, and added to the malting process at 5 X 10^4 cfu/g barley.

2. Micro-malting

2.1 Method and program

Barley (500 g) was mixed with 800 mL distilled water and added to specially designed vessels. Two 5 kg plastic containers were placed into one another. An airflow outlet was provided in the bottom container by coupling rubber tubes to an aquarium air pump. Steeping and germination were conducted in these vessels, referred to as Steep-germination-vessels (SGVs).

Samples were steeped for two days with two alternating wet stand (9 h, 14 h), and air rest (14 h, 6 h) periods. Air was supplied during steeping to prevent anaerobic respiration. Germination followed for 84 h, after which samples were kilned for 24 h in separate units (Table 1). Rootlets were removed after kilning for malt analysis.

2.2 Malt analysis

Malt analysis was conducted on green malt and kilned samples. For green malt analysis, samples (90 g) were blended with a food-blender for 1 minute. To each sample, 360 mL sterile distilled water was added and mashed in an Industrial Equipment Corporation (IEC) mash bath (Fig. 1). Malt analysis for kilned samples was done according to the European Brewing Convention (EBC) method. In brief, this entails the grinding of 50 g kilned malt in a disc mill (*Bühler-Miag* 4000, *Bühler* Ltd., Milano, Italy) (gap setting 0.2 mm), after which 400 mL distilled water is added and placed in the IEC mash bath. The mash of kilned and green malt was filtered through filter paper and analysis was conducted on wort. Green malt samples were stored at – 20 °C until analysis. Beta-glucan content and Free-amino nitrogen (FAN) content of the green malt and kilned malt were determined with a Skalaar.

3. Enzyme analysis

Enzyme activities were measured on green and kilned malt. Green malt samples were dried for 24 h at 50 °C, after which both green and kilned malt were milled with a disc mill (gap setting 0.2 mm) before analysis. The α-amylase activity was analysed with the Cerelpha Megazyme kit (K-CERA 08/05, Megazyme). Beta-glucanase was measured using the Azo-barley glucan method (K-MBGL 03/11, Megazyme) at 30 and 60 °C to distinguish between malt and microbial β-glucanases, respectively. Xylazyme AX tables were used to assay xylanase activity (T-XAX200 10/2008, Megazyme). Xylanase enzyme extraction was performed for 15 min at room temperature in sodium acetate buffer (25 mM, pH 4.7), with continuous stirring.

4. Statistical analysis

Micro-maltings were conducted in duplicate and the average results presented. Statistical analysis was done with Statistica (v. 10, StatSoft, Inc.). The students'-test was performed at 95 % confidence levels. Significant differences ($p = 0.05$) are indicated by an asterisk (*).

5. Micro-malting trials

5.1 Trial 1 (Optimization of addition time and spore state)

A total of 15 micro-maltings were conducted with SSG 564 barley cultivar, each with different parameters (Table 2). The starter culture cocktail consisted of 3 *G. candidum* strains (1173, 2590, and 4964) and 1 *L. plantarum* strain (B.S1.6), each prepared separately and then combined. Starter

cultures were sprayed onto kernels at the start of germination. During 1st or 2nd wet stand additions, the starter culture cocktail was mixed with 800 mL distilled water and then added to the respective SGVs.

5.2 Trial 2 (Optimization of starter culture combination)

Micro-maltings were conducted with Erica barley cultivar. Non-activated *G. candidum* strains (1173, 2590 and 4964) were added in different combinations, while *L. plantarum* B. S1.6 was present in each starter culture cocktail (Table 3). Cultures were added at the start of germination. Malt- and enzyme analysis were conducted on kilned samples.

5.2.1 Microbial analysis

Kernels were sampled at T 84 of germination and after kilning to evaluate the penetration of starter cultures during malting. Sterilized kernels were homogenised for 5 min with 45 mL sterile saline in a stomacher blender, after which it was serially diluted with sterile saline and plated out onto the desired selective media.

Lactobacillus plantarum B.S1.6 was enumerated on MRS supplemented with 200 mg/L Delvocid, 100 mg/L cyclohexamide and 10 mg/L Ciprofloxacin (Difco Laboratories) to inhibit fungi and slime producers (Bujalance et al., 2006). *Geotrichum candidum* strains were counted on YPD agar supplemented with 0.01 % (w/v) tetracycline and 0.01 % (w/v) chloramphenicol.

5.3 Trials 3 and 4 (Time-course malt analysis)

Nine micro-maltings were conducted with different starter culture combinations on Erica (Trial 3) and SSG 564 (Trial 4) barley varieties, respectively (Table 4). Gibberellic acid was added as a positive control at a concentration of 0.11 ppm. FANs and β-glucans were measured at T 24, T 48, T 72, T 84 of germination, and after kilning (Tkiln). Malt enzyme activities were measured at T 0, T 48 and T 84 of germination. Microbial analysis was conducted as described in 5.2.1.

5.4 Trial 5 (Optimization of malting procedure)

The most promising starter culture from preliminary trials, *G. candidum* 1173 + *L. plantarum* B.S1.6, was added to Erica and SSG 564, respectively. Two different malting procedures were also investigated: Normal = as performed during Trials 1 – 4, and Cargill: Method suggested by Cargill where the steeping period has no air rest (Table 5). Malt- and enzyme analysis were conducted on kilned malt.

5.4.1 Microbial analysis

Full microbial analysis without surface sterilization was conducted during germination (T 0, T 48, and T 84) and after kilning (Tkiln). Aerobic heterotrophic bacteria were enumerated on Plate Count Agar (PCA) (Biolab) and *Pseudomonas* spp. on C-F-C agar (Oxoid Ltd., Basingstoke, UK) at 30 °C for 2 - 3 days. To reduce fungal overgrowth, 0.001 % (w/v) cycloheximide was added. *Enterobacteriaceae* was counted on Violet Red Bile Agar (VRBA) (Biolab) with added cycloheximide (0.001 %, w/v) after incubation at 37 °C for 24 h. Yeast counts were determined on YPD agar supplemented with chlorotetracycline (0.01 %, w/v) and chloramphenicol (0.01 %, w/v) (Difco Laboratories). These plates were incubated for 3 days at 26 °C. Bacterial and yeast counts were expressed as colony forming units per gram barley (cfu/g) and spores/g barley, respectively.

5.5 Trial 6 (Addition of filamentous fungi with high enzyme activities)

Aspergillus sp., *Rhizopus* sp. and *T. reesei*, as well as their supernatants, were added to Erica cultivar. *Lactobacillus plantarum* B.S1.6 was added to each starter culture SGV (Table 6). All fungal cultures were received from the Department of Plant Pathology (University of Stellenbosch). Malt- and enzyme analysis were conducted on kilned malt.

Results and discussions

Geotrichum candidum is an anamorphic/nonsexual fungus included in the *Hemiascomycetes* (*Galactomyces candidus* = teleomorphic state) (deHoog and Smith, 2004; Pottier et al., 2008). Its asexual reproduction cycle switches between the extension of mycelia (somatic phase) and the fragmentation of hyphae to produce cells called arthroconidia or arthrospores (Caldwell and Trinci, 1973; Park and Robinson, 1969; Quinn et al., 1981). Germination is the link between the two phases and is initiated when arthrospores become swollen and germ tubes arise. When the length of these germ tubes are greater or equal to the greatest dimension of the swollen cells, spores are considered germinated (Dantigny et al., 2006). Germinated spores are generally metabolically more active than non-germinated spores and are referred to as being “activated”. It can be speculated that germinated spores will be more effective in breaking down the barley structure than non-germinated spores. This theory has been proven by Noots et al. (2001) who illustrated that malt modification could be enhanced if *Rhizopus* VII was activated before addition to the malting process.

During Trial 1 it was investigated if germinated *G. candidum* spores will also perform better in the malting environment than non-germinated spores. Preliminary germination studies were conducted to establish the most efficient induction media in terms of germination tempo and percentage. It was also investigated whether a correlation between % germination and pH could be established.

The process conditions were optimized to ensure maximal germination efficiency. Firstly, the initial spore concentration was kept low as crowding of spores is known to inhibit/delay germination. Secondly, 2-day-old spores were used, as aged spores have a longer lag phase of germination. Lastly, germination was conducted on a rotary shaker (120 rpm) to enhance O_2 availability, as it correlates directly with germination efficiency (Park and Robinson, 1970; Robinson et al., 1989).

Fig. 2 illustrates the pH and % germination during incubation of the three *G. candidum* strains. All strains reacted similarly on Malt Extract and molasses. In the first 90 min, a latency phase was observed after which germination increased exponentially until maximal germination was reached at ± 200 min. During the latency and exponential period a gradual decrease in pH was observed, which continued after germination stabilized. A rapid pH fluctuation occurred between 240 – 300 min. The reason for this is not known and has not been described in literature. The pH fluctuation took place approximately 30 min after maximum germination was reached and appeared independent of germination itself. It is also evident when the hyphae length of *G. candidum* 1173 between 180 min and 300 min is considered (Fig. 3). The majority spores were germinated at 180 min, after which hyphael elongation occurred. The pH fluctuation may be the result of hyphael elongation and/or preparation for new spore development. In future studies it will be valuable to conduct HPLC analysis to determine whether this fluctuation is due to fungal metabolites, or a change in media components. This phenomenon could nevertheless be used as a tool to assess whether germination has occurred.

Malt Extract resulted in a lower pH than molasses after the pH fluctuation. The cause of this difference is unknown and is probably due to the difference in media components. Both media types were good inducers, as > 80 % germination was achieved in all cases. Molasses was chosen for further studies as it is the most economically convenient media for up-scaling purposes. Water was an unsuitable induction media since it resulted in no germination and extensive pH fluctuations (Results not shown). Park and Robinson (1970) noted that *G. candidum* will only germinate in this nutrient-poor substrate if the spore concentration was very low. The spore concentration in water was thus too high to allow germination.

The first malting trial was conducted to identify the optimal starter culture addition time during malting. It was also investigated whether activated spores could improve malt quality as opposed to non-activated spores. Various parameters are generally used to evaluate malt modification, although only FAN and β-glucan content was assessed in this study. These parameters are not only reliable, but also fundamental indicators of malt quality (Bamforth, 1982; Fox et al., 2003; Wang et al., 2004). Beta-glucan accounts for 70 % (w/w) of the starchy endosperm cell wall and its hydrolysis is essential to allow access to enzymes which degrade the starchy endosperm. Inadequate degradation of this polysaccharide will result in poor malt quality, associated with reduced filtration rates and the

presence of gels and hazes in the final beer product (Bamforth, 1985, 1994; Forrest et. al., 1977). FAN content illustrates the extent by which proteolysis occurred during malting and mashing. High FAN content is desired as it is essential for maximal biosynthetic and fermentative performance of brewers' yeast (Lowe et al., 2004).

The starter culture consisted of a complex cocktail of three *G. candidum* strains (1173, 2590 and 4964) and *L. plantarum* B.S1.6. The majority starter culture SGVs resulted in better FAN and/or β-glucan content than the control (Table 7). In contrast to *Rhizopus* VII (Noots et al., 2001), activated *G. candidum* spores did not significantly improve malt quality parameters compared to non-activated spores.

Malt quality was greatly influenced by starter culture addition time. FAN and β-glucan content were significantly improved compared to the control when starter cultures were added at the start of germination (SGV 5, 6, 10 and 14) while wet stand additions resulted in poor malt quality (SGV 1 - 4, 7, 8). These results suggest that the starter cultures are most effective when added during germination. This statement is supported by the fact that kernels are more prone to microbial attack during germination than the first wet stands (Laitila et al., 2006; Noots et al., 1998; O'Sullivan et al., 1999). Non-malted kernels are inaccessible to microbial attack, as no internal modification has occurred. During the alternating wet stand periods, moisture content increases to 42 – 44 % and allows germination to commence (Noots et al., 1999). The internal enzyme systems hydrolyze the kernel structure to nourish the growing embryo, softening the rigid surface of the husk. This allows the internal endosperm to be more accessible for microbial degradation (Flannigan et al., 1982; Petters et al., 1988). Germination is therefore regarded as the stage where maximal viable microbes are reached throughout the process (Flannigan et al., 1982; Laitila et al., 2006a; Noots et al., 1999; Petters et al., 1988).

The majority starter cultures will adhere to kernels during germination, because it is sprayed directly onto its surface. This is not the case for wet stand periods. Low adherence is expected as the majority cultures are washed away when steep water is drained for air rest periods (Briggs and McGuinness, 1992; Noots et al., 2001). Germination is also longer than wet stands, allowing more time for cultures to penetrate and modify the kernel structure.

The mentioned statements theoretically support results found in this study, although literature contains contrasting data. *Rhizopus* VII was more effective when added during the 1st wet stand than the start of germination. Spores could also successfully adhere to kernels at this early stage (Noots et al., 2001). It has also been demonstrated that *G. candidum* are more efficient when added during steeping (Foszczynska et al., 2004). It can thus be postulated that each microbial strain has its own optimal addition time, which depends on factors such as mode of adherence, penetration and proliferation.

Consecutive starter culture additions resulted in poor β-glucan degradation (SGV 7 - 9, 11 - 13), except for SGV 10 and 14. Multiple starter culture additions might reduce germination efficacy, as it correlates directly with β-glucan content. Kernels are not granted enough time to respire and germinate between addition intervals, as the starter culture competes with the structure for O_2 and H_2O. This competition is a general phenomenon (Doran and Briggs, 1993; Etchevers et al., 1977; Kelly and Briggs, 1992; Lowe, 2005) but no literature could be found to support these claims since this is the first study where starter cultures were added consecutively during malting. Further studies are needed to confirm these statements. The FAN content was nonetheless unaffected in these cases and might be due to the proteolytic activity of the cultures.

Scanning electron microscope analysis was conducted on SSG 564 [SGV 6 and 15 (control)] and a Cargill variety to visualize surface attachment of starter cultures and starchy endosperm modification. Fig. 4 illustrates that *G. candidum* and *L. plantarum* were present in abundance on the surface of the husk. Improved FAN and β-glucan content, accompanied by such dense starter culture colonization suggests that the presence of these cultures did not interfere with respiration. No difference in modification was, however, observed between the endosperm structure of the control and SGV 6. Scanning electron microscope analysis is nevertheless a poor representative of barley modification as only one kernel is evaluated.

Endosperm modification in SSG 564 was poor compared to the Cargill barley variety. The latter contained no protein matrix and pores were present in starch granules, whereas SSG 564 still harboured a dense protein matrix, accompanied by large starch granules. In general, SSG 564 is difficult to modify and might explain these findings (personal communication, SAB Maltings).

With knowledge gained from Trial 1, non-activated starter cultures were added during germination in further studies. The aim of Trial 2 was to evaluate if a greater enhancement in malt quality could be observed on Erica, which is easier to modify than SSG 564. Different starter culture combinations were also added to assess whether all 3 *G. candidum* strains are needed to improve modification.

It was assumed that starter cultures would be more effective on Erica than SSG 564, although none of the SGVs delivered malt with significantly enhanced FAN and β-glucan content (Table 8). The successful combination from Trial 1 (SGV 6) did also not deliver good results. Beta-glucan content was higher than the control in all cases and it was consequently not possible to identify the most promising combination.

Geotrichum candidum 1173 and 2590 had proteolytic activity with preliminary plate assays (Chapter 3). These cultures were thus expected to enhance FAN content, although it was not observed (Table 8). No correlation between their proteolytic capabilities and FAN content could also be found.

Contribution to proteolytic enzymes might not have been significant enough during germination, or the microbial enzymes did not survive kilning for further degradation during mashing.

Kernel colonization of *G. candidum* and *L. plantarum* was assessed after germination and kilning to evaluate if abovementioned results were due to poor starter culture adherence and/or proliferation. Kernels were surface sterilized prior to analysis, and results are based on cells that penetrated the structure. LAB are known to proliferate extensively during germination and therefore MRS medium was selective for only *L. plantarum* (Booysen et al., 2002; Bujalance et al., 2006; Laitila et al., 2006a; Raulio et al., 2009).

Geotrichum candidum spp. and *L. plantarum* B.S1.6 were viable throughout the process and successfully penetrated the structure (Fig. 5). These cultures are not naturally present on this barley variety as their counts were negligibly low in the control. It has been demonstrated that LAB and *G. candidum* are rarely detected in native barley, although it may become dominant during germination (Laitila et al., 2006b; Noots et al., 1999; Petters et al., 1988). *Geotrichum candidum* and *L. plantarum* was added at 1 X 10^6 spores/g barley and 5 X 10^4 cfu/g barley, respectively. At the end of germination *G. candidum* was present at ± 10^6 spores/g barley, while *L. plantarum* reached levels of 10^8 cfu/g barley. Competition between cultures did also not affect growth, as no significant difference in cell counts was observed between SGVs. It has been demonstrated that *G. candidum* may in fact stimulate the growth of LAB (Noots et al., 1999). This high colonization of starter cultures on kernels might have interfered with barley respiration, resulting in poor FAN and β-glucan content.

Viable cell counts decreased after kilning and were expected, as microbes struggle to survive such high temperatures. The survival of *G. candidum* and *L. plantarum* during kilning has also been demonstrated by other authors and is mainly due to the protection of cells by the outer layers of kernels (Booysen et al., 2002; Dziuba et al., 2000; Laitila et al., 2006a; Laitila et al., 2006b; Lowe et al., 2005; Noots et al., 1999; Petters et al., 1988). High cell concentrations on kilned malt are, however, of concern and further studies will have to be conducted to evaluate if the presence of these cultures will affect the brewing process. Numerous authors have demonstrated that microbial activity on malt can negatively influence yeast flocculation (O'Sullivan et al., 1999; Van Nierop et al., 2004). Although viable cell counts are destroyed during mashing and wort boiling, microbial metabolites can survive the process and affect the final beer product. Negative effects include cloudiness, haziness and off-odours and -flavours (Fleet, 1992; Loureiro and Querol, 1999; Walker et al., 1997).

FAN and β-glucan content is nevertheless not the only parameters by which malt quality can be assessed. Malt enzymatic potential is also valuable and predicts the extent to which substrates will be hydrolyzed during mashing. Xylanase-, α-amylase and β-glucanase activity was consequently evaluated.

Each starter culture combination contained *L. plantarum* B.S1.6 with membrane-bound and extracellular α-amylase activity (Chapter 4). In all cases amylase activity was higher than the control, although only SGV 1 proved significant (Table 8). It can be theorized that the strain does not produce amylase in such a harsh environment, or that the contribution of its activity is not good enough to significantly enhance amylase activity. Lowe et al. (2005) did also not observe improved amylase activity by an amylolytic *L. plantarum.* Lactic acid bacteria are known to decrease malt pH due to lactic acid production (Engelmann and Reichert, 1991; Laitila et al., 2006a; Lewis, 1998) and these authors attributed poor amylase activity to an unfavourable low pH environment created by this metabolite. The pH was not monitored in this study and it is hence unknown whether it influenced amylase activity. It is unlikely as *G. candidum* is known to de-acidify substrates by catabolizing LAB lactic acid, especially in cheese (Boutrou and Gueguen, 2005; Fox et al., 1990; Lucey and Fox, 1993).

Beta-glucanase activity was determined at both 30 and 60 °C to distinguish between malt and microbial activity. Malt β-glucanase functions optimally at 30 °C and is largely inactivated at 60 °C, while microbial β-glucanase functions best at such high temperatures (Home et al., 1993; Laitila et al., 2006a). At both temperatures only SGV 1 and 7 resulted in enhanced activity, although all *G. candidum* strains demonstrated β-glucanase activity with preliminary assays (Chapter 3). Furthermore, β-glucan levels are usually related to β-glucanase activity (EtpKAkpan, 1993; Whang et al., 2004), although no correlation between degree of degradation and activity levels were observed. Isolate 1173 was the only *G. candidum* strain that had xylanase activity with preliminary plate assays (Chapter 3), but no enhancement in xylanase activity was seen in SGV 1, 4, 5 and 7, where this isolate was present. All xylanase activities were in fact lower than the control. This inability to correlate *in vitro* β-glucanase and xylanase activities of *G. candidum* with associated malt enzyme activities has also been demonstrated by Noots et al. (2001). Another study nevertheless demonstrated that a β-glucanase *G. candidum* producer enhanced malt β-glucanase activity (Foszcynska et al., 2004). This contrasting literature, together with data collected from this study suggests that the ability of *G. candidum* to contribute to these enzymes in the malting environment is strain specific. It is also possible that the enzyme contribution was adequate, but did not survive the kilning process. No definite conclusion could be drawn at this point.

The results obtained from Trials 1 and 2 suggest that the starter culture has the ability to enhance malt quality in SSG 564, but not Erica. This is in contrast to what was expected, as Erica is easier to modify that SSG 564. It was however possible that the starter culture caused faster germination in Erica, but that a point of saturation was reached where after no significant difference in malt quality could be observed. To investigate this statement, malt quality was monitored at different intervals during germination in Trial 3 (Erica) and Trial 4 (SSG 564). All parameters were kept the same as for Trial 2, but in addition, the effect of gibberellic acid was evaluated.

FAN content was significantly enhanced in SGV 1, 2 and 3 during the first 84 h of germination in Erica (Trial 3) (Fig. 6). This effect was not so pronounced in Trial 4 (SSG 564), and supports the assumption that the starter culture might enhance modification in Erica during germination (Fig. 8). Isolate 4964 (SGV 3) did not demonstrate proteolytic activity on skim milk with preliminary trials (Chapter 3), although FAN content were enhanced in Trial 3 and 4. It can consequently be speculated that its protease enzymes might be more specific for barley proteins. In the majority starter culture SGVs from both trials, the FAN content dropped drastically after kilning. This occurrence might be explained if culture viability is considered. Kernel penetration and colonization was similar in both cultivars and demonstrated good survival after kilning. These cultures might have utilized the available FAN content in the period between kilning and malt analysis as proliferation can occur during the early stages of kilning (Petters et al., 1988; Stars et al., 1993). As a result, a drastic drop in FAN content was observed, where as it increased in the control and gibberellic acid SGVs. If this statement holds true, it also explains the poor FAN content observed in Trial 2.

A similar pattern of β-glucan degradation was observed for both cultivars. High β-glucan content at T 24 was followed by extensive degradation at T 48. The β-glucan content did not change drastically between T 72 and T kiln, suggesting that β-glucan modification was already saturated at an early stage of germination. Beta-glucan degradation was nevertheless not enhanced during germination in Trial 3 or 4. Only SGV 2 in Trial 3 resulted in significantly low β-glucan content at T 72, and correlated well with the high β-glucanase activity found in this SGV (Fig. 7). Beta-glucanase was not enhanced in Trial 4 (Fig. 9) and explains why no enhanced β-glucan degradation was observed.

Negligible low α-amylase levels was observed at the end of steeping (T 0 germination) in both Trial 3 and 4 after which it drastically increased. This is in accordance with literature, which states that α-amylase is non-detectable in barley and only develops as germination commences (Bathgate and Palmer, 1973). No starter culture could enhance α-amylase activity in Trials 3 or 4, illustrating the inability of *L. plantarum* to increase α-amylase activity, as noted in Trial 2. Gibberellic acid nevertheless successfully improved activity levels.

The results from Trials 3 and 4 suggest that the starter cultures enhanced FAN content during germination in Erica as initially speculated, but only when *G. candidum* strains were added separately. The same could not be concluded for any other malt quality parameters. Enhanced malt quality for SSG 564 in Trial 1 could not be repeated in Trial 4, and no correlation between starter culture enzyme activity and malt quality could be established. The malting environment is extremely complex, and factors such as temperature and moisture content influence malt quality (Noots et al., 2003). These trials were conducted on unsterilized barley, open vessels, at room temperature and different seasons, which probably explains the inconsistent data collected. At this stage it was thus not possible to conclude if the starter culture is effective or not.

In a last attempt, Trial 5 was conducted to evaluate if the malting process could be optimized for the starter culture. Starter culture cocktail *G. candidum* 1173 and *L. plantarum* B.S1.6 was added to both Erica and SSG 564. In addition to the normal malting program, a second program where the steeping stage has no air rest period was employed. This program was suggested by Cargill and is consequently referenced thereafter. The evolution of barley microflora was also monitored during germination in the normal malting method to evaluate if the starter culture has the ability to enhance the microbiological stability of malt.

The FAN content was significantly enhanced on SSG 564 and Erica, and both malting methods, compared to their respective controls (Fig. 10). The Cargill method was more effective than the normal method as it generated higher FAN content in both cultivars. Beta-glucan degradation was nevertheless not improved, and no significant difference between methods was observed.

The Cargill method produced significantly enhanced enzyme activities, especially on Erica cultivar (Table 9). Similar to previous trials, no enhancement was observed with the normal method in neither cultivar, although the normal method produced higher activity levels in general. Starter culture performance can thus be optimized by the malting method, although the Cargill method cannot replace the normal method as it adversely affects enzyme activities.

Various authors have demonstrated the ability of *G. candidum* and LAB to enhance malt quality by suppressing the growth of certain microbial groups (Laitila et al., 2006a; Laitila et al., 2006b; Lowe et al., 2005; Noots et al., 1999). The proliferation of *Pseudomonas* spp., *Enterobacteriaceae*, aerobic heterotrophic bacteria and yeasts during germination and kilning was investigated for the normal steep method to evaluate if it could also be accomplished in this study. Aerobic heterotrophic bacteria are known to compete with the grain for oxygen, and are therefore largely unwanted during the malting process (Kelly and Briggs, 1992). *Enterobacteriaceae* and *Pseudomonas* spp. are the predominant bacteria in the malting environment (Laitila et al., 2007; Noots et al., 1999; O'Sullivan et al., 1999; Petters et al., 1988) and are infamous for the production of exopolysaccharides (EPS), which result in biofilms on barley kernels (Raulio et al., 2009; Morris and Monier, 2003). The presence of these cultures is alarming as EPS inhibits grain germination and retards mash filtration and wort separation (Haikara and Home, 1991; Kreisz et al., 2001; Laitila et al., 1999; Morris and Monier, 2003). Yeast cells can also have negative impacts in malting because it creates factors which influence yeast flocculation during brewing. Its metabolic activity in the production chain may also lead to off-odours, off-flavours and haziness of the final beer product (Fleet, 1992; Loureiro and Quarol, 1999; Van Nierop et al., 2004).

No difference in microbial proliferation was observed throughout germination and kilning (Figure 11). At the start of germination all bacterial groups and yeast were present at 10^8 cfu/g and 10^7 spores/g respectively. These values are in accordance with data collected by other authors (Laitila et

al., 2006; Laitila et al., 2007; Raulio et al., 2009). Viable bacterial counts increased during germination and are comparable with results found by Lowe et al. (2005) and Petters et al. (1988). This progressive increase throughout germination is largely attributed to the release of readily metabolizable components by the enzymatic activity in germinating kernels (Lowe et al., 2005; Noots et al., 1999; Petters et al., 1988). Kilning decreased cell numbers in all cases, although it remained considerably high. No significant difference was observed between starter and control SGVs, suggesting that *G. candidum* 1173 and *L. plantarum* B.S1.6 could not restrict the growth of these microbial groups. The inability of *G. candidum* to suppress the growth of aerobic heterotrophic bacteria has been demonstrated (Dziuba et al., 2000), although *L. plantarum* is a well-known inhibitor of these bacterial groups (Laitila et al., 2006; Raulio et al., 2009).

Trials 1 – 5 suggest that *G. candidum* and *L. plantarum* starter cultures cannot improve the quality or microbial stability of malt. Filamentous fungi are known to produce copious amounts of extracellular enzymes and therefore the potential of *T. reesei*, *Rhizopus* sp., and *Aspergillus* sp. as starter cultures were evaluated in Trial 6. Supernatant of these fungi with known β-glucanase, α-amylase, xylanase and cellulase activities were also added during germination. Erica cultivar was used in this study, and *L. plantarum* was added in all test SGVs.

All test SGVs generated significantly improved FAN content compared to the control, while only the supernatant was effective in β-glucan degradation (Fig. 12). Both supernatant and live cultures resulted in enhanced enzyme activities in the majority SGVs (Table 10). The effect of *Aspergillus* sp. and *Rhizopus* sp. have been evaluated by Noots et al. (2001) who also demonstrated that these cultures enhance malt enzyme activity.

The DNS-assay demonstrated that *Aspergillus* sp. supernatant harboured the highest xylanase activity, while very low levels was recorded for *Rhizopus* sp. supernatant (Table 11). Malt xylanase activity was however higher in SGVs where *Rhizopus* sp. supernatant was added than *Aspergillus* sp. supernatant. *Rhizopus* sp. also produced very low β-glucanase activity, although it resulted in the highest malt β-glucanase activity at 30 and 60 °C. It can thus be speculated that *Rhizopus* sp. enzymes are more effective on barley than the other fungi. The efficiency of microbial enzymes is consequently not only based on activity level, but also substrate specificity.

Malt α-amylase activity was significantly enhanced in all SGVs. The supernatants of *Rhizopus* sp. and *Aspergillus* sp. contained amylolytic enzymes and therefore it could not be concluded whether the contribution in these SGVs was from *L. plantarum* B.S1.6 or the fungi. A correlation between fungal- and malt α-amylase activity was nevertheless observed. *Rhizopus* sp. supernatant contained the highest activity and its SGV also delivered the highest malt α-amylase activity. As *T. reesei* did not produce α-amylase enzymes, *L. plantarum* B.S1.6 most likely contributed to the enhanced activity.

No significant enhancement was found when *L. plantarum* was added in conjunction with *G. candidum* in previous trials, suggesting that *G. candidum* might have suppressed its activity.

Fungal supernatant was more effective than live cultures. To our knowledge, this is the first study where fungal supernatant was used to augment modification, and the success thereof is a major breakthrough. The use of supernatant is a convenient alternative to fungal starter cultures as it eliminates various difficulties. Since no living cultures are involved, process conditions will not significantly by altered in terms of vessel preparation and cleaning. In addition, enzymes will result in a more constant malt product because the activity can be determined and adjusted prior to the malting process. It is not possible for living cultures, as certain environmental stresses might change its activity. In addition, the risk of posing a health hazard to workers due to exposure to high levels of spores is also eliminated especially in immune compromised people. Starter cultures can also produce various metabolites which can negatively influence the brewing process and are eliminated when supernatant is used.

Since culture supernatant was chosen as the final additive it was necessary to optimize growth conditions to ensure maximal enzyme production in the most economical manner. Spores of each fungal isolate were inoculated into 2 % molasses, 2 % Malt Extract, and YNB + 1 % CMC + 0.5 % Malt Extract. Enzyme production was monitored over an 8-day period. In all cases YNB proved to be the most efficient induction medium, and it was chosen to harvest spores after 7 days (Fig. 13).

In the industry 400 L H_2O is sprayed onto 130 ton barley as it is transported from steeping- to germination vessels. On a micro-malting scale this equals 1.53 mL H_2O for 500 g barley. With all trials 20 mL supernatant was sprayed onto kernels and is too much when up-scaling is considered. It was consequently necessary to concentrate fungal enzymes with freeze-drying. The activity of the supernatant of each strain was measured before and after drying. All enzymes demonstrated good survival (Table 12), although it will be necessary to determine how long it stays stable after drying.

Conclusion

Geotrichum candidum and *L. plantarum* starter cultures could not significantly contribute to malt modification, although they had the ability to produce extracellular hydrolytic enzymes with *in vitro* assays. Enhanced quality parameters could not be correlated with microbial enzyme activities and was also not repeatable. It was consequently inconclusive whether starter cultures were more effective on Erica than SSG 564. In was however observed that the malting schedule could be optimized for starter culture performance as cultures were more effective with the Cargill method than the normal cycle. The Cargill method nevertheless adversely affected the enzymatic potential of malt

and could therefore not replace the normal method. Further optimization of the malting program in which both barley germination and starter culture performance is favoured might prove these cultures successful.

Aspergillus sp., *Rhizopus* sp., and *T. reesei* as starter culture was a tremendous breakthrough as both live cultures and supernatants significantly enhanced malt quality. This is the first study to our knowledge that demonstrated improved malt modification by the addition of culture supernatants. This concept is not only more convenient as starter cultures, but will also deliver a more stable malt product because enzyme activities can be quantified prior to addition.

Optimization of enzyme production and freeze-dry processes is currently underway, and future studies will focus on the effect of higher enzyme concentrations on malt quality. It will also be valuable to combine these supernatants to broaden the spectrum of enzymes delivered to the germinating kernel.

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Table 1. Malting program

	Time (hours)	**Temperature (°C)**
Steeping		
1st wet stand	9	25
1st air rest	14	25
2nd wet stand	14	25
2nd air rest	6	25
Germination		
1st stage	72	25
2nd stage	12	25
Kilning		
1st stage	3	50
2nd stage	5	60
3rd stage	12	70
4th stage	4	80
5th stage	2	35

Table 2. The addition times and budding stages of starter cultures in each of the 15 SGVs in Trial 1

SGV	Starter culture addition time[a]			Starter culture stage
	1st wet stand	2nd wet stand	Germination	
1	Y	N	N	Spores
2	Y	N	N	Germinated
3	N	Y	N	Spores
4	N	Y	N	Germinated
5	N	N	Y	Germinated
6	N	N	Y	Germinated
7	Y	Y	N	Spores
8	Y	Y	N	Germinated
9	N	Y	Y	Spores
10	N	Y	Y	Germinated
11	Y	N	Y	Germinated
12	Y	N	Y	Spores
13	Y	Y	Y	Germinated
14	Y	Y	Y	Spores
15	N	N	N	Spores

[a]Y = Yes; N = No

Table 3. Starter culture cocktails added to SGVs in Trial 2

SGV	Starter culture cocktail
1	*G. candidum* 1173 + *L. plantarum* B.S1.6
2	*G. candidum* 2590 + *L. plantarum* B.S1.6
3	*G. candidum* 4960 + *L. plantarum* B.S1.6
4	*G. candidum* 1173 + 2590 + *L. plantarum* B.S1.6
5	*G. candidum* 1173 + 4960 + *L. plantarum* B.S1.6
6	*G. candidum* 2590 + 4960 + *L. plantarum* B.S1.6
7	*G. candidum* 1173 + 4964 + 2590 + *L. plantarum* B.S1.6
8	None (control)

Table 4. Starter culture cocktails added to SGVs in Trial 3 and 4

SGV	Starter culture cocktail and/or hormone added
1	*G. candidum* 1173 + *L. plantarum* B.S1.6
2	*G. candidum* 2590 + *L. plantarum* B.S1.6
3	*G. candidum* 4960 + *L. plantarum* B.S1.6
4	*G. candidum* 1173 + 2590 + *L. plantarum* B.S1.6
5	*G. candidum* 1173 + 4960 + *L. plantarum* B.S1.6
6	*G. candidum* 2590 + 4960 + *L. plantarum* B.S1.6
7	*G. candidum* 1173 + 4964 + 2590 + *L. plantarum* B.S1.6
8	None (Negative control)
9	Gibberellic acid (Positive control)

Table 5. Malting program, barley cultivar and starter culture added to each SGV in Trial 5

Malting program	Barley cultivar	SGV	Starter culture added
Normal	Erica	1	*G. candidum* 1173 + *L. plantarum* B.S1.6
		2	Control
	SSG 564	3	*G. candidum* 1173 + *L. plantarum* B.S1.6
		4	Control
Cargill	Erica	5	*G. candidum* 1173 + *L. plantarum* B.S1.6
		6	Control
	SSG 564	7	*G. candidum* 1173 + *L. plantarum* B.S1.6
		8	Control

Table 6. Starter cultures of Trial 6

SGV	Culture added
1	*Aspergillus* sp. + *L. plantarum* B.S1.6
2	*Rhizopus* sp. + *L. plantarum* B.S1.6
3	*T. reesei* sp. + *L. plantarum* B.S1.6
4	*Aspergillus* sp. filtrate + *L. plantarum* B.S1.6
5	*Rhizopus* sp. filtrate + *L. plantarum* B.S1.6
6	*T. reessei* sp. filtrate + *L. plantarum* B.S1.6
7	None (control)

Table 7. FAN and β-glucan content of SGVs added with starter cocktail *G. candidum* (1173, 2590 and 4964) + *L. plantarum* B.S1.6 in Trial 1. Included are the respective starter culture additive times and budding stages

	Addition time					
SGV	**1st wet stand addition**	**2nd wet stand addition**	**Start of germination**	**Starter culture stages**	**FAN**	**β-glucans**
1	Yes	No	No	budding	178.5 ± 2.1	85.0 ± 5.7*
2	Yes	No	No	No	154.0 ± 5.7	115.0 ± 2.8
3	No	Yes	No	budding	199.0 ± 4.2	94.0 ± 1.4
4	No	Yes	No	No	188.0 ± 2.8	89 ± 4.2
5	No	No	Yes	budding	253.0 ± 7.1*	72.5 ± 0.7*
6	No	No	Yes	No	242.5 ± 0.7*	87.5±2.1*
7	Yes	Yes	No	budding	195.5 ± 6.4	180.5 ±
8	Yes	Yes	No	No	187.0 ± 4.2	112.5 ± 3.5
9	No	Yes	Yes	budding	199.5 ± 2.1*	202.5 ± 9.2
10	No	Yes	Yes	No	201.0 ± 1.4*	54.0 ± 2.8*
11	Yes	No	Yes	budding	173.0 ± 7.1	132.5 ± 3.5
12	Yes	No	Yes	No	277.5 ±	176.5 ± 6.4
13	Yes	Yes	Yes	budding	206.5 ± 3.5*	287.0 ± 8.5
14	Yes	Yes	Yes	No	228.0 ± 5.7*	74.0 ± 5.7*
15	No	No	No	No	181.0 ± 4.2	110.5 ± 6.4

Table 8. FAN, β-glucan content and enzyme activities of starter culture SGVs from Trial 2

SGV	Starter combination	β-glucan (ppm)	FAN (ppm)	α-amylase (CU/g malt)	β-glucanase (30 °C) (U/Kg malt)	β-glucanase (60 °C) (U/Kg malt)	Xylanase (U/g malt)
1	**1173**	99.21 ± 14.14	156.65 ± 5.66	132.69 ± 7.87	227.89 ± 9.47*	250.28 ± 1.35*	0.24 ± 0.02
2	**2590**	122.50 ± 9.19*	167.51 ± 14.85	161.87 ± 0.53*	191.01 ± 7.37	207.32 ± 11.77	0.27 ± 0.00
3	**4964**	109.26 ± 29.70	152.23 ± 5.66	142.68 ± 13.25	200.67 ± 9.54	182.35 ± 8.66	0.25 ± 0.01
4	**1173 + 2590**	111.36 ± 26.87	155.50 ± 0.71	138.56 ± 0.08	200.82 ± 1.42	177.28 ± 3.38	0.28 ± 0.02
5	**1173 + 4964**	120.56 ± 4.24*	145.25 ± 3.54	134.68 ± 0.97	211.34 ± 9.61	204.17 ± 1.62	0.26 ± 0.01
6	**2590 + 4964**	95.51 ± 7.78	157.29 ± 2.83	155.56 ± 7.86	174.13 ± 14.34	174.53 ± 11.87	0.26 ± 0.01
7	**1173 + 4964 + 2590**	101.54 ± 23.33	160.25 ± 6.36	134.26 ± 16.06	209.95 ± 1.56*	224.16 ± 2.57*	0.27 ± 0.03
8	**control**	91.23 ± 4.24	157.69 ± 4.24	127.31 ± 8.22	178.98 ± 9.10	189.70 ± 8.02	0.31 ± 0.05

Table 9. Enzyme activities of kilned malt from Trial 5

Malting program	Barley cultivar	SGV		α-amylase (U/g malt)	β-glucanase(U/Kg malt) (30 °C)	β-glucanase (U/Kg malt) (60 °C)	Xylanase (U/g malt)
Normal	Erica	1	1173	165.72 ± 19.14	206.84 ± 21.10	131.19 ± 23.23	0.25 ± 0.02
		2	Control	151.39 ± 18.41	155.39 ± 2.94	77.81 ± 3.55	0.22 ± 0.05
	SSG 564	3	1173	165.44 ± 14.76	157.04 ± 27.80	122.65 ± 35.31	0.25 ± 0.04
		4	Control	164.08 ± 21.74	198.87 ± 22.01	79.32 ± 5.88	0.24 ± 0.00
Cargill	Erica	5	1173	159.89 ± 5.00*	117.01 ± 0.12*	51.33 ± 2.5*	0.22 ± 0.01
		6	Control	100.24 ± 8.57	63.63 ± 12.47	20.62 ± 1.82	0.21 ± 0.03
	SSG 564	7	1173	196.55 ± 5.00*	61.98 ± 0.81	56.21 ± 2.0*	0.22 ± 0.0
		8	Control	120.01 ± 5.00	80.20 ± 2.06	16.46 ± 3.0	0.19 ± 0.02

Table 10. Enzyme activities of kilned malt from Trial 6

SGV	Starter culture	α-amylase (U/g malt)	β-glucanase (U/Kgmalt)(30°C)	β-glucanase (U/Kg malt)(60°C)	Xylanase (U/g malt)
1	*Aspergillus* sp.	354.72 ± 14.58*	413.84 ± 32.16*	274.21 ± 39.88*	0.29 ± 0.06
2	*Rhizopus* sp.	372.80 ± 0.00*	281.60 ± 40.99*	202.68 ± 24.35*	0.33 ± 0.08
3	*T. reessei* sp.	337.69 ± 31.57*	347.33 ± 12.78*	227.79 ± 5.28*	0.30 ± 0.03
4	*Aspergillus* sp. sup	314.19 ± 34.50*	323.86 ± 26.88*	200.88 ± 12.48*	0.39 ± 0.02*
5	*Rhizopus* sp. sup	364.53 ± 17.95*	342.88 ± 7.91*	242.64 ± 35.01*	0.46 ± 0.03*
6	*T. reessei* sp. sup	346.10 ± 9.57*	292.58 ± 45.76	189.05 ± 25.57*	0.37 ± 0.01
7	Control	151.38 ± 18.41	155.39 ± 2.94	77.81 ± 3.55	0.22 ± 0.05

Table 11. Enzyme activities of fungal supernatant in Trial 6

Fungal isolate	β-glucanase (nkats/mL)	Cellulase (nkats/mL)	Xylanase (nkats/mL)	α-amylase (CU/mL)
***Aspergillus* sp.**	7.39 ± 0.65	4.64 ± 0.35	217.58 ± 12.65	33.66 ± 5.23
T. reesei	10.84 ± 0.56	17.62 ± 2.65	53.87 ± 4.32	0.00
***Rhizopus* sp.**	0.55 ± 0.01	0.8 ± 0.05	0.59 ± 0.023	171.56 ± 8.177

Table 12. Enzyme activities of fungal supernatants before and after freeze-drying

Fungal isolate	Xylanase (nkats/mL)		β-glucanase (nkats/mL)		Cellulase (nkats/mL)		α-amylase (CU/mL)	
	Before	After	Before	After	Before	After	Before	After
***Aspergillus* sp.**	217.58 ± 12.65	214.58 ± 11.69	7.39 ± 0.65	6.39 ± 0.95	4.64 ± 0.35	4.24 ± 0.15	33.66 ± 5.23	30.66 ± 7.23
T. reesei	53.87 ± 4.32	50.87 ± 6.32	10.84 ± 0.56	9.84 ± 1.23	17.62 ± 2.65	14.82 ± 2.15	0.00	0.00
***Rhizopus* sp.**	0.59 ± 0.023	0.51 ± 0.013	0.55 ± 0.01	0.47 ± 0.05	0.8 ± 0.05	0.76 ± 0.09	171.56 ± 8.17	165.16 ± 9.21

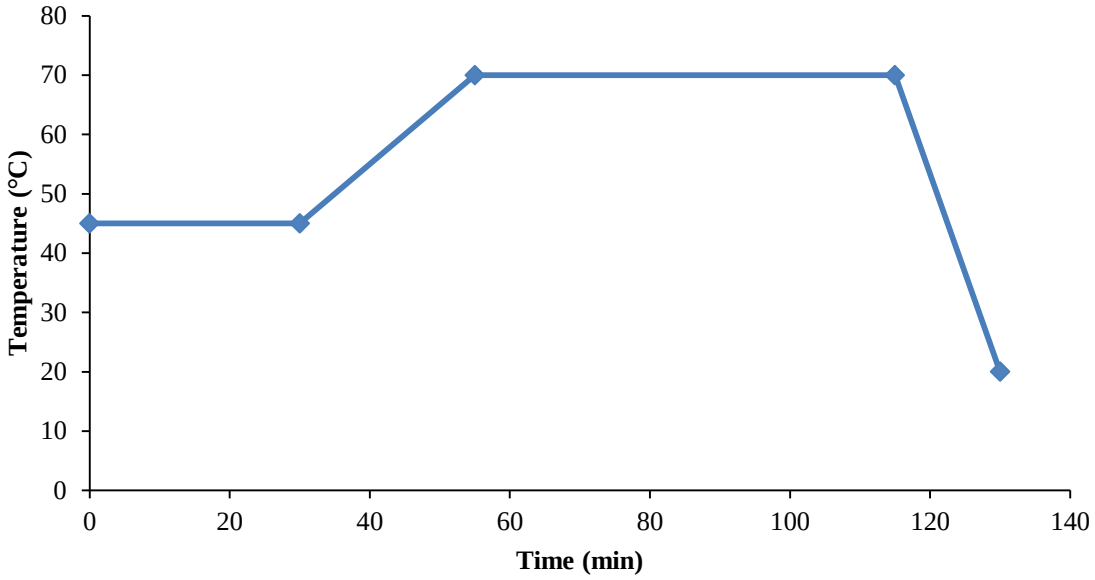

Fig. 1. Mash cycle

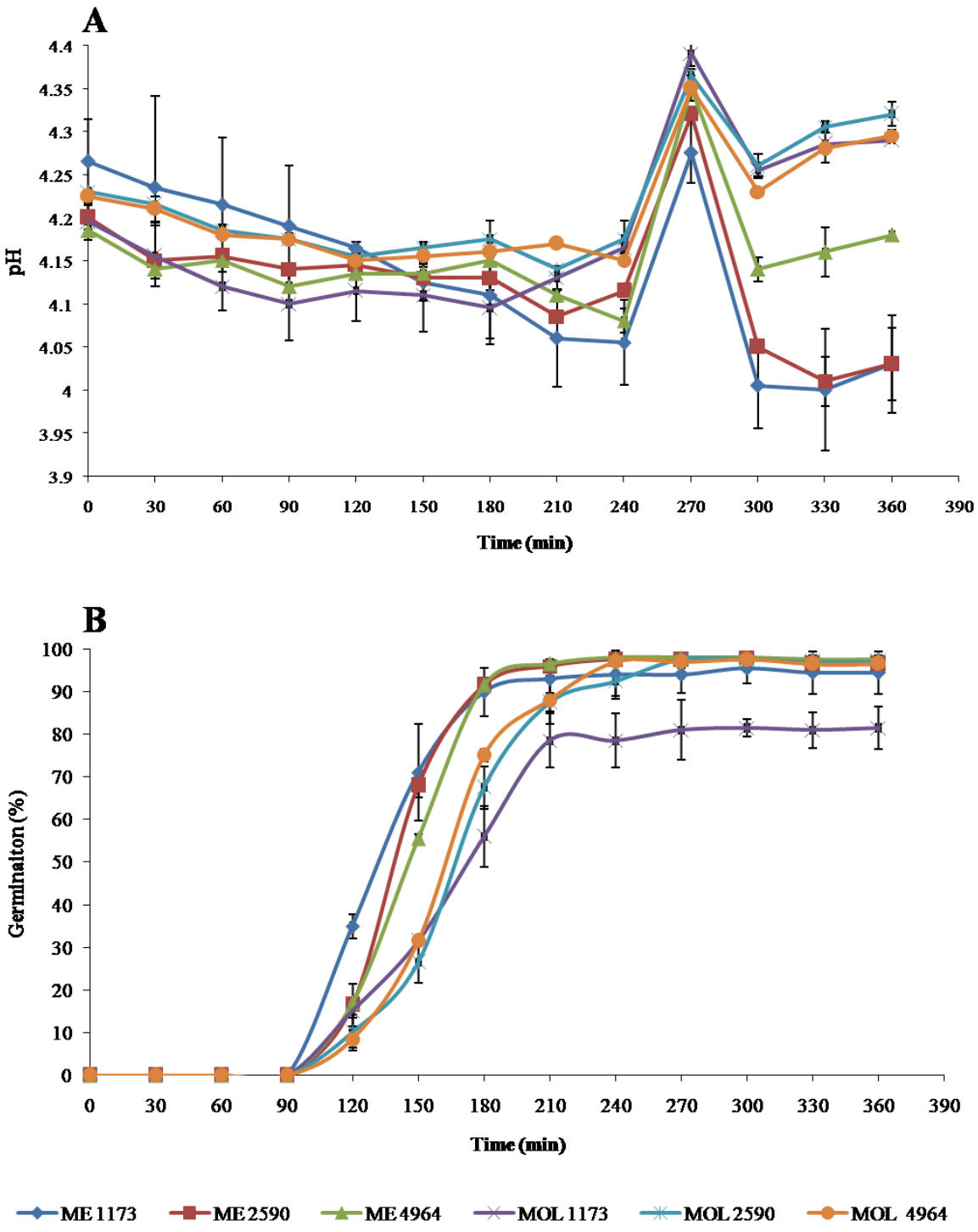

Fig. 2. The evolution of (A) pH and (B) germination percentage during the 360 min germination study of 3 *G. candidum* strains (1173, 2590 and 4964) in 2 % molasses (MOL) and 2 % Malt Extract (ME), respectively

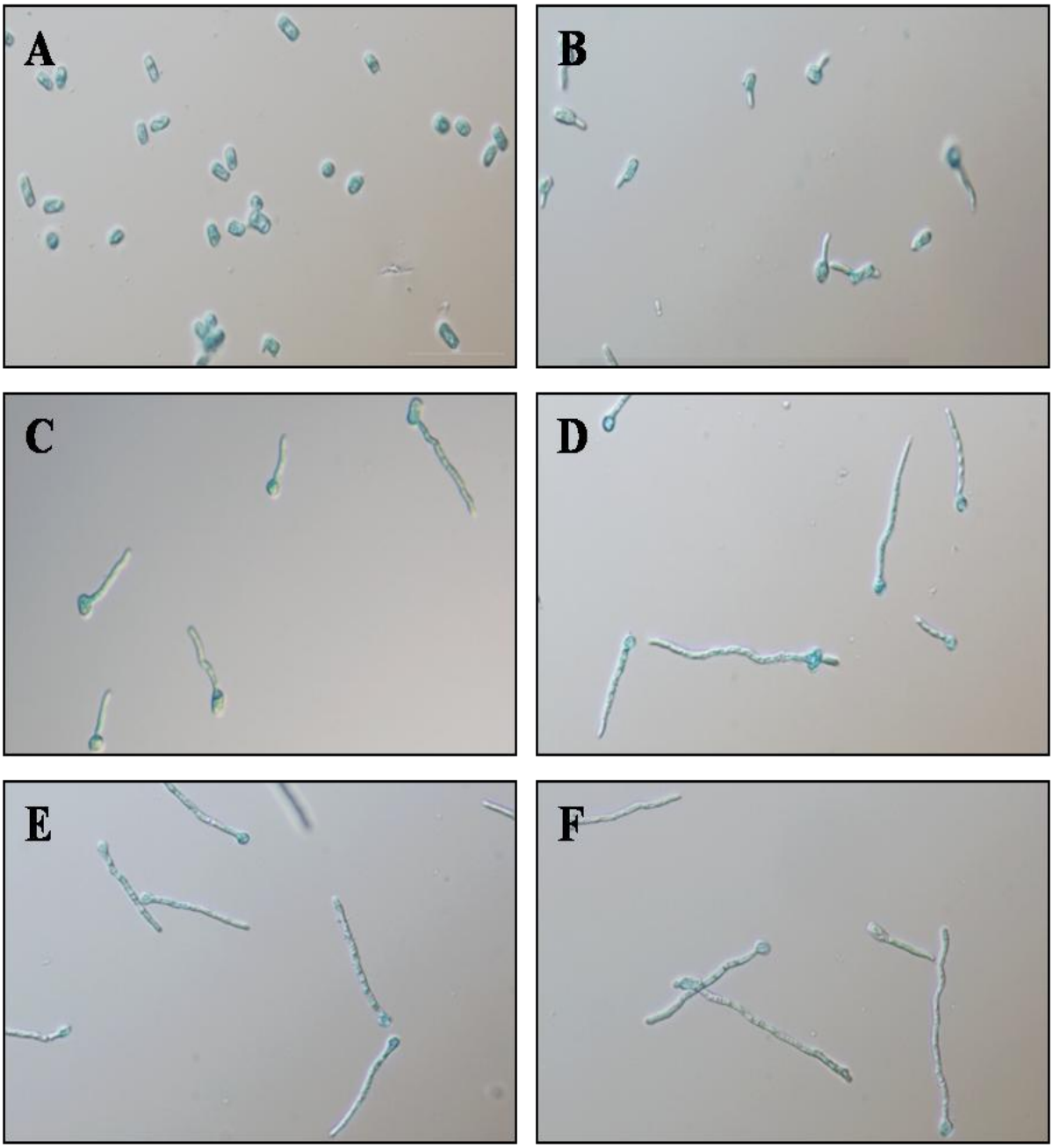

Fig. 3. Germination and hyphael elongation during the 360 min germination study of *G. candidum* 1173 in 2 % molasses. A = 0 min; B = 120 min; C = 180 min; D = 240 min; E = 270 min; F = 300 min

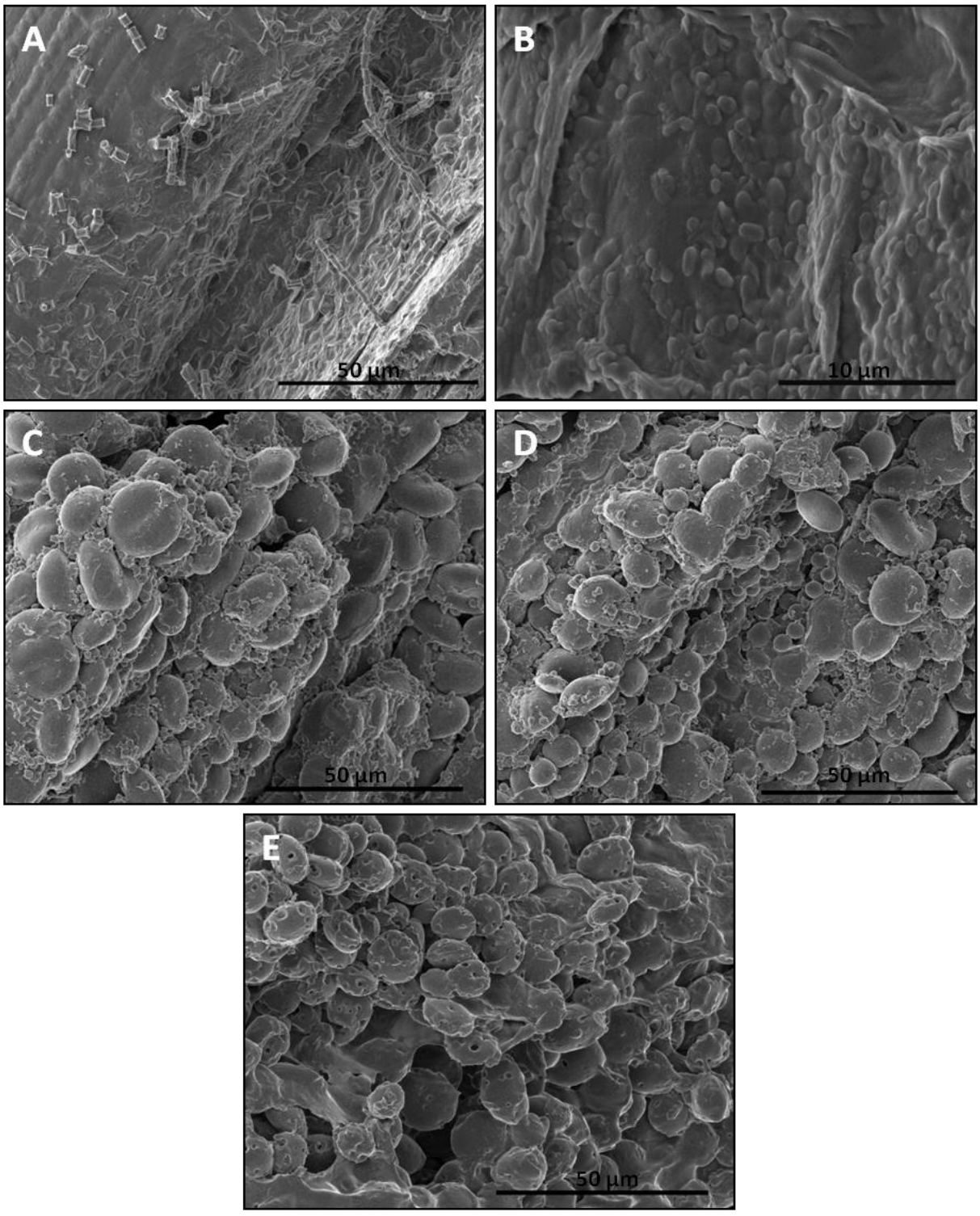

Fig. 4. SEM micrographs of malt from Trial 1. Panel A: *G. candidum* hyphae on the surface of a kernel from SGV 6. Panel B: Rod-shaped bacterial cells, resembling *L. plantarum* B.S1.6 on surface of a kernel from SGV 6. Panel C + D + E: Starch granules and protein matrix in the starchy endosperm of kernels from SGV 6, SGV 15 (Control) and a Cargill cultivar, respectively

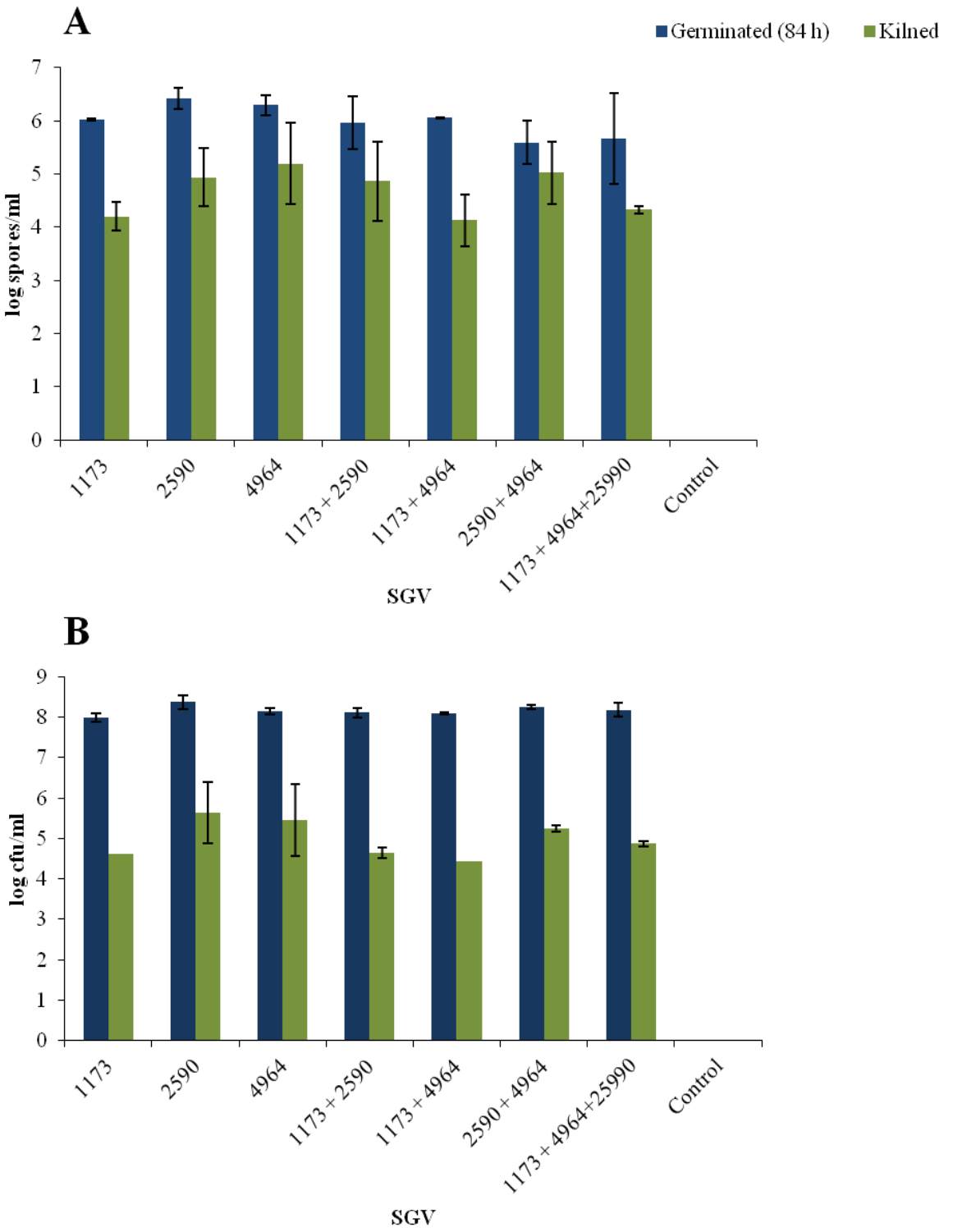

Fig. 5. Proliferation of (A) *G. candidum* and (B) *L. plantarum* after germination (84 h) and after kilning for Trial 2

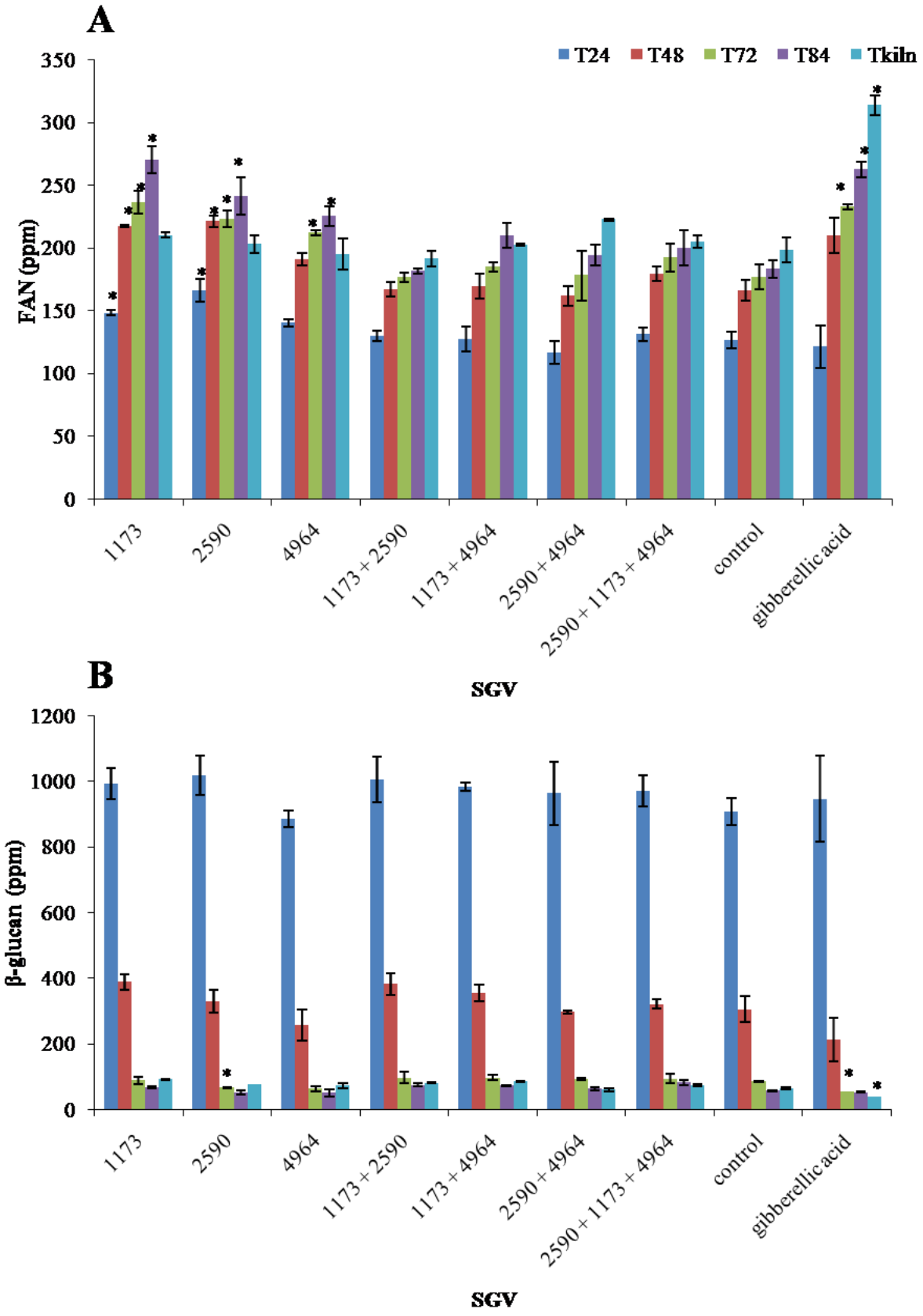

Fig. 6. Evolution of (A) FAN and (B) β-glucan content during germination and after kilning in malt from Trial 3

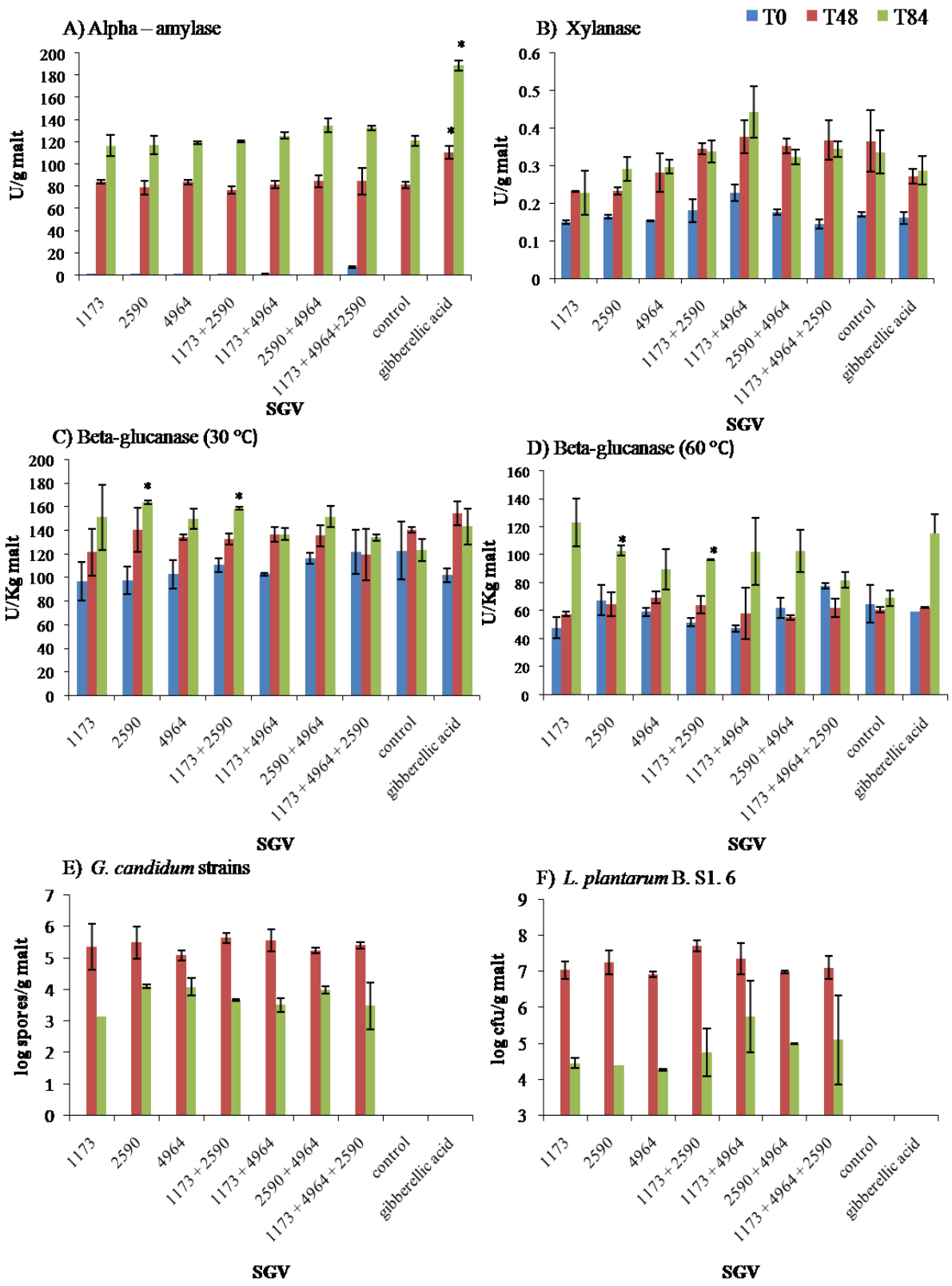

Fig. 7. Evolution of (A-E) enzyme activities and (E-F) starter culture penetration during germination and kilning of Trial 3

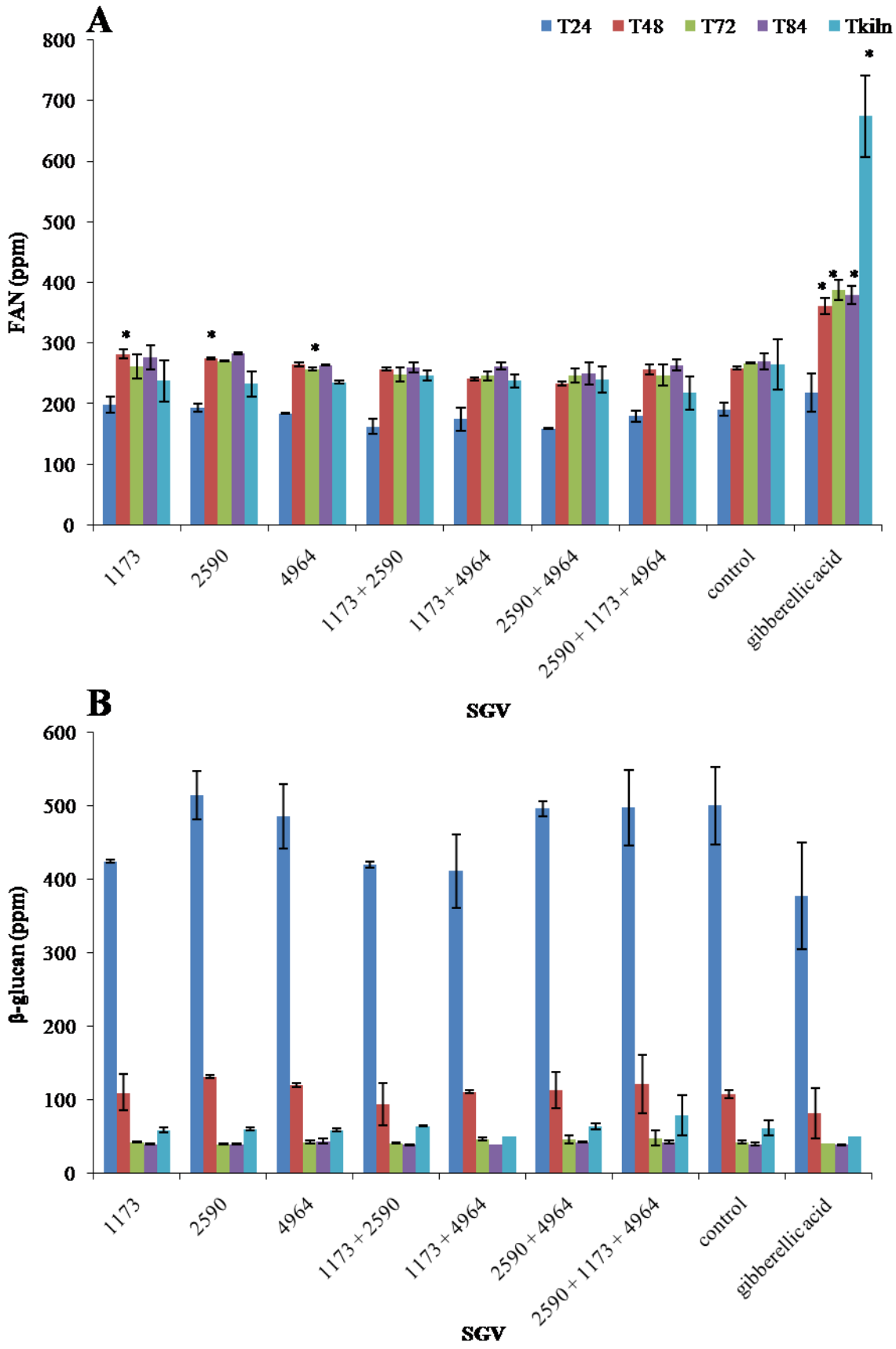

Fig. 8. Evolution of (A) FAN and (B) β-glucan content during germination and after kilning in malt from Trial 4

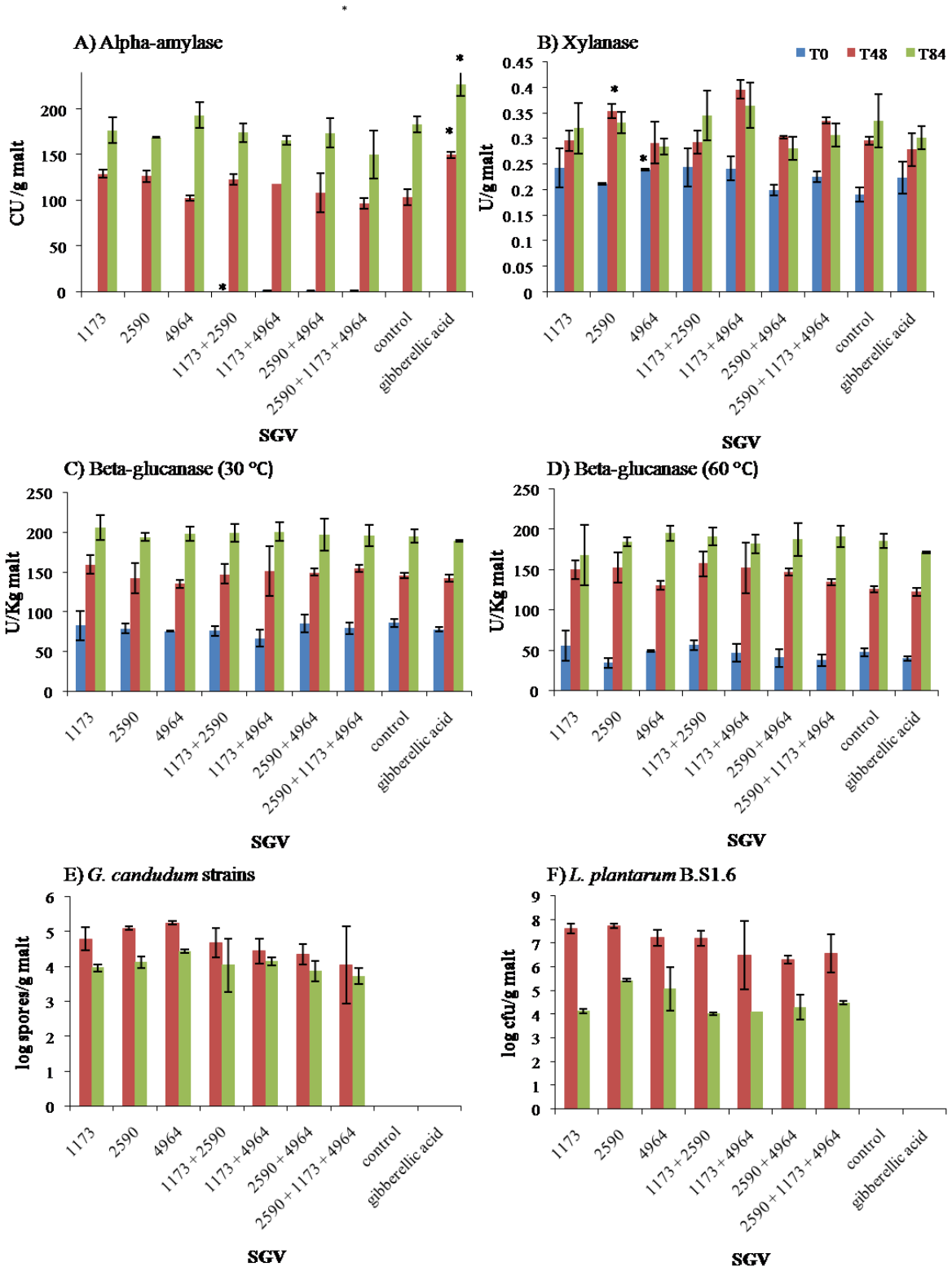

Fig. 9. Evolution of (A-E) enzyme activities and (E-F) starter culture penetration during germination and kilning of Trial 4

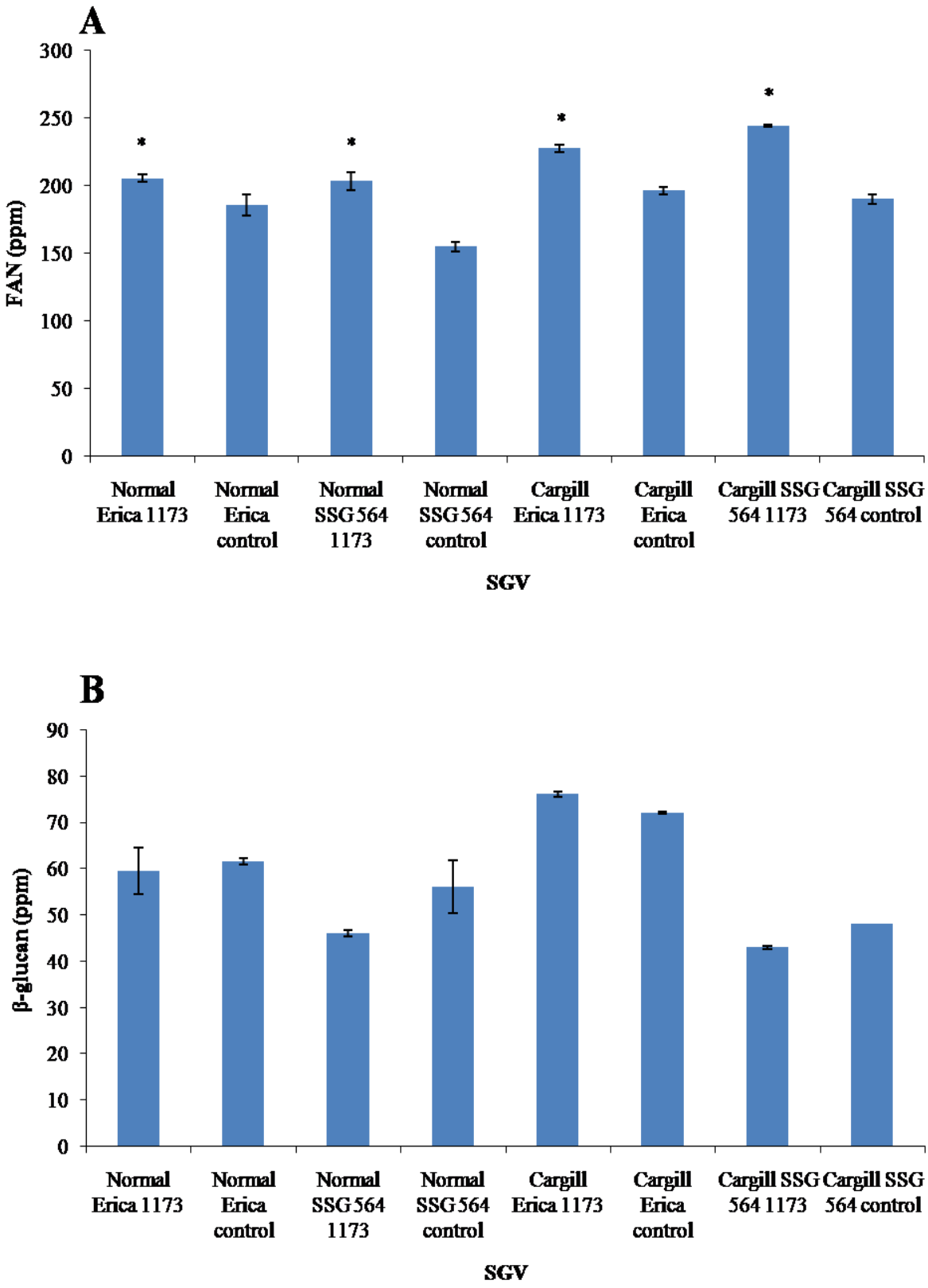

Fig. 10. FAN (A) and β-glucan content (B) of kilned malt from Trial 5

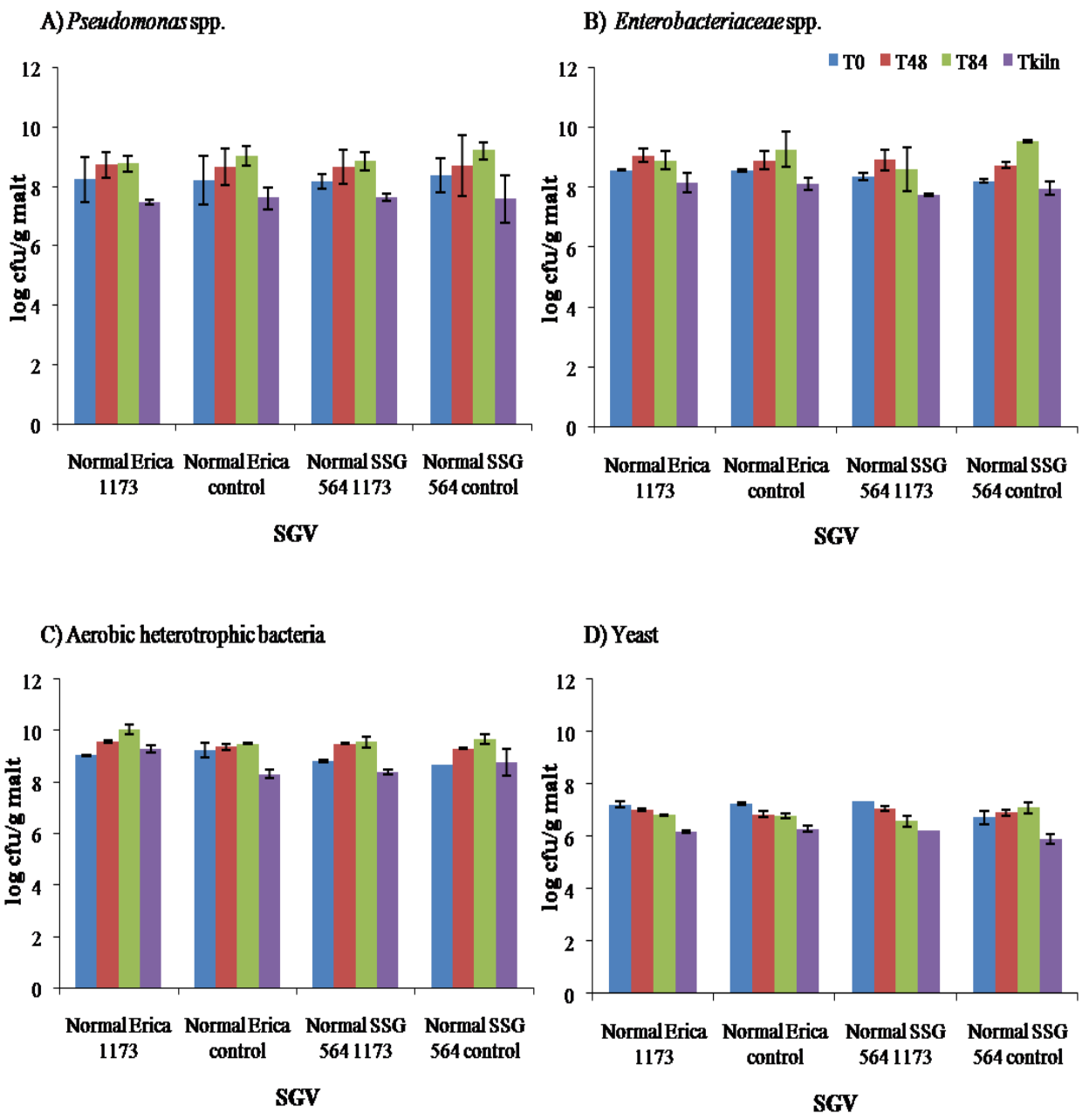

Fig. 11. Proliferation of native microbial communities during germination and after kilning in Trial 5

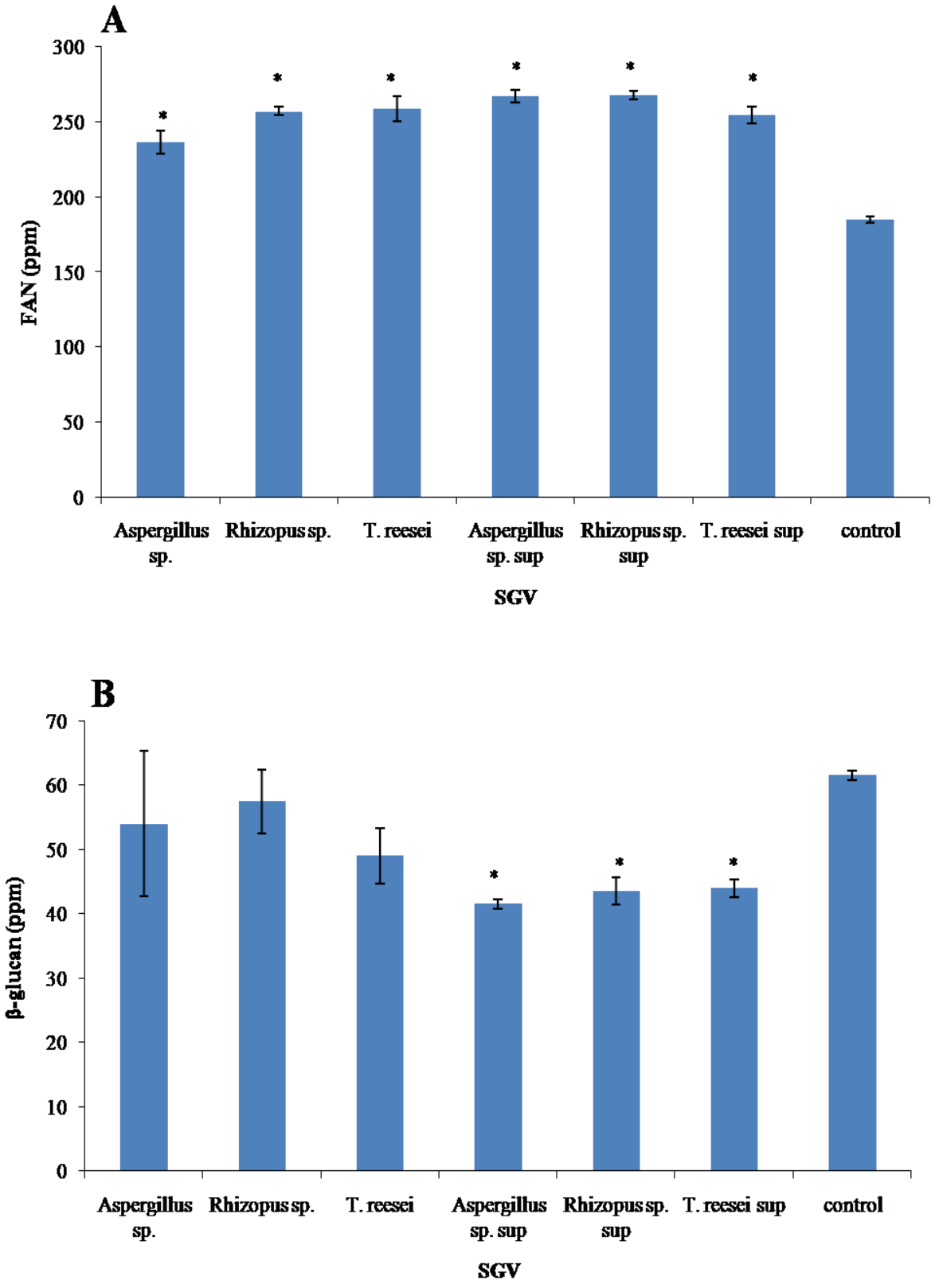

Fig. 12. FAN (A) and β-glucan content (B) of kilned malt from Trial 6

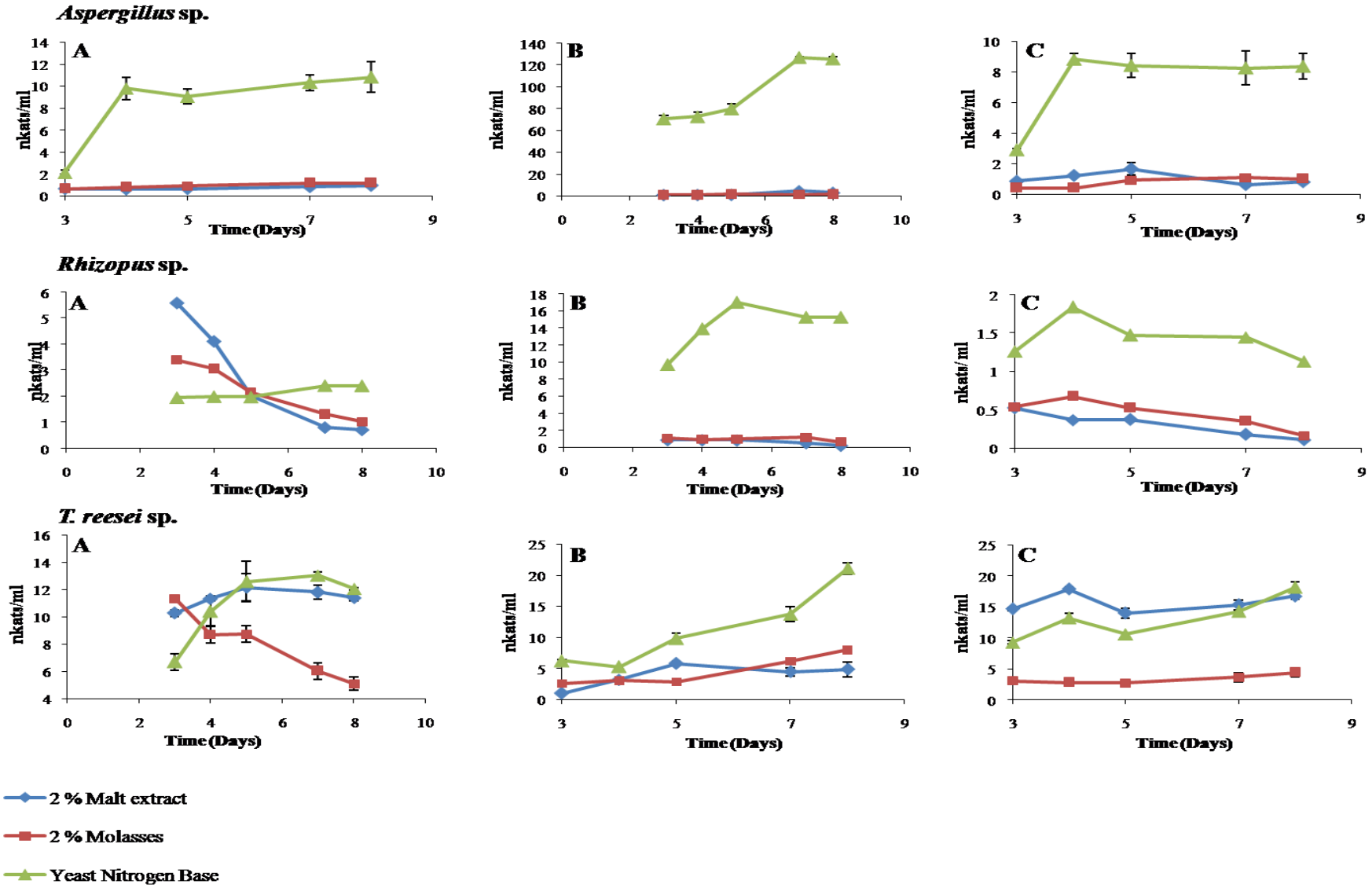

Fig. 13. Enzyme activities of fungal supernatants. (A) β-glucanase, (B) cellulase, (C) xylanase

Chapter 6

General Discussion and Conclusions

General Discussion and Conclusions

Malt quality depends on the generation of high enzyme levels for sufficient substrate hydrolysis during germination and mashing (Laitila et al., 2007; Noots et al., 1998). It is often difficult to sustain malt of high quality due to inconsistent malt batches and poor germination capacities of dormant barley. External additives such as chemicals and gibberellic acid have been used to overcome these difficulties, but are unwanted in the beverage industry (Hough, 1985). Microbes produce diverse enzymes which can contribute to barley germination (Biovin and Malanda, 1997; Foszczynska et al., 2004; Haikara et al., 1995; Noots et al., 1998). The development of such starter cultures might provide a natural and economically feasible alternative to augment barley germination. Starter culture technology has been employed in the malting industry, although the main focus has been to improve the microbial stability of malt, rather than enhancing malt production (Laitila et al., 2007; Linko et al., 1998; Lowe et al., 2005). Only one group of researchers have added a *Rhizopus oligosporus* with the specific aim to contribute to malt modification, and consequently this field is largely unexplored (Noots et al., 1993; Noots et al., 1999; Noots et al., 2001). The aim of this study was to assess whether starter cultures with known hydrolytic capabilities can enhance germination for improved malt quality.

Geotrichum spp. harboured diverse hydrolytic capabilities as all twelve strains had β-glucanase and cellulase activity, nine strains displayed extracellular protease activity and three degraded xylan. None were able to degrade starch. These results correlate with results from the literature as β-glucan and cellulose degradation appears to be a general ability of these isolates, while only a few authors identified amylase producing strains (Laitila et al., 2006; Subash et al., 2005; Piegza et al., 2005).

Two amylase producing *L. plantarum* strains were isolated from barley and both harboured extracellular- and cell-bound amylase. To our knowledge, this is the first report on amylase producing lactic acid bacteria from barley. Extracellular amylase from both cultures was unique compared to known amylolytic *L. plantarum* strains, and resembled characteristics of other amylase producing lactic acid bacteria species (Guyot et al., 2001; Dunican and Seeley, 1962; Lindgren and Refai, 1984). Genes encoding these enzymes were most likely acquired though plasmid exchange or horizontal gene transfers. Valuable insight regarding the activity and regulation of amylase synthesis will be gained in future studies once the position and sequence of the respective genes have been identified.

G. candidum and *L. plantarum* starter cultures proved unsuccessful to enhance the quality of SSG 564 or Erica malt. Improved malt parameters could not be correlated with microbial enzyme activities and was also not repeatable. Preliminary plate assays could thus not be used as a measure to predict enzyme

production in a malting environment. Characterization of *L. plantarum* amylase demonstrated poor activity at low malting temperatures and activity was subjected to catabolic repression, possibly explaining why no enhanced malt amylase activity was observed. Enzyme activities of *G. candidum* strains were not characterized although different parameters during malting such as pH, low temperature, microbial competition, low water activity and poor oxygen and substrate availability might have obstructed enzyme production. In future studies it will be valuable to screen possible starter culture isolates on similar substrates and environmental conditions to effectively predict enzyme production.

Aspergillus sp., *T. reesei* and *Rhizopus* sp. are known for their exceptionally high extracellular enzyme production and their effect on malt modification was also evaluated. Supernatants were more effective in enhancing malt quality than live cultures, although a significant improvement was observed in both cases. Spectrophotometric assays demonstrated that enzyme production by these cultures was significantly higher than recorded for *Geotrichum* spp. and *L. plantarum*. The enzyme capabilities of the latter isolates might therefore not have been sufficient enough to enhance malt quality. To our knowledge, this is the first study where a fungal supernatant was used to augment the modification process, and the success thereof is a major breakthrough. Supernatant is a more convenient alternative to starter cultures as it eliminates various difficulties. Since no living cultures are involved, process conditions will not significantly be altered in terms of vessel preparation and cleaning. In addition, a more constant malt product may be produced because enzyme activity can be determined and adjusted prior to the malting process. This is not possible for live cultures, as certain environmental stresses might change its enzyme activity. In addition, the risk of posing a health hazard to workers due to exposure to high levels of spores is also eliminated, especially in immune compromised people.

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